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Original Article

Secang wood (*Caesalpinia sappan* L.) nanoemulgel activity as antiaging through suppressing the MMP-1 expression and the collagen degradation

[Actividad del nanoemulgel de secang wood (*Caesalpinia sappan* L.) como antienvejecimiento mediante la supresión de la expresión de MMP-1 y la degradación del colágeno]

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Abstract

Context: Aging is closely related to reactive oxygen species (ROS). ROS increases the collagenase enzyme (MMP-1) levels and collagen degradation that causes skin wrinkling. Secang wood (*Caesalpinia sappan* L.) containing brazilin and brazilein has been shown to have photoprotective and antioxidant properties.

Aims: To evaluate the activity of *C. sappan* nanoemulgel as antiaging agent against the target protein, matrix metalloproteinases (MMPs), especially MMP-1, MMP-3, and MMP-9 by *in silico* assay and using *in vivo* assay through MMP-1 and collagen expression parameter.

Methods: *C. sappan* nanoemulgel was made by mixing the gel base with *C. sappan* nanoemulsion from heartwood extract. The *C. sappan* nanoemulsion was formulated using the Self Nanoemulsifying Drug Delivery System method. *In vivo* testing was conducted with a post-test-only control group design and used male Wistar rats. MMP-1 expression was examined using immunohistochemical techniques, and the amount of dermal collagen was observed with Picro Sirius Red staining. *In silico* assay using a computational method with Autodock 4.2 program.

Results: *C. sappan* nanoemulgel concentrations of 0.0625, 0.125, and 0.25% obstruct the expression of MMP-1 and collagen degradation. The bond energy value to MMP-1, MMP-3, and MMP-9 were -8.04, -10.40, and -8.70 kcal/mol (for brazilin); -8.82; -10.99, and -8.51 kcal/mol (for brazilein).

Conclusions: Nanoemulgel containing *C. sappan* nanoemulsion has a potential activity as an antiaging agent by repressing MMP-1 expression and dermal collagen degradation. *C. sappan* nanoemulgel 0.25% showed the best result as antiaging. Brazilin and brazilein from *C. sappan* inhibit the MMP-1, MMP-3, and MMP-9 by *in silico* assay.

Keywords: antiaging; collagen; *in silico*; *in vivo*; MMP-1.

Resumen

Contexto: El envejecimiento está estrechamente relacionado con las especies reactivas de oxígeno (ROS). ROS aumenta los niveles de la enzima colagenasa (MMP-1) y la degradación del colágeno que causa las arrugas en la piel. Se ha demostrado que la madera de secang (*Caesalpinia sappan* L.) que contiene brasilina y brasilina tiene propiedades fotoprotectoras y antioxidantes.

Objetivos: Evaluar la actividad de *C. sappan* nanoemulgel como agente antienvejecimiento contra la proteína diana, las metaloproteinasas de matriz (MMP), especialmente MMP-1, MMP-3 y MMP-9 mediante ensayo *in silico* y usando ensayo *in vivo* a través de MMP-1 y parámetro de expresión de colágeno.

Métodos: El nanoemulgel de *C. sappan* se elaboró mezclando la base del gel con la nanoemulsión del extracto de duramen de *C. sappan*. Esta se formuló utilizando el método del sistema de liberación de fármaco autonanoemulsionante. Las pruebas *in vivo* se realizaron con un diseño de grupo de control solo posterior a la prueba y se utilizaron ratas Wistar macho. La expresión de MMP-1 se examinó mediante técnicas inmunohistoquímicas y la cantidad de colágeno dérmico se observó con tinción con Picro Sirius Red. El ensayo *in silico* utilizó un método computacional con el programa Autodock 4.2.

Resultados: Las concentraciones de *C. sappan* nanoemulgel de 0,0625; 0,125 y 0,25% inhiben la expresión de MMP-1 y la degradación del colágeno. El valor de la energía de enlace para MMP-1, MMP-3 y MMP-9 fue -8,04; -10,40 y -8,70 kcal/mol (para brasilina); -8,82; -10,99 y -8,51 kcal/mol (para brazilein).

Conclusiones: El nanoemulgel que contiene nanoemulsión de *C. sappan* tiene una actividad potencial como agente antienvejecimiento al reprimir la expresión de MMP-1 y la degradación del colágeno dérmico. *C. sappan* nanoemulgel 0,25% mostró el mejor resultado como antienvejecimiento. Brazilin y brazilein de *C. sappan* inhiben MMP-1, MMP-3 y MMP-9 mediante ensayo *in silico*.

Palabras Clave: antienvejecimiento; colágeno; *in silicio*; *in vivo*; MMP-1.

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INTRODUCTION

Skin aging is a complex biological process influenced by a combination of intrinsic and extrinsic factors. Intrinsic aging occurs naturally with age. In contrast, extrinsic factors that cause aging are chronic sun exposure, air pollution, cigarettes, alcohol, and poor nutrition. This irrelevant factor works together with the intrinsic factor so that skin aging occurs earlier than the age it should be. The main extrinsic factor causing skin aging is the chronic exposure to sun ultraviolet (UV) radiation, known as photoaging. About 80% of the exposure to UV radiation brings about facial skin aging (Zhang and Duan, 2018).

Structural and functional changes in the extracellular matrix components, especially the degradation of collagen, which is the main component of the skin's extracellular matrix, accompany photoaging. A decrease in collagen creates significant changes in the skin's connective tissue, which give rise to the skin wrinkling, roughness, and sagging more quickly (Hwang et al., 2011; Wongrattanakamon et al., 2018). Intracellular ROS contribute significantly to skin aging and photoaging, where these intracellular ROS will activate the mitogen-activated protein kinase (MAPK) pathway, which will form transcription factor activator protein-1 (AP-1). AP-1 transcription plays an important role in the transcriptional regulation of matrix metalloproteinases (MMPs), especially MMP-1 (collagenase type-1), MMP-3 (stromelysin-1), and MMP-9 (gelatinase-B) which work to degrade collagen types I, III, and IV. The three types of collagen are synthesized mainly as the composition of the extracellular matrix of human skin. Inhibition of these MMPs enzymes can overcome the problem of skin photoaging (Pittayapruek et al., 2016).

The field of dermatology currently focuses on preventing skin aging with UV protection, such as antioxidants, anti-free radical creams, and skin protection products, such as sunscreens (Bayerl, 2016). One of the compounds that can prevent aging is retinoids (retinol). Based on Kong et al. (2015) research, retinol has antiaging activity by increasing collagen synthesis in the skin by inhibiting UV induction of matrix metalloproteinases (MMPs). However, retinol cause side effects such as skin erythema, pruritus, stinging or burning, and peeling (Mukherjee et al., 2011). Therefore, it is necessary to develop antiaging compounds with side effects that are safer to use in the long term, for example, natural compounds (Karim et al., 2014). Antiaging therapy from natural compounds has been widely developed because of the minimal side effects caused (Mukherjee et al., 2011).

The mechanism of natural ingredient extracts in protecting the skin from aging is through reducing ROS reactivity, inhibiting the oxidation process, inhibiting MMPs enzymes, and increasing the amount of collagen. This mechanism occurs because of the alleged presence of phenolic compounds, flavonoids, and triterpenoids responsible for protecting the skin. Both phenolics and flavonoids have a phenol ring in the presence of hydroxyl substituents to inhibit ROS, reduce metal ions, and modulate protein phosphorylation associated with inhibition of enzyme activity and lipid peroxidation (Karim et al., 2014; Pouillot and Polla, 2011). One of the evolvements and utilization of natural medicine is secang wood (*Caesalpinia sappan* L., family *Leguminosae*). *C. sappan* is reported to contain phenolic components, with the main active compounds being brazilin and brazilein. *C. sappan* has many pharmacological activities for the skin, such as a very strong antioxidant with an IC_{50} smaller than ascorbic acid using the DPPH scavenging assay, as a sunscreen with SPF 19.53, as a skin lightening agent by inhibiting tyrosinase, tyrosinase related protein-1 (TRP-1), and dopachrome tautomerase (Laksmiani et al., 2019; 2020; Niu et al., 2020). The development of *C. sappan* as a natural antiaging agent is auspicious.

