The increasing of catalase activity in dimethylbenz-α-anthracene (DMBA) induced rat treated by Hibiscus sabdariffa L extract

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WORD COUNT 3988
TIME SUBMITTED 05-JUN-2018 02:47PM
PAPER ID 37861440

The increasing of catalase activity in dimethylbenz-α-anthracene (DMBA) induced rat treated by *Hibiscus sabdariffa* L extract

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Abstract: The objective of the present study was to characterize the activity and expression of catalase enzymes in *Hibiscus sabdariffa* L (rosella) extract treated rat induced by dimethylbenz-α-anthracene (DMBA) and to evaluate the relationship between the catalase activity and histopathological characteristics of liver organ. The 25 animals were divided randomly into 5 groups: the normal group, the negative control group, and treated groups, which treated by rosella extract with variation of dose of 10, 50 and 100mg/kgBW/day for 35 days. On day36 the animals were given with DMBA in dose of 75 mg/kgBW single dose. After one week, the animals were sacrificed and the catalase activity was measured from liver homogenate by the decomposition of H_2O_2 and followed directly by the decrease in absorbance at 240 nm. The expression of catalase gene was observed using RT-PCR. The results showed that treatment of rosella extract increases the activity of catalase, significantly ($P<0.05$). The increasing of catalase activity was found in dose dependent manner. The catalase gene expression was also found to increase in rosella extract treated groups. The histopathological observation of liver organ was found normal. Rosella extract increase the catalase activity and expression of catalase antioxidant gene. It was concluded that rosella extract increase activity and gene expression of catalase *in vivo*.

Keywords: *Hibiscus sabdariffa*, catalase activity, antioxidant, free radical, gene expression.

INTRODUCTION

The activity of intrinsic antioxidant enzymes are essential for celluler protection against reactive oxygen species (ROS). The overwhelming of ROS could affect many biological processes. Free radicals are highly reactive molecule as having one or more unpaired electrons. They will restore the balance by seeking electrons from other molecules (Pham-huy *et al*. 2008). The overwhelming of free radical can cause oxidative stress and plays an important role in the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases. Oxidative stress could promote by reactive oxygen species which produced from internal metabolism or external exposure.

Polycyclic aromatic hydrocarbon (PAH) is an environmental pollutant that can be generated by the combustion of fuel, wood and other organic materials. PAHs are also found in cigarette smoke, grilled food, motor vehicle pollution (Gao *et al*. 2005). DMBA is a prototype of PAH that reported to be carcinogenic and immunosuppressives (Gao *et al*. 2008; Shimada & Fujii-Kuriyama 2004; Buters *et al*. 2003). DMBA also reported to be hepatotoxic (Singh *et al*. 2011; Ali *et al*. 2013; Koul *et al*. 2014; El-gerbed 2013).

The human body produces antioxidants to fight oxidative stress. These antioxidants called the endogenous antioxidants, including superoxide dismutase (SOD), catalase and glutation peroxidase (GPx)(Blokina *et al*. 2003). In the excess of oxidant, exogenous antioxidant supplied externally through food and supplements are needed. Endogenous and exogenous antioxidants act to prevent and repair damage caused by reactive oxygen and nitrogen. They can increase the immune system and reduce the risk of cancer and degenerative diseases (Valko *et al*. 2006; Parthasarathy *et al*. 1999)

Catalase, one of endogenous antioxidant, catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species. The defect on catalase activity has been implicated in different physiological and pathological conditions(Kodydková *et al*. 2014).

Rosella (*H. sabdariffa*) has been reported to have antioxidant effects (Anokwuru *et al*., 2011), (Al-hashimi 2012). In the *in vivo* treatment, rosella extract treatment could decrease malondialdehyde (MDA) in highly MDA level rat induced by used cooking oil (Suwandi 2012). This antioxidant effect was expected to protect the liver damage caused by PAH. The objective of this research was to characterize the activity and expression of catalase enzymes in *Hibiscus sabdariffa* L (rosella) extract treated rat induced by dimethylbenz-α-anthracene (DMBA) and to evaluate the relationship between the catalase activity, gene expression and histopathological characteristics of liver organ.

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MATERIALS AND METHOD

Material

Rosella calyx were collected from Madiun, East Java, Indonesia. The specimen was identified in Laboratory of Biology, Ahmad Dahlan University, Yogyakarta, Indonesia. Rosella calyx was dried and grounded to powder. Extraction was carried out by 70% ethanol, followed by evaporation to get concentrated extract.

