

Test on the Antioxidant Activities of Methanol Extract of Bidara Leaves (*Ziziphus spina-christi* L.) using the DPPH Radical Immersion Method

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Abstract

Bidara (*Ziziphus spina-christi* L.) is a tropical tree originating from Sudan in which it is commonly known as "Nebeq" in Saudi Arabia. It is of a bioactive compound - a flavonoid compound, which is the potential to be used as an antioxidant. It is capable of inhibiting any cell damages caused by free radicals. This study aimed to measure the activities of the free radicals in methanol extracts of *Z. spina-christi* leaves. The process of extracting the *Z. spina-christi* leaves was carried out through the maceration method using methanol as a solvent. The qualitative analysis of chemical compounds with certain eluents using thin-layer chromatography (TLC) was purposely to determine the groups of active compounds in extracts. The measurement of antioxidant activities was carried out using the 1,1-Diphenyl-2-picryl Hydrazyl (DPPH) immersion method in which absorption was measured at a maximum wavelength of 513 nm. The results showed that the methanol extract of *Z. spina-christi* leaves had a very weak antioxidant activity with the IC₅₀ value of 466.804 µg/ml. The results of the bioautographic profile showed the presence of flavonoid compounds, phenols, saponins, and tannins.

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INTRODUCTION

Bidara (*Ziziphus spina-christi* L.) is one of plants used as herbal medicine. It has been used as Traditional Chinese Medicine to treat various diseases such as digestive disorder, fatigue, liver disease, obesity, urinary problems, diabetes, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anemia, diarrhea, insomnia and cancer (Almeer *et al.*, 2018). In Indonesia, *Z. spina-christi* plant is mostly found in Sumenep, Madura Island (Cahyaningsih *et al.*, 2017). The main compounds contained in this plant include flavonoid, alkaloid, triterpenoid, saponin, lipid, and protein. Its leaf contains botulinic, seanoctic acids, various flavonoid compounds, saponins, tannins, and tritepenoids (Asgarpanah &

Khoshkam, 2012). Study by Kusriani & Az Zahra (2015) revealed that the extract of *Z. spina-christi* leaves with ethanol solvent contains alkaloid, flavonoid, saponin, tannin, quinolones and steroid/triterpenoid that are potential to be used as antioxidant that is capable of preventing any free radicals.

Antioxidants are electron donor compounds or reductants – the compounds with small molecular weights, but able to inactivate the development of oxidation reactions by preventing any radical formation (Kurutas, 2016). Antioxidants are also compounds that can inhibit the oxidation reactions by binding the very reactive free radicals and molecules resulting in the inhibition of cell damage caused by free radicals (Phaniendra *et al.*, 2015; Lobo *et al.*, 2010). In the body,

reactive oxygen compounds or free radicals are not always harmful. In certain state, its existence is even necessary, for example to exterminate bacteria entering the body. Its existence, therefore, must be controlled by the oxidant system in the body (Nita & Grzybowski, 2016; Phaniendra *et al.*, 2015).

Antioxidants can be in the form of enzymes (e.g. superoxide dismutase or SOD, catalase, and glutathione peroxidase), vitamins (e.g. vitamins E, C, A and β -carotene), and other compounds (e.g. flavonoids, albumin, bilirubin, or ceruloplasmin). Enzymatic antioxidants are the primary defense system against oxidative stress conditions. They work by preventing any formation of new free radical compounds (Kurutas, 2016).

There are also non-enzymatic antioxidants, which can be nutritional or non-nutritional compounds. These two groups are also called secondary antioxidants for being able to be obtained from food intake, such as vitamin C, E, A and β -carotene. Glutathione, uric acid, bilirubin, albumin and flavonoids are also included in this group. These compounds function to capture oxidants and prevent any chained reactions (Pham-Huy *et al.*, 2008).

