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In vivo effect of sub-chronic administration of ethanol extract of Rosella (*Hibiscus sabdariffa* L.) calyx on total blood cholesterol, triglyceride level, and heart histopathologic profile

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Abstract

Rosella (*Hibiscus sabdariffa* L.) has been used as traditional medicine. The use of rosella for various therapeutic outcome become increasingly. This research objective was to observe the effect of sub-chronic administration of ethanol extract of rosella calyx on total blood cholesterol, triglyceride level, and the histopathologic profile of the heart. The result showed that treatment of the rosella calyx ethanol extract of 50 mg/kgBW and 100 mg/kgBW for 35 days were significantly decreased the total cholesterol in rat ($p<0.05$), while treatment with 200 mg/kgBW was not revealed the total cholesterol significantly ($p>0.05$). The levels of triglyceride blood was also decreased significantly after treatment with the rosella calyx ethanol extract at 50 mg/kgBW, 100 mg/kgBW, and 200 mg/kgBW dose ($p<0.05$) respectively. It seem that the effect of lowering total cholesterol level was reversible, but the decreasing effect of triglyceride level was not reversible after two weeks observation. It was found that there were no changes on the heart histopathologic.

Introduction

Rosella (*H. sabdariffa* L.) has been used as a traditional medicine, especially the calyx of H. Sabdariffa that contains anthocyanins, flavonoids, and polyphenols (Lin *et al*., 2007). Various of flavonoids were found in the calyx: gossypetin, sabdaretin, hibiscetin and anthocyanins (Ologundudu *et al.*, 2009). Previous researches showed that rosella extract has antioxidant and hypolipidemic effect in alloxan induced rat (Farombi and Ige, 2007). It has been also reported to have a hepatoprotective effect (Sunilson *et al*., 2008; Nurkhasanah and Rahardhian, 2015). The roselle calyx could be used to prevent development of atherosclerosis and cardiovascular complications (Parthasarathy *et al*., 1999).

The use of rosella extract for various therapeutic outcome become increasingly following the abundant report of activities. For this objective, the safety aspect becomes very important. Some toxicity study of rosella extract showed that in the acute toxicity test, there is no toxicity observed for 7 days after oral administration of high doses (15 g/kg BW) of ethanol and water extract of H. Sabdariffa calyx in mice (Reanmongkol and Itharat, 2007). There is no significantly changes in SGPT (serum glutamate pyruvate transaminase), SGOT (serum glutamate oxaloacetate transaminase) and ALP (alkaline phosphatase) after a single treatment with high dose of rosella extract (Sari *et al.*, 2016). Sub-acute effects of H. Sabdariffa aqueous extract on hepatic cytochrome P450 (CYP) was reported that two doses (250 and 1000 mg/kg BW/day) of the extract administered orally to rats for 30 days showed that no significant on total CYP contents and the activities of some isomers of cytochrome CYP 1A1, 1A2, 2B1/2, 2E1 and 3A (Prommetta *et al.*, 2006).

The objective of this research was to observe the effect and toxicity of rosella extract treatment in the sub-chronic administration on biochemical parameters. The research used effective dose reported in the previous research (Nurkhasanah and Rahardhian, 2015).

Materials and Methods

Materials

Roselle calyx was obtained from Kediri, East Java, Indonesia. The experimental animals used were found from Animal house of Universitas Gadjah Mada, strain Sprague Dawley rats with 8 weeks of age and weighing ± 200 grams.

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Preparation of ethanol extract

A total of 800 grams of rosella calyx powder was extracted using 70% ethanol in the ratio (1: 5) with maceration method. Maserat was then evaporated with a rotary evaporator at 50°C and followed by evaporation over the water bath to obtain a concentrated or viscous extract.

Qualitative test of polyphenols

The ethanol extract of rosella was added by $FeCl_3$. The occurrence of blue-green color indicate the presence of polyphenols.

Qualitative test of flavonoids

An aliquot of ethanol extract of rosella calyx was dissolved in 70% ethanol and then dropped on the filter paper. The filter paper is passed through ammonia vapor. The more intensive yellow color indicated the flavonoid content.

Determination of total phenol content

Determination of total phenol content was carried out using Folin-Ciocalteau reagent and gallic acid was used as standard. A total of 10.0 mg of ethanol extract of rosella calyx diluted to a volume of 10.0 ml with a mixture of ethanol : distilled water (1:1). The 3.0 ml of the extract solution was transferred and added with 1.5 ml of Folin-Ciocalteau reagent. After settling for 3 minutes, the solution was added by 1.2 ml of 7.5% Na_2CO_3 solution and allowed to stand on a range of operating time at room temperature. The absorbance of the extract solution was measured by UV-Vis spectrophotometer (Anesini *et al*., 2008).