Nanoemulsions are very attractive for application in cosmetics because they are more stable and visually transparent, and their high surface area allows effective delivery of the active ingredients to the skin (Gutierrez et al., 2008). The combination of nanoemulsions in the form of a gel is called nanoemulgel, which can increase the viscosity to be comfortable when applied. This study aimed to evaluate antiaging activity of *C. sappan* nanoemulgel preparations by using *in vivo* assay and investigate *C. sappan* ability as antiaging *in silico* with molecular docking.

MATERIAL AND METHODS

Material and instrument

MMP-1 (PDB ID: 966C); MMP-3 (PDB ID: 1G4K); MMP-9 (PDB ID: 2OW0) for *in silico* which was downloaded from http://www.rcsb.org/pdb/home/home.do. Then, the 3-dimensional structure of the brazilin and brazilein as the active compound in *C. sappan* were downloaded from https://pubchem.ncbi.nlm.nih.gov/compound/. Ethyl acetate (Merck), n-hexane (Merck), methanol (Merck), *C. sappan* powder, olive oil, Cremophor RH 40 (Asian Chemical), PEG 400 (Asian Chemical), and Lubrajel II XD (DKSH, Indonesia) for *C. sappan* nanoemulgel preparations. *In vivo* assay utilized 10% buffered formalin, DAB Chromogen kit (Dako, Denmark), anti-mouse

MMP-1 (BIOSS, USA), Picro Sirius Red stain kit (USA), hematoxylin gill (Merck), male Wistar rats (Laboratory of Animal, Department of Pharmacy, Mathematic and Natural Science Faculty, Udayana University, Indonesia). The equipment used *in silico* test was a computer set with Windows 10 64bit specifications equipped with Autodock 4.2 program, Chimera 1.10.1, and Hyperchem 8. UV-Vis spectrophotometer (UV Mini-1240) Shimadzu was worked to evaluate the physical characteristic of *C. sappan* nanoemulsion. The *in vivo* test used TL-K UV-B Lamp 40 watt (Philips), UV light (Lutron, YK-35UV type), and Olympus CX41 Microscope (Japan).

Plant material

C. sappan heartwood powder was collected and identified from Center for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT), Karanganyar Regency, Central Java, Indonesia (7°66'37.722"S, 111°13'48.873"E) with the register number KM.04.01/2/1055/2022.

Samples extraction

Samples extraction and fractionation of *C. sappan* process according to Laksmiani et al. (2020). *C. sappan* powder was extracted using methanol by maceration. Extraction was carried out in a 100 g sample: 1 L solvent for 24 hours with three replications. The obtained macerate was filtered and then concentrated at a temperature of 50°C using a vacuum evaporator to get a dry extract. Then the extract was fractionated through liquid-liquid extraction using n-hexane solvent first to remove non-polar components, then re-extracted with ethyl acetate solvent, and the ethyl acetate fraction of *C. sappan* was obtained.

C. sappan nanoemulsion and nanoemulgel preparation

Ethyl acetate fraction of *C. sappan* with a concentration of 0.5% w/w was mixed in olive oil with a magnetic stirrer speed of 600 rpm for 15 minutes, then added with PEG 400 as a co-surfactant stirred again with a magnetic stirrer speed of 600 rpm for 15 minutes. Cremophor RH 40 was added as a surfactant and mixed with a magnetic stirrer speed of 600 rpm for 2 hours. After that, the oil phase was placed in a sonicator for 1 hour. The mixture of the oil phase was then added to deionized water. The comparison of an oily phase: deionized water = 1:5, then stirred to form a nanoemulsion. Formulas were made of 3 types based on the percentage of olive oil as carriers, Cremophor RH 40 and PEG 400 (1:7:2**,** 1:7:3, and 1:8:1) (Tables 1 and 2). Evaluation of physical stability, clarity test, zeta potential, and particle size of each formula was carried out to select the best formulation for making *C. sappan* nanoemulgel using Lubrajel II XD and propylene glycol.

C. sappan nanoemulsion characterization

Physical stability test

The physical stability of nanoemulsions was carried out by using a centrifugation test. The nanoemulsion of ethyl acetate fraction of *C. sappan* was centrifuged at a speed of 12000 rpm for 15 minutes, and then observations were made. Stable nanoemulsions can be observed by no separation between the oil and water phases (Bernardi et al., 2011).

Clarity test

Observing the clarity of the nanoemulsion formed was evaluated by measuring the % transmittance parameter using visible spectrophotometry at a wavelength of 650 nm with deionized water as a blank. A good nanoemulsion has clear visuals with a transmittance percent value of 90-100% (Costa et al., 2012).

Droplet size measurement

The nanoemulsion droplet size and polydispersity index were determined using Photon Correlation Spectroscopy. One gram of *C. sappan* nanoemulsion was dispersed in 5 mL of deionized water and measured. *C. sappan* nanoemulsion must meet the nanoemulsion droplet size criteria, which is <100 nm (Forgiarini et al., 2001; Tadros et al., 2005).

Table 1. Formulation and comparison of each component of *C. sappan* nanoemulsion.

Formula	Ethyl acetate fraction of *C. sappan* % w/w	Comparison of each component in 12 g nanoemulsion			
		Oily phase			Water phase
		Olive oil	Cremophor RH 40	PEG 400	Deionozation water
F1	0.5	1	7	2	5
F2	0.5	1	7	3	5
F3	0.5	1	8	1	5

Table 2. Formulation of *C. sappan* nanoemulgel.

Ingredient	Function	Composition
Nanoemulsion of *C. sappan* (FA; FB; FC)	Active compound	12%
Propilenglycol	Humectan	10%
Lubrajel II XD	Gelling agent	20%
Deionozation water	Solvent	ad 100%

Nanoemulsion FA: Formula A contain 0.0625% *C. sappan* nanoemulsion; FB: Formula B contain 0.125% *C. sappan* nanoemulsion, and FC: Formula C contain 0.25% *C. sappan* nanoemulsion.

Zeta potential test

Zeta potential was determined using electrophoretic light scattering. One gram of ethyl acetate fraction of *C. sappan* nanoemulsion was dispersed in 5 mL of deionized water. The zeta potential value obtained as a nanoemulsion is -30 mV to +30 mV (Kale and Deore, 2017).

Physical characterization of C. sappan nanoemulgel

The *C. sappan* nanoemulgel was placed at room temperature 25 ± 2°C RH 60% for 30 days, then evaluated for stability on day-0 and day-30, followed by organoleptic, homogeneity, pH, viscosity, and spread test.

In silico assay

The MMP-1, MMP-3, and MMP-9 target proteins were structurally selected in an active form that binds to native ligands. Target protein preparation was carried out using the Chimera 1.11 program. The target protein preparation step begins with removing a water molecule on the target protein that is not involved in the interaction. The next step is to separate the native ligand from the target protein to provide a pocket cavity (Ferreira et al., 2015). Validation of the method was done by redocking the native ligand of each target protein on the target protein whose native ligand had been removed. The parameter validation method for molecular docking is the RMSD (Root Mean Square Deviation) value, which in molecular docking was to be valid if the RMSD is still in the range of 3 (Jain and Nicholls, 2008). The structure of brazilin and brazilein as active compounds in *C. sappan*, in their 3-dimensional form, was optimized using the HyperChem 8 program, complete with hydrogen atoms. Optimization of the 3-dimensional structure of brazilin and brazilein compounds using the AM-1 semi-empirical computational method. Optimization of the active compound in 3-dimensional form aims to calculate the energy and optimize the molecular geometry of the active compound by adjusting the bond length, bond angle, and torsion angle to achieve an equilibrium value (Mukesh and Rakesh, 2011).

Analysis of in silico testing

The results obtained from molecular docking are the bond energy and the type of hydrogen bond between the compound and the target protein. The bond energy value indicates the affinity (bond strength) between the active compound and the target protein. The binding energy between brazilin and brazilein with MMP-1, MMP-3, and MMP-9 is negative, indicating that brazilin and brazilein have an affinity for MMP-1, MMP-3, and MMP-9 (Mukesh and Rakesh, 2011). If the energy of the active compound (brazilin and brazilein) bond is lower than the native ligand bond energy, then *C. sappan* can be an antiaging agent. The active compound bond's energy was also compared with the ascorbic acid bond energy. The ascorbic acid was a positive control that was proven as an antiaging agent.

