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Original Research Article

In-silico and In-vitro Studies of Antioxidant and Sun Protection Activities of Sappan Wood (*Caesalpinia sappan* L.)

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ABSTRACT

Sappan wood which contains brazilin and brazilein is known to have antioxidants potential because of its hydroxyl groups. The use of antioxidants in sunscreen preparations increases photoprotective activity. The purpose of this study was to evaluate the antioxidant activity of sappan wood, to determine the affinity of brazilein and brazilin for glutathione peroxidase (GPX) and catalase protein targets *in silico*, and describe the ability of sappan wood as an *in vitro* sun protection agent. The antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferrous ion chelating (FIC) assays, while the *in silico* test was carried out by using molecular docking with the autodock 4.2 programs. Sunscreen activity was tested by determining the sun protection factor (SPF) using the spectrophotometric method. Sappan wood was found to have very high antioxidant activity, with IC_{50} values of 0.88 ppm and 1.75 ppm for the ethyl acetate fraction and methanol extract, respectively, which were lower than that of ascorbic acid (7.8 ppm) in the DPPH scavenging assay. In the FIC assay, the IC_{50} values were 69.46, 62.59, and 10.21 ppm for the extract, the fraction and EDTA, respectively. SPF value of the extract and the fraction of sappan wood were 17.91 and 19.53 at a 200 ppm concentration each. Brazilein and brazilin in sappan wood can induce GPX and catalase. Sappan wood has great potential to be developed as a sunscreen with a dual function as antioxidants and as UVB rays absorber.

Keywords: Antioxidant, Docking, DPPH, FIC, Sappan wood, Sun protection.

Introduction

Indonesia is one of the countries with a tropical climate, which means that Indonesians are exposed to more solar radiations every year than in countries that are not crossed by the equator.[1-3] Solar radiation are dangerous because it can trigger free radicals. Thus, due to the risks of solar radiation on human skin, it is necessary to have antioxidant compounds in every sunscreen.[4-6]

Antioxidants work by donating one electron to free radicals or reactive oxygen species (ROS) so that the antioxidant inhibit the activity of these oxidant compounds. These free radicals are reactive in looking for their electron pair. It produces new free radicals, which will make the number of free radicals continues to increase.[7-10] In the body, there is intracellular ROS scavenging system that protects us from those oxidation processes. For example, superoxidase dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are major enzymes that breakdown ROS.[11,12] However, sometimes, the body is exposed to free radicals in an amount that exceeds the body's antioxidant defense capacity. Therefore, our bodies need essential antioxidants substances in order to protect the body from free radical attacks by reducing their harmful effects and preventing photoaging.

Sunscreens are cosmetic ingredients that physically or chemically block the penetration of UV rays into the skin.[13] There are also natural sunscreens, such as phenolic compounds found in plants and can be used to protect the skin against the damaging effect of solar radiation.[14] Some plants contain flavonoids, and phenolic compounds that have antioxidant benefits are potentially great as antioxidant cosmetics and sunscreens.[15-18]

Caesalpinia sappan L, from the Caesalpiniaceae family is commonly found in Indonesia. Sappan wood is empirically known to have many healing properties and is often consumed by the public as a herbal drink. Sappan wood contains the main compounds, namely brazilin, and brazilein.[19] Based on the research results, water extract from sappan wood had been shown to have higher antioxidative index compared to commercial antioxidants Butyl Hydroxy Toluene (BHT) and Tertiary Butyl Hydroxy Quinon (TBHQ), even though both BHT and TBHQ are synthetic compounds that had relatively high antioxidant activity.[20,21] The methanol extract of sappan wood, increases SOD, and CAT in test animals.[22] However, no research had discussed the ability of sappan wood in different solvents (methanol and ethyl acetate) as a DPPH free radical scavenging agent, iron-chelating agent, and its capacity as a sunscreen. The purpose of this research is to evaluate the ability of sappan wood as an antioxidant *in vitro* (DPPH scavenging and ferrous ion chelating method), and *in silico* induction of GPX and catalase, and also to evaluate its ability as a sunscreen.

Materials and Methods

Materials

Sappan wood was purchased and identified from Center for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT) Tawang - mangu with the register number YK.01.03 / 2/1381/2018. Sodium hydroxide (Merck), 98% gallic acid (MP Biomedicals), 2,2-diphenyl-1-picrylhydrazyl or DPPH (Sigma Aldrich), ascorbic acid (Sigma-Aldrich), Folin-Ciocalteu reagent (Sigma-Aldrich, USA), sodium carbonate (Sigma-Aldrich, USA), iron

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(III) chloride (Sigma-Aldrich, USA), aquadest (Bratacho, Indonesia), ferrozine (Hach, USA), ferrous sulfate heptahydrate (Sigma-Aldrich, USA), ethylene diamine tetra acetic acid (EDTA) disodium salt, quercetin (Merck, Germany), methanol (Merck, Germany), ethyl acetate (Merck, Germany), silica gel TLC plate GF 254 nm (Merck) for phytochemical screening and TLC profiling of extract and fraction, chloroform (Merck, Germany) and hexane (Merck, Germany). The equipment used consists of a UV-Visible (Shimadzu) spectrophotometer, measuring pipette, and bulb filler for *in vitro* studies. Computer set with Windows 10 64 bit specifications equipped with Autodock 4.2 program, Chimera 1.10.1, and Hyperchem 8 for *in silico* assay.

