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Antioxidant Activity and Bioactive Compounds of Ethyl Acetate Fractions from *Syzygium cumini* Wood Stem

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Abstract. *Syzygium cumini* is one of the endemic plants in Indonesia that has the potential to be developed. The fruit was known to have potential as a diabetes drug. However, a few studies to determine of medicinal potential and *Syzygium cumini* wood stem has never been studied before. Therefore, this study aimed to determine the antioxidant activity and content of compounds in *Syzygium cumini* wood stem ethyl acetate fraction. The ethanol crude extract was prepared from the wood stem powder of *Syzygium cumini* in ethanol using maceration. Furthermore, it was carried out by the liquid-liquid fractionation process using solvents of different polarity. Antioxidant activity of the crude extract, hexane fraction, and ethyl acetate fraction was determined by spectrophotometric methods using 1,1-diphenyl-2-picrylhydrazyl (DPPH). Analysis of bioactive compounds by using Thin Layer Chromatography (TLC). Identification of compounds functional groups was carried out to support the results of TLC analysis by using Fourier-transform infrared (FTIR). Ethyl acetate fraction showed the highest antioxidant activity (IC₅₀ 13,62 µg/mL), followed by ethanol extract (IC₅₀ 19,64 µg/mL) and hexane fraction (IC₅₀ 61,25 µg/mL). The antioxidant activity of the acetate fraction was lower than ascorbic acid with an IC₅₀ value of 2,85 µg/mL. However, this fraction has very strong antioxidant activity. The TLC analysis results showed that the sample was thought to contain an alkaloid, phenolic, flavonoid, and terpenoid. The assumption on agreement with FTIR analysis which showed functional groups were C-H, O-H, N-H, C=O, and C=C aromatics as constituents of these compounds.

Keywords : antioxidant, bioactive, DPPH, ethyl acetate fraction, *syzygium cumini*

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Introduction

ROS (Reactive Oxygen Species) are formed naturally during the process of oxidative metabolism in the body and are needed if normally available, such as playing a role in the process of hormone biosynthesis, fertilization, and cellular signals. However, ROS that increases in excess (oxidative stress) can lead to many illnesses including degenerative diseases such as cardiovascular disease, inflammation, cancer, diabetes, and others [1]. Several types of antioxidants synthetic known role as free radical scavengers that include BHT (butylated hydroxytoluene), propyl gallate, TBHQ (ter-butyl hydroquinone), and BHA (butylated hydroxyl anisole). However, the use of them can present risks to humans. Various studies confirmed that using synthetic antioxidant have carcinogenic effects on liver and kidney damage, so the best alternative is to use natural antioxidants [2].

Natural antioxidants have been widely recognized as a substance that can protect the effects of hyperglycemia, increase glucose metabolism, and a source of treatment for diabetes mellitus. Flavonoids are one of the antioxidants that role-playing as free radical-reducing agents [3]. In addition, the presence of phenolic compounds and high total phenol content in the seed extract of *Syzygium cumini*, also able to influence its activity as an antibacterial. The seed extract with a concentration of 22.59 mg GAE/g, inhibited *S. aureus* by forming an inhibition zone with a diameter of 24.5 mm [4].

Syzygium cumini (Figure 1) is a rare endemic plant in Indonesia but is useful as a source of medicine. Various studies have been conducted on the bioactivity of the seed and fruit extracts of *Syzygium cumini*, but the study on the potential of the *Syzygium cumini* wood stem as a source of antioxidants and the identification of bioactive compounds resulting from the fractionation has never been studied before. Based on several studies reported by Panche et.al [5], the secondary metabolites compounds known have a potential as antioxidants are widely distributed in every part of the plant. It is hoped that the study of the *Syzygium cumini* wood stem has the potential as a new natural antioxidant, so the benefits of *Syzygium cumini* can be explored. This study aimed to evaluate the antioxidant activity of the extract and fraction of *Syzygium cumini* wood and to de-

termine the components of the bioactive compounds from the most active fraction that acted as antioxidants.



