

# Protective effect of *Caesalpinia sappan* L. extract against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress on HaCaT and its formulation as antioxidant cream

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**ABSTRACT:** Oxidative stress arises from excessive generation of reactive oxygen species and it is known to be the major factor that accelerates skin aging and involved in the development of skin inflammatory disorders. Therefore, there is a need to develop cosmetics products containing active ingredient with potent antioxidant effect. One of medicinal plants that is potential to be utilized as an active ingredient for cosmetics is *Caesalpinia sappan* L. Previous studies demonstrated the potent antioxidant activity of *C. sappan* in numerous *in vitro* scavenging free radicals assay. The present study aims to evaluate the antioxidant of *C. sappan* in a biological system and further formulates the extract in cream formulation. Powdered *C. sappan* was macerated with 96% ethanol and the dried extract was assessed for the cytotoxic and antioxidant effect on human keratinocytes cell line using MTS assay. Several cream formulations containing non-toxic concentration of *C. sappan* extract were made and evaluated. Cytotoxicity assay showed that *C. sappan* extract had an IC<sub>50</sub> of 146.8±2.6 µg/ml. At a concentration of 15.625 µg/ml, the extract exhibited a protective effect against oxidative stress induced by H<sub>2</sub>O<sub>2</sub> that was comparable to vitamin E. All of the formulated creams were o/w cream type, homogenous and had white-pinkish color. pH were in a range of 5.07±0.01 – 6.51±0.04 with shear-thinning properties. Following freeze-thaw cycle, the formulas were found to be stable and no significant changes were observed, except for viscosity. This study demonstrates that several cream formulations of *C. sappan* extract have been successfully formulated with proven antioxidant property.

**KEYWORDS:** *Caesalpinia sappan* L.; antioxidant; cream; cytotoxicity; human keratinocytes cell line.

## 1. INTRODUCTION

Reactive oxygen species (ROS) at low concentrations play an important role in maintaining cellular signaling for normal physiological responses. However, excessive generation of ROS results in oxidative stress that lead to cell damage and has been implicated in the development of various diseases [1]. In the skin, ROS that originate from extrinsic factors, such as UV light, tobacco and air pollution, are the major cause of premature skin aging that is characterized by increased pigmentation, sagging, wrinkling, and skin dryness [2, 3]. The oxidative stress also causes damage to macromolecules, including protein, lipid, and DNA, leading to carcinogenesis. In addition, overproduction of ROS triggers signaling cascades that modify cytokine release, contributing to skin inflammation disorders [2, 4]. The innate antioxidant mechanism in our skin is unable to overcome oxidative stress caused by excessive ROS generation, therefore the development of cosmetic products containing antioxidant is required.

*Caesalpinia sappan* L. is one of medicinal plants that is known to have a strong antioxidant activity [5, 6]. The plant belongs to Leguminosae family and geographically distributed in Africa, America, and Southeast Asia [5]. The pharmacological activities of *C. sappan* have been reported in earlier studies, these include immunomodulation, anti-inflammation, hepatoprotection, hypoglycemic, and antibacterial activity [7]. Antioxidant activity of ethanolic extract of *C. sappan* has been tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and ferric reducing antioxidant power (FRAP) assay with an IC<sub>50</sub> of 101.47, 26.70 and 11.37 ppm, respectively [8]. Further, *C. sappan* extract had an EC<sub>50</sub> of 6.65±0.07 µg/ml on superoxide anion radical scavenging activity assay which was comparable to ascorbic acid and rutin, demonstrating the potent antioxidant activity [5]. Strong

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antioxidant activity of *C. sappan* was also observed on nitric oxide scavenging activity with an  $EC_{50}$  value of  $4.24 \pm 0.14 \mu\text{g/ml}$  [5]. Although the antioxidant activity of *C. sappan* has been tested in numerous scavenging free radicals *in vitro* method, the antioxidant activity tested on biological system is still lacking.

Various flavonoids and phenolic compounds that are known for their antioxidant activity have been successfully isolated from *C. sappan*, making *C. sappan* as an excellent source of antioxidant for cosmetic purposes. These compounds include coumarin, xanthone, gallic acid, chalcones, flavones, homoisoflavonoids, brazilin, and brazilein [5, 9]. Brazilin demonstrated highest ferric reduction activity and DPPH scavenging activity compared to vitamin E and other compounds isolated from *C. sappan*, such as sappanchalcone, brazilein, protosappanin B and C [9].

To evaluate the cytotoxicity and antioxidant activity of *C. sappan* ethanolic extract on biological system, human keratinocytes (HaCaT) cell line was used. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) was used to evoke oxidative stress and the cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. A non-toxic, yet effective concentration was used in cream formulations. Several cream formulations were prepared by heating the oil-soluble and water-soluble ingredients to  $70^\circ\text{C}$  separately and mixed using homogenizer. The formulations were then evaluated for their stability and physical characterizations, namely freeze-thaw cycle, centrifugation test, organoleptic, type of cream, pH, homogeneity, and viscosity. The result of the study will establish the antioxidant activity of *C. sappan* on biological system and address the need for cosmetic products with antioxidant activity.

