ISSN 0975-2366
DOI:https://doi.org/10.31838/ijpr/2020.12.02.0010

Research Article

Antioxidant Activities and Identification of Compounds in Ethyl Acetate Fraction, Dichloromethane Fraction And Ethanol Extract Of *Garcinia mangostana* L. Leaves

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Received: 06.11.19, Revised: 03.12.19, Accepted: 14.01.20

ABSTRACT

Mangosteen leaves (*Garcinia mangostana* L.) are a good source of antioxidants. The compounds contained therein are flavonoids and xanthone. This study aims to compare antioxidant activity and identify active compounds and to determine the types of flavonoids from ethanol extract, dichloromethane fraction and ethyl acetate fraction of *G. mangostana* leaves. The maceration method was used as extraction with solvent 70% ethanol. The thick extract was hydrolyzed using 2 N hydrochloric acid, then partitioned with dichloromethane and ethyl acetate. Ethanol extract, dichloromethane fraction, and ethyl acetate fraction were tested for antioxidant activity using DPPH method with UV-Vis spectrophotometer and quercetin as standard. The IC_{50} value of ethanol extract, dichloromethane fractions and ethyl acetate fraction were 0.819 µg/mL, 0.723 µg/mL, 0.025 µg/mL. Flavonol Quercetin as a standard has IC_{50} of 0.131 µg/mL. It indicates that the extract and fraction of *G. mangostana* leaves have potent antioxidant activity and ethyl acetate fraction has higher antioxidant activity than other solvents.

Keywords: Garcinia mangostana L, antioxidant activity, ethyl acetate fraction, ethanol extract

INTRODUCTION

Naturally, the body has a fortress that can prevent free radical attacks called antioxidant. The most well-known antioxidant synthetic compounds are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are widely used in the food and beverage industry [(1)]. However, these antioxidant compounds have undesirable side effects, which are potentially carcinogenic. Therefore, the new natural antioxidant types must continue to be sought to reduce free radicals that can damage the human body.

Based on research conducted by [(2)] on the test of antioxidant activity, ethanol extract of *G. mangostana* peel has an IC_{50} value of 5.03 µg / ml, ethyl acetate extract with IC_{50} value of 41.56 µg / ml, fractionation of ethyl acetate shows value IC_{50} 2.78 µg / ml and n-hexane fraction has IC_{50} of 22.33 µg / ml. It indicates that *G. mangostana* peel extract and fraction has robust antioxidant activity and ethanol extract has antioxidant activity compared to other solvents.

The results of the chromatographic profile showed that the *G. mangostana* leaf ethanol extract contained flavonoid compounds [(3)]. Flavonoid levels in *G. mangostana* leaf ethanol extract were two times greater than the degrees in *G. mangostana* pericarp ethanol extract [(4,5)]. Solvents are very influential on the extraction of active compounds so that by using different enzymes it can be seen that the water has the highest anti-hyperuricemia and antioxidant activity and the smallest IC_{50} value. So the researchers were encouraged to research the antioxidant activity of dichloromethane fraction and ethyl acetate fraction from the ethanol extract.

METHOD

Preparation of ethanol extract of *G. mangostana* leaves.

Plants obtained from the Banyumas, Central of Java, which is thought to be a plant of *G mangostana* was determined at the Environmental Laboratory, Faculty of Biology, Jenderal Soedirman University. Dry powder of *G. mangostana* leaves weighed +500 grams then macerated. Maceration is done by soaking the dried *G. mangostana* leaves powder in the solvent. Improving the effectiveness of extraction carried out stirring and re-maceration, macerated for 3 x 24 hours by comparison between powdered leaves and 70% ethanol was 1:10 for the first day, 1: 4 for the second day, 1: 4 for the third day. The method is that 500 grams of dried *G. mangostana* powder are macerated with 70% ethanol as much as 5 liters, then poured and squeezed. The pulp obtained is again macerated with 70 liters of ethanol as much as 2 liters and is

poured. Then the second day of maceration dregs was macerated again with 70% ethanol as much as 2 liters. Ethanol juice is then evaporated with a rotary evaporator (*vacuum evaporator)* at a maximum temperature of 50˚ C until thick consistency can still be poured. Then the thickened ethanol extract was poured in a porcelain cup and evaporated using a water bath. Then weigh the extract if the consistency is thick [6].