Figure 1. *Syzygium cumini*

Experimental

Extraction and fractionation

Syzygium cumini wood stem was taken from Gedebage, Bandung, Indonesia. The authentication process was carried out at Bogoriense Herbarium, Research Center for Biology, Indonesian Institute of Sciences, Indonesia. The fresh samples were used in these studies. The samples were cleaned, cut into smaller pieces, and then dried. The dried samples were ground into powder. The sample powder was extracted by ethanol 96% as solvent using the maceration method twice consecutively for 72 hours. The crude extract was evaporated with a rotary evaporator at 40 OC [4]. The liquid-liquid fractionation was taken using solvents which have different polarity. The ethanol crude extract was first dissolved in aquadest and then fractionated with hexane (1:1) and ethyl acetate (1:1), to give ethyl acetate fraction, hexane fraction, and water fraction. They were collected and evaporated [6].

Antioxidant activity assay by DPPH method

Determination of the antioxidant activity of ethanol extract, hexane fraction, and ethyl acetate fraction was carried out using the method of DPPH reduced. Each sample was made with a concentration of 1000 ppm in methanol, then diluted

until 5 variations in concentration (5-100 µg/mL) were obtained. Each test solution was taken and added the DPPH solution in methanol, homogenized, and then incubated for 30 minutes in a dark room at room temperature. The absorbance measurement of the test samples were carried out using a UV-Vis Spectrophotometer (Infinite M200 PRO Multimode Reader from Tecan) at a wavelength of 515 nm. A total of 1 mL of methanol solution in the DPPH solution was used as a blank, then was measured for absorbance. The same procedure was applied for ascorbic acid (Merck) as a positive control. The percentage of inhibition is calculated based on the calculation of the IC50 value [7].

TLC analysis of ethyl acetate fraction

TLC analysis was carried out on the test sample that had the highest antioxidant activity to identify the content of bioactive compounds. The TLC plate used a type of silica G60 F254 (Merck, Germany). The ethyl acetate fraction that had been dissolved was then spotted using glass capillaries on the previously activated TLC plate. The plates were dried then eluted using a different mobile phase which has been pre-saturated in the elution chamber. The mobile phases used were chloroform: acetone (7:3), chloroform: methanol: water (6:3:1), and chloroform: ethyl acetate (1:9), as the mobile phases which produced the best spots separation. The result of spots were detected under UV light at 254 nm and 366 nm. Spray reagents such as dragendorff, 10% H₂SO₄, and AlCl₃ were applied to the plates to identify the presence of secondary metabolites of alkaloids, terpenoids, and flavonoids. Each spot resulted was calculated the value of R_f.

Analysis of bioactive compounds using FTIR

A total of 0.2 mg of ethyl acetate fraction were weighed that used an analytical balance then analyzed by FTIR (Thermo, NICOLET 380) to identify the functional groups present in the sample. The analysis was carried out in the instrument laboratory at the Faculty of Mathematics and Natural Sciences, Garut University.

Results and Discussion

The sample used was obtained from the Gedebage Bandung area with the results of deter-

mination showing the species *Syzygium cumini* (L.) Skeels. *Syzygium cumini* wood stem was the object of research which tested its antioxidants and was identified the compounds contained in the sample.

Extraction and fractionation of *Syzygium cumini* wood stem

The extract yield of *Syzygium cumini* wood stem was 135.64 grams (6.78%). There are parameters that affect the extract obtained, including based on the type of solvent, the solubility of the compound, and the type of extraction method. Ethanol has the same polarity level as secondary metabolite compounds so the solvent can to dissolve most non-polar, semi-polar, and the polar compounds [8]. The use of maceration is a beneficial extraction method in the isolation of natural products. Extracts produced using maceration can provide higher antimicrobial and antioxidant activity compared to other methods due to the high solubility of compounds such as phenols, flavanols, tannins, and flavonoids. The crude extract obtained was then fractionated gradually using hexane followed by ethyl acetate, so that the hexane fraction, ethyl acetate fraction, and water fraction. Table 1 shows the fractionation results of 4 grams of ethanol crude extract.

