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Flavonoids Analysis using HPLC of Ethanol and Chloroform Fractions of *Alstonia Scholaris* (R. Br) Bark and Their Antioxidant Potency

Zuraida [a]*, Sulistiyani [b], Dondin Sajuthi [c], Irma H. Suparto [d]

[a] *Forest Research and Development Agency, Jalan Gunung Batu No 5, Bogor 16610, Indonesia*

[b]*Biochemistry Departement, Faculty of Mathematics and Natural Science, Bogor Agricultural University, Dramaga, Bogor 16680, Indonesia*

[c]*Veterinery Medicine Departement, Faculty of Veterinery Medicine, Bogor Agricultural University, Dramaga, Bogor 16680, Indonesia*

[c]*Primate Research Center, Bogor Agricultural University, Jalan Lodaya II/5, Bogor 16151, Indonesia*

[d]*Chemistry Departement, Faculty of Mathematics and Natural Science, Bogor Agricultural University, Dramaga, Bogor 16680, Indonesia*

Email: zuraidaus21@gmail.com

Abstract

Alstonia scholaris R.Br is one of the medicinal forest plant in Indonesia. Antioxidant activity was considered to be correlated with atherosclerosis by LDL oxidation inhibition. *Alstonia scholaris* R.Br bark has been reported as potential antioxidant because of its alkaloid content, however the potency of its flavonoids as antioxidant has not been investigated. The research objective was to investigated the antioxidant activity, and to identify flavonoids compounds from *Alstonia scholaris* R Br. Crude extract 70% ethanol of *Alstonia scholaris* bark, was fractionated by liquid-liquid fractionation using n-hexane, chloroform and ethanol. Ethanol, and chloroform fraction were analyzed for antioxidant activity through radical scavenge DPPH method, while identification of flavonoids was investigated by HPLC method. Results showed that yield of ethanol, and chloroform fraction are 2.52% and 0.83% respectively. Ethanol fraction had stronger antioxidant activity than that of chloroform fraction with IC_{50} value 73.53 µg/mL, and 445.96 µg/mL respectively. Identification of flavonoids through HPLC showed that ethanol fraction contain rutin (5.01 mg/g), and quercetin (0.05 mg/g) higher than that in chloroform

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* Corresponding author.

E-mail address: zuraidaus21@gmail.com.

fractions with rutin content (0.94 mg/g) and quercetin (0.04 mg/g). Rutin is dominant in both fractions, and rutin in ethanol fraction was higher than that in ethanol fraction. Quercetin, and rutin are flavonoids compounds act as antioxidants.

Keywords: *Alstonia scholaris*; antioxidant; flavonoids, quercetin; rutin

1. Introduction

Atherosclerosis is a disease in which plaque builds up inside arteries, which is made up of fat, cholesterol, calcium, and other substances found in the blood. The plaque hardens and narrows arteries, limits the flow of oxygen-rich blood to organs and other parts of our body. This condition can lead to serious problems, including heart attack, stroke, or even death. Atherosclerosis is a disease caused by many factors, and one of them is caused by oxidative stress. Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Reactive oxygen species is one of free radicals, which a molecule that have one or more unpaired electrons in their outer orbital, that it is highly reactive [1-2]. The important role of free radicals in the body is as a mediator in the natural process such as cytotoxicity, controlling blood vessel flow, and as neurotransmitters [1]. However, excessive production of free radicals compare to capacity of antioxidant production in the body will result in oxidative stress [2]. Oxidative stress can lead to degenerative diseases such as aging, cancer, atherosclerosis, diabetes, cardiovascular disease, and other neurodegenerative diseases [3]. Free radicals come from metabolic processes of the body, drugs, alcohol, pollution, stress, and poisoning [1].

