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The Effect of Adding Ethanol Extract Fraction of Secang Wood (*Caesalpinia sappan* L.) on the Antioxidant Activity of Serum Preparations

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ABSTRACT

Free radicals can trigger cell damage, premature aging, and even degenerative diseases. One preventive measure is the use of antioxidants. Sappan wood (Caesalpinia sappan L.) is known to contain bioactive compounds, such as flavonoids, tannins, and brazilin, which help neutralize free radicals. This study aims to evaluate the antioxidant activity of various fractions of sappan wood ethanol extract and to assess changes in its activity after formulation into a serum preparation. Extraction was carried out by maceration with 96% ethanol, followed by liquid-liquid fractionation with n-hexane, ethyl acetate, and water. Antioxidant activity testing was carried out using the FRAP (Ferric Reducing Antioxidant Power) method using quercetin as a comparator. Serum was formulated from each fraction and evaluated for its physical properties, including organoleptic, homogeneity, pH, viscosity, spreadability, adhesiveness, and drying time. The results showed that the ethyl acetate fraction had good antioxidant activity with an IC_{50} value of 7.29 µg/mL. After being formulated into a serum, the IC_{50} value was 6.65 µg/mL and met all physical quality parameters for topical preparations. Thus, the ethyl acetate fraction of sappanwood has the potential to be developed as an active ingredient in antioxidant serum preparations.

INTRODUCTION

Free radicals are molecules that have one or more unpaired electrons, making them highly reactive and can damage body cells through oxidation reactions (Himawan et al., 2018). Antioxidants play a crucial role in combating free radicals that can damage cells, and one method for measuring their potential is using the IC50 value (Isnaeni, 2021). The content of sappan wood, which is rich in flavonoids and tannins, has been shown to have significant antioxidant activity, with the IC50 value indicating its effectiveness in fighting oxidative damage. Nurullita & Irawati (2022) and Tanzaq et al (2019) confirmed that sappan wood extract (Caesalpinia sappan L.) has strong antioxidant activity, as evidenced by its low IC_{50} values of 56.32 ppm and 74.44 µg/mL,

respectively. Serums are cosmetic formulations containing high concentrations of active ingredients and designed for deeper penetration into the skin (Suleman et al., 2023). Serums allow for faster and more optimal effects and are easier to spread across the skin's surface (Handayani et al., 2023).

METHODS

1. Materials and Equipment

Sappan wood (*Caesalpinia sappan* L.) obtained from the Tawangmangu area, 96% ethanol, n-hexane, ethyl acetate, distilled water, $FeCl_3$ solution, 1% $K_3Fe(CN)_6$, TCA, acetate buffer pH 6.6, quarcetine, carbomer, triethanolamine, sodium benzoate, propylene glycol, glycerin, Mg Powder, Dragendroff Reagent, Wagner Reagent, Folin-Ciocalteu Reagent, Liebermann-Burchard Reagent, Concentrated HNO3, analytical balance, measuring cylinder (Pyrex), beaker (Pyrex), separating funnel, rotary evaporator (B-One), UV-Vis spectrophotometer (Shimadzu), Silica Gel F-254, Brookfield viscometer, pH meter, spreadability, adhesion tester, stopwatch.

2. Sample Preparation

1200 grams of the sample was sorted and ground using a grinder. Then, it was sieved using a No. 40 sieve.

3. Extraction

1000 g sample was added to 96% ethanol at a 1:5 ratio, then stirred every 8 hours. Extraction using the maceration method was carried out for 3 x 24 hours.

4. Fractionation

The thick extract was dissolved in ethanol, then fractionated successively with n-hexane and ethyl acetate (1:1) using a separating funnel. Each fraction was separated, evaporated using a rotary evaporator, and then evaporated at 50°C.

5. Thin Layer Chromatography

Flavonoid compounds were confirmed by TLC using quercetin as the standard. The sample and standard were spotted onto a silica gel 60 F-254 plate, then eluted with a mobile phase of butanol:glacial acetic acid:water (4:1:5).

