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Administration of Sugar Apple Leaf Extract (*Annona Squamosa* L.) Decreased Levels of Interleukin-6 (IL-6) and Increased Levels of Endothelial Nitric Oxide Synthase (eNOS) in Serum of Male Wistar Rat Exposed to Cigarette Smoke

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ABSTRACT

Background: Cigarette smoke is a source of free radicals that are often encountered. Smoking causes a decrease in Nitric Oxide (NO) for active and passive smokers which results in various health problems including hypertension and atherosclerosis. Endothelial Nitric Oxide Synthase (eNOS) keeps blood pressure low and prevents the formation of platelets on blood vessel walls. Sugar apple leaves contain various antioxidants that can be used to counteract oxidative stress in the body by stabilizing free radicals and preventing the formation of new free radicals, including interleukin-6 (IL-6).
Methods: This research was an experimental study with pretest-posttest control group design. Twenty-six male rats (*Rattus norvegicus*), Wistar strain, 2-3 months old, weight 200-220 grams were divided randomly into two groups. The control group was given 2 cc aquadest 30 minutes before induction to cigarette smoke and the treatment group was given 350 mg/kgBB/day of sugar apple leaf extract (*Annona squamosa*) 30 minutes before induction to cigarette smoke. IL-6 and eNOS levels were measured before and after 28 days treatment using ELISA method.
Results: Control group and treatment group for eNOS levels pretest compared to posttest were 20,63 ± 2,19 and 19,23 ± 2,43 vs. 23,47 ± 1,94 and 25,06 ± 1,90, respectively and for IL-6 levels were 7,47 ± 1,70 and 7,69 ± 1,57 vs. 7,12 ± 0,86 and 6,51 ± 0,53, respectively.
Conclusion: Sugar apple (*Annona squamosa L.*) leaf extract can prevent a decrease in Endothelial Nitric Oxide Synthase (eNOS) levels and can prevent an increase in Interleukin-6 (IL-6) levels in the serum of male Wistar rats exposed to cigarette smoke.

Keywords: sugar apple leaf extract, eNOS, IL-6, cigarette smoke

INTRODUCTION

Aging is one of the physiological stages that can be felt by living things. Increasing age causes a decrease in the function of several organs of the body. With increasing age, the risk of cardiovascular disease also increases. In some individuals, the aging process has begun to be found which takes place more quickly, such as the occurrence of early vascular aging (EVA) syndrome. EVA syndrome occurs when there is stiffness of the arteries. Hypertension is a risk factor for the occurrence of the EVA process, apart from that, several studies have shown the presence of non-hemodynamic factors that cause EVA such as glucose metabolism

disorders, chronic inflammation, and oxidative stress [1].

Based on theory of aging, structural damage where the loss of organ function during aging is caused by the accumulation of oxidative damage to macromolecules (lipids, DNA and proteins) due to reactive oxygen and nitrogen species (RONS). Oxidative stress is caused by the presence of free radicals which trigger the formation of superoxide radicals or peroxinitrit which ultimately affect the emergence of various diseases [2,3].

Oxidative stress can trigger an imbalance in lipid metabolism with an immune response, causing inflammation in blood vessels. This triggers the production of proinflammatory cytokines such as Interleukin 6 (IL-6). Inflammation that occurs because Interleukin-6 is involved in the occurrence of atherosclerosis. Interleukin-6 is a proinflammatory cytokine that can be found in acute or chronic inflammation [4].

Endothelial nitric oxide synthase (eNOS) is an enzyme in the vascular endothelium that plays a role in the formation of nitric oxide (NO). Nitric oxide has an important role in relaxing the arteries and blood vessels and increasing blood pressure. During the aging process there can be an imbalance in decreased NO production or increased ROS production which causes endothelial dysfunction and can develop into hypertension or atherosclerosis. A decrease in endothelial nitric oxide synthase (eNOS) causes endothelial dysfunction so that eNOS levels can be used as a marker of vascular aging [5,6].