These components are equally important in inducing the antioxidant status of body. Isoflavone, for instance, is one of many flavonoid components found in soybeans and their processed products. This compound, as reported, have many antioxidant roles (Panche *et al.*, 2016). There are still many other food ingredients also containing isoflavones, such as tea, ginger, jelly leaves, coffee, or spices (Carlsen *et al.*, 2010). *Ziziphus spina-christi* is another plant that contains other polyphenol compounds. This plant contains phenolics and flavonoids rich in biological benefits, such as antioxidant, anti-inflammatory, antimicrobial, and antifungal. It is also able to prevent tumors (Abdoul-Azize, 2016).

Based on the description above, an antioxidant activity test was carried out from 96% ethanol extract of *Z. spina-christi* leaves that aimed to observe the antioxidant activity based upon the binding activity against 1,1-Diphenyl-2-picryl Hydrazyl (DPPH). Antioxidants will prevent any free radical reactions in lipid oxidation. The inhibited compounds, i.e. free radicals continuously formed and accumulated in the body, are potential to inactivate various enzymes, oxidize the fat and disrupt the body's DNA causing cell mutations as the beginning of cancer (Rahal *et al.*, 2014). It is expected that the results of this study can provide scientific information in the field of chemical natural biological substances and pharmacy as an effort to utilize antioxidant compounds from *Z. spina-christi* leaves.

MATERIALS AND METHODS

Material sampling

The samples of *Z. spina-christi* leaves were obtained from Pekalongan, Central Java. They were collected and sorted to remove the parts of the plant undesired. The samples were then washed in the flowing water and dried in an oven at a temperature of 40-50°C prior to be pollinated.

Extraction

A total of 60 g of *Z. spina-christi* leaves powder was extracted using the maceration method (stirred and soaked in a certain solvent) by using a 150 ml methanol solvent in a glass beaker. They were then left for three to four days. Once the first extraction process was completed, the pulp was again macerated with a 150 ml methanol. The extracts collected were evaporated with a rotary vacuum evaporator until obtaining a thick extract.

Phytochemical screening

Alkaloids

The alkaloids in extract solution were tested using reagents of Mayer, Wagner, and Dragendorff. The

positive test results of alkaloids using Mayer's reagent showed the formation of white precipitate, using Wagner's reagent showed the formation of brown precipitate, and using Dragendorff's reagents showed the formation of orange precipitate. Before adding the reagent, the samples were added with HCl considering that alkaloids were alkaline in nature and it needed to be extracted using acid solvents (Nurwidayati, 2012).

Flavonoids

The extract solution was tested based upon the flavonoid test by Baud *et al.* (2014) by adding 5 ml of 95% ethanol to the test sample. Subsequently it was added with the powder of metal Mg, and ten drops of concentrated HCl. Identification showed the positive results if it produced red, yellow or orange.

Phenols

The simple detection of phenol compounds was conducted through the addition of iron (III) chloride solution. This was quoted in Robinson (1995) stating that phenol and its derivatives with iron (III) chloride could produce a bluish green or deep black.

Saponins

The detection of saponin was conducted by adding water to the extract and then was shaken within a minute. If it produced foam, HCl was then added. As reported by Kareru *et al.* (2008), the positive extract contains saponins for the formation of foam that can last for a minute with a height of 1 cm.

Tannins

Adamczyk *et al.* (2017) stated that tannin can be identified through precipitation using a gelatin solution with NaCl that could produce a white precipitate at the base.

Identification with TLC

Flavonoids

The extract of *Z. spina-christi* leaves and the comparison of quercetin were bottled on the activated TLC plates. It

was then eluted with an eluent which is the mixture of methanol : chloroform (3 : 3). Then, it was observed in UV light with the wavelength of 254 and 366 nm and the spots were observed.

Phenols

The extract of *Z. spina-christi* leaves and gallic acid as the comparison were bottled on the activated TLC plates. They were then eluted with an eluent which is the mixture of methanol : chloroform (3 : 3). Subsequently, it was observed in UV light 254 and 366 nm and the spots were observed.

