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Hydroxyl Radical Scavenging Activity of *Stachytarpheta jamaecensis* Root Extract Using In Vitro Deoxyribose Degradation Assay

Juliyatin Putri Utami*, Yusrinie Wasiaturrahmah, Deby Kania Tri Putri
Department of Biomedic, Faculty of Dentistry, Universitas Lambung Mangkurat, Indonesia

ABSTRACT

Antioxidant of *S. jamaecensis* roots has a potential role as antiinflammatory, antibacterial and anticancer to treat various diseases. Antioxidant reduces the process of free radical formation or preventing the continuous and chain reaction of free radicals. The deoxyribose degradation assay is used to evaluate the hydroxyl (OH) radical-scavenging ability of *S. jamaecensis* because it was suitable for all types of antioxidants. This study aims to determine the antioxidant activity of *S. jamaecensis* root extract used the deoxyribose degradation method. Dry roots of *S. jamaecensis* (250 g) were soaked with ethanol 96% for 3x24 hours at room temperature. The liquid extract obtained is evaporated by rotary evaporator and water bath till viscous extracts are obtained. Then to extract performed phytochemical screening by a quantitative and qualitative method. The antioxidant activity, in general, will be evaluated using in vitro deoxyribose degradation assay. The extract concentrations were varied, namely 125, 250, 500, and 1000 ppm. The data obtained was performed a one-way ANOVA test. The results of the phytochemical screening showed the extract contains flavonoids, alkaloids, saponins, phenols, and tannins. The results obtained were significant values from the antioxidant test using deoxyribose degradation assay, the largest average value of root extract was 60.606% at a concentration of 1000 ppm. The results of spectrophotometric measurements showed that the extract has IC_{50} was 683.5294 µg/ml, while vitamin C has IC_{50} lower value (251.700 µg/ml).

Keywords: A*ntioxidant; Hydroxyl radical scavenger; Stachytarpheta jamaecensis; Deoxyribose Degradation Assay*

INTRODUCTION

The natural ingredients of plants play a big role in the protection of human immunity and against diseases or damage to human cells. In addition, natural products have mild side effects and low cost. Antioxidants are inhibitors to delay the oxidation of other molecules in food and biological systems. Antioxidants are compounds that can delay, slow down, and prevent the process of lipid oxidation (Ahmad, 2012). These natural antioxidants which are widely distributed in food and medicinal plants exhibit a wide range of biological effects, including anti-inflammatory, anti-aging, anti-atherosclerosis, and anticancer (Xu *et al*, 2017). The majority of the diseases are mainly linked to oxidative stress due to free radicals (Chandrasekara and Shahidi, 2017).

A free radical is a highly reactive molecule that has unpaired electrons in its outer orbital. These radicals tend to create a chain reaction which, if they occur in the body, can cause continuous and continuous damage. The human body has an endogenous defense system against free radical attack, which mainly occurs through normal cell metabolism events and inflammation (Wahdaningsih, 2011). Some free radicals can be Reactive oxygen species (ROS), reactive nitrogen species (RNS), or both such as hydroxyl (OH), nitric oxide (NO), superoxide (O^{-2}), hydrogen peroxide (H_2O_2), atomic oxygen (O) and so on (Ghimire et al, 2018).

Antioxidant activity, in general, will be evaluated using the hydroxyl free radical scavenging method. The free radical scavenging method provides information on the reactivity of the compound being tested with a stable radical. Hydroxyl radical (OH), known as the most biologically active free radical, is formed in vivo under hypoxic conditions. Reactive hydroxyl radicals cause hydroxylation of various biomolecules by reducing the unsaturated bonds present in their structures (Lipinski, 2011). Hydroxyl radicals have this very high reactivity because hydroxyl radicals (HO (O2 • -) have an electron a potential value of 940 Eo '/ mV (pH). Hydroxyl radicals can damage three important compounds that function to maintain cell integrity, namely lipids, proteins, and DNA (Treml and Smejkal, 2016; Borra *et al.*, 2014). OH produced from the lipooxygenase pathway in arachidonic acid metabolism plays an important role in the cytotoxicity of Natural Killer (NK) cells (Suthantiran, 1984). In Rojasanakul's (1993)

***Corresponding author : Juliyatin Putri Utami**
Email :

study, it has been shown that macrophages exposed to hydroxyl radicals show changes in intracellular Ca_{2+} levels to be 3-5 times higher than basal levels. Reducing the process of free radical formation or preventing the continuous and chain reaction of free radicals can be done by providing exogenous antioxidants from outside the body.

