

# Study of Anthocyanins Activity from Purple Sweet Potato for Reducing Apoptotic Cells Expression of The Cerebellum On Ischemic Stroke Rats

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## ABSTRACT

This study was aimed to determine anthocyanin antioxidant performance with a dose of 2 cc/day based on the expression of apoptotic cells in ischemic stroke rats cerebellum. The research was conducted using ischemic stroke rats (*Rattus norvegicus*) that prepared by ligated for 3 h on the Common Carotid Artery (CCA) and External Carotid Artery (ECA) followed by reperfusion that is commonly known as MCAO (Middle Cerebral Artery Occlusion). The anthocyanin extract was characterized by LC-MS and its IC<sub>50</sub> was measured by DPPH method. The rats were divided into five groups 1) negative control; 2) reperfusion 1 h; 3) reperfusion 72 h; 4) reperfusion 24 h, with anthocyanin therapy; 5) reperfusion 72 h, with anthocyanin therapy. The results of LC-MS showed that anthocyanin from purple sweet potato extracts contained Petunidin-3,5-O-diglucoside (Pt-DG) with an IC<sub>50</sub> value of 22.16 µg/mL, categorized as a very strong antioxidant. The results showed that apoptotic cells expression of cerebellum decreased significantly ( $p < 0.01$ ) after 72 h reperfusion with anthocyanins therapy until 2.42%. The current work proved that anthocyanin extract effectively suppresses the apoptotic cell's expression of the cerebellum on stroke ischemic rats.

Key word: anthocyanin, purple sweet potato, ischemic stroke, MCAO, apoptotic cells

## INTRODUCTION

Purple sweet potato (*Ipomoea batatas L.*) is one of the most abundant coarse crops with the amount of production reaches 2.4 billion ton/year. However, optimizing the purple sweet potato specifically indigenous from Bali as the derived product or herbal medicine has not been utilized and explored yet. Based on the literature, purple sweet potato contains high levels of antioxidant from anthocyanins group [1]. Anthocyanins are widely known as a powerful natural antioxidant to down-regulate the inflammation, oxidative stress, free radicals, and apoptotic cells [2]. Overproduction of reactive oxygen species (ROS) or free radicals in biological systems leads to the production of lipid peroxidation, protein oxidation, and DNA damage, eventually produce excessive oxidative stress [3]. Oxidative stress plays role in the enlargement of various diseases such as neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and ischemic stroke [4]. Ischemic stroke occurs as a consequence of a deflation blood brain flow that leads to apoptotic cells [5,6].

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In order to understand the mechanism of ischemic stroke and how to preserve it, the animals model need to be used. Wistar rats (*Rattus norvegicus*) were commonly used as the animal model [7,8,9], in particular on ischemic stroke model because of the similarity of brain anatomy and physiology with the human brain. Among all the ischemic stroke model, MCAO is the most widely used experimental paradigms [10]. MCAO (Middle Cerebral Artery Occlusion) was carried out by ligating left common carotid artery (CCA) and external carotid artery (ECA) using surgical thread. The wistar rats were decapitated after 3 hours of occlusion followed by reperfusion [2,11]. During MCAO, damage in the blood vessel triggers ischemic penumbra because of the oxidative stress in the core region after the occlusion. Ischemic stroke occurs because the brain consumes a large quantity due to ROS after the reperfusion and terminated by apoptotic cells [12]. Therefore, anthocyanins from purple sweet potato have been chosen to suppressed ROS that may cause ischemic stroke rats.

## EXPERIMENT

### Materials

The material used in this study consists of extract anthocyanin from purple sweet potato indigenously from Bali produced by Sweet Imo; 1,1-Diphenyl-2-picrylhydrazyl (DPPH), trolox, potassium phosphate mono and dibasic were purchased from Sigma-Aldrich; methanol was obtained from Merck; xylazine 2% was purchased from interchemie; ketamin was produced by PT. Lucas Djaya; sodium chloride 0.9% was purchased from PT. Widatra Bhakti; formaldehyde 37% was purchased from Polysciences, Inc., and *in situ* apoptotic detection kit ab206386 was obtained from Abcam.

### Experimental Animals and Research Design

A number of 25 male rats (*Rattus norvegicus*) (body weights of 250 – 300 g) were housed at room temperature ( $\pm 25$  °C), moisture of  $\pm 40\%$ , and were illuminated for 12 h in the animal house of Biosains Institute, Brawijaya University, with alternate cycles of 12 h light and dark. This research used ischemic stroke rats induced by MCAO. The surgical began with performing anaesthesia to rats using xylazine: ketamin= 0.3 mL : 0.7 mL. Rats were ligated for 3 h on the CCA and ECA sections, followed by reperfusion. Rats were divided into five groups and each group consisted of five rats. The first group was negative control. Second and third groups were treated without anthocyanin followed by reperfusion for 1 h and 72 h, respectively. Fourth and fifth groups were treated with anthocyanin followed by reperfusion for 24 h and 72 h, respectively. After all treatment, rats were sacrificed and their cerebellum were taken for apoptotic cells analysis. Cerebellum was stored immediately in buffer formalin with the ration of formaldehyde 37% : sodium chloride 0.9% : potassium phosphate monobasic:potassium phosphate dibasic:distilled water =10 mL : 0.8 g : 0.4 g : 0.65 g : 90 mL at 25 °C, followed by preparation of cerebellum slides. All conditions and handling of rats were conducted following the protocols approved by Ethics Committee of Brawijaya University No: 651-KEP-UB.