Antioxidants test

Preparation of DPPH solutions

The 100 µg/ml DPPH solution was made by weighing 5 mg of DPPH and dissolved in 50 ml of methanol in a measuring flask.

Preparation of sample solutions

A stock solution of *Z. spina-christi* leaves extract of 1000 µg/ml was made, i.e. 50 mg in 50 ml of methanol. Furthermore, it was diluted using a methanol solvent by making the series of concentrations of 100, 250, 500, 750, and 1000 µg/ml.

Measurement of antioxidant capacity

Measurement of blank antioxidant capacity was conducted by measuring 2 ml of DPPH added with 3 ml of methanol, incubated at 37°C for 30 minutes. The maximum wavelength was then measured in the range of 510 to 520 nm.

The measurement of antioxidant capacity of methanol extract of *Z. spina-christi* leaves was quantitatively carried out by piping 1 ml of sample solution at various concentrations, each replicated three times. Afterwards, 2 ml of DPPH and 2 ml of methanol were added to concentration respectively. They were incubated at 37°C for 30 minutes. The measurement of the absorbance showed the wavelength of 513 nm.

Determination of IC₅₀ values

The analysis of DPPH method antioxidant testing was done by observing the color changes of each sample. If all DPPH electrons were paired with electrons in the extract sample, there would be a color change in the sample from dark purple to bright yellow. Then, the absorbance value of the sample was measured using a UV-Vis spectrophotometer at the maximum wavelength of 513 nm (Kedare & Singh, 2011).

RESULTS AND DISCUSSION

Antioxidants are the compounds that are able to cope with any oxidative damages caused by free radicals in the body. Thus, the antioxidants play a role in preventing various diseases. In testing the antioxidant, it began by conducting the phytochemical test to find out the bioactive components contained. This phytochemical test was able to detect the components of bioactive compounds produced by secondary metabolites for groups of compounds including flavonoids, phenols, alkaloids, saponins and tannins (Alfian & Susanti, 2012). It was then continued with the test on the anti-free radical using DPPH.

Sample preparation

Preparing the sample was the initial phase in this research purposely to facilitate the maceration process by minimizing the size of sample to create more contacts between the sample and the solvents and to accelerate and maximize the maceration process of the sample. The phase of sample preparation included washing, drying and pollinating activities (Zhang *et al.*, 2018). Washing aimed to remove any impurities attached on the *Z. spina-christi* leaves; the drying was done to remove the water level contained in the *Z. spina-christi* leaves to prevent any microbial development and the growth of fungi. Drying was done by means of an oven at a temperature of 40-50°C. Meanwhile, pollination was conducted to

equalize the sample size (i.e. in the size of 60 mesh) and widen the surface area of the sample.

Moisture content analysis

Moisture content determination functions to find out the best storage method for simplicia samples and the estimation in the number of samples required. The simplicia sample was dried under the sunlight or by the heat of oven. Here, the value of the moisture content of the simplicia powder used reached 4.00%. The analysis of moisture content in dry samples aimed to identify the quality of simplicia used for the water content contained. Water is a medium for fungi to grow and develop. The requirements of simplicia moisture content in accordance to the applicable standard parameters are not more than 10% (Manalu & Adinegoro, 2016). The analysis of moisture content was carried out using a moisture meter by inserting the amount of simplex powder into it. The results of the analysis with a moisture meter showed that the water content of *Z. spina-christi* leaves simplicia reached 4.00% (w/w). Thus, it can be seen that simplicia is quite safe from fungal contamination during the storage process.

Extraction

The extraction was carried using the maceration method. The extraction technique with the maceration method was done by immersing the simplicia with a certain extract fluid. The extraction process occurs in view of the differences in concentration outside and inside the cells in which the liquid will penetrate the cell wall and enter the cell cavity containing active compounds or substances that, in turn, will dissolve (Sasidharan *et al.*, 2011). Due to differences in concentration between the solution of active substances inside and outside the cells, then the concentrated solution is pushed out. This event occurs repeatedly leading to a continuous concentration between the solution outside and inside the cells (Lodish *et al.*, 2000). The extraction of active compounds of *Z.*

spina-christi leaves samples extracted by maceration obtained from 60 g of dried *Z. spina-christi* leaves samples using 300 ml of 96% ethanol solvent produced 12.96 g of concentrated and thick green extracts with the yield of 21.6%.