In vivo assay

Antiaging activity using *in vivo* study has received approval from the Research Ethics Commission of the Faculty of Medicine, Udayana University, with No. 205/UN14.2.2.VII.14/LT/2022 was conducted with a post-test only control group design and used 35 male Wistar rats as research subjects. Subjects were divided into two groups, control and treatment groups. The control group received a placebo in the form of nanoemulgel as a base material without a *C. sappan* nanoemulsion. The treatment group consisted of 4 treatment groups. The treatment group received a nanoemulgel with a 0.0625, 0.125, 0.25% *C. sappan* nanoemulsion content, and 3% ascorbic acid gel as positive control. Each group consisted of 7 rats. Nanoemulgel was administered topically before and after UV-B irradiation with a total dose of 840 mJ/cm2, which was administered three times a week for four weeks. The expression of MMP-1 was assessed using immunohistochemical techniques, and the amount of dermal collagen was shown with Picro Sirius Red staining. The results obtained are processed using the software.

Matrix metalloproteinase-1 (MMP-1) expression staining and calculation of the amount of its expression

Immunohistochemical staining was performed using the DAB chromogen kit (Dako, Denmark) immunohistochemical technique using MMP-1 anti-mouse primary antibody (BIOSS, USA). Before painting, the slides go through a process of deparaffinization and rehydration. Next, antigen retrieval was carried out. Namely, the slide was immersed in trisodium citrate buffer, then heated in the microwave for 10 minutes using 800 watts of power, cooled, and then washed with PBS for 2 × 5 minutes. After that, endogenous peroxidase was blocked, then 100 L of the primary antibody was dripped overnight. After one night, the preparations were washed with PBS 1× for five minutes in a glass jar while shaking and repeated twice. Dropped with labeled polymer-HRP and allowed to stand for 30 minutes in a closed box. Fibroblasts containing HRP can turn brown, indicating the presence of the MMP-1 enzyme. Then in the following process, DAB was dripped until it turned brown and then washed with PBS 1× until clean and dried. After that, Haematoxylin Gill was added, left for five minutes, washed with running water for 5 minutes, then washed with water for 2x 5 minutes. Process followed by immersion in alcohol 70, 95, and 100% for 2 × 2 minutes, then soaked into xylene twice for five minutes each. Then the slide was mounted with a xylene-based medium (Entellan) and closed with a cover glass.

The stained samples were split into three fields of view with a magnification of 400 times using an Olympus CX41 microscope, and then microphotography was performed using an Optilab Pro camera. Photos in JPEG format were analyzed using Optilab Viewer 1.0 and Image Raster 2.1 software. Counts were performed manually on the number of fibroblast cells stained with brown in the cytoplasm and interpreted as cells expressing MMP-1. The total number of fibroblast cells was also counted, whether brown or not, in the cytoplasm (fibroblasts that did not express MMP-1).

$$\text{MMP-1 expression (\%)} = \frac{\text{Fibroblast expressing MMP-1}}{\text{Total number of fibroblasts}} \times 100\% \quad [1]$$

Dermal collagen staining and measurement of the amount of dermal collagen

Collagen staining was performed using Sirius Red. Tissue still containing paraffin was deparaffinized with xylene for 2 × 5 minutes and hydrated using 100, 95, and 70% alcohol for 2 × 2 minutes, respectively. The step was followed by staining with Picro Sirius Red for 1 hour. Wash with acid water two times. Remove excess water physically by shaking gently, then dehydrated with 100% ethanol three times, cleaned in xylene liquid, mounting with Entellan medium, and covered with cover glass.

The amount of collagen in the preparation was calculated using a digital analysis method with a magnification of 10× the eyepiece and 40× the objective lens, using an Olympus CX41 microscope (Japan) photographed with an Optilab Pro camera (Miconos, Indonesia). Each slide was photographed three times using JPEG format using Optilab Viewer 1.0 software (Miconos, Indonesia). The amount of dermal collagen is calculated using Adobe Photoshop CS3 software, and Image J. Collagen tissue that appears bright red is selected using the Magic Wand function by Adobe PhotoShop CS3.

$$\text{Dermal collagen (\%)} = \frac{\text{Collagen pixel area}}{\text{Total pixel in one field of view}} \times 100\% \quad [2]$$

Statistical analysis

The data obtained from the antiaging activity of *C. sappan* nanoemulsion in nanoemulgel were statistically analyzed using the SPSS 16.0 for Windows application. The data was then tested for normality of the data with the Shapiro-Wilk test and homogeneity with the Levene test with a confidence level of 95%. If the data distribution was normal and homogeneous ($p>0.05$), the analysis was continued with the one-way ANOVA statistical test to determine the differences between each group and the LSD test with a 95% confidence level to determine which groups gave significant differences. Suppose the data were not normally distributed or homogeneous. In that case, a non-parametric test using the Kruskal-Wallis test was performed to see if there was a significant difference between the experimental groups. If it was known that there is a considerable difference, further analysis is carried out using the Mann-Whitney test to determine the different groups with a 95% confidence level. *C. sappan* nanoemulsion has antiaging activity if there was a significant difference between the control and treatment groups ($p<0.05$).

RESULTS

Characterization of *C. sappan* nanoemulsion

Particle characterization of the nanoemulsion is necessary because it determines the drug delivery system's *in vivo* distribution and targeting ability. Particle size will affect the delivery and stability of nanoparticles (Mukubwa et al., 2020). Nanoemulsion characterization was carried out to determine the characteristics of the resulting nanoemulsion based on physical stability, clarity, droplet size, and zeta poten-

tial. A good and stable nanoemulsion is a nanoemulsion that does not undergo separation between the oil phase and the water phase. Nanoemulsion has a transmittance value of close to 100% or at least 90%. The droplet size is 100 nm, and the zeta potential value is -30 mV to +30 mV. Table 3 expresses the characterization of *C. sappan* nanoemulsion.

Table 3 showed that the formula that complied with an excellent and stable nanoemulsion was F1 with a composition of 1:7:2 (olive oil: Cremophor RH 40: PEG 400). Formula 1 was selected to make nanoemulgel preparations and tested *in vivo* for antiaging activity.

Physical characterization of *C. sappan* nanoemulgel

Physical stability parameters include the absence of organoleptic changes during storage, homogeneity, and evenly distribution. This test was carried out by applying a nanoemulgel to a piece of glass or other suitable transparent material. The preparation must show a homogeneous arrangement, and no coarse grains are visible. Homogeneous pharmaceutical preparations will be evenly distributed to the skin and produce an excellent pharmacological effect. The three formulas showed stable and homogeneous pharmaceutical preparations.

Another parameter is the spreadability of the preparation. The spreadability test describes the distribution of the gel and its ability to spread when applied to the skin. Besides that, the spreadability can describe the viscosity of the preparation made. The dispersion is inversely proportional to the viscosity of the semisolid preparation. The lower the viscosity, the higher the dispersion (Garg et al., 2002). Nanoemulgel must be able to disperse with slight pressure so that it is easy to apply on the skin surface. In addition, the distribution of active substances on the skin is more evenly distributed so that the pharmacological effects of the active substances become more optimal (Chellapa et al., 2015). The evaluation results of the spreadability of the three *C. sappan* nanoemulgels met the requirements for a good gel preparation dispersion of 5-7 cm (Garg et al., 2002).

A good topical preparation has a pH between 4-8. If the pH is outside the 4-8 range, it can cause skin irritation and cause dry skin. Meanwhile, in the viscosity test, the nanoemulgel met the gel requirements with a viscosity value between 2000-4000 cps (Nurman et al., 2019). Table 4 showed the three nanoemulgel preparations had a stable dan good formula. The three nanoemulgels with a concentration of 0.0625, 0.125, and 0.25% *C. sappan* nanoemulsion can be used for *in vivo* antiaging activity testing.

