Phytochemical Screening of Phenolic Levels from Extracted Bitter Mustard Leaves *(Brassica Juncen L. Czern.)* using UV-Visible Spectrophotometer

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Abstract: To determine the total phenolic content of the ethyl acetate extract of bitter mustard leaf. To determine the total phenolic levels, the Folin – Ciocalteu method with gallic acid comparison compounds using ethyl acetate solvents using UV-Visible spectrophotometer is expected to provide information and scientific evidence to develop new medicines from this plant. Antioxidants are inhibitors of oxidation reactions due to free radicals that can cause damage to unsaturated fatty acids, cell wall membranes, blood vessels, DNA bases, and lipid tissue causing disease. Phytochemical screening was using $FeCl_3$ 5% to identify phenolic compound. Total phenolic is gotten by many process such maceration and partition. Analysis of total phenolic was doing by TLC analysis. Total phenolic was analysed on plate of thin layer chromatography with used chloroform and methanol with 70:30 comparison. Antioxidant test was measured by UV-Visible spectrophotometer at 516 nm wavelength. Bitter mustard leaf extract contains phenolic compounds based on phytochemical screening results. Weight total phenolic content o 39.7252 ± 0.7326 mg GAE/g extract, the total phenol content determined according to the Folin-Ciocalteu medthod is not an absolute level, but principally based on the reduction capacity of the material being tested against an equivalent reduction of gallic acid. Calibration curve measurement with a concentrations of 200, 225, 250, 275 and 300 µg/ml. All solutions were measured at a wavelength of 739.50 nm. Phytochemical screening results show that the extract of bitter mustard leaf (Brassica juncea L.), contains chemicals, flavonoids, tannins, saponins, glycosides, steroids / triterpenoids, anthraquinone and polyphenols. The acetyl extract of bitter mustard leaf has a total phenolic activity value (39.7252 ± 0.7326) mg GAE / g extract. Penol compounds are susceptible to oxidation at higher temperature in extractions that are to long can provide opportunities for phenol compounds to oxidize more, but measured total phenol levels can be lower.

1 INTRODUCTION

Brassica juncea L. commonly known as Indian mustard belongs to family brassicaceae. B. juncea is an economically important plant widely used as an oil source, a green vegetable and also having a medicinal value. This species has been described in traditional remedies in the ancient literature (Manohar et al., 2009). Indian mustard is consumed as leafy vegetable and is a source of various micronutrients as well as antioxidants, vitamin c and e, β-carotenoids etc. B. juncea is believed as eco-friendly source for various nutraceuticals or drugs which are used to prevent and cure of wide range of non-communicable diseases in present time (Kumar et al., 2011). Food preparation of Indian mustard leaves is helpful in lowering the cost for diabetic patients suffering with comorbid anxiety disorders (Thakur et al., 2013). Plants of genus Brassica are also known for the production of various volatile organic compounds like ketones, aldehydes, esters, alcohols, terpenes and glucosinolates.

The leaves of B. juncea are utilized to produce medicines which act as stimulants, diuretics and expectorants (Farrell et al., 1985). Indian mustard is also known for its therapeutically pharmacological uses due to its active bio-constituents (Kumar et al., 2011). Glucosinolates and isothiocyanates are reported to be very active in B. juncea (Hill et al., 1987; McNaughton and Marks, 2003) which act as anti-cancerous and anti-microbial compounds (Luciano and Holley, 2009; Okulicz, 2010; Zhang et

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al., 2010). Leaves of Indian mustard were also reported to have anti-depressant effects during diabetes (Thakur et al., 2014). The presence of different brassinosteroids namely castasterone, teasterone, 24-epibrassinolide and typhasterol have been reported from B. juncea (Kanwar et al., 2015).

Indonesia has the potential to grow vegetables. Among the various types of vegetables that can be cultivated, mustard *(Brassica juncea L.)* is one of the vegetables that has commercial value and high prospects. Mustard greens include leaf vegetable plants from the Cruciferae family or cabbage plants which have high economic value because it is rich in fiber, high in nutritional content and has medicinal properties. Some of the results of epidemiological studies, it is known that eating vegetables from the genus Brassica can reduce the risk of several types of cancer, namely breast, prostate, kidney, colon, bladder and lung cancer. Vegetables included in the Brassicaceae tribe are mustard greens, bitter mustard greens, cabbage, broccoli (Sulihandari, 2013).

