



Research Article

Protective Effect of Merbau (*Intsia bijuga*) Extract on Hydrogen Peroxide-Treated HaCaT Human Keratinocytes and Its Formulation as Antioxidant Cream

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Abstract

Background: The excessive generation of ROS in the skin results in oxidative stress that can contribute to premature skin aging, inflammation, and skin carcinogenesis. To prevent these detrimental effects, the development of herbal medicine with a potent antioxidant activity into cosmetic products is required. This study aims to formulate cream that contains a safe and effective concentration of merbau (*Intsia bijuga*), which has been shown to have a strong antioxidant activity.

Methods: Powdered merbau wood was macerated with methanol and the dried extract was evaluated for its cytotoxic effect and antioxidant activity on human keratinocytes cell line using MTS assay. Five cream formulations containing the extract were made and subjected to stability and physical evaluations, including organoleptic, types of cream, pH, viscosity, and homogeneity.

Results: Cytotoxicity assay revealed that merbau extracts had an IC_{50} of 181.3 $\mu\text{g/mL}$ (95% confidence interval (CI): 165.4 - 200.1 $\mu\text{g/mL}$). At a concentration of 31.25 $\mu\text{g/mL}$, the extract exhibited a protective effect against H_2O_2 -induced oxidative stress, comparable to vitamin E. Five cream formulas that were developed demonstrated good physical properties that fulfilled the evaluation parameters, including o/w type of cream, homogenous, and stable based on centrifugation and freeze-thaw cycle tests. The pH values were between 5.65 ± 0.067 - 7.4 ± 0.050 , while the viscosity values were between 131 ± 1.249 - $56,011 \pm 2,729.27$ mPa.s. All cream formulas exhibited shear-thinning properties upon increasing shear stress.

Conclusion: Overall, this study has successfully formulated several cream formulations containing merbau extract at a concentration that shows antioxidant activity.

Introduction

Skin aging is a natural process that occurs in the human body. The process of aging involves intrinsic and extrinsic factors, such as physiological and environmental factors, respectively. The excess free radicals and reactive oxygen species (ROS) from environmental sources, such as ultraviolet (UV) radiation and cigarette smoke, increase oxidative stress. ROS formation is the main cause of skin aging which is characterized by increased wrinkling, sagging, laxity, skin dryness, and pigmentation of the skin.^{1,2}

Oxidative stress produced by ROS also contributes to the development of various diseases and skin disorders.^{3,4} ROS triggers melanogenesis in the skin, and it is known that melanocytes undergoing melanogenesis have a much lower capacity to repair DNA against oxidative damage, leading to skin carcinogenesis. ROS also triggers downstream

signaling cascades that lead to changes in cytokines release and thus further exacerbates skin inflammation disorders. Although skin has its own mechanism to protect against oxidative stress, the body's protection mechanism cannot counteract the damage caused by excess ROS formation.^{1,3,5} Thus, there is a need to develop cosmetic products with antioxidant activity to alleviate oxidative damage.

One of the plants that has the potential to be developed into a cosmetic product with antioxidant activity is merbau (*Intsia bijuga*). Merbau grows in coastal and mangrove areas of South East Asia and it is considered as one of the highly valued timbers due to its durability and resistance to decomposition and termites.^{6,7} Traditionally, its bark and leaves are used to treat dysentery, rheumatism, and urinary tract infection.⁶ Screening results of 35 Indonesian medicinal plants showed that merbau wood methanolic extract had an excellent antioxidant activity with an IC_{50}

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of 6.6 ± 0.13 $\mu\text{g/mL}$ on 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.⁸

To address the need of developing cosmetic products with antioxidant activity to prevent the damaging effects of ROS, merbau wood was extracted and used as an active ingredient in a cream. In this study, the cytotoxicity and antioxidant activity of merbau methanolic extract were characterized on human keratinocytes (HaCaT) cell line and the cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. An effective and non-toxic concentration of merbau was determined and used in the cream formulation. Five cream formulations were prepared and subjected to physical and stability evaluations, including organoleptic, types of cream, homogeneity, pH, viscosity, centrifugation, and freeze-thaw (FT) cycle test. Finally, this study successfully developed stable cream formulations containing potent antioxidant yet non-toxic merbau extract.

