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Original Research Article

Effect of rosella (Hibiscus sabdariffa L) extract on glutathione-S-transferase activity in rats

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Abstract

Purpose: *To determine the effect of rosella (Hibiscus sabdariffa L) extract on glutathione-S-trasferase (GST) activity and its hepatoprotective effect.*
Methods: *A total of 25 rats were divided randomly into 5 groups (5 rats per group). Group I served as the baseline, group II was the negative control group, while groups III, IV and V were treated with rosella extract at doses of 10, 50 and 100 mg /kg /day, respectively for 35 days. On day 36, the animals were given a single dose of dimethyl benz(a)anthracene (DMBA) orally. After one week, blood was taken from the sinus orbitalis for measurement of serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) activities by a kinetic method with DIasys® kit. The activity of GST was measured in liver homogenate using 1-choloro-2,4-dinitrobenzene (CDNB) method, while the expression of GST gene was determined by reverse transcriptase polymerase chain reaction (RT-PCR).*
Results: *Treatment with rosella extract at 10, 50 and 100 mg /kg for 35 days led to significant increases in GST activity relative to the control group. In addition, serum SGPT and SGOT activities were significantly decreased. There were significant increases in the expression of GST gene as evidenced by increased GST band intensity.*
Conclusion: *These results indicate that rosella possesses significant hepatoprotective effect against hepatic injury caused by DMBA treatment. Thus, rosella may be useful for the prevention of oxidative stress caused by free radicals produced from pollutants and foods.*

Keywords: *Hibiscus sabdariffa, Rosella, Glutamate pyruvate transaminase (SGPT), Glutamate oxaloacetate transaminase (SGOT), glutathion-S-transferase (GST)*

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INTRODUCTION

Free radicals are highly reactive molecules since they have one or more unpaired electrons. Their reactivities are reduced by electron donation from other molecules. Oxidative stress plays a major role in the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, cardiovascular disease and neurodegenerative disease [1]. The human body produces endogenous antioxidants to fight oxidative stress. However, in the presence of excess levels of pro-oxidants, exogenous antioxidants from food and supplements are needed. Endogenous and exogenous antioxidants act to prevent and repair damage caused by reactive oxygen and nitrogen species; they boost the immune system and reduce the risk of cancer and degenerative diseases [1,2].

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants generated by the combustion of fuel, wood and other organic materials. They are also found in cigarette smoke, grilled food and motor vehicle exhaust fumes [3,4]. Dimethyl benz (a) anthracene (DMBA), a prototype of PAH, is a carcinogenic and immunosuppressive agent [5, 6,]. It is also a hepatotoxic agent [7,8]. Damaged liver cells release various enzymes into the blood. These enzymes such as SGPT and SGOT are important indicators of liver damage [9].

Rosella (*H. sabdariffa*) possesses antioxidant properties [10]. Previous studies reported that rosella exerted hepatoprotective effect against toxicity from paracetamol [11], carbon tetrachloride [12] and streptozotocin [13]. It has also been reported that rosella extract treatment reduced SGPT, SGOT and ALP activities after DMBA exposure [14]. The objective of this research was to investigate the protective effect of rosella against DMBA-induced hepatotoxicity with regards to its effect on GST activity and GST gene expression.

EXPERIMENTAL

Plant material

Rosella calyx was obtained from East Java. The specimen was identified at the Laboratory of Biology, Ahmad Dahlan University by Assoc Prof. Hadi Sasongko on May 2014 (identification no. B/B.12/1030/VII/2014). The calices were dried and ground into powder. Extraction was carried out with 70 % ethanol, followed by evaporation in a rotary evaporator at 50 ^{o}C to get the concentrated extract.

Determination of total flavonoids

The total flavonoid content of the extract was determined using quercetin as standard [15]. The extract (1gram) was dissolved in 100 mL of 70 % ethanol. Then 3 mL of the solution was made up to 10 mL with 70 % ethanol. To 0.5 mL of this extract solution was sequentially added 1.5 mL of ethanol, 0.1 mL 10% $AlCl_3$; 0.1 mL of 1 M Na acetate; and 2.8 mL of distilled water. The resultant solution was mixed to homogeneity, and then read at 430nm in a UV-VIS spectrophotometer.