Cigarette smoke is a source of free radicals that are often encountered. Inhaling cigarette smoke can cause oxidative stress when the body experiences an imbalance between free radical levels and the body's ability to neutralize free radicals. Smoking is known to increase levels of free radicals which trigger DNA damage and various oxidized bases. Under conditions of oxidative stress, the total reactive oxygen species (ROS) in the body will increase. ROS are very reactive oxidants. The negative impact of ROS is that it can damage cell components which are very crucial in maintaining cell integrity due to the activity of these compounds. Cigarette smoke contains ROS which can enter the bloodstream and damage endothelial cell macromolecules. Cigarette smoke contributes to oxidative blood vessel damage thereby accelerating the vascular aging process [3,7].

Adequate and optimal consumption of antioxidants (exogenous antioxidants) can control excessive oxidative stress. Indonesia has a variety of plant natural resources which are very rich in antioxidant content. Sugar apple leaves (*Annona squamosa*) contain natural antioxidant compounds, namely flavonoids (especially quercetin and eugenol) but have not been widely used by Indonesian people as a source of natural antioxidants. Sugar apple leaves can be found in several countries such as Southeast Asia, America, Brazil, and Egypt [8].

Sugar apple leaves contain various antioxidants that can be used to counteract oxidative stress in the body by stabilizing free radicals and preventing the formation of new free radicals. However, the effectiveness of sugar apple leaf extract in suppressing oxidative stress due to exposure to cigarette smoke is not known with certainty and there has been no further research on the antioxidant activity of sugar apple leaf extract in experimental animals under conditions of oxidative stress caused by exposure to cigarette smoke through the Endothelial Nitric Oxide Synthase (eNOS) and Interleukin-6 (IL-6) indicator.

MATERIALS AND METHODS

This research is an animal experimental study with a pretest-posttest control group design. The subjects were 26 male rats Wistar strain, 2-3 months old, and weight 200-220 grams. All rats were adapted for 7 days before treatment and then divided randomly into 2 groups. The control group was given 2cc aquadest 30 minutes before

induction of cigarette smoke and the treatment group was given sugar apple leaf extract (*Annona squamosa*) 350 mg/kg/day 30 minutes before induction of cigarette smoke. Before induction of cigarette smoke the blood, samples were taken for all groups to check eNOS and IL-6 pretest levels. The treatment was carried out for 28 days. After the treatment was completed in both groups, it was continued by taking blood samples to examine eNOS and IL-6 posttest levels. After the research was completed, the rats anesthetized with ketamine/xylazine, were killed and taken to an incinerator to be burned.

In this study, eNOS and IL-6 were examined using *ELISA* method. The procedure using standard procedure written for ELISA kit. All data collected was tested for data normality with Shapiro Wilk and homogeneity test using Levene's test. Furthermore, descriptive analysis and comparative analysis will be carried out using parametrical statistic tests with paired t-test (inter group) and independent sample t-test (between group).

RESULT

*Endothelial Nitric Oxide Synthase (*eNOS)

Table 1. eNOS Comparison Before and After Treatment

Group	n	eNOS Pre (Mean±SD)	eNOS Post (Mean±SD)	P*
Control	13	20,63±2,19	23,47±1,94	0,003
Treatment	13	19,23±2,43	25,06±1,90	< 0,001
P**		0,136	0,046	

p* = uji t-paired; p = uji t-independent**

The result of this study (Table 1) showed that before the treatment, the average of eNOS level in both the control group and treatment group were insignificantly different (20,63±2,19 and 19,23±2,43 respectively, p=0,136). After being treated for 28 days, the average eNOS level in the treatment group increased significantly (p<0,001) became 25,06±1,90 and the control group also increased significantly (p=0,003) became 23,47±1,94. The average of eNOS level after treatment in both the control group and treatment group significantly different (23,47±1,94 and 25,06±1,90 respectively, p=0,046).

The graph of the eNOS level inter-group comparison is shown in Figure 1.