Hydrolysis and Fractionation

G. mangostana leaves (50 mg) was hydrolyzed with 2N HCl: Methanol with a ratio of 1: 1 as much as 200 ml, then refluxed at a temperature of 100 ° C for 30 minutes [7]. The results of hydrolysis were included in a separating funnel and then fractionated using dichloromethane solvent with a ratio of 1: 1 of 400 ml in a separating funnel, allowed to form until 2 layers were formed namely dichloromethane fraction layer and dichloromethane fraction residue layer, then the dichloromethane layer was washed with aquadest 3 times then layer the top is taken, and anhydrous Na Sulfate is added then filtered. The upper layer is removed, and the bottom layer is heated in a fume hood while the residual thickness of dichloromethane is re-vaccinated using ethyl acetate in a ratio of 1: 1, then left to form two layers, namely ethyl acetate and 70% ethanol. The ethyl acetate layer was then washed with aquadest three times so that two layers formed. The lower layer is taken, and then anhydrous Na sulfate is then filtered. The upper layer is removed while the bottom layer is heated in a fume hood. The results of the dichloromethane and ethyl acetate fractions which have been evaporated are then used as test samples [8].

Identification of compound was grouped by thin layer chromatography method.

Identification of chemical compounds from the chromatographic profile of TLC was carried out by giving spotting reagents to flavonoid compounds. The results are identified by looking at the color of spots on visible light or UV light 366.

Detection of flavonoid compounds is carried out as follows:

Stationary phase: Cellulose

Mobile phase: 30% glacial acetic acid

Spotting reagents: Ammonia and sitroborate vapors

The appearance of patches: Ammonia and sitroborate vapor are then heated at 105 ° C for 5 minutes. If positive contains flavonoids, it will fluoresce yellow-green in UV light 366 [9].

Measurement of Antioxidant Activity.

A total of 1 ml ethanol extract, dichloromethane fraction and ethyl acetate fraction (0.5 μg / ml, 0.3 μg / ml, 0.1 μg / ml and 0.05 μg / ml) was added to 2 ml DPPH 0.004%. The mixture was then shaken and incubated at room temperature for 30 minutes in a dark place. This solution is then measured for absorbance at the wavelength that has been obtained in the previous stage using UV-Vis spectrophotometer. The same treatment is also carried out for a blank solution (DPPH solution which does not contain test material). The new solution consisted of 2 ml DPPH 0.004% and one mL methanol as the initial Zeroing was carried out using three mL methanol pro analysis [2]. Quercetin, natural antioxidant was used as positive control with concentration of 0.20 μl / ml, 0.12 μl / ml, 0.04 μl / ml, 0.02 μl / ml

Data analysis.

Free radical inhibition of DPPH in percent inhibition was calculated using the formula:

I% = ((blank absorbance - sample absorbance)/ blank absorbance) X 100

Blank absorbance is the absorbance of the control reaction (containing all reagents except the sample), and the absorbance of the sample is the absorbance of the tested compound. The concentration of ethanol extract and fraction which showed 50% resistance (IC_{50}) was calculated from the percentage relationship resistance curve with sample concentration. Natural antioxidant compounds used as positive controls were quercetin. All tests are carried out with a triple [11].

Data obtained from measurements of free radical scavenging activity in the form of percent inhibition (I%) were further analyzed to determine the IC_{50} price, using linear regression equation on the relationship curve between percent inhibition and the concentration of the test sample. Then processed using SPSS 23 program by looking at the normality test and homogeneity test which is used as a condition for testing one-way variance analysis to see the average difference of two or more treatment groups. If one of the requirements for the ANOVA test is not met, then the Kruskal-Wallis test with a 95% confidence level is used to see the difference, then the Mann-Whitney test is performed [12].

RESULT

Making ethanol extract of mangosteen leaves used maceration method, because it was a simple method that was done by soaking the powdered leaves in the solven. Maceration was carried out for three days using 70% ethanol. Three days is

enough time for the active substance to dissolve and exit the cell. The powdered leaves of *G. mangostana* was soaked and stirred every 6 hours so that the macerate was not saturated with the active material because if the liquid was already saturated, it was difficult for the fluid to take the active ingredient from the powder. The solven was used 70% ethanol, because flavonoids can dissolve in them other than that non-toxic ethanol, the absorption is excellent, and the heat needed for concentration was less [13].

The ratio between the powder and the solvent solution was 1:10. The powdered leaves 500 grams extracted with 5 liters 70% ethanol. Then proceed with the re-maceration process for 24 hours, this process is carried out two times to get the active substance that is still left in the powdered leaves because the solven was saturated. Comparison of powder with 1: 4 seaweed solution with a total of 70% ethanol as much as 2 liters. Viscous extract was obtained 130.05 grams (Table 1) and were colored chocolate old, smelly typical, bitter taste (Figure 1).