Animal treatments

Animal handling in this research was approved by the Research Ethical Committee of Ahmad Dahlan University (approval ref no. 011505043). The care and use of animals was in line with the Guide For The Care and Use of Laboratory Animals published by National Research Council of the National Academies [16]. Male Sprague Dawley rats (25) were divided into 5 groups, each group consisting of 5 male rats. The animals were acclimatized for 1 week to the experimental environment. Group I, the normal group, received feed and drinking water *ad libitum*. Group II was treated with a single oral dose of DMBA at 75 mg/kg. Groups III, IV, V were treated with the extract at doses of 10, 50 and 100 mg /kg, respectively daily for 35 days. All treatments were administered orally. After 35 days, rats in the extract groups were treated with a single oral dose of DMBA at 75 mg/kg. Blood samples were collected from orbital sinus after 1 week for SGPT and SGOT analyses. The rats were thereafter sacrificed under CO_2 gas anaesthesia, and their liver were excised for GST analysis.

Determination of SGPT and SGOT activities

The activities of SGPT and SGOT were assayed colorimetrically using Diasys® kits as per manufacturer's instructions.

Determination of GST activity

The activity of GST was measured in liver homogenates using 1-choloro-2,4-dinitrobenzene (CDNB) method (17). Liver tissue (100 mg) was homogenized with 1 mL of 0.1 M phosphate buffer, pH 7.5. The homogenate was centrifuged at 10,000 rpm and 4 °C for 30 min. The supernatant was then centrifuged further at 15,000 rpm for 30 min. at 4 °C. The supernatant from the second centrifugation was the cytosolic fraction containing GST. It was stored at -20 °C and used for GST assay. In the assay, 702.5 µL of 0.1 M phosphate buffer pH 6.5 was added to 17.5 µL of the liver cytosol fraction, 15 µL, 50 mM GSH, and 15 µL of 50 mM CDNB (dissolved in ethanol) in a1-mL cuvette. The GS-DNB 194 conjugate product was measured at 340 nm from 0 to 3 min in a spectrophotometer. The GST activity was stated as average rate of absorbance per minute.

GST gene expression by RT-PCR

The expression of GST gene was monitored by RT-PCR. Liver tissue (100 mg) was extracted with 1000 µL of Trizol®, re-suspended 20-30 times, and kept at room temperature for 5 min. The mixture was added to 200 µL of chloroform, incubated at room temperature for 15 min and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant (400 µL) was taken and mixed with 500 µL of isopropanol, shaken for 5 seconds and

then centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was then discarded and the pellet was washed and shaken gently with 1 mL of 75% ethanol. The mixture was vortexed and centrifuged at 9800 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was dried at room temperature and re-suspended in RNase-free water followed by incubation at 60 °C for 10 min. It was thereafter stored at -80 ºC as RNA Stock.

Synthesis of cDNA

The RNA stock (5 mg) was added to 1.0 mL of a universal primer (Oligo dT) and made up to a volume of 12 µL with RNase-free water (RFW). This was the RNA template. The master mix solution was prepared by mixing 4 µL of 5x reaction buffer, 1 µL of ribolock RNase inhibitor, 2 µL of 10 mM dNTP mix, and 1µl RevertAidM-MuLV reverse transcriptase. After gentle mixing, it was centrifuged at 3500 rpm for 1 min. Then, each master mix was added to RNA template and mixed gently. Each sample was incubated in a thermal cycler at 42 °C for 60 min and terminated at 70 °C for 5 min.

Polymerase chain reaction (PCR)

The process of PCR was initiated with the preparation of PCR Master Mix by putting into a 25-mL microtube 0.5 DreamTaq green PCR, 1 µL of 10 µM GST forward primer, 10 µM of 1 µL GST reverse primer, 4 µL of cDNA template and 19 µL of RFW, followed by gentle vortex-mixing. The mixture was then placed on the PCR thermal cycler machine. Initiation step (initial denaturation) was at a temperature of 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 sec (denaturation), 55 °C for 30 sec (annealing), 72 °C for 1 min (elongation or automated extension), and at 72 °C for 10 min (final extension). The GSTp bp (C to T) used as primers (IDT, Madison, AL, Singapore) were:

GSTP 1 – 5′-CAT GCC TAT GAT ACT GGG ATA-3′;

GSTP 2 – 5′-CTA CTT GTT ACT CCA TTG GGC -3’.

