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Article

Antioxidant Activity of *Averrhoa bilimbi* Linn. Leaves Extract Using Two Different Types of Solvents

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Abstract— **Exposed to the pollution has led to generation of reactive oxygen species (ROS) in human skin. ROS generated cause many skin diseases such as skin-aging, inflammation, melanogenesis and skin cancer. ROS is a family of oxygen-based free radicals that contains or capable of producing an unpaired electron. Antioxidant is a molecule that can inhibit the reaction of free radical from ROS by donating its electron. *Averrhoa bilimbi* Linn. (AVBL) is one of the potent natural antioxidant belongs to the group of Oxalidaceae which can be widely found in Asia including Malaysia. Traditionally, this plant has been used to treat many diseases such as cough, itchiness, pimple, fever and inflammation. As a result, much attention has been directed towards the studies regarding the potential of this plant in treating disease. The present study was undertaken to assess the antioxidant activity of AVBL leaves extract. The AVBL leaves were extracted using sonicator with ethanol and distilled water as two different types of solvent. The total phenolic content (TPC) and flavonoid content (TFC) of this study were determined by using Folin-Ciocalteu reagent and aluminium chloride colometric assay. Antioxidant activity of the plant extract was tested using 2,2- diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing power (FRAP). From the analyses, water extract of AVBL possessed greater extraction yield (11.231%) as compared to ethanolic extract (5.358 %). However, ethanolic extract of AVBL leaves revealed higher result of TPC (126.4±0.35 mg/g gallic acid equivalent), TFC (32.80±0.37 mg/g quercetin equivalent), DPPH (0.0019±0.0003) and FRAP (41.81±0.45 mg/g gallic acid equivalent). The results of TPC and TFC have strongly positive correlation with antioxidant capacity (r = 1). Thus, it can be concluded that this plant is a potent source of natural antioxidant.**

Keywords— **Reactive Oxygen Species; *Averrhoa bilimbi* L leaves.; Antioxidant activity; Potent antioxidant**

I. INTRODUCTION

The increase in number of vehicle, urbanization, industrialization and domestic combustion of organic materials has led to air pollution [1-3]. Air pollution is one of the world's largest health and environmental problems. Indeed, World Health Organization (WHO) estimates that around 7 million people die every year from exposure to polluted air. The main sources of pollutions are polycyclic aromatic hydrocarbons (PAHs), ozone, and heavy metals, volatile organic compounds (VOCs), particulate matter, nitrogen and sulfur oxide and carbon monoxide [4]. Increase in air pollution can cause many health problems. Recent research reported that air pollution is harmful to human skin and can lead to skin aging, skin cancer, melanogenesis and inflammation [5-7]. Air pollutants damage the skin by inducing oxidative stress. Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products [8].

ROS is a family of oxygen-based free radicals that contains or capable of producing an unpaired electron. ROS includes the superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2), an alkoxy radical ($RO^{\bullet}$), peroxy radical ($ROO^{\bullet}$), singlet oxygen (O_2) and excited carbonyl (RO*) [9]. ROS generated from air pollution caused skin diseases such as skin aging, skin inflammation and skin cancer [10].

Antioxidant is a molecule that can inhibit the reaction of free radical from ROS by donating its electron. Natural antioxidant is considered safe, biodegradable, environmentally friendly and low cost because it is derived from plants, fruits and other natural resources. It consists of many beneficial bioactive compounds such as flavonoids, phenol and tannins which help to protect human body against damage by ROS and also act as an antioxidant [11]. Recent research reported that, antioxidant nutrients and related bioactive compounds common in fruits and vegetables can protect against environmental toxic insults [12]

Averrhoa bilimbi Linn. (AVBL) (Figure 1) is one of the examples of natural antioxidant resources. AVBL belongs to the group of Oxalidaceae and it is normally found in Indonesia, Malaysia, Myanmar, Bangladesh and Sri Lanka. Agroforestry database reported the distribution of AVBL which can be found in all continents except Antartica. AVBL possess many beneficial bioactive compounds such

as flavonoids, tannins and phenols which can play an important role as an antioxidant [13]. A few decades ago, AVBL was used in traditional medicine to treat cough, itchiness, pimple and many more [14]. Previous research shows the importance of AVBL fruit in various biological activities such as antioxidant activity, catalytic activity, antihyperlipidemic properties, nitric acid inhibition, anti-diabetic and antimicrobial and cytotoxic properties [15-20]. Phytochemical screening of an AVBL leaves shows the presence of primary metabolites like aldehyde, sugar and protein as well as secondary metabolites such as cardiac glycoside, flavonoid, alkaloid, phenol, tannin and coumarin [21].

