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Acute toxicity profile and Sun Protection Factor (SPF) nanoemulgel combination of C-phenylcalix[4] resorcinaryl octacinnamate, C-methylcalix[4] resorcinaryl octabenzoate, and quercetin in vitro and in vivo

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ABSTRACT

Background: The ageing process (photoaging) can be caused by sun exposure, especially ultraviolet light. Organic and inorganic sunscreen products are commercially available. Two calixarene organic compounds, namely C-phenylcalix[4]resorcinaryl octacinnamate and C-methylcalix[4]resorcinaryl octabenzoate, have been successfully synthesized. Besides, the antioxidant quercetin can be potentially combined with these two compounds since ultraviolet rays also cause reactive oxygen species. This study aimed to evaluate the acute toxicity profile *in vitro* by cell line Vero and to develop the optimal activity of the product in New Zealand rabbit skin.

Methods: Optimal formulation of three formulas nanoemulgel of sunscreen was using *D Optimal Mixture Design*. Acute cytotoxicity test *in vitro* by culture cell line Vero was using *randomized post-test only control group design*. The activity of the product was measured by the value of Sun Protection Factor (SPF) in vivo using *randomized post-test only control group design*. Data of acute toxicity in vitro test (IC50 value) was analyzed using probit analysis and activity sun protection factor was analyzed using one-way ANOVA on SPSS version 20 for Windows.

Results: The *in-vitro* toxicity test of formula 1, 2, 3 nanoemulgel were 2,940.569 µg/mL, 13,489.728 µg/mL, and 6,289.248 µg/mL respectively. The formula 1 nanoemulgel sunscreen products were produced with the three highest SPF values. SPF *in vivo* test showed that the nanoemulgel protection capability of the formula 1 with three different doses were 34; 36; dan 43 respectively.

Conclusion: It can be concluded that the nanoemulgel sunscreen products were successfully formulated with high in vivo SPF value and can be potentially developed as organic sunscreens in the future because it is not toxic in culture cell.

Keywords: C-phenylcalix[4]resorcinaryl octacinnamate, C-methylcalix[4]resorcinaryl octabenzoate, quercetine, nanoemulgel, acute toxicity, SPF

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INTRODUCTION

Sunlight has a vital role as the source of life for living things on earth. On the other hand, sun exposure, unusually excessive ultraviolet rays can cause skin damage.[1-4] Ultraviolet B (UVB) rays with a wave range of 280 to 320 nm are most predominantly causing adverse effects in humans.[5] UVB rays can penetrate the superficial part of the epidermis to the basal part of the epidermis which has the potential to cause the formation of reactive oxygen species (ROS) or reactive nitrogen species (RNS), inflammation, sunburn, premature ageing and even cancer.[5-7]

Sunscreen is an inorganic and organic material that can be used to avoid the harmful effects of sunlight by absorbing or reflecting sunlight. A good ultraviolet absorbent in cosmetics must have several requirements that must be met include: non-toxic, high ultraviolet absorbance over a wide range of wavelengths, no damage caused by ultraviolet light and have excellent compatibility with cosmetic preparations.[8] The efficacy of a sunscreen is also determined by the Sun Protection Factor (SPF) which is defined as a value that describes the ultraviolet energy needed to cause a minimal dose of erythema to protect the skin.[9-11] Organic sunscreen is increasingly in demand because it does not cause DNA damage, high efficacy, and does not cause whitish colour when applied. Organic

sunscreens still have weaknesses that are less stable. A high photostability is needed for the effectiveness and safety of an organic sunscreen.[12,13]

Photostable organic sunscreens, C-methylcalix [4] resorcinaryl octabenzoate and C-phenylcalix [4] resorcinaryl octacinnamate have been successfully synthesized by the Chemistry Department of the Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. Both of these compounds have these characteristics, namely C-methylcalix [4] resorcinaryl octabenzoate compound has absorption spectrum at three ultraviolet wavelengths which are UVC (at wavelength 248 nm), UVB (at wavelength 280 nm), UVA (at wavelength 353 nm) with SPF results in vitro spectroscopy 35. In contrast, C-phenylcalix compound [4] resorcinol octacinamic has an absorption spectrum at three wavelengths which is UVC (at wavelength 274 nm), UVB (at wavelength 295 nm), UVA (at wavelength 367 nm) with SPF results in vitro 123.[14] The combination of these two compounds is expected to increase the absorption spectrum of ultraviolet light, increase SPF and increase the stability of sunscreen.

