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Research Article

Antihypertension Effect from Bakasang's Peptide Extract Based on MDA Levels in Sera and iNOS Expression in Cardiac Tissue of Rats Hypertensive Model

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ABSTRACT

Hypertension is an abnormality of cardiovascular condition, marked by high-blood pressure over normal condition. One of the factors that causes hypertension is altered equilibrium of Renin-Angiotensin-Aldosteron system, which commonly caused by genetic disorder, could produces excess of Angiotensin II which known as vasoconstrictor. Angiotensin II elevates body's blood pressure and stimulates production of free radicals causes hypertension and oxidative stress. This research conducted to explore potential antihypertension effect from bakasang's peptide extract made from fermented skipjack tuna (Katsuwonus pelamis L.) based on MDA levels in sera and iNOS expression in cardiac tissue from hypertension rats (Rattus norvegicus) induced by DOCA-salt. Rats were divided into five groups, they were: (1) negative control group, (2) hypertensive control group, (3) hypertension with captopril therapy with dose of 5 mg/kg of body weight (BW), (4,5) bakasang's peptide extract therapy with dose of 200 mg/kgBW, and 300 mg/kgBW respectively. The results showed that bakasang's peptide extract with dose of 200 mg/kgBW significantly decrease ($p<0.05$) MDA level in sera and iNOS expression in cardiac tissue. MDA level decreased up to 32.77%, and iNOS level of expression decreased up to 34.46 % after therapy using bakasang's peptide extract on hypertension rats. It can be concluded that bakasang's peptide extract has antihypertension effect (ACE-inhibitor), and capable to decrease MDA levels in sera and iNOS expression in cardiac tissue on hypertension rats induced by DOCA-salt.

Keywords: Bakasang, Hypertension, iNOS, MDA.

INTRODUCTION

Hyertension is a cardiovascular disorder which indicated by the elevation of blood pressure in the body, reaches ≥140 mmHg for systolic pressure and ≥ 90 mmHg for diastolic pressure, compared with normal blood pressure in human is <120 mmHg for systolic and <80 mmHg for diastolic[1]. The number of patients with hypertension in Indonesia is almost 27,5% of the total population and increase significantly through the years, based on 2004 survey[2]. With the most common type of cases is essential hypertension, nearly 95% of total cases[1]. Essential hypertension, which caused by genetical disorder, plays significant role in the development of hypertension. One of the factors that leads to hypertension, is disruption of the balance system of Renin-Angiotensin-Aldosteron System (RAAS). RAAS which normally plays a role in regulating blood pressure and fluid balance in the body, will produce excess volume of Angiotensin II, a potent vasoconstrictor, which induces sodium and water retention in kidney leads to elevation of body's blood pressure[3]. In addition, Angiotensin II also stimulates the secretion of Aldosterone which causes activation of NADPH oxidase (NOX)[3,4]. Activation of NOX will increase the production of free radicals (Radical Oxygen Spesies / ROS) leads to oxidative stress after imbalance concentration between free radicals and antioxidants in the body's[4].

Deoxycorticosterone Acetate (DOCA)-salt, is a synthetic mineralcorticoid which commonly used in the production of animal model of hypertension, leads to an endocrine hypertension model. Induction of DOCA-salt could form a severe hypertension model by quickly, followed by increasing of oxidative stress. This endocrine type of hypertension allows to be treated using bioactivate peptides as a therapy[4].

It has been known that dietary protein is a rich-source of bioactive component which has beneficial effect for health accordance to the sequence of it's amino acid[5]. In this research, Bakasang (traditional food from maluku and north sulawesi) which made from fermented skipjack tuna was used as therapeutic agent. Research shown that used of dried skipjack tuna's meat (Bonito), had potential of antihypertensive effect as an ACE (Angiotensin Converting Enzyme)-inhibitor[6,7].

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This study is conducted to prove the potential of antihypertension effect from bakasang's peptide extract, based on MDA (Malondialdehyde) level in sera and iNOS expression in cardiac tissue of hypertensive rat (*Rattus norvegicus*) induced by DOCA-salt.