Materials and Methods

Materials

Merbau wood was purchased from Astro Concept and botanical determination was done in Institut Pertanian Bogor, Indonesia. Methanol used for Merbau powder extraction was technical grade. Methylparaben, propylparaben, glycerin, and Tween 80 were obtained from Solvay Specialty Chemicals Asia Pacific Pte Ltd, Singapore, while Span 80 was obtained from Kolb Distribution Ltd, Switzerland. Sandalwood essential oil and olive oil were obtained from Darjeeling Perfumery and Oils, Italy. Mineral oil, cetyl alcohol, triethanolamine (TEA), and stearic acid were purchased from Xi'an Virgin Biological Technology Co., Ltd, China. H_2O_2 and dimethyl sulfoxide (DMSO) were purchased from Merck, Germany. HaCaT cells were generously provided by Dr. Ng Kee Woei (Nanyang Technological University, Singapore). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and trypan blue were purchased from Thermo Fisher Scientific, USA. Penicillin-streptomycin and vitamin E were purchased from Sigma-Aldrich, USA while MTS reagent was purchased from Promega, USA.

Merbau extraction

300 g of Merbau wood powder was immersed in 1.2 L of methanol for 24 hours. The extract was filtered and collected. The maceration process was repeated 3 times. The collected filtrate was concentrated using a rotary evaporator (R-100, Buchi) and kept in a desiccator until further use.

Phytochemical screening

The dried methanolic merbau extract was screened for the presence of alkaloids, flavonoids, saponins, quinones, tannins, and steroids/triterpenoids using standard phytochemical screening methods as previously described.^{9,10}

Cell culture

HaCaT cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin antibiotics. The cells were maintained in an incubator with a temperature of 37°C and 5% CO_2 .

Cytotoxicity test

Cytotoxic effect of merbau extract was tested on the HaCaT cell line. HaCaT cells were seeded on a 96-well plate at a cell density of 1×10^4 cells/well and grown in 150 μL DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The plate was then incubated overnight at 37°C with 5% CO_2 . Merbau stock was prepared in DMSO and further diluted with the media to yield a concentration of 750, 500, 250, 125, 62.5, 31.25, 15.625, and 7.826 $\mu\text{g/mL}$ where the highest concentration of DMSO in the dilution was 0.75%. Three controls were used in the experiment: (1) media only was used as a blank, (2) cells maintained in the media as a control, and (3) cells incubated in 50% DMSO in the media as a positive control.¹¹ The seeded cells were treated with various concentrations of merbau extract and incubated for 24 hours.

The cells were then washed and 100 μL of media was added, followed by the addition of 20 μL MTS reagent solution into each well. After 3-3.5 hours of incubation, the absorbance was measured using a plate reader (Nanoquant Plate™ Infinite® M200, Tecan) at 490 nm as described in previous study.¹² Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{E - B}{C - B} \times 100\% \quad \text{Eq. (1)}$$

Where E is the absorbance of extract, C is the absorbance of the control, and B is the absorbance of the blank. IC_{50} represented the concentration of a drug required to reduce cell viability to 50% and was determined by plotting data points over a range of concentration. The IC_{50} was calculated on Prism version 8.3.0 (GraphPad Software, U.S.A) by using non-linear regression analysis.

Antioxidant test

Antioxidant activity of merbau extract was determined against oxidative stress induced by H_2O_2 . HaCaT cells were seeded on a 96-well plate at a density of 1×10^4 cells/well and incubated overnight. Subsequently, seeded HaCaT cells were incubated with (a) different concentrations of H_2O_2 and (b) a fixed concentration of H_2O_2 and different concentrations of merbau extract for 24 hours.¹³ Untreated cells were used as the control and cells treated with 0.25 mg/mL of vitamin E was used as the positive control. Cell viability was then determined by MTS assay as stated above.

Merbau cream preparation

Five formulations (F1-5) of merbau cream were prepared according to Table 1. Briefly, the creams were made by

preparing both the oil and water phases. The oil phases were prepared by mixing olive oil, mineral oil, Span 80, cetyl alcohol, stearic acid, and tocopherol acetate as stated in the formulas. Whereas, the water phases were prepared by mixing propylparaben, methylparaben, Tween 80, merbau extracts, glycerin, and TEA. The optimal proportion of Span and Tween 80 was determined by calculating the required Hydrophile-Lipophile Balance (HLB). This was done by adding the required HLB of oil and fat components as stated in the literature proportional to the total number of each component used in the formulation.¹⁴

Both of the phases were heated until they reached temperature of 70°C and the oil phase was slowly added to the water phase while it was being mixed using a homogenizer on top of a hot plate.¹⁵ Homogenizer was set at 10 rpm and every 2 minutes the rpm was increased by 10 rpm until it reached 40 rpm. After removing the mixture from the hot plate, sandalwood oil was added and the mixture was mixed homogeneously.

