

## ORIGINAL ARTICLE

## Combination of aerobic exercise and *Hibiscus sabdariffa* Linn. increased nitric oxide in rats

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### ABSTRACT

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#### BACKGROUND

Hypertension and myocardial infarction account for the high rate of mortality globally. *Hibiscus sabdariffa* (HS) Linn. is rich in antioxidants and previous studies have demonstrated its anti-hypertensive effects. Several studies show that regular physical activity is an important component to reduce cardiovascular mortality. The objective of this study was to evaluate the effects of a combination of aerobic exercise and HS extract on nitric oxide (NO) and endothelin-1 (ET-1) in rats.

#### METHODS

An experimental study was conducted on 36 male Wistar rats, aged 4 weeks and 60-70 g in weight. The interventions were aerobic exercises and HS at 400 mg/kg BW/day administered for 4, 8 and 12 weeks. The rats were randomized into 12 groups: 3 control groups (C4, C8, C12), 3 aerobic exercise groups (A4, A8, A12), 3 HS groups (H4, H8, H12), and 3 combination groups [aerobic exercise and HS] (HA4, HA8, HA12). After 4, 8, and 12 weeks, the rats were sacrificed and their abdominal aorta was collected for determination of nitric oxide and ET-1 concentrations. One way ANOVA was used to analyze the data.

#### RESULTS

There was a significant difference in NO levels between all groups, with the 4-week aerobic exercise group (A4) showing the highest NO levels compared to the other eleven groups ( $p < 0.05$ ). In contrast, the ET-1 levels were not significantly different between all groups.

#### CONCLUSIONS

This study demonstrated that the combination of HS supplementation and aerobic exercise increases NO in rats, and provided further evidence to the traditional use of the plant as an antioxidants agent.

**Keywords:** Aerobic exercise, *Hibiscus sabdariffa* Linn., nitric oxide, endothelin-1, rats

## INTRODUCTION

Cardiovascular disease (CVD) and stroke produce immense health and economic burdens in the United States and globally. It has been projected that by the year 2030, 41.4% of US adults will have hypertension (an increase of 8.4% from 2012 estimates) and 43.9% of the US population will have some form of CVD. Cardiovascular disease is one of the three principal causes of death in America.<sup>(1)</sup>

One of the underlying pathological processes of cardiovascular disease is atherosclerosis, which is initiated by endothelial dysfunction occurring in childhood due to various risk factors. According to the study of Hong,<sup>(2)</sup> 50% of American children aged 10–14 years show initial signs of atherosclerosis as a result of oxidative stress. In the present study, to prevent endothelial dysfunction in 5-week old rats (which are the equivalent of 10-year old children),<sup>(3,4)</sup> the animals were subjected to aerobic physical exercises and *H. sabdariffa* L. administration, separately or in combination.

Regularly performed physical exercise may improve endothelial dysfunction through increased blood flow, which results in higher vascular shear stress. The latter increases the phosphorylation of endothelial NO synthase (eNOS), leading to increased eNOS activity. Therefore the bioavailability of nitric oxide (NO) is improved, so increasing NO production and inhibiting the production of endothelin-1 (ET-1).<sup>(5-7)</sup> Oxidative stress may cause a decrease in the bioavailability of NO, so causing hypertension and atherosclerosis. The decreased NO production increases binding of ET-1 to its receptors (ET<sub>A</sub>/ET<sub>B</sub>), so increasing calcium levels in vascular smooth muscle cells, resulting in vasoconstriction and ultimately hypertension.<sup>(6,7)</sup> One herb that has a strong antioxidant effect and can prevent oxidative stress is *Hibiscus sabdariffa* (HS) Linn.<sup>(8,9)</sup> Antioxidants act as electron donors, so preventing oxidation. *Hibiscus sabdariffa* Linn. contains anthocyanins that act as antioxidants.<sup>(8)</sup>

*Hibiscus sabdariffa* Linn. (Malvaceae) (common name: roselle) is used in traditional medicine as a diuretic and for treating hypertension, fever, and gastrointestinal and hepatic disorders. The fresh or dried buds, calyces, flowers, leaves, and petals of HS are also used as refreshing beverages, food items, or lotions.<sup>(10,11)</sup> The antihypertensive activity of HS could be possibly via the inhibition of angiotensin-converting enzyme activity (ACE),<sup>(11)</sup> or by incrementing the production of NO an endothelium-derived relaxation factor (EDRF).<sup>(12)</sup>

