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Original Article

IN VITRO AND *IN SILICO* ANTIOXIDANT ACTIVITY OF PURIFIED FRACTIONS FROM PURPLE SWEET POTATO ETHANOLIC EXTRACT

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ABSTRACT

Objective: Purple sweet potato (*Ipomoea batatas* L.) contains antioxidant compounds like anthocyanins (cyanidin and peonidin). Therefore, the current study was conducted to obtain anthocyanins fractions from purple sweet potato with evaluation of its antioxidant activity following ferrous ion chelating (FIC) method and comparing its activity with Na_2EDTA. Furthermore, the oxidative molecular mechanism of purified fractions was investigated by *in silico* molecular docking to superoxide dismutase (SOD) and glutathione peroxidase (GPX).

Methods: Evaluating antioxidant activity by using FIC method performed by observing the absorbance of a mixed solution of $(NH4)_2$ $Fe(SO_4)_2$, ferrozine and purified fraction measured using a UV-Vis spectrophotometer. Linear regression was used to calculate the IC_{50} value. Molecular docking was performed using 4.2 Autodock program. Data obtained in the form of docking score. Lower binding energy value show the more stable bond between the active compound and its target protein, SOD and GPX.

Results: Anthocyanin purified fraction has strong antioxidant capabilities with IC_{50} value of 74.44±1.29 µg/ml, but was significantly lower with Na_2EDTA ($p<0.05$). The other mechanism is its ability to induce the target protein such as SOD and GPX intracellular defense body to capture free radicals. But it still lower affinity than native ligand to the target protein GPX is-4.28 kcal/mol while the native ligand with GPX of-7.12 kcal/mol. While the bond between peonidin with SOD is greater affinity (-4.21 kcal/mol) than the native ligand (-0.86 kcal/mol).

Conclusion: Anthocyanin purified fraction of purple sweet potato has a very potent antioxidant activity through two mechanisms as well as the metal chelating by using *in vitro* assay and free radical scavenger by inducing SOD.

Keywords: Anthocyanin purified fraction, FIC, SOD, GPX, Antioxidant

INTRODUCTION

Antioxidant agents act as free radicals or reactive oxygen species (ROS) scavenger by terminating the radical chain reactions [1-2]. ROS can trigger lipid peroxidation as a beginning of the oxidation process. In the body, there is a system of intracellular ROS scavengers to inhibit further oxidation processes; and these include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and others [3-4]. In addition to the natural antioxidants, there are also some synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are commercially available and currently used in industrial processes. However, the use of these industrial antioxidants in food, cosmetics and pharmaceutical products has declined because of their alleged promotion of carcinogenesis and other side effects. Lately, various natural antioxidants derived from vegetables, fruits, leaves, plant cereals, bark, roots, spices and herbs become the people's choice [5].

Research on the antioxidant activities from plants began to multiply readily. Among the phytochemicals with potent antioxidative effect, phenolic compounds proved to be powerful [6]. Of special interest, flavonoids are phenolic compounds that have an antioxidant activity [7]. One of the plants with potential antioxidant activity is the purple sweet potato (*Ipomoea batatas* L.) due to its high content of anthocyanins particularly cyanidin and peonidin [8]. Anthocyanin levels were positively correlated with antioxidant activity referring to the possibility that peels and purple yam tubers of purple sweet potato may have higher antioxidant activity [9-10]. Thus, the current study aimed at screening the antioxidant activity of acidified ethanolic extract of purple potato peel and tuber following *in vitro* ferrous ion chelating (FIC) method. Moreover, the molecular mechanisms involved in such activity would be investigated using *in silico* molecular docking analysis to superoxide dismutase (SOD) and glutathione peroxidase (GPX) as protein target.

MATERIALS AND METHODS

Materials

Purple sweet potatoes (peels and yam tubers) were obtained from Sidemen Village on Karangasem Regency. The chemical agents such as ferrozine, toluene, ethyl acetate were purchased from the Sigma-Aldrich co. USA. $(NH4)_2$ $Fe(SO_4)_2$, ethylene diamine tetra-acetic acid (EDTA) disodium salt, ethanol 70%, HCl 0,005%, $AlCl_3$ 5%, $FeCl_2$ 2%, NH_3, n-buthanol, aqua dest, glacial acetic acid and formic acid were purchased from Kurniajaya Multisentosa, Surabaya, Indonesia. Silica gel 60, silica gel F_{254} TLC plate were purchased from Merck, Pvt. Ltd., Germany.