Fig. 1 The *Averrhoa bilimbi L.* leaves and fruits

Extraction is the main process by which bioactive compounds may be obtained from biomass materials. The goal of the extraction process is to optimise the quantity of target compounds and achieve the maximum biological activity of these extracts. Solvent is one of the important factor to obtain optimize bioactive compound. Choosing a suitable solvent is important to extract maximum bioactive compound. Polar solvent such as methanol, acetone, ethanol and water normally used to extract phenolic compound [22]. Recent research reported on the effect of extraction solvent on antioxidant activity [23-25]. Water and ethanol are considered as green solvent because it is environmentally friendly, not hazardous and also produced from green source [26-27]. Thus, in this study, these two solvents was chosen because it is a green solvent and have ability to extract phenolic compound. The objectives of the present study are to extract the phenolic compound from AVBL leaves using two types of green solvents (ethanol and water) and determined the antioxidant activity.

II. MATERIALS AND METHOD

A. Plant Material and Extraction

The fresh leaves of AVBL were collected and washed with distilled water to remove all dirt. The leaves then were dried at room temperature for 72 h until dry and were blended using blender to obtain powder form. The AVBL dried powder was proceeded to the extraction process using Ultrasonic Assisted Extraction with distilled water and ethanol as a solvent.

An amount of 50 g powdered sample and solvent (ethanol and distilled water) (1:10, sample to solvent ratio) were placed in the beaker and sonicated at 30 ˚C for 30 minutes. The solvent surface in the beaker was kept at the same level of water in the ultrasonic bath, and was monitored by thermometer. The change in temperature in the ultrasonic bath was observed. The beaker was covered with the aluminium foil to avoid and minimize the evaporative loss of ethanol. Obtained extract was then filtered through Whatman No. 1 filter paper and the filtrate was concentrated in a rotary evaporator under controlled vacuum. The concentrated extract was then kept in the 4 °C chiller until further analysis. The physical properties of the extracts were recorded, and the percentage yield of the samples was calculated as shown below:

$$\% \text{ Yield} = \frac{\text{Mass of Extract (g)}}{\text{Initial Sample Weight (g)}} \text{ X } 100 \qquad (1)$$

B. Antioxidant Activity Determination

A stock solution of the sample extract was prepared by dissolving 1 g of the extract in 100 mL of 99% ethanol and was sonicated for 20 min at 40 °C. The test sample was stored in an amber bottle for further analysis.

I. Total Phenolic Content (TPC)

The TPC of the extracts was determined using the Folin Ciocalteu reagent, following the method described by Chandra et al. (2014) [28]. 100 µl sample extract (2000 ppm) was mixed with 1 mL of 10% Folin-Ciocalteu's phenol reagent. The mixture then was shaken well and 2 mL of 7.5 % sodium carbonate solution (Na_2CO_3) was added to the mixture. The mixture was kept in the water bath at 40 °C for 30 minutes. The absorbance of blue colour from different samples was measured at 760 nm with gallic acid as a standard against reagent blank. The phenolic content was calculated as gallic acid equivalents (GAE)/ g of dry plant material on the basis of a standard curve of gallic acid (50–250 mg/L). All determinations were carried out in triplicate. The total phenolic contents in all sample will be calculated using equation 2:

$$C = c \times \frac{V}{m} \qquad (2)$$

where C = total phenolic content mg GAE/g dry extract, c = concentration of gallic acid obtained from calibration curve in mg/mL, V = volume of extract in mL, m = mass of extract in gram.