Alstonia scholaris R. Br is one of medicinal forest plants in Indonesia that have farmacological effect. It can cure many kinds of disease such as cancer, bacterial, inflammation, and diabetes. Almost all parts of *Alstonia* plants are usually used such as leaves, stems, barks, and leaves. Saxena and his colleagues [13] reported that ethyl acetate bark extract contained flavonoids, alkaloids, saponins, and terpenoids. Khanum [14], reported that chloroform bark extract has antioxidant activity with IC_{50} value 47.7 μg/mL. According to Dhruti and his colleagues [15], *Alstonia* bark is widely used as a herbal component formula, while in Indonesia research about flavonoid antioxidant activity of *Alstonia scholaris R Br bark is* still limited. The objectives of this paper are to analyze antioxidant activity of *Alstonia scholaris* R.Br bark and to identify the flavonoids content of the plant part. Body itself has a defense mechanism to fight against free radicals. Antioxidants are molecules that capable of stabilizing, inhibiting, and deactivating free radicals before reacting [4-5]. Antioxidants have several mechanisms to inhibit the free radicals such as scavange species that initiate peroxidation; metal chelate metal ions so that they can not produce reactive species and lipid peroxides; decompose, eliminating O_2- radicals to prevent formation of peroxides, breaking the chain reaction auto oxidative, and reduces O_2 [6].

Antioxidants are divided into endogenous antioxidants and exogenous antioxidants. Endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), play important role in maintaining good cellular function and health. However, because of oxidative stress, body needs exogenous antioxidants to compensate it [5,7]. Exogenous antioxidants are divided into natural antioxidants and synthetic antioxidants. However, the use of synthetic antioxidants (BHA, BHT, propyl gallate) are not recommended in pharmacology due to safety concerns [8]. Therefore, many natural antioxidants source from fruits and plants are

to be developed. Compounds that act as natural antioxidants are vitamins, phenolics, flavonoids, carotenoids, and thiols [9-10].

Flavonoids are the most effective antioxidants because they have several hydroxyl groups. Flavonoids can reduce oxidation of metal transitions by donating their hydrogen atoms. In addition, flavones and some flavanons are able to bind metals to the 5-OH and 4-OXO groups [6]. Based on the degree of unsaturation and substitution patterns, flavonoids are divided into flavones, flavonols, flavanons, flavan-3-ol, anthocyanin, dihydroflavonols, and isoflavones [11]. Andersen and Markham [12], flavonoids can be separated, analyzed quantitatively, and identified using high performance liquid chromatography (HPLC).

2. Material and Methods

2.1 Material and tools

Main material used in this research is *Alstonia scholaris* bark powder with the size of 100 mesh obtained from Center for International Forestry Research (CIFOR), Bogor, Indonesia. Other materials used are vitamin C, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), rutin, ethanol, n-hexane, chloroform, glacial acetic acid 5%, $AlCl_3$ 2%, HCl 25%, hexamethyltetramine (HMT) 0.5%, acetone, ethyl acetate, acetonitrile, methanol and tert-butihydroquinone (TBHQ) .

Main tools used are Scale AND GR-200 series analytical balance, Zorbax SB-C18 column (4.6 x 150 mm), HITACHI UV/VIS Spectrophotometer U2800 BRUKER, and EPOCH Microplate Spectrophotometer. Supporting tools used are glassware, upright cooler, Thermo Scientific 10 µL, 100 µL, and 1000 µL micropipets, Falcon microplate, BRANSON B1510 sonicator.

2.2 Extraction

Alstonia scholaris bark simplisia was extracted by maceration in ethanol (1:10) at various concentrations of 0%, 30%, 50%, 70%, 80%, and 90% respectively. Maceration was carried out for 3x24 hours to obtain the filtrate. Filtrate was concentrated with vacuum rotary evaporator at 50 ºC to obtain a crude extract.

2.3 Total flavonoids content

Flavonoid content method was describe by NA-DFC [16]. Quercetin as standard was dissolved with 10 ml glacial acetic acid 5% v/v and, prepared in 0.1, 0.5, 1, 5 and 10 µg/mL concentration. Each of these was added in 1 mL $AlCl_3$ 2%. Furthermore, it was incubated for 30 minutes and, absorbance was measured at wavelength 425 nm.

Ethanol crude extract of *Alstonia scholaris* bark was weighed as much as 200 mg and fed into Erlenmeyer flask, then 2 mL of HCl 25%, 1 mL of HMT 0.5% w/v, and 20 mL of acetone were added. Then, it was shaken, and refluxed at 90 ºC for 30 minutes. Thereafter, acetone was added and filtered on a 100 mL flask. The remaining residue was dissolved with acetone and filtered again. Filtrate was collected in 100 mL flask and marked with acetone. Furthermore, as much as 20 mL of filtrate was taken and poured into a separating funnel, then 20 mL of

distilled water and 15 mL of ethyl acetate were added. Thereafter, it was extracted to separate the ethyl acetate fraction from water fraction. Ethyl acetate fraction was collected until the volume reached 50 mL in the measuring flask. Ethyl acetate fraction was taken as much as 10 mL and was put into a 25 mL measuring flask. Thereafter, 1 mL of $AlCl_3$ 2% was added and marked with glacial acetic acid 5% v/v. Sample solution was made in triplets manner. Blank solution was prepared from 1 mL of $AlCl_3$ 2% poured into a 10 mL measuring flask and was marked with glacial acetic acid 5% v/v. Then it was incubated for 30 minutes, and absorbance was measured at wavelength 425 nm.