The PCR results were subjected to agarose gel electrophoresis. Agarose gel was prepared from agarose gel (Invitrogen, New Zealand) 2 % with 1x TBE (Tris Boric EDTA) buffer and 5 µL ethidium bromide. Electrophoresis was carried out at 150 V for 30 min. The electrophoresis result was captured by photographing the ethidium bromide flourescense in a dark room.

Statistical analysis

Data were expressed as mean±SD of three replications. Quantitative data were compared with control values and analyzed statistically using one-way ANOVA with SPSS software. The significance level was set at $p < 0.05$.

RESULTS

Total flavonoid content

The total flavonoid content of roselle extract was 0.371 ± 0,011 %. Antocyanin was one of abundant flavonoids in rosella calyx.

Effect of rosella extract on SGPT and SGOT activities

The rosella extract led to significant decreases in DMBA-induced elevations in the activities SGPT and SGOT (Table 1).

Table 1: Levels of SGPT and SGOT after treatment with rosella extract and DMBA

Group	SGPT (U/L)	SGOT (U/L)
Normal	22.58±0.62*	18.69±0.63*
Negative control	26.58±1.07	21.73±0.83
Rosella (10 mg/kg)	24.28±1.05*	18.69±0.63*
Rosella (50 mg/kg)	22.46±0.46*	18.21±0.62*
Rosella (100 mg/kg)	22.09±0.27*	18.09±0.46*

**Significantly different from negative control group ($p < 0.05$). Data were expressed as mean ± SD of three replications*

The results showed that DMBA was hepatotoxic, as indicated by the significant increases in SGPT and SGOT levels after DMBA treatment [12,17]. Treatment with rosella extract at 10, 50 and 100 mg /kg for 35 days decreased the activities of SGPT to normal levels.

Effect of rosella extract on GST activity

Treatment with DMBA decreased GST activity in the liver. However, these decreases were reversed by the rosella extract, which resulted in increases in the activities of GST (Table 2).

Table 2: Effect of rosella extract on hepatic activity of GST in DMBA-treated rats

Group	Activity (mU/mg)
Normal	11.233 ± 7.332
Negative control	6.533 ± 2.838
Rosella (10 mg/kg)	7.333 ± 1.528
Rosella (50 mg/kg)	6.333 ± 1.155
Rosella (100 mg/kg)	14.500 ± 3.536*

**Significantly different from negative control group ($p < 0.05$). Data were expressed as mean ± SD of three replications*

Treatment with rosella at a dose of 100 mg /kg significantly increase GST activity, but not at lower doses.

Effect of rosella extract on GST gene expression

The purity of RNA was determined by the ratio of absorbances at 260 nm and 280 nm ($A_{260/280}$) (Table 3). The best ratio was 1.8-2.0. The ratio obtained for the isolated RNA was 1.8-3.6, which indicated some DNA impurity, since $A_{260/280}$>2.0, but no protein impurity ($A_{260/280}$>1.8). It was also revealed that there was an increasing level of GST gene expression in the rosella extract-treated groups (Figure 1). Thus, treatment with rosella for 35 days (sub-chronic exposure) influenced the level of GST at the molecular level.