Brazilin and brazilein have affinity and inhibit the target protein (MMP-1, MMP-3, and MMP-9) using *in silico* assay

This study evaluated the potential of brazilin and brazilein as the main components in *C. sappan* as antiaging agents *in silico* by inhibiting the MMP-1, MMP-3, and MMP-9 proteins. The three target proteins (MMP-1, MMP-3, and MMP-9) activate the collagen degradation process so that the skin begins aging. In the molecular docking process, preparation and optimization of the 3D structure of the brazilin and brazilein test compounds and ascorbic acid as positive control were carried out. The purpose of optimization is to obtain a more stable compound structure, which is characterized by low total energy. Optimizing the 3-dimensional brazilin, brazilein, and the ascorbic acid structure includes single-point calculations and geometry optimization. Single-point calculations were carried out to determine the total energy of the compound configuration. Geometry optimization aims to minimize energy and find the best conformation of the molecule.

Table 3. *C. sappan* nanoemulsion characterization.

Formula	Physical stability	Clarity (% transmittance)	Droplet size (nm)	Zeta potential (mV)
F1	Good and stable emulsion	93.27 ± 0.23	31.57 ± 0.11	-1.12 ± 0.07
F2	Good and stable emulsion	87.93 ± 0,15	38.04 ± 0.46	-0.56 ± 0.05
F3	Good and stable emulsion	88.01 ± 0.17	35.66 ± 0.28	-0.41 ± 0.06

Data are expressed as mean ± SEM (n = 5).

Table 4. Physical characterization of *C. sappan* nanoemulgel.

Formula	Organoleptic	Homogeneity	pH	Spreadability (cm)	Viscosity (cps)
A	Light yellow, odorless	Homogeneous	7.18	5.21 ± 0.07	3023.67 ± 12.50
B	Light yellow, odorless	Homogeneous	7.32	5.36 ± 0.04	3210 ± 27.00
C	Light yellow, odorless	Homogeneous	7.38	5.65 ± 0.05	3413 ± 4.93

Formula A: Formula contain 0.0625% *C. sappan* nanoemulsion; Formula B: formula contain 0.125% *C. sappan* nanoemulsion; and Formula C: formula contain 0.25% *C. sappan* nanoemulsion. Data are expressed as mean ± SEM (n = 3).

Table 5. Optimization energy of active compound in *C. sappan.*

Active compound	Single-point calculation (kcal/mol)	Geometry optimization (kcal/mol)
Brazilin	-3899.06	-3921.58
Brazilein	-3740.72	-3777.36
Ascorbic acid	-2023.93	-2037.97

The total energy calculated from the single-point calculation of brazilin, brazilein compounds and ascorbic acid were -3899.06 kcal/mol, -3748.80 kcal/mol, and -2023.93 kcal/mol (Table 5). A decrease in the energy of the brazilin, brazilein and ascorbic acid structures indicated the compound structure was more stable. Fig. 1 shows the compound's shift structure as the structure in the most stable geometry. The energy obtained from the geometric optimization results were -3921.58 kcal/mol (brazilin), -3775.88 kcal/mol (brazilein), and -2037.97 kcal/mol (ascorbic acid). Based on the resulting total energy, the optimization of the test compound in the three-dimensional structure was successful because the total energy of the optimization result had a lower energy value than the single-point calculation. The lower energy obtained after geometric optimization, the more stable the compound will be because the energy required to break the molecular bonds of the compound will be greater. The interaction is in the form of powerful attraction between atoms, while the repulsion between atoms becomes minimal, so the confirmation of the compound obtained is more stable (Mukesh and Rakesh, 2011).

Target protein preparation is the second step in the molecular docking method. The target protein will be docked with the optimized test compound. Protein preparation aims to separate the native ligand from the target protein, providing space or a binding site that is used during the docking process. Protein preparation involves selecting a chain that binds to the appropriate native ligand. Using a single chain makes it easier to determine the space coordinates (pocket cavities) of the ligands to be used for docking.

In protein preparation, water molecules (H_2O) are removed from each target protein whose no native ligand. Removing the water molecule is to leave only amino acid residues on the target protein so that later those that interact with the test compound, namely quercetin, are amino acids. Removing water molecules in each target protein chain also maximizes the energy between the test compound and the target protein (Huey et al., 2012; Kitchen et al., 2004). The results obtained from this preparation are proteins without native ligands and the structure of native ligands. Which results from this protein preparation will be used in the validation stage of the molecular docking method. Fig. 2 shows the results of the target protein preparation.

The validation stage of the analytical method is important to ensure that the method used in the research is acceptable, validated, and meets the requirements for its use. The molecular docking method was validated by redocking the native ligand of each target protein to the target protein without a native ligand. The validation parameter of the molecular docking method is the RMSD (Root Mean Square Deviation) value from the docking results. RMSD is the deviation of the position of the native ligand that binds to the protein after docking compared to the position of the true native ligand binding before being separated. The larger the RMSD value, the greater the deviation, which indicates the greater the prediction error of the ligand interaction with protein. The

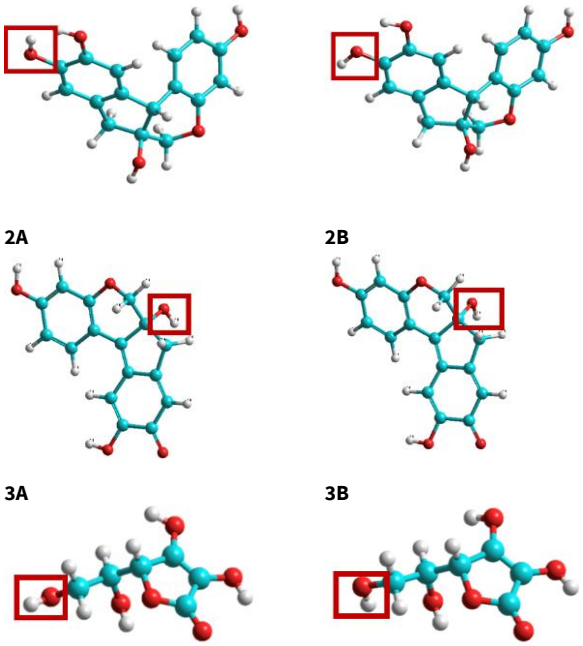

Figure 1. 2D Brazilin and brazilein structure.

(1A): Brazilin structure using single point calculation; **(1B):** Geometry optimization of brazilin; **(2A):** Brazilein structure using single point calculation; **(2B):** Geometry optimization of brazilein; **(3A):** Ascorbic acid structure using single point calculation; **(3B):** Geometry optimization of ascorbic acid.

Red box: Compound conformation changes after geometry optimization.