2.4 Fractionation of ethanolic crude extract

Fractionation method following Andersen and Markham [12]. Powder extracted by maceration using 70% ethanol at a ratio of 1:10. Maceration was carried out for 3x24 hours until the filtrate was obtained. Filtrate was evaporated at 50 ºC until the volume reached 1/10 of initial volume. Filtrate was fractionated with n-hexane to obtain fractions of n-hexane and ethanol. Ethanol fraction was then fractionated back with chloroform to obtain fractions of chloroform and ethanol. Ethanol fraction and chloroform fraction were concentrated with evaporator at 50 ºC.

2.5 Antioxidant analysis using DPPH method

Chloroform and ethanol fraction were dissolved with ethanol and made concentrations became 400, 200, 100, 50, 25, and 12.5 µg/mL in microplate. Vitamin C as positive control was dissolved with ethanol and made concentrations became 20, 10, 5, 2.5, 1.25 and 0.62 µg/mL in microplate. As much as 100 µL of DPPH 125 µM was added to microplate and incubated in dark room for 30 min. Thereafter, absorbance of fraction and vitamin C was measured by microplate spectrophotometer at wavelength of 517 nm. Antioxidant analysis described by Aranda *et al.* [17].

2.6 Flavonoids identification of Alstonia scholaris bark with HPLC method

Flavonoids identification descibed by Hertog *et al.* [18]. Quercetin and rutin were used as standard with 10 µg/mL and 1000 µg/mL respectively. As much as 20 µL of standard was injected by using a Zorbax SB-C18 (4.6 x 150 mm) column into an HPLC system with a column temperature of 30 ºC. There were two phases of motion used, e.g. first-motion phase that contained 25% of acetonitrile in 0.025 M KH_2PO_4 (pH 2.4), and second-motion phase that contained 45% of methanol in 0.025 M KH_2PO_4 (pH 2.4) with a flow rate of 0.9 mL/min. The peak was identified by UV-vis at λ = 370 nm.

Ethanol and chloroform fraction of *Alstonia scholaris* bark was weighed as much as 0.25 g, then 20 mL of tert-butihydroquinone (TBHQ) in methanol 62.5% (1 g/L) and 5 mL HCl 6 M was added. Thereafter, the extract solution was refluxed for 2 hours at temperature of 90 ºC. Extract solution was filtered with filter paper in a 25 mL measuring flask and the volume was adjusted with TBHQ (1 g/L) in methanol 62.5% (1 g/L) so the concentration became 10000 µg/mL. Extract solution was filtered again with Whatman 0.45 µm filter paper, then it was injected as much as 20 µL with a flow rate of 0.9 mL/min. The peak of the sample was identified

with UV-vis at λ = 370 nm. Thereafter, the retention time and the area of the sample were compared with the standard to determine the concentration of quercetin in the sample.

2.7 Statistical analysis

Results were analyzed using one-way ANOVA followed by Duncan's test at 5% of significance. Analysis were performed with SPSS programme.

3. Results

3.1 Total Flavonoids Content of crude extract

Extraction of *Alstonia scholaris* bark used maceration method with various ethanol concentration: 0%, 30%, 50%, 70%, 80%, and 90%. Maceration was done in triplo manner with ratio of solute to solvent of 1:10 to obtain yield. Figure 1 shows the yields of *Alstonia scholaris* bark with various ethanol concentrations ranging from 2.32% to 6.47%. The results show that the crude extract with solvent of 90% ethanol resulted in the highest percentage of yield (6.47%), and vice versa with solvent of 30% ethanol which had the lowest percentage of yield (2.32%).