Two systematic reviews have been published on the effectiveness of HS for the treatment of hypertension, but both reviews found that the evidence available at the time of review was inconclusive.<sup>(13,14)</sup>

Nitric oxide (NO) is often recognized as an important indicator of vascular health. This compound plays an important role in blood pressure regulation due to its vasodilating potency,<sup>(15)</sup> as well as its ability to inhibit aggregation of platelets and proliferation of vascular smooth muscle cells (VSMCs).<sup>(16)</sup> Vascular smooth muscle cells participate in the pathogenesis of hypertension, and their proliferation contributes to increased peripheral resistance by decreasing arterial diameters.<sup>(17)</sup> Amongst the several VSMC growth inhibitors are nitric oxide (NO), cyclic guanosine monophosphate (cGMP), transforming growth factor-beta (TGFβ), and adenosine monophosphate activated protein kinase (AMPK).<sup>(18)</sup> A study examining the effects of a crude methanolic HS calyx extract on vascular reactivity in isolated aortas from spontaneously hypertensive rats, showed that HS increased NO in these animals.<sup>(12)</sup> However, another study showed that administration of HS did not clearly increase NO levels.<sup>(19)</sup>

One study on the administration of polyphenols from *Hibiscus sabdariffa* calyces at 125 mg/kg BW/day for 4 weeks to patients with metabolic syndrome showed a non-significant reduction in endothelin-1 levels.<sup>(20)</sup> However, no information could be obtained regarding the

effect of a combination of physical exercise and HE on nitric oxide and endothelin-1 levels in Wistar rats.

The study by Padilla et al.<sup>(21)</sup> found that the abdominal aorta in rats is more susceptible to atherosclerosis than the thoracic aorta.

The purpose of the present study was to evaluate the effect of a combination of physical exercise and HS on nitric oxide and endothelin-1 levels in rats at stages equivalent to childhood, adolescence, and adulthood in humans.

## METHODS

### Research design

The study was carried out at the Experimental Animal Laboratory, Center for Biomedical and Basic Health Technology, Agency for Health Research and Development, Ministry of Health, Republic of Indonesia, and at the Integrated Laboratory, Faculty of Medicine, University of Indonesia from July to November 2015.

### Animals

The experimental animals were 36 healthy male Wistar strain rats (*Rattus norvegicus*), 4 weeks of age and weighing around 60–70 grams. Before performing aerobic physical exercises (groups A and HA), the rats were acclimatized by running treadmills for 1 week. Before and during treatment, the rats were prevented from falling ill. The rats were fed a standard diet at portions corresponding to their age, and water ad libitum, and were kept in cages, with each cage containing 3 rats. The cages were kept clean and subjected to 12 hour light-and-dark cycles. The environmental temperature was kept at  $23 \pm 1$  °C. All other conditions were maintained in accordance with the ethics code of the commission on management and utilization of experimental animals.

The sample size was determined with the Federer formula:  $(t-1)(n-1) \geq 15$ , where  $t$  = number of experimental groups (12);  $n$  = number of animals per group. The calculated sample size was 3

animals per group, giving a total of 36 animals. The 5-week old male Wistar strain rats were randomized into 12 groups, i.e. groups 1 to 3 (4-, 8- and 12-week controls), groups 4 to 6 (aerobic physical exercise for 4, 8 and 12 weeks, respectively), groups 7 to 9 (HS at 400 mg/kgBW/day for 4, 8 and 12 weeks, respectively), groups 10 to 12 (combination of aerobic physical exercise and HS administration at 400 mg/kgBW/day for 4, 8, and 12 weeks, respectively).

### Intervention

The aerobic physical exercises were performed in treadmills at gradually increasing speeds and maintained at moderate intensities in accordance with the protocol of Hsu et al.<sup>(22)</sup> as adapted by Utami.<sup>(23)</sup> The initial speed of the aerobic physical exercises for the groups of rats aged 5, 7 and over 9 weeks was 12 m/min, 15 m/min, and 20 m/min, respectively, with each speed being maintained for 2 weeks. The frequency of aerobic physical exercise was 5 times per week, with a duration of 20 minutes and 90 seconds rest for each 5-minute run, to prevent fatigue.<sup>(23)</sup>