Extraction of plant sample

A total of 500 g of sappan wood powder was macerated in methanol (5 L) for three days with occasional stirring. The extraction process was repeated three times. The macerate was filtered, then evaporated using a rotary evaporator until a thick extract was obtained. Meanwhile, to obtain the ethyl acetate fraction of sappan wood, fractionation of 5 g methanol extract of sappan wood was carried out with a liquid-liquid extraction method using hexane and ethyl acetate solvents. The ethyl acetate fraction of sappan wood was collected and evaporated.

Determination of Total Phenolic Content

Sappan wood methanol extract and sappan wood ethyl acetate fraction were weighed (3.8 mg each), and dissolved in 10 mL ethanol (test solution). 0.1 mL test solution was pipetted, then 0.9 mL distilled water and 0.5 mL of Folin-Ciocalteu reagent were added, then shaken and left to stand for 5 min. 2.5 mL of 7% Na_2CO_3 solution was added to the mixture, shaken homogeneously, and allowed to stand for 26 min at room temperature. The absorbance of the mixture was measured at 759 nm using a UV-Vis spectrophotometer. The determination was done in triplicate. The phenolic content was obtained from gallic acid calibration curve (Y= 0.1168x + 0.2401), and expressed as mg gallic acid equivalent/g of sample extract.[23]

Determination of total flavonoid content

15 mg of the samples were weighed and dissolved in 10 mL ethanol, to obtain a concentration of 1500 ppm. 1 mL of 2% $AlCl_3$ solution and 1 mL of potassium acetate (120 mM) were added. Samples were incubated for one hour at room temperature. The absorbance was determined using the UV-Vis spectrophotometric method at a maximum wavelength of 435 nm. Samples were made in three replicates for each analysis and the mean absorbance value was recorded. The concentration of total flavonoid content in the test samples were obtained from the calibration curve (Y= 0.0965x-0.0156) and the value expressed as mg quercetin equivalent/g of sample extract.[24]

TLC Profiling of extract and fraction of sappan wood

Sample solutions were prepared by dissolving the extract and fraction in methanol so that a concentration of 100 ng/µL was obtained. Then the extract and fraction were spotted with different volumes, namely 2, 4, 6, and 8 µL respectively using an automatic TLC sampler 4 (ATS 4) and eluted with a mixture of hexane: chloroform: methanol (1: 7: 2 v/v). The plate was inserted into the chamber that had previously been saturated with the mobile phase. After elution, the plates were dried at 60°C for 5 min on a hotplate. The plates were scanned using the TLC-Scanner 3 Camag at a wavelength of 210 nm, and the peak spectrum was scanned at 200-700 nm.

Determination of DPPH radical scavenging activity

A total of 200 µL of test samples from each concentration (0.1, 0.5, 5, 7, and 10 µg/mL) were added to 800 µL of DPPH Solution (200 µM in methanol). The mixture was shaken and incubated in the dark at room temperature for 30 min. This method was repeated twice. The absorbance of the mixture solution was measured at a wavelength of 517 nm using a spectrophotometer. The positive control used was Vitamin C (ascorbic acid) treated the same way as in the sample.[25,26]

The percentage DPPH radical-scavenging activity was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100) \quad \text{.....} \quad (1)$$

Where A_0 = the absorbance value of control (DPPH without test solution)
A_1 = the absorbance value of the test solution (DPPH + samples or ascorbic acid).[26]

Determination of ferrous ion chelating activity

FeSO4 0.25 mg/mL (1 mL) was mixed with 2 mL of sample (methanol extract of sappan wood or ethyl acetate fraction of sappan wood) and EDTA (standard), then 3 mL of 0.02 M ferrozine was added. The mixture was incubated for 10 min, and the absorbance (A) was measured at a wavelength of 562 nm. The test sample solution concentration was in the range of 3.33-83.33 µg/mL. This range of concentration variations was repeated three times.[27] The percentage Chelating ability was calculated using the following equation:

$$\text{Chelating ability (\%)} = [(A_0 - A_1) / A_0] \times 100 \quad \text{.....} \quad (2)$$

Where A_0 = absorbance of control (Ferrozine-FeSO4 solution)
A_1 = Absorbance of the test solution or standard absorbance

In-vitro sun protection activity assay

Determination of the protective activity against UV rays was carried out *in vitro* using a UV-Vis spectrophotometer. The absorbance of a 200 µg/mL test solutions (extract and fraction of sappan wood and standard UV-B absorber, namely benzophenone) were measured with a UV-Vis spectrophotometer at a wavelength of 290-320 nm using ethanol as a blank. The absorbance value was observed at interval of 5 nm.[28,29] The absorbance results were recorded and the SPF (Sun Protecting Factor) was calculated using the following equation:

$$\text{SPF} = \text{CF} \times \sum_{290}^{320} \text{EE}(\lambda) \times \text{I}(\lambda) \times \text{abs}(\lambda) \quad \text{------} \quad (3)$$

Where:
CF: correction factor (=10); EE (λ): erythemal effect spectrum; I (λ): solar intensity spectrum (as a constant); abs (λ): absorbance of the test solution.