6. Preparation of 0.2M Phosphate Buffer Solution, pH 6.6

The solution was prepared by dissolving 2 g of NaOH in CO_2-free distilled water to 250 mL, and 6.8 g of KH_2PO_4 in CO_2-free distilled water to 250 mL. A total of 16.4 mL of NaOH solution was mixed with 50 mL of KH_2PO_4 solution, the pH was adjusted to 6.6, and then the volume was topped up with CO_2-free distilled water to 200 mL.

7. Preparation of Quercetin Stock Solution

A standard quercetin solution was prepared at 1000 ppm by dissolving 50 mg of quercetin in ethanol to a final volume of 50 mL. This stock solution was diluted to 100 ppm (2.5 mL of stock solution in a 25 mL flask). A series of solutions was then prepared: 10 ppm (2.5 mL in a 25 mL flask), 15 ppm (3.75 mL in a 25 mL flask), 20 ppm (5 mL in a 25 mL flask), 25 ppm (6.25 mL in a 25 mL flask), and 30 ppm (7.5 mL in a 25 mL flask).

8. Determination of Maximum Wavelength

The maximum wavelength was determined using a 30 ppm quercetin standard solution. A total of 1 mL of quercetin solution was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$, vortexed for 5 minutes, incubated at 50°C for 5 minutes, then 1 mL of 10% TCA was added. The mixture was centrifuged at 3000 rpm for 5 minutes, then 1 mL of the upper layer was taken and mixed with 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. After being left for 30 minutes, The maximum wavelength obtained was 675 nm.

9. Operating Time

The procedure was carried out by mixing 3 mL of FRAP reagent and 1 mL of distilled water, then measuring the absorbance at the maximum wavelength every 1 minute using a UV-Vis spectrophotometer until the value stabilized. The operating time was determined when the absorbance reached stability.

10. Determination of Quercetin Antioxidant Activity

1 mL of quercetin standard solutions (10, 15, 20, 25, and 30 ppm) was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$, vortexed for 5 minutes, incubated at 50°C for 5 minutes, and then added 1 mL of 10% TCA. The mixture was centrifuged at 3000 rpm for 5 minutes, then 1 mL of the upper layer was taken and mixed with 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. After 30 minutes, the absorbance was measured at 675 nm using a UV-Vis spectrophotometer.

11. Determination of Antioxidant Activity of Fractions

A 10 mg fraction of the ethanol extract of sappan wood was dissolved in 10 mL of 96% ethanol to obtain a 1000 ppm solution. 5 mL of this solution was diluted with 10 mL of 96% ethanol to obtain a 500 ppm solution. Next, 1 mL of the 500 ppm solution was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$, vortexed for 5 minutes, incubated at 50°C for 20 minutes, and then 1 mL of 10% TCA was added. The mixture was centrifuged at 3000 rpm for 10 minutes. 1 mL of the upper layer was removed, 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ were added, allowed to stand for 25 minutes, and the absorbance was measured at 675 nm. This procedure was performed in triplicate.

12. Formulation of Serum

Ingredient	F1 (%)	F2 (%)	F3 (%)	F4 (%)
Aqueous Fraction	–	–	1%	–
Ethyl Acetate Fraction	–	–	–	1%

Quercetin	–	1%	–	–
Carbomer	0.4%	0.4%	0.4%	0.4%
Triethanolamine (TEA)	0.04 %	0.04 %	0.04 %	0.04 %
Sodium Benzoate	0.2%	0.2%	0.2%	0.2%
Propylene Glycol	10%	10%	10%	10%
Purified Water	ad 100 mL	ad 100 mL	ad 100 mL	ad 100 mL

The serum formulations were prepared using a gel-based system. Initially, carbomer was dispersed gradually into a sufficient amount of purified water under continuous stirring until a homogeneous dispersion was obtained. The mixture was allowed to hydrate completely for approximately 24 hours to ensure optimal swelling of the carbomer. Separately, sodium benzoate was dissolved in purified water, followed by the addition of propylene glycol as a humectant. The resulting solution was stirred until completely homogeneous. Quercetin or the active fractions (aqueous fraction or ethyl acetate fraction of *Caesalpinia sappan* L.) were then dissolved or dispersed in the propylene glycol phase according to the formulation design.