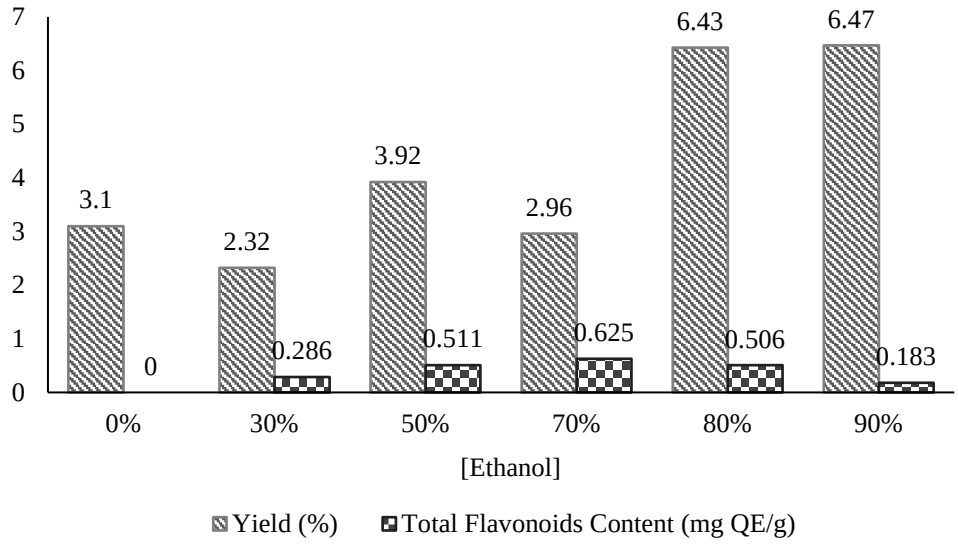

Figure 1: Yield and total flavonoids content of extract of *Alstonia scholaris* bark at various concentrations of ethanol

Crude extract was then measured its total flavonoids content by using quercetin as the standard. Absorbance of the crude extract obtained was directly proportional to the quercetin contained in the extract. The higher the absorbance, the higher the total flavonoids content. Total flavonoids content of crude extract tested started from undetectable (0) to 0.625 mg EQ/g of extract (Figure 1). The results show that the crude extract of the *Alstonia* bark with 70% ethanol has the highest total flavonoid content, indicating that 70% ethanol is the best solvent in extracting flavonoid of *Alstonia* bark. But the opposite is on the crude extract with 0% ethanol (water) as solvent.

3.2 Yield of ethanol and chloroform fraction

Alstonia scholaris R Br bark extraction was performed again by 70% ethanol because it has the highest total flavonoid content. Table 1 shows that the percentage of yield obtained from the ethanol fraction (2.52%) was higher than that of the chloroform fraction (0.83%).

3.3 Antioxidant activity of ethanol and chloroform fraction of Alstonia scholaris bark

Antioxidant activity was presented through IC_{50} (Inhibition Concentration) values shown in Table 1. The IC_{50} values are inversely proportional to their antioxidant activity. The smaller the value of the IC_{50} have the stronger antioxidant activity. Vitamin C as a positive control has an IC_{50} value of 4.14 µg/mL which is grouped as very strong antioxidants (IC_{50} <50 µg/mL). Ethanol fraction had a lower value of IC_{50} (73.53 µg/mL) than chloroform fraction (445.96 µg/mL). Ethanol fraction based on its strength is grouped into strong antioxidant (IC_{50} <100 µg/mL), whereas the chloroform fraction had no antioxidant activity (IC_{50} > 200 µg/mL) [19].

Table 1: Yield of fractionation and IC_{50} of ethanol and chloroform fraction of *Alstonia scholaris* bark

Sample	Yield (%)	IC_{50} (µg/mL) ± SD
Chloroform fraction	0.83	445.96^{c} ± 48.77
Ethanol fraction	2.52	73.53^{b} ± 5.16
Vitamin C	–	4.14^{a} ± 0.45

[a]n=3 for antioxidant assay, Numbers in column that are followed by same letters are not differ significantly, p>0.05 (ANOVA/Duncan)

3.4 Flavonoids identification of ethanol and chlorofrom fraction of Alstonia scholaris bark

Identification of quercetin and rutin compounds on the ethanol and chloroform fraction were performed by high performance liquid chromatography (HPLC) method. Quercetin and rutin contents in the fractions were obtained by comparing the standard area with fraction that it had the highest peak and the same or almost the same standard of retention time. Results of the identification in Table 2 show that the quercetin content of ethanol fraction (0.05 mg/g) is greater than chloroform fraction (0.04 mg/g). Similarly, Table 3 shows that rutin content of ethanol fraction (5.01 mg/g) is greater than chloroform fraction (0.94 mg/g). Rutin content dominated in the two tested fractions.