MATERIALS AND METHODS

Isolation of Bioactive Peptides from Bakasang

Bakasang samples was obtained from the island of Banda Naira, Maluku, and weighed 200 grams before dissolved in 1000 mililiters of distilled water (Solvent ratios of 1:5 (w/v)). Sample was homogenized using a magnetic stirrer until homogenous (about 15 minutes) and then deactivated in 90^0C for 5 min. Bakasang mixture's then centrifuged (4^0C) for 15 min at 6000 rpm and the supernatant was taken and added with ethanol absolute (1:1 (v/v)), then mixed it before stored overnight at -20^0C. Sample then centrifuged at 10000 rpm (4^0C) and the pellet's was obtained and dried. Then it was added with Tris-HCl buffer (pH 6.8) (1:1 (v/v)) and stored at -20^0C.

Preparation of Hypertensive Rats induced DOCA-salt

The use of animal model in this research was approved by Ethical Clearance Committee of Brawijaya University (KEP-440-UB/2016). Animals which used in this research was Rat (*Rattus norvegicus*) and divided into five groups, there are: (1) Normotensive control group, (2) Hypertensive control group, (3) *Captopril* treated group (5 mg/kg of body weight (BW)), (4) Bakasang's peptide extract (200 mg/kgBW), (5) Bakasang's peptide extract (300 mg/kgBW). Rats were adapted for one week before used. Rats were given standard feed AD II (containing water 12%, proteins's crude 15%, lipid's crude 3-7%, fiber 6%, maximum ash 7%, calcium 0.9-1.1% and phosphorous 0.6-0.9%).

Induction period was 2 times a week for 5 weeks (10 injections), which first five injections contained 20 mg/kgBW and then the next five injections contained 10 mg/kgBW. DOCA-salt was dissolved in corn oil and injections were carried out at subcutan area of cervical section. Rats were given drinking 2% of NaCl (w/v) during induced by DOCA. Blood pressure was measured using the tail-cuff method (CODA tail-cuff Blood Pressure System, Kent Scientific) once a week, until the end of the study (necropsy).

Preparation of Bakasang's Peptide Extract as a Therapy

The theraupetic dose of bakasang's peptde extract were 200 mg/kgBW and 300 mg/kgBW. The extract was dissolved in distilled water and was carried out by oral administration once daily for four weeks (28 days).

Preparation of Captopril as a Therapy

The theraupetic dose of bakasang's peptide extract were 5 mg/kgBW. Captopril was dissolved in distilled water and was carried out by oral administration once daily for four weeks (28 days).

Measurement of MDA Level in Sera

After all the process has completed, necropsy will be performed and followed by analysis to determine MDA level in sera and observation of iNOS expression on cardiac tissue using immunoshistochemistry technique.

For the measurement of MDA level in sera, each sera from all groups was pippeted 100 µL and added with 550 µL aquadest. The mixture was homogenized and then added with 250 µL of Tri-Chloroacetic Acid (TCA 10%), 250 µL of HCl 1N and 100 µL of Na-thiobarbituric Acid solution, respectively with homogenized after each addition. The mixture then centrifuged at 500 rpm for 10 minutes and supernatant was taken. Supernatant was heated at 100^0C in waterbath for 30 minutes, then cooled at room temperature before measured using visible-spectrophotometer at spesific wavelenght[8].

Analysis of iNOS expression in Cardiac Tissue using Immunohistochemistry Method

Cardiac tissue were stored in PFA (paraformaldehyde) 10% and then sectioned and attached on object glass (slides) before de-parrafinized and re-hydrated. Slides later was washed by PBS (Phosphate Buffer Saline) solution then added using Hydrogen Peroxide (H_2O_2) and incubated for 20 minutes before washed by addition of PBS. Blocking was performed using addition of BSA (Bovine Serum Albumin 5% (w/v)) in PBS solution and incubated 30 minutes at room temperature. Slides was washed using PBS solution and then added by iNOS antibody as primary antibody. Slides later incubated with addition of secondary antibody-biotin labelled and then added using SA-HRP (Strep-Avidin Horse Radish Peroxidase) and DAB (diamino benzedine) solution respectively. Counterstasining was performed by addition of Mayer Hematoxylin before being closed by coverglass. Slides was observed under the microscope and later was analyzed by Immunoratio software

Table 1: MDA Levels From Sera.