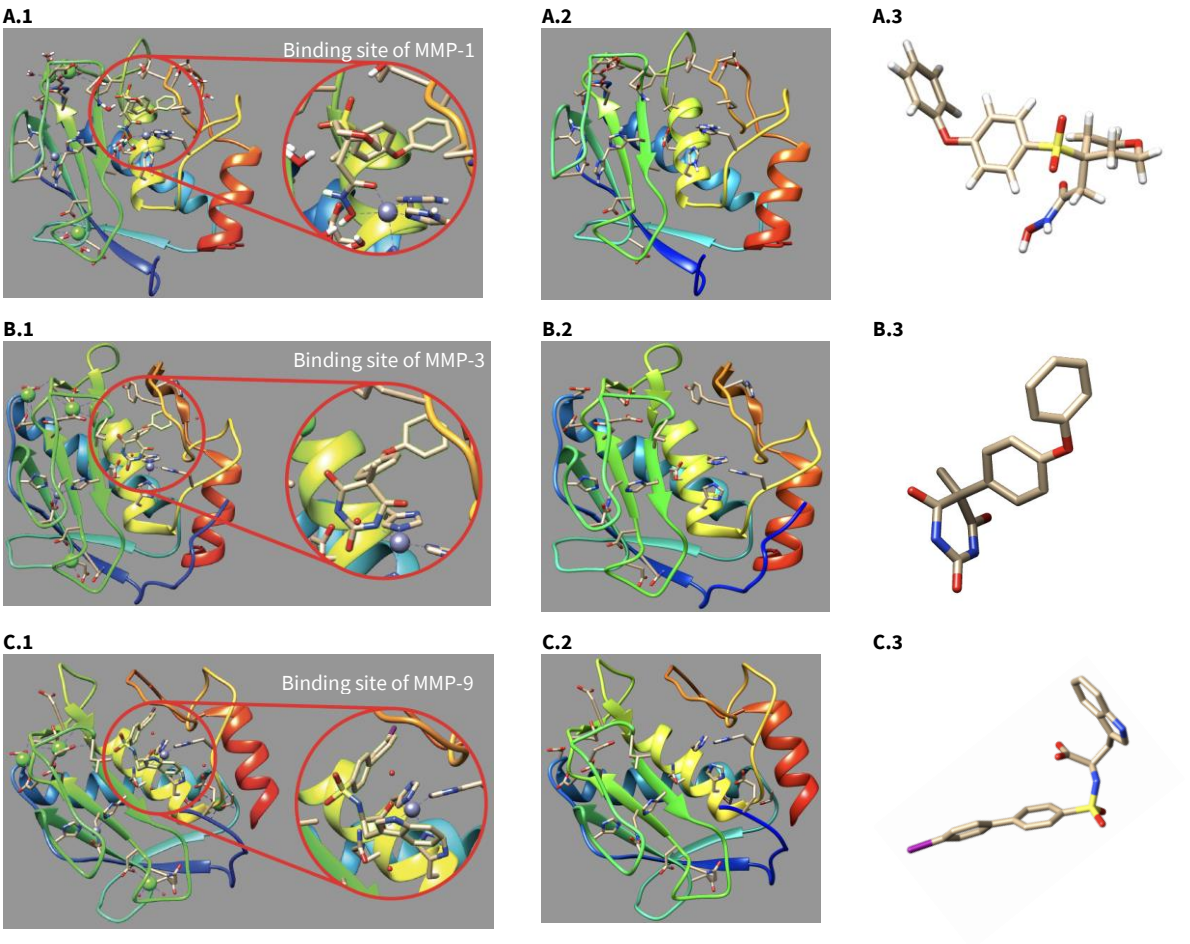

Figure 2. The target protein (MMP-1, MMP-3 and MMP-9) preparation.

(A.1): The structure of MMP-1 with native ligand (PDB ID: 966C); **(A.2):** MMP-1 structure without native ligand; **(A.3):** Native ligand (N-hydroxy-2-[4-(4-phenoxy-benzenesulfonyl)-tetrahydro-pyran-4-YL]-acetamide) structure; **(B.1):** The structure of MMP-3 with native ligand (PDB ID: 1G4K); **(B.2):** MMP-3 structure without native ligand; **(B.3):** Native ligand (5-methyl-5-(4-phenoxy-phenyl)-pyrimidine-2,4,6-trione) structure; **(C.1):** The structure of MMP-9 with native ligand (PDB ID: 2OW0); **(C.2):** MMP-9 structure without native ligand; **(C.3):** Native ligand (N-[(4'-Iodobiphenyl-4-YL)sulfonyl]-D-tryptophan).

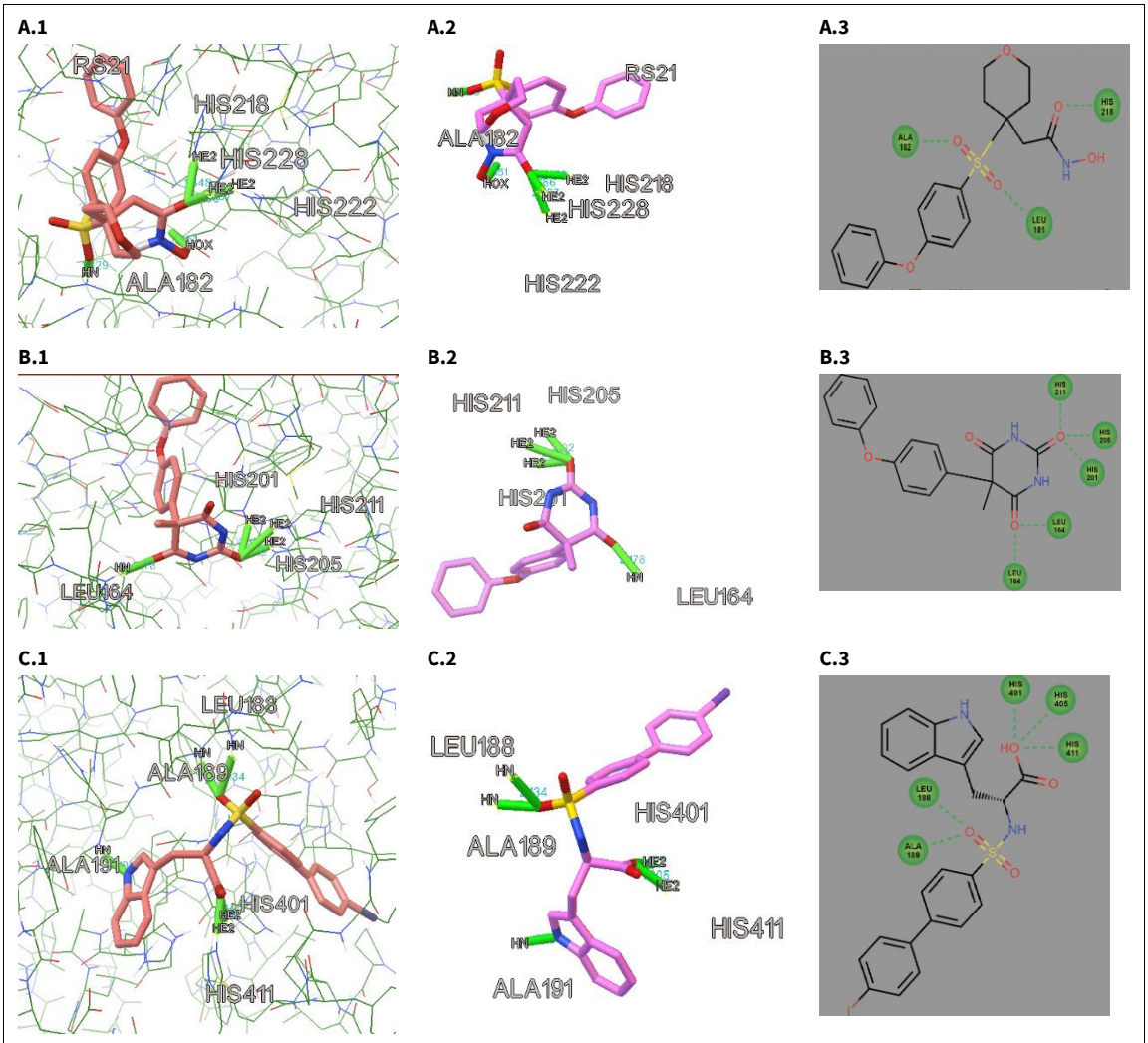

Figure 3. Redocking visualization between the target protein (MMP-1, MMP-3, and MMP-9) and native ligand.