Antioxidants are the ability to capture free radicals. Radicals contained in biological systems can oxidize nucleic acids, proteins, lipids or DNA and cause degenerative diseases. Antioxidant components found in plants such as phenolic acids, polyphenols and flavonoids will capture free radicals such as peroxide, hydroperoxide or peroxyl lipids and also inhibit oxidative mechanisms that cause degenerative diseases (Prakash, 2001). Extraction is needed to obtain the desired compound in the bitter mustard greens. Selection of the right solvent can increase extraction efficiency. Things that need to be considered in the selection of solvents include selectivity, toxicity, polarity, ease of evaporation and the price of solvents. Ethyl acetate is a solvent with low toxicity that is semi-polar so it is expected to attract polar and nonpolar compounds from bitter mustard leaves (Akbar, 2010). Phytochemical screening needs to be done to determine the class of compounds contained in the extract used. In this study phytochemical screening was conducted to see the class of compounds in the ethyl acetate extract of bitter mustard leaves so that it can also be known the ability of the ethyl acetate solvent to attract compounds contained in bitter mustard leaves (Akbar, 2010).

Phenolic compounds are natural compounds which are widely used at present. Its ability as an active biological compound gives a large role to human interests. One of them is as an antioxidant, for the prevention and treatment of degenerative diseases, cancer, premature aging and immune system disorders in the body (Apsari, 2011). This study aims to determine the total phenolic content of the ethyl acetate extract of bitter mustard leaves. To determine the total phenolic content, the Folin-Ciocalteu method with gallic acid comparison compound using ethyl acetate solvent using UV-Visible Spectrophotometer is expected to provide information and scientific evidence to develop new medicines from this plant (Andarwulan, 2012).

2 METHOD

Place of research, The extract making is carried out in Chemical laboratory, at Pharmacy Faculty, Institut Kesehatan Medistra Lubuk Pakam. Research time, This research is carried out on the month (May 2019 to September 2019). Material, Fresh bitter mustard leaves, ethyl acetate extract from bitter mustard leaf. Gallic acid, Hydrochloric acid, Sulfuric acid, Iron (III) chloride, Sodium carbonate, Sodium hydroxide, Mayer reaents, Bouchardate reagents, Liebermann reagents Burchard, Folin – Ciocalteau reagents and Methanol. Equipment: Beaker Glass, Macerator, Separate Funnel (Schoot Duran), Rotary evaporator (Heidolph), Steaming waterbath (Memmert), TLC plate, Chamber, Incubator (Memmert) and UV-Visible Spectrophotometer (Shimadzu. Sample Processing, this research was carried out sequentially in laboratory with the following research scheme. Process started from maceration and screening test, then continued to evaporate solvent. Solid extract is soluted by water to remove the lipid. Then filtrated the fraction that soluted in water. Filtrate is partitied using ethyl acetate conducted partition using n-hexane by separate funnel. TLC is done to analysis of total phenolic compound and measure of antioxidant activity. The scheme of research is showed in Figure 2.

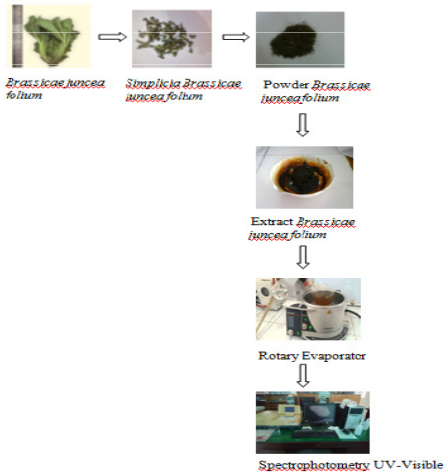

Figure 2. Scheme of Research

Phytochemical Screening:

This research was conducted in the laboratory of organic chemistry of natural materials, Institute Medical Lubuk Pakam, Faculty of pharmacy to determine the presence of phenolic compounds in the leaves of the Bitter Mustard plant. A preliminary test was carried out, phytochemical screening where 10 g fresh leaves of Bitter Mustard plant that had been blended with a blender macerated with methanol and then filtered. The filtrate was tested by adding 3 drops of 5% $FeCl_3$ reagent solution, forming a black precipitate if Bitter Mustard extract is positive contained phenolic compound (Eko BM, 2015). Sample as 1000 g of Bitter Mustard leaves powder which had been dried and finely macerated for ± 24 hours with methanol as much as 5 liters at room temperature. Macerate was filtered and a extract of Bitter Mustard leaves was obtained. Maceration was repeated using methanol as a solvent until the methanol extract obtained gave a negative test result with 5% $FeCl_3$ reagent. The methanol extract obtained was concentrated by rotary evaporator at a temperature of 60°C with a rotation of 80 rpm. The following is the determination of the wavelength of gallic acid with the addition of the Flin Ciohalteu reagent, measured in wavelength and Operating Time using a UV-Visible Spectrophotometer. This is shown in Figure 3.