Quercetin (3,3 ‘, 4’, 5,7-pentahydroxyflavone) is a polyphenolic flavonoid group which is a powerful antioxidant added to this formulation.[15] Quercetin has various biological activities such as antioxidant, anti-inflammatory, anticancer, antiviral and antimicrobial.[16,17] Its ability as a catcher of intracellular free radicals is expected to work synergistically with the compound C-methylcalix [4] resorcinaryl octabenzoate and C-phenylcalix [4] resorcinaryl octacinamate to increase protection against ultraviolet B.

Based on those mentioned above, this study aims to determine the combination of the C-methylcalix[4] resorcinaryl octabenzoate, C-phenylcalix [4] resorcinaryl octacinamate, and quercetin can be useful as a sunscreen that is guaranteed safe and efficacious through acute toxicity testing (in-vitro) and the determination of the activity of the combination of these compounds in-vivo in New Zealand rabbit.

MATERIALS AND METHODS

Materials

Photostable organic sunscreens, C-methylcalix [4] resorcinaryl octabenzoate and C-phenylcalix [4]resorcinaril octacinnamate synthesized by the Chemistry Department of the Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. Quercetin was purchased from Sigma. Candlenut oil was purchased from CV Orizo (Indonesia). Tween 80, PEG 400 and distilled water were purchased from Brataco (Indonesia). Ethanol was purchased from Merck. New Zealand Rabbit was purchased from Animal Laboratory Department Pharmacology and Therapy Universitas Gadjah Mada.

Production of Nanoemulgel

The output of nanoemulgel of the three compounds requires a combination of candlenut oil, tween 80 and PEG 400. The formula homogenized with a vortex mixer for 5 minutes and then followed with sonication for 15 minutes. And then use magnetic stirrer with 300 rpm for 1 hour. This process uses Design Expert®ver.7.1.5 methods with D-optimal mixture to gain the optimum formula. The optimum formula adds with Carbopol as the basis of the gel.

Toxicity Test In Vitro Using Vero Cell Line

Cytotoxicity tests were carried out using Vero cells grown on DMEM culture media. The test materials given were formula 1 optimum nanoemulgel, formula 2 optimum nanoemulgel, formula 3 optimum nanoemulgel, as positive controls were Parasol®SPF 33 and carbopol gel 1%. The basis of this cytotoxicity test is the cell's ability to survive due to the presence of toxic compounds given. The general method used for cytotoxic testing is the MTT assay method. This method is a colourimetric method, in which MTT reagents are tetrazolium salts which can be broken down into formazan crystals by succinate tetrazolium reductase which are present in the cell respiration pathway in active living mitochondria. Formazan crystals will give a purple colour that can be read absorbed by using an ELISA reader with a wavelength between 550-595 nm. The parameter used for the cytotoxic test is the value of Inhibition Concentration (IC50). IC50 values indicate concentration values that result in inhibition of cell proliferation by 50% and show the potential for the oxidation of a compound to cells. This value is a benchmark for conducting cell kinetics observation tests. IC50 values can indicate the possibility of a compound as cytotoxic. The higher value of IC50 means the compound is more non-toxic.

Sun Protection Factor (SPF) Activity Test

In-vivo, sunscreen protection activity test was carried out on rabbit skin which was smeared with optimum formula 1 nanoemulgel with three dosage variations: 1 mg/cm2, 2 mg/cm2, 4 mg/cm^2. Parasol was used as a positive control of the study. The first step of this study was to determine the minimum radiation dose for UVB through a

preliminary test (dose variations of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mJ/cm2). The radiation dose that causes the erythema response is 40 mg /cm2. The minimal erythema dose was used as the initial dose used in this study. The radiation dose range used in this study was 40, 80, 160, 320, 640, 1280, 1400, 1600, 1800 and 2000 mJ/ cm2. The optimum formula 1 nanoemulgel is applied to the rabbit's back and waited 30 minutes so that the sunscreen blends with the skin horn layer before irradiating with UVB. The SPF value of sunscreen is determined based on the minimum erythema dose (MED) produced. SPF value in vivo is the optimum ratio between MED formula 1 nanoemulgel compared to MED without nanoemulgel, which is observed after 24 hours after radiation. Erythema calculation is observed visually by dividing from degree 0 to degree 4 (zero degrees = no redness, first-degree pink, second degree = erythema, third-degree = very red, 4th degree red with the extended area). The degree of erythema is observed visually.

