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Research Paper

Antioxidant activity assay of extracts and active fractions of kasturi fruit *(Mangifera casturi* Kosterm.*)* using 1, 1-diphenyl-2-picrylhydrazyl method

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ABSTRACT

In-vitro antioxidant activity assay was conducted on methanolic extracts and its fractions (*n*-hexane fraction, ethylacetate fraction, and methanol fraction) of *Mangifera casturi* fruit using DPPH (1,1-diphenyl-2- picrylhydrazyl) method. The assay began by observing the chromatographic profile and phytochemical constituent analysis. The analysis showed that methanolic extracts of *M. casturi* fruit and its fractions contained compounds of terpenoid and polyphenol groups. Methanolic extracts of *M. casturi* had antioxidant activity with an IC_{50} value of 112.4µg/ml, while *n*-hexane, ethylacetate, and methanolic fractions had IC_{50} values of 193.0µg/ml, 6.0µg/ml, and 538.9µg/ml respectively.

Keywords: Kasturi; *Mangifera casturi*; Antioxidant; IC_{50}.

INTRODUCTION

Decrease in body stamina can facilitate the onset of diseases where the free radicals as infectious and toxin agents will easily penetrate the defense of body. According to American Medical Association (AMA), most people do not gain sufficient antioxydant intakes from foods consumed. Antioxydant is an substance required by body to neutralize the free radicals and prevent the resultant damages to normal cell, protein and fat. Free radicals are unstable atoms or chemical molecules that can cause damages in cells as a consequence of the unbalance between reactive oxygene species (ROS) and antioxydant enzyme generation. Antioxydant stabilizes the free radicals by completing lack of electron and impedes the occurence of chain reaction resulted from the establishement of free radicals that can cause oxidative stress (Pardo-Andrew, et al., 2006).

At present the discovery of antioxydant drugs from natural, semi-synthetic and synthetic matters continuously develops. Natural compound with antioxydant activity include compound in phenolic, flavonoid, and vitamin group (Gupta and Sharma, 2006). Phenolic compound contained in plants is a main souce in oxydant rection

(free radicals) and antioxydant (free radical scavenger). It is resulted from oxydation-reduction property as an agent acting as hidrogen donor. Phenolic antioxydant activity plays an important role in the adsorption or neutralization of free radicals (Ercisli, et al., 2008). In this study, investigation of phytochemical composition and evaluation of antioxydant in vitro from the methanol extract of *M. casturi* fruit and its fractions, i.e. *n*-hexane, ethyl acetate and methanol fractions are conducted.

The investigation of antioxydant activity from the methanol extract of *M. casturi* fruit and it factions is done for the first time. It is very interesting because the plant of *M. casturi* is one of the Borneo-typical vegetations (the local name is Borneo mango) and the existence began to be scarce. The kasturi plants are spread in South Borneo such as Martapura, Kandangan, and Tanjung. Moreover, they are spread also in Central Borneo and East Borneo such as Kutai and Tenggarong Sebrang (Kostermans & Bompard, 1993). The juice of kasturi fruit is reported as having activity as antioxydant (Edyson, et al., 2008) and the fruits have the total flavonoid content of 30.0 ± 1.2μg/ml (Suhartono, et al., 2012). Sutomo et. al. (2013) conducted an isolation of terpenoid compound from a *n*-hexane fraction of kasturi fruit, i.e. lupeol. Here we observed activity of the methanolic extract of *M. casturi* fruit and its fractions as antioxydant.

MATERIALS AND METHOD

Plant Materials: *Mangifera casturi* fruits were collected from Banjar District, Southern Borneo, Indonesia. The sample of ripe fruits of *M. casturi* was collected from December 2010 to January 2011. Plant identification was conducted at Pharmaceutical Biology Laboratory of Gadjah Mada University, Yogyakarta. Fruit Parts used in the research, were the fruit flesh and fruit bark (isolated from the seed), desiccated under 50C for 3 days, made into powder and then weighed and extracted.

Extract Preparation: Methanolic extracts of *M. casturi* fruit were prepared by weighing 1 kg of powder and extracting the power with maceration method by using 96% methanol eluent. Extraction was conducted under ambient temperature and the eluent was replaced every 24 hours. The extraction process (eluent replacement) was repeated 3 times. The macerate was evaporated by vacuum rotary evaporator under a temperature of 50°C to produce thick extracts to process and use for the next phase.