(A.1): Redocking conformation of MMP-1; **(A.2):** 3D interaction of amino acid residues from MMP-1 with native ligand; **(A.3):** 2D interaction of amino acid residues from MMP-1 with native ligand; **(B.1):** redocking conformation of MMP-3; **(B.2):** 3D interaction of amino acid residues from MMP-3 with native ligand; **(B.3):** 2D interaction of amino acid residues from MMP-3 with native ligand; **(C.1):** Redocking conformation of MMP-9; **(C.2):** 3D interaction of amino acid residues from MMP-9 with native ligand; **(C.3):** 2D interaction of amino acid residues from MMP-9 with native ligand.

smaller the RMSD value indicates that the predicted pose is closer to the true native ligand conformation. The best conformation has the lowest RMSD value. The lowest RMSD means the native ligand coordinates are close to or almost identical to the initial native ligand position before being separated. A large RMSD value indicates that the coordinates of the native ligand after redocking are getting farther away or different. The RMSD value required to state that the molecular docking method used is valid is in the range of 3 (Jain and Nicholls, 2008). The RMSD values from the validation process for MMP-1, MMP-3 and MMP-9 proteins were 0.98; 1.07; 1.05 Å. The visualization of the results of the molecular docking validation process is shown in Fig. 3. The results were obtained from the docking process between the test compound with MMP-1, MMP-3, and MMP-9 in the form of bond energy and hydrogen bonds between brazilin and brazilein compounds with amino acid residues in the target protein. Table 6 describes the bond energy values between the test compound and the target protein compared to native ligands. The bond energy in the molecular docking process indicates the magnitude of the affinity between the test compound and the three target proteins. Affinity is the ability of an active component to bind to the target protein (Morris et al., 2012). The bond energy produced from brazilin and brazilein as the test compound and ascorbic acid as reference drug to the three target proteins had a negative value. Negative bond energy indicates the affinity between the test compound and the target protein. A negative energy value shows a reaction takes place spontaneously and a stable system that allows the formation of bonds, while a positive energy value means that a system has very little or no tendency for a reaction to occur so that bonds are not formed (Mukesh and Rakesh, 2011).

Table 6. Comparison of bond energies of the active compound in *C. sappan* and native ligand with the target protein (MMP-1, MMP-3, and MMP-9).

Ligand	The target protein	Bond energy (kcal/mol)	Amino acid residues	Hydrogen bond protein-ligand
Native Ligand	MMP 1	-10.85	GLU219	HOX-OE2
			HIS218	HE2-O31
			HIS228	HE2-O31
			HIS222	HE2-O31
			ALA182	HN-O25
	MMP 3	-10.29	HIS201	HE2-O2
			HIS205	HE2-O2
			HIS211	HE2-O2
			LEU164	HN-O6
	MMP 9	-12.30	HIS411	HE2-OXT
			ALA191	HN-NE1
			LEU188	HN-OBE. OAP
			ALA189	HN-OAP
			HIS401	HE2-OXT
Brazilin	MMP 1	-8.04	LEU181	HN-O
			HIS228	HE2-O
	MMP 3	-10.40	-	-
	MMP 9	-8.70	GLN402	HE22-O
Brazilein	MMP 1	-8.82	GLU219	OE2-O
	MMP 3	-10.99	TYR223	HN-O
			HIS237	HD1-O
			ARG179	HH21-O
			GLN283	HE21-O
			CYS285	HN-O
	MMP 9	-8.51	GLN402	HE22-O
Ascorbic acid	MMP 1	-5.22	TYR237	O-O
			THR241	O-O
			ALA234	O-O
			THR241	HN-O
	MMP 3	-5.63	LEU222	O-O
			HIS224	HN-O
			THR215	O-O
			HIS224	HE2-O
			HIS224	O-O
	MMP 9	-5.54	ALA417	O-O
			ARG424	HN-O
			TYR420	O-O

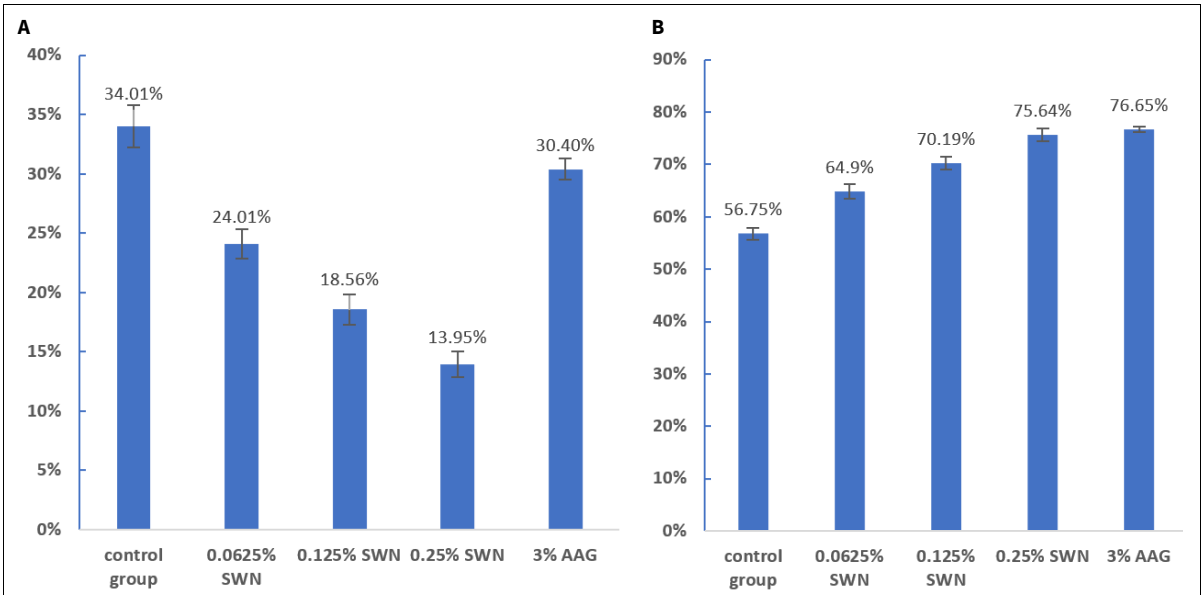

Figure 4. Nanoemulgel containing *C. sappan* nanoemulsion (SWN) and ascorbic acid gel (AAG) inhibit the MMP-1 expression and increase the collagen dermis.

(A): Percentage of MMP-1 expression after exposure of UV-B irradiation; **(B):** Percentage of collagen dermis after exposure of UV-B irradiation (with a total dose of 840 mJ/cm^2) three times a week for a month. Data are expressed as mean ± SEM (n = 7).

C. sappan nanoemulgel obstructs the MMP-1 expression and encourages collagen production using *in vivo* assay

In vivo assay was carried out for four weeks, and then a biopsy was performed on the skin tissue of the back of the rat. The mean percentage of MMP-1 expression in the placebo group was 34.01%, while in the treatment group, the content of 0.0625, 0.125, and 0.25% *C. sappan* nanoemulsion in nanoemulgel was 24.10, 18.56, and 13.95% (Fig. 4A), respectively. These results indicate a difference of 9.91 to 20.06%. The difference was significant with $p<0.05$. Therefore, *C. sappan* nanoemulgel decreased MMP-1 expression in male Wistar rats exposed to UV-B light.

The average percentage of dermal collagen in the control group showed results of 56.75%, while in the group that received 0.0625, 0.125, 0.25% nanoemulgel of *C. sappan* nanoemulsion was 64.90, 70.19, and 75.64% (Fig. 4B). There is a difference of 8.15% to 18.89%, with a $p<0.05$. The data expressed that *C. sappan* nanoemulgel increased the amount of dermal collagen in male rats exposed to UV-B light. The histopathological examination of the tissue obtained is shown in Figs. 5 and 6.

DISCUSSION

Antiaging is a part of cosmetics that contains ingredients to reduce wrinkles (wrinkles) and increase the skin's moisture level (moisture). The primary function of antiaging preparations is to reduce wrinkles and spots. Today there are more and more developments in delivery systems in cosmetics to increase penetration and optimize the cost of using active ingredients and therapeutic effectiveness. The main concern in cosmetics is reaching skin cells. Nanoemulsion is a delivery system that can increase the efficacy and bioavailability of active ingredients. The nanoemulsion system provides greater penetration than conventional emulsions because it has a smaller particle size and surface tension. Nanoemulsions were prepared using the Self-Nanoemulsifying Drug Delivery System (SNEDDS) (Sokolov, 2014). SNEDDS is a drug delivery method by making isotropic mixtures of oil, surfactants, cosurfactants, and active substances that spontaneously form oil-in-water nanoemulsions and produce nanometer-scale droplets (Makadia et al., 2003). The use of nanoemulsions through the topical route, one of which is through a gel dosage form (nanoemulgel).