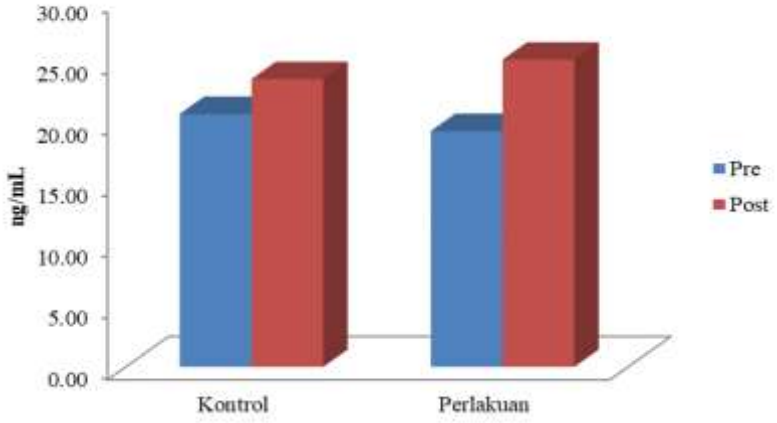

Figure 1. eNOS level inter-group comparison

Interleukin 6 *(*IL-6)

Table 2. IL-6 Comparison Before and After Treatment

Group	n	IL-6 Pre (Mean±SD)	IL-6 Post (Mean±SD)	P*
Control	13	7,47±1,70	7,12±0,86	0,545
Treatment	13	7,69±1,57	6,51±0,53	0,023
P**		0,743	0,039	

p* = uji t-paired; p = uji t-independent**

The results (Table 2) showed that before the treatment, the average of IL-6 level in both the control group and treatment group were insignificantly different (7,47±1,70 and 7,69±1,57 respectively, p=0,743). After being treated for 28 days, the average IL-6

level in the treatment group decreased significantly (p=0,023) became 6,51±0,53 and the control group was insignificantly different (p=0,545) became 7,12±0,86. The average of IL-6 level after treatment in both the control group and treatment group significantly different (7,12±0,86 and 6,51±0,53 respectively, p=0,039).

The graph of the IL-6 level inter-group comparison is shown in Figure 2.

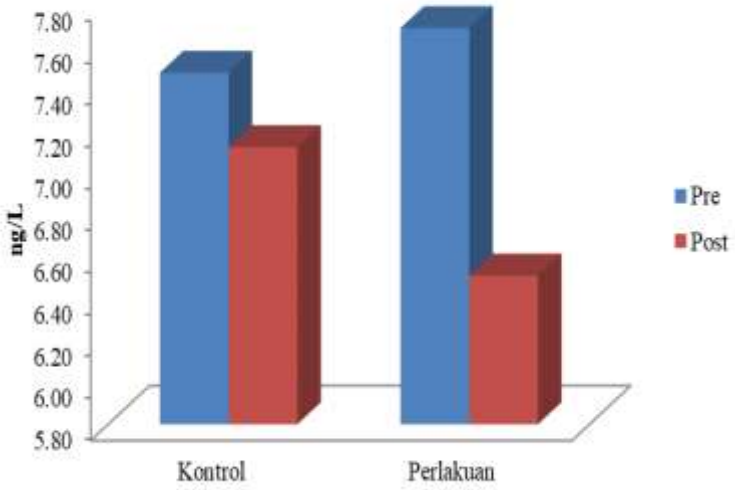

Figure 2. IL-6 level inter-group comparison

DISCUSSION

Effect of exposure to cigarette smoke on eNOS and IL-6

In this study, rats were exposed to cigarette smoke as much as 2 cigarettes per day for 28 days, causing oxidative stress which triggers atherosclerosis. Cigarette smoke can also trigger cell inflammation so that free radicals are formed indirectly in the body where the amount of oxidants in the body is higher than the amount of available antioxidants[9]. Several previous studies have shown that exposure to cigarette smoke can increase ROS production, causing oxidative stress [10].