Extraction and purification of anthocyanin from peels and yam tubers of purple sweet potatoes

One kg small piece of purple sweet potatoes was ground and extracted with 1 liter 0,005% HCl in 70% ethanol solution (pH 3) for 24 h and the process was repeated twice. The extracts were filtered through Whatman No. 2 filter papers and concentrated in vacuum at 50°C using a rotary evaporator to obtain 110. 64 g of extract (yield 11,06% based on a sample).

Part of the extract (420.8 g) was separated by solid phase extraction (SPE) column on silica gel 60 by elution with ethyl acetate, toluene, water and formic acid (12:3:0.8:1.2) v/v and it was continued by elution using ethanol under acidic condition. The first eluent was used to separate the cyanidin and peonidin with the other classes of anthocyanin and eliminate the impurities compound from cyanidin and peonidin. Second elution using acidic ethanol was conducted 5 times with a volume of 5 ml to obtain the clear fraction.

The anthocyanin purified fraction identification and characterization were determined using thin layer chromatography with silica gel F_{254} as the stationary phase and n-buthanol: glacial acetic acid:

water (4:1:2) v/v as mobile phase. The fraction was monitored at λ 210 nm by means of a scanning pass in λ range of 200-700 nm.

Metal chelating ability of ferrous ions

The chelating of ferrous ions by anthocyanin purified fraction of ethanol extract 0.005% HCl from purple sweet potato was estimated by the method of Hinneburg *et al.* [11]. The different concentration of fraction was added to a solution of 0.027 mg/ml $(NH4)_2$ $Fe(SO_4)_2$ (0.75 ml). The reaction was initiated by the addition of 0.027 M (0.75 ml) and the mixture was shaken vigorously and left at 37 °C for 10 min and the absorbance was measured spectro-photometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA disodium salt as standard. This assay was held in triplicate. The lower absorbance at 562 nm indicated a stronger chelating effect. Those absorbance data were calculated chelating ability percentage by using formula:

$$\text{Chelating ability (\%)} = [A_0\text{-}A_1] \times 100\%$$

Where A_0 was the control absorbance (complex between ferrozine and Fe) and A_1 was the mixture containing the fraction absorbance or the standard absorbance.

Molecular docking

Molecular docking was performed to get the drug-receptor binding energy. The PDB files obtained from Protein Data Bank (PDB) (www. rcsb. org) is a worldwide repository for processing and distribution of 3D biological macromolecular structure data [12]. The structure of SOD (1MFM) and GPX (2F8A) proteins were downloaded from PDB and prepared for docking by removing the heteroatoms and water molecules in them.

The software used were as follows: Autodock 4.2. for molecular docking, Chimera 1.10.1 for protein and ligand preparation. The three-dimensional structure optimization by using HyperChem 8 by semi-emphasis AM1 computation and calculate with a single point and geometry optimization. The downloaded PDB file of SOD and GPX were first read in Chimera 1.10.1, added waters removed, and polar hydrogens were added. The structure of peonidin was drawn by using Hyper Chem 8. Next running docking protein target and ligand in file Autodock 4.2. The configuration then the ligand's binding energy to target protein score was compared to native ligands.

Statistical analysis

Each sample was tested first distribution by Shapiro-Wilk Test and homogeneity with Lavene Test. The data were normally distributed and homogeneous ($p>0.05$) can be analyzed statistically with Paired T-Test with a 95% confidence level. If Paired T-Test results provide significant value less than 0.05 ($p<0.05$), then proceed with the statistical analysis of test Independent T-Test to determine which of the test samples provide a significant difference ($p<0.05$). For data were not normally distributed and homogeneous, statistically analyzed with Kruskal-Wallis test followed by Mann-Whitney test [13].

RESULTS

Fractionation and identification of anthocyanin fraction from purple sweet potato

Anthocyanin fraction is the fraction of the ethanol elution with the acidic condition. The whole anthocyanin fraction was collected and the solvent was evaporated. Anthocyanin fraction obtained in brown colour with a combined weight fraction yield amounted to 51.5%. The result of identification assay was shown in spectra. The spectra resulting from measurements in the wavelength range of 200-700 nm is shown in fig. 1. The spectra showed that the maximum absorbance is at a wavelength of 280 nm, and there is also a peak at a wavelength of 325 nm and 540 nm. Depending on appropriate literature on the identification of anthocyanin extract, the spectra from fractions showed the spectrum anthocyanin acyl group [14-19].