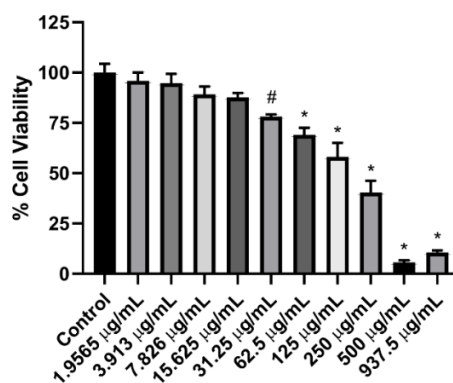
## 2. RESULTS

### 2.1. Extraction and phytochemical screening

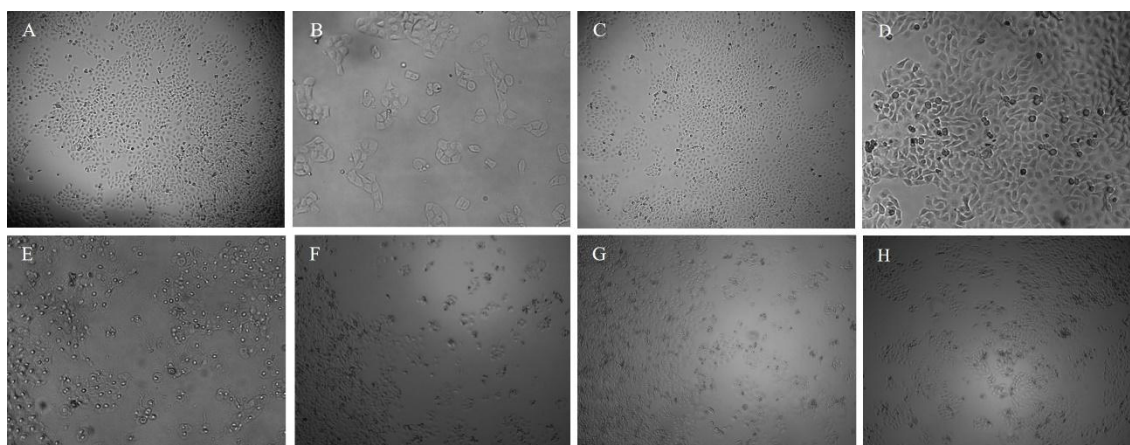
Extraction of *C. sappan* powder using 96% ethanol yielded 5.38% dried extract. To assess chemical compound groups presence in the extract, phytochemical screening was performed. Flavonoids, quinones and steroids/triterpenoids were detected in the extract as evident by a color change after the addition of the reagents.

### 2.2. Cytotoxic effect of *C. sappan* ethanolic extract

Initially, HaCaT cells were treated with different concentrations of *C. sappan* extract and cell viability was determined using MTS assay. The extract produced cytotoxicity in a concentration-dependent manner with an  $IC_{50}$  value of  $146.8 \pm 2.6 \mu\text{g/ml}$ . The extract evoked significant damage to HaCaT cells at concentrations from  $31.25 \mu\text{g/ml}$  above (Figure 1,  $p < 0.05$ ) while at lower concentrations, the extract did not cause any damage. The damaging effect was even more profound in the range concentrations of 125 to  $937.5 \mu\text{g/ml}$  (Figure 1,  $p < 0.0001$ ). At  $500 \mu\text{g/ml}$ , *C. sappan* extract reduced the cell viability to  $5.6 \pm 1.1\%$ . *C. sappan* extract at  $937.5 \mu\text{g/ml}$  resulted in higher cell viability of  $11 \pm 1.1\%$  compared to  $500 \mu\text{g/ml}$ , however, the difference was not significant ( $p = 0.99$ ). The microscopic observation showed that high concentration of the extract resulted in cell damage as evident by cell swelling, nucleus denaturation, and the presence of cell debris (Figure 2E). Dimethyl sulfoxide (DMSO) was used to dissolve the extract and the highest concentration of DMSO used in the experiment was 0.75%. The use of 0.75% DMSO had no effect on the cells as the cells exhibited similar characteristics as the healthy cells as shown by visible membrane integrity and clear nucleus (Figure 2A and 2C).



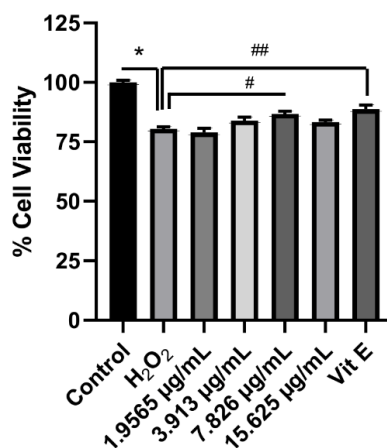
**Figure 1.** Cytotoxic effect of *Caesalpinia sappan* ethanolic extract. Data was expressed as mean  $\pm$  SEM ( $n = 5-9$ ). #  $p < 0.05$  and \*  $p < 0.0001$  compared to control group.