II. Total Flavonoid Content (TFC)

The TFC was determined based on the formation of flavonoid-aluminium complex which having the absorbtivity maximum at 430 nm [29]. 2 mL of sample extracts (2000 ppm) was mixed with 2 mL of 2% aluminium chloride-6-hydrate solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured using spectrophotometer at 430 nm. Quercetin was used as a standard to plot the calibration curve. The amount

of flavonoids was expressed as mg quercetin equivalent (QE)/g of dried plant material. All the determinations were carried out in triplicate. The total phenolic contents in all samples were calculated using equation 3:

$$C = c \times \frac{V}{m} \quad (3)$$

C= total flavonoid content mg QE/g dry extract, c = concentration of quercetin obtained from calibration curve in mg/mL, V = volume of extract in mL, m = mass of extract in gram.

III. 2,2- diphenyl-1-pirylhydrazyl (DPPH)

The free radical scavenging activity of the fraction was measured based on the scavenging activity of the stable 2,2-diphenyl-1-pirylhydrazyl (DPPH) assay [30]. 100 µl of the extracts were added to 2.9 mL of a 0.004 % ethanol solution of DPPH (0.0037g in 100mL ethanol). After a 15 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The percentage of inhibition of free radical DPPH by the extracts was calculated using equation 4 and the concentration required for 50% inhibition of viability (IC_{50}) was determined

$$\text{Inhibition (\%)} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100 \quad (4)$$

where A_{blank} = the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} = the absorbance of the test compound.

IV. Ferric Reducing Power (FRAP)

The reduction power as FRAP reactivity was determined using the method of Huyut et al., (2017) with slight modification [31]. Different volume of solutions (20, 40, 60, 80, 100 µg/mL) were prepared from the 10 mg/mL stock solutions of the ethanolic and water extract. An amount of 100 µl (2 mg/mL) of both sample extract was added to the freshly prepared FRAP reagent. This reagent contains of acetate buffer solution (pH 3.6, 0.3M), iron (III) chloride anhydrous (20 mM) and 10 mM of 2,4,6-tripyridyl-s-triazine (TPTZ) with the ratio 10:1:1. After incubation for 10 min, the absorbance of the mixture was read at 593 nm against the blank. Acetate buffer was used as a blank control sample and gallic acid was used as a standard. The result was expressed as mg GAE/g of dry extract

V. Statistical Analysis

The results are expressed as mean values and standard deviation of mean X ± SD. Samples were carried out in duplicate. The Pearson's correlation was carried out using SPSS statistical program to study the relationship between antioxidant activity and total phenolic and flavonoid content.

III. RESULTS AND DISCUSSION

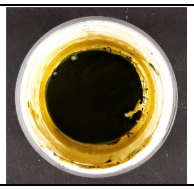
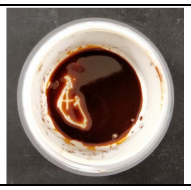
A. Extraction

The characteristic of the extract from two different solvents (ethanol and distilled water) is shown in Table 1. Both extracts were compared between their physical and chemical properties. Water and ethanol are widely used as extraction solvent because they safer, low toxicity and low cost.

From the table, it can be concluded that the use of different solvents resulted in the variation of colour, odour and also extraction yield. The variation is due to the nature and amount of secondary metabolites extracted. The extract of AVBL using ethanol shows the green colour of extract while distilled water resulted orange to brown extract in colour. However, both of extract are gel type extract. The use of distilled water as a solvent give higher extraction yield compared to ethanol which is 11.231% to 5.358%.

This result illustrated that the extraction yield increase with increase in polarity of the solvent. Distilled water has higher polarity compared to ethanol (10.2 and 4.3). During extraction, compounds other than phenolics such as proteins and carbohydrates may have been extracted and contributed to higher yield. This may be attributable to the higher solubility of proteins and carbohydrates in water than in ethanol. The results of this study are in agreement with the extraction yields of some medicinal plant [32-34].

TABLE 1:
THE CHARACTERISTIC OF AVBL EXTRACT ACCORDING TO THEIR SOLVENT.