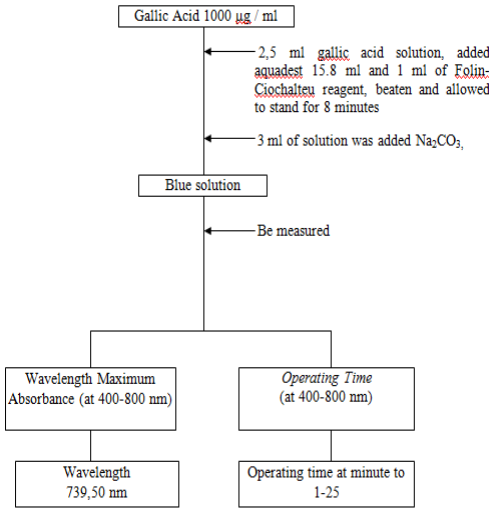

Figure 3: Flow Chart Determination of Maximum Wavelength and Operating Time

The following is the determination of the standard acid gall curve using the Folin-Ciohalteu reagent by dissolving the raw parent sample solution from bitter mustard leaves, where the wavelength is measured using a UV-Visible Spectrophotometer tool. This is shown in Figure 4.

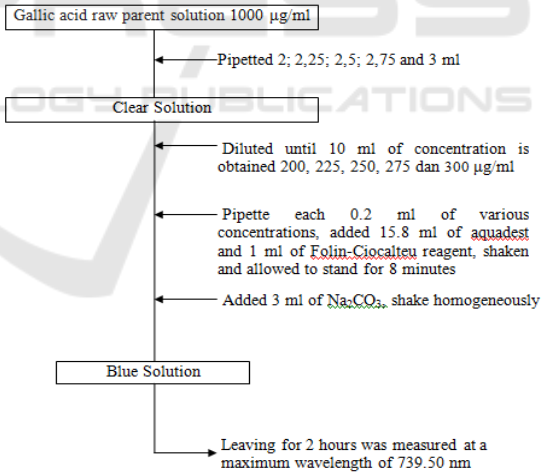

Figure 4: Flow Chart of Determination of Raw Acid Gallic Curve with Folin-Ciocalteu Reagent

The following is the determination of the total phenolic content of bitter mustard ethyl acetate extract by dissolving a bitter mustard leaf sample soltion, which is measured in wavelength using a UV-Visible Spectrophotometer tool as shown in Figure 5.

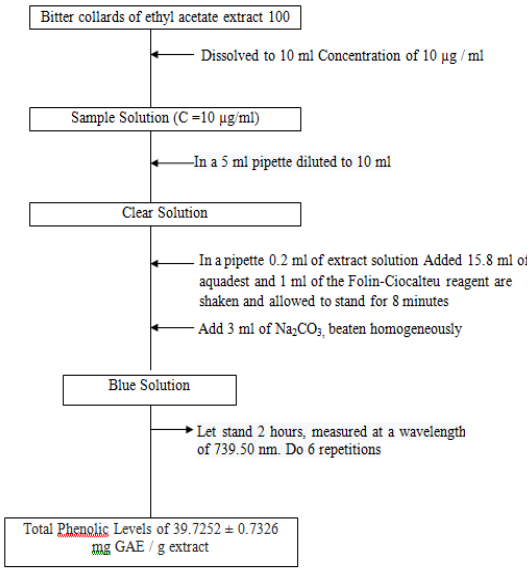

Figure 5: Flowchart Determination of Total Phenolic Content of Bitter Mustard Extract Ethyl Acetate

Bitter mustard leaf is considered dry if it is fragile (when squeezed it will be easily destroyed), then dried bitter mustard leaf is made into powder by blending, then powder which has become weighed dry weight and obtained as much as 1 kg of powder weight. Simplicia powder that has been weighed is then put into a dry plastic bag that is well closed, protected from sunlight and protected from heat. A total of 500 g of simplex powder of bitter mustard leaves were macerated using ethyl acetate solvent. For the first treatment 3750 ml of ethyl acetate was used in a tightly closed container and protected from sunlight for 5 days, the maserat could be separated into another container, the pulp was macerated again with 1250 ml of ethyl acetate for 2 days, then filtered so that it was obtained by the maserate. The first and second maserates obtained were transferred to another container which was tightly closed and then evaporated in a rotary evaporator so that a thick extract was obtained.

