The Effects of Rosella Extract (*Hibiscus sabdariffa*) against the *n*carboxymethyl-lysine, NF- $\kappa\beta$, TNF- α in the Rats Heating Food Diets

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Abstract

The food processing by heating can increase the formation of AGEs (Advanced Glycation End Products). AGEs are compounds that formed from a non-enzymatic continuous glycation reaction between proteins and sugar residues. The CML (N-carboxymethyl-lysine) is used as a marker for AGEs cause most commonly found in vivo. The bond of AGEs and RAGE (reseptor for AGEs) induce various signaling pathways that trigger inflammation and increase oxidative stress. The AGE and RAGE interaction activate the transcription factor of NF-KB. NF-KB activate gene transcription to release proinflammatory cytokines such as TNF-a. Anthocyanins are compounds that can prevent the formation of AGEs and muffle the adverse effects of AGEs. Rosella contains anthocyanin such as : delphinidin-3-O-glucoside, delphinidin-3-Osambubioside, and cyanidin-3-O-sambubioside. This study is to determine whether the daily intake of Rosella extract can reduce the levels of *n*-carboxymethyl-lysine in serum, expression of NF- $\kappa\beta$ and TNF α in the rats fed with heated food. This study applied experimental post test control using Rattus norvegicus Wistar strain. The samples were divided into 5 groups: KN (negative control/fed without heating), KP (positive control/fed heated food but not treated by Rosella extract), R₁ (fed heated food and treated by 200 mg.kg⁻¹BW Rosella extract), R₂ (fed heated food and treated by 300 mg.kg⁻¹BW Rosella extract), and R₃ (fed heated food and treated by 400 mg.kg⁻¹BW Rosella extract). The *n*carboxymethyl-lysine levels were measured by using the ELISA, the expression of NF- $\kappa\beta$ is analyzed by using immunofluorescence, and expression of TNF - α is observed by immunohistochemistry. There was significantly decreased the levels of *n*-carboxymethyl-lysine in all groups which were treated by Rosella extracts (R₁,R₂,R₃); p= 0.000, $\alpha = 0.05$ (p< α). Decreased activation of NF- $\kappa\beta$ in all groups which were treated by Rosella extract is significant (p = 0.000), and decreased expression of TNF α in all groups which were treated by Rosella extract is also significant (p = 0.000). Rosella extract can reduce the levels of *n*-carboxymethyl-lysine, expression of NF- $\kappa\beta$, and TNF α .

Keywords: AGEs, anthocyanin, Rosella flower extract, *n*-carboxymethyl-lysine, NF- $\kappa\beta$, TNF α .

INTRODUCTION

There are two sources of AGEs (Advanced Glycation End Products) namely exogenous derived from consumed food and beverages and endogenous formed in the body as part of normal metabolism. Raw foods derived from plants and animals containing AGEs, however within the limits that can be tolerated by the body considering the body that has homeostatic system.

Foods processing that contain sugar, lipid or fat, and protein through a heating process such as fried, grilled, and roasted can increase AGEs. The processing using dry heat methods can increase the formation of AGEs up to 100-fold,

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compared to the amount of AGEs found in raw foods.

The amount of AGEs of food consumption is greater than the total amount of AGEs normally contained in plasma and tissue [1]. The average total AGEs dietary exposure to healthy people which did not cause adverse effects was approximately 16,000 AGEs kU.day⁻¹ [2]. Consumption of processed foods with warming will lead to increased exposure to AGEs, thus exceeding 20,000 kU.day⁻¹ [3].

The accumulation of AGEs in the body, will cause oxidative stress and chronic inflammation, which in turn will further enhance the endogenous formation of AGEs. Accumulation of AGEs will also increase the susceptibility to damage tissue [4].