The plant that has potential as an antioxidant is *Stachytarpheta jamaecensis*. This plant belongs to the Verbenaceae family and comes from tropical America. *S. jamaecensis* has benefits as an analgesic, antacid, anti-inflammatory, antispasmodic, antiulcerogenic, digestive, diuretic, febrifuge, gastroprotective, hepatoprotective, hypoglycemic, hypotensive, sedative, and tonic (Liew and Yong, 2016). The phytochemicals contained are flavonoids, triterpenes, monoterpenes, iridoids, phytosterols, aromatic acids, GABA, dopamine, and alkanes. This plant is often used as herbal therapy. The phytochemical screening of the root of this plant revealed the presence of alkaloids, tannins, saponins, glycosides, steroids, and phenols (Chinonye, 2018). Usually, it is often used as a medicine to treat sore throat, menstrual pain, smooth blood, hemostatic, anti-inflammatory, strengthens the organs that manage the body's toxins such as the liver and kidneys. A previous study about CH2Cl2 crude of *S. jamaecensis* extract exhibited potent cytotoxicity against oral cancer cell lines (Khummueng. W., et al., 2020). Also known, there was an antibacterial activity of S. jamaecensis. It showed in The zones of inhibitions *S. jamaecensis* to microorganisms such as *B. cereus*, *P. vulgaris,* and *S. pyogenes* were larger than their positive control except for *S. typhii* (Thangiah, 2019). *S. jamaecensis* contains glycoside compounds that are usually found only in Verbenaceae group plants, namely iridoid ipolamiide and the phenylethanoid glycoside acteoside as anti-inflammatory, antioxidant and antibacterial compounds (Schapoval, 1998; Li, *et al* 2018,).

Leaves and several plant organs contain active compounds including flavonoids, alkaloids, tannins, saponins, terpenoids, phenols, and ascorbic acid (Ololade et al, 2017, Liew and Yong, 2016). The hydroxyl radical-scavenging assay is suitable for all types of antioxidants. The deoxyribose degradation assay is widely used to evaluate the hydroxyl (OH) radical-scavenging ability of food or medicines. Degradation of deoxyribose by OH radicals produced from Fenton-type reactions has measured the conditions underlying free radical initiated oxidative stress and antioxidant activity of various test compounds (Asamari *et al.,* 1996; Halliwell *et al.,* 1994). The previous study that proven the hydroxyl radical-scavenging activity of 25 antioxidant samples suggested that there was an approximately 9-fold difference between assay results for the ethanol solution and residue samples (Li *et al.,* 2013). Based on the description above, in this study, the antioxidant activity of the root of *S. jamaecensis* will be evaluated using the hydroxyl radical scavenging method.

METHODOLOGY

Materials

The materials used to make the extract of *S. jamaecensis* were the root of *S. jamaecensis*, distilled water, 96% ethanol, filter paper, NaOH, 2N HCl, 0.1% FeCl3, chloroform, H2SO4, disodium hydrogen phosphate dihydrate (Na2HPO4. 2H2O), sodium. Dihydrogen phosphate monohydrate (NaH2PO4. H2O), NaCl, 70% ethanol, 10 mM ferric chloride, 1mM EDTA, 20 mM hydrogen peroxide, 30 mM deoxyribose, 1% TBA and 5% trichloroacetic acid.

The tools used were a spectrophotometer (Shimadzu®), rotary evaporator (Heodolph®), oven (Memmert®), water bath, analytical scale (Precisa®), desiccator, glass tools, crucible cups, maceration vessels, and weigh bottles.

Methods

Sampling and Processing of Samples

The roots *S. jamaecensis* were taken from the edge of the forest on the road of Kiram village, Cempaka sub-district, Banjarbaru district, South Kalimantan province. The roots were taken in the morning. An adult plant with sufficiently large roots was taken. The roots were then separated from the stem and then collected, washed, and finely chopped. Then the roots were dried by being aerated in the open and protected from direct sunlight. The dried roots were stored in a dry and tightly closed container.