Determination of water content is done by the azeotropic method (toluene distillation). The device consists of a 500 ml round bottom flask, a container, a ball cooler, a connecting tube and a 0.1 ml scale receiving tube. A total of 200 ml of toluene and 2 ml of distilled water were put into a round bottom flask, a container was installed and cooled, then distilled for 2 hours. The distillation is stopped and allowed to cool, then the volume of water in the receiver tube is read as the initial volume of water with an accuracy of 0.05 ml. In a round bottom flask containing saturated toluene, 5 g of driedia bitter mustard leaf powder which has been carefully weighed is then carefully heated for 15 minutes. Toluent to boil, the droplet speed is set to 2 drops per second until as distilled water, then the distillation speed is increased to 4 drops per second, after all the water is distilled, the inside of the cooler is rinsed with saturated toluene. The distillation is continued for 5 minutes, then the receiver tube is allowed to cool at room temperature, after the water and toluene separate completely, the volume of water is read as the final volume with accuracy of 0.05 ml. The second difference in the volume of water reads according to the water content contained in the material being examined.

As much as 2.5 ml of 1000 µg gallic acid solution was put into a 10 ml volumetric flask, then stirred up to the mark with ethanol, then pipetted 0.2 ml put into a 25 ml flask, then added 15.8 ml of distilled water and 1 ml of Folin-reagent Ciocalteu, then shaken and allowed to stand for 8 minutes. To the solution was added 3 ml of Na_2CO_3 solution, shaken until homogeneous and allowed to stand for 2 hours at room temperature, then the absorbance was measured at a wavelength of 400-800 nm. As much as 2.5 ml of 1000 µg gallic acid solution was put into a 10 ml volumetric flask, then stirred up to the mark line, then pipetted 0.2 ml put into a 25 ml flask, then added 15.8 ml of distilled water and 1 ml of Folin-Ciocalteu reagent, then shake until homogeneous and sit for 8 minutes. To the solution was added 3 ml of Na_2CO_3 solution, then shaken homogeneously and allowed to stand for 2 hours at room temperature, measured its absorbance in the span of 1-25 minutes wavelength 739.50 nm. From the gallic acid mother liquor concentration of 1000 µg / ml pipetted 2, 2, 25, 2, 5, 2, 75 and 3 ml then put into a 10 ml flask, then stirred to the mark line with ethanol. From each pipette 0.2 ml was put into a 25 ml flask and then added 15.8 ml of aquadest and 1 ml of the Folin-Ciocalteu reagent were shaken until homogeneous, allowed to stand for 8 minutes. To the solution was added 3 ml of Na_2CO_3 solution then shaken homogeneously, allowed to stand for 2 hours at room temperature. The absorbance was measured at a wavelength of 739.50 nm to obtain concentrations of 200, 225, 250, 275 and 300 µg / ml.

A total of 100 mg of bitter mustard extract was dissolved with 10 ml of distilled water to obtain a concentration of 10 mg / ml. From a concentration of 10 mg / ml pipette 5 ml put into a 10 ml flask, then stirred up to the mark line, then pipette 0.2 ml extract put into a 25 ml flask then added 15.8 ml of distilled water and 1 ml of Folin-Ciocalteu reagent,

shaken homogeneously let stand for 8 minutes then add 3 ml of Na_2CO_3 solution. allowed to stand for 2 hours at room temperature. The absorbance of the extract solution was measured with a UV-Visible Spectrophotometer.

3 RESULT AND DISCUSSION

The results of phytochemical screening of bitter mustard seed ethanol extract. Phytochemical screening uses 5% FeCl3 reagent where the sample was previously dissolved with methanol solvent repeatedly. In this case, the extract becomes a black precipitate after decreasing 5% FeCl3 while the previous extract is green. The screening results are tested in a test tube. The black sediment in question is bitter mustard leaf which contains phenolic. The maceration process is processed on a sample of bitter mustard leaf powder in macerator. Maceration is treated repeatedly to maximize the extract produced. Samples as macerated solid extracts in methanol solvents were obtained at 300 g. This method is carried out by inserting suitable plant powders and solvents into a humidly closed container at room temperature. The principle of maceration method is based that samples soaked using organic solvents will break down the cell walls and membranes due to pressure differences found outside and inside the cell so that secondary metabolites contained in the cytoplasm will dissolve into organic solvents. The extraction process is stopped when a balance is reached between the concentration of the compound in the solvent and the concentration in the plant cell18.