**Figure 2.** Microscopic images of HaCaT cells after 24 hours treatment. HaCaT were treated with the following: (A) no treatment, (B) 50% DMSO, (C) 0.75% DMSO, (D) 1.9565 µg/mL *C. sappan* extract, (E) 937.5 µg/mL *C. sappan* extract (F) 500 µM H<sub>2</sub>O<sub>2</sub>, (G) 15.625 µg/mL *C. sappan* and 500 µM H<sub>2</sub>O<sub>2</sub>, and (H) 0.25 mg vitamin E and 500 µM H<sub>2</sub>O<sub>2</sub>. Pictures were taken in 100x magnification (Image A, C, F-H) and 200x magnification (image B, D, E) using Zen Software Blue Edition (Carl Zeiss Microscopy GmbH).

### 2.3. Antioxidant activity of *C. sappan* ethanolic extract

Non-toxic concentrations of *C. sappan* extract (1.9565-15.625 µg/ml) were further used for the antioxidant assay. Cells treated with 500 µM of H<sub>2</sub>O<sub>2</sub> for 24 hours had a significant reduction in cell viability compared to control cells (Figure 3,  $p < 0.0001$ ). This result was corroborated by microscopic observation, showing more detached cells with denatured nucleus compared to control cells (Figure 2A and 2F). Treatment with *C. sappan* extract at 7.826 µg/ml ameliorated the decrease in cell viability caused by 500 µM H<sub>2</sub>O<sub>2</sub> (Figure 3,  $p < 0.05$ ), demonstrating protective effect of the extract. Similarly, microscopic observation revealed that cells treated with 15.625 µg/ml *C. sappan* extract had less cell detachment and had more cells with clear nucleus compared to cells treated with 500 µM of H<sub>2</sub>O<sub>2</sub> (Figure 2G). The protective effect of 15.625 µg/ml *C. sappan* extract against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage was similar as 0.25 mg/ml vitamin E since no statistical significance was observed ( $p = 0.92$ ).



**Figure 3.** Antioxidant activity of *Caesalpinia sappan* ethanolic extract against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in HaCaT cells. Data was expressed as mean±SEM (n = 5). \*  $p < 0.0001$  compared to control group. #  $p < 0.05$  and ##  $p < 0.005$  compared to cells treated with H<sub>2</sub>O<sub>2</sub> only.

### 2.4. Formulation and evaluation of *C. sappan* creams

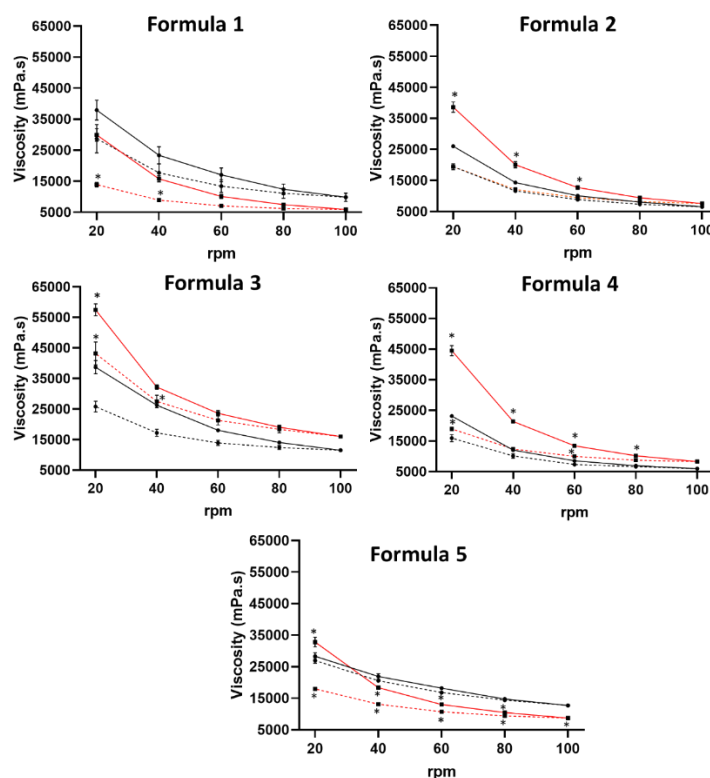
Five formulations of *C. sappan* creams were developed as stated in Table 1. The concentration of *C. sappan* ethanolic extract was kept at 15.625 µg/ml, based on the cytotoxic and antioxidant results stated above. The evaluation data was presented in Table 2. All creams were appeared to be white-pinkish in color with sandalwood scent and homogenous. It was evident that all formulas were oil in water (o/w) type of cream, with pH ranging from 5.07±0.01 – 6.51±0.04. Centrifugation testing right after cream preparation showed no

phase separation occurred, which indicated the stability of all formulated creams. It was found that all creams exhibited shear-thinning properties, with viscosity values ranging between 5,957 - 38,733 mPa.s, as shown in Figure 4 (round marker).