### Procedure

#### Bioactive compound analysis by LC-MS

Anthocyanin was characterized by LC-MS to determine compounds inside it. The instruments used consist of LCMS (LC conditions; column hypersil gold, solvent A 0.1% formic acid in distilled water, solvent B 0.1% formic acid in Acetonitrile with gradient elution 0-0.6 min 5% B, 2.5-5.0 min 75% B, 5.5-7.0 min 5% B, flow rate 300  $\mu$ l/min, UHPLC ACCELLA type 1250 by Thermo Scientific. Mass spectrometry was conducted

using TSQ QUANTUM ACCESS MAX by Thermo Finnigan, the ionized source using ESI (electrospray ionization) in positive mode.

### Determination of Antioxidant Level using DPPH Assay

The antioxidant activity was determined using DPPH assay adapted from the procedure described by Dudonne [13]. A 1 mL of Fresh DPPH solution (0.05 mM) was mixed with 2.5 mL anthocyanins extract, and then mixture was incubated in 37°C for 20 min. The absorbance at 515 nm was measured using UV-Vis spectrophotometer. 2.5 mL of methanol 2.5 mL was mixed with 1 mL DPPH solution, and this mixture was used as a blank solution. The standard solution was made from trolox. The percentage of antioxidant activity on inhibiting free radicals was calculated by the following formula:

$$\% \text{ inhibition} = (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100$$

where  $IC_{50}$  is maximum inhibitor concentration in half reaction used to measure the total samples needed to inhibit free radicals.

### Apoptotic Cells Analysis Using *In situ* Apoptosis Detection

The *in situ* apoptotic detection kit protocol (from Abcam) was used in the examination of apoptotic expression by the immunohistochemistry reaction. All procedures for *in situ* apoptotic detection kit ab206386 Abcam based on manual standard copyright by Abcam [14].

## RESULT AND DISCUSSION

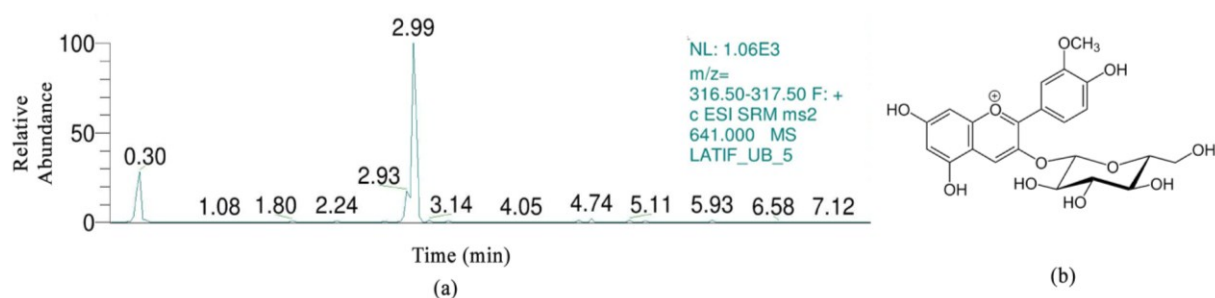
### Bioactive Compounds of Anthocyanin Extracts

In this study, anthocyanin extracts were characterized by LC-MS to determine the specific compounds that play role in decreasing the apoptotic expression. The result of LC-MS data was presented in **Table 1**. In addition, the LC-MS chromatogram was shown in **Figure 1**.

**Table 1.** Interpretation of LCMS result for anthocyanin compounds

Peak	Fragment Ions (m/z)	[M] <sup>+</sup> (m/z)	Standard Fragment Ions (m/z)	Prediction of Anthocyanin compounds
2.99	316.50-317.50	641	317	Petunidin-3,5-O-diglucoside
4.63	300.50-301.50	463	301	Peonidin-3-O- glucoside
5.14	330.50-331.50	493	331	Malvidin-3-O- glucoside
6.10	302.50-303.50	465	303	Delphinidin-3-O- glucoside
3.26	302.50-303.50	611	303	Delphinidin-3-O-(6-O-koumaroil)- glucoside

According to **Table 1**, anthocyanin extracts contained at least five anthocyanin compounds. Furthermore, analysis of LC-MS spectra gave the highest chromatogram peak at 2.99 min with the molecular weight of 641 *m/z*. This peak was identified as ion-molecule of C<sub>28</sub>H<sub>33</sub>O<sub>17</sub> and proved the presence of Petunidin-3,5-O-diglucoside (Pt-DG). The structure compound of Pt-DG was depicted in **Figure 1**. Pt-DG has conjugated electrons that means this compound has strong antioxidant activity. Therefore, it can be used for ischemic stroke model therapy by free radical deactivation.