Groups	Average MDA Levels (µg/mL)	Increased Levels Against Negative Hypertension Group (%)	Decreased Levels Against Positive Hypertension Group(%)
Negative Hypertension	1.012 ± 0.244^{a}	-	-
Positive Hypertension	2.137 ± 0.138^{c}	111.21	-
Captopril Therapy 5 mg/kgBW	1.901 ± 0.223^{c}	-	11.04
Bakasang Therapy 200 mg/kgBW	1.437 ± 0.198^{ab}	-	32.77
Bakasang Therapy 300 mg/kgBW	1.712 ± 0.204^{bc}	-	19.89

Note: Different superscripts notations indicate significantly different effect ($p<0.05$).

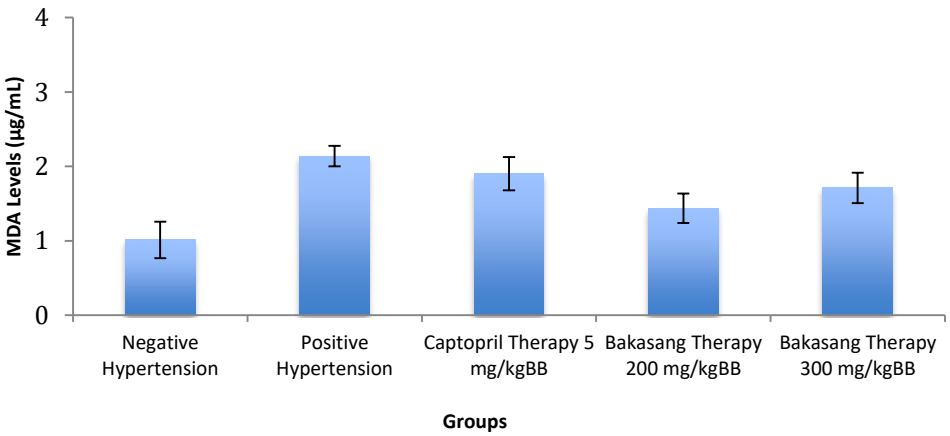

Figure 1: MDA Levels From Each Group.

Statistical Analysis

Statistical analysis was performed using SPSS application for Mac (Version 20) with ANOVA (Analysis of Variance) method and Tukey's post hoc test ($p<0.05$).

RESULTS

Based on the results of MDA level in sera (Table 1), it was found that the positive group of hypertension had the higest level of MDA by 2.137 ± 0.138 µg/mL. It shown that in the positive group of hypertension, MDA level had significantly increase compared to negative hypertension group, which had MDA level by 1.012± 0.244 µg/mL or increased 111.21%. In captopril group of therapy, MDA level was 1.901± 0.223 µg/mL or decreased 11.04% from postive hypertension group. For bakasang group of therapy, MDA levels were 1.437± 0.198 µg/mL or decreased 32.77% from postive hypertension group and 1.712 ± 0.204 µg/mL or decreased 19.89% from postive hypertension group, with dose of 200 and 300 mg/kgBW respectively.