Giving AGEs dietary may increase its circulation thus exceeding the normal limit that can be tolerated. The increase of AGEs in circulation will activate RAGE (Receptor of AGEs). AGE and RAGE interaction causes the activation

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of MAPKs and PI3-K, which will then activate the transcription factor of NF- $\kappa\beta$. Once activated, NF- $\kappa\beta$ moves from the cytoplasm to the nucleus and activate gene transcription to release proin-flammatory cytokines, growth factors and adhesive mole-cules, such as TNF- α , IL-6 and VCAM1 [5,6].

CML (*N-carboxymethyl-lysine*) is a type of AGEs which is mostly used as a marker for the formation of AGEs. CML is a type of AGEs which is most commonly found in vivo, best known for its characterizations [5] and most representable structure of AGEs both quantitative and pathophysiologic [7].

AGEs's bad consequences can be avoided by prevent the formation, build up on the network, and overcome the inflammatory reactions and oxidative stress of AGEs. The use of ingredients that work as AGE inhibitors and breakers can prevent the formation and accumulation of AGEs in the body tissues [8,9].

Anthocyanin is a type of flavonoid polyphenols in the group known as antioxidants. The mechanism of anthocyanin which can be indicated as inhibitor formation of AGEs is through the inhibition of auto-oxidation monosacaride [10] which prevent lipid peroxidation and inhibition of polyol pathway [11]. Anthocyanins also inhibit the binding of AGEs to its receptor (RAGE) [12].

Rosella contains phenolics, organic acids, sterols, terpenoids, polysacharides and some minerals. Its fenolic content consists of anthocyanins as delphinidin-3-O-glucoside, delphinidin-3-O- sambubioside, and cyanidin-3-O-sambubioside [13]. This study was conducted to determine whether daily intake of Rosella extract could reduce the levels of CML in serum, activation of NF- $\kappa\beta$, and expression of TNF α in Wistar rats' aorta fed by heating food diets.

MATERIALS AND METHODS Object Study

This study was an experimental research laboratories used *Rattus norvegicus* Wistar strain and designed *post test control group*. The study used 25 male rats, ages 3-4 months old with 100-150 gram initial body. The rats is randomly devide into 5 groups, KN (negative control), KP (positive control), R₁ (treated by 200 mg.kg⁻¹BW Rosella extract) R₂ (treated by of 300 mg.kg⁻¹BW Rosella extract), and R₃ (treated by 400 mg.kg⁻¹ BW Rosella extract). KN fed without heating, while KP, R₁, R₂, and R₃ fed by heating food for 8 weeks. The Rosella extract is given once a day.

Rosella that used in this study was purchased from Materia Medika Batu. Rosella extract is the result of Rosella flower extraction using 70% ethanol solvent.

Data Collection

The levels of CML was examined after 8 weeks. The feeding for all groups was continued until 12 weeks. At the end of the treatment, the mice were fasted, anaesthetized and then dissected. The serum was taken to measure CML level by using ELISA method (used ELISA kit for CML , No. CATALOG E1374Ra). The aorta was taken to analyze the activation of NF- $\kappa\beta$ by using immunoflouroescence method (using primary antibody NF- $\kappa\beta$ P65 ThermoFisher no catalog MA5-15160) and to observe the expression of TNF- α by using immunohisto-chemistry method (dyes by DAB and Mayer's Hematoxilen).

Data Analysis

Data were statistically analyzed. Test for normality using the Shapiro-Wilk test, homogeneity test using Levene's Test. Data comparison using One-way ANOVA, followed by LSD (Least Significant Difference) 5%. Comparisons between groups using multiple comparison test. The test results said to be significant if the p value <0.05.

RESULT AND DISCUSSION

The Effect of Rosella Extract against CML Levels

The results of ANOVA was obtained p-value for 0000 which was smaller than α =0.05 (p<0.05). It can be concluded that there was significant influence of Rosella extract on the levels of CML. The LSD 5% test results showed that the significant decrease of CML's levels occurred in all groups which were treated by Rosella extract. The lowest average levels of CML was obtained in R₁. The highest average level of CML was obtained in KP and R₂, average value of CML levels were not significantly different with KN. On the other words, the provision of rosella extract at a dose of 300 mg.kg⁻¹BW can lower the levels of CML to the rats given feed without heating (Fig. 1).