After maceration, the partition was carried out using ethyl acetate to obtain a 600 g solid extract. The last partition was carried out using n-hexane to partition non-polar compounds from phenolic compounds. the actual total phenolic content was 39.7252 ± 0.7326 mg GAE / g extract, the total phenol content determined according to the Folin-Ciocalteu method was not an absolute level, but in principle based on the reduction capacity of the material being tested against an equivalent reduction of gallic acid. Bitter mustard leaf extract from the partition contains total phenolic because it reacts positively to the FeCl3 reagent when we do the filtering again. In the liquid-liquid partition process, the two phases of solution have different solubility. Separate funnel shocks during partition aim to expand the contact surface area between insoluble solvents. The solvent requirements for the partition method have suitable polarity for the extracted material and must be separated after shaking. Total phenol levels are influenced by the type of solvent. Phenol is a polar compound so that its solubility is highest in a semi-polar solvent. Polar solvents are able to dissolve phenols so that the levels in extracts are high. Phenol compounds are susceptible to oxidation at high temperatures so that they experience degredation while extraction that is too long can provide an opportunity for phenol compounds to oxidize more, but the total phenol levels measured can be even lower.

Phytochemical screening tests were conducted to determine the class of chemical compounds contained in bitter mustard leaves using ethyl acetate extract. Based on phytochemical screening conducted on ethyl acetate extract is a low toxicity solvent that is semi polar so that it can attract polar and non polar compounds from bitter mustard leaves. Bitter mustard leaves contain flavonoids, alkaloids, glycosides, tannins, saponins, steroids / triterpenoids and anthraquinones. On examination of flavonoids with the addition of hydrochloric acid to the Mg powder, it gives a red color which indicates the presence of flavonoid compounds. Alkaloide examination with Mayer, Dragendorff and Bouchardat reagents results in turbidity and deposition. The examination of anthraquinone glycosides with the addition of NaOH to the simplicia powder gives a positive red color, while the extract does not form red. Tannin examination with the addition of $FeCl_3$ solution will show blue or blackish green discoloration (Harborne, 1996). Determination of total phenolic levels using the spectrophotometric method of visible light with the Folin-Ciocalteu reagent is the simplest, easiest method, using a relatively small number of samples and a shorter processing time. Total phenolic testing begins with the measurement of the maximum wavelength of gallic acid solution with a concentration of 1000 µg / ml in ethanol using a Visible spectrophotometer to obtain a wavelength of 739.50 nm with an absorbance of 0.340.

The color of the Folin-Ciocalteu reagent standard solution is usually less stable so it is necessary to find the right working time to take measurements because the amount of absorbance in the spectrophotometry of the visible light is strongly influenced by the color. Determination of working time is done by using a standard solution of the Folin-Ciocalteu reagent accompanied by the addition of an extract which aims to find a stable measurement time when the sample reacts completely with a color reagent, measured at a wavelength of 739.50 nm. Operating Time

measurement results obtained results in minutes 23-25.

According to Rahmawati research, 2015. Analysis of flavonoid and phenolic levels was done using the Chang method, then the wavelength optimization was performed to determine the maximum wavelength to be used in measurements on UV-Visible Spectrophotometer. From the measurement results obtained a maximum wavelength of 415 nm for flavonoids and 730 nm for phenolics. Quantitative analysis of flavonoids was carried out by making a series of regular standard solution concentrations of 3.75 ppm, 5 ppm, 6.25 ppm, 7.5 ppm, 8.75 ppm and 10 ppm, from each concentration in a 1 ml pipette, then add 3 ml of 95% ethanol 0.2 ml of aluminum chloride 10% 0.2 ml (to make a shift towards a longer wavelength, thus changing the standard wavelength routine to enter the Spectrophotometer of UV-Visible wavelength range).