In-silico antioxidant activity assay

This test described and evaluated the ability of sappan wood using brazilein and brazilin as major compounds and biomarkers in sappan wood as antioxidants binding ability to GPX and CAT as target proteins. In the *in silico* test, the optimization of the structure of brazilin, brazilein, and ascorbic acid (as positive control) was carried out with hyperchem 8; GPX and CAT target protein preparation was done with chimera 1.11 software; validation of the method (reflected by the root mean square distances / RMSD ≤ 3.0 Å).[30] Then, brazilein and brazilin docking with the target protein was performed using autodock tools (autodock 4.2 and autogrid). The final step was to analyze the data, including the bond energy and the types of hydrogen bonds formed.[31] The result of molecular docking was recorded as the bond energy (kcal/mol) and the type of hydrogen bond formed between the compound and the target protein. The type of hydrogen bond formed between brazilein and brazilin with the target protein was used for the analysis of the interaction model formed between the test compounds and the amino acid residues of the target protein. Meanwhile, the bond energy obtained was used for the affinity analysis of brazilein and brazilin against the target protein. A negative bond energy results indicated that the compound has an affinity for the target protein.

Statistical analysis

The IC_{50} and SPF data from the test samples were statistically analyzed using oneway ANOVA, followed by the LSD Post Hoc test.

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The comparison between the TPC or TFC levels of ethanol extract and ethyl acetate fraction of Sappan wood was analyzed by independent t-test. The relationship between antioxidant activity (IC_{50}) and TPC and TFC levels was analyzed by using the Pearson Correlation test. Significant differences were stated with p-value < 0.05.

Results and Discussion

The total phenolic contents of sappan wood

Phenolic compounds are the most abundant components produced by secondary plant metabolism. Some researchers claimed that there is a linear relationship between the content of phenolic compounds and antioxidant activity.[32-34] The total phenolic content of sappan wood ethanol extract was determined by the Folin-Ciaocalteu method, and gallic acid was used as the standard. In principle, the hydroxyl group in phenolic compounds can form complexes with molybdate compounds from the Follin-Ciocalteu reagent to produce a blue complex.[35] In this research, a linear regression of standard gallic acid with Follin-Ciocalteu reagent had been successfully made, the equation is, $y = 0.1168x + 0.2401$ with a correlation coefficient value (r) = 0.9981. The results of the analysis of total phenol content is presented in Table 1. The total phenolic content of ethyl acetate fraction of sappan wood has a greater value than the methanol extract of sappan wood, but the amount is not statistically significant ($p > 0.05$).

The total flavonoid contents of sappan wood

The main components of sappan wood extract were brazilin and brazilein.[36] Brazilin and brazilein are flavonoids. Flavonoids consist of a large group of polyphenolic compounds with a benzo-gamma-pyrone structure and are found in various types of plants. The hydroxyl functional groups in flavonoids provide antioxidant effects that could scavenge free radicals and bind metals.[37] Some studies have stated that flavonoids had a protective effect against various infections (diseases caused by bacteria and viruses), degenerative diseases such as cardiovascular disease, cancer, and other diseases associated with aging.[37-39] Flavonoid contents in sappan wood extract and fraction is shown in Table 1. The total flavonoid content of ethyl acetate fraction of sappan wood has a significantly greater value than its methanol extract ($p < 0.05$).

TLC profiling of methnol extract of sappan wood and ethyl acetate fraction of sappan wood

Sappan wood had two major compounds, brazilein and brazilin. These two active compounds were used as biomarkers in sappan wood. Figure 1 showed that the TLC profile and the chromatogram of the extracts and fractions were identical. According to the UV spectrum of extracts and fractions with spots at Rf 0.47, it was suspected to be brazilein compound. Both of them had a similar spectrum as shown in figure 1D-F. The brazilein spectrum was obtained from Laksmiani *et al*. 2017.[40]

Methanol extract of sappan wood and ethyl acetate fraction of sappan wood as a potent DPPH radical scavenger

The working principle of the DPPH method is due to the presence of hydrogen atoms from antioxidant compounds that bind to free electrons in radical combinations, it caused a change from free radicals (diphenylpicrylhydrazyl) to non-radical compounds (diphenylpicrylhydrazine). DPPH method is characterized by a change in colour from purple to yellow because the presence of antioxidants reduced free radical compounds.[41]

Determination of antioxidant activity with the DPPH method used the IC_{50} parameter, which is the 50% sample concentration needed to capture DPPH radical. Table 1 and Figure 2 showed the antioxidant test results by DPPH method for sappan wood methanol extract, ethyl acetate fraction, and ascorbic acid with IC_{50} value 1.75 ppm, 0.88 ppm, and 7.8 ppm, respectively. The smaller the IC_{50} value, the stronger the antioxidant activity. Sappan wood, both in extracts and fractions, has very strong antioxidant activity with minimal IC_{50} value.[41] The IC_{50} value were below 50 ppm and even lower than that of ascorbic acid as a positive control. Antioxidant activity (IC_{50}) of ascorbic acid, methanol extract, and ethyl acetate fraction of sappan wood ($n = 3$) were significantly different ($p < 0.05$). The IC_{50} value of sappan wood showed that sappan wood might be developed into a natural antioxidant agent.