The results showed that there was significant decline of the CML levels in all groups of mice which were treated by Rosella extract. This could occur because the Rosella extract contained anthocyanin. These results were consistent with the previous studies which stated that the antioxidant is a type of flavonoid that can inhibit the formation of AGEs [12]. This result also showed that anthocyanin in the Rosella extract

could break the CML. This fits with previous research which states that the phenolic antioxidant can function as a *breaker* AGEs [13].



- Figure 1. The average levels of CML (ELISA method) using ANOVA test.
 - Description:
 - KN = negative control/fed without heated
 - KP = positive control/fed heated food but not treated by Rosella extract
 - R₁ = fed heated food, treated by 200 mg.Kg⁻¹BW Rosella extract
 - R₂ = fed heated food, treated by 300 mg.Kg⁻¹BW Rosella extract
 - R₃ = fed heated food, treated by 400 mg.Kg⁻¹BW Rosella extract.
 - p-value = 0.000

This study found that in the higher dose of Rosella extract, its ability become low for reducing the levels of CML. This might be because this study were still used a crude of Rosella extract rather than the type of anthocyanins of Rosella that have been isolated. The crude extract of rosella that had been used had many other compounds besides the anthocyanin, i.e. fatty acids (saturated and unsaturated), protein, carbohydrates, minerals, organic acids (citric, hydroxycitric, Hibiscus, malik, tartaric, oxalate, and ascorbate), anthocyanins, polysaccharides and flavonoids [10,14].

With the wide range of content in the Rosella ekstract, an increase in dosage also increases the activity of another material, thus the effect of anthocyanins reduced. Previous research suggested that there are many precursors to form the CML, among others, Polysaturated fatty acid (PUFA), and ascorbic acid. CML formation pathways other than through the Maillard reaction also through the reaction between PUFA or ascorbic acid with lvsine residues derived from proteins. Researchers suspect that when the dose of Rosella is increased likely not due to the inability of anthocyanins to break the CML that causes its levels increased, but rather because there have been more CML formation in the body through a reaction between lysine and PUFA, as well as ascorbic acid. So then, although anthocyanins have the ability to break down the CML but since its number is formed mounting so that the CML levels tend to remain high.

The Effect of Rosella Extract against the Activation of NF- $\kappa\beta$

Based on the results of ANOVA, p-value was obtained for 0000, smaller than α = 0.05 (p<0.05). The LSD 5% test results indicated that the lowest activation of NF- $\kappa\beta$ was in KN and the highest in KP. There was significant decrease of the activation of NF- $\kappa\beta$ in all groups which were treated by Rosella extract compared with KP. The lowest average of activation of NF- $\kappa\beta$ was obtained in rats which were treated by 200 mg.kg⁻¹ BW Rosella extract (R₁) (Fig. 2).





Description:

- KN = negative control/fed without heated
- KP = positive control/fed heated food but not treated by Rosella extract
- R₁ = fed heated food, treated by 200 mg.Kg⁻¹BW Rosella extract
- R₂ = fed heated food, treated by 300 mg.Kg⁻¹BW Rosella extract
- R₃ = fed heated food, treated by 400 mg.Kg⁻¹BW Rosella extract.
- p-value = 0.000

These results indicated a decline in the activation of NF- $\kappa\beta$ significantly in all groups which were treated by Rosella extract. This happened because the extract of Rosella flower containing anthocyanin.