In this study, an evaluation of the antiaging activity of a *C. sappan* nanoemulsion was carried out utilizing an *in vivo* assay. Before testing *in vivo*, it is necessary to determine the nanoemulsion formula of *C. sappan* to deliver active substances to skin cells as antiaging. Based on physical characteristics, including physical stability, clarity, droplet size, and zeta potential value, the best formula for *C. sappan* nanoemulsion could be determined. The formula with the composition of olive oil: Cremophor RH40: PEG 400 (1:7:2) is the best because only the 1:7:2 formula with % transmittance meets the requirements. A good nanoemulsion has clear visuals with a 90-100% transmittance value. Droplet size significantly affects the physical appearance of the nanoemulsion. Suppose the nanoemulsion has a tiny droplet size, and

light can pass through. In that case, the light beam is passed on so that the physical appearance of the solution looks transparent, and the percentage of transmittance produced is more remarkable (Costa et al., 2012).

The nanoemulgel preparations containing *C. sappan* nanoemulsion with concentrations of 0.0625, 0.125, and 0.25% have met the criteria for an adequate nanoemulgel preparation. Research on the antiaging effectiveness of *C. sappan* nanoemulgel against mice can be continued. The antiaging activity of topical preparations in the form of nanoemugel from *C. sappan* was carried out by evaluating the ability of *C. sappan* nanoemulgel to prohibit MMP-1 production and collagen degradation after UV-B exposure. Increased production of MMP-1 and collagen degradation is one of the causes of photoaging.

Exposure to UV light on the skin will stimulate the formation of ROS. Although ROS are continuously produced by the body and are involved in physiological processes, continuous exposure to UV light can lead to ROS accumulation in high concentrations. Two weeks of UV-B exposure can cause inflammation, and ten weeks of cumulative exposure will lead to the formation of significant wrinkles on the skin of mice (Lin et al., 2019). Antioxidants produced by the skin to overcome the adverse effects of ROS often cannot overcome the high accumulation of ROS, resulting in oxidative stress (Naidoo and Birch-Machin, 2017). ROS synthesis due to exposure to UV light will cause ROS aggregation and pro-inflammatory cytokines (Varma et al., 2017). Aggregation of ROS also impacts the induction of fibroblast damage, where there will be collagen degradation and decreased collagen synthesis (Lin et al., 2019; Xiao et al., 2018). Fibroblasts are the main cell component in the dermis, where collagen fibers, elastin fibers, and other matrix components are secreted by these cells (Xiao et al., 2018). Overexposure to ROS will induce cytokines responsible for the breakdown of the matrix, where IL-6 can stimulate the production of MMP-1. As a result, wrinkles, sagging skin, and other photoaging changes are formed (Varma et al., 2017).

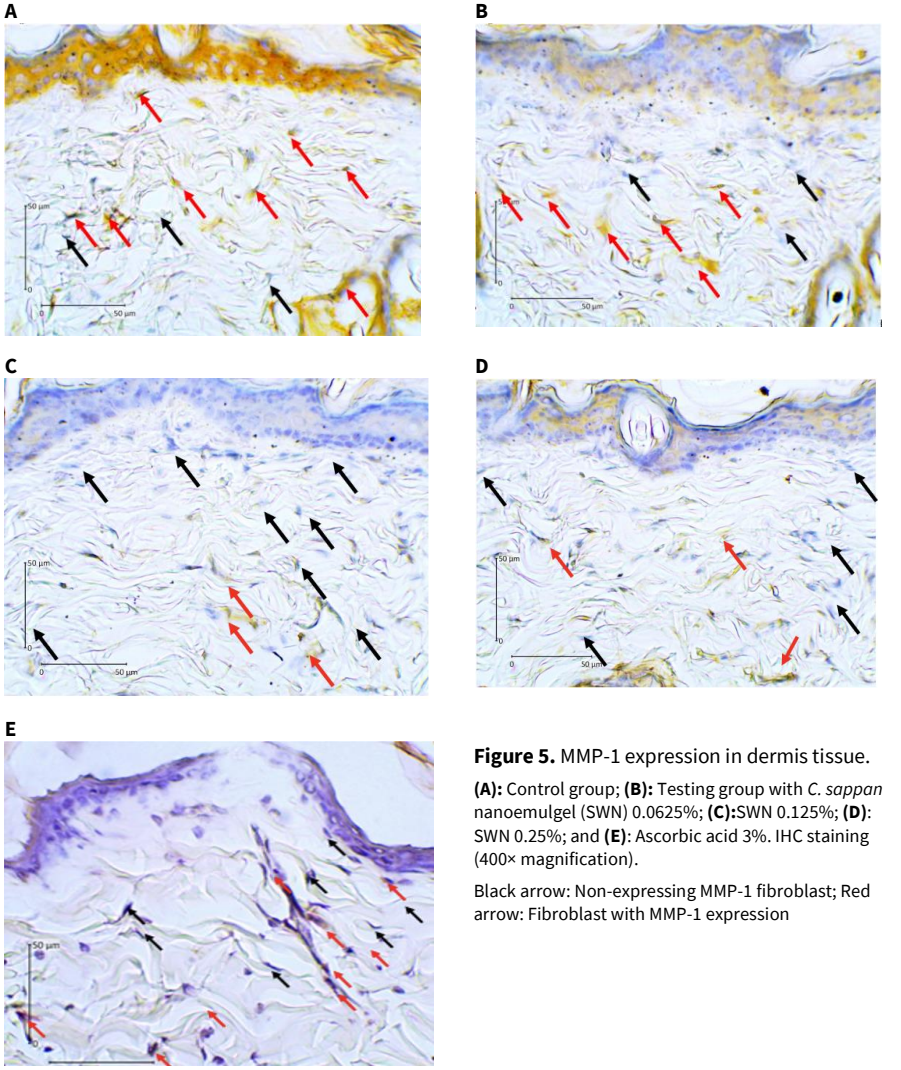

Figure 5. MMP-1 expression in dermis tissue.

(A): Control group; **(B):** Testing group with *C. sappan* nanoemulgel (SWN) 0.0625%; **(C):**SWN 0.125%; **(D)**: SWN 0.25%; and **(E)**: Ascorbic acid 3%. IHC staining (400× magnification).

Black arrow: Non-expressing MMP-1 fibroblast; Red arrow: Fibroblast with MMP-1 expression