**Table 2.** Physical properties of *C. sappan* cream before and after freeze-thaw cycles.

Parameter	Formula									
	F1		F2		F3		F4		F5	
	Before	After	Before	After	Before	After	Before	After	Before	After
Organoleptic	White, sandal-wood scent	Yellowish, sandal-wood scent	White, sandal-wood scent	Yellowish, sandal-wood scent	Pinkish, sandal-wood scent	Yellowish, sandal-wood scent	White, sandal-wood scent	Yellowish, sandal-wood scent	White, sandal-wood scent	White, no odor
Type of cream	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w
pH <sup>a</sup>	5.07±0.01	5.65±0.04	5.15±0.02	5.59±0.05	6.51±0.04	6.94±0.02	5.31±0.02	5.56±0.03	5.14±0.03	5.35±0.03
Homogeneity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Centrifugation testing	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable	Stable

<sup>a</sup> Data was expressed as mean ± SEM (n = 3).



**Figure 4.** Viscosity profile of *C. sappan* cream formula 1-5 before and after freeze-thaw cycles. Data was expressed as the mean of three independent measurements±SEM. Black line, round marker: before freeze-thaw cycles; Red line, square marker: after freeze-thaw cycles; Solid line: ascending shear rate values; Dash line: descending shear rate values. \**p* < 0.05 compared to before freeze-thaw cycles.

The stability testing was done by three times freezing and thawing cycles at 4°C and 40°C, followed by several evaluations to address the effect of freeze-thaw cycles towards the physical properties of creams. Organoleptic testing results showed that all creams had yellowish discoloration with less scent of sandalwood. Moreover, the pH values of cream were found to be slightly higher after freeze-thaw cycles which fell in the range of 5.35±0.03 - 6.94±0.02. The viscosity measurement revealed that freeze-thaw cycles significantly affected the viscosity profile of all creams (Figure 4, square marker, *p* < 0.05). The viscosity range was then shifted into 5,911 - 57,528 mPa.s after accelerated stability testing. Despite those changes, all formulas were able to sustain o/w type of creams and were stable after centrifugation testing, as shown by the absence of phase separation.

### 3. DISCUSSION

#### 3.1. Cytotoxicity and antioxidant activity of *C. sappan* ethanolic extract

*C. sappan* has been traditionally used as a medicinal plant to treat various diseases and its decoction is often consumed as healthy drinks [6, 7]. In this study, cytotoxicity and antioxidant activity of *C. sappan* ethanolic extract were evaluated on human keratinocytes cell line and the extract was further used as an active ingredient for antioxidant cream formulation. The formulations made were evaluated for their physical characteristics and stability.

Cytotoxicity of the extract was assessed to ensure that the concentration of the extract used in cream formulation did not have any detrimental effect on skin. Keratinocytes are the predominant cells that comprise nearly 90% of the cells within the skin epidermis, therefore HaCaT is an excellent model to evaluate changes of the skin [10, 11]. According to an earlier study conducted by Srisawat *et al.* [12], IC<sub>50</sub> values that fall within 21-200 µg/ml are regarded as moderately toxic. Since *C. sappan* extract had an IC<sub>50</sub> value of 146.8 ± 2.6 µg/ml, *C. sappan* extract is considered as moderately toxic on HaCaT cells. Several studies have reported cytotoxicity of *C. sappan* and its isolated compounds on various cancer cell lines, demonstrating the potential of *C. sappan* as a source of anticancer [13-15]. However, the selectivity of the extract must also be taken into consideration as this study showed that higher concentrations of *C. sappan* caused substantial damage to HaCaT cells.

The antioxidant assay was conducted on concentrations that did not cause any damage to HaCaT cells. Treatment with H<sub>2</sub>O<sub>2</sub> induced the generation of ROS on HaCaT, leading to stress oxidative damage that causes cell deaths [10]. The addition of 15.625 µg/ml *C. sappan* extract on H<sub>2</sub>O<sub>2</sub>-treated cells significantly reduced the cell death, indicating the protective effect of *C. sappan*. The antioxidant activity of *C. sappan* has also been reported in previous studies where ethanolic extract of *C. sappan* exhibited a potent antioxidant activity on DPPH, ABTS, superoxide anion, nitric oxide, and FRAP assay [5, 6, 8]. The present study also revealed that the antioxidant activity of *C. sappan* extract was similar to vitamin E. The antioxidant activity of *C. sappan* most likely attributed by polyphenolic compounds and flavonoids contained in *C. sappan* [6, 8, 16]. Febriyanti *et al.* [6] reported that the total phenolic compounds of ethanolic extract *C. sappan* was equivalent to 71,144 g gallic acid per 100 g of extract. Furthermore, gallic acid, brazilin, brazilein, and protosappanin A with proven antioxidant activity have been successfully isolated from *C. sappan* [6, 17]. Taken together, *C. sappan* is indeed potential to be used as an active ingredient of herbal supplement or in cosmetics to overcome ROS.

