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The Effect of Ethanol Extract of *Kuca* (*Allium schoenoprasum* L.) Bulbs on Serum Nitric Oxide Level in Male Wistar Rats

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Abstract: The aim of this study was to determine the effect of ethanol extract of *kucai* bulbs on serum nitric oxide (NO) levels in rats, in an attempt to study the mechanism of antihypertensive action. The measurement of NO level was conducted by Griess colorimetric assay. The male Wistar rats were divided randomly into three groups; the first group was treated with *kucai* extract of 100 mg kg⁻¹ body weight (*kucai*-treated group); the second group with isosorbide dinitrate (ISDN) of 0.18 mg kg⁻¹ body weight (ISDN-treated group) and the third group with vehicle only (control group). The results showed that at 30 min (T₃₀) the mean NO level in *kucai*-treated group increased significantly (138.69±169.38%) compared to baseline (T₀); similarly, in ISDN-treated group, the mean NO level increased (212.08±140.01%) significantly. At T₉₀, average NO levels in *kucai*-treated group was significantly higher than those in control group (p = 0.018), while those in ISDN-treated group were not significantly higher than those in control group (p = 0.436). Compared to *kucai*-treated group, NO levels in ISDN-treated group at T₉₀ was significantly different (p = 0.028). From this study it can be concluded that ethanol extract of *kucai* bulbs increased significantly serum NO level in experimental rats. Therefore, it is preassumed that antihypertensive effect shown by *kucai* is, one of which, due to mechanism of vasodilator action. The present results showed that *kucai* increased NO levels and this might be one mechanism of blood pressure lowering action of *kucai*.

Key words: *Allium schoenoprasum* L., *kucai*, nitric oxide, vasodilator, high blood pressure

INTRODUCTION

Nitric oxide (NO) is a chemical compound that plays a significant role in pathogenesis of several diseases, including hypertension. A physiological function of NO was discovered in the vasculature when it was shown that the endothelium-derived relaxing factor (EDRF) described by Furchgott and Zawadzki in 1980 is accounted for by formation of NO by endothelial cells (Furchgott, 1983; Sun *et al.*, 2003). NO is produced by endothelium cell or nitrate group of drugs, is the endogenous activator of soluble guanylate cyclase and plays mainly in this mechanism. NO is biosynthesized from L-arginine amino acid by at least three different isoform types of NO synthase, that is, neuronal NO synthase (nNOS), endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) (Balbatun *et al.*, 2003; Gharavi and El-Kadi, 2003). Free-radical NO produced will immediately react with molecular oxygen and water to form a variety of end-products, including nitrite (NO₂⁻), nitrate (NO₃⁻) and

S-nitrosothiol (RSNO) (Gharavi and El-Kadi, 2003; Brovkovich *et al.*, 1999). Free-radical NO is promptly inactivated by superoxide, Fe(II) and Fe(III), that are all in significant amount in biological system. The endothelium produced NO will diffuse into smooth muscle and activate guanylate cyclase enzyme (atrial natriuretic peptide receptor A). This enzyme stimulates cyclic guanosine 3',5'-monophosphate (cGMP) synthesis and then activates a number of protein kinase-dependent phosphorylations in smooth muscles and eventually produces dephosphorylation of myosin chain of the smooth muscles (Fig. 1). Calcium ion release will further trigger smooth muscle cell relaxation, reducing peripheral resistance, hence vasodilation occurs (Katzung and Chatterjee, 2007). By vasodilation the blood pressure will decrease.

Kuca (*Allium schoenoprasum*, L.) is a medicinal plants that has been used traditionally in Indonesia for lowering blood pressure. *Kuca* possesses a good prospect to be developed as phytopharmaceutical

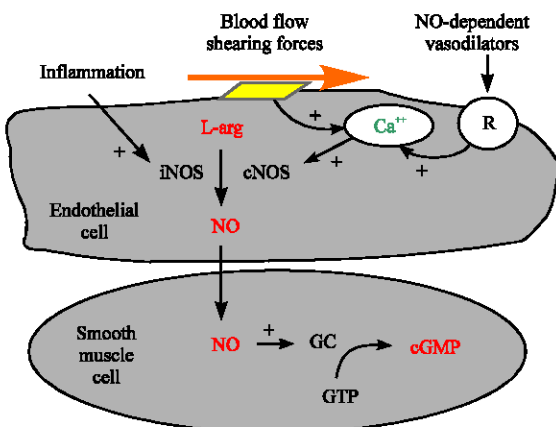


Fig. 1: Biosynthesis of nitric oxide (adapted from Cardiovascular Physiology Concepts, Klabunde, RE). NO is biosynthesized from L-arginine amino acid in endothelial cell by at least three different isoform types of NO synthase, that is, neuronal NO synthase (nNOS), endothelial NO synthase (eNOS) and inducible NO synthase (iNOS). Free-radical NO produced will activating guaianylate cyclase enzyme, therefore stimulating cGMP synthesis and then activates a number of protein kinase-dependent phosphorylations in smooth muscles and eventually produces dephosphorylation of myosin chain of the smooth muscles. Calcium ion release will further make smooth muscle cell relaxation, reducing peripheral resistance, therefore vasodilation occurs