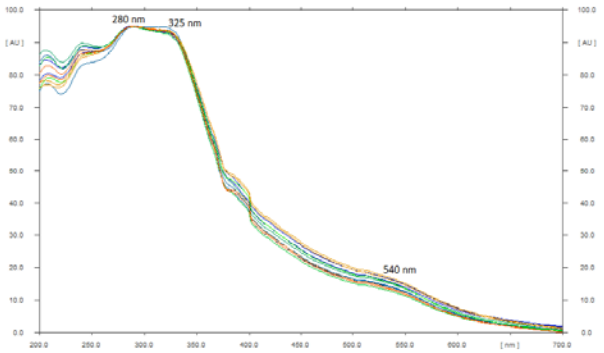


Fig. 1: Fraction spectra of anthocyanin fraction from purple sweet potato on λ 200-700 nm

Maximum λ a band I is at 540 nm, and therefore the wavelength of 540 nm chosen as the maximum wavelength of the aglycone group. Further TLC plate is scanned at a wavelength of 540 nm and a wavelength range of 200-700. The scans at a wavelength of 540 nm were data chromatograms, and spectra could be seen in fig. 2. These results suggest that the peak of the chromatogram 1 is caused by anthocyanin compound.

Anthocyanin fraction of purple sweet potato chelates the ferrous ion by using ferrous ion chelating

FIC method is a method to measure the ability of antioxidant compounds to compete with ferrozine to form chelates with iron ions [20]. The formed Fe ferrozine complex can be characterized by the formation of intense purple color in the solution. At the time of addition of the metal chelate compounds capable of the intensity of the purple color will begin to decrease [21]. Metal chelating measurement results of Na_2EDTA as a positive control and anthocyanin fraction with three repetitions show the chelating effects of anthocyanin fraction and Na_2EDTA on ferrous ions increase with increasing concentrations (fig. 3).

Based on calculation (fig. 3), the average IC_{50} value of anthocyanin fraction from purple sweet potato amounted to 74.44±1.29 µg/ml and IC_{50} of Na_2EDTA is 5.00±0.25 µg/ml. Then performed statistical analysis of the data to the IC_{50} anthocyanin fraction of purple sweet potato and Na_2EDTA. However, after tests of normality and homogeneity, the data obtained is not homogeneous. So that continued analysis using nonparametric test of Mann-Whitney with a 95% confidence level. Results of the analysis states that the value of IC_{50} fraction of purple sweet potato anthocyanins different significantly with IC_{50} value of the positive control, namely Na_2EDTA ($P = 0.05$).

Anthocyanin fraction with peonidin as active compound potentially induces the target protein SOD and GPX

Molecular docking was performed to explore the binding mechanism, and it's docking score to predict the activity of an active compound that showed the binding affinity between the target protein and compound. In this study, the docking of peonidin as the active compound in anthocyanin fraction to SOD and GPX binding site was observed, using ascorbic acid as a standard.

The docking analysis described the interaction of peonidin with antioxidant enzyme SOD and GPX. Before the molecular docking analysis was done, the method of docking with Autodock 4.2 was confirmed to be good and valid to qualify the RMSD value 1-3 Å. SOD with ligands has RMSD value of 2.98 Å, while GPX with ligands shows RMSD value 2.63 Å (table 1). Lower RMSD value means better docking method that used. In terms of the value of RMSD the molecular interaction could be continued to the following step. The interaction between peonidin or native ligan with GPX and SOD showed in table 1 and 2 and fig. 4.