#### 3.2. *C. sappan* cream formulation and evaluation

In this study, 5 different cream formulas containing *C. sappan* ethanolic extract were developed and examined. The concentration of *C. sappan* ethanolic extract in the formulation was determined based on the antioxidant activity result, which did not produce any detrimental effect on the skin (Figure 1), while also showed good antioxidant properties against H<sub>2</sub>O<sub>2</sub>-induced oxidation (Figure 2). The cream formulas were varied on the amount or type of oil, stearic acid, triethanolamine (TEA) or the ratio between Tween 80 and Span 80 used. Their HLB values were calculated to be 10.25, 10.25, 10.95, 10.8 and 11.95, respectively for F1 until F5. This indicated that all cream formulas were expected to be o/w type of cream.

All *C. sappan* creams were appeared to be all white to pinkish in color, with sandalwood scent due to the added fragrance (Table 2). After freeze-thaw cycles, F2, F3 and F4 creams were turned yellowish, with faint smell of sandalwood. The odor was even completely disappeared for F5 cream. Nonetheless, all formulas were found to be stable during centrifugation testing after freeze-thaw cycles, which indicated that all cream formulas were stable even after three times freezing and thawing cycles.

All formulated creams were shown to be o/w type of cream based on the sudan red testing, and the results were consistent before and after freeze-thaw cycles, as presented in Table 2. Aligned with that, all applied creams were easily washed by flowing water without leaving oily residue on the skin. The results were as expected, as they were matched with the calculated HLB values. All creams were also found to be homogenous based on the microscopic evaluation even after freeze-thaw cycles.

The pH of *C. sappan* creams before freeze-thaw cycles was observed to be in the range of 5.07±0.01 – 6.51±0.04 (Table 2). This variation resulted from different materials employed in the formula. F1 had the lowest pH due to lower amount of Tween 80 and Span 80 included. Meanwhile, F3 had the highest pH observed due to incorporation of TEA, which is slightly basic. It has been noted that after freeze-thaw cycles, the pH value of every cream was slightly increased. This changes could indicate the initial instability of the formula, despite the results observed in the centrifugation testing. Theoretically, a cream with pH ranging from 4-6 was recommended in a skincare formulation, which was calculated based on the normal pH range of skin [18].

Accordingly, almost all cream formulas complied to the pH requirement, even after stability testing, except for F3.

The viscosity evaluation was done before and after freeze-thaw cycles to observe the viscosity profile and to investigate the effect of freezing and thawing treatment towards the viscosity of the cream. The results were presented in Figure 4 as the comparison between the viscosity of cream before and after freeze-thaw cycles (round marker and square marker, respectively). Based on the results, all creams exhibited shear-thinning properties, in which viscosity decreased with increasing shear strength (*i.e.* rpm) [19]. The viscosity of the creams before freeze-thaw cycles were found to be in the range of 5,957-38,733 mPa.s. F3 exhibited the highest viscosity values among the others, which was attributed to the additional stearic acid in the oil phase formula. In contrast, F4 had the lowest viscosity values due to higher concentration of Tween 80 and Span 80.

Following freezing and thawing cycles, however, the final viscosity values were significantly shifted into 5,911-57,528 mPa.s. As presented in Figure 4, F1 and F5 showed lower viscosity profile after freeze-thaw cycles, while F2, F3 and F4 showed the opposite results. The exact reasons for this obscurity are yet to be elucidated, but it was hypothesized to be attributed by the interaction between the cream bases themselves. The statistical analysis showed significant differences between viscosity of cream before and after freeze-thaw cycles, with  $p < 0.05$ . Viscosity is one of the important properties affecting stability of cream by acting as a mechanical barrier to prevent coalescence [20]. Recently, Banerjee, Thiagarajan and Thiagarajan [21] have reported the viscosity values of standard topical emulsion cream to be around 27,000 mPa.s at 5 rpm to 8,000 mPa.s at 200 rpm. In regards to the standard, F5 showed the closest viscosity values before and after freeze-thaw cycles. Additionally, it has been observed that there was hysteresis in the viscosity measurement upon ascending and descending of shear rate. This characteristic suggested that all creams had thixotropic behavior [21].

#### 4. CONCLUSION

In this study, our data showed that *C. sappan* extract was able to dampen the oxidative damage evoked by H<sub>2</sub>O<sub>2</sub> on HaCaT Cells. Although the extract had a comparable antioxidant activity with vitamin E, the extract possessed moderate cytotoxic activity. As such, the concentration used in cosmetics and herbal product should consider the cytotoxic effect of the extract. All cream formulas were found to be stable, based on the type of cream, pH, homogeneity, and centrifugation testing. Viscosity evaluation revealed that all creams had shear-thinning or thixotropic behavior. Among them, F5 was shown to have a good pH and viscosity values before and after three cycles of freezing and thawing. Hence, the *C. sappan* cream may serve as a promising antioxidant cream that helps to protect against oxidative stress.