Determination of total flavonoids content

Determination of total flavonoid content was carried out using $AlCl_3$ and quersetin as standard. A total of 1 gram of ethanol extract of rosella calyx was dissolved in 70% ethanol, add the solvent to 100.0 ml. The solution was pipetted 0.5 ml and added with 0.5 ml of 10% $AlCl_3$ and 0.5 ml Na-acetate solution and allowed to stand for operating time. The absorbance of the extract solution was measured by UV-Vis spectrophotometer (Chang *et al*., 2002).

Experimental animal treatment

The animal treatment had an ethical approval from ethical committee of Ahmad Dahlan University with ethical approval number 011505043. The 30 animals of female Sprague Dawley rats were divided into 5 groups, the control group was given by CMC-Na 0.5% as a solvent of extract, and each member of treated groups were administered by ethanol extract of rosella with doses of 50 mg/kgBW, 100 mg/kgBW, and 200 mg/kgBW. The reversible group was carried out to determine whether the effects are reversible or not after waiting for 14 days. The extract treatment was given orally. The blood was collected from orbital sinus of eye on the day 36, and the animal was sacrificed for heart observation. In the reversible group, the blood sampling was carried out again in the day 50 for observing the reversibility of changes after subchronic treatment. The cholesterol and triglyceride level found in the day 36 was compared to the level on day 50.

Blood sampling

The blood sample was collected from the orbital sinus of the eye using the microhematocrit (± 2 ml), then transferred to Eppendorf tubes. The blood is then centrifuged for 15-20 minutes at 3000 rpm, to separate the serum.

Determination of total blood cholesterol levels with CHOD-PAP

The 10 µl of serum was reacted with 1000 µl of reagent Diasys® and homogenized using vortex then incubated at 37^{O}C for 10 minutes. Absorbance was measured with a spectrophotometer at 500 nm. The cholesterol level was calculated by comparing with a standard.

Determination of triglycerides levels with GPO-PAP

The 10 µl of serum was reacted with 1000 µl of reagent Diasys® and homogenized using vortex then incubated at 37^{O}C for 10 minutes. Absorbance of the solution was measured by a spectrophotometer at 500 nm. The triglyceride level was calculated by comparing with standard.

Histopathologic observation of heart

The animals were anesthetized using carbondioxyde (CO_2) gas and wait for a few minutes until showed no movement. The mice was sacrificed by cervical dislocation. The heart organs was separated immediately and washed by physiological saline solution and transfereed to 10% formalin solution for preservation. The organ was then made paraffin block for slicing and followed by staining using Haematoxylin and Eosin..

Results and Discussion

Flavonoid and phenolic content of rosella extract

Anthocyanin, one of the flavonoids group member is the major compound of *H. sabdariffa* and various color fruit and vegetables. Flavonoids have been reported to have an antioxidant effect (Einbond *et*

Table 1. Total cholesterol land triglyceride levels of rats on the day 36 that has been treated by the ethanol extract of rosella calyx for 35 days

Groups	Total cholesterol (mg/dl)	Triglyceride (mg/dl)
Negative control	119,25 ± 7,23	90,05 ± 12,23
50 mg/kgBW	91,58 ± 11,38*	59,36 ± 7,79*
100 mg/kgBW	62,95 ± 10,99 *	48,24 ± 9,12*
200 mg/kgBW	116,54 ± 15,42	72,57 ± 7,48*

*= significant different from control (p<0,05)

Table 2. Total cholesterol and triglyceride levels of rats on 14 days observation after 35 days treatment of the ethanol extract of rosella

Groups (treated by)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)
Control (reversible)	75,79 ± 3,26	89,01 ± 14,81
200 mg/kgBW (reversible)	79,32 ± 5,41*	32,02 ± 5,62*

*=significant different from control (reversible) (p<0,05)

al., 2004; Guerrero *et al*., 2010; Miguel, 2011). This research also found the high content of flavonoid and phenolic content in rosella extract. The result showed that the blue color after reacting with $FeCl_3$ indicated the presence of polyphenol compounds. The high content of flavonoids also was shown by intensive yellow color after vaporizing with ammonia.

The quantitative analysis showed that total phenolic content of rosella extract was 1.96 ± 0.04%. The total phenolic content of rosella extract was higher than previously reported (Alfian and Susanti 2012). It could be caused by the difference of solvent. Ethanol solvent produced the higher total phenol content than methanol solvent. The total flavonoid content was 0.52 ± 0.02%. This concentration was higher than reported by Munim *et al.* (2008). Munim *et al*. (2008) used methanol for solvent extraction and resulted the lower content of total flavonoids of rosella extract compare to ethanol extract of rosella.