Characteristic	**Type of Solvent**	
	Ethanol (AVBE)	**Distilled Water (AVBW)**
Polarity of solvent	4.3	10.2
Colour	Green	Orange to Brown
Nature	Gel Type	Gel Type
Odour	Nature	Nature
Extraction Yield (%)	5.358%	11.231%
Sample		

B. Antioxidant Activity Determination

I. Total Phenolic Content (TPC)

Table 2 and Figure 2 show the TPC of the extracts measured using the Folin Ciocalteau method. TPC values were obtained from the calibration curve y = 2.7261x + 0.022 with R^2 = 0.9942, where x is the absorbance and y is the concentration of gallic acid solution (µg/mL) expressed as mg GAE/g of dry extract of AVBL. From

the table, the TPC value of the extract ranged from 126.4±0.35 for ethanolic extract and reduce to 65.94±2.18 for water extract. This may also due to the content of more nonphenolic compounds such as carbohydrate and terpene in water extracts than in ethanolic extract. It may also be caused by the possible complex formation of some phenolic compounds in the extract that are soluble in ethanol. These phenolic compounds may possess more phenol groups or have higher molecular weights than the phenolic in the water extract [26]. The differences can be explained by the variance of the solvent polarities that selectively extract various hydrophobic or hydrophilic phenolic compounds from the sample

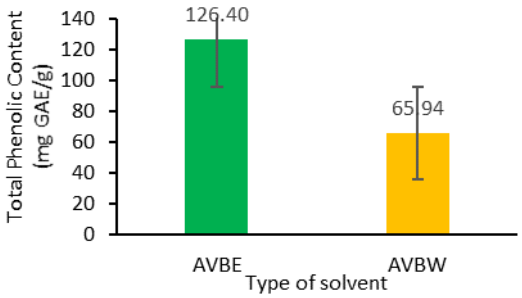


Fig. 2 Comparison of total phenolic content of two different solvents. Mean TPC content of AVBL. leaves extracts. Results were expressed as gallic acid equivalent (GAE). The values were expressed as mean standard deviation (n=2).

TABLE 2:
RESULTS OF TOTAL PHENOLIC, FLAVONOIDS AND ANTIOXIDANT ACTIVITY OF TWO TYPES OF SOLVENTS.

Type of Solvent	Total Phenolic Content[a]	Total Flavonoid Content[b]	Antioxidant Activity	
			DPPH[c] (IC_{50})	FRAP[a]
Ethanol (AVBE)	126.4±0.35	32.80±0.37	0.0019± 0.0003	41.81± 0.45
Distilled Water (AVBW)	65.94±2.18	13.84±0.11	0.0039± 0.001	20.85± 1.59

[a]Expressed as mg gallic acid/g of dry material
[b]Expressed as mg quercetin/g of dry plant material
[c]Expressed as g/mL

Phenolic compounds are known as powerful chain breaking antioxidants [35]. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups and may contribute directly to antioxidative action. Folin Ciocalteau (F-C) reagent is made up of a mixture of tungsten and molybdate which can detect the changing of colour by transferring of electron. The reduction process changes the colour of F-C from yellow to blue as shown in Figure 3 [36]. The presence of phenolic compounds in AVBL extract able to change the colour of F-C reagent thus, shows the positive results for TPC.

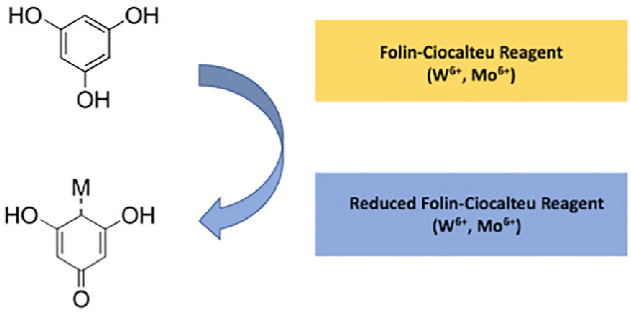


Fig. 3 Reduction of Folin Ciocalteau reagent.

II. Total Flavonoids Content (TFC)

Apart from that, the same trend also can be seen for TFC results (Table 2 and Figure 4). TFC values were obtained from the calibration curve y = 35.49x - 0.0283 with R^2 = 0.9853, where x is the absorbance and y is the concentration of quercetin solution (µg/mL) expressed as mg QE/g AVBL. From Table 2 and Figure 4, the results illustrated that, the ethanolic extract has higher TFC value compared to the water extract (32.80±0.37 to 13.84±0.11). The principle of aluminium chloride colorimetric assay is that aluminum chloride used in the colorimetric assay will react with flavone and flavonol group in either C-4 keto group, C-3 or C-5 hydroxyl group, or the ortho dihydroxyl group in the A- and B-ring group (Figure 6) and create a colour signature complex (Figure 5) [37].