Determination of Water Content

A total of 5 grams of simplicia were carefully weighed and put into a closed porcelain crucible which had been heated at 105° C for 30 minutes and had been tared. Simplicia was smoothed in a porcelain crucible by shaking the crucible until evenly distributed. Put it in the oven, open the lid of the crucible, heat it at a temperature of 100° C to 105° C, weigh and repeat heating until you get a boarding weight.

S. jamaecensis Root Extraction

This extract was made using the maceration method and was carried out based on research conducted by Anggriawan *et al.,* (2018). The root simplicia powder of Stachytarpheta jamaicensis

was put into a maceration vessel and 96% ethanol was added until the root powder was immersed. Extraction was carried out for 3x24 hours and stirred occasionally, changing the solvent with the same volume every 24 hours. Stored in a place protected from direct sunlight, then filtered and stored in a container to get the filtrate. The resulting filtrate was then evaporated with a rotary evaporator and thickened with a water bath to obtain a thick extract.

Quantitative Phytochemical Screening

Alkaloid

The extract solution was 3 mL plus 1 mL 2 N HCl and 6 mL distilled water. Then heated over a water bath for 2 minutes, cooled, and filtered. A total of 3 drops of the filtrate was transferred to the watch glass, then checked for the presence of alkaloid compounds by adding 2 drops of Mayer and Dragendroff reagents each. The presence of alkaloids is characterized by the formation of white deposits with Mayer reagent and red precipitate with Dragendorff reagent (Marliana *et al.,* 2005).

Flavonoid

A total of 3 mL of sample was evaporated, washed with hexane until clear. The residue was dissolved in 20 mL ethanol and filtered. The filtrate was divided into 3 parts A, B, and C. Filtrate A was blank, filtrate B was added with 0.5 ml of concentrated HCl then heated in a water bath, if there was a change in dark red to purple color shows positive results (Bate Smith-Metchalf method). The filtrate C was added with 0.5 ml of HCl and Mg metal and then observed the color change that occurred (Wilstater method). Red to orange color was given by flavone compounds, dark red color is given by flavonols or flavonones, green to blue colors are given by aglycones or glycosides (Harborne, 1987).

Saponin

The extract solution of 1 ml was added to 10 ml of distilled water and shaken vigorously for 10 minutes. The result is positive if the foam is stable for not less than 10 minutes, as high as 1cm to 10cm. With the addition of 1 drop of 2 N HCl, the foam does not disappear (Marliana, *et al.,* 2005).

Terpenoid

A total of 0.5 g of extract added 2 ml of chloroform. A total of 3 ml H_2SO_4 was added carefully to form a layer. Changes in reddish-brown color between layers indicate the presence of terpenoids (Ayoola *et al.,* 2008; Wachidah, 2013).

Tannin

A total of 3 ml of extract was extracted with 10 ml of hot distilled water then cooled. After that, 5 drops of 10% NaCl were added and filtered. Then the filtrate was added with gelatin salt, and the changes were observed. The formation of sediment indicates the presence of tannins or tanning substances (Wachidah, 2013).

Qualitative Phytochemical Screening

Flavonoid Determination

Natural ingredients in extract form, weigh 0.02 g, dissolved in 10 ml of solvent (distilled water and ethanol). For Standard curve creation Weigh the quercetin respectively, namely 10 mg, 20 mg, 30 mg, 40 mg, and 50 g then each dilute 10 mL with aquadest. Then take using a micropipette of 500 µL of each standard concentration and pour into a test tube and add 2 mL of distilled water. Add 150 µL of 5% $NaNO_2$ and let stand 6 minutes. Adding 150 µL of 10% $AlCl_3$ and let stand for 6 minutes. Adding 2 mL of 4% NaOH dilute with aquadest until the volume of the tube reaches 5 ml and let stand 15 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 520 nm.

Determination of Total Flavonoids

The natural ingredients have been prepared using a 500 µL micropipette and pour into a test tube and add 2 ml of aquadest. It was followed by adding 150 µL of 5% $NaNO_2$ and let stand for 6 minutes. After that, there was an addition of 150 µL of 10% $AlCl_3$ and let stand for 6 minutes again. Two mL of 4% NaOH dilute with aquadest until the tube volume solution reaches 5 ml and let stand for 15 minutes. Finally, the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 520 nm.