Total cholesterol and triglyceride levels

The safety aspect is very important in the usage of herbal medicines in therapeutical of disease. Rosella was reported to have therapeutic effect as antihyperlipidemic and clinically proved as antihypertension (Herrera-Arellano *et al.*, 2007). The utilization of rosella in community was increasingly as immunomodulator (Nurkhasanah, 2015) for maintaining health status.

The total cholesterol and triglyceride level were important biochemical parameters to evaluate the safety aspect of the ethanol extract of rosella. The long administration of the ethanol extract of rosella could cause the changes in some biochemical parameters. The total cholesterol and triglyceride level after 35 days treatment of rosella extract was shown in Table 1.

It was found that treatment with rosella extract with 50 mg/kgBW and 100 mg/kgBW dose for 35 days respectively decreased cholesterol level significantly compare to control group, by which 0.5% CMC-Na (solvent) was given to the group. In case the higher dose (200 mg/kgBW) given, the cholesterol level was not decreased. Carvajal-zarrabal *et al*. (2005) has also found the same case, in term of treatment with a lower concentration of rosella extract in the rat was fed by high containing cholesterol could reduce the cholesterol level more effective. The cholesterol in the body comes from two sources, food consumed (exogenous cholesterol) and produced by the body (endogenous cholesterol) (Nelson and Cox, 2008) According to Yokozawa *et al.* (2002), polyphenol compounds could lowering plasma cholesterol levels by inhibiting the absorption of cholesterol in the intestines and increase the formation reaction of bile acids of cholesterol and then excreted through fesses.

Decreasing of total cholesterol level might be due to anthocyanin content that has a strong antioxidant activity. This antioxidant effect can inhibit the oxidation of low density lipoprotein (LDL) (Hirunpanich *et al*., 2005). In a subsequent study proved that the water extract of roselle has the ability as an antioxidant and hypocholesterolemic by lowering the amount of TBARS (Thiobarbituric Acid Reactive Substances) in mice fed a high cholesterol diet (Hirunpanich *et al*., 2006).

The treatment of ethanol extract of rosella for 35 days at 50 mg/kgBW, 100 mg/kgBW and 200 mg/kgBW doses respectively was also found to decrease the triglyceride level significantly compare to control. The result was is in line with the previous report that treatment of the ethanol extract of rosella for 4 weeks on hyperlipidemic rat in doses of 5%, 10% and 15% reduced the triglyceride levels (Carvajal-Zarrabal *et al*., 2005). But the decreasing triglyceride levels caused by treatment of ethanol extract of rosella in this research still in a normal range, (26-145 mg/dl (Gani *et al*., 2013).

The decreasing level of total cholesterol was found reversible and no significant difference compare to control in the same time after 14 days observation (Table 2), but the triglyceride level was still low after 14 days observation. Sireeratawong *et al*. (2013) have also reported that no toxicity was detected after chronic administration of water extract

Table 3. Histopathologic profile of heart after treatment with rosella extract for 35 days

Groups	Outcome
Negative control	No damage
50 mg/kgBW	No damage
100 mg/kgBW	No damage
200 mg/kgBW	No damage
200 mg/kgBW (Reversible)	No damage

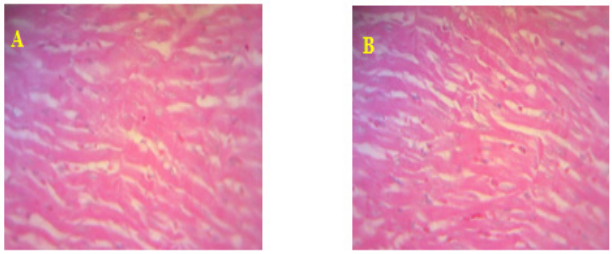

Figure 1. Histopathology heart with HE staining and magnification of 400x (A) control group (B) treatment group dose of 200 mg/kgBW.

of rosella including biochemical parameter and weight of organ.

Histopathological profile of heart

The histopathologic observations were carried out to investigate any damage of the cellular level, whether changes in cholesterol and triglyceride levels lead to changes in heart. Moreover, cholesterol level always to be involved in the heart failure. The histopathological observation of the heart after rosella extract treatment for 35 days was shown in Table 3 and depicted in Figure 1. The result showed that all of heart is normal.

Conclusion

Treatment of ethanolic extract of roselle calyx for 35 days can lowering total cholesterol and blood triglyceride levels in rats and does not cause any damage to the heart organ. This effect is reversible on total cholesterol levels but not reversible in blood triglyceride levels.

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