Furthermore, rabbit skin is given UVB rays with 10 different dose ratings, according to the MED results. The distance between UVB rays and irradiation is 30 cm. Twenty-four hours after radiation, the presence or absence of erythema was observed in the radiated area. The SPF value is determined based on a minimum erythema dose without sunscreen compared to a minimum erythema dose with sunscreen. MED values were measured 24 hours after radiation.

Data Analysis

All of the data regarding the acute toxicity in-vitro test (IC50 value) was analyzed using probit analysis. Besides, the activity of the sun protection factor was analyzed using one-way ANOVA on SPSS version 20 software for Windows.

RESULTS

Formula optimum of nanoemulgel

Results of the optimal formula 1 sunscreen contains candlenut oil: 1,250 mL, Tween 80: 6,308 mL and PEG 400: 2,442 mL.The optimal formula 2 contains candlenut oil : 1,250 mL, Tween 80: 7,350 mL and PEG 400: 1,400 mL. The optimal formula 3 contain 2,500 mL candlenut oil, Tween 80: 5,066 mL and PEG 400: 2,434 mL. The optimal formula 1 contains a combination of quercetin 10 mg; C-phenilcalix [4] resorcinol octacinamate 10 mg, the optimal formula 2 containing a combination of quercetin 5 mg, C-phenilcalix [4] resorcinaril octacinamate 10 mg and C methylcallic [4] resorcinaril octabenzoate 5 mg. The optimal formula 3 contains a combination of C-phenilcalix [4] resorcinaril octasinamat, and C-phenilcalix [4] resorcinaryl octabenzoate 10 mg.

Acute Toxicity Test In-Vitro

I n-vitro cytotoxicity tests were performed on all three nanoemulgel formulas to determine the toxicity of nanoemulgel in typical cell cultures. The parameters used for the cytotoxic analysis are the value of Inhibition Concentration (IC50). IC50 values indicate concentration values that result in the inhibition of cell proliferation by 50% and suggest the toxic potential of a compound to cells. This value is a benchmark for conducting cell kinetics observation tests. The morphology of Vero cells observed 24 hours shortly before MTT staining can be seen in Figure 1, and the results of the cytotoxicity test are shown in Table 1. The results obtained indicate that the three nanoemulgel formulations are not toxic to Vero cells. Based on the comparison between IC50 values against Vero cells, nanoemulgel formula 2 has the highest safety value (Table 1). Also seen compared to carbopol gels, the three nanoemulsions added to carbopol gels provide a protective value against Vero cells which are shown to be higher IC50 values than carbopol gels. The results of this test also showed that the hydrogel material used was also not toxic, with an IC50 value of 595,138 µg / mL. All three test materials have high IC50 values, and this proves the third test material is quite safe to use as a sunscreen (Table 1). Cytotoxic test results show that the three nanoemulgel formulations can be developed as sunscreen. Also, the results showed positive control of Parasol®33 had an IC50 value of 5,818.0 µg / mL close to the IC50 value of the optimum formula 3 (Table 1).

Figure 1 shows the morphology of Vero cells which was observed 24 hours shortly before MTT staining showed that live cells were glowing and transparent. An ingredient can be categorized as toxic in-vitro if the IC50 value is in the range 0-100 µg/mL and is very toxic if the IC50 value is in the range <5µg/mL (Figure 1). In-vitro cytotoxicity research is the basis for continuing the safety of an ingredient used as a sunscreen by continuing in-vivo toxicity testing both acute, subacute and chronic as well as examining the organs involved in it. In-vitro cytotoxicity research has an advantage over other biocompatibility tests because it requires a relatively short time, can be standardized, and can be controlled. However, this study has a limitation that is not able to know the complex interactions in causing biological responses.