This decreased activation of NF- $\kappa\beta$ agrees with the theory that the intake of anthocyanins may decrease the activation of the transcription factor NF- $\kappa\beta$ and the expression of several cytokines and proinflammatory mediators [15]. These results are also consistent with several other studies which stated that the Rosella flower extract has an effect as an antioxidant, and the compound suspected to have effect as an antioxidant and inhibits the activation of NF- $\kappa\beta$ is its fenolic content which is flavonoidcompound anthocyanin [16]. The mechanism of inhibition on the activation of NF- $\kappa\beta$ is to prevent loss of p50 and p65 binding so there is no translocation of p50/p65 into the nucleus. Moreover, it can be worked as an inhibitor of NADPH oxidase which produces superoxide anion radicals. Anthocyanins can inhibit the activation of NF- $\kappa\beta$ by inhibiting the degradation of I $\kappa\beta$, and also inhibition on the activation of I $\kappa\beta$ kinase enzyme that prevent phosphorylation of NF- $\kappa\beta$ (p50 and p65) dimer result which do not separate each other, and subsequently p50 and p65 translocation into the nucleus will not occur.

The results of this study are also consistent with other studies mentioned that the anthocyanins in the dried petals of Rosella extract (H. sabdariffa Linn) were able to inhibit oxidative stress-induced activation of NF-κβ [17]. Anthocyanins and flavonoids have been proven to inhibit LDL oxidation and the activation of NF-κβ, and decrease inflammation. Mechanism of anthocyanin in inhibiting Ox-LDL is to fight free radicals, eliminate oxidant product, inhibite the oxidation of lipids, lipoproteins, liposomes and DNA, and suppress production of radicals and their precursors both in vivo and in vitro. In addition, anthocyanins can increase the production of endogenous antioxidants such as superoxide dismutase, Gluthation Peroxidase and Catalase. The ability of anthocyanins to reduce the activation of NF-κβ can be solved through AGEs/CML. The breakdown of AGEs reduces the amount of AGEs so then it was inadequate to carry out the binding to RAGE. Moreover, it can be done through binding of AGEs/CML with its receptor which is RAGE.

From the various doses of Rosella which are given, it turns out that the most effective dose that lowers the activation of NF- $\kappa\beta$ is a dose of 200 mg.kg⁻¹ BW Rosella extract. This study shows that with the addition dose of Rosella extract, its extract ability to decrease activation of NF- $\kappa\beta$ decreases. This may be due to the formation of AGEs/CML increases with the increasing doses of Rosella extract so that the amount of AGEs/CML which binds to RAGE increases. The increasing amount of AGEs which binding to RAGE will increase the number of activation of NF- $\kappa\beta$.

The Effect of Rosella Extract against TNF- α Expression

Based on the results of ANOVA, p-value was obtained for 0000, smaller than α = 0.05 (p<0.05). The LSD 5% test results showed that KP had the highest average expression of TNF- α . The

decreased expression of TNF- α were significantly demonstrated in all group treated by Rosella extract.

In comparison of KN with the R₁ and R₂, pvalue was obtained greater than 0.05 (p>0.05). This means there was no significant difference in the average expression of TNF- α between KN and R1 and R2. In comparison of KP with R1, the pvalue of R_2 and R_3 was obtained less than 0.05. This proved that the group that treated by Rosella extract at the dose of 200, 300, and 400 mg.kg⁻¹BW can lower the expression of TNF- α significantly. KP had the highest average value of the expression of TNF- α , while the average expression of TNF- α decreased in all groups which were treated by Rosella extract. The lowest average expression of TNF- α was obtained in rats which were treated by Rosella extract 200 mg.kg⁻¹BW (R₁) (Fig. 3).