Subsequently, the active-containing solution was slowly added to the hydrated carbomer dispersion under continuous stirring to obtain a uniform mixture. Triethanolamine (TEA) was added dropwise to the mixture to neutralize the carbomer and adjust the viscosity, resulting in the formation of a clear and stable serum. Finally, purified water was added to adjust the total volume to 100 mL. The serum was stirred gently until a homogeneous and bubble-free preparation was achieved. The final product was then evaluated for physical appearance and stored in a tightly closed container at room temperature for further analysis.

13. Determination of Antioxidant Activity of Serum Fractions

1 mL of serum was dissolved in 10 mL of 96% ethanol to obtain a concentration of 1000 ppm. 5 mL of this solution was diluted with 10 mL of 96% ethanol to obtain a concentration of 500 ppm. Next, 1 mL of 500 ppm solution was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$, vortexed for 5 minutes, incubated at 50°C for 20 minutes, then 1 mL of 10% TCA was added. The mixture was centrifuged at 3000 rpm for 10 minutes, 1 mL of the upper layer was taken, added with 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$, left for 25 minutes, then the absorbance was measured at 675 nm. The procedure was carried out in three replications.

14. Serum Preparation Characteristics Test

a. Organoleptic Test

Observe the serum preparation organoleptically, including its shape, odor, and color.

b. Homogeneity Test

A small amount of the serum preparation is taken and ensured to be homogeneously mixed before testing. The preparation is then applied to the surface of a glass slide using a spatula or pipette. Afterward, observations are made to assess the presence of coarse particles, clumps, or uneven distribution within the preparation.

c. Viscosity Test

Viscosity testing is performed by attaching spindle number 3 to the spindle hanger and then lowering it until the spindle is submerged in the sample.The viscometer is turned on at 6 rpm and allowed to rotate for 1 minute. The viscosity value is then read from the display on the instrument

d. pH Test

The pH test procedure is performed by weighing approximately 0.5 g of serum and dissolving it in 5 mL of distilled water. A pH meter calibrated with a standard buffer solution is used to measure the pH of the solution.

e. Spreadability Test

A 0.5 g of serum is placed on a glass surface and left for 1 minute, then the spread diameter is measured. Afterward, a 150 g load is placed on top of the serum and left for 1 minute, then the spread diameter is measured again.

f. Adhesion Test

A 0.5 g serum sample is taken, placed between two glass objects, and a 250 g load is applied to the sample for 5 minutes. Afterward, the time it takes for the serum to begin to detach from the glass surface is measured

g. Drying Time Test

This test is performed by applying a consistent amount of serum to the skin surface, usually the back of the hand or wrist. After application, the time is measured from the time the preparation is applied until it is completely dry, indicated by the disappearance of the sticky or wet sensation on the skin.

h. Stability Testing

The stability chamber is set to the desired test conditions, namely, for accelerated testing, a temperature of 40°C ± 2°C and a relative humidity of 75% ± 5%. The sample is positioned so that its entire surface is evenly exposed to temperature and humidity.

15. Data Analysis

IC_{50} data were analyzed using SPSS software. Normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. If the data met the assumptions of normality and homogeneity ($p > 0.05$), differences among groups were analyzed using One-Way ANOVA followed by Tukey's post hoc test. If the assumptions were not fulfilled, the Kruskal–Wallis test was applied. Statistical significance was set at $p < 0.05$ with a 95% confidence level.

RESULT AND DISCUSSION

Result

1. Extaction and Fractination

This study used the maceration method, which involves soaking sappanwood powder in 96% ethanol (1:5) for 72 hours with periodic stirring. This method was chosen to maintain thermolabile compounds and maximize the extraction of active compounds. After maceration, the solvent was evaporated with a rotary evaporator and water bath at 50°C, producing 97.3297 g of extract with a yield of 9.73%, meeting the Indonesian Herbal Pharmacopoeia standard (≥8.1%). From 35 grams of thick extract, 12.09 grams of water fraction, 7.96 grams of ethyl acetate fraction, and 1 gram of n-hexane fraction were obtained.