Fractionations: The thick methanolic extracts were fractionated using *n*-hexane, ethylacetate, and methanol eluents. Fifty grams of methanolic extracts were suspended with 10ml of aquadest, and the put into isolation tube. *N*-hexane soluble constituents were drawn with liquid-liquid method. One hundred milliliters of *n*-hexane were added into isolation tubes that contained methanolic extract suspension and then stirred well. The *n*-hexane soluble constituents were isolated; extractions were repeated 7 times. *N*-hexane insoluble layers were re-extracted using ethylacetate eluent under the same treatment as the previous ones. Ethylacetate insoluble layers were re-extracted using methanol eluents, then centrifuged and isolated between the solution and deposition. Methanolic fractions are methanol soluble constituents. All fractions were evaporated to get thick extracts.

Antioxidant activity assay with qualitative analysis using TLC: Methanolic extracts, *n*-hexane fractions, and ethylacetate fractions were dissolved in methanol eluent. Each solution was splattered on solid gel plate 60 F_{254}. The plates were eluted using motion phase of *n*-hexane-ethylacetate (8:2 and 6:4)v/v. The spots were observed using a UV lights with wavelengths (λ) of 254 and 366nm. Spot visibility test (specifically for antioxidant compounds) was conducted using a syringe reactor, namely 0.4mM b/v

DPPH, in methanol eluent. Compounds of flavonoid group, terpenoid group, and the compound components generally used $FeCl_3$, $AlCl_3$, ammoniac favor, anisalaldehyde, and $CeSO_4$ reagent (Wagner and Bladt, 1996).

Quantitative antioxidant activity assay with DPPH method: Procedure of DPPH radical scavenging activity was measured by using described by Nanjo et al. (1996) with slight modifications. The methanolic extracts, *n*-hexane fractions, ethylacetate fractions, and methanolic fractions were dissolved in methanol pa eluent with a concentration of 0.01%b/v. The DPPH reagent of 0.4mM was prepared by dissolving 15.7mg of DPPH powder into methanol pa eluent in a 100ml flask. The test solution was measured for antioxidant activity by adding 1ml of 0.4mM DPPH solution into 5ml flasks. Each flask was added with test solution (methanolic extracts, *n*-hexane fractions, ethylacetate fractions, methanolic fraction, and quercetin) at dosage of 0.5µg, 1.0µg, 2.0µg, 4.0µg, 8.0µg, 16.0µg, and 32.0µg respectively. Then, methanol pa eluent was added to get 5ml of solution and vortexed for 30 seconds. The mixture was let for 20 minutes (to achieve operating time). Then, the solution absorbance was read using a UV-Vis spectrophotometer with a wavelength of 517nm. Quercetin was used as a positive control. Antioxidant activity of the sample was presented in the reduction ratio of DPPH absorbance (%) that is calculated using the formula:

Reduction ratio of DPPH absorbance = [(abs.blanco–abs.sample)/abs.blanco]x100%

Statistical analysis: Determining IC_{50} value through antioxidant test was conducted with linear regression, followed by one-way analysis of variance (ANOVA) version 16.0 and tukey analysis at a Confidence Interval (CI) of 95% (P<0.05) to determine the significance between the test sample and positive control.

RESULTS

Plants material: The treatment of 10kg flesh and skin resulted in dry simplisia powder of 1.25kg (12.5%).

Extract preparation: The dry simplisia of *M. casturi* fruit (1kg) that was extracted by using methanol solution was 379.5g (37.95%).

Fractionation: The fractionations of methanol extract by *n*-hexane, ethylacetat, and methanol were 5.30%, 8.35% and 82.68% respectively.

Qualitative analysis with TLC: A qualitative analysis shows that the methanol extract of *M. casturi* fruit and its fractions contain the compounds of polyphenol and terpenoid group (Figures 1 and 2).

Quantitative analysis: Antioxydant activity test was quantitatively carried out by using DPPH method. The antioxydant activity of ethyl acetate>methanol extracts > *n*-hexane > methanol fractions (table 1). The analysis of each treatment is continued by using ANOVA at 95% confidence interval (P<0.05). Result of the analysis show that at the same range of concentration, there was no significant difference at the activity of free radical scavenger in DPPH quercetin (positive control) with ethyl acetate fraction, but significantly different on the activities of methanol extract, *n*-hexane fraction, and methanol fraction. It show that the compounds contained in ethylacetate fraction is the stronger antioxydant ones compared with *n*-hexane and methanol fractions. The antioxydant activity of ethylacetate fraction was almost equal to the capacity of quercetin in scavenging DPPH free radicals.