### Preparation of *Hibiscus sabdariffa* Linn. ethanol extract

The plant material was obtained from the Research Station for Spice and Medicinal Plants (*Balai Penelitian Tanaman Rempah dan Obat*, BALITRO). The HS calyces were dried and then ground to a degree of fineness of 3-4 mm. One kilogram of the ground sample was mixed with 5 liters of 96% ethanol for 3 hours. The mixture was shaken for 2–3 hours, then kept for 24 hours at room temperature, filtered and the filtrate dried in a Rotavapor at 50°–60°C, yielding a concentrated extract, which was stored in the refrigerator until further use. An aqueous suspension was prepared by dissolving suitable amounts of the ethanolic HS extract to obtain the desired concentration. The HS solutions were prepared freshly each time and administered by gavage using a cannulated syringe. The dosing schedule used was once per day. The HS extract was administered for 5 days per week according to group, at an oral dose of 400 mg/kgBW/day.

### Measurements and laboratory analysis

The NO levels were determined by means of a nitric oxide colorimetric assay kit (BioVision, catalog #K262-200). The kit measures total nitrites in the sample in 2 stages, i.e. conversion of nitrates into nitrites using nitrate reductase, then conversion of nitrites into dark-violet colored azo compounds using Griess reagent.

The materials and reagents used were homogenates of rat abdominal aortas, nitrate standard solution, assay buffer, nitrate reductase, enzyme cofactor, enhancer, Griess Reagent R1, and Griess Reagent R2.

Subsequently the standard or calibration curve was constructed as follows: The standard working solution was 5 mL (100mM) of standard solution mixed with 495 mL of assay buffer. For the concentration of 0 nmol/well, 85 mL of assay buffer was used. For the concentration of 2 nmol/well, 2 mL standard solution mixed with 83 mL assay buffer. For the concentration of 4 nmol/well, 4 mL standard solution mixed with 81 mL assay of buffer. For the concentration of 6 nmol/well, 6 mL standard solution mixed with 79 mL assay buffer. For the concentration of 8 nmol/well, 8 mL standard solution mixed with 77 mL assay buffer. For the concentration of 10 nmol/well, 10 mL standard solution mixed with 75 mL assay buffer.

The assay buffer to be used as blanks, at a volume of 200 mL was pipetted into the wells, then standards (0, 2, 4, 6, 8, 10) and samples at 85 mL each were pipetted into the wells, 115 mL of assay buffer was added to each well (standards and samples), then 5 mL each of nitrate reductase and enzyme cofactor were successively added to each well (standards and samples). The plate was covered and incubated at room temperature for 1 hour to convert nitrate into nitrite. Then 5 mL of enhancer was added to each well and the plate was incubated for 10 minutes (standards and samples). In addition, 50 mL of Griess reagent R1 50 mL of Griess reagent R2 were successively added to each well (standards and samples), after which the plate was incubated at room temperature for 10 minutes, and the absorbance read at the wavelength of 540 nm.

After construction of the standard curve, the values of  $a=0.1535$ ,  $b=-0.0004$ , and  $r=0.9998$  were obtained. The nitric oxide concentrations were calculated from the standard curve. The NO concentrations were divided by the protein concentrations in mg to give the protein concentrations in nmol/mg.

The ET-1 concentrations were assayed with the USCNK ELISA kit (catalog no. CEA482Ra). Specific monoclonal antibody against ET-1 was pre-coated on the microplate. A competitive inhibition reaction then occurs between biotin-labeled ET-1 (standard) or non-labeled ET-1 (sample) and the specific anti-ET-1 antibody coating on the microplate. After an incubation period, the unbound conjugate was washed away. Then HRP-conjugated avidin was added to each well and the plate again incubated. The amount of bound HRP conjugate is inversely proportional to the ET-1 concentration in the sample. After the addition of the substrate, the color intensity is inversely proportional to the ET-1 concentration in the sample.

The materials and reagents used were homogenates of rat abdominal aortas, 500 pg/mL of standard stock solution (standard mixed with 0.5 mL of standard diluent), detection reagent A, detection reagent B, wash solution, and tetramethylbenzidine (TMB) substrate.

Construction of the standard curve: For the standard solution concentration of 0 pg/mL, 3.90625 pg/mL of standard solution was used. For the standard solution concentration of 15.625 pg/mL, 300  $\mu$ L of standard solution at a concentration of 31.25 pg/mL was mixed with 0.6 mL standard diluent. For the standard solution concentration of 31.25 pg/mL, 300 mL of standard solution at a concentration of 62.5 pg/mL was mixed with 0.6 mL standard diluent. Similarly, for the standard solution concentrations of 62.5 pg/mL and 125 pg/mL, 300 mL standard solutions at concentrations of 125 pg/mL and 250 pg/mL, respectively, were each mixed with 0.6 mL standard diluent. Finally, for the standard solution concentration of 250 pg/mL, 300 mL of standard stock solution was mixed with 0.6 mL standard diluent.