Fig.1: Thick extract G. mangostana Leaves

Table 1: Randemen Mangosteen Leaf Extract

Powder (gram)	Container weight + thick extract (gram)	Empty container weight (grams)	Extract weight (grams)	Randemen (%)
500	270.60	140.55	130.05	26.01

Results of Hydrolysis and Fractionation of Mangosteen Leaf Ethanol Extract

Ethanol extract (50 mg) was hydrolyzed using 2N HCl: 200 ml of Methanol (1: 1), then refluxed at 100 ° C for 30 minutes [7]. According to Hartono and Wahyudi [14] to reduce the activation energy (lower the reaction temperature) and accelerate the course of the hydrolysis reaction a catalyst is needed. In micro, the mechanism of catalyst work can be explained as the occurrence of collisions between electrons which results in changes in electron configuration so that new elements can be obtained which eventually produce new compounds. Adding an acid catalyst can create an acidic condition and an appropriate pH. The effectiveness of catalyst work is also strongly influenced by temperature and concentration. One of the acid catalysts that can be used is HCl to break down flavonoids into their aglycones and break the sugar from flavonoid aglycones. Aglycones generally have higher antioxidant and radical capture power than flavonoid glycosides because of the flavonoid glycosides phenolic hydroxyl groups which are active groups of antioxidants or radical catchers have bound sugar groups [15]. Flavonoids are commonly found in plants, bound to sugar as flavonoid glycosides and aglycones which may be present in one plant in several forms of a combination of glycosides [13]. The results of hydrolysis are then partitioned with ethyl acetate to separate the flavonoid aglycones from the sugar so that the flavonoid aglycones will be in the ethyl acetate fraction, while the sugar will be in the water fraction [9].

Flavonoid aglycones are polyphenols because that have character chemistry compound phenol that is slightly acid so that could come late in the base but when left out in solution base and condition there is oxygen, so much who will decompose. Flavonoid is polar compounds are generally late enough in polar solvents like ethanol, methanol, butanol, acetone, dimethyl-sulfoxide, dimethylformamide, water, etc. [9]. There is sugar bound on flavonoids (shape commonly found) tend cause more flavonoids easy late in a polar solvent and with thereby mixing ethanol solvent is a suitable solvent for glycosides. Conversely, the less extreme aglycone isoflavones, flavanones, and flavone and flavonol are methoxylated tend easier late in a solvent like an ethyl acetate, dichloromethane, ether and chloroform [9].

The aglycone will be in the dichloromethane fraction and ethyl acetate fraction while the sugar will be in the mangosteen leaf ethanol extract. The concentrated extract produced from dichloromethane fraction was 280 mg and 570 mg ethyl acetate fraction, then the results of fraction and ethanol extract of mangosteen leaves were analyzed by thin layer chromatography.

Identification Results of Compound Groups with Thin Layer Chromatography Methods

Ethanol extract of mangosteen leaves, dichloromethane fraction, and ethyl acetate fraction were then identified using thin layer chromatography to find out the compounds contained therein. Thin layer chromatography method is one of the qualitative techniques to identify a compound that is carried out by removing a sample in the stationary phase using the appropriate mobile period.

The use of TLC has several advantages; namely, separation can be done quickly, substances that are acidic or strong bases can be used, analysis can be done more sensitive with simple tools so that their use is easy. Besides this method is simple, fast in separation, responsive and easy to retrieve the separated compounds [16].

The stationary phase used is cellulose and 30% glacial acetic acid as the mobile phase. The chromatogram produced was then observed under UV light 366. The color of the spot after being found under UV light 366 showed a lavender color indicating that the sample contained flavonoids [9]. Then evaporated with ammonia in an example formed in yellow or green, after spraying with sitroborate in the sample still form yellow or green indicating that this sample contains flavonoid aglycones (flavone or flavonol) [9].

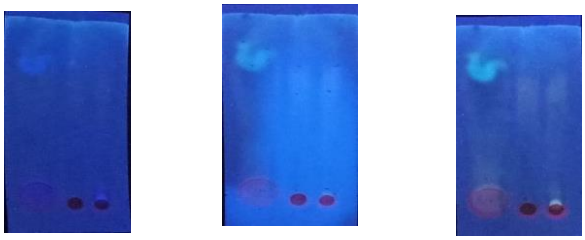

Fig.2: Chromatogram Profile with Stationary Phase Cellulose and Mobile Phase of Glacial Acetic Acid 30% (1) EE (2) DF (3) EAF, observed (A) under UV366 nm (B) under UV366 nm light vaporized with ammonia (C) seen under UV366 nm light and sprayed with sitroborat