Merbau cream evaluation

Organoleptic characteristics

All formulations were evaluated for their physical appearances, including color, phase separation, and odor.¹⁶

Types of cream

The types of cream were determined using red sudan and washability test.¹⁷ Briefly, a small amount of red sudan was added into the cream and stirred. The cream was considered as an oil in water (o/w) type of cream if the red sudan formed red spots on the cream base instead of coloring the whole cream base. In the washability test, a sufficient amount of the cream was spread on the dorsal

side of the palm and the cream was rinsed with water to evaluate the ease of cream removal.

Determination of pH

1 g of each formulation was dispersed in 10 mL of deionized water and the pH was measured using a digital pH meter (Pb-11, Sartorius).¹⁶

Centrifugation test

A sufficient amount of cream was transferred into microcentrifuge tubes and subjected to centrifugation at 5,000 rpm for 10 minutes (Eppendorf Centrifuge 5424).¹¹ Any phase separation was observed for instability.

Homogeneity testing

About 0.25 g of each of the cream samples were taken from the top, middle, and bottom part of the container using a pipette. Four samples were taken from each part. The samples were placed on glass microscope slides and the cover glasses were gently pressed on top of the samples. Any appearances of oil globules or coarse particles were observed.¹⁶

Determination of viscosity

B-one Touch Lamy Rheology viscometer was used to determine the viscosity and rheology of each formulation.¹⁶ The F2-F5 formulas were measured using L-4 spindle while the F1 formula was measured using L-3 spindle. The spindle was rotated at 20, 40, 60, 80, and 100 rpm in an ascending and descending manner where each speed was maintained for 60 seconds. All measurements were done in triplicate.

Table 1. Five formulas of cream containing merbau wood methanolic extract.

Components	Amount (% w/w)				
	F1	F2	F3	F4	F5
Merbau wood methanolic extract	31.250 µg/mL	31.250 µg/mL	31.250 µg/mL	31.250 µg/mL	31.250 µg/mL
Olive oil	10	10	10	10	-
Mineral oil	-	-	-	-	10
Tween 80	5.562	6.674	6.674	9.072	7.15
Span 80	4.438	5.326	5.326	5.928	2.85
Cetyl alcohol	8	8	8	8	8
Stearic acid	-	-	3	-	-
TEA (Triethanolamine)	-	-	1	-	-
Methylparaben	0.18	0.18	0.18	0.18	0.18
Propylparaben	0.02	0.02	0.02	0.02	0.02
Glycerin	10	10	10	10	10
Vitamin E	0.05	0.05	0.05	0.05	0.05
Sandalwood oil	125 µL	125 µL	125 µL	125 µL	125 µL
Deionized water	up to 100	up to 100	up to 100	up to 100	up to 100

Stability test

The cream stability was tested in 2 cycles of FT.¹¹ Briefly, a cycle was completed when the creams were stored at 4°C for 24 hours, followed by subsequent storing at 40°C for another 24 hours. After 2 cycles of FT, the cream formulas were re-evaluated based on the parameters mentioned above.

Data analysis

Data analysis was performed using GraphPad Prism version 8.3.0 (GraphPad Software, USA). Statistical analysis was performed using one-way ANOVA, followed by Tukey's test as a *post-hoc* test. Data were obtained from at least three independent measurements and were presented as the mean \pm standard mean error (SEM). The viscosity of creams was evaluated using two-way ANOVA with Bonferroni's *post-hoc* test on Prism version 8.0.1.