Determination of total flavonoid

The total flavonoid content was assayed using quercetin as standard (Chang *et al.* 2002). The extract was weighed of 1 gram and dissolved in 100 ml of 70% ethanol. The 3ml of extract solution was added with ethanol until 10,0ml. From this solution it was taken of 0.5ml and diluted with 1.5ml of ethanol, and added with 0.1ml 10% $AlCl_3$; 0.1ml Na acetate; and 2.8ml of distilled water. The solution was mixed until homogeneous and measured by UV-Vis spectrophotometer at 430nm. The flavonoid content was expressed as quercetin equivalent.

Determination of total anthocyanincontent (TAC)

The total anthocyanin content (TAC) was determined by the pH-differential method at pH 1.0 and pH 4.5 (Lee *et al.* 2005). Anthocyanin will form oxonium structure and give intensive colour at pH 1.0. The hemiketal form (colorless) will found at pH 4.5. Briefly, transfer 1mL extracted solution into 10mL volumetric flask for preparing two dilutions of the sample. First sample was added with potassium chloride buffer to adjust pH 1.0, and the other was added with sodium acetate buffer, pH 4.5. The solution was measured after 15 minutes by spectrophotometer at the 510 and 700 nm.

The different of absorbance at two wavelngt (A) was measured as follows:

$A = (A_{510} - A_{700})pH\ 1.0 - (A_{510} - A_{700})pH\ 4.5$

The antocyanin concentration (C) were calculated with formula:

$C = A \times MW \times DF \times 1000 / \varepsilon x1$

Where MW is the molecular weight of anthocyanin expressed as cyanidin-3-glucoside (449.2), DF is the dilution factor, and ε is the molar absorptivity of anthocyanin (26900).

Animal treatment

The animal studies had ethical clearance from ethical committee of Ahmad Dahlan University. The male Sprague Dawley rats of 25 were divided into 5 groups, each group consisting of 5 male rats. The animals were acclimatized for 1 week. Group I was the normal group were only given food and drink *adlibitium*. Group II is the group was given. DMBA (75mg/kgBW), Group III, IV, V were the treated groups which were given the extract with dose variation of 10, 50 and 100mg/ kgBW respectively, once daily for 35 days and administered orally.

After administration of the extract for 35 days, mice were treated byDMBA75mg/kgBW. The animal was sacrified and liver organ was taken for analysis.

Catalase activity assay

The catalase activity was measured using procedure reported by (Iwai *et al.* 2002) with modifications. Catalase activity was measured as the amount of the hydrogen peroxide reduction from the liver homogenate. The 0.05mL of liver supernatant added by 2000 µl of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM hydrogen peroxide. The amount of hydrogen peroxide reduction was measured with spectrophotometer at 240 nm every 15 seconds for one minute. The catalase activity was calculated using the slope value.

Catalase gene expression

The catalase gene expression was observed by the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method (Attia *et al.* 2014).

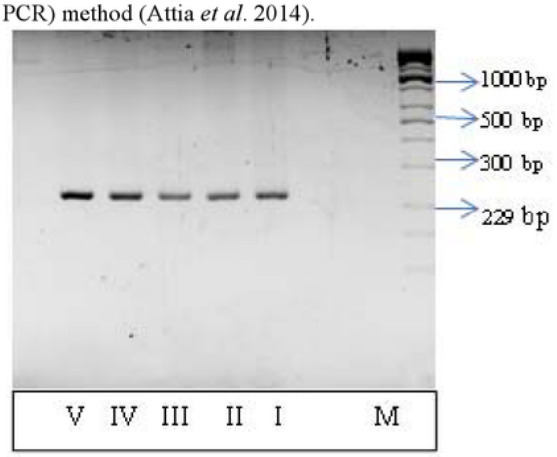

Fig. 1: Agarose gel electrophoresis of RT-PCR products of liver organ of rosella extract and DMBA treated rat. from total RNA isolated using Trizol. The gels were stained with ethidium bromide and photographed using Polaroid camera I: normal group, II: negative control, III: 10mg/kg BW, IV: 50mg/kg BW, V: 100mg/kg BW, M: DNA ladder.

RNA extraction

RNA extraction was carried out using TRIZOL reagent. The liver organ of 100 mg was homegenated with 1000 mL of Trizol. After that, it was added by 200mL of chloroform, stirring until thoroughly for 15 seconds, incubated at room temperature for 15 minutes and centrifugated at 12,000 rpm 4°C for 15 minutes. The 400 mL of the supernatant was taken and mixed with isopropanol (1: 1) and mixed for 5 seconds, and centrifuged at 12,000 rpm, 4°C for 5 minutes and the

supernatant was discarded. The pellets was washed with 1mL of 75% ethanol and mixed and centrifugated slowly at a 9,800 rpm, 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspensed in RNase-free water and incubated at 50ºC for 15 minutes. The RNA was stored at deep freezer with a temperature of-70ºC.