Table 2: Quercetin content of ethanol and chloroform fractions

Sample	Retention time	Area	[Sample] mg/g	[Sample] %
Ethanol fraction	15.844	30425	0.05	0.005
Chloroform fraction	15.680	24336	0.04	0.004
Quercetin standard	15.870	646927		

Table 3: Rutin content of ethanol and chloroform fractions

Sample	Retention time	Area	[Sample] mg/g	[Sample] %
Ethanol fraction	2.509	841897	5.01	0.501
Chloroform fraction	2.543	157216	0.94	0.094
Rutin Standard	2.447	16800322		

4. Discussion

4.1 Ethanol crude extract of Alstonia scholaris bark

Extraction aims to separate active components such as secondary metabolites from plants using selective solvents [20]. The aimed to make various concentration solven 0%, 30%, 50%, 70%, 80%, and 90% ethanol is to determine the best solvent to produce high total flavonoid content. Crude extract yield from various ethanol concentrations ranged from 2.32%-6.47%. The extracted yield hasn't yet in accordance with the standards set by national agency of drug and food control [16] because they are still less than 6.6%. The amount of yield show that the *Alstonia* bark extracted with 90 % ethanol produced the highest yield of 6.47%. This yield is lower than result of Ismiyah *et al.* [21] amount 11.47% that using 95% ethanol. There are many factors affecting the quantity of secondary metabolites such as extraction method, extraction time, temperature, solvent concentration, and solvent polarity [22].

Maceration was chosen in this study because it has a simple extraction procedure. The principle immersion of the material into a solvent is to break cell walls of the plant so that the bioactive component can be extracted [20]. In addition, this maceration extraction can protect compounds that are not resistant to heat treatment. In addition to the extraction method, solvent selection is also an important factor in extraction. Water and ethanol are chosen because they are safe as solvents of medicines or herbal products. Water is a universal solvent capable of extracting polar compounds, while ethanol is capable of extracting compounds with various polarities because it has a polar hydroxyl group and a non-polar alkyl group [23]. Ethanol is also known to penetrate the cell wall of plants easily, making it more efficient in degrading plant cell walls [22].

The crude extract obtained was tested for its total flavonoids to determine the best solvent with total quercetin flavonoids as parameter. *Alstonia* bark extracted with 70% ethanol showed the highest total flavonoid was 0.625 mg EQ/g extract. This indicates that 70% ethanol is the best solvent in attracting flavonoid compounds equivalent to quercetin on *Alstonia* bark. This is also consistent with Tiwari *et al.* [22] suggesting that 70% ethanol is a solvent capable of extracting high volume of flavonoid bioactive components. However, the total flavonoid from the crude extract of 70% ethanol was lower than methanol extract in Ramachandra *et al.* [24] study was 20.16 mg EQ/g extract. In addition, Dhruti *et al.* [15] also proved that total flavonoid from water extract and methanol extract of *Alstonia* bark were 37.51 and 26.38 mg EQ/g extract, respectively which were higher than the result of this study. The difference in total flavonoids can be affected by extraction method, extraction solvents, measurement method, and environmental factors such as temperature, UV light, nutrients, water availability, and CO_2 content [23].

4.2 Yield of ethanol and chloroform fraction

Yield of ethanol fraction (2.52%) was higher than that of the chloroform fraction (0.83%). This is due to the initial solvent used in the maceration process was 70% ethanol which tends to be polar because it contain 30% water. Therefore, the fractionation process of the crude extract of 70% ethanol, yields ethanol fraction higher than that of the chloroform fraction because of higher polarity of the ethanol fraction. The yields of this study consistent result with Thara and Zuhra [26], that extract of *Alstonia* bark that was extracted by staratified maceration by different solvent polarity resulted in ethanol fraction is higher, than chloroform fraction

Fractionation of a crude extract of 70% ethanol with n-hexane is aimed at separating non-polar compounds such as lipids. The fractionation is continued with chloroform to attract compounds that are less polar, while the more polar compounds will be attracted to the ethanol fraction [12]. One of the compounds that can be extracted into ethanol and chloroform solvents is flavonoid [12,26].