product, so that it is expected as an alternative medication for hypertension. For this purpose, various steps of testing through both preclinical and clinical trials must be performed to study the efficacy and safety of *kucai* as antihypertensive agents. This study was conducted to determine the mechanism of *kucai* action on vascular relaxation (vasodilation) in experimental animals. There are several methods of such mechanism, one of them is by measuring the NO level in animal serum. NO is produced by various types of cells in picomolar to nanomolar amount and its half-life ($T_{1/2}$) is very short, i.e., 5 sec in biological fluid. Therefore, it is very difficult to measure NO products directly; instead, analysis of nitrate (NO_3^-) and nitrite (NO_2^-) are used to estimate NO level in biological fluid and cell culture medium due to their stability.

Several methods have been developed in assessment of NO level by nitrite measurement; i.e., Griess colorimetric assay, chemiluminescence analysis, gas chromatographic analysis, hemoglobin-trapping and fluorometric method. Apart from that, there are also ion-exchange, reverse-

phase ion pairing, reverse-phase high-performance liquid chromatography method developed to measure nitrite and nitrate in biological system, using absorbance of UV-visible, conductivity, electrochemical or fluorescence (Gharavi and El-Kadi, 2003). In this study, determination of NO level was performed through measurement by Griess colorimetric assay. This method is considered adequately simple due to it is wide used to analyze some amount of biological samples, including plasma (serum), urine, cerebrospinal fluid, saliva and cell culture media (Sun *et al.*, 2003). This study would determine NO level of rat serum as one of the methods to establish the mechanism of vasodilator action of *kucai*. In a earlier study on ethanol extract from *kucai* bulbs, an active ingredient presumed having antihypertensive effect had been identified as tetramethyloxamide (Fidrianny *et al.*, 2001). For this reason, this study used ethanol extract from fresh *kucai* bulbs.

MATERIALS AND METHODS

This study was performed in Pharmacology Laboratory-School of Pharmacy Institut Teknologi Bandung and Prodia Laboratory, Bandung-Indonesia at January 2008.

Determination of nitric oxide level was conducted by Griess colorimetric method. In colorimetric Griess method, nitrite was first treated with diazotizing reagent e.g., sulfanilamide (SA) in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with N-naphthylethylenediamine (NED) to form a stable azo compound. The intense purple color of the product allows nitrite assay and can be used to measured nitrite concentration as low as $\sim 0.5 \mu\text{M}$. The absorbance of this adduct at 540 nm is linearly proportional to nitrite concentration in the sample.

Preparation of *kucai* extract: Fresh *kucai* bulbs was purchased from local grocery market at Andir in Bandung, West Java, identified by botanists in the Herbarium Bandungense-School of Life Science and Technology, Institut Teknologi Bandung-Indonesia. The *kucai* extract was prepared with maseation-percolation method with 95% ethanol solutions. The extract then was vaporized with rotavapor until a concentrated extract was obtained.

Chemicals

Griess reagent: Griess reagent was purchased from Oxford Biomedical Research, USA (Product #NB 88). Griess reagent consists of granulated cadmium (25 g cadmium beads), ZnSO_4 solution (2 mL of 30% (w/v)

ZnSO₄), microcentrifuge tubes (50×1.5 mL microcentrifuge tubes), Color Reagent No. 1 (7 mL sulfamamide dissolved in 3 N HCl), Color Reagent No. 2 (7 mL N-[1-naphthyl] ethylenediamine dihydrochloride dissolved in deionized H₂O), nitrite standard (1.5 mL 500 pmol μL⁻¹ NaNO₂, equivalent to 500 μM NO), microtiter plate (One 96 well low protein binding plate with flat-bottom well), microtiter plate template (one printed template for duplicate assay), reagent reservoirs (three plastic troughs for dispensing and pipetting reagents), cadmium bead washing solutions (125 mL of 0.1 M HCl and 125 mL of 0.1 M NH₄OH).

Drugs: Isosorbide dinitrate (ISDN) tablets, produced by PT. Kimia Farma Tbk. (Bandung, Indonesia) was used in this study. ISDN was prepared as aqueous suspension and diluted with distilled water.