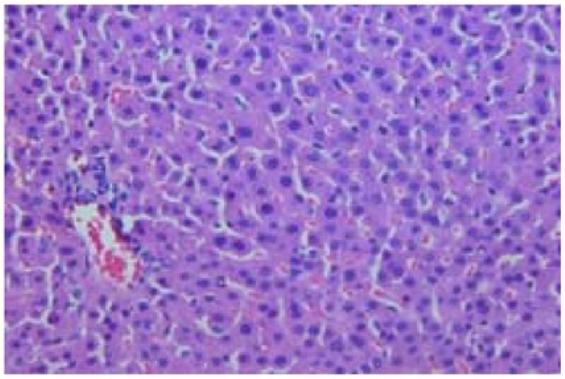

Fig. 2: The histopathological observation using Haematoxylin-eosin staining of negative control group. Single dose treatment of DMBA caused the reversible damage (hydropic degeneration).

RT-PCR

The process of reverse transcription was carried out using Thermo Scientific products Revert Aid First Strand cDNA Synthesis Kit #K1621, #K1622. The 5ug of RNA was added on 1.0mL Cu-Z catalase primer (Integrated DNA Technologies), 1.0mL of Oligo (dT) (Thermo Scientific) and 4.0mL of RNase-free water. The reaction starts at a temperature of 65ºCfor 5 minutes and then stored at a temperature of -70ºC.The master mix component 4.0mL first-strand buffer (Thermo Scientific), 1mL ribolock RNase inhibitor, 2.0mL DNT (10mM, Thermoscientific), and 1.0mL Revertaid) was mix gently and centrifuge at 3500 rpm 30 seconds. The mixture was then mixed with the RNA template and incubated at 42ºC for 60 minutes and 70°C for 5 minutes. The cDNA was stored at a temperature of-70ºC.

cDNAs were amplified using specific primers for rats CAT (*Forward* : CAT1-5'GCA GAT ACC TGT GAA CTG TC-3'; *Reverse*: CAT2-5'GTA GAA TGT CCG CAC CTG AC 3')(de Oliveira e Silva *et al.* 2012). Gene expression was assayed according to the manufacturer's instruction. The PCR program cycles were set as follows: initial denaturing at 95°C for 3 minutes, followed by 35 cycles (95°C for 30 s, 54°C for 30 s, 72ºC for 60s, 72ºC for 15 minutes). The PCR product was analyzed using electrophoresis on a 2% agarose gel in Tris-borate-EDTA buffer and visualized over a UV Trans-illuminator. The ethidium bromide-stained gel bands were captured images and the band intensities were quantified.

RESULTS

Total flavonoids and total anthocyanin content

The total flavonoid contents of the extracts in terms of quercetin equivalent was 0.74±0.011% and the total anthocyanin content of the extract was 0.37±0.045%. Flavonoid is also reported to have a hepatoprotective activity (Al-jumaily & Abdulla 2014).

Catalase activity

Catalases are protective enzymes responsible for the degradation of hydrogen peroxide before it can damage cellular components. They are present in virtually all aerobic organisms and many anaerobic organisms (Switala & Loewen 2002).

The result showed that DMBA treatment decrease the catalase activity significantly compared to baseline group. DMBA is one of polycyclic aromatic hydrocarbons will metabolize into the ultimate carcinogen DMBA dihydrodiol epoxide (DMBA-DE), which can mediate neoplastic transformation by inducing DNA damage and form reactive oxygen species, as well as mediate the chronic inflammatory process (Manoharan *et al.* 2013). The reactive species inactivate the endogenous antioxidants including catalase enzyme, the absence of exogenous antioxidant caused the decreasing of cathalase activity (Pham-Huy *et al.*, 2008). Treatment with rosella extract increase the catalase activity significantly ($p<0.05$) (Table 1). This study found that increasing of rosella extract dose treatment did not increase catalase activity significantly.

Table 1: The Catalase activity on Dimethylbenz-α-anthracene (DMBA) induced rat treated by *Hibiscus sabdariffa* L extract

Groups	Catalase activity
Normal group	9.320±0.268(b)
Negative control	6.571±0.289(a)
Extract 10 mg/kgBW	8.312±0.618(b)
Extract 50 mg/kgBW	7.840±0.506(a)(b)
Extract 100 mg/kgBW	7.830±0.366(a)(b)

(a) significantly different with normal group ($p<0.05$),
(b) significantly different with negative control group($p<0.05$)

Table 2: The absorbance ratio (A260/280) of RNA on liver organ of rosella extract and DMBA treated rat

Groups	Rasio (R)
Normal group	2.136
Negative control	1.832
Extract 10 mg/kgBW	2.839
Extract 50 mg/kgBW	3.600
Extract 100 mg/kgBW	2.694

Catalase gene expression

The observation of catalase gene expression in this study was performed by RT-PCR of mRNA isolated by Trizol. The purity of RNA was performed by absorbance ratio on 260/280 nm and showed on Table II. This study found that absorbance ratio A260/280 was 1.8-3.6, indicate that there is no protein impurity, but there is a small amount of DNA in the isolate. According to (Sambrook & Russel 2001), contamination of proteins and other organic materials are characterized by low value of the ratio of A260/280 (<1.8). This RNA isolate was then copied to cDNA and the catalase gene was amplified using RT PCR method. The PCR product was then visualized by agarose gel electrophoresis. The electrophoresis of PCR product was shown on fig. 1.