Phytochemical screening

The identification of phytochemical contents was conducted to qualitatively observe the content of secondary metabolites contained by *Z. spina-christi* leaves extract. **Table I** presents the identification results of phytochemical screening.

Table I. Results of phytochemical screening of *Z. spina-christi* leaves

Compounds	Reagent	Results	Conclusion
Alkaloids	Dragendorff	No red precipitate	-
	Mayer	No white – yellowish precipitate	-
	Wagner	No brown precipitate	-
Flavonoids	Mg + HCl	Orange color	+
Phenols	FeCl ₃	Bluish green solution	+
Saponins	Aquadest, HCl	Stable foam	+
Tannins	Gelatin + NaCl	White precipitate	+

Identification with TLC

Another qualitative test carried out was by using TLC test. The separation of the methanol extract compound of *Z. spina-christi* was carried out using the TLC method by using a mixture of eluents of methanol : chloroform (3 : 3). The samples to be analyzed for separation were spotted on G₆₀F₂₅₄ silica chromatography plates as a stationary phase with the size of 5 x 10 cm. The TLC plate to be used was firstly preheated in an oven at a temperature of 100°C for 30 minutes to eliminate the water content contained in the plate (Bele & Khale, 2010). Then, it was spotted using 5-10 samples (in the same place) using capillary tubes. After the eluent was saturated, the TLC plate was eluted until the eluent reached the threshold. The elution then produced the spots.

The appearance of spots was then analyzed using UV light with wavelength of 254 and 366 nm. Then based on the resulted spots, they were analyzed quantitatively by measuring the distance traveled by the spots compared to those taken by the eluent. By so doing, they could be expressed as the degree of retention or R_f to determine the position of the sample after development or elution. (Cai, 2014).

The observation process with UV light with the wavelengths of 254 and 366 nm showed the spots occurred due to the interaction between UV light and chromophore groups bound by auxochrome on the stain. The visible light fluorescence refers to the light emission by these components when electrons are excited from the basic energy level to a higher one and then returns to be stable to release energy (Cai, 2014). The results of TLC identification are presented in **Figure 1**.

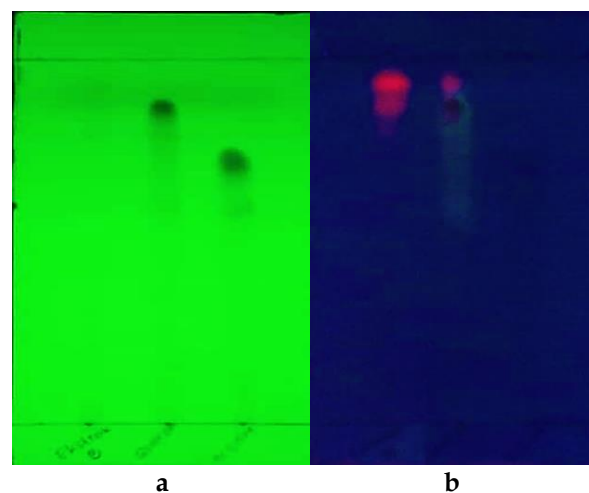


Figure 1. The results of the TLC identification of *Z. spina-christi* leaves extracts under UV light observations of 254 (a) and 366 nm (b)

The results of the compounds separation in *Z. spina-christi* leaves extract by TLC using the eluent of methanol : chloroform (3 : 3) produced a spot per row of spots. There are three types of spots to be observed including ethanol extract of *Z. spina-christi* leaves, as well as a comparative compound, i.e. quercetin and gallic acid. Quercetin is a comparing compound to the presence of flavonoids in the sample and gallic acid compounds is a

comparison to the presence of phenols. This observation found the value of Rf for extract of 0.975, Rf for quercetin of 0.938, and Rf for gallic acid of 0.8. Based on the Rf values, if the Rf values or patches were close together, it had the same or almost the same chemical structure. It can then be seen that the extract of *Z. spina-christi* leaves contains compounds almost equal to quercetin and phenol compound.