Protection Activity of Optimum Formula 1 Nanoemulgel Sunscreen In-Vivo

In Table 2, it appears that the change in skin colour to erythema in the negative control group has begun at a dose of 40 mJ/cm^2. Whereas in the group given formula 1 the optimum nanoemulgel dose of 1 mg/cm^2 erythema colour change occurs at a UVB dose of 1280 mJ/cm^2. This shows that the optimum formula 1 nanoemulgel given to rabbit skin can prevent erythema even at larger UVB doses. The data also showed that rabbit skin in the negative control group experienced a more considerable colour change after UVB exposure than in the group given the optimum formula 1 nanoemulgel (Table 2). This shows an increase in erythema in the negative control group and increases when the UVB dose is increased. The UVB dose needed to cause erythema in the optimum 1 nanoemulgel formula group was higher than that of the Parasol®33 groups, which had a consequence of an in vivo SPF value in that group better (Table 2). The image of rabbit skin erythema observed visually after exposure to UVB rays at various doses of UVB rays can be seen in Figure 2.

SPF values in vivo for optimum formula 1 nanoemulgel can be seen in Figure 3. Based on Figure 3, the optimum formula 1 nanoemulgel has potential protection activity (SPF in vivo) on

Table 1 IC_{50} values of nanoemulgel formulas

Cytotoxic Test Material	IC_{50} value (µg/mL)
Optimal formula 1 sunscreen	2,940.569
Optimal formula 2 sunscreen	13,489.728
Optimal formula 3 sunscreen	6,289.248
Parasol	5,818.000
Carbopol gel	595.138

Table 2 Minimal Erythema Dosage and SPF Value of Formula 1 Optimum Nanoemulgel *in vivo*

		Radiation energy(mJ/cm^2) Minimal erythema dose (simulation dosage according to orientation results)										
Sample	**Rabbit**	**40**	**80**	**160**	**320**	**640**	**1280**	**1400**	**1600**	**1800**	**2000**	**SPF**
F1 Optimal dose 1 mg/cm^2	*K1*	0	0	0	0	0	1	2	2	2	2	32
	K2	0	0	0	0	0	0	1	2	2	2	35
	K3	0	0	0	0	0	0	1	1	2	2	35
	K4	0	0	0	0	0	0	1	1	2	2	35
	K5	0	0	0	0	0	0	1	2	2	2	35
F1 Optimal dose 2 mg/cm^2	*K1*	0	0	0	0	0	0	1	1	1	1	35
	K2	0	0	0	0	0	0	1	1	2	2	35
	K3	0	0	0	0	0	0	0	1	1	1	40
	K4	0	0	0	0	0	0	0	1	1	1	40
	K5	0	0	0	0	0	0	1	1	2	2	35
F1 Optimal dose 4 mg/cm^2	*K1*	0	0	0	0	0	0	1	1	1	1	40
	K2	0	0	0	0	0	0	1	1	1	1	40
	K3	0	0	0	0	0	0	1	1	1	1	40
	K4	0	0	0	0	0	0	0	0	1	1	45
	K5	0	0	0	0	0	0	0	0	0	1	50
Parasol dose 2 mg/cm^2	*K1*	0	0	0	0	0	0	1	2	2	2	35
	K2	0	0	0	0	0	1	1	2	2	2	32
	K3	0	0	0	0	0	0	1	1	1	1	35
	K4	0	0	0	0	0	1	1	1	1	1	32
	K5	0	0	0	0	0	0	1	1	1	1	35
Negative control without nano-emulgel	*K1*	1	1	1	2	2	2	2	3	4	4	-
	K2	1	1	1	2	2	2	2	3	4	4	-
	K3	1	1	1	2	2	3	3	3	4	4	-
	K4	1	1	1	2	2	3	3	4	4	4	-
	K5	1	1	1	2	2	3	3	4	4	4	-