Table 1. The fractions yield of *Syzygium cumini* wood stem

Samples	Yield (grams)
hexane fraction	0.76
ethyl acetate fraction	0.83

Fractionation is carried out to separate the various components of a compound based on their relative solubility in the two immiscible liquids. Each compound with a different polarity will separate into two phases. The water fraction yield was highest than the other fractions because some of the compounds contained in the wood stem of *Syzygium cumini* were polar. Compounds contained in polar solvents may be simple macromolecular sugars and saponins which have complex structures and larger molecular weights [9].

DPPH radical-scavenging activity

In this study, the determination of antiox-

inant activity was based on the principle of DPPH radical scavenging activity. This method is used because its the simplest, easiest, fastest method, and not require too many samples in testing, so this method is widely used to determine antioxidant activity from natural product [10].

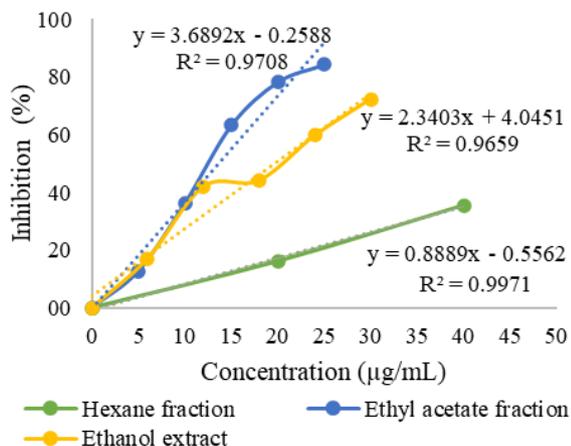


Figure 2. Antioxidant activity of ethanol extract, hexane fraction and ethyl acetate fraction

In [Figure 2](#) shows the inhibition of DPPH radical by extract and fractions samples. The IC₅₀ of the samples were calculated based on the linear regression equations, that were $y = 0.8889x - 0.5562$, $R^2 = 0.9971$ (hexane fraction), $y = 3.6892x - 0.2588$, $R^2 = 0.9708$ (ethyl acetate fraction), and $y = 2.3403x + 4.0451$, $R^2 = 0.9659$ (ethanol extract). The IC₅₀ value for ascorbic acid was calculated based on the linear regression equation ($y = 19.474x - 5.4757$, $R^2 = 0.99$). The calculation results obtained that the IC₅₀ value of each sample is shown in [Table 2](#).

Table 2. Antioxidant activity of ethanol crude extracted hexane fraction, ethyl acetate fraction, and ascorbic acid against DPPH free radical

Samples	IC ₅₀ (µg/mL)
ethanol extract	19.64
hexane fraction	61.25
ethyl acetate fraction	13.62
ascorbic acid	2.85

DPPH includes stable radicals and purple in solution. The color changes from purple to yellow, when the electrons of the DPPH structure

pair with the presence of a donated proton. This DPPH color change is a result of the scavenging of free radicals by antioxidant compounds in the presence of reactions between DPPH molecules and hydrogen atoms released by antioxidant compounds from the sample to form diphenylpicrylhydrazin[11]. The ethanol extract and ethyl acetate fraction were classified as very strong. An antioxidant is very strong if the IC₅₀ result is less than 50 µg/mL. The hexane fraction has a strong antioxidant activity because it has an IC₅₀ value of 50-100 µg/mL [12]. The results showed that the antioxidant activity assay was similar to that of the ethanol extract of *Syzygium malaccense* stem wood. Both of these plants belong to the *Myrtaceae* family with similar strength of antioxidant activity [13]. The antioxidant activity of the samples was related to the compounds contained in each of these samples. The methanol and ethanol extract of the *Severinia buxifolia* showed the content of alkaloids, flavonoids, phenols, terpenoids providing high antioxidant activity [14].

Antioxidant activity of ethyl acetate fraction in this study had an IC₅₀ value of 13.62 µg/mL. This shows that the ethyl acetate fraction of *Syzygium cumini* wood stem at a concentration of 13.62 µg/mL was able to inhibit DPPH free radicals by 50%. The antioxidant activity of the ethyl acetate fraction was still low when compared to the positive control ([Figure 3](#)), but it still has very strong.