2. IC_{50} Value of Fraction and Quarcetine

Fraction Type	IC_{50} Value ± SD (n=3)	Category
Quercetin	2.1073 ± 0.0907	Very strong (< 50)
Ethyl Acetate Fraction	7.2859 ± 0.3629	Very strong (< 50)
Aqueous Fraction	9.5243 ± 0.1694	Very strong (< 50)
n-Hexane Fraction	21.8559 ± 7.5736	Very strong (< 50)

3. IC_{50} Value of Serum Preparation

Sample Type	IC_{50} Value ± SD (n=3)	Category
Serum containing Aqueous Fraction	8.1683 ± 0.0405	Very strong (< 50)
Serum containing Ethyl Acetate Fraction	6.6499 ± 0.3995	Very strong (< 50)
Serum containing Quercetin	1.5786 ± 4.0617	Very strong (< 50)

Discussion

Extraction and Fractination

Extraction aims to obtain secondary metabolites from samples, influenced by the method, solvent, particle size, and soaking time. This study used the maceration method, which involves soaking sappanwood powder in 96% ethanol (1:5) for 72 hours with periodic stirring. This method was chosen to maintain thermolabile compounds and maximize the extraction of active compounds. Fractionation aims to separate compounds based on differences in polarity using two solvents with different polarities. This process utilizes differences in polarity and density to separate the resulting fractions (Pratiwi et al., 2016). Fractionation of sappanwood ethanol extract was carried out to separate bioactive compounds based on their polarity so that each fraction could be analyzed specifically for its antioxidant content and activity. This process also aims to concentrate polar compounds such as flavonoids and reduce the influence of inactive compounds.

Thin Layer Chromatography

The presence of flavonoids in the sample was confirmed by thin layer chromatography (TLC) using quercetin as a reference compound, because sappanwood is suspected to contain flavonoids. The mobile phase used was Butanol: Glacial Acetic Acid: Water (4:1:5). The reason for using eluent B:A:A (4:1:5) in the flavonoid test on TLC is because flavonoid compounds have relatively polar properties, so a mobile phase that is also polar is needed to achieve a balanced interaction between the mobile phase and the compounds. This similarity in polarity helps separate flavonoid compounds effectively, because the compounds can move well from the stationary phase (silica gel which is also polar) to the mobile phase, resulting in a clearly separated spot pattern (Nuari et al., 2017). The maximum wavelength is the wavelength at which electronic excitation produces the highest absorbance. Measurements at this wavelength provide the most significant absorbance change per concentration, thereby increasing analytical sensitivity (Apriliyani et al., 2018). Determining the maximum wavelength aims to determine the point at which the absorbance change with concentration is greatest, thus achieving optimal analytical sensitivity (Rantung et al., 2021). Test results show a maximum wavelength of 675 nm. Operating time aims to determine the measurement time when the compound's absorbance reaches its most stable state. Measurements are performed by monitoring changes in the solution's absorbance to ensure the correct observation time, thereby minimizing measurement errors (Suharyanto & Prima, 2020). Based on observations, the absorbance of the FRAP reagent began to stabilize at 25 minutes. This condition indicates that the reaction between the reagent components has completed, resulting in stable absorbance values.

Serum Evaluation

1. Organoleptic testing

Organoleptic testing showed that all serum preparations—both base and those containing quercetin, ethyl acetate fraction, and water fraction—had a uniform, slightly viscous texture, indicating that the active ingredient did not visually alter the viscosity. The color of the preparations changed from clear (base) to pale yellow to dark yellow in the formulations containing the active ingredient, likely due to the quercetin compound and other components in sappan wood extract. All preparations also had no strong odor, making them comfortable to use without disturbing odors.