Animals: Fifteen male Wistar rats (weighing 250 to 300 g) were randomly selected and divided into three groups. The first group received a dose of 100 mg kg⁻¹ of body weight of *kucai*, second group with a dose of 0.18 mg kg⁻¹ body weight of isosorbide dinitrate (ISDN) and third group received only vehicle. Blood samples were taken before treatment and 10, 30, 60 and 90 min after the treatment (Sumino *et al.*, 2000). This study was conducted in accordance with the Guideline for Care and Use of Animals Laboratory of School of Pharmacy Institut Teknologi Bandung.

Samples preparation: Blood sample was incubated at room temperature for 45 min, then centrifuged at 3000 rpm for 15 min. The serum was stored in 0.3 mL tube and kept at -20°C until use. 10 to 50 μL of defrozen serum was adjusted to 190 μL with distilled water and then added with 10 μL of 30% (w/v) ZnSO₄ solution to make the total volume 200 μL. Final solution was then mixed by vortexing, incubated for 15 min at room temperature and centrifuged at 3,000-4,000 rpm for 5 min. The resulting supernatant was transferred to microcentrifuge tubes containing 0.5 g granulated cadmium (about 6-7 beads) and incubated at room temperature overnight with agitation. The sample solution was transferred to clean microcentrifuge tube, recentrifuge and the supernatant was tested for NO₂ content.

Standard solutions preparation: Standard solution was prepared by diluting nitrite standard containing 1.5 mL 500 pmol μL⁻¹ NaNO₂ (equivalent to 500 μM NO). From the solution, serial nitrite standard solutions were prepared with NO concentration of 100, 50, 25, 10, 5, 1 and 0.5 μM (added with deionised water). Calibration curve thus made was used to determine nitrite levels.

Procedure: Cadmium beads were washed in their individual microtubes with H₂O (2×1 mL); 0.1 M HCl (2×1 mL) and 0.1 M NH₄OH, pH 9.6 (2×1 mL) before used. Standards and samples were prepared as described before. Samples were added to wells in duplicate, 20-100 mL depending on the NO concentration in sample, distilled water was added to make a total volume of 100 mL. Fifty milliliter Color Reagent No. 1 was added followed by brief shaking. Fifty milliliter Color Reagent No. 2 was then added and shaken for 5 min at room temperature. Absorbance was read at 540 nm in microtiter plate reader. The standard curve was plotted and the sample concentration was estimated from the curve.

Statistical analysis: Statistical analysis was carried out by 2-way analysis of variance (ANOVA) by post-hoc test. Data are shown as mean values±SD (standard deviation). A value of p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Isosorbide dinitrate (ISDN) have been widely used as vasodilators to treat acute myocardial ischemia, their biological effects being due to the release of NO. ISDN have longer terminal elimination half life than other nitrovasodilator (20 min intravenously, 64 min sublingually) and has a high first-pass metabolism, broken down to 5-isosorbide-mono-nitrate and 2-isosorbide-mono-nitrate which are both more active than their parent compound. ISDN has been used as a drug standard in several studies on determination of nitric oxide in both *in vivo* and *in vitro* systems (Balciogiu *et al.*, 1998; Baluchnejadmojarad *et al.*, 2003).

The dose of extract used in this study was at dose of 100 mg kg⁻¹ body weight. This dose was chosen on the basis of a earlier study suggesting that the *kucai* dose to have antihypertensive effect was 100 mg kg⁻¹. While the dose of ISDN i.e., 0.18 mg kg⁻¹ body weight was derived from conversion of human therapeutic dose.

The level of NO among the control, *kucai*-treated and ISDN-treated groups were not significantly different. Up to 10 min (T₁₀) NO levels in *kucai*-treated, ISDN-treated and control groups were decreased compared to those of baseline (T₀), but the decrease was not significant. After 10 min, NO levels increased in ISDN-treated and *kucai*-treated groups to their peaks (Table 1, Fig. 2).