Table 1 showed that bakasang's peptide extract therapy significantly decrease MDA level ($p<0.05$) with dose of 200 mg/kgBW as effective dose for decreasing MDA level in sera. Based on observations of iNOS expression using Immunoratio software in each group (Table 2), it was found that the positive group of hypertension (Figure 3 B) had the higest level of iNOS expression by 49.62 ± 3.633 %. It shown that in the positive group of hypertension, iNOS expression had significantly increase compared to negative hypertension group (Figure 3 A), which had iNOS expression by 5.975 ± 2.075 %. or increased 730,46%. In captopril group of therapy (Figure 3 C), iNOS expression was 43.06 ± 2.157 %, or decreased 13.22 % from postive hypertension group. For bakasang group of therapy, iNOS expression were 32.52 ± 4.219 % or decreased 34.46% from postive hypertension group (Figure 3 D) and 35.88 ± 2.039 % or decreased 27.69% from postive hypertension group (Figure 3 E), with dose of 200 and 300 mg/kgBW respectively. Bakasang's peptide extract significantly decrease ($p<0.05$) in iNOS expression in cardiac tissue ($p<0.05$), with dose of 200 mg/kg BW is the effective dose for decreasing iNOS expression in cardiac tissue (table 2).

DISCUSSION

The bakasang's peptide extract, which obtained from extraction of bakasang (fermented skipjack tuna (*Katsuwonus pelamis* L.)), has potential as an alternative antihypertensive therapy (ACE-inhibitor) by in vitro study and had good ACE-inhibitory activity with average of 68.80%. Bakasang's peptide extract has better ACE-inhibitory activity compared with another ACE-inhibitor sources, such as *douchi* (traditional Chinese fermented food from soybean) which has ACE-inhibitory activity of

Table 2: Levels of iNOS Expression From Each Group.

Groups	Average iNOS Expression (%)	Increased Levels Against Negative Hypertension Group (%)	Decreased Levels Against Positive Hypertension Group(%)
Negative Hypertension (A)	5.975 ± 2.075[a]	-	-
Positive Hypertension (B)	49.62 ± 3.633[d]	730.46	-
Captopril Therapy 5 mg/kgBW (C)	43.06 ± 2.157[cd]	-	13.22
Bakasang Therapy 200 mg/kgBW (D)	32.52 ± 4.219[b]	-	34.46
Bakasang Therapy 300 mg/kgBW (E)	35.88 ± 2.039[bc]	-	27.69

Note : Different superscripts notations indicate significantly different effect ($p<0.05$).