Cigarette smoke contains a variety of free radicals that can cause damage to tissue structures associated with inflammatory responses. The presence of Reactive Oxygen Species (ROS) in cigarette smoke and also phenol-rich glycoproteins which provide a direct stimulus to macrophages thereby triggering the production of proinflammatory cytokines, one of which is interleukin-6 (IL-6)[11]. Interleukin-6 is regulated in several tissues which has an important role in increasing atherogenesis in aging. Therefore, IL-6 blockade is effective in treating atherosclerosis in aging[12].

Oxidative stress triggered by cigarette smoke can also cause vascular endothelial dysfunction. Reactive oxygen species (ROS) cause a decrease in the bioavailability of NO through the oxidation of BH4, causing the release of uncoupling eNOS. eNOS uncoupling causes a decrease in NO bioavailability[13]. One of the functions of NO is to regulate vascular smooth muscle tone, when a disturbance occurs it can cause endothelial dysfunction. Endothelial dysfunction is an early stage of atherosclerosis and cardiovascular disease. With the improvement of the endothelium, it is hoped that it can prevent cardiovascular disease[14].

Based on the theoretical basis above, this research is appropriate where it shows that exposure to cigarette smoke can reduce eNOS levels and increase IL-6 levels.

Effect of Sugar Apple Leaf Extract on eNOS and IL-6

In the treatment group, the mean posttest eNOS levels were higher than the control group. From the comparability test it can be concluded that administration of sugar apple leaf extract can increase eNOS levels and decrease IL-6 levels. This analysis is supported by the results of the posttest comparability analysis where there is a significant difference between the control group and the treatment group. In the treatment group, an increase in eNOS levels and a decrease in IL-6 levels were inhibited by giving sugar apple leaf extract at a dose of 350 mg/kgBW/day.

This study showed that for the treatment group there was a significant decrease between the pretest and posttest of IL-6 levels (p=0.023). From these results, it can be concluded that administration of sugar apple leaf extract for 28 days not only inhibited the increase in IL-6 levels but could even reduce IL-6 levels. The treatment group also showed an increase in the mean eNOS levels (pretest and posttest) after 28 days of treatment. In the treatment group, eNOS levels showed a significant increase (p<0.001). So from these results it can be concluded that ethanol extract of sugar apple leaves can inhibit endothelial dysfunction which is part of the vascular aging process.

Result of this study is along with research from Hendawy et al. which stated that the administration of sugar apple leaves ethanol extract at a dose of 300 mg/kg BW proved to have an anti-inflammatory effect in examining the brains of rats that experienced neuroinflammation and had an effect on reducing the occurrence of apoptosis in nerve cells[15].

Another study that is along with this research is Safira et al. which stated that sugar apple leaves can reduce atherogenic risk factors associated with cardiovascular disease such as inflammation, oxidative stress, total cholesterol, triglycerides and LDL[16].

While research from Kumar et al. stated that the ethanol extract of sugar apple leaves at a dose of 350 mg/kg in Wistar rats with diabetes could reduce total cholesterol, LDL and triglyceride levels. The lipid profile, as it is known, can reduce the risk factors for atherosclerosis. So far, researchers have not found research on sugar apple leaves having an antioxidant effect on eNOS levels by exposure to cigarette smoke in rats[8].

Based on the theory that free radicals cause an increase in ROS production so that they can also induce the formation of eNOS uncoupling. Flavonoids inhibit oxidative stress by regulating the balance of oxidants and antioxidants. Bioflavonoids work as hydrogen or electron reducers which inhibit or reduce the toxicity of free radicals[17]. Free radicals from cigarette smoke can cause eNOS levels to decrease but in this study, it can be seen in the control group's average eNOS level pretest 20.63 ± 2.19 and posttest 23.47 ± 1.94 with p = 0.003 where there is no decrease in eNOS levels. numbers and the number posttest treatment group with a value of p = 0.049. This can be caused by the frequency and intensity of exposure, the type of cigarette used, the length of time of exposure, the type of animal strain, the age of the experimental animal, and many other factors that can affect research results, such as in the study of Hartono et al. stated that giving certain doses of ciplukan leaf extract to male Wistar strain rats exposed to cigarette smoke for 30 minutes per day for 28 days decreased eNOS levels in the control group[18]. Each cigarette takes 6 minutes to burn completely[19]. So that 30 minutes of giving the number of cigarettes given is about 5 cigarettes per day.