At T₃₀, mean NO levels of *kucai*-treated group increased significantly (% changes were 138.69±160.38) compared to T₀ (p = 0.044); similarly, that of ISDN-treated group increased significantly (% changes were 212.08±140.01, p = 0.026), while in control group the increase was not significant (% changes were

Table 1: Serum Nitric Oxide (NO) levels changes before and after administration of ethanol extract of *Kuca*

Time	Control group (n = 5)		<i>Kuca</i> 100 mg kg ⁻¹ (n = 5)		ISDN 0.18 mg kg ⁻¹ (n = 5)	
	NO (µM)	p	NO (µM)	p	NO (µM)	p
T ₀	245.90±98.85		215.06±81.70		148.50±71.03	
T ₁₀	197.73±83.50		210.90±77.52		113.90±19.64	
T ₃₀	209.78±122.11	0.512 ^a	469.46±232.12	0.044 ^a	394.88±65.59	0.026 ^a
T ₉₀	168.93±25.60	0.167 ^a	436.03±156.95	0.229 ^a 0.018 ^b	202.94±78.19	0.139 ^a 0.436 ^b

ISDN = Isosorbide dinitrate, ^a Compared to T₀; ^b Compared to control group, a value of p<0.05 was considered statistically significant. At T₃₀ NO level of *kucai*-treated group compared to ISDN-treated group p = 0.818 and at T₉₀, p = 0.028

Table 2: Percentage changes of serum NO levels at T₃₀-T₀ between different groups

Group	NO level at T ₀ -T ₃₀	
	Changes (%)	p
Control	21.74±27.42	-
<i>Kuca</i> 100 mg kg ⁻¹	138.69±169.38	0.198 ^a 0.495 ^b
ISDN 0.18 mg kg ⁻¹	212.08±140.01	0.037 ^a

ISDN = Isosorbide dinitrate, ^a Compared to control group, ^b Compared to ISDN-treated group, a value of p<0.05 was considered statistically significant. Changes were counted comparing to baseline (T₀) serum NO level

Table 3: Comparison of serum NO levels changes between different groups

Group	T ₀ -T ₃₀		T ₃₀ -T ₉₀	
	Δ NO level (µM)	p	Δ NO level (µM)	p
Control	44.17±28.55	-	92.37±84.59	-
<i>Kuca</i> 100 mg kg ⁻¹	261.66±238.59	0.812 ^a 0.179 ^b	221.26±206.34	0.899 ^a 0.373 ^b
ISDN 0.18 mg kg ⁻¹	297.90±75.12	0.005 ^b	238.70±165.72	0.213 ^b

ISDN = Isosorbide dinitrate, ^a Compared to ISDN-treated group, ^b Compared to control group

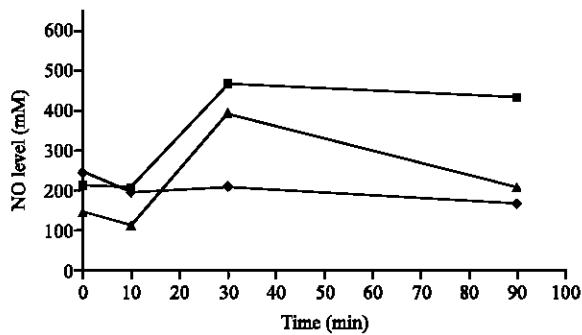


Fig. 2: Effect of ethanol extract of *kucai* administration to NO serum level. Male Wistar rats divided randomly into three groups: control group (◆), *kucai*-treated group (■) and ISDN-treated group (▲). T₀ as baseline (before administration of *kucai*, ISDN or vehicle); n = 5 (each group). Blood samples were taken before treatment and 10, 30, 60 and 90 min after the treatment

21.74±27.42%, p = 0.512) (Table 1, 2). After T₃₀, average NO level in *kucai*-treated and ISDN-treated groups reduced down to certain level at T₉₀ (Table 1, Fig. 2).

At T₉₀, compared to control group, average levels of NO was significantly higher (p = 0.018) in *kucai*-treated group; while that in ISDN-treated group was not significantly different (p = 0.436). Meanwhile compared to *kucai*-treated group, NO level in ISDN-treated group at T₉₀ were significantly lower (p = 0.028) (Table 1). It means that *kucai*-treated group serum keep remain NO levels compared to that of ISDN-treated group. *Kuca* showed longer effect up to 30 min, while ISDN showed shorter effect up to 90 min.

During the interval between T₃₀-T₉₀, average NO levels in ISDN-treated group decreased by 238.70±165.72 µM, while in *kucai*-treated group this figure was 221.26±206.34 µM. Although the decrease in ISDN-treated group almost reach the initial level, comparison between the two groups didn't showed significant difference, p = 0.899 (Table 3).

The effect of *kucai* in increasing serum NO level was presumably due to amide group in its active compound, namely, tetramethyloxamide (Fidrianny *et al.*, 2001). The mechanism of action was presumably resembles that of ISDN.

CONCLUSION

The study results suggested that ethanol extract of *kucai* bulbs increased NO level significantly in experimental rats. Therefore, it is preassumed that the antihypertensive effect shown by *kucai* was, one of which, due to its mechanism of action as vasodilator. The effect of *kucai* on the increased NO level was comparable to that of ISDN.

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