The ferrous ion chelating (FIC) activity of sappan wood

The FIC method was a method used to measure the antioxidant ability by metal chelating process. In this method, there was a competition between ferrozine and antioxidant compounds in chelating metal ions. The metal ion used in this study was Fe^{2+} from $FeSO_4$. An intense purple colour indicated the formation of the complex between Fe^{2+} and ferrozine in the solution. When a metal chelating compound was added, the purple colour intensity would begin to decrease.[42,43] Decreased purple colour intensity happened on addition of the sappan wood test solution, in which the higher the sappan wood extract concentration added to the purple $FeSO_4$, the more the colour fades. The antioxidant activity of sappan wood was compared with the metal chelating activity of EDTA. EDTA is a chelating ligand that had a strong affinity for forming complexes with metals.

Sappan wood in the form of extracts and fractions could compete with ferrozine to bind Fe metal. Table 1 and Figure 3 showed that the IC_{50} of the methanol extract of sappan wood, ethyl acetate fraction of sappan wood, and EDTA were 69.46 ppm, 62.59 ppm, and 10.21 ppm, respectively. The IC_{50} value of the test solution was classified as a compound that has antioxidant activity with a strong metal chelating mechanism because the concentration of strong antioxidants ranged from 50-100 ppm. The antioxidant activity of sappan wood was significantly different from EDTA. So sappan wood's ability to chelate Fe^{2+} is substantially lower than EDTA.

Brazilein and brazilin from sappan wood induced GPX and catalase activity in silico

In this study, we evaluated the ability of sappan wood active components; brazilein and brazilin to induce the GPX and CAT target proteins by *in silico* molecular docking with the autodock program 4.2. Before the docking process started, it was necessary to validate the molecular docking method from the RMSD (Root Mean Square Deviation) value generated in the validation process. An RMSD value of ≤3.0 Å is generally used as the requirement for the docking method success. The RMSD values for GPX and CAT were 1.93 and 0.51 Å, respectively, which means that the docking process produced valid data. Table 2 and Figure 4 demonstrated that brazilein and brazilin had potentials as antioxidants by inducing the GPX and CAT enzymes. This could be seen from the active compound which had negative bond energy so that the bonds that occured did not require any energy to bond, therefore the formed bonds were stable. Besides, compared to ascorbic acid that is known for its antioxidant ability as the positive control, brazilin and brazilein have lower energy. The binding energy value reflected that sappan wood could activate GPX and CAT so that further oxidation processes in the body could be inhibited.

Sun protection activity of sappan wood

The determination of protection against UV rays of the test compound was carried out *in vitro* using a UV spectrophotometer. In the positive control treatment, benzophenone was used because it is the most widely used ingredient in sunscreen preparations. Benzophenone is a chemical sunscreen that protects the skin by absorbing UV radiation energy which make the molecules to be excited to the higher energy level. And when these molecules return to their ground state, the emitted energy is in a lesser condition than the absorbed energy. Benzophenone is a compound that absorbs UV light at a wavelength of 290-320 nm in the UVB region. Table 1 showed that ethyl acetate fraction had a high SPF value than the methanol extract of sappan wood. The SPF value and the antioxidant activity produced by the extract and fraction of sappan wood was proportional. Both extract and fraction of sappan wood at a 200 ppm concentration had a moderate level of protection, with a range of SPF 15-30. The SPF value for methanol extract and ethyl acetate fraction of sappan wood

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was 17.91, and 19.53, respectively. In contrast, benzophenone at a 200 ppm concentration had higher protection with SPF of 30.81. Statistically, the SPF values of these three components at 200 ppm concentration were significantly different.

This study also evaluated the relationship between total phenolic contents (TPC), total flavonoid contents (TFC) and sappan wood antioxidant activity. Statistically, it was shown that TFC correlated with the antioxidant activity of sappan wood ($p < 0.05$). The higher the test compound concentration, the greater the flavonoid content resulted in a stronger antioxidant activity. The antioxidant activity referred to the ability to ward off DPPH radicals and chelate metals. Sappan wood had the potential to be developed into an active ingredient in cosmetics with a dual function, as a scavenger agent to free radicals, metal chelator so that the oxidation process is prevented and can absorb UV B. The combination of these two mechanisms of antioxidant and sun protection action of sappan wood could inhibit the aging process.

Table 1: Antioxidant activity, total phenol contents, total flavonoid contents, sun protection factor of sappan wood

No	Testing Sample	DPPH scavenging (IC_{50}) ppm	Ferrous ion chelating (IC_{50}) ppm	TPC	TFC	SPF (200 ppm
1	Methanol extract of sappan wood	1.75 ± 0.27	69.46 ± 3.36	126.25 ± 6.69	253.85	17.91 ± 0.29
2	Ethyl acetate fraction of sappan wood	0.88 ± 0.031	62.59 ± 1.15	140.65 ± 6.65	288.91	19.53 ± 0.14
3	Ascorbic acid as positive control in DPPH scavenging	7.8 ± 0.1	-	-	-	-
4	EDTA as positive control in FIC assay	-	10.21 ± 0.82	-	-	-
5	Benzophenone as positive control as sunscreen	-	-	-	-	30.81 ± 0.46

Value are calculated as mean ± SD, (n = 3).