Description:

- KN = negative control/fed without heated
- KP = positive control/fed heated food but not treated by Rosella extract
- R₁ = fed heated food, treated by 200 mg.Kg⁻¹BW Rosella extract
- R₂ = fed heated food, treated by 300 mg.Kg⁻¹BW Rosella extract
- R_3 = fed heated food, treated by 400 mg.Kg⁻¹BW Rosella extract.
- p-value = 0.000

The results showed that the expression of TNF- α in all groups of mice which were treated by Rosella extract decreased. The type of anthocyanins such as cyanidin and delphinidin may inhibit the expression of inflammatory mediators. Anthocyanins included in a group of polyphenolic compounds, especially the type of flavonoid compounds. Some studies suggest that flavonoids have a tendency to scavenge free radicals, so it does not form excessive ROS. ROS can stimulate phosphorylation of $\kappa\beta$ Inhibitor (I $\kappa\beta$). Iκβ serves to bind NF-κβ, so then it remains inactive in the cytoplasm. If Ικβ is

phosphorylated, the bond of NF- $\kappa\beta$ and I $\kappa\beta$ aparts, so that NF- $\kappa\beta$ active and migrate to the nucleus. In the nucleus, NF- $\kappa\beta$ will activate transcription by binding to DNA sequences on a target gene, thus triggering the release of inflammatory cytokines such as TNF α . Therefore, when ROS was inhibited, then the activation of NF- $\kappa\beta$ can be inhibited. Inhibition of activation of NF- $\kappa\beta$ will also inhibit the secretion of inflammatory cytokines such as TNF α .

 $TNF\alpha$ is an important mediator in the inflammatory process, it plays a role in increasing the inflammatory response of endothelial cells [18]. This study proved that rosella flower extract may decrease the activation of NF- $\kappa\beta$, so that the expression of TNF α on endothelial decreased. The highest reduction is in mice fed a rosella flower extract 200 m.kg⁻¹. This study showed that with increasing the doses of extract of Rosella, there was a decline in the ability to reduce the expression of TNF α . This might be due to along with increasing dose, there was an increase on the formation of CML. When the number of CML which binds to RAGE increased, the activation of NF-κβ increased. The incerased activation of NF- $\kappa\beta$ resulted in increasing the secretion of TNF α .

Relationship Levels of CML, Activation of NF- $\kappa\beta$, and TNF- α by Using Correlation Analysis

The results of testing the correlation between the levels of CML and the activation of NF- $\kappa\beta$ was obtained correlation coefficient of 0598 with a pvalue of 0.002. p<0.05 indicated that there was a significant relationship between the levels of CML and the activation of NF- $\kappa\beta$. The correlation coefficient was worth 0598 showed the quite strong level of the relationship. The correlation coefficient was positive indicated that there was a positive relationship between levels of CML and the activation of NF- $\kappa\beta$. The increasement levels of CML would also be followed by the increased activation of NF- $\kappa\beta$ and vice versa.

The results of testing the correlation between the expression levels of CML and TNF- α was obtained correlation coefficient of 0687 with a pvalue of 0.000. p<0.05 indicated that there was a significant relationship between levels of CML and TNF- α expression. The correlation coefficient was worth 0687 showed the strong level of relationship. The correlation coefficient was positive indicated that there was a positive relationship between levels of CML and TNF- α . The increasement levels of CML would also be followed by the increase levels of TNF- α and vice versa. The results of testing the correlation between the activation of NF- $\kappa\beta$ and the expression of TNF- α was obtained correlation coefficient of 0753 with a p-value of 0.000. p<0.05 indicated that there was a significant correlation between the activation of NF- $\kappa\beta$ and the expression of TNF- α . The correlation coefficient was worth 0753 showed the strong level of relationship. The correlation coefficient was positive indicated that there was a positive relationship between the activation of NF- $\kappa\beta$ and the expression of TNF- α .

and vice versa.

Rosella ethanol extract can reduce the levels of *N-Carboxymethyl-Lysine* in serum, activation of NF- $\kappa\beta$ and expression of TNF α in aorta Wistar rats fed heated significantly. There are close relationship on the decreased levels of CML, ekspression of NF- $\kappa\beta$, and expression of TNF α .

The increasement activation of NFkB, would also

be followed by the increased expression of TNF- α

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