Results and Discussion

Merbau extraction and phytochemical screening

Merbau wood extraction using methanol resulted in a yield of 15.01% dried extract against the merbau powder. Phytochemical screening showed that the merbau extract had flavonoids, saponins, tannins, and steroids/triterpenoids. The phytochemical contents of merbau wood have not been reported previously. Previous studies reported the phytochemical contents of other parts of merbau.^{6,18} One study had successfully isolated flavonoids from the bark of merbau, namely romadendrin, luteolin, and apigenin.¹⁸ Meanwhile phytochemical screenings of its leaves detected the presence of anthrones, flavonoids, glycosidic flavonoids, phenolic compounds, tannins, and steroids/triterpenoids.⁶

Cytotoxicity of merbau extract

To assess the safety of merbau extract, the cytotoxicity of the extract was determined on the HaCaT cell line. The extract significantly reduced HaCaT cell viability starting from a concentration of 125 $\mu\text{g/mL}$ (Figure 1, $p < 0.005$). At concentrations higher than 125 $\mu\text{g/mL}$, the extract caused a more profound reduction in cell viability (Figure 1, $p < 0.0001$). The IC_{50} of merbau extract was 181.3 $\mu\text{g/mL}$ (95% CI: 165.4-200.1 $\mu\text{g/mL}$). Concentrations of the extract lower than 125 $\mu\text{g/mL}$ were further used on the antioxidant assay against H_2O_2 -induced oxidative damage as these concentrations did not reduce cell viability of HaCaT.

Antioxidant activity of merbau

H_2O_2 was used to induce cell injury through the generation of ROS, including free radical superoxide anions ($\text{O}_2^{\cdot-}$) and hydroxyl radicals (OH^{\cdot}).¹⁹ The accumulation of ROS alters the dynamic equilibrium between free radical production and cellular antioxidant defense systems, leading to lipid peroxidation, disruption of nucleic acid structure and function, activation of proapoptotic gene expressions, and cellular damage.^{20,21} This method has been widely used to evaluate antioxidant activities and has advantages

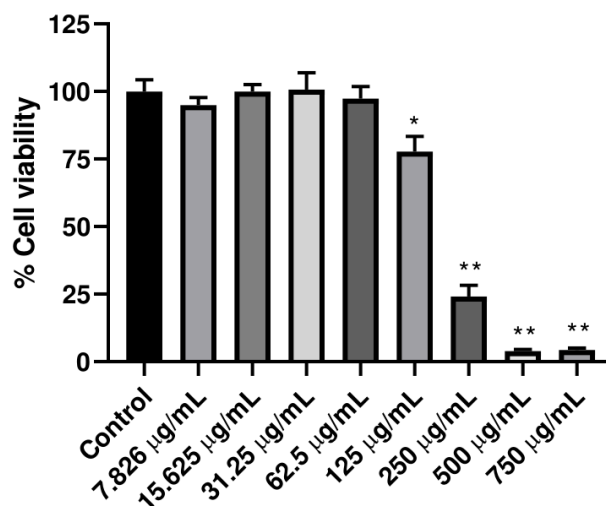


Figure 1. The effect of merbau wood methanolic extract on HaCaT cells viability. Data was expressed as mean \pm SEM ($n = 5 - 9$). * $p < 0.005$ compared to control group and ** $p < 0.0001$ compared to control group.

over the other *in vitro* assays, including DPPH, ferric reducing antioxidant power (FRAP), and trolox equivalent antioxidant capacity (TEAC).^{2,22-23} Keratinocytes are the major cells that make up the skin epidermis, which have a similar topology as the outer layer of the skin. Therefore, the antioxidant assay on HaCaT cells is more representative than the other *in vitro* methods.²⁴

To optimize the concentration of H_2O_2 that evokes oxidative damage, increasing concentrations of H_2O_2 were applied to HaCaT cells. H_2O_2 decreased HaCaT cells viability in a dose-dependent manner. Significant reduction of cell viability was observed upon exposure of 500 and 1,000 μM of H_2O_2 compared to control group (Figure 2A, $p < 0.005$ and $p < 0.0001$, respectively). 500 μM of H_2O_2 was further selected for the antioxidant assay as this concentration already produced significant damage, but not too excessive so that the cells could still recover from the damage.