#### 5. MATERIALS AND METHODS

##### 5.1. Materials

*C. sappan* wood was purchased from Research Centre of Spices and Medicinal Plants, Bogor, Indonesia. *C. sappan* identification and authentication was conducted by Arifin Surya Dwipa Irsyam B.Sc, M.Sc. from Herbarium Bandungense and Zoology Museum, Bandung Institute of Technology. Propylparaben, methylparaben, tween 80, and glycerin were obtained from Solvay Specialty Chemicals Asia Pacific Pte Ltd (Singapore). Sandalwood essential oil and olive oil were obtained from Darjeeling Perfumery and Oils (Italy). Span 80 and MTS reagent were purchased from Kolb Distribution Ltd (Switzerland) and Promega (U.S.A.), respectively. Mineral oil, stearic acid and cetyl alcohol were purchased from Xi'an Virgin Biological Technology Co., Ltd (China). HaCaT cells were a generous gift from Associate Professor Ng Kee Woei, School of Materials Science & Engineering, Nanyang Technological University, Singapore. DMEM, FBS, and trypan blue were from Thermo Fisher Scientific (U.S.A) while penicillin-streptomycin and vitamin E were from Sigma-aldrich (U.S.A). H<sub>2</sub>O<sub>2</sub> and DMSO were purchased from Merck (Germany). All of the chemicals used in phytochemical screening were obtained from Sigma-aldrich (U.S.A) and Merck (Germany).

##### 5.2. Extraction of *Caesalpinia sappan*

*C. sappan* wood was grounded into powder and the powder was macerated using 96% ethanol with a ratio of 1:4 for 3 x 24 hours. The extract was decanted and filtered using Whatman paper no. 1. The filtered extract was collected and dried using R-100 rotary evaporator from Buchi (Switzerland). The collected dried extract was kept in desiccator until further use. Yield of the dried extract was calculated based on weight/weight (w/w) of the *C. sappan* powder.

### 5.3. Phytochemical screening

The dried extract of *C. sappan* was screened for the presence of alkaloids, flavonoids, saponins, quinones, tannins, and steroids/triterpenoids as previously described with minor modification [22]. Briefly, alkaloids were detected using Mayer and Dragendorff reagents. The formation of white or yellow sediment after the addition of Mayer's reagent and formation of orange deposits after the addition of Dragendorff's reagent indicates the presence of alkaloids. For flavonoids detection, magnesium powder and concentrated HCl were added onto the extract that had been dissolved in methanol. Orange, red, or yellow color formation indicates the presence of flavonoid. For saponins detection, froth test was conducted where a stable lather formed after the addition of 2 N HCL indicated the presence of saponin. For quinones detection, about 5 ml of the solution obtained from the identification of flavonoids was transferred into a test tube. A few drops of 1 N NaOH solution was added and the formation of red color indicated the presence of quinones. To detect tannins, the extract was mixed with hot 0.9% NaCl solution, filtered and divided into 3 equal portions. NaCl solution was added to one portion of the test tube, 1% gelatin solution to a second portion and a gelatin-salt reagent (1:1 ratio) to a third portion. Precipitation formation with the latter reagent or with both the second and third reagent indicates the presence of tannins. Positive tests were confirmed by the addition of FeCl<sub>3</sub> solution to the mixture and should result in a characteristic blue, blue-black, green or blue-green color and precipitation. For steroids/triterpenoids detection, the extract was dissolved in water and ether was added into the test tube. The mixture was mixed and the ether layer was taken and allowed to dry. Two drops of acid anhydrous acetate and 1 drop of concentrated sulfuric acid were added. Orange, red, or yellow formation indicates the presence of terpenoids while green formation indicates the presence of steroids.

### 5.4. Cell culture

Normal HaCaT cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C with 5% CO<sub>2</sub> and passaged twice a week. For cytotoxicity and antioxidant test, the cells were seeded in 96-well plates at a concentration of 10,000 cells/well and grown in 150 µL DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were then incubated at 37°C overnight in an incubator with 5% CO<sub>2</sub> atmosphere.

### 5.5. Cytotoxicity test

To evaluate whether *C. sappan* extract had toxic effect on HaCaT cells, cytotoxicity test was performed. A stock solution of *C. sappan* extract was freshly prepared with a concentration of 125 mg/mL in DMSO. The stock solution was further diluted with DMEM to yield a concentration of 937.5, 500, 250, 125, 62.5, 31.25, 15.625, 7.826, 3.913, and 1.9565 µg/ml where the highest concentration of DMSO in the dilution was 0.75%. Following overnight incubation, the seeded cells were treated with various concentrations of *C. sappan* extract and incubated for 24 hours. Three controls were used in the experiment, namely media only as a blank, cells maintained in the media as a control, and cells maintained in 50% DMSO in DMEM as a positive control.

Media was removed from each well and washed with 150 µL of media. 100 µL of media was added, followed by the addition of 20 µL MTS reagent solution into each well and incubated for 3-3.5 hours. The absorbance was measured using a plate reader (Nanoquant Plate™ Infinite® M200, Tecan, Switzerland) at 490 nm. Cell viability was calculated using the following equation:

$$\% \text{ Cell viability} = (A_{\text{extract}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\% \quad [\text{Eq. 1}]$$

IC<sub>50</sub> was defined as drug concentration that reduced cell's viability to 50% and was determined by plotting data points over a range of concentration. The IC<sub>50</sub> was calculated via regression analysis using Prism version 8.3.0 (GraphPad Software, U.S.A)

### 5.6. Antioxidant assay

Antioxidant assay was performed against oxidative damage induced by H<sub>2</sub>O<sub>2</sub> in HaCaT cells. After overnight incubation, the seeded cells were incubated with H<sub>2</sub>O<sub>2</sub> 500 µM or various non-toxic concentrations of *C. sappan* extract added with H<sub>2</sub>O<sub>2</sub> 500 µM or vitamin E 0.25 mg/ml added with H<sub>2</sub>O<sub>2</sub> 500 µM for 24 hours. Cell viability was determined using MTS assay as stated on 5.5 subsection.