DISCUSSION

Fruit is one of the parts of vegetation largely containing water, so does *M. Casturi* fruit. Of 10kg flesh and skin, the dry simplisia was 1.25kg. It shows that the existence

of water component in fruit is very dominant (87.5%). Extraction by maceration using methanol solution produces the extract of 37.95%. The results of fractionation using *n*-hexane, ethyl acetate, and methanol were 5.30%, 8.35%, and 82.68%, respectively. Compounds soluble in *n*-hexane fraction are possibly terpen and fatty acid groups, while those soluble in etilacetat fraction are polyphenol and flavonoid groups. Many compounds soluble in methanol fraction are possible in glicoside, carbohidrate, and sugar contained in kasturi fruit.

A qualitative test was carried out on methanol extract, *n*-hexane fraction, and ethylacetate fraction by KLT method. From the test of compound group by a specific reagent, it can be known that kasturi fruit contained several compounds, including terpenoid and polyphenol group. Compounds of terpenoid group result in change in chromatogram color after being sprayed by anisaldehyde and cerium sulfate reagents, i.e. blue spotted color, then reddish brown after being heated (Figures 1 and 2). Compounds of polyphenol group are indicated by change in chromatogram color, i.e. purple red spotted color, then dark blue at chromatogram after being sprayed by $FeCl_3$ (Harborne, 1998). In the antioxydant activity test of methanol extract, *n*-hexane fraction, and ethylacetate fraction, it can be known that the three were indicated as antioxydant. The antioxydant property was shown by the occurence of change in yellow color of compounds after being sprayed by DPPH reagent of 0.004%b/v with purple background at chromatogram (Figures 1 and 2).

The quantitative antioxydant activity test of sample was done by DPPH method by an ultraviolet-visible spectrophotometry (UV Vis). DPPH method was mostly frequent used in antioxydant activity test. Several advantages of DPPH method were that the test treatment was relatively simple, fast, and sensitive for the antioxydant activity test of certain compounds or plant extracts (Koleva, et al., 2002; Prakash, et al., 2010). The working mechanism was based on reactions involving the role of DPPH as electron scavenger atau free hydrogen radical scavenger. The reaction resulted in stable dimagnetic compound and neutralized free radicals of DPPH whose reaction can be seen in Figure 3.

The operating time required in the study was 20 minutes with change in solution color from purple to yellow. The strength of antioxydant property was based on the calculation of IC_{50} value, where the more the proton (H^+) detached by the tested compounds, the stronger the antioxydant activity or the lower the IC_{50} value. The absorbance measured was that of remnant DPPH solution with no reaction on antioxydant compound. For comparison, quercetin compound well-known as antioxydant was used as a potent radical scavegner. The results of the quantitative antioxydant test are shown in table 1.

From table 1, it can be known that methanol extract, *n*-hexane fraction, ethylacetate fraction, and methanol fraction are indicated as antioxydants. With the same concentration, ethylacetate fraction had stronger antioxydant activity. Quercetin compounds as positive control have the strongest antioxydant activity with IC_{50} value of 2.96µg/ml. The more the hydroxil cluster of moleculer compounds with potential as antioxydant, the higher the capacity in reducing the free radicals.

Based on the results of qualitatively phytochemical test and quantitative test by DPPH method, it can be predicted that compounds of polyphenol group is one of the compounds with antioxydant properties. Chromatogram in Figures 1 and 2 are the indication of the presence of compounds in polyphenol group that are contained in ethyl acetate fraction of kasturi fruit. The antioxydant properties are based on the

presence of several hydroxy clusters in compounds of polyphenol group that can react against DPPH radicals (Chen, et al., 2008).

CONCLUSION

The methanol extract of *M. casturi* fruit and its fractions contained the compounds of polyphenol and terpenoid group. The largest antioxydant activities are those of ethylacetate fraction with IC_{50} value of 6.00μg/ml, then of methanol extract, *n*-hexane fraction, and methanol fractions with IC_{50} values of 112.43; 193.02; and 538.97 μg/ml, respectively. The activity of ethylacetate fraction was equal to the positive control (quercetin), where there was no significant difference at $P < 0.05$.

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Table-1: Antioxidant activities of methanol extract, fractions, and quercetin (positive control) free radical scavenging assay

Sample	Concentration (µg/ml)							IC_{50}
	0.5	1.0	2.0	4.0	8.0	16.0	32.0	
Methanol extr.	1.08[a]	1.47[a]	3.05[a]	3.34[a]	7.66[a]	19.94[a]	28.09[a]	112.43[b]
***n*-hexane frac.**	0.10[a]	0.79[a]	1.18[a]	2.26[a]	5.60[a]	14.93[a]	16.80[a]	193.02[b]
Ethylacetat frac.	4.32[a]	8.64[a]	15.13[a]	28.29[a]	69.25[a]	84.58[a]	84.87[a]	6.00[b]
Methanol fract.	0.18[a]	1.17[a]	3.05[a]	4.42[a]	5.80[a]	12.47[a]	14.25[a]	538.97[b]
Quercetin	20,43[a]	25,84[a]	38.41[a]	54.42[a]	66.48[a]	88.41[a]	90.52[a]	2.96[b]