Saponin Determination

The extract was poured into a separating funnel and taken a layer of water. 60 mL of n-butanol mixed into the extract and n-butanol. It was washed by 10 mL of 5% NaCl then evaporated. The sample is dried in an oven until a constant weight was obtained.

Tannin Determination

One gram of extract dissolved with aquadest, then take 50 µL. Add 400 µL $K_4Fe(CN)_6$ 0.8mM + 400 µL $FeCl_3$ in 0.1 M HCl. Adding distilled water into the solution until the solution becomes 10 ml was needed in the next step. After 7 minutes, then measure the absorbance at λ = 700 nm.

Table I. Phytochemistry Screening Result of *S. jamaecensis* extract

Secondary metabolite	Reactor	Result	Information
Alcaloid	*Mayer & Dragendorff*	Sediment form	+
Flavonoid	HCl concentrated	Deep Red	+
Saponin	Forth	froth	+
Terpenoid	Kloroform	Reddish brown	+
Tanin	$FeCl_3$	Brownish green	+

Keterangan: (+) = exist, (-) = none

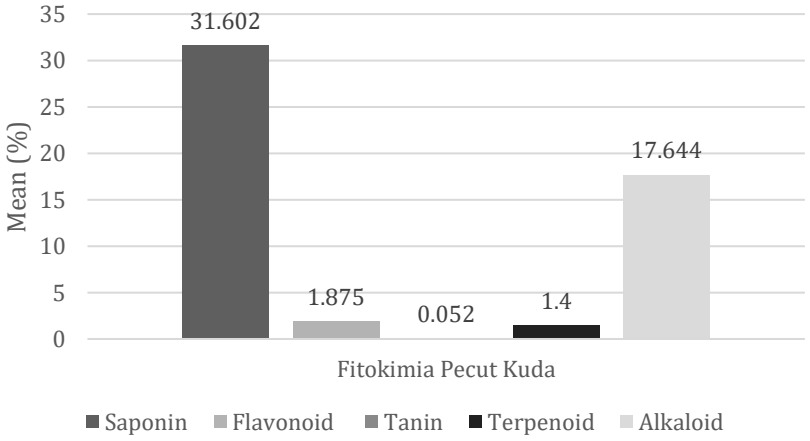

Figure 1. Quantitative Phytochemistry Screening of *S. jamaecensis* extract A. Saponin; B. Flavonoid; C. Tanin; D. Terpenoid; E. Alkaloid

Terpenoid Determination

Two grams of sample are inserted into a beaker. Then 10 mL of ethanol was added to a boil and filter, then take 5 mL of extract. Two mL of chloroform and 3 mL of concentrated sulfuric acid were added too then observe the changes.

Alkaloid Determination

Ten grams of natural ingredients addition into 250 mL beaker glass. Then added 200 mL of 10% acetic acid in ethanol. Close the beaker and let stand for 4 hours then strain. A quarter of the extract was evaporated with a water bath. After that, added ammonium hydroxide dropwise and then precipitate. The precipitation was washed by dilute ammonium hydroxide, then filter off. The residue was an alkaloid. The residue was steamed until constant weight.

Antioxidant Activity Test using Deoxyribose Degradation Assay

The hydroxy radical scavenger activity test used the deoxyribose degradation assay. 100 µL of the extract of the test solution (*S. jamaecensis*) with various concentrations (125, 250, 500, and 1000 ppm), added several solutions, namely 150 µL 10 mM ferric chloride, 150 µL EDTA 1mM, 150 µL hydrogen peroxide 20 mM, 150 µL vitamin C 1 mM, 150 µL deoxyribose 30 mM in phosphate buffer pH 7.4 to 3 mL. Then the mixture was incubated for 30 minutes at 37° C. Then performed the addition of 0.5 mL of 1% TBA and 0.5 mL of 5% trichloroacetic acid at 80° C for 30 minutes. After cooling, the absorption of the solution was read with a spectrophotometer at a wavelength of 532 nm (Luqman and Kumar, 2012).