**Figure 1.** LCMS Chromatogram (a) and Structure compound (b) of Petunidin-3,5-O-diglucoside (Pt-DG)

The result of  $IC_{50}$  from anthocyanin extract that used in this experiment was 22.16  $\mu\text{g/mL}$ . In agreement with LC-MS interpretation, DPPH analysis showed similar results, that anthocyanin can be classified as strong antioxidant capacity proved by the  $IC_{50}$  value which was less than 50  $\mu\text{g/mL}$  [15].

**Table 2.**  $IC_{50}$  Value Classification

Intensity	$IC_{50}$ Value
Very Strong	< 50 $\mu\text{g/mL}$
Strong	50-100 $\mu\text{g/mL}$
Medium	250-500 $\mu\text{g/mL}$
Weak	> 500 $\mu\text{g/mL}$

### Apoptotic Cells of Cerebellum After Anthocyanin Extract Therapy

Effects of anthocyanin treatments in reducing apoptotic cells expression on ischemic stroke rats are shown in **Table 3**. This data indicates that antioxidant levels in anthocyanin extracts provide positive influences on decreasing apoptotic cells expression in cerebellum.

**Table 3.** Apoptotic cells expression by Immunohistochemistry Technique

Code	Group	Average		
		Apoptotic cells expression (%)	Increasing (%)	Decreasing (%)
KN	Negatif control	8.42 <sup>a</sup> $\pm$ 0.56	-	-
K1	Reperfusion 1 hour	22.46 <sup>b</sup> $\pm$ 1.39	166.60	-
K2	Reperfusion 72 hour	17.52 <sup>c</sup> $\pm$ 0.68	-	21.97
T1	Treatment 24 hour	5.48 <sup>d</sup> $\pm$ 0.30	-	75.58
T2	Treatment 72 hour	2.42 <sup>e</sup> $\pm$ 0.19	-	89.22

Mean  $\pm$  standard deviation of quintuplicate analyses. Different letters in the same column indicate highly significant difference at ( $p < 0.01$ ).

Apoptotic cells expression was given in **Table 3**. The rats achieved the highest level of apoptotic cells expression after 1 h of reperfusion with average apoptotic cells expression of 22.46%  $\pm$  1.39. In comparison to negative control, apoptotic cells expression after 1 h reperfusion increased to 166.60%. However, without anthocyanin extract treatment, rats showed self-recover. This has been proven by decreasing in apoptotic cells expression from 22.46%  $\pm$  1.39 to 17.52%  $\pm$  0.68. Nevertheless, significant effects of treatment by anthocyanin extract was shown after 72 h reperfusion, shown by decreasing of apoptotic

expression until  $2.42\% \pm 0.19$ . Apoptotic cells expression after 72 h decreased in total to 71.25%. This means treatment with anthocyanin extracts has significant effects to reduce apoptotic cells expression.

The results showed that the critical period of apoptotic cells expression due to MCAO occurs an hour after reperfusion. According to literature, the performance of MCAO made rats suffered from ischemic stroke by triggering post-reproduction oxygen burst. The oxygen burst occurs due to the high rate of blood flow, therefore oxygen supply suddenly enters cells and mitochondria. This condition triggers the formation of excessive free radicals that damage the membranes, proteins, and cell DNA, thus activating the apoptotic pathway. Hence, the critical period occurs an hour after reperfusion. After 24 h, the oxygen flow has begun to stabilize, as a result oxygen explosion phenomenon has diminished. This causes apoptotic cells expression decreases naturally without therapy. Nevertheless, since apoptotic cells expression is in the range of  $17.52\% \pm 0.68$ , this means that self-recovery may not be carried out completely; and thus therapy is still needed. Administration of anthocyanin extracts is proven to decrease apoptotic cells expression up to  $2.42\% \pm 0.19$  or 71.25% from the normal condition.

## CONCLUSION

Extract from purple sweet potato indigenous from Bali contained at least five anthocyanin compounds with Pt-DG as the highest peak based on LC-MS. In addition, this compound is classified as strong antioxidant capacity, with  $IC_{50}$  value 22.16  $\mu\text{g/mL}$ . It is proven that anthocyanin extract reduced apoptotic cells expression to 71.25%, after 72 h therapy in cerebellum ischemic stroke rats.

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