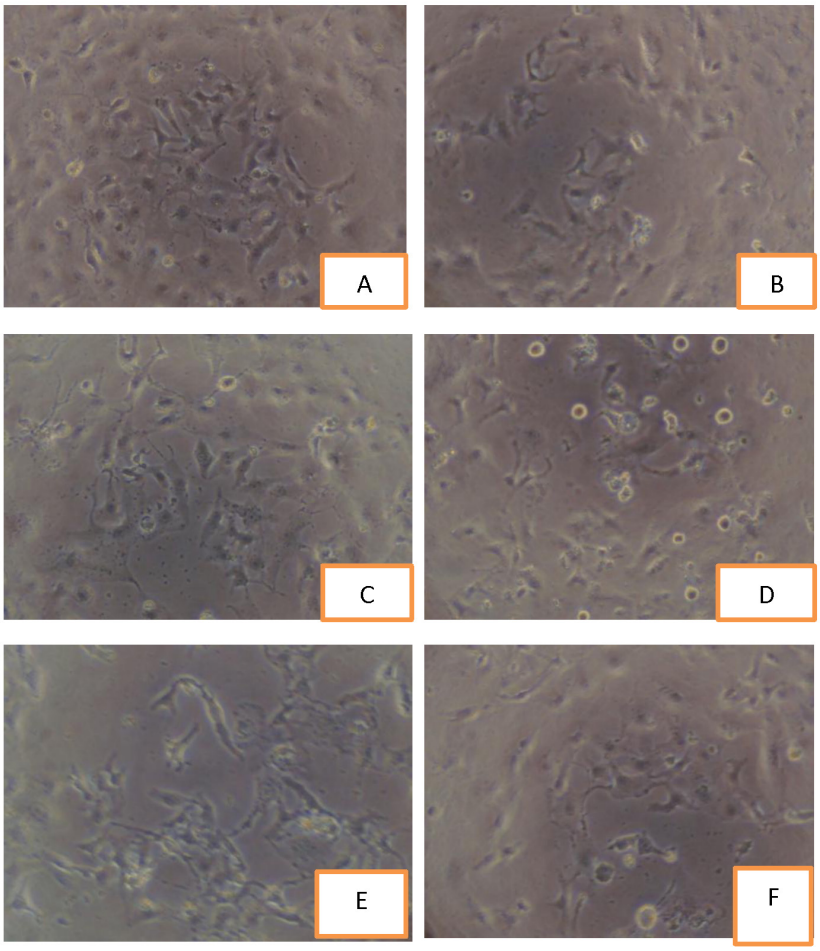

Figure 1 Morphology of vero cells observed 24 hours shortly before MTT staining. Inverted microscope 10X magnification. (A) Negative control. Living cells appear bright and transparent; (B) Vero cells were given the optimum formula 1 nanoemulgel. The living cells appear bright and transparent. (C) Vero cells were given the optimum formula 2 nanoemulgel. Live cells appear glowing and transparent with reduced density compared to negative controls; (D) Vero cells were given the optimum formula 3 nanoemulgel. Live cells appear to be glowing and transparent and appear to be some cells contracting; (E) Vero cells given positive control of Parasol® 33. Appear living cells glow and are transparent and appear to be some cells contracting; and (F) Vero cells were given positive control of 1% carbopol gel. Live cells appear to be glowing and transparent and appear to be some cells contracting

rabbit skin. The SPF value of optimum formula 1 nanoemulgel at doses 1, 2, and 4 mg/cm^2 had a flat average SPF of 34.40 ± 1.34; 36.00 ± 2.23 and 43.00 ± 4.47. Parasol® SPF 33 dose 2 mg/cm^2 shows SPF in vivo 33.8 ± 1.64. It appears that the optimum formula 1 nanoemulgel at 4 mg/cm^2 dose has the best in vivo SPF (Figure 3).

The test table of homogeneity of variances shows the variants of the four groups are the same so that the ANOVA test is valid for this relationship. ANOVA test showed a significant difference (p (0.00) <0.05) in the experimental group between the optimum formula 1 nanoemulgel group and Parasol dose 2 mg/cm^2. The results of the post hoc test with Tukey HSD showed that groups that showed a significant difference in the SPF values were seen in the optimum formula 1 nanoemulgel group at a dose of 2 mg/cm^2, at a dose of 1 mg /cm^2 and a dose of 4 mg/cm^2, a dose of 4 mg/cm^2 was significantly different from all groups. The Parasol group 2 mg/cm^2 were significantly different with the optimum formula 1 nanoemulgel dose 4 mg/cm^2.