Table 2: Docking score of brazilein and brazilin bonds to target proteins

No	Target proteins	RMSD (Å)	Ligand	Binding energy (kcal/mol)	Hydrogen bond	(Ligand-protein) group
1	GPX (2F8A)	1.93	Brazilein	-5.33	GLN 82	H-O
					ASP 144	H-O
			Brazilin	-6.02	GLN 82	H-O
					ARG 180	H-O
			Ascorbic acid	-5.23	TRP 160	H-O
					ARG 179	H-O
					TRP 160	O-N
					GLN 82	H-O
					GLY 48	H-O
2	Catalase (1QQW)	0.51	Brazilein	-9.68	ARG 112	H-O
					HIS 362	H-O
			Brazilin	-10.20	ARG 72	H-O
					VAL 74	H-O
					SER 114	O-O
			Ascorbic acid	-6.52	ARG 72	H-O
					ARG 112	H-O
					HIS 362	H-O
					SER 114	H-O

GLN 82: Glutamine with position 82; ASP 144: Aspartic acid 144; ARG 180: Arginine 180; TRP 160: Tryptophan 160; GLY 48: Glycine 48; HIS 362: Histidine 362; VAL 74: Valline 74; SER 114: Serine 114; O-H: Interaction between oxygen atom from ligand and hydrogen from protein group.

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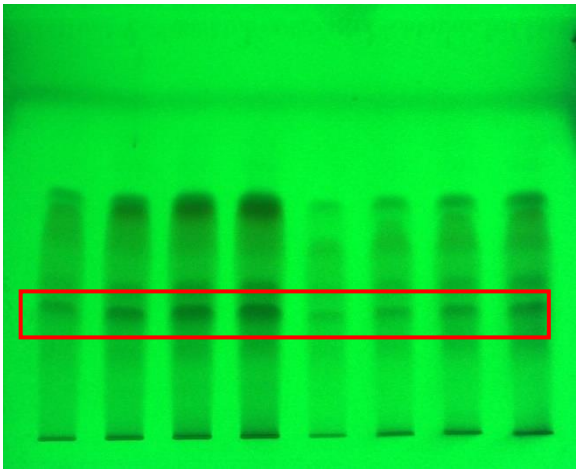

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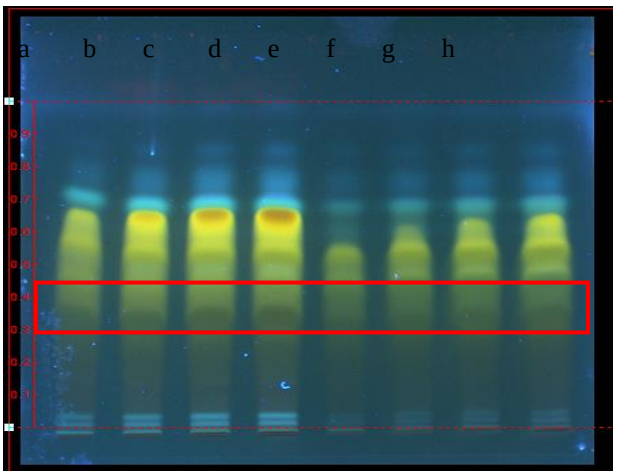

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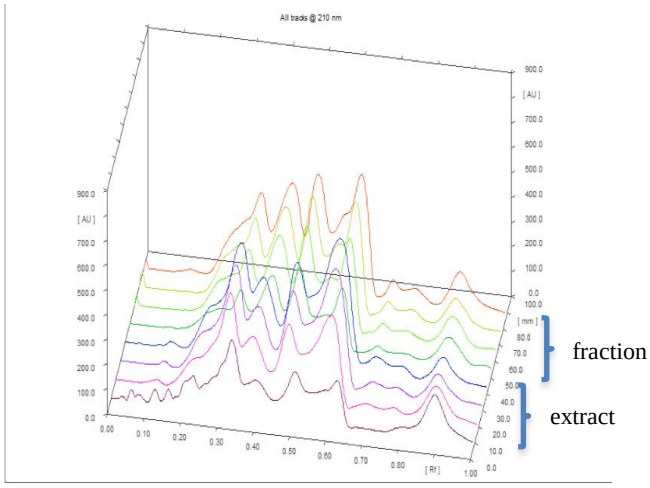

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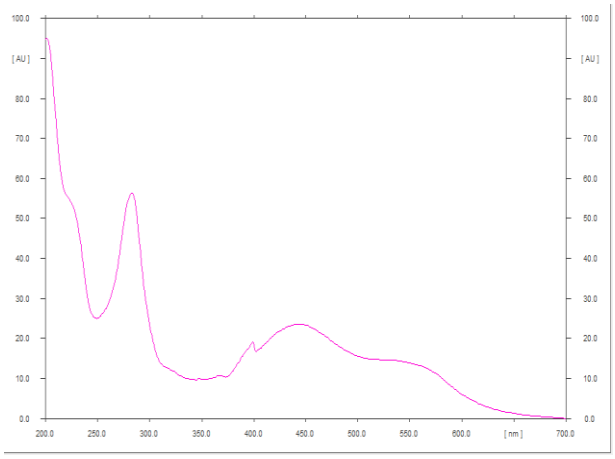