Free radicals from cigarette smoke can cause direct stimulation of macrophages thereby triggering the production of proinflammatory cytokines, one of which is interleukin-6 (IL-6). In this study, it could be seen in the mean IL-6 level in the control group pretest 7.47 ± 1.70 and posttest 7.12 ± 0.86 with p = 0.545 where there was no difference between before and after

treatment. This can be caused by the type of animal strain, the frequency and intensity of exposure, the length of time of exposure, the type of cigarette used, the age of the experimental animals, and many other factors that can affect research results, such as in the study of Kennedy-Feitosa et al. stated that giving eucalyptol to C578BL/6 rats exposed to 12 cigarette smoke per day for 5 days resulted in an increase in IL-6 levels in the control group[19]. Meanwhile, in the study by Mohammadtursun et al. stated that giving loki zupa to male sprague-dawley rats exposed to cigarette smoke for 24 weeks resulted in an increase in IL-6 levels in the control group[20].

In the results of this study, administration of sugar apple leaf extract increased eNOS and decreased IL-6. Results that were not significant in the control group could be due to the frequency and intensity of exposure which required more and longer exposure times.

Benefits of Sugar apple Leaf Extract (*Annona squamosa*) as an Anti-Aging Medicine

The content of bioactive compounds in sugar apple leaf extract as an anti-aging medicine can be seen from the results of the phytochemical examination which contained flavonoids 546.81 mg QE/100mL, tannins 1402.03 mg TAE/100mL, phenol 7335.72 mg GAE/100mL, antioxidant capacity 2539, 68 mg GAEAC/L and IC50 78.80 ppm so they have antioxidant and anti-inflammatory effects. In previous research Hartono *et al*. used ciplukan leaf extract which contained 1804.68 mg/100g of flavonoids, 3010.83 mg/100g TAE of tannins, 3589 mg/100g of GAEA phenols and 1431.02 mg/L of GAEAC antioxidant capacity which was proven to be able to prevent a decrease in eNOS levels and research Nurzalia *et al* which contains 168 mg/100 g of flavonoids has been shown to be able to prevent increased levels of IL-6[18,21].

From the theory explained by giving sugar apple leaf extract can neutralize ROS from exposure to cigarette smoke so as to prevent oxidative stress. When compared to the results of other phytochemicals that have been studied which have proven to have the potential to prevent oxidative stress, the content of sugar apple leaves can be an antioxidant that functions to prevent aging due to oxidative stress from exposure to cigarette smoke in experimental animals. This proves that sugar apple leaf extract has a role in Anti-Aging Medicine by preventing and inhibiting aging thereby improving the quality of human life.

CONCLUSION

Sugar apple leaf extract (*Annona squamosa*) can prevent an increase in Interleukin-6 (IL-6) and a decrease in Endothelial Nitric Oxide Synthase (eNOS) levels in the serum of male Wistar rats exposed to cigarette smoke. A similar study is needed with a longer time span and more exposure intensity to see the significance of the antioxidant and anti-inflammatory effects of sugar apple leaf extract in male Wistar rats exposed to cigarette smoke.

AUTHOR CONTRIBUTION

All authors have the same contribution in writing the report on the results of this study, from the stage of proposal preparation, data search, and data analysis, to the interpretation of research data and presentation of the final report.

Declaration by Authors

Ethical Approval: This research has been approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, Universitas Udayana with number B/14/UN14.2.9/PT.01.04/2023.

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Conflict of Interest: The authors declare no conflict of interest.

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