DISCUSSION

The development of a sunscreen material (product) requires preclinical evaluation to determine the toxic potential of a substance in normal tissue. The acute toxicity test conducted in this study is acute toxicity *in-vitro*. A cytotoxicity test was carried out to determine the toxic effects of the three nanoemulgel formulations on normal tissue on cell culture (Vero cells). This cytotoxicity test with MTT is based on the ability to live cells to reduce salts of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT assay) which are yellow and dissolve to formazan deposits which are blue and insoluble. This method has the advantage that it does not require cell transfer, semi-automatic, simple and fast to assess the number of cells. The reduction of tetrazolium salts occurs intracellularly and involves enzymes from the endoplasmic and mitochondrial reticulum. The number of living cells can be measured formazan products dissolved in the solvent, and the colour intensity was measured with a wavelength spectrophotometer 595 nm. The more concentrated the colour, the higher the absorbance value, which indicates more living cells. The IC50 value of nanoemulgel formula shows that nanoemulgel formula 2 has the highest IC50 value compared to formula 1, formula 3, Parasol. Carbopol as a gel base also has a high IC50 value. Based on research conducted by Caamal-Fuentes et al., a material is classified as cytotoxic if the IC50 value is <5 µg/mL (very strong), 5-10 µg/mL (strong), 10-20 µg/mL (moderate), 20-100 µg/mL (weak), > 100 µg/mL inactive.[18] The results showed a non-toxic number of all substances tested. The material was strongly toxic if the IC50 value was below 10 µM.[19]

The activity of sunscreen preparations is evaluated through the protection ability of nanoemulgel formulations against sunlight, especially ultraviolet B. The protection ability of the nanoemulgel formulation was assessed based on the SPF value. SPF value is obtained by looking at the erythema

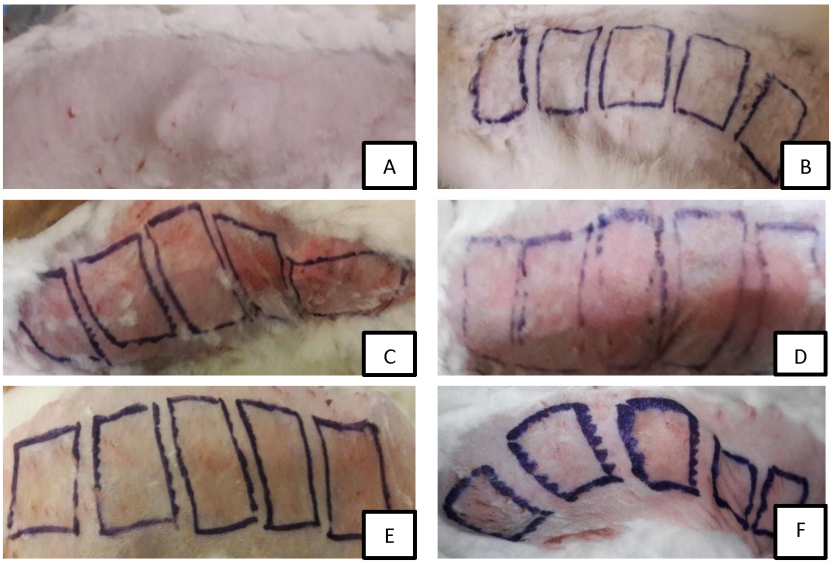

Figure 2 Image of rabbit skin erythema observed visually after UVB exposure. A) rabbit skin that is ready to be treated B) rabbit skin that has not been shined C) negative control rabbit skin that has been exposed to UVB D) negative control rabbit skin that has been irradiated with UVB, it appears that the expansion of erythema E) rabbit skin given Parasol 2 mg /cm^2 is exposed to UVB starting dose 1280 mJ /cm^2, F) rabbit skin given nanoemulgel formula 1 optimum 2 mg/ cm^2 is exposed to UVB starting at 1280 mJ/cm^2

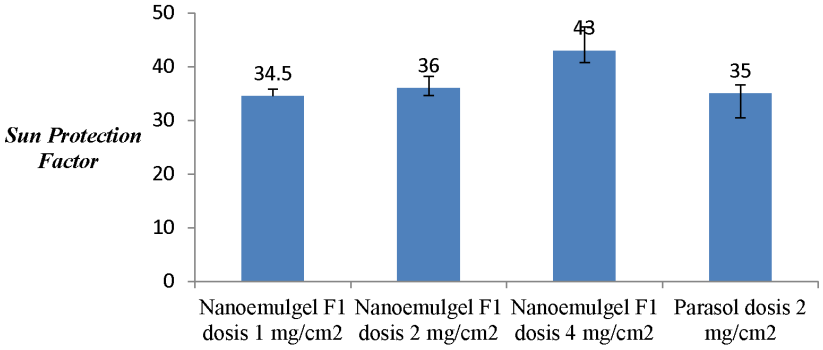

Figure 3 SPF Value in vivo of optimum formula 1 nanoemulgel. Tests were carried out in groups given nanoemulgel formula 1 with varying doses of 1, 2,and 4 mg/cm^2, and positive control of Parasol® 33 was observed after 24 hours