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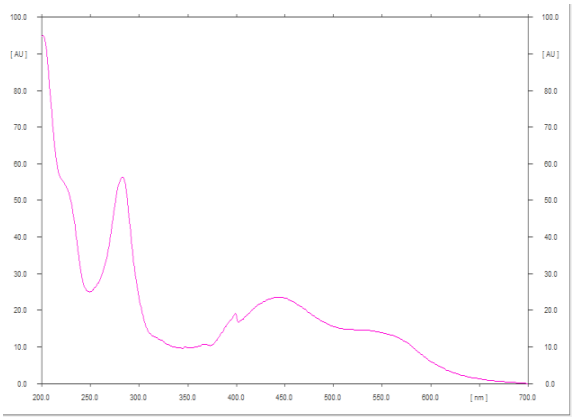

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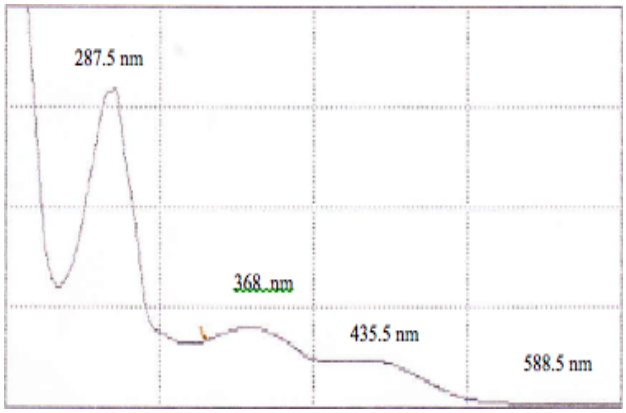

F

Figure 1: TLC chromatogram of methanol extract and ethyl acetate fraction of sappan wood at UV 254 nm **(A)** and UV 366 nm **(B)**. Methanol extract of sappan wood 200 ng/µL (a), 400 ng/µL (b), 600 ng/µL (c), 800 ng/µL (d). Ethyl acetate fraction of sappan wood 200 ng/µL (e), 400 ng/µL (f), 600 ng/µL (g), 800 ng/µL (h); TLC-chromatogram using densitogram comparison between extract and fraction of sappan wood **(C)**; UV spectrum of spot with Rf 0.47 nm from methanol extract of sappan wood **(D)**; UV spectrum of spot with Rf 0.47 nm from ethyl acetate fraction of sappan wood **(E)**; UV spectrum of brazilein isolated from sappan wood according to Laksmiani *et al.*, 2017[40] **(F)**.

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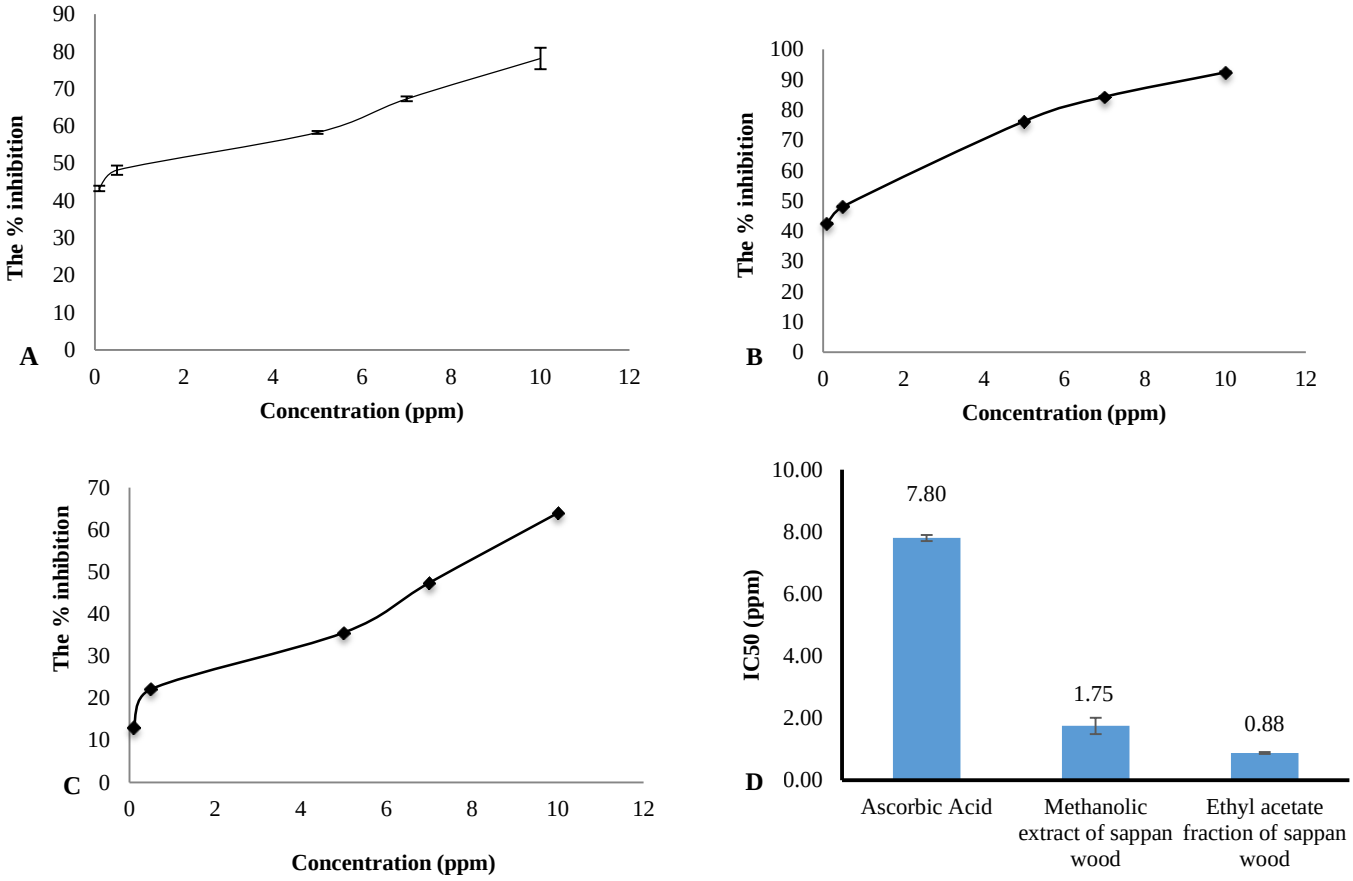