The protective effect of merbau extract against H_2O_2 -induced oxidative damage in HaCaT cells was evaluated at concentrations that did not show any cytotoxic effect (Figure 1). Co-treatment of merbau extract increased cell viability in a dose-dependent manner compared to the H_2O_2 -treated group (Figure 2B). Furthermore, cells co-treated with 31.25 $\mu\text{g/mL}$ of merbau extracts resulted in a significant increase of cell viability ($p < 0.005$) compared to cells treated with H_2O_2 only, which was comparable to the protective effect of 0.25 mg/mL vitamin E (Figure 2B). High antioxidant activity of merbau wood methanolic extract also has been demonstrated by Batubara *et al.*⁸ where it was shown that the merbau extract had an IC_{50} of 6.6 ± 0.13 $\mu\text{g/mL}$ in DPPH assay. The antioxidant activity of merbau extracts might arise from several bioactive components such as flavonoids and tannins. These compounds have been extensively studied for their antioxidant properties.²⁵⁻²⁷ The potent antioxidant property of merbau may indicate the potential of merbau as a source

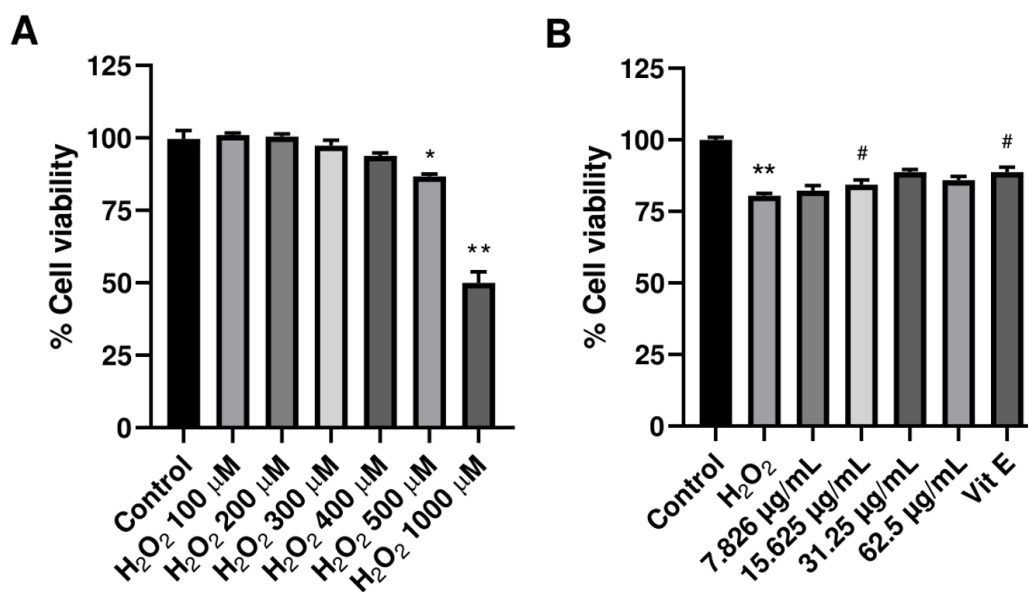


Figure 2. Protective effects of merbau wood methanolic extract against H₂O₂-induced oxidative stress on HaCaT cells. (A) Optimization of H₂O₂ concentration. (B) Antioxidant assay of various concentrations of merbau extract in HaCaT cells treated with 500 µM H₂O₂. Data was expressed as mean \pm SEM (n = 5 - 6). * p < 0.005 compared to control group, ** p < 0.0001 compared to control group, and # p < 0.05 compared to H₂O₂-treated group.

of novel antioxidant compounds, however, further studies are needed to determine which compounds are responsible for the antioxidant activity of merbau.

Merbau cream evaluation

Based on the antioxidant testing result, 5 different cream formulas of merbau extract were developed. The concentration of merbau extract used in the final formulation was kept at 31.25 µg/mL (Table 1), as this concentration did not result in any damage to the cells, yet it had a potent antioxidant activity (Figure 2B). Calculation was done to determine the required HLB where the HLB for F1-5 were found to be 10.25, 10.25, 10.95, 10.8, and 11.95, respectively.

The prepared cream formulations were then subjected to several evaluations before undergoing stability testing. Table 2 showed the result of physical properties of F1-5 before and after FT cycles. Before FT cycles, all formulas showed white-yellowish color, with a smell of sandalwood oil. After FT cycles, there was a slight change in the color of F2, F3, and F4 where they turned yellowish. Nevertheless, all formulas maintained the sandalwood scent after FT cycles.

All formulas were confirmed to be o/w type based on the red sudan testing, which showed red spots on the cream base (Table 2). After FT cycles, all formulas constantly showed o/w type of cream. These results were also supported by the data obtained from the washability test. All the formulas were easily washed by water without leaving any oil residue, indicating easy removal of the creams.