Determination of total phenolic levels using the spectrophotometric method of visible light with the Folin-Ciocalteu reagent is the simplest, easiest method, using a relatively small number of samples and a shorter processing time. Total phenolic testing begins with the measurement of the maximum wavelength of gallic acid solution concentration of 1000 µg / ml in ethanol using a Spectrophotometer of UV-Visible to obtain a wavelength of 739.50 nm with an absorbance of 0.340. According to Gandjar and Rohman (2007) the blue color will produce maximum absorbance at a maximum wavelength of 400-800 nm.

Calibration curve measurements were carried out with different solution concentrations pipetted from gallic acid solution at a concentration of 1000 µg / ml. Obtained concentrations of 200, 225, 250, 275 and 300 µg / ml, put into a volumetric flask added 1 ml of Folin-Ciocalteu reagent and shaken after it is allowed to stand for 8 minutes, each solution plus 3 ml of Na_2CO_3 beaten homogeneously and allowed to stand for 2 hours at room temperature to perfect the reaction. All solutions were measured at a wavelength of 739.50 nm, then a calibration curve was made between the concentration of gallic acid (µg / m) and absorbance. Calibration curve measurements were carried out with concentrations of different solutions pipetted from gallic acid solution at concentrations of 1000 µg/ml with concentrations of 200, 225, 250, 275 and 300 µg/ml. The results of the standard absorbance of gallic acid by using a sample of bitter mustard leaf extract based on the concentration and absorbance of the regression equation measured with wavelength using the Spectrophtometer of UV-Visible tool.

Table 1: Standard Absorbance of Gallic Acid

Sampel	Konsentrasi	Absorbansi	Persamaan Regresi
Ekstrak Daun Sawi Pahit	0,00	0,000	Y = 0,08659 x + 0,00046
	2,00	0,175	
	2,25	0,197	
	2,50	0,216	
	2,75	0,235	
	3,00	0,262	

From this table a calibration curve is obtained as shown in the following figure:

The following are the results of the calibration curve for gallic acid compounds which were measured using Spectrophtometer of UV-Visible at a maximum wavelength of 515 nm. The regression equation used in determining the total phenolic compound content is Y = 0.08569 x + 0.00046 with a correlation coefficient of 0.999940. The linearity value shows the correlation between the concentration and the absorbance produced. Testing the total phenolic content is calculated by substituting the absorbance value (y) of the sample at the maximum wavelength into the linear regression equation y = ax + b obtained from the gallic acid calibration curve so that the concentration (x) is obtained. The value of x is then substituted in the formula for calculating total phenol levels. Measurement of total phenol levels was carried out by repetition 6 times and the average was taken as presented in table 2 below.

From the results of the above study, the actual total phenolic content was 39.7252 ± 0.7326 mg GAE / g extract, the total phenol content determined according to the Folin-Ciocalteu method was not an absolute level, but in principle based on the reduction capacity of the material tested against an equivalent reduction of gallic acid. Total phenol levels are influenced by the type of solvent. Phenol is a polar compound so that its solubility is highest in a semi-polar solvent. Polar solvents are able to dissolve phenols so that the levels in extracts are high. Phenol compounds are susceptible to oxidation at high temperatures so that they experience degredation while extraction that is too long can provide an opportunity for phenol compounds to oxidize more, but the measured levels of total phenol can be lower. Testing the total phenolic content is calculated by substituting the absorbance value (y) of the sample at the maximum wavelength into the

linear regression equation y = ax + b obtained from the gallic acid calibration curve so that the concentration (x) is obtained. The value of x is then substituted in the formula for calculating total phenol levels. Total phenol levels are influenced by the type of solvent. Phenol is a polar compound so that its solubility is highest in a semi-polar solvent. Polar solvents are able to dissolve phenols so that the levels in extracts are high. Phenol compounds are susceptible to oxidation at high temperatures so that they experience degredation while extraction that is too long can provide an opportunity for phenol compounds to oxidize more, but the measured levels of total phenol can be lower.

4 CONCLUSIONS

After maceration and partition, a total phenolic compound is obtained from saputangan leaves as 18.25 g. Results of thin layer chromatography analysis of total phenolics using the chloroform : methanol as eluent showed that total phenolic has 3 spots that have an Rf of 0.44 ; 0.29 and 0.22. The total phenolic compound is able to act as a strong antioxidant by having an IC_{50} value of 15.22 ppm. This antioxidant test was carried out using a DPPH (2,2-diphenyl-1- picrilhidrazil) which was measured using a UV-Visible spectrophotometer at a wavelength of 516 nm.

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