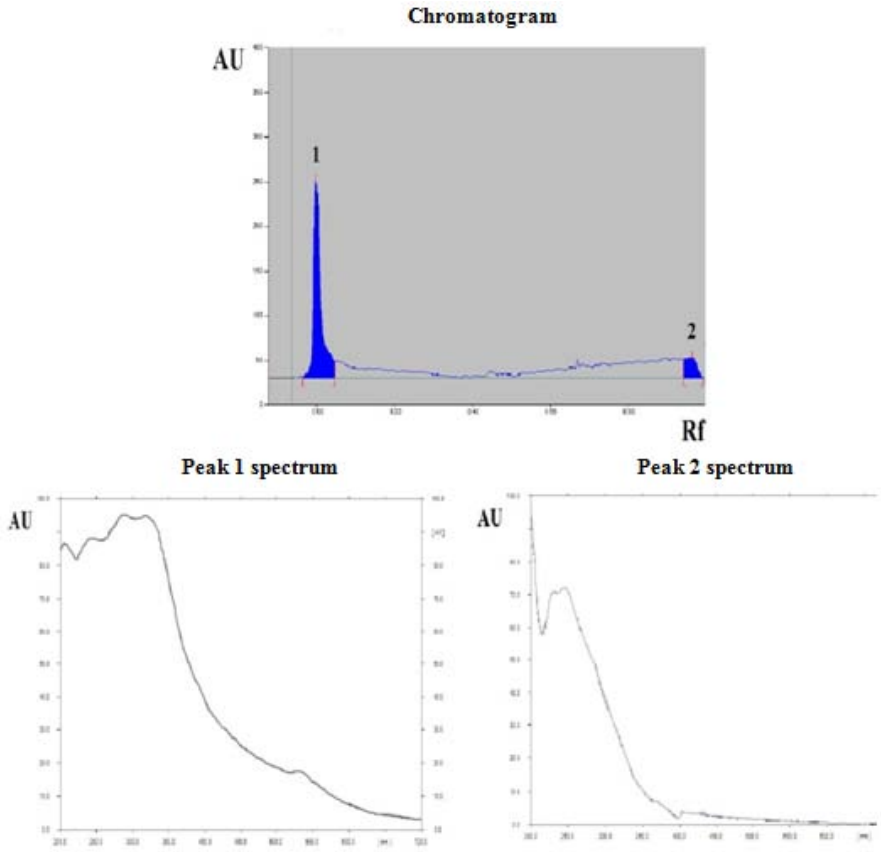
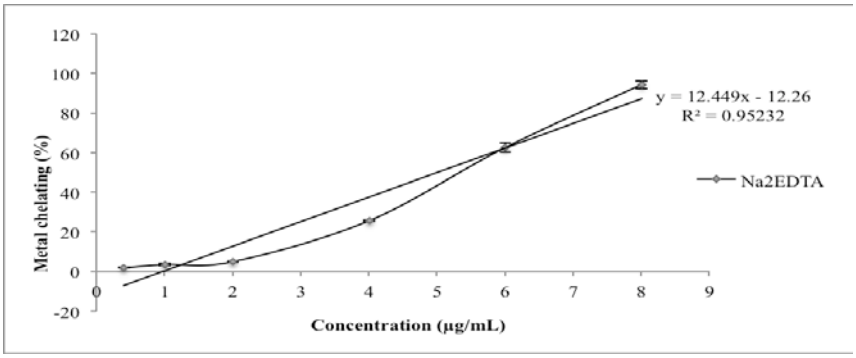
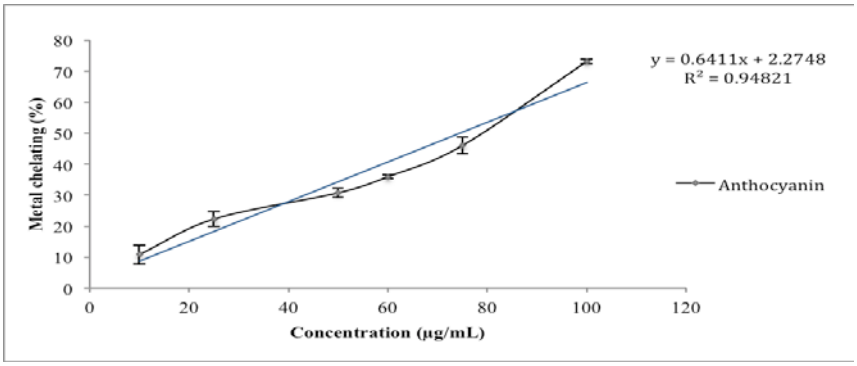


Fig. 2: Chromatograms and spectra of each peak in anthocyanin fraction of purple sweet potato at λ 540 nm



A



B

Fig. 3: Chelating activity of ferrous ions by Na_2EDTA as a positive control (A) and anthocyanin fraction from purple sweet potato (B). Results are shown as mean±SD (n=3)

Table 1: Docking score of interaction between peonidin to the target protein

	Score docking (kcal/mol)	
	SOD (1MFM)	GPX (2F8A)
RMSD (Root mean square deviation)	2.98 Å	2.63 Å
Native ligan	-0.86	-7.12
Peonidin	-4.21	-4.28
Ascorbic acid	-3.64	-3.65

Table 2: Amino acid residue and bonding formation of interaction peonidin and the target protein

Target proteins	Compound	Amino acid residue	Bonding formation
GPX (2F8A)	Native ligan	Arg 180	Hydrogen Bond
		Arg 179	Hydrogen Bond
	Peonidin	Arg 180	Hydrogen Bond
		Lig 1	Hydrogen Bond
	Ascorbic acid	Thr 143	Hydrogen Bond
SOD (1MFM)	Native ligan	Lig 1	Hydrogen Bond
	Peonidin	Lig 1	Hydrogen Bond
	Ascorbic acid	Lig 1	Hydrogen Bond