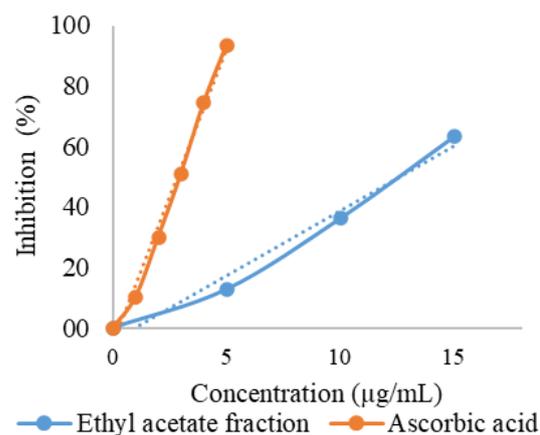


Figure 3. Antioxidant activity of ethyl acetate fraction and ascorbic acid

The fractionation process provides the highest antioxidant activity of a test sample. this is evidenced by the ethyl acetate fraction which has

higher antioxidants than ethanol extract [15]. This is probably because in the crude extract there are compounds that can suppress the ability to reduce DPPH, but after being fractionated with certain solvents such as ethyl acetate, it is possible that these compounds will not be fractionated so that the damping reaction by ethyl acetate fraction [16]. The types of compounds distributed in ethyl acetate have more hydroxy groups such as flavonoid and phenolic compounds. The higher phenolic compounds content in the ethyl acetate fraction can increase antioxidant activity up to two times than the crude extract [17].

TLC profile of ethyl acetate fraction

The ethyl acetate fraction obtained from the liquid-liquid fractionation produced the strongest antioxidant activity compared to the ethanol extract and hexane fraction. TLC analysis accompanied by the use of sprayer reagent was used to identify the distribution of secondary metabolites found in this fraction. Various eluents are used in TLC analysis to obtain the best separation results based on the number of spots that are separated, clearly visible, round, and tail-less.

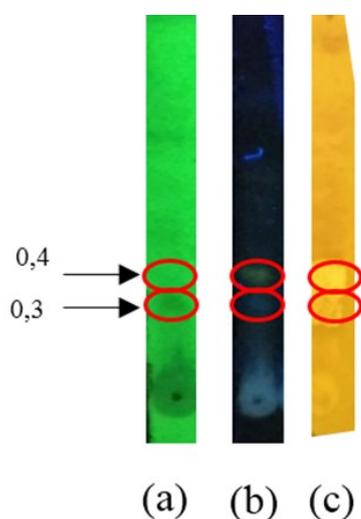


Figure 4. Chromatogram for ethyl acetate fraction developed with an eluent system of chloroform: acetone (7:3): viewed under (a) UV at 254 nm, (b) viewed under UV at 366 nm, and (c) sprayed with Dragendorff

Elution using the mobile phase of chloroform: acetone (7: 3), obtained several spots on the TLC plate which were observed under UV light of

254 nm and 366 nm. The plate sprayed with dragendorff reagent resulted in an orange spot that was observed visually which indicated a strong suspicion of alkaloid with Rf values of 0.3 and 0,4 (Figure 4). Alkaloids react with dragendorff's reagent involving ligand replacement in which nitrogen which has a lone pair in the alkaloid compound forms a coordinate covalent bond with the K^+ ion from potassium tetraiodobismuthate to produce a potassium-alkaloid complex that is orange to brownish red [18].

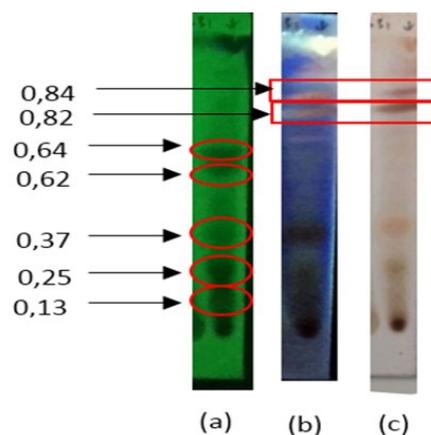


Figure 5. Chromatogram for ethyl acetate fraction developed with an eluent system of chloroform: methanol: water (6:3:1) (a) viewed under UV at 254 nm, (b) viewed under UV at 366 nm, (c) sprayed with 10% H_2SO_4

The elution results using the mobile phase of chloroform: methanol: water (6:3:1) obtained several spots (Figure 5). Dark spots appeared on observations under 254 nm UV light (Figure 5a) which indicated a suspected phenolics compound (Rf 0.13; 0.25; 0.37; 0.62; 0.64). The terpenoids compounds were detected using 10% H_2SO_4 reagent and then heated, visually showed reddish-brown spot with an Rf value of 0.82 and magenta with an Rf value of 0.84, while orange spots were viewed in 366 nm UV light [19].