2. Homogeneity

Homogeneity testing showed that all serum preparations—both base and those containing quercetin, ethyl acetate fraction, and water fraction—mixed evenly and consistently across all three repetitions. No phase separation, clumps, or mixing imperfections were observed. Good homogeneity is essential for maintaining physical stability and ensuring even distribution of active ingredients during application. Therefore, all preparations meet homogeneity standards for topical cosmetics.

3. Viscosity

Viscosity testing on various serum preparations before

and after the stability test showed that the viscosity values remained within the specified range (230–3000 cps). Statistical analysis using a t-test revealed no significant difference between the viscosity before and after the stability test ($p > 0.05$). This indicates that the serum preparations had good viscosity stability and did not experience significant physical changes during the testing period.

4. pH

Ph testing on various serum preparations before and after the stability test showed that the pH values remained within the specified range (4.5–6.5). Changes in pH during storage were relatively small, and a Wilcoxon statistical test showed no significant difference between the pH before and after the stability test ($p > 0.05$). This indicates that all preparations had good pH stability without significant chemical changes during the testing period.

5. Spreadability

Spreadability testing on various serum preparations before and after the stability test showed that the values remained within the acceptable range (5–7 cm). Although there were slight changes after the stability test, these changes were not statistically significant. A t-test showed no significant difference between spreadability before and after testing ($p > 0.05$). This indicates that all serum preparations had good spreadability stability and maintained their physical characteristics throughout the testing period.

6. Adhesion

Adhesion testing of various serum preparations before and after the stability test showed that all preparations met the requirements, with an adhesion time of more than 1 second. However, there was a significant decrease in adhesion values after the stability test ($p < 0.05$). This change is likely influenced by factors such as active ingredient concentration, storage temperature, stirring method, pH, particle size, and viscosity of the formulation, all of which can affect the physical properties of the serum and its adhesion to the skin.

7. Drying Time

Drying time testing of various serum preparations before and after the stability test showed that all preparations met the requirements, with a drying time of less than 5 minutes. Although there were slight changes in some preparations, these changes were not statistically significant ($p > 0.05$). This indicates that all preparations have good drying time stability during storage, so that the serum's ability to dry on the skin is maintained.

IC_{50} Value of Fraction and Quarcetine

This study shows that the IC_{50} values of sappanwood extract fractions based on the FRAP method are 7.2859 µg/mL for the ethyl acetate fraction, 9.5243 µg/mL for the water fraction, and 21.8559 µg/mL for the n-hexane fraction. Quarcetine as a standard has an IC_{50} value of 2.1073 µg/mL. According to the principle of antioxidant activity, a lower IC_{50} value indicates a higher antioxidant capacity. Thus, the ethyl acetate fraction has the strongest antioxidant activity compared to other fractions, although it is still below the strength of quarcetine.

IC_{50} Value of Serum Preparation

Based on the Games-Howell test, the antioxidant activity of the aqueous fraction serum and ethyl fraction serum was not significantly different compared to the original fraction ($p > 0.05$). This indicates that the formulation in serum form did not significantly reduce antioxidant activity. The aqueous fraction serum had the highest IC_{50} value, indicating the lowest antioxidant activity among the other preparations. In conclusion, the ethyl acetate fraction and the aqueous fraction of the ethanol extract of sappan wood showed very strong antioxidant activity, especially after being formulated into serum. Evaluation of the physical quality of serum containing fractions showed that all formulas were physically stable, including organoleptic, viscosity, homogeneity, pH, spreadability, drying time, and adhesiveness, although there was a slight decrease in adhesiveness after the stability test.

CONCLUSION

The ethyl acetate fraction of sappanwood extract showed the highest antioxidant activity with an IC_{50} value of 7.29 µg/mL. After being formulated into a serum preparation, its antioxidant activity decreased to 6.65 µg/mL, but remained very potent. Furthermore, the formulated serum preparation showed good physical stability during storage, as evidenced by organoleptic, pH, viscosity, spreadability, and homogeneity tests that still met the established criteria.

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