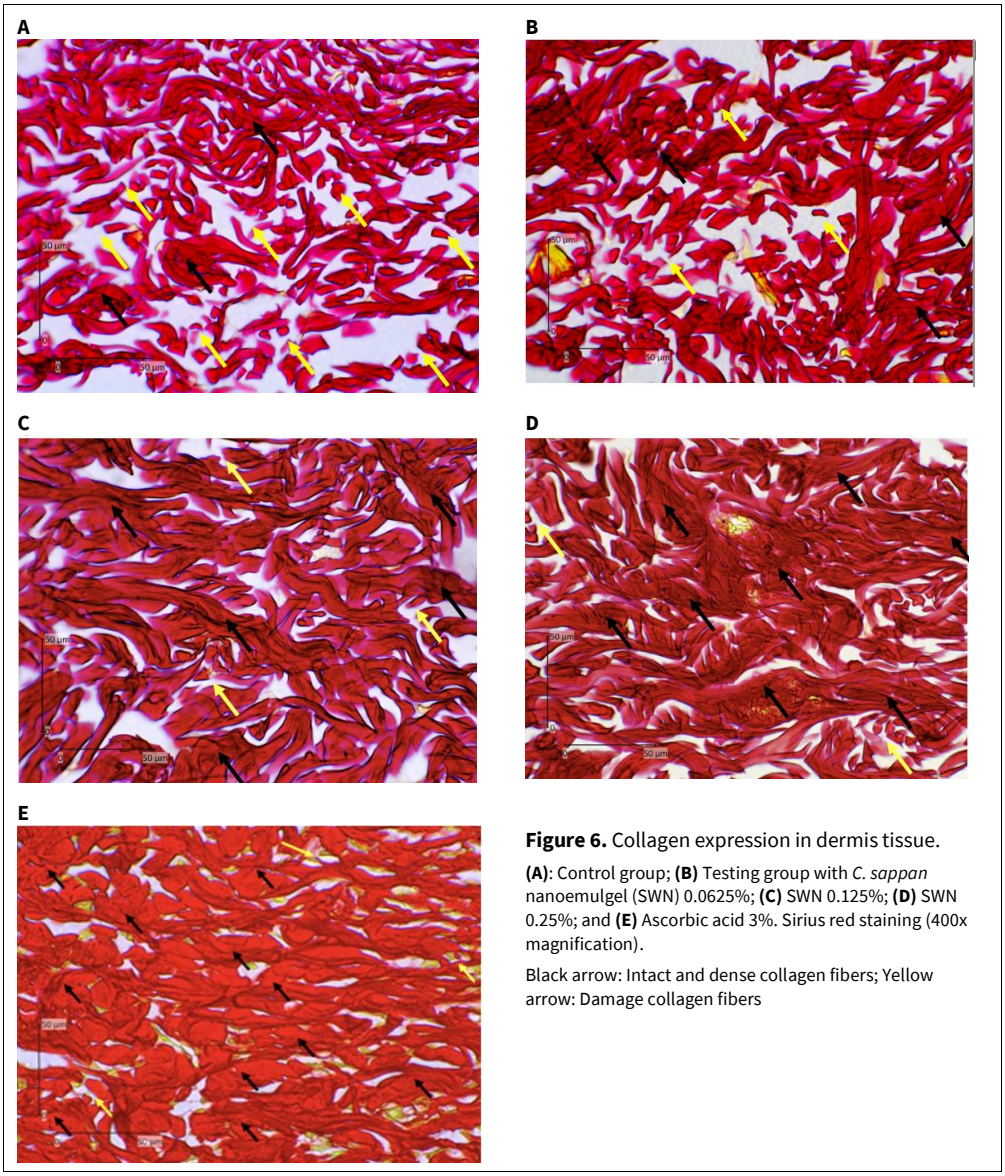

Figure 6. Collagen expression in dermis tissue.

(A): Control group; **(B)** Testing group with *C. sappan* nanoemulgel (SWN) 0.0625%; **(C)** SWN 0.125%; **(D)** SWN 0.25%; and **(E)** Ascorbic acid 3%. Sirius red staining (400x magnification).

Black arrow: Intact and dense collagen fibers; Yellow arrow: Damage collagen fibers

Collagenase, or MMP-1, is an enzyme responsible for collagen degradation. The balance of the MMP-1 enzyme levels is always maintained in the human body. This balance begins to be disturbed because, with age, collagen production decreases, and exposure to UV rays that cause ROS makes the body produce more MMPs (Mulyani et al., 2017). These things will make the skin look wrinkled.

MMP-1 expression in skin fibroblasts was significantly increased 12 hours after exposure to UV-B light, and at 72 hours after exposure, MMP-1 expression increased 4.5-fold compared to the control group that did not get exposure (Xiao et al., 2018). This study found that exposure to UV-B rays for four weeks with a total dose of 840 mJ/cm2 could increase MMP-1 expression in the control group, who only received a placebo by 34.01%. In contrast to the group that received nanoemulgel with *C. sappan* content of 0.0625, 0.125, and 0.25%, the expression of MMP-1 was only 24.01, 18.56, and 13.95%, respectively. The percentage of MMP-1 due to treatment was analyzed statistically. The data were normally distributed and homogeneous ($p>0.05$), so it was continued with the One-way ANOVA and LSD tests. Based on the ANOVA data, there were significant differences in the treatment group ($p<0.05$), and the LSD test explained that each variation in the concentration of *C. sappan* nanoemulsion on nanoemulgel against the control group gave significantly different effectiveness ($p<0.05$). The 0.25% *C. sappan* nanoemulgel has the best activity in repressing and overcoming the overexpressing of MMP-1.

Ascorbic acid has been shown to inhibit photoaging for topical preparations with a concentration of 3-10% by interrupting collagen degradation and increasing collagen synthesis (Firas and Nicole, 2017; Wang et al., 2022). Ascorbic acid was used as a positive control with anti-photoaging activity in this research. Fig. 5 shows that ascorbic acid with a concentration of 3% was not able to disrupt MMP-1 expres-

sion as though the *C. sappan* treatment group. However, ascorbic acid prevented collagen degradation after exposure to UV with the amount of dermal collagen that was not significantly different ($p>0.05$) from the administration of *C. sappan* nanoemulsion with a concentration of 0.25%.

The phenolic and flavonoid components, namely brazilin and brazilein, contained in a *C. sappan* have been known to have significant antioxidant benefits. Phenols play a role through electron transfer mechanisms, while flavonoids through their ability to reduce free radicals and metal-chelating. The hydroxyl group of polyphenols interacts with the functional chain of collagenase. The hydrophobic interaction between the benzene ring of polyphenols and collagenase can render this enzyme ineffective. Polyphenols and flavonoids, which act as metal chelators, can also bind to Zn ions on the active site of the collagenase chain, thus preventing the breakdown of enzymes (Pientaweeratch et al., 2016).

Free radicals induced by UV exposure will trigger the upregulation of AP-1 and NF-κB and suppress the regulation of transforming growth factor-β. These proteases will simultaneously upregulate MMP, responsible for collagen degradation and suppressing collagen production (Al-Niaimi and Chiang, 2017). In this study, it was seen that the *C. sappan* nanoemulsion in the nanoemulgel inhibiting MMP-1 expression also increased the amount of dermal collagen. There was a significant collagen amount difference between the placebo and treatment groups ($p<0.05$).

Molecular evaluation of the active content of *C. sappan,* namely brazilin and brazilein, against MMPs target proteins (MMP-1, MMP-3, and MMP-9) was also conducted to determine the binding affinity between the active substance and the target protein. The docking of the active substance with the target protein also showed the presence of hydrogen bonds formed from the interaction of brazilin and brazilein with these proteins. Chemical bonds other than hydrogen bonds can also occur due to flexible ligands interacting with receptors. Other interactions besides hydrogen bonds that contribute to the ligand-receptor's bond energy value (ΔG) can be hydrophobic and electrostatic interactions formed from Van der Waals and ionic bonds (Mukesh and Rakesh, 2011). The interaction formed involves the nearest amino acid residue at the binding site of the target protein.

A comparison of the bond energy results between native ligands with brazilin and brazilein in MMP-1, MMP-3, and MMP-9 can be seen in Table 6. Thus, the affinity of the three native ligands for the target protein was greater than the active substance (except MMP3). However, the negative bond energy still indicates that the compound has an affinity for MMP-1, MMP-3, and MMP-9. The affinity of the active substance to the target protein was greater than ascorbic acid. *In silico* assay data was appropriate with *in vivo* assay as ascorbic acid was less able to inhibit MMP 1 expression than *C. sappan*. The presence of this affinity illustrates that brazilin and brazilein compounds can provide the potential for inhibiting MMP-1, MMP-3, and MMP-9, minimizing the occurrence of collagen degradation as one of the causes of aging. The potential activity of *C. sappan* can also be seen from the *in vivo* assay, where *C. sappan* nanoemulsion on nanoemulgel suppressed MMP-1 and increased the amount of collagen after exposure to UV-B.

CONCLUSION

Brazilin and brazilein have an affinity to three target proteins that play an important role in breaking down collagen (MMP-1, MMP-3, MMP-9). The bond energy between brazilin and brazilein as active compounds in *C. sappan* produces a negative value that indicates *C. sappan* potential as an antiaging agent. In *in vivo* testing, the *C. sappan* nanoemulsion in the nanoemulgel preparation is able to act as antiaging in preventing and slowing down the aging process that occurs due to exposure to UV-B rays. The antiaging activity of *C. sappan* is reflected in the low expression of MMP-1 and the high amount of dermal collagen in the treatment group.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

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Contribution	Laksmiani NPLL	Leliqia NPE	Paramita NLPV	Arijana IGKN	Wijayanti NPAD	Adiwibawa PI	Putra IMH	Pratama IPAAC
Concepts or ideas	x			x	x			
Design	x	x	x	x	x	x	x	x
Definition of intellectual content	x	x	x	x	x	x	x	x
Literature search	x	x						
Experimental studies	x	x						
Data acquisition	x	x	x	x	x	x	x	x
Data analysis	x	x	x	x	x	x	x	x
Statistical analysis	x	x						
Manuscript preparation	x	x	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x	x

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