Table 2: Identification of Flavonoids

Sampling sample	hRf	Observation on UV light 366		Information
		With ammonia vapor	After spraying sitroborat	
Ethanol extract	81.25	Green	Green	+ flavonoids
Dichloromethane fraction	62.5	Orange-red	Change color to green	+ flavonoids
Ethyl acetate fraction	Point 1 = 6.25 Point 2 = 62.5	Bright yellow	Color changes become darker	+ flavonoids

Based on table 2, dichloromethane fractions flourosity red or orange when given steam ammonia possible flavonoid types contained inside it that was compound chalcon containing 2- and 4- OH-free. While on ethyl acetate fraction and extract ethanol chromatogram, the second sample flourosity green yellow after given steam ammonia possible flavonoid types contained inside it that is compound 5-OH flavone or flavonol, 5-OH flavonone and 4'-OH chalcones [9].

Antioxidant Activity Test Results

In determining the maximum wavelength of DPPH, the method used is a spectrophotometric method with the reason that this method has the advantage of providing a simple way to determine the smallest amount of substances and at a cost that is not too expensive. Determination of the maximum wavelength of 0.004% aims to identify the wavelength with the maximum absorbance value in UV-vis spectrophotometer. The maximum wavelength is the wavelength where electronic excitation occurs which gives maximum absorbance. The reason for the measurement is the change in absorbance for each unit of concentration at the maximum at the maximum wavelength so that a maximum analytical sensitivity is obtained. The antioxidant activity test of ethanol extract and its fractions was carried out by 2 ml of 0.004% DPPH observed its absorption in the wavelength range of 400-800 nm using methanol blanks. The result shows that the maximum wavelength of DPPH solution is at 514.0 with an absorbance value of 0.5146. It means that absorbance in the antioxidant activity test is carried out at a wavelength of 514.

The antioxidant activity test with DPPH method is

intended to strengthen the activity of a compound in plants (mangosteen leaves) as an antioxidant because as is well known antioxidant power can be carried out with various methods. DPPH is a free radical that is stable at room temperature and is often used to evaluate the antioxidant activity of several compounds or extracts of natural ingredients. DPPH accepts electrons or hydrogen radicals to form a stable diamagnetic molecule. The interaction of antioxidants with DPPH either electron transfer or hydrogen radicals in DPPH will neutralize the free radical character of DPPH. If all electrons in DPPH free radicals become paired, then the color of the solution changes from dark purple to bright yellow and absorbance at a wavelength of 514 nm will disappear. This change can be measured by stoichiometry according to the number of electrons or hydrogen atoms captured by the DPPH molecule due to the presence of antioxidants.

Testing of antioxidant potential in samples was carried out at a wavelength of 514 nm. The variation of concentration used is 0.5; 0.3; 0,1; 0.05 μg / ml. The samples tested for antioxidant activity were ethanol extract, dichloromethane fraction and ethyl acetate fraction. And quercetin as a comparison sample was used at a concentration of 0.2; 0.12; 0.04; 0.02 μg / ml. In the measurement of the potential for antioxidant activity, DPPH 0.004% solution was used. It is essential because the control solution is useful as a comparison in determining the antioxidant potential of the sample. Also, the control solution serves to determine the radical absorbance of DPPH before being reduced by the sample. The absorbance difference that has been reduced by DPPH with absorbance control is the residual DPPH radical that is read on a UV-Vis spectrophotometer.

Table 4: Antioxidant Activity Test Results

Material	Concentration (μg / ml)	% inhibition	Average IC_{50} (μg / ml) ± SD
Ethanol extract	0.5	46.433	0.8193 ± 0.031
	0.3	43.774	
	0.1	42.736	
	0.05	40.531	
Dichloromethane fraction	0.5	47.211	0.7231 ± 0.019
	0.3	44.876	
	0.1	42.866	
	0.05	41.245	
Ethyl acetate fraction	0.5	66.601	0.025 ± 0.003
	0.3	59.014	
	0.1	56.549	
	0.05	52.140	
Quercetin	0.2	52.269	0.1314 ± 0.002
	0.12	50.648	
	0.04	46.952	
	0.02	43.709	