## 5.7. Preparation of *C. sappan* cream

*C. sappan* creams were prepared according to the formulation stated in Table 1. Briefly, the oil phase was made by mixing all oil-soluble ingredients, including mineral oil, olive oil, Span 80, stearic acid, cetyl alcohol, and tocopherol acetate at 70°C. Meanwhile, the water phase was made by mixing all water-soluble ingredients, including *C. sappan* ethanolic extracts, glycerin, TEA, methylparaben, propylparaben, and Tween 80 at 70°C. Afterwards, the oil phase was slowly added into the water phase with a continuous mixing using homogenizer (Witeg, WiseTis HG-15D) at elevated speed up to 40 rpm on a 70°C hot plate for 8 minutes. The mixing was continued for another 2 minutes without hot plate. Then, sandalwood oil was added and mixed homogeneously to obtain the final *C. sappan* cream.

**Table 1.** Formulations of *C. sappan* creams.

Components	Amounts (% w/w)				
	F1	F2	F3	F4	F5
<i>C. sappan</i> ethanolic extract	15.625	15.625	15.625	15.625	15.625
	µg/ml	µg/ml	µg/ml	µg/ml	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	-	-
Methyl paraben	0.18	0.18	0.18	0.18	0.18
Propyl paraben	0.02	0.02	0.02	0.02	0.02
Glycerin	10	10	10	10	10
Tocopherol acetate	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

## 5.8. Evaluation of *C. sappan* cream

### 5.8.1. Organoleptic assessment

*C. sappan* creams were evaluated for their color, odor and phase separation.

### 5.8.2. Type of cream

The type of cream was determined by mixing the cream with a small amount of red sudan until homogenous. The cream is oil in water (o/w) type if the red sudan forms spots on the cream basis.

In addition, the type of cream was also examined by using washability test. Briefly, a sufficient amount of cream was spread evenly on the dorsal side of the palm. Afterwards, the cream was rinsed with flowing tap water. The cream is o/w type if the cream is readily washed without leaving oily residue.

### 5.8.3. pH measurement

pH of the creams was measured by a digital pH meter (Pb-11, Sartorius). 1 g of each formula was dissolved in 10 ml of deionized water before measurement.

### 5.8.4. Centrifugation testing

Each cream formula was placed in a 1.5 mL microcentrifuge tube before underwent centrifugation at 5,000 rpm for 10 minutes using Eppendorf Centrifuge 5424. Then, the cream was observed for any phase separation.

### 5.8.5. Homogeneity testing

The cream formulas were subjected under a microscope to examine their homogeneity.



#### 5.8.6. Viscosity measurement

The viscosity of the cream was evaluated by using B-one Touch Lamy Rheology viscometer with L-4 spindle. The spindle was rotated from 20-100 rpm in ascending and descending manner with increment or decrement of 20 rpm every 60 seconds. All measurements were done in triplicate.

#### 5.8.7. Accelerated stability testing

The stability of cream was evaluated in 3 cycles of freezing and thawing test where alteration from 4°C in refrigerator for 24 hours to 40°C in incubator for 24 hours was counted as one cycle. After 3 cycles, the cream formulas were re-evaluated for organoleptic, type of cream, pH, centrifugation, homogeneity, and viscosity.

### 5.9. Statistical analysis

Prism version 8.3.0 was used for data analysis. Data were obtained from at least three independent measurements and were presented as the mean  $\pm$  Standard Mean Error (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. The stability data of cream were evaluated using two-way ANOVA with Bonferroni's post-hoc test by Prism version 8.3.1.