Phenolic compounds (tannins) were also identified based on chromatograms (Figures 6a and 6b) which showed several spots when using the mobile phase of chloroform: ethyl acetate (1: 9). Dark brown spots with Rf values of 0.25 and 0.48 appeared when viewed under UV at 254 nm and 366 nm, which indicated attenuation occurred by these lights [20]. Phenolic compounds are aromatic compounds so they show strong absorption at the 254 nm UV spectrum [21]. Analytical TLC showed other spots that the suspected terpenoid compounds were marked with purple (Rf 0.38 and

0.68) and pink (Rf 0.86) when sprayed by 10% H_2SO_4 then heated and viewed visually (Figure 6d). All three reddish-yellow spots when viewed in 366 nm UV light (Figure 6e). This principle based on the reducing properties of sulfuric acid which destroys the chromophore at the compound from the sample so that the wavelength will shift to a longer direction so that the spots become visible visually. The terpenoid will give the pink to violet coloring after being sprayed by 10% H_2SO_4 , red/reddish-yellow was viewed when the plate was heated and detected under 366 nm UV light [22]. The presence of flavonoid was shown in Figure 6b which resulted in a blue fluorescent spot when detected under 366 nm UV light with a dark-colored plate. This argument was supported by spraying the AlCl_3 reagent against the TLC plate and was viewed under 366 nm UV light which resulted in yellow (Rf 0.95) and green (Rf 0.85) spots as shown in Figure 6c [23]. The appearance of these spots is caused by the interaction of UV rays with the chromophore groups bound by auxochromes. The compound component in the spots emits light emission in the form of light fluorescence when electrons are excited from a basic energy level to a higher energy level then return to their original energy level by releasing energy [24].

Analysis of bioactive compounds through FTIR spectrum

Identification of functional groups of compounds from ethyl acetate fraction was carried

out using FTIR. This was done to support the results of TLC analysis which showed the presence of phenolics, flavonoids, terpenoids, and alkaloid compounds in the fraction. The FTIR spectrum (Figure 7) showed several absorption peaks in both the fingerprint area and the $4000\text{--}1400\text{ cm}^{-1}$ wave number area.

Two absorption peaks at $3634,50\text{ cm}^{-1}$ and $3662,89\text{ cm}^{-1}$ indicated the presence of alcohols and phenols due to O-H stretching [25]. Absorption peak was widened at $3339,41\text{ cm}^{-1}$ is caused by the O-H stretch (H-bonded) and also the N-H [26]. The peaks at $2925,15\text{ cm}^{-1}$ and $2854,74\text{ cm}^{-1}$ revealed the presence of a sharp aliphatic C-H absorption. The absorption of the C=O (carbonyl) appears at $1721,47\text{ cm}^{-1}$ which indicated the general characteristics of the flavonoid compounds. The absorption peak at $1457,3\text{ cm}^{-1}$ indicated aromatic C=C absorption (stretching) which strengthens the presumption of the flavonoids [27]. In addition, at $1609,21\text{ cm}^{-1}$ and $1509,86\text{ cm}^{-1}$ showed the presence of N-H (primary amines). Absorption peaks also appear at $1268,18\text{ cm}^{-1}$ and $1116,05\text{ cm}^{-1}$ which indicated the presence of the C-N stretch (aliphatic amine) [28]. The alkane C-H uptake also appears at the $730,78\text{ cm}^{-1}$ [29]. The functional groups identified as C-H, O-H, N-H, C=O, C=C aromatics are thought to be the constituents of the alkaloid, phenol, tannin, flavonoid, and terpenoid class compounds. Several similar functional groups were detected in methanol extract of *Syzygium guineenses* leaves such as O-H, C-H (alkanes), C = O (carbonyl) and C = C alkenes provides infor-