Figure 2: DPPH Radical Scavenging activity of sappan wood and Ascorbic acid. DPPH inhibitory activity of sappan wood methanol extract **(A)**, DPPH inhibitory activity of ethyl acetate fraction of sappan wood **(B)**, DPPH inhibitory activity of ascorbic acid **(C)**, Comparison of antioxidant activity (IC_{50} values of DPPH assay) between test samples **(D)**. Percentage of DPPH inhibition were expressed as mean ± SEM, (n = 3).

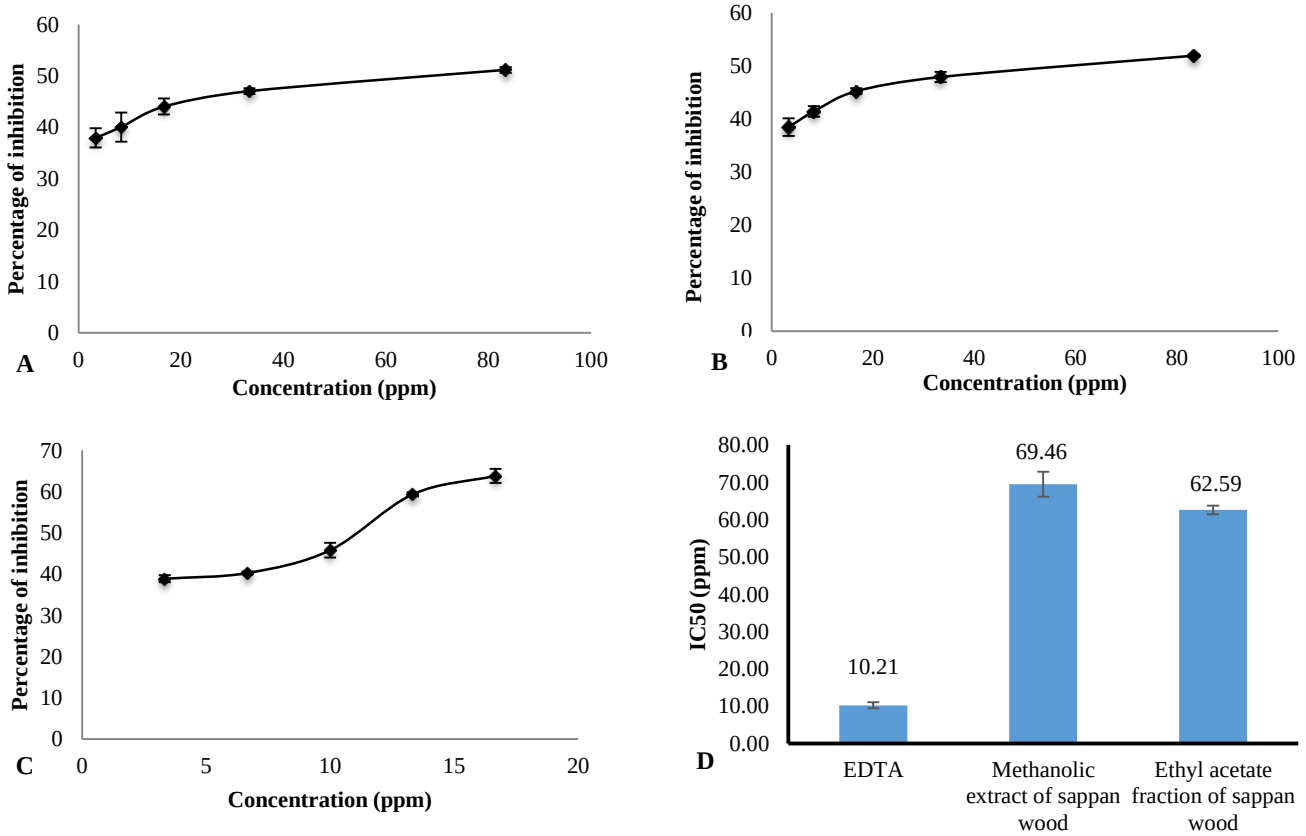

Figure 3: FIC activities of sappan wood with different solvent. FIC activity of sappan wood methanol extract **(A)**, FIC activity of ethyl acetate fraction of sappan wood **(B)**, FIC activity of EDTA **(C)**, Comparison of antioxidant activity (IC_{50} of FIC assay) between test samples **(D)**. Percentage of FIC activity were expressed as mean ± SEM (n=3).