% Antioxidant activity = (A control – A sample) / A control x 100%

RESULT AND DISCUSSION

Extraction of Plant Material

Plant extraction is carried out using maceration techniques because it is feared that the active compounds contained in the simplicia are unstable on heating. In this study, it was used ethanol 96% solvent due to the high level of effectiveness in taking the target active compound. The thick extract obtained was 18.05 g, with a yield of 12.03% for dry simplicia.

Phytochemical Screening

Table I showed that 96% ethanol extract from *S. jamaecensis* root contains compounds classified as alkaloids, flavonoids, saponins,

Table II. Hydroxyl radical scavenging activity and IC50 of *S. jamaecensis* Extract

Concentration of *S. jamaecensis* (µg/mL)	**Antioksidan Activity (%)**	**IC50 (ppm)**
125	21.212	
250	33.333	683.5294
500	48.485	
1000	60.606	

Table III. Hydroxyl radical scavenging activity and IC50 of Vitamin C

Concentration of Vitamin C (µg/mL)	**Antioxidant activity (%)**	**IC50 (ppm)**
125	44.444	
250	50.000	251.700
500	61.111	
1000	81.97	

terpenoids, and tannins. Phytochemical screening of the plant showed the highest value of all compounds is saponin. The bioactive components in Figure 1 that have antioxidative potential are saponin compounds, with the highest compound content, namely 31.602%, and alkaloids 17.644%. flavonoids were 1.875%, terpenoids were 1.4%, and tannins were 0.052%. Saponins are high molecular weight amphiphilic compounds having aglycon as lipophilic moiety and sugars as hydrophilic moiety (Singh and Chaudhuri, 2017). The polar and non-polar portions of the saponins determine the number of rates of movement of the molecules. The rate of diffusion of saponins into the liquid phase depends on the mass transfer rate of the solute from the plant material to the solvent (El Aziz *et al.*, 2019. The concentration gradient between the solid and liquid phases is the driving force for the diffusion of saponins into 90% ethanol solvent. The presence of saponins, flavonoids, alkaloids, terpenoids, and tannins is likely to be responsible for the free radical scavenging effects observed. Saponins showed profitable antioxidant activity and can be used as a source of natural antioxidants.

Antioxidant Activity Test Using Deoxyribose Degradation Method

The process of degradation of deoxyribose by hydroxyl radicals occurs in several stages. These steps occur when the reaction mixture consisting of Fenton's reagent (FeCl3, EDTA, H2O2, vitamin C) and deoxyribose is incubated at 37^{o}C for 30 minutes. The steps of this reaction are the reaction of hydroxyl radical formation from the Fenton reaction and the degradation of deoxyribose by hydroxyl radicals (Halliwell and Gutteridge, 1999). Furthermore, the hydroxyl free radical scavenging process occurs in the presence of interactions with the bioactive components of the *S. jamaecensis* root extract.

Figure 2 showed a percentage of antioxidant activity with a concentration of 1000 ppm produces the highest percentage, namely 60.606%. By plotting the concentration value on the X axis against the percent inhibition (reduction) on the Y axis, the equation Y = 20.95 + 0.0425 X, and R2 = 0.956282463 is obtained. With the regression equation, the IC50 value of *S. jamaecensis* root extract can be calculated, which was 683.5294 ppm. At a concentration of 683.5294 ppm, it means that the extract was able to reduce free radicals by 50%. The method of hydroxyl radical scavenging has been widely used as free radicals to evaluate a compound capable of reducing hydroxyl free radicals. As shown, the *S. jamaecensis* extract has an IC50 of 683.5294 ppm. While the measurement results of Vitamin C at a maximum wavelength of 515.30 nm, namely y = 0.042x + 39.304 with a correlation coefficient of 1,000 have an IC50 value of 251,700 ppm.

The IC50 value (Table 3) of the *S. jamaecensis* extract indicated that the extract had less strong antioxidant activity (more than 50 µg / mL). The antioxidant activity of *S. jamaecensis* was lower than that of vitamin C as a positive control because the smaller the IC50 value, the higher the antioxidant activity. This is because vitamin C is a pure compound, while the *S. jamaecensis* extract consists of several compounds.