- [a]percentage inhibition of DPPH radical; [b]inhibitory activity was expresed as the mean of 50% inhibitory concentration

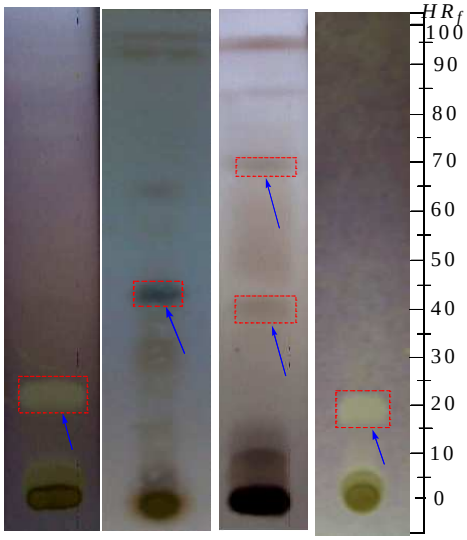
Chromatogram	Description
	A, B, and C = methanolic extracts D = *n*-hexane fractions A and D spot of DPPH 0.004% b/v B spot of $FeCl_3$ C spot of $Ce(SO_4)_2$. Eluents : *n*-hexane-ethylacetate (8 : 2) v/v Yellow spot in chromatogram A with a HR_f value 21 and D with HR_f value of 19 indicating antioxidant compounds (arrow) Blue spot in chromatogram B with HR_f value of 45 indicating polyphenol compound (arrow) Reddish-brown spot in chromatogram C with HR_f values of 8; 41; and 74 indicating terpenoid/steroid compounds (arrows)

Figure-1 : Chromatograms of methanolic extracts and *n*-hexane fractions of *M. casturi* fruit

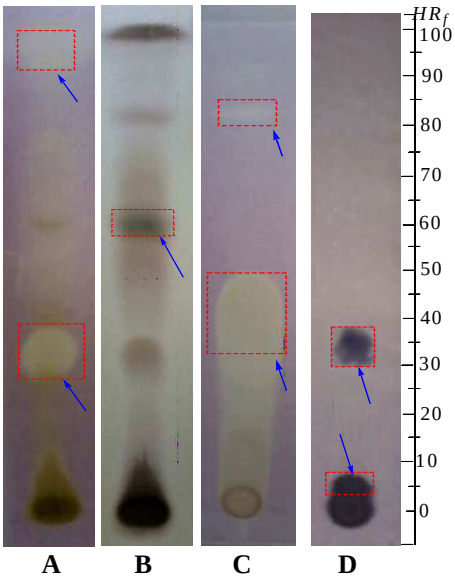
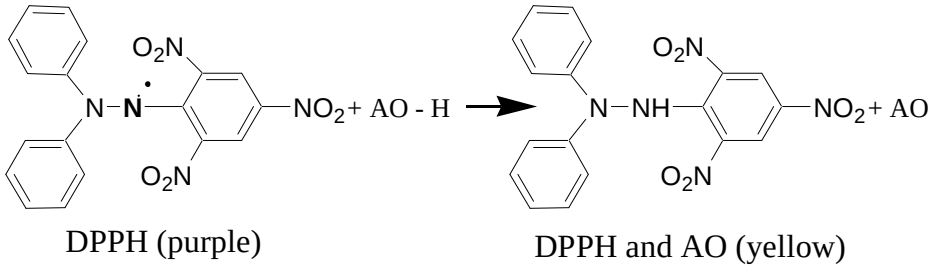
Chromatogram	Description
	A and B = methanolic extracts C and D = ethylacetate extract A and C spot of DPPH 0.004% b/v B and D spot of $Ce(SO_4)_2$. Eluents : *n*-hexane-ethylacetate (6 : 4)v/v Yellow spot in the chromatogram A with HR_f values of 33 and 93 and C with HR_f values of 37 and 83 indicating antioxidant compounds (arrow) Blue spot in the chromatogram B with HR_f value of 58 indicating polyphenol compound (arrow) Blue spot in the chromatogram D with HR_f values of 4 and 35 indicating polyphenol compounds (arrow)

Figure-2 : Chromatograms of methanolic extracts and ethylacetate fractions of *M. casturi* fruit



DPPH (purple) DPPH and AO (yellow)

Figure-3: DPPH reaction with antioxidant compounds (Prakash, et al., 2010)