Histopathological observation of liver organ

Catalase enzyme is one of antioxidant enzymes are found mostly located in the liver. A decrease in the activity of catalase enzyme can be used as a sensitive indicator to indicate the presence of liver damage. In this present study found the reversible damage (hydropik degeneration) in the normal group, negative control group and treated group. Indicating that rosella treatment is nontoxic as well as single dose treatment of DMBA could not cause the permanent damage in the liver. The histopathological profile was shown on fig. 2.

DISCUSSION

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 antioxidant effect (Miguel 2011; Einbond *et al*. 2004). The antioxidant properties of flavonoids were considered to deactivate the reactive metabolite of DMBA.

The previous research reported the high level of flavonoid and phenolic compound on *H. sabdariffa* L extract. It has been well recognized that some flavonoid compounds show high antioxidant activity. The mechanisms of action of flavonoids are through scavenging or chelating process (Nijveldi *et al*. 2001).

Catalase is an antioxidant that plays a role in regulation of the immune system. Increasing of reactive oxygen species led to an increasing use of endogenous antioxidants (such as catalase) to neutralize ROS. The imbalance of oxidant and antioxidant will occurred in oxidative stress if increasing ROS cannot encounter by endogenous antioxidant capacity.

Anthocyanin is one of flavonoid family member content in the rosella extract might be responsible to this antioxidant effect (Jadwiga & Ryszard 1988; Birben *et al*. 2012). Conjugated double bond system in anthocyanins could stabilize the free radicals. The presence of hydroxyl (OH) group in this compound was increase the antioxidant effect through scavenging free radicals. The very active free radical could cause the chain reaction and produce the new free radicals. Catalase is an important enzyme for inactivating free radicals. Catalase responsible in the decomposition of hydrogen peroxidase, one of intermediate product in deactivating of free radical.

Hydrogen peroxide could be produced by various sources including the process of electron transport in mitochondria by cytochrome oxidase and reduces O_2^- and super oxide O_2^- reaction catalyzed by superoxide dismutase (Noori 2012). Hydrogen peroxide has the ability to diffuse across cell membranes and cause cell damage. It can also be a precursor of other toxic species. It can penetrate to cell membranes and therefore its site of action can be different and far from its site of production. H_2O_2 can react with transition metals such as Fe^{2+} and Cu^{2+} generating highly reactive OH in the extra cellular environment (Limaye *et al*. 2003). Based on the structure of catalase, it could also be oxidized by free radicals and ROS, resulted the damage of protein structures and followed by loss catalase activity to decompose hydrogen peroxide.

The catalase activity was affected by its gene expression. It was reported that activity of CAT and SOD in liver tissues increased after repeated administration of the *H. opuntia* aqueous extract in CCl_4 induced rat. Treatment with the *H. opuntia* extract also resulted a higher catalase gene expression compared with that observed in the CCl_4-treated group (de Oliveira e Silva *et al*. 2012).

The study found that treatment with rosella extract increase the catalase gene expression. Treatment with rosella extract for 35 days affect the gene expression, and might be involved the activating of antioxidant responsive element (ARE). The previous studies reported that antioxidant compounds exert their chemopreventive activity by inducing expression of phase II enzymes and endogenous antioxidants that protect cells from oxidative stress damage or reactive carcinogenic intermediates (Zhang 2006; Jeyapaul & Jaiswal 2000). The promoter regions of the phase II genes contain specific DNA sequences, termed the antioxidant response elements. The activation of ARE has important role in chemopreventife mechanism of chemopreventife compounds against oxidative stress or electrophiles (Jeyapaul & Jaiswal 2000; Nioi *et al*. 2003). Activation of ARE led to actifation of Nrf2 transcription factor in modulating the expression of phase II detoxificationenzymes and endogenous antioxidants.

CONCLUSION

The treatment with rosella extract could increase the activity and gene expression of catalase in DMBA treated rats.

ACKNOWLEDGEMENT

The authors thank to Ministry of Higher Education of Indonesia Republic for funding the research through Postgraduate research team scheme.

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