Assay procedure: 50 mL volumes of standard solution at concentrations of 0; 3.90625; 15.625; 31.25; 62.5; and 125 pg/mL, were each pipetted into their respective wells. Then 50 mL of detection reagent A was rapidly added to each well, the plate covered with plate sealer and shaken using the microplate shaker and incubated for 1 hour at 37°C. Then the plate was washed 3 times, after which 100 mL of detection reagent B was added to each well and the plate incubated for 30 minutes at 37°C. After incubation, the plate was washed 5 times, then 90 mL of substrate solution was added to the wells and the plate incubated for 25-25 minutes at 37°C. Finally, 50 mL of stop solution was added to the wells. The absorbances were read as soon as possible at a wavelength of 450 nm.

After construction of the standard curve, the values of  $a=-0.4334$ ,  $b=-0.3568$ , and  $r=0.9871$  were obtained. The ET-1 concentrations were divided by the protein values in mg to obtain protein values in nmol/mg.

### Statistical analysis

Data analysis was performed by one-way ANOVA using SPSS version 21 and  $\alpha=0.05$ .

### Ethical clearance

This study received ethical clearance from the Health Research Ethics Committee, Faculty of Medicine, University of Indonesia, under no. 796/UN2.F1/ETIK/2015.

## RESULTS

### NO concentrations

A comparison of NO and ET-1 levels in the control groups (C) and treatment groups (A, H, and HA) for 4, 8, and 12 weeks is presented in Table 1. There were significant differences in NO levels between the control and treatment groups after 4 weeks and 12 weeks of intervention, but not after 8 weeks of intervention.

The ET-1 concentrations in the control groups, in the physical exercise groups, and in the HS groups tended to increase progressively,

but the increases were not significantly different. A comparison of NO and ET-1 levels in the 12 treatment groups is presented in Table 2. There were significant differences in NO but not in ET-1 concentrations between all groups.

The NO levels in the aerobic physical exercise groups (A4, A8, A12) tended to decrease with increasing duration of the physical exercises. The NO levels in the aerobic physical exercise groups were higher than those in the controls. The highest NO level was in the 4-week aerobic physical exercise group (A4), while the lowest NO level was in the control groups after 12 weeks of treatment (C12).

The results of the multiple comparison analysis showed that the groups with significant differences were C4 and A4, C4 and A8, C4 and HA8, C8 and A4, C8 and A8, C8 and HA8, C12 and A4, C12 and A8, C12 and HA8, A4 and A12, A4 and H4, A4 and H8, A4 and HA4, A4 and HA12, A8 and A12, A8 and H4, A8 and H8, A8 and HA4, A8 and HA12, A12 and HA8, H4 and HA8, HA4 and HA8 ( $p<0.05$ ) (data not presented).

## DISCUSSION

The NO levels in the control groups (C4, C8, and C12) tended to decrease with increasing age. This agrees with the results of a study carried out by Puzserova et al.<sup>(24)</sup> who explained that the aging process is closely related to a decrease in NO production and bioavailability. The NO levels in the aerobic physical exercise groups (A4, A8, and A12) tended to decrease with increasing age and duration of the physical exercises. Lantz et al.<sup>(25)</sup> and Trinity et al.<sup>(26)</sup> explained that increasing age causes a reduction in shear stress, due to greater vessel diameter.

The NO levels in the aerobic physical exercise groups were higher than those in controls. This result is in agreement with the study by Utami<sup>(23)</sup> who found higher NO levels in the aerobic physical exercise groups in comparison with the controls in juvenile rats. This is presumably because of increased eNOS mRNA

Table 1. NO and ET-1 levels after 4, 8 and 12 weeks of intervention, by treatment groups

	C	A	H	HA	P
<b>After 4 weeks</b>					
NO (nmol/mg)	0.123 ± 0.049	1.351 ± 0.771	0.169 ± 0.082	0.090 ± 0.041	0.011
ET-1 (pg/mg)	0.015 ± 0.011	0.003 ± 0.0015	0.014 ± 0.013	0.006 ± 0.009	0.453
<b>After 8 weeks</b>					
NO (nmol/mg)	0.117 ± 0.029	1.316 ± 0.807	0.465 ± 0.553	0.892 ± 0.556	0.128
ET-1 (pg/mg)	0.036 ± 0.042	0.016 ± 0.013	0.019 ± 0.028	0.017 ± 0.009	0.784
<b>After 12 weeks</b>					
NO (nmol/mg)	0.037 ± 0.006	0.163 ± 0.075	0.687 ± 0.276	0.241 ± 0.221	0.011
ET-1 (pg/mg)	0.057 ± 0.088	0.030 ± 0.034	0.035 ± 0.026	0.002 ± 0.001	0.609