Antioxidant Activities

Free radicals commonly used as a model in measuring the free radical capacity include DPPH, a stable free radical compound with the absorbance values in the range of 510 to 520 nm (Najafabad & Jamei, 2014). The method used in testing the antioxidant activity is the DPPH free radical reduction method based on the reduction of a solution of colored DPPH free radical methanol by inhibiting the free radicals. When the purple DPPH solution meets the electron donor material, the DPPH will be reduced, fading the purple color and replaced by yellow from the picryl group (Rahman *et al.*, 2015).

The measurement of the antioxidant activity of the sample was carried out using a UV-Vis spectrophotometer at a wavelength of 513 nm as the maximum wavelength of DPPH with a concentration of 100 µg/ml (Kedare & Singh, 2011). The presence of antioxidant activity from the sample will cause a change in the color of DPPH solution in which it was originally violet and then turned into pale yellow (Akar *et al.*, 2017). The number of antioxidant activities is indicated by the IC₅₀ value, which is the concentration of the sample solution required to inhibit 50% of DPPH free radicals. The effective concentration value is a number showing the extract concentration (µg/ml) that is able to inhibit 50% of oxidation. The calculation of the effective concentration value or IC₅₀ (Olugbami *et al.*, 2015).

Furthermore, the calculation results were converted into the regression equation with the extract concentration (µg/ml) as abscissa (x-axis) and the value of % antioxidant inhibition as the ordinate (y-axis). The IC₅₀ value is calculated when the %inhibition value is 50% using the equation $y = bx + a$. From the test, the data were taken to do processing to make the data possible to be analyzed. The results of the test on the antioxidant with DPPH method are shown in Table II. The results of the IC₅₀ value was averaged and it was found that the IC₅₀ value of methanol extract of *Z. spina-christi* leaves from three replications was 466.804 µg/ml. The IC₅₀ values that are greater than 250 µg/ml are said to have very weak antioxidant activity (Molyneux, 2004). Thus, it can be stated that the antioxidant activities of *Z. spina-christi* leaves were very weak.

Table II. Data on the values of antioxidant percentage in the methanol extract of *Z. spina-christi* leaves

Concentration (µg/ml)	Replication					
	I		II		III	
	Abs (Å)	%IC	Abs (Å)	%IC	Abs (Å)	%IC
100	0.844	19.772	0.893	15.114	0.897	14.734
250	0.703	33.175	0.709	32.605	0.709	32.605
500	0.463	55.989	0.472	55.133	0.490	53.422
750	0.176	83.269	0.178	83.079	0.179	82.848
1000	0.166	84.221	0.172	83.650	0.177	83.175
Linear regression equation	$y = 0.0773x + 15.093$ $R^2 = 0.9446$		$y = 0.0807x + 11.959$ $R^2 = 0.9389$		$y = 0.0805x + 11.485$ $R^2 = 0.9388$	
IC₅₀ (µg/ml)	451.578		471.388		478.447	
Average IC₅₀ (µg/ml)	466.804					

Note: Blank abs.: 1.052 Å

CONCLUSION

The chemical contents in the ethanol extract of *Z. spina-christi* leaves include flavonoids, phenols, saponins, and tannins. *Ziziphus spina-christi* leaves extracted using ethanol 96% has the very weak antioxidant activities with the value of IC₅₀ of 466.804 µg/ml. Further studies can be done by comparing the antioxidant activity obtained with *Z. spina-christi* which grow elsewhere, as well as with other plant parts besides leaves.

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