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### REFERENCES

- [1] Yoon Y, Lee YM, Song S, Lee YY, Yeum KJ. Black soybeans protect human keratinocytes from oxidative stress-induced cell death. *Food Sci Nutr*. 2018; 6(8): 2423-2430. [CrossRef]
- [2] Gragnani A, Cornick SM, Chominski V, de Noronha SM, de Noronha SA, Ferreira LM. Review of Major Theories of Skin Aging. *Adv Aging Res*. 2014; 3: 265-284. [CrossRef]
- [3] Lan CC, Hung YT, Fang AH, Ching-Shuang W. Effects of irradiance on UVA-induced skin aging. *J Dermatol Sci*. 2019; 94(1): 220-228. [CrossRef]
- [4] Woodby B, Penta K, Pecorelli A, Lila MA, Valacchi G. Skin Health from the Inside Out. *Annu Rev Food Sci Technol*. 2020; 11: 235-254. [CrossRef]
- [5] Saenjum C, Chaiyasut C, Kadchumsang S, Chansakaow S, Suttajit M. Antioxidant activity and protective effects on DNA damage of *Caesalpinia sappan* L. extract. *J Med Plant Res*. 2010; 4(15): 1594-1608. [CrossRef]
- [6] Febriyenti F, Suharti N, Lucida H, Husni E, Sedona O. Karakterisasi dan Studi Aktivitas Antioksidan dari Ekstrak Etanol Secang (*Caesalpinia sappan* L.). *J Sains Farm Klin*. 2018; 5(1): 23-27. [CrossRef]
- [7] Mitani K, Takano F, Kawabata T, Allam AE, Ota M, Takahashi T, Yahagi N, Sakurada C, Fushiya S, Ohta T. Suppression of melanin synthesis by the phenolic constituents of sappanwood (*Caesalpinia sappan*). *Planta Med*. 2013; 79(01): 37-44. [CrossRef]
- [8] Setiawan F, Yunita O, Kurniawan A. Uji Aktivitas Antioksidan Ekstrak Etanol Kayu Secang (*Caesalpinia sappan*) Menggunakan Metode DPPH, ABTS, dan FRAP. *MPI*. 2018; 2(2): 82-99. [CrossRef]
- [9] Nirmal NP, Rajput MS, Prasad RG, Ahmad M. Brazilin from *Caesalpinia sappan* heartwood and its pharmacological activities: A review. *Asian Pac J Trop Med*. 2015; 8(6): 421-30. [CrossRef]
- [10] Domínguez-Perles R, Guedes A, Queiroz M, Silva AM, Barros AI. Oxidative stress prevention and anti-apoptosis activity of grape (*Vitis vinifera* L.) stems in human keratinocytes. *Food Res Int*. 2016; 87: 92-102. [CrossRef]
- [11] Ramanauskienė K, Stelmakiene A, Majienė D. Assessment of lemon balm (*Melissa officinalis* L.) hydrogels: quality and bioactivity in skin cells. *Evid Based Complement Alternat Med*. 2015; 2015: 1-7. [CrossRef]
- [12] Srisawat T, Chumkaew P, Heed-Chim W, Sukpondma Y, Kanokwiroon K. Phytochemical screening and cytotoxicity of crude extracts of *Vatica diospyroides* symington type LS. *Trop J Pharm Res*. 2013; 12(1): 71-76. [CrossRef]

- [13] Jenie RI, Handayani S, Susidarti RA, Udin Z, Meiyanto E. Cytotoxic and Antimetastasis Effect of Ethyl Acetate Fraction from *Caesalpinia sappan* L. on MCF-7/HER2 Cells. *IJCC*. 2017; 8(1): 42-50. [[CrossRef](#)]
- [14] Bukke AN, Hadi FN, Babu KS. In vitro studies data on anticancer activity of *Caesalpinia sappan* L. heartwood and leaf extracts on MCF7 and A549 cell lines. *Data Brief*. 2018; 19: 868-877. [[CrossRef](#)]
- [15] Hung TM, Hai NX, Nhan NT, Quang TT, QUAN TL, Cuong TD, Dang NH, Dat NT. Cytotoxic activity of new phenolic compounds from Vietnamese *Caesalpinia sappan*. *Biosci Biotechnol Biochem*. 2013; 77(12): 2378-82. [[CrossRef](#)]
- [16] Nirmal NP, Panichayupakaranant P. Antioxidant, antibacterial, and anti-inflammatory activities of standardized brazilin-rich *Caesalpinia sappan* extract. *Pharm Biol*. 2015; 53(9): 1339-1343. [[CrossRef](#)]
- [17] Batubara I, Mitsunaga T, Ohashi H. Brazilin from *Caesalpinia sappan* wood as an antiacne agent. *J Wood Sci*. 2010; 56(1): 77-81. [[CrossRef](#)]
- [18] Chen M X, Alexander K S, Baki G. Formulation and evaluation of antibacterial creams and gels containing metal ions for topical application. *J Pharm (Cairo)*. 2016; 2016: 1-10. [[CrossRef](#)]
- [19] Walters K., Jones WM. Measurement of Viscosity. In: Boyes W. *Instrumentation Reference Book* 4th edition. Butterworth-Heinemann, Oxford, 2010, pp.69-75.
- [20] Simões A, Veiga F, Vitorino C. Developing cream formulations: renewed interest in an old problem. *J Pharm Sci*. 2019; 108(10): 3240-3251. [[CrossRef](#)]
- [21] Banerjee K., Thiagarajan N, Thiagarajan P. Formulation and characterization of a *Helianthus annuus*-alkyl polyglucoside emulsion cream for topical applications. *J Cosmet Dermatol*. 2019; 18(2): 628-637. [[CrossRef](#)]
- [22] VH ES, Utomo SB, Syukri Y, Redjeki T. Phytochemical screening and analysis polyphenolic antioxidant activity of methanolic extract of white dragon fruit (*Hylocereus undatus*). *Indones J Pharm*. 2012; 23(1): 60-64. [[CrossRef](#)]

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