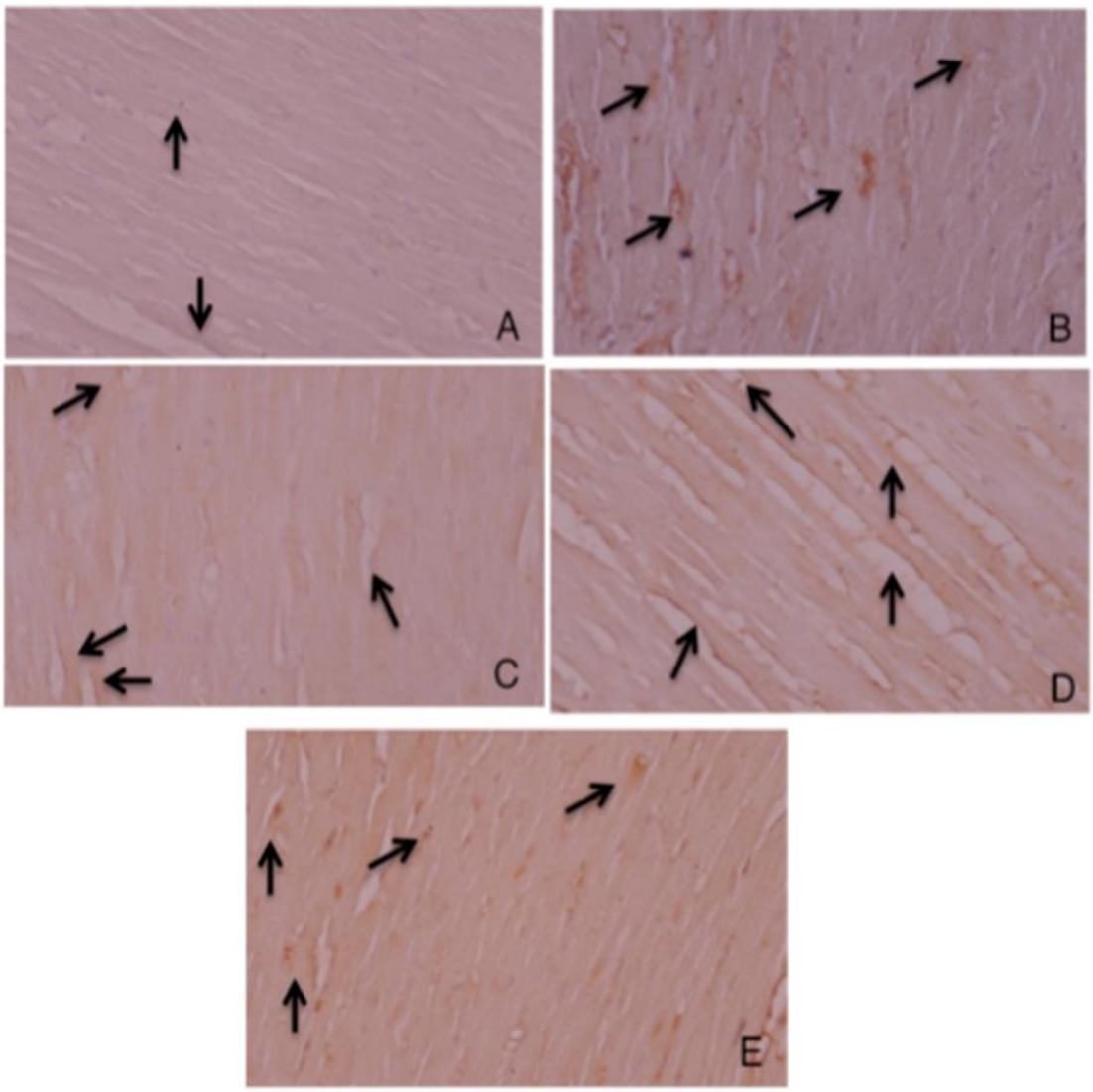

Figure 2: iNOS Expression in Cardiac Tissue After Immunohistochemistry Method.
Note: (A) Negative hypertension, (B) Positive Hypertension, (C) Captopril therapy 5 mg/kgBW, (D) Bakasang therapy 200 mg/kgBW, (E) Bakasang therapy 300 mg/kgBW. (→) Location of iNOS expression.

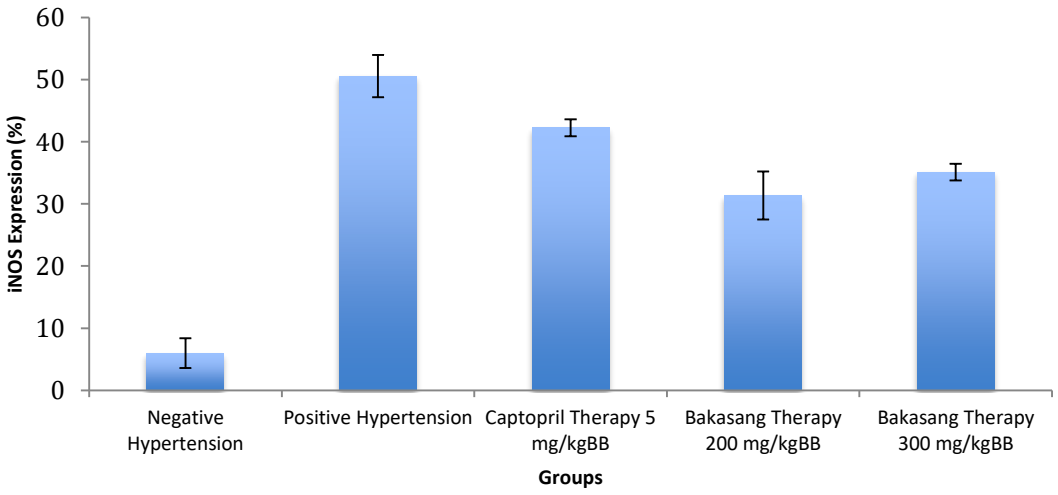

Figure 3: Levels of iNOS Expression From Each Group.