Legend: C = control groups, A = aerobic exercise groups, H = *Hibiscus sabdariffa* Linn. groups, HA = groups treated with a combination of aerobic exercise and *Hibiscus sabdariffa* Linn. NO : nitric oxide; ET-1 : endothelin-1; Data are presented as mean ± SD

Table 2. NO and ET-1 levels between all control and treatment groups

	C4	A4	H4	HA4	C8	A8	H8	HA8	C12	A12	H12	HA12	P
NO levels (nmol/mg protein)	0.123 ± 0.049	1.351 ± 0.771	0.169 ± 0.082	0.090 ± 0.041	0.117 ± 0.029	1.316 ± 0.807	0.465 ± 0.553	0.892 ± 0.556	0.037 ± 0.006	0.163 ± 0.075	0.687 ± 0.276	0.241 ± 0.221	0.002
ET-1 levels (pg/mg protein)	0.011 ± 0.0015	0.003 ± 0.0015	0.014 ± 0.013	0.006 ± 0.009	0.036 ± 0.042	0.016 ± 0.013	0.019 ± 0.028	0.017 ± 0.009	0.057 ± 0.088	0.030 ± 0.034	0.085 ± 0.026	0.002 ± 0.001	0.714

Legend: C = control groups, A = aerobic exercise groups, H = *Hibiscus sabdariffa* Linn. groups, HA = groups treated with a combination of aerobic exercise and *Hibiscus sabdariffa* Linn. ; NO : nitric oxide; ET-1 : endothelin-1; Data are presented as mean ± SD.

expression in the aortas after long-term physical exercise. Consistent results were obtained in the study by Ajay et al.<sup>(12)</sup> who showed that HS administration at 0.3 mg/mL increased NO levels in the aortic ring from spontaneously hypertensive rats. These data suggests that the relaxant effects of HS involved activation of endothelium-derived nitric oxide (EDNO)/cyclic guanosine monophosphate (cGMP)-relaxant pathway activity in addition to the increased synthesis/release of EDNO.

The NO levels in the HS groups (H4, H8, and H12) tended to increase with increasing age. This was presumably due to the prevention of oxidative stress, which improved NO bioavailability, so as to increase NO production.<sup>(6,7)</sup>

The NO levels in the HS groups were higher compared to those in the controls in all age groups. However, the NO levels in groups H4 and H8, respectively, were lower as compared to those in groups A4 and A8, respectively, presumably reflecting the inadequacy of the administered HS dose. The NO levels in the groups with a combination of HS administration and aerobic physical exercises (HA4, HA8, and HA12) were found to be higher in adolescents as compared with children and adults. This is presumably due to the higher ET-1 levels in adolescents. The NO level in group HA4 was lower than that in the controls, probably because of competitive inhibition. According to this study, the ET-1 levels in the control groups tended to increase progressively, but non-significantly. This is due to the decreased NO bioavailability so as to cause increased ET-1 production from physical inactivity.<sup>(6,7)</sup>

HS extracts are generally considered to have a low degree of toxicity. Studies demonstrate that HS consumption does not adversely effect liver and kidney function at lower doses, but may be hepatotoxic at extremely high doses. In addition, electrolyte levels generally are not effected by ingesting HS extracts despite its diuretic effects.<sup>(27)</sup> HS has great potential to reduce risk factors associated with cardiovascular disease and merits further study.

The present findings explain the anti-oxidant effect of *Hibiscus sabdariffa* Linn. *in vivo*, and provide further evidence for the traditional use of the plant as an anti-oxidant agent.

## CONCLUSIONS

This study demonstrated that HS supplementation at 400 mg/kgBW and aerobic exercise increases NO concentration in rats.

## CONFLICT OF INTEREST

Competing interests: no relevant disclosures.

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## CONTRIBUTORS

DAKM, MS, and DISS contributed to the concept and design of the study, data acquisition, data analysis and/or interpretation. DAKM, MS, and DISS drafted and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.



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