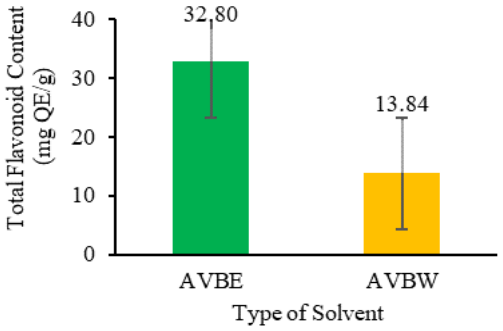


Fig 4 Comparison of total flavonoid content of two different solvents. Mean total flavonoid content of AVBL leaves extracts. Results were expressed Quercetin equivalent (QE). The values were expressed as mean standard deviation (n=2).

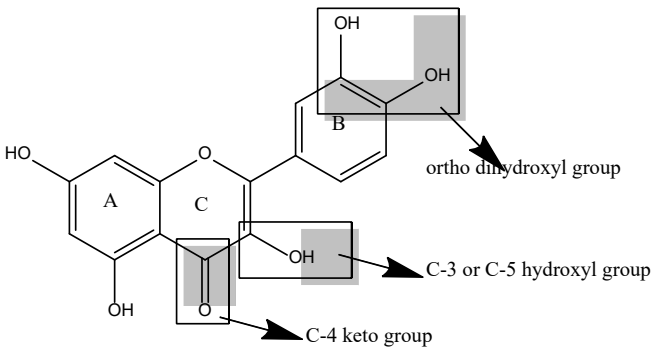


Fig. 5 Structure of flavonoid

III. DPPH

Figure 6 shows the results of DPPH radical scavenging activity of *Averrhoa bilimbi* L. leaves when extracted using different solvents. The DPPH radical scavenging activity differs considerably according to solvent (ethanol and water) with the concentration of sample ranging from 0.5 mg/mL to 3 mg/mL. From the graph, ethanolic extract of *Avherroa bilimbi* L. leaves (AVBE) has higher DPPH level compared to water extract (AVBW). The similar result was found in the leaves of Limnophila aromatic where ethanolic extract shows the highest DPPH activity compared to water extract [26]. A higher DPPH activity was found in the ethanol and methanol extracts of Lepisanthes alata (Blume) Leenh leaves than in the water extract [38]

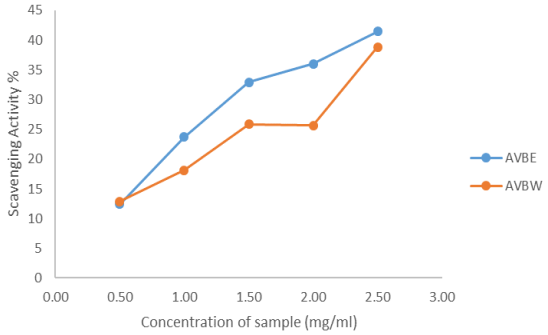


Fig. 6 Scavenging activities of the ethanolic extract and water extract of AVBL leaves expressed as mean ± standard deviation (n=2).w

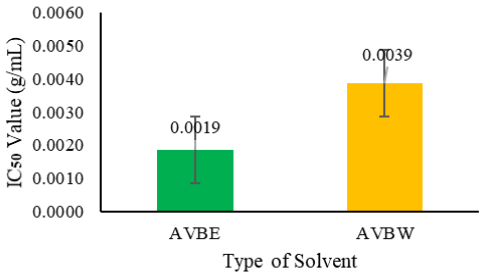


Fig. 7 IC_{50} value of the eethanolic and water extract of AVBL leaves

The use of DPPH to detect antioxidant activity is because it is able to obtain an antioxidant-donated hydrogen atom (H·) to form a stable DPPH-H molecule. (Figure 8). Visually, the antioxidant activity of the extract can be noticed by discoloration of DPPH from purple to yellow. AVBL extract able to change the colour of DPPH from purple to yellow. The change in colour indicated that antioxidants in these extracts were capable of scavenge free radicals and reducing oxidants.