56.8 – 76.3% and bekasam (fermented fish product, Indonesia) which has ACE-inhibitory activity of 55.17%. This ACE-inhibitory activity from bakasang's peptide extract, obtained due to the fermentation process of skipjack tuna's meat by lactic acid bacteria (LAB) such as, *Lactobacillus casei*, *Lactobacillus farciminis* dan *Lactobacillus plantarum*. LAB have the ability to break down the long-chained protein into short-chained fragments (peptides) that could provide positive benefit for the body, one of them as an ACE-inhibitor[9].

Bakasang's peptide extract has the ability to decrease MDA level in sera besause of its ACE-inhibitory activity. ACE-inhibitor is able to inhibit ACE activity to convert angiotensin I into angiotensin II, which is a potent vasoconstrictor, so that the blood pressure is not get elevated and beside that, production of ROS also become declined. Because, apart as a potent vasoconstrictor, angiotensin II also contributes to stimulate the production of superoxide (O_2^-) in large quantities. The decline in production of free radicals causes reduced level of oxidative stress, thus the endogenous antioxidants could provide therapeutic effect to the body. This is accordance with the research of Khora (2013) and Wenno et al. (2016), which state that the protein hydrolisate from skipjack tuna's, even from bonito and bakasang, have potential as an antihypertensive therapy (ACE-inhibitor)[7,9]. Captopril therapy is also give a decreasing in MDA level in sera, although not giving significantly different with the positive hypertension group, caused by relatively small dose of captopril. According to Pechanova (2007), that the admistration dose of captopril about 50 mg/kgBW could lowering the MDA level in rat[10].

MDA is a biomarker to measure the level of oxidative stress due to lipid peroxidation in cell. The formation of MDA is initiated when the hydroxil radicals can perform an electron abstraction of PUFA (*Poly Unsaturated Fatty Acid*) which generated lipid radicals (L^-), and later could binds with oxygen to form a peroxyl radicals (LOO^-). If these peroxyl radicals are can't be reduced by antioxidants, it radicals will attack another PUFA and form a chain reaction that lead to the formation of MDA[11]. MDA level can be observed in vitro using TBA (Thio-Barbituric Acid) method, this method is based from condensation reaction between one molecule of MDA and two molecules of TBARS (Thio-Barbituric Acid Reactive Substances) in acidic condition. This reaction will produce a pink color that can be observed using visible-spectrophotometer[11].

Table 2 showed that bakasang's peptide extract therapy significantly decrease iNOS expression ($p<0.05$) in rat's cardiac tissue. ACE-inhibitor from bakasang's peptide extract will inhibit activity of ACE enzyme, resulting no conversion from angiotensin I to angiotensin II, which causes decreasing in production of ROS due to unactivated NOX. In positive hypertension group, high production of ROS as result from the activation of NOX will activate NF-κB to encoding iNOS gene[12].

The increasing expression of iNOS, will iead to excess formation of NO (Nitrogen Oxide) and later could bind with superoxide radicals (O_2^-) to form peroxynitrite radicals ($ONOO^-$) which is a very reactive cytotoxic substances. The increasing expression of iNOS also produces superoxide radicals (O_2^-) which can transform to hydrogen peroxide (H_2O_2) spontaneously or by catalitic reaction with superoxide dismustase (SOD). It has been known that the presence of peroxynitrite radicals and hydrogen peroxide has implication to tissue damage and organ dysfunction, including the heart[13].

According to the previous research of Sun et al. (2005), although it was known that the inhibition of iNOS did not have a directly effect on lowering blood pressure and left vebtricular hypertrophy, however, the inhibition and lowered expression of iNOS was able to gave therapeutic effect on left ventricular contractile tissue caused by decreased level of oxidative stress, as well known that angiotensin II are able to increase the iNOS expression and oxidative stress which causes reducing performance and function of the heart[14].

We conclude that the bakasang's peptide extract has potential to be an alternative therapy for antihypertension indicated by reduced MDA level in sera and iNOS expression from cardiac tissue in animal's model of hypertension induced by DOCA-salt.

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