Percent (%) of antioxidant activity is one parameter that shows the ability of an antioxidant to inhibit free radicals. The higher percent (%) of antioxidant activity indicates the number of hydrogen atoms given by active compounds to radical DPPH, so DPPH is reduced to DPPH-H [17]. Ethanol extract, dichloromethane fraction and ethyl acetate fraction changed color after incubation. The measurement results of the sample when added DPPH solution had a color change from purple to faded purple to yellow. This color change indicates that each test sample has the ability as an antioxidant. The reduction in color intensity that occurs is related to the number of DPPH electrons that capture hydrogen atoms from antioxidant compounds. Cut in the strength of dark purple (DPPH) indicates an increase in the ability of antioxidants to capture free radicals. Testing of antioxidant activity of mangosteen leaves was done on 70% ethanol extract, dichloromethane fraction and ethyl acetate fraction using quercetin standard. Quercetin was chosen as a positive control because it has been shown to have free radical scavenging activity. Quercetin is the largest compound of the flavonol group. When flavonol quercetin reacts with free radicals, quercetin donates its protons and becomes a radical compound, but the unpaired electrons produced are delocalized by resonance. It makes organic quercetin compounds have the

deficient energy to become reactive radicals. The purpose of this antioxidant test is to find out how strong the antioxidant potential is in the extract. If the% of the antioxidant activity of the sample is the same or close to the value of the related antioxidant activity, it can be said that the sample has the potential as an alternative antioxidant [18]. Percentage (%) of antioxidant activity from samples and standards was shown in table 4.

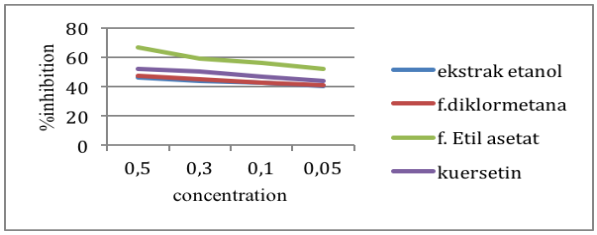

Fig.5: Chart Test Activity Antioxidants of ethanol extract, dichloromethane fraction, ethyl acetate fraction and quercetin

Value of IC $_{50}$ was concentration substrate that can cause a 50% reduction in DPPH activity. Getting less small IC_{50} value signify increasingly great activity antioxidants so that the required concentration for muffle radical DPPH by 50% increasingly small. Based on on table 4, was known that percent (%) activity antioxidants (IC_{50} value) of ethanol extract, dichloromethane fractions and ethyl acetate fraction were 0.819 μg/mL, 0.723 μg/mL, 0.025 μg/mL. Flavonol Quercetin as a standard has IC $_{50}$ of 0.131 μg/mL. The results showed that ability of extract ethanol and its fractions to scavenge free radical DPPH was very strong. Based on figure 5, ethyl acetate fraction has the ability as highest antioxidants when compared with extract ethanol and fraction dichloromethane, possible because there was some compound that contains aglycon flavonoid on ethyl acetate fraction.

There is womb flavonoid compounds on extract ethanol, dichloromethane fraction and ethyl acetate fraction cause extract that have % activity enough antioxidants good. It due to flavonoid compounds are class compound polyphenols that have many group hydroxy (OH). Hydrogen atom from group hydroxy that could donate on compound radical so that compound that could be stabilized. Fitriyani [19] mentions that flavonoids are class compound potentially active as antioxidants natural.

IC $_{50}$ value of quercetin equal to 0.131 μg/ml, extract ethanol amounting to 0.819 μg/ml, dichloromethane fraction amounting to 0.723 and ethyl acetate fraction amounting to 0.025 μg/ml, this show that ability extract ethanol and its fractions for muffle radical free very strong. Compound with IC $_{50}$ value <50 μg / ml has power free radical scavenger very strong. Said strong if IC $_{50}$ values between 50-100 ppm, whereas IC $_{50}$ values of 100-150 μg /ml then power arrest first free medium and compound with IC value $_{50}$ between 150-200 then have power arrest free radical weak [20].

IC $_{50}$ data were analyzed by using SPSS 23 for a test there is no difference activity inhibition radical free DPPH on concentration and type different solvents. The results of normality and homogeneity analysis showed data did not meet terms for ANOVA test (*analysis of variance*) because the significant value was 0,016 (P <0.05) exists difference, so the data is not homogeneous. Datas were continued with Kruskal Wallis nonparametric test. The results of Kruskal-Wallis test showed not significant with the value was 0.016 (p <0.05) so could follow up with use test Mann-Whitney. The results were showed Mann Whitney test with value not significant amounting to 0.037 (p <0.05), so could conclude there is difference meaningful between IC_{50} value on the fourth data [12].

CONCLUSION

Based on the research that has been done it can be concluded that ethanol extract, dichloromethane fraction and ethyl acetate fraction of mangosteen leaves have potent antioxidant activity against DPPH free radicals. From the results of the research obtained, it is recommended to do antioxidant testing with other methods.

ACKNOWLEDGEMENT

We would like to thank the Ministry of Research, Technology and the Higher Education Republic of Indonesia for the research funding.

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