It was confirmed that all formulas were homogenous before and after FT cycles (Table 2), although there were slightly larger size globules observed in F1 compared to the others (data is not presented). There were no visible coarse

particles found in any of the cream samples from the top, middle, and bottom part of the container, showing that all excipients and merbau extract were evenly dispersed or dissolved.

The pH values of merbau creams were found to be in the range of 5.65 ± 0.04 - 7.4 ± 0.03 (Table 2). This variation was a result of different compositions of the cream formulas. Lower pH of F1 was attributed to a lower proportion of Tween 80 and Span 80, while higher pH of F3 was due to the presence of TEA, which was basic (Table 1). Generally, it was recommended to formulate a cream with pH around 4 to 6 based on the normal pH range of skin.²⁷ In accordance, only F1 met the pH requirement, while the others showed higher pH than the desired pH range. Moreover, after FT cycles, the pH values were slightly increased. Nevertheless, Gupta *et al.* (2013) reported that formulated cream with pH ranged from 6.5 - 7 did not cause sensitivity reaction nor irritation on the skin.²⁸ Ajala *et al.* stated that stratum corneum tolerates pH of 3 to 9.²⁹ Therefore, the pH of formulated merbau creams in this study were still considerably accepted.

The viscosity of each cream formula was measured before and after FT cycles to determine their viscosity profile and to investigate whether the freezing and thawing process significantly affect the viscosity. Viscosity is one of the important properties of a cream, as it influences the stability by acting as a mechanical barrier for coalescence.¹⁴ The graphs shown in Figure 3 suggested that all cream formulas exhibited shear-thinning properties, in which an increased in the shear strength resulted in a decreased viscosity.²⁹ The viscosity before FT cycles was found to be in the range of 131 ± 1.249 - $56,011 \pm 2,729.27$ mPa.s. Meanwhile, after FT cycles, the viscosity values were shifted to the range of $6,881 \pm 182.78$ - $64,424 \pm 3,504.85$ mPa.s. Apparently,

Table 2. Physical properties of merbau creams.

Parameter	Formula 1		Formula 2		Formula 3		Formula 4		Formula 5	
	Before	After	Before	After	Before	After	Before	After	Before	After
Organoleptic	White- yellowish, sandalwood scent	White- yellowish, sandalwood scent	White- yellowish, sandalwood scent	Yellowish, sandalwood scent	White- yellowish, sandalwood scent	Yellowish, sandalwood scent	White- yellowish, sandalwood scent	Yellowish, sandalwood scent	White- yellowish, sandalwood scent	White- yellowish, sandalwood scent
Type of cream	oil in water	oil in water	oil in water	oil in water	oil in water	oil in water	oil in water	oil in water	oil in water	oil in water
pH^a	5.65 ± 0.04	5.87 ± 0.02	6.44 ± 0.03	6.58 ± 0.03	7.4 ± 0.03	7.57 ± 0.03	7.12 ± 0.02	7.42 ± 0.04	6.44 ± 0.02	6.98 ± 0.02
Homogeneity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Centrifugation testing	Separated	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable

^a Data was expressed as mean ± SEM (n = 3).