This antioxidant activity can be derived from the content of saponins, flavonoids, terpenoids, alkaloids, and tannins. Antioxidants can form combinations of transition metal ions, which result in repression of OH group production and inhibit damage processes such as lipid peroxidation, protein, and other biological molecular mechanisms. The ability of saponins as

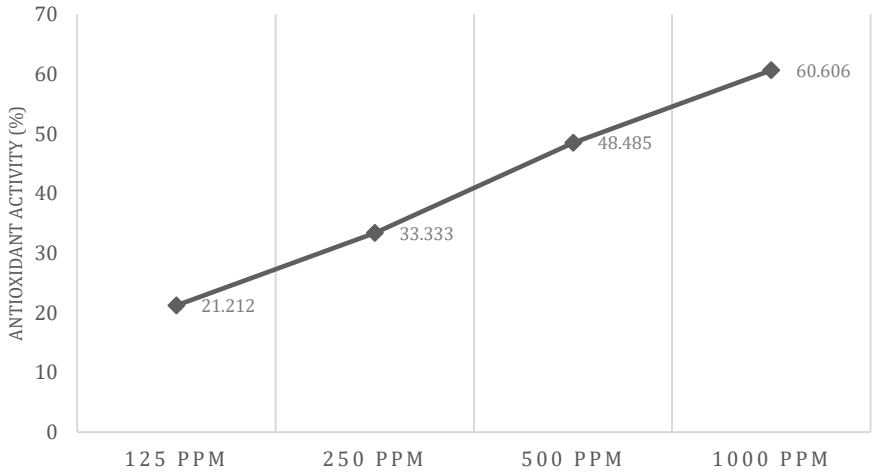

Figure 2. Percentage of Antioxidant Activity

free radical scavengers in this study, which was conducted in vitro, may be suspected by donating electrons and being captured by free radicals and then converting unstable free radicals into a stable form. In the biological environment of the body, the ability of saponins to reduce free radicals, apart from being able to release and donate electrons, is likely that saponins can bind metal ions that cause free radicals to form and can capture free radicals or react directly with free radical products and turn them into a more stable form. so that its radical character is reduced. In Nafiu's (2017) study, saponin extract from *Dianthus basuticus* can donate protons and has the potential to act as a free radical inhibitor and can act as a primary antioxidant. Meanwhile, Ashraf *et al*. (2013) stated that the total saponin fraction of *Chlorophytum borivilianum* showed higher chelating iron ion formation compared to crude extract.

In addition, alkaloids can efficiently break free radical chain compound reactions. Other compounds that have the potential to act as antioxidants are flavonoids, which are polyphenolic compounds that can donate hydrogen atoms to free radical compounds, so the antioxidant activity of polyphenol compounds can be generated in free radical neutralization reactions or at the termination of chain reactions. the last is tannins which can reduce radicals through the formation of hydroperoxide intermediates to prevent biomolecular damage. (Valko *et al.,* 2007). Flavonoids and tannins are thought to be compounds that are responsible for antioxidant activity. The ability of flavonoids and tannins as antioxidants has been proven by Pratiwi *et al.,* (2013) in their study of the antioxidant activity of mekah leeks (*Eleutherine americana* Merr). The structural elements of the flavonoid molecule have the best capture effect through the hydroxylation of the B ring and the C2-C3 double bonds connected to the C-3 hydroxyl and C-4 carbonyl groups (Treml and Smejkal, 2016). In the research of Raju *et al.,* (2015) plant extracts of *Vinca rosea*, *Gymnema sylvestre*, *Tinospora cordifolia* and *Emblica officinalis* and the combination of all these plants show that terpenoids are the highest content that can capture hydroxyl free radicals, superoxides, and peroxides. The occurrence of these structures in *S. jamaecensis* might be responsible for the comparable antioxidant activity with that of known antioxidant ascorbic acid.

CONCLUSION

In this study, the ethanol extract of *S. jamaecensis* has identified flavonoids, alkaloids, saponins, terpenoids, and tannins. In comparison with vitamin C, the IC50 value of 96% ethanol extract of *S. jamaecensis* root was 683.53 ppm and vitamin C was 251,70 ppm. This extract has antioxidant potential using in vitro deoxyribose degradation assay.

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