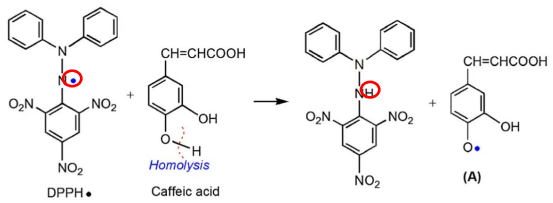


Fig. 8 Reaction of caffeic acid to scavenge DPPH·

In addition, the amount of plant extract needed to decrease the initial DPPH• concentration by 50%. (IC_{50}) is a parameter widely used to measure the antioxidant activity. Usually IC_{50} is defined as the moles of phenolic compounds divided by moles of DPPH• necessary to decrease by 50% the absorbance of DPPH•. The lower the IC_{50} value , the higher the antioxidant power. Table 2 and Figure 8 illustrated that the value of IC_{50} of AVBE is lower than AVBW. The value of IC_{50} for AVBE is 0.0019±0.0003 while AVBW 0.0039±0.001. This indicated that AVBE has higher antioxidant power.

Antioxidant activities of plant extracts were usually linked to their phenolic content. Hydrogen donating characteristics from hydroxyl group of the phenolic compounds is responsible for the inhibition of free radical to scavenge free radicals and give oxygen species such as singlet oxygen, superoxide free radicals and hydroxyl radicals [39]

IV. Ferric Reducing Activity Power (FRAP)

The FRAP assay was used to determine the reduction potential ($Fe^{3+} \longrightarrow Fe^{2+}$) of AVBL. The antioxidant compounds in the extract are responsible for the reduction of ferric (Fe^{3+}) form to ferrous (Fe^{2+}) form. The addition of $FeCl_3$ to the ferrous form led to the formation of blue coloured complex form. The colour changes of the FRAP solution with the sample from brown to blue can be observed after incubated for 15 minutes. The findings on this work show that extracts of AVBL possess antioxidative potential.

Figure 9illustrates that the FRAP value of AVBL varied among the extracts, but the values are lower than the standards. Ethanolic extract shows a higher reductive potential than the water extract. The reductive potential of AVBE extracts at a concentration of 2000 µg/mL are found to be higher compared to AVBW with values of 41.81±0.45 and 20.85±1.59, respectively (Table 2). The differences in impact of solvents on antioxidant capacity of AVBL extract in the current study can be explained by the variation of bioactive groups extracted by different solvents.

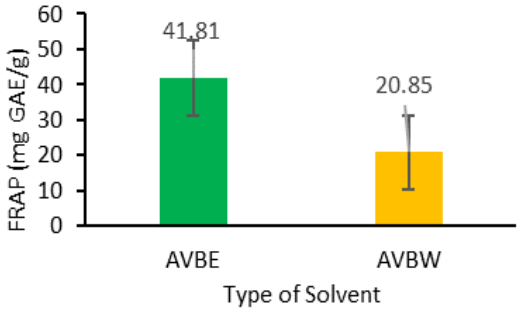


Fig. 9 Comparison of total FRAP value of AVBL of two different solvents expressed as mean± standard deviation (n=2).

IV. CONCLUSIONS

The results indicated a direct correlation between antioxidant activity and phenolic content of the extract, which might be the foremost contributors to the antioxidant activity of the plant. The present study indicated that, ethanolic extract of AVBL shows higher result of total phenolic and flavonoids content compared to water extract. It also shows higher performance of antioxidant activity of DPPH and FRAP. From the results, it is confirmed that ethanol is the best solvent to extract phenolic compound from AVBL. Ethanol as a solvent is a polar molecule with OH group. It has high electronegativity which allow hydrogen bonding to take place with other molecules. OH group in ethanol attract polar molecules and C_2H_5- attract non-polar substances. Thus, ethanol can dissolve in both polar and non-polar compounds. Apart from that, from the analyses of antioxidant activity, it can be concluded that AVBL leaves extract is a potential source of natural antioxidant.

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