response observed 24 hours after the radiation is done. The negative control group showed an erythema response occurring at a UVB dose of 40 mJ/cm^2. The nanoemulgel formula one group with a dose of 1 mg/cm^2 showed erythema which occurred at a dose of 1280 mJ/cm^2. Something similar is shown in the formula one dose of 2 mg/cm^2 and 4 mg/cm^2. Formula one nanoemulgel has a very protective effect against UVB light exposure, which is evident from the difference in radiation dose that causes erythema between sun-protected and non-sun-protected skin. This study proves that excessive UVB (290-300) radiation can cause skin damage.[1,4] Skin damage that occurs is erythema shown in this study. Sunscreen proves it can minimize the damage that occurs where erythema is seen at higher radiation doses. Sunscreen can minimize UVB exposure by absorbing, reflecting or spreading light.[20] Erythema that occurs on rabbit skin is caused by radiation rays that penetrate the epidermis, which can potentially cause ROS. ROS are formed due to lipid peroxidation in the skin cell membrane. Lipid peroxidation is one generation of superoxide anion. ROS will cause changes in intracellular signals that can activate nuclear factor kappa B (NF-kB), which causes the production of inflammatory mediators.[21,22] Nanoemulgel sunscreen is a combination of organic sunscreen containing cinnamic group and quercetin. Action activity is predicted that organic sunscreens can transfer their energy into dynamic electronics. Solar energy can be eliminated through internal conversion through molecular vibrations through collisions with surrounding molecules.[14] The highest SPF value was obtained in nanoemulgel formula with a dose of 4 mg/cm^2. It is estimated that the dose given is quite high, and the content of sunscreens containing cinnamic groups with calyx [4] structures resorcinarene and quercetin. C-phenylcalix [4] resorcinaryl octacinamate has a functional double bond conjugated with the carbonyl group (C-O) and the aromatic group. Calyx [4] resorcinarene is stable at high temperatures, so it will not be damaged if exposed to UVB light. Cinnamate molecules can absorb sunlight by binding to calyx [4] resorcinarene, which will make sunscreen have good characteristics. The combination of cinnamate with calyx [4] resorcinarene makes the combination of sunscreen as an absorbent of sunlight with high stability.[14,23]

CONCLUSION

The development of a sunscreen material requires preclinical evaluation to determine the toxic potential of a substance in normal tissue. The acute toxicity test conducted in this study is acute vitro toxicity with MTT, which is based on the ability to live cells to reduce salt 3- (4,5-dimethylthiazol-2yl) -2,5-diphenyl-tetrazolium bromide (MTT assay). All three formulas had IC50 values high enough, and the results of the study showed non-toxic numbers on all the ingredients tested. The ability to protect against sunlight in the first formula, which is composed of C-phenylcalix [4] resorcinaryl octacinnamate and quercetin has a high enough value. Nanoemulgel product can improve the characteristics of the three sunscreen ingredients. This research shows that the nanoemulgel product

produced high SPF value and is not toxic in vitro. It is hoped that it can be developed on a large scale industry and commercialized as an Indonesian sunscreen product.

CONFLICT OF INTEREST

There is no competing interest regarding manuscript.

ETHICAL CONSIDERATION

This study obtained the ethical clearance from the Ethical Commission in Research of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia with approval number: KE/FK/513/EC/2016.

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AUTHOR'S CONTRIBUTION

Data gathering and idea owner of this study was conducted by Agung Wiwiek Indrayani, Martodihardjo Suwaldi, Radiono Sunardi, Jumina, I Gusti M Ngurah Budiana, Mustofa. Writing and submitting manuscript was carried out by Agung Wiwiek Indrayani, I Gusti Ayu Artini, Martodihardjo Suwaldi, Radiono Sunardi, Jumina, I Gusti M Ngurah Budiana, Dewa Ayu Arimurni, Made Dwi Pradipta Wahyudi, Mustofa. In addition, editing and final draft approval was conducted by Martodihardjo Suwaldi, Radiono Sunardi, Jumina, Mustofa, Lutfi Chabib, IGA Artini, Ni Wayan Sucindra.

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