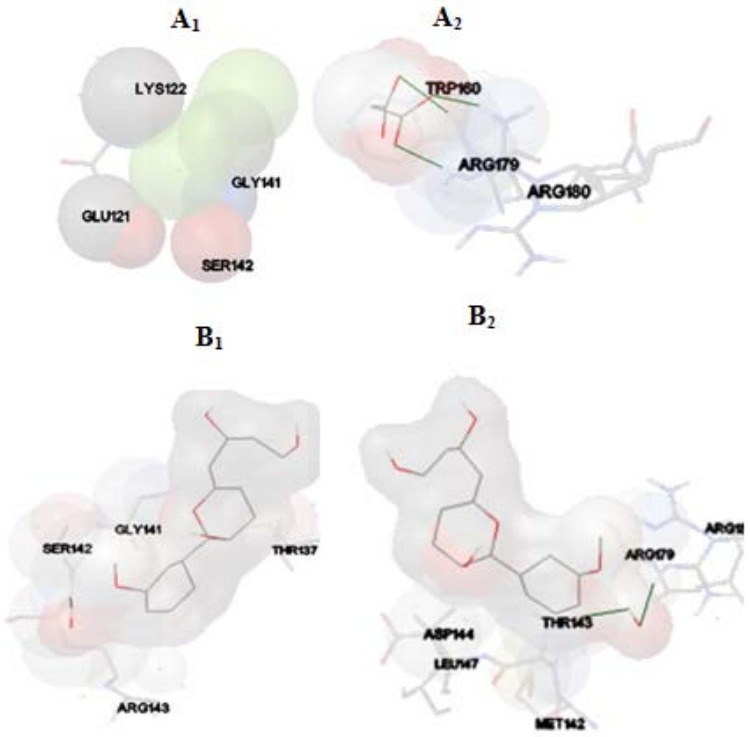


Fig. 4: Docking representations of ligan and peonidin inactive binding site to SOD and GPx target proteins using Autodock 4.2. The 3D interaction between native ligan and active site of SOD (1MFM)(A_1); native ligan and active site of GPX (2F8A)(A_2); peonidin and active site of SOD (1MFM) (B_1); peonidin and active site of GPX (2F8A) (B_2)

DISCUSSION

Ferrous ion chelating activity assay used as Na_2EDTA a comparison or positive control. Na_2EDTA is the salt form of EDTA where the compound EDTA is a chelating ligand which has a strong affinity for forming complexes with metal [22]. Fig. 3 shows the correlation between the concentrations of Na_2EDTA with chelating ability percentage. This is because of EDTA which has a high affinity for forming complexes with metal.

In this study, the addition of the anthocyanin fraction to a solution of Fe-Ferrozine, purple color diminishing with increasing concentration of fraction added. So that it could be interpreted that the anthocyanin fraction of purple sweet potato able to compete with the chelate iron ions ferrozine. Anthocyanin fraction included in compounds that have potent antioxidant activity through the metal chelating mechanism. Antioxidant compounds said to be strong if IC_{50} value are in the range of 50-100 µg/ml.

Na_2EDTA has a strong chelating ability, but Na_2EDTA could not be used as a chelator agent on the body. Because It has a very strong ability to bind all metals, Including metals needed by the body. When all the metal in the body are bound by EDTA, of course, this will interfere with the body's metabolic processes. So that the compounds could be used as an alternative to natural materials such as anthocyanin fraction of purple sweet potato as a chelator agent.

Molecular docking analysis demonstrated that peonidin as the active compound in anthocyanin fraction of purple sweet potato potentially induced SOD and GPX. Both of these enzymes play a role in ROS scavenging. Peonidin has lower affinity than native ligand to the target protein GPX (4.28 kcal/mol) while the native ligand with

GPX is-7.12 kcal/mol. Score docking between peonidin with SOD protein is higher affinity (-4.21 kcal/mol) than the native ligand is-0.86 kcal/mol. This shows the anthocyanin fraction of purple sweet potato has a very potent antioxidant potency through two mechanisms as well as the metal chelating by using *in vitro* assay and free radical scavenger by using *in silico*.

CONCLUSION

Peonidin and cyaniding, as the active compound in anthocyanin fraction of purple sweet potato, have potent antioxidant efficiency as ferrous ion chelating and ROS scavenger.

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CONFLICT OF INTERESTS

Declared none

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