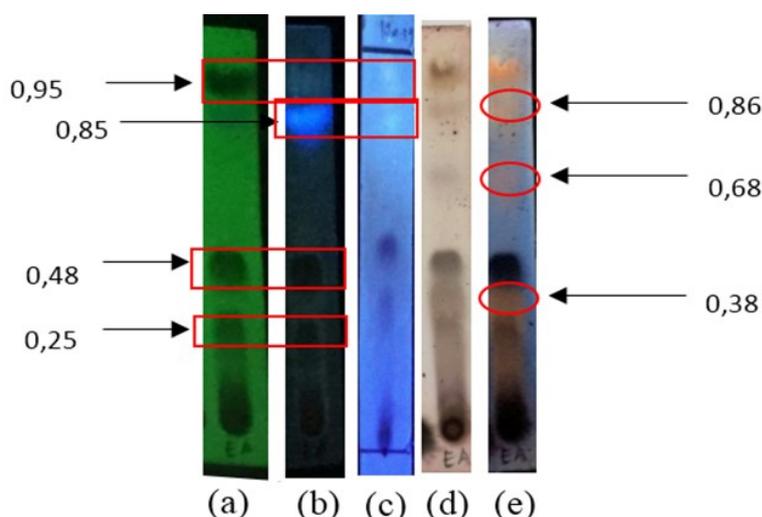


Figure 6. Chromatogram for ethyl acetate fraction developed with an eluent system of chloroform: ethyl acetate (1:9) (a) viewed under UV at 254 nm, (b) viewed under UV 366 at nm, (c) sprayed with AlCl_3 + viewed under UV at 366 nm (d) sprayed with 10% H_2SO_4 that viewed visually, dan (e) sprayed with 10% H_2SO_4 + viewed under UV at 366 nm.

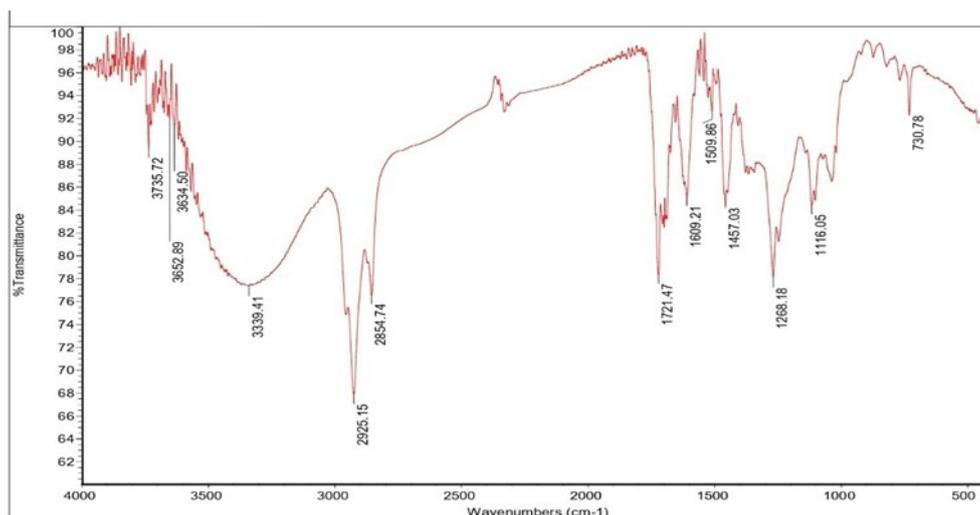


Figure 7. FTIR spectrum of ethyl acetate fraction

mation on the presence of phenols, flavonoids, alkaloids, and reducing sugars [30].

Conclusion

Syzygium cumini wood stem has the potential as a new natural antioxidant through testing of its extract and fractions which can reduced DPPH free radicals. The highest antioxidant activity was given by ethyl acetate fraction (IC₅₀ value of 13.62 µg/mL). The results of TLC analysis and identification of functional groups using FTIR showed that the ethyl acetate fraction of *Syzygium cumini* stem wood was thought to contain secondary metabolites such as phenolics, alkaloids, flavonoids, tannins, and terpenoids.

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