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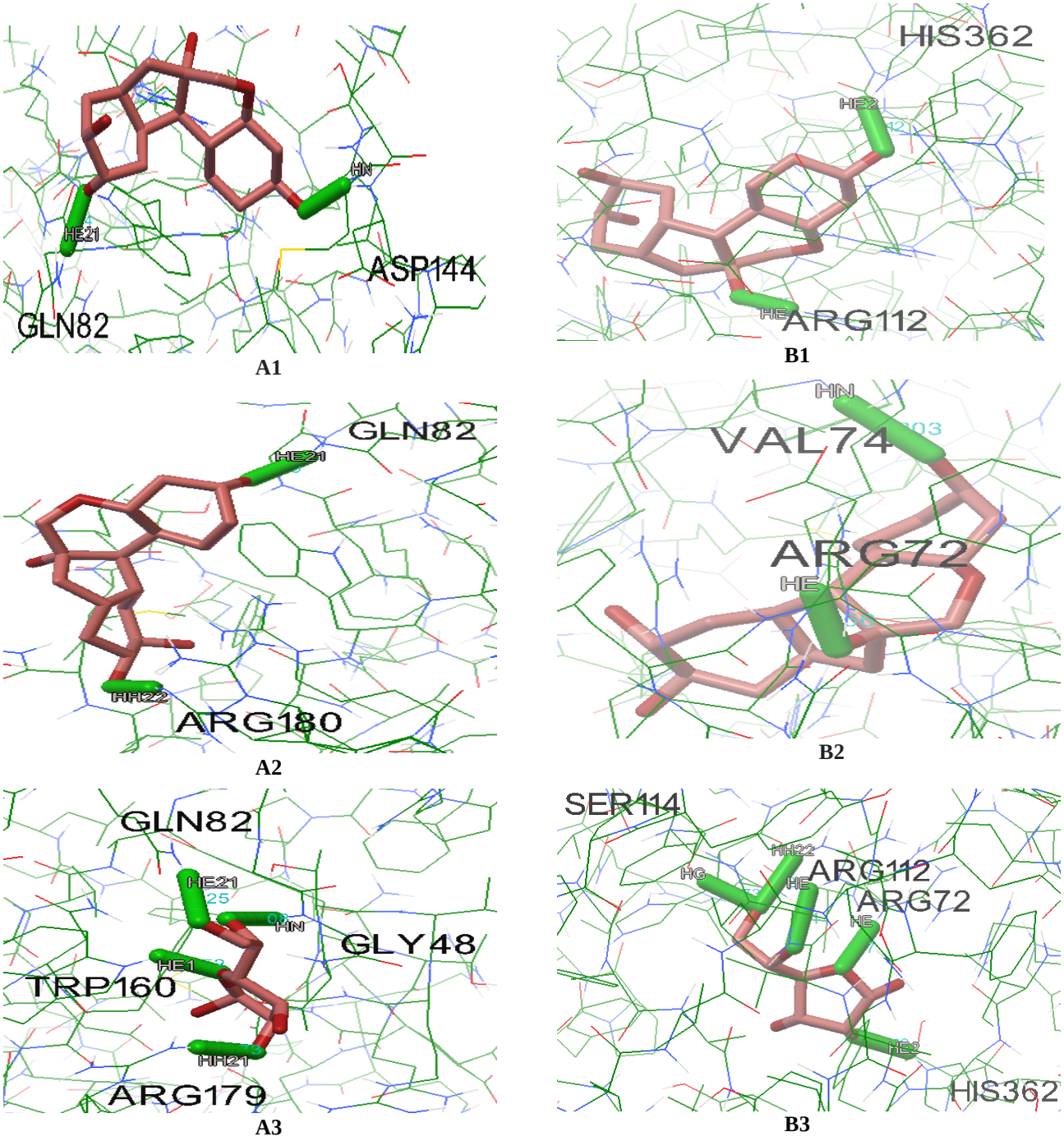

Figure 4: Interaction between of brazilein, brazilin, and ascorbic acid with target proteins (antioxidant enzymes) with amino acid residue on target protein. **A1:** interaction of brazilein with GPX; **A2:** interaction between brazilin with GPX and **A3:** interaction of ascorbic acid with GPX. **B1** interaction of brazilein with CAT; **B2:** interaction between brazilin with CAT and **B3:** interaction of ascorbic acid with CAT.

Conclusion

The ethyl acetate fraction of sappan wood had higher antioxidant activity than the methanol extract of sappan wood. Both exhibited potent antioxidant activity by inducing glutathione peroxidase and catalase, scavenged free radicals and chelate metals. Sappan wood could be developed as a sun protection agent due to its ability to absorb UVB rays.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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