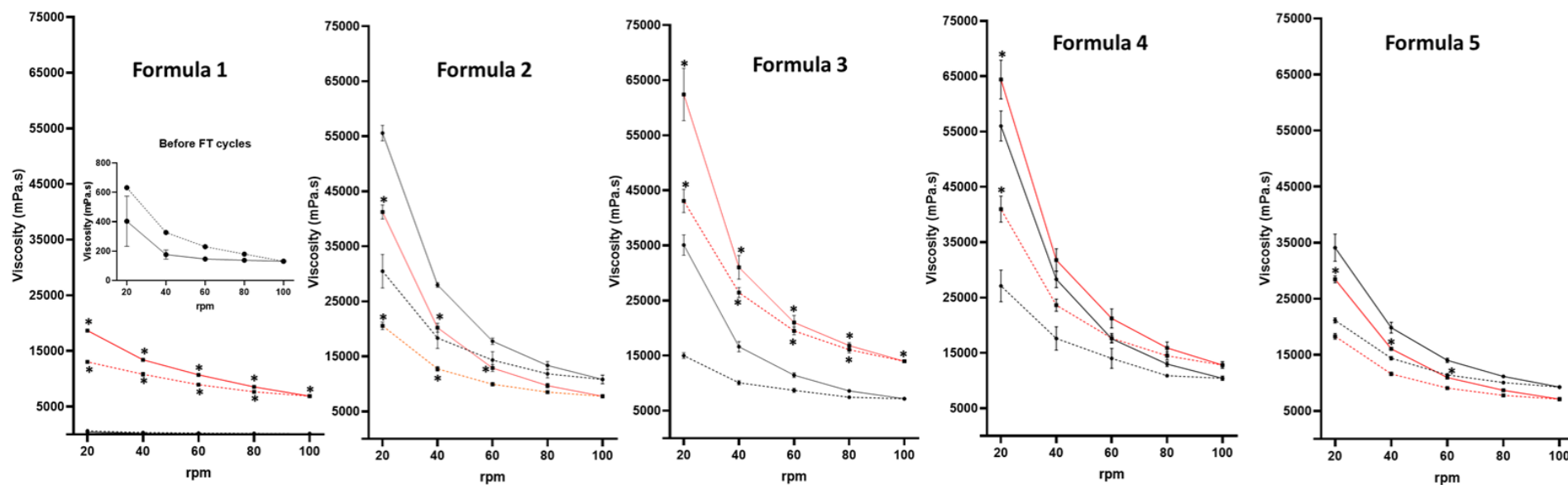


Figure 3. Viscosity of Merbau cream F1-5 before and after FT cycles. Insertion: viscosity of F1 before FT cycles. Data was expressed as the mean ± SEM (n = 3). Round marker, black line: before FT cycles; Square marker, red line: after FT cycles; Solid line: ascending shear rate values; Dash line: descending shear rate values. *p < 0.05 compared to before FT cycles.

there is no standard for viscosity value of cream that is internationally recognized. Nevertheless, the viscosity evaluation of standard topical cream was reported to be around 27,000 mPa.s in 5 rpm to 8,000 mPa.s in 200 rpm.³⁰ Based on this standard, F5 exhibited similar viscosity values with the standard reference.

It was also found that the viscosity values were significantly different before and after the FT cycles ($p < 0.05$). However, there was no clear trend whether the viscosity values were increased or decreased after FT cycles, as they were increased in F1, F3, and F4, and were decreased in F2 and F5. The exact reason is yet to be elucidated, but most probably attributed by the interaction between the cream bases themselves. There was also hysteresis observed in the viscosity measurement upon ascending and descending shear rates, which was reflected the thixotropic behavior of shear-thinning creams.³¹

Lastly, centrifugation test was done to evaluate the physical stability of creams before and after FT cycles. Before FT cycles, almost all creams were not separated by centrifugation (Table 2). Exception was observed for F1 where there was a separation into two distinct phases before FT cycles. This instability was aligned with very low viscosity of F1 before FT cycles, which in turn decreased the ability of F1 to retard the movement of oil phase. Surprisingly, after FT cycles, there was no separation found in any formulas. This data indicated that all formulas were able to resist separation caused by centrifugation.

Conclusion

Our results demonstrated that methanolic extract from merbau wood at 31.25 µg/mL exhibited protective effect against H₂O₂-induced oxidative stress on HaCaT cells; an effect that was similar to the protection afforded by 0.25 mg/mL of vitamin E. Merbau wood might offer a potential alternative for a source of novel antioxidant compound. Nevertheless, merbau extract exhibited cytotoxic effect as evident by significant decrease in cell viability at concentrations higher than 125 µg/mL. In this study, merbau extract at concentration of 31.25 µg/mL was successfully formulated into five stable o/w creams. Albeit the pH of the creams was slightly higher than the recommended pH ranges, the pH was still considered as acceptable, with all creams demonstrated shear thinning properties. Accelerated stability testing using FT cycles significantly affected the physical properties of creams, with F5 exhibited the closest viscosity profile to the standard. Nevertheless, all formulas were stable on centrifugation after FT cycles without any phase separation observed. This study concluded that it is possible to develop cream containing merbau methanolic extract which can be used as a potent antioxidant cosmetic. Further studies are suggested to evaluate the effectiveness of antioxidant merbau cream in animal or human volunteers.

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Conflict of Interest

The authors claim that there is no conflict of interest.

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