4.3 Antioxidant activity of ethanol and chloroform fractions of Alstonia scholaris bark

DPPH test aims to analyze the antioxidant potency of ethanol fraction and chloroform fraction of *Alstonia* bark to inhibit DPPH as free radicals. The inhibition activity is expressed in inhibition concentration 50 (IC_{50}) was the amount of sample concentration that can inhibit 50% of DPPH radicals. The ethanol fraction has lower IC_{50} value (73.53 µg/mL) than the chloroform fraction (445.96 µg/mL). These results indicate that the ethanol fraction has stronger antioxidant activity than the chloroform fraction because the ethanol fraction is more polar. The polar extract has a hydroxyl group so that it can donate the hydrogen atom. The values of IC_{50} of ethanol fraction and chloroform fraction are lower than those of Ramachandra *et al.* [24] who stated that the inhibition of 50% DPPH from methanol extract of *Alstonia* bark was at a concentration of 600 µg/mL. This suggests that both fractions of this study have stronger antioxidant activity. The antioxidant activity differences can be caused by different in phytochemical components contained, solvent concentration, type of solvent and extraction technique [27].

DPPH method was chosen in this test because it has a simple procedure and DPPH radical is stable. DPPH will provide maximum absorption at 517 nm wavelength. The principle of this test is antioxidant that reacts with the DPPH will donate its hydrogen atoms so that, DPPH will be reduced to a non-radical diphenyl picryl hydrazine (DPPH-H). The reaction will cause the color change from purple to yellow [8]. Vitamin C is used as a positive control in this test because vitamin C requires very small concentration in inhibiting DPPH radicals. In addition, vitamin C is able to regenerate radicals from other antioxidants such as vitamin E radicals and glutathione radicals. While vitamin C can easily regenerate itself from vitamin C radicals through NADH or NADPH-dependent reductase [8].

4.4 Flavonoids identification of ethanol and chloroform fraction of Alstonia scholaris bark

This analysis aims to identify the content of quercetin and rutin flavonoids on ethanol fraction and chloroform fraction of *Alstonia* bark by using high performance liquid chromatography (HPLC) method. The ethanol fraction has quercetin content of 0.05 mg/g which is not differerent significantly from the chloroform fraction of

0.04 mg/g. Meanwhile, the rutin content on the ethanol fraction (5.01 mg/g) is higher than that of the chloroform fraction (0.94 mg/g), because rutin is a polar glycoside flavonol that it has a sugar group, so that it is more abundantly found in the ethanol fraction which is more polar. In addition, glycoside flavonol are also widespread in plant tissues [25]. While quercetin is an aglicon flavonol which is less polar because it does not have sugar groups, so that quercetin content in ethanol fraction is less than chloroform fraction. The quercetin content in the chloroform fraction should be higher than that of the ethanol fraction, because the chloroform fraction is less polar. This can be due to an imperfect process of fractionation or because the quercetin content in *Alstonia* bark is less.

Jain *et al*. [28] had identified quercetin 3-0-galactoside and quercetin 3-0-glucoside, anthocyanidins and kaempferol on *Alstonia scholaris* bark. The compounds that are also found in the methanol extract of *Alstonia* bark are lupeol (terpenoid), and epicatechin (flavonoid) [25]. Reddy [29] also identified ecitamidin-N-oxide-19-0-β-glucopyranoside (alkaloid) from 95% ethanol extract of *Alstonia* bark. Quercetin and rutin which are detected in ethanol, and chloroform fraction are suspected to play important role as antioxidant that inhibited the DPPH radical in the previous test. It is also reported by Mothlanka *et al.* and Siddique *et al*. [30-31] that quercetin and rutin had antioxidant activity as good as vitamin C in capturing DPPH radicals.

5. Conclusion

Crude extract of 70% ethanol of *Alstonia scholaris* bark produced the highest total flavonoid content. Ethanol fraction (IC_{50} 73.53 µg/mL) had stronger antioxidant activity than chloroform fraction was (IC_{50} 445.96 µg/mL). Identification of flavonoids showed that ethanol fraction contain rutin (5.01 mg/g), and quercetin (0.05 mg/g) higher than that in chloroform fractions with rutin content (0.94 mg/g) and quercetin (0.04 mg/g). Rutin is dominant in both fractions, and rutin in ethanol fraction was higher than that in ethanol fraction.

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