Table 3: $A_{260/280}$ ratio of RNA isolated from rosella extract-treated, DMBA-exposed rats

Group	Ratio ($A_{260/280}$)
Normal	2.136
Negative control	1.832
Rosella (10 mg /kg)	2.839
Rosella (50 mg /kg)	3.600
Rosella (100 mg /kg)	2.694

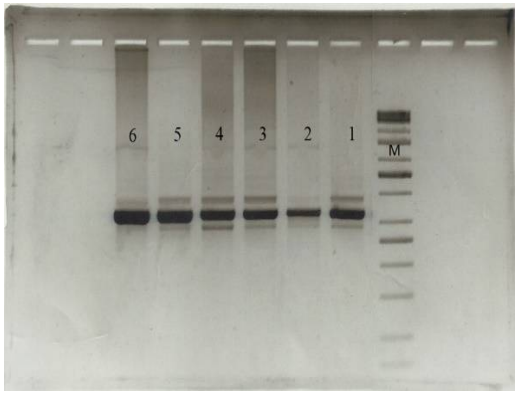

Figure 1: GST gene expression from RT-PCR of rosella extract-treated, DMBA-exposed rats. M: marker; (1) normal; (2): negative control, (3) 10 mg/kg, (4) 50 mg/kg, (5) 100 mg/kg, (6) rosella-treated, 100 mg/kg control without DMBA induction

DISCUSSION

Rosella has been reported to have antioxidant effects [10]. Antioxidant property is important for protection of the liver from damage caused by free radicals. Studies have shown that rosella is hepatoprotective against liver damage from exposure to paracetamol, [11]; carbon tetrachloride [12], and streptozotocin [13]. Flavonoids are known to possess antioxidant properties. This antioxidant property is expected to neutralize the damaging effects of free radicals, including DMBA. The hydroxy phenolic groups of flavonoids scavenge free radicals by donating their lone pair of electrons. Enzymatic activation of PAHs leads to the generation of active oxygen species such as peroxides and superoxide anion radicals, which induce oxidative stress in the form of lipid peroxidation. Formation of reactive oxygen species (ROS) in hepatocytes is deleterious to the liver because oxidative stress plays an important role in liver injury [9].

Many of the members of the PAH family are toxic, carcinogenic and immunotoxic [5]. DMBA is metabolized in the liver to a more toxic form which is involved the activation of cytochrome P450 to more reactive intermediates such as epoxides, dihydrodiol and radical cations. Epoxides and dihydrodiols bind covalently to amino groups of purines to form stable and unstable DNA adducts. The cytochrome P450 enzyme CYP1A1 or CYP1B1, and microsomal hydrolase metabolize DMBA to DMBA-3,4-diol-1,2-epoxide (DMBA-DE). These enzymes are located in hepatic cells and extra-hepatic tissues such as lung, mammary gland, spleen, kidney, prostate, uterus, and heart [4].

The elimination of DMBA-DE from the body is accelerated in the presence of the antioxidant enzyme glutathione-S-transferase (GST) [6]. PAHs are metabolized by cytochrome P450 monooxygenases (CYPs). Enzymes of the CYP1A sub-family are involved in phase I biotransformation of xenobiotics in many vertebrates. Substrates for CYP1A are of planar conformation, and the expression of CYP1A mRNA is regulated by binding of planar aromatic hydrocarbons to the cytosolic aryl hydrocarbon receptor (AhR). The second step in the metabolism of PAH is catalyzed by phase II biotransformation enzymes, amongst which is GST, which is also regulated by the AhR [18].

The increased activity of GST in the extract-treated group is an indication of the hepatoprotective effect of rosella against DMBA damage, and its ability to reverse the oxidative stress caused by DMBA. In the treated groups, the increase in activity of GST implied enhanced capacity to detoxify DMBA to DMBA-DE. This is supported by the concomitant decreases in levels of SGPT and SGOT. Previous reports showed that dietary quercetin significantly decreased the antioxidant activities of phase II hepatic enzymes [18].

In the present study, results from PCR showed significant decreases in GST expression after

DMBA treatment, which is an indication that DMBA has adverse effect on GST gene expression. However, treatment with rosella extract reversed this effect. In a previous study, it was shown that oral administration of anthocyanin-rich rosella extract increased the activities of SOD, catalase and GPx. Thus, the results obtained in the study can be attributed to the radical-scavenging effects of the antioxidant principles in the extract.

CONCLUSION

The findings of this study indicate that rosella extract has hepatoprotective effect against DMBA-induced liver injury as seen from the reversal of decreases in GST gene expression, increases in GST activity and reduction in SGPT and SGOT levels.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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