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Effect of white tea (*Camellia sinensis*) leaf extract on cigarette smoke and high-fat diet-induced atherosclerosis in Wistar rats

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ABSTRACT

Atherosclerosis is a complex pathological process begins with endothelial dysfunction, one of which is triggered by an inflammatory process due to increased levels of low-density lipoprotein (LDL) cholesterol and exposure to cigarette smoke. White tea leaf (*Camellia sinensis*) shows an anti-inflammatory effect which has not been known for its effect on the atherosclerotic process. The aim of this study is to determine the effect of *Camellia sinensis* leaf extract on IL-6 levels, foam cell count, and the ratio of intima-media thickness of cigarette smoke and high-fat diet-induced atherosclerosis in Wistar rats. Thirty male Wistar rats were randomly divided into 5 groups namely HC (healthy control), NC (negative control), WT100, WT200, and WT400 (treated with white tea leaf extract 100, 200, and 400 mg/kgBW/day, respectively). IL-6 levels were measured by the ELISA method. At 400x microscope magnification, foam cell count and intima-media thickness ratio were seen in aortic tissue. Administration of graded doses of white tea leaf extract in groups WT100, WT200, WT400 significantly reduced IL-6 levels, foam cell count, and intima-media thickness ratio of abdominal aorta compared to the NC group. These results suggest that white tea leaf extract may decrease levels of IL-6, foam cell count, and intima-media thickness ratio in atherosclerosis-induced Wistar rats.

INTRODUCTION

Atherosclerosis is a complicated pathological process that primarily results from cholesterol buildup, endothelial dysfunction, infiltration of inflammatory cells, and migration of vascular smooth muscle cells [1]. It is characterized by structural abnormalities in the intima and media arteries. Certain factors or behaviors, such as high levels of low-density lipoprotein (LDL) cholesterol, low blood levels of high-density lipoprotein (HDL), hypertension, smoking, diabetes mellitus, obesity, and sedentary lifestyles that increase the risk of vascular endothelial dysfunction and atherosclerosis, may increase the likelihood of developing atherosclerosis [2]. This increases the risk of stroke, myocardial infarction, and other cardiovascular diseases.

One of the key risk factors for the early initiation of atherosclerosis, which starts the inflammatory reactions in the microvascular system, is hypercholesterolemia and exposure to cigarette smoke [3–5]. The release of pro-inflammatory cytokines like interleukin-6 (IL-6) is linked to abnormalities in blood flow and endothelial cell stretching. Vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) expression rises in response to IL-6 binding to its receptors [6]. Monocytes are drawn in by the expression of VCAM and ICAM-1, and they later

develop into macrophages in the subendothelial layer where they capture oxidized LDL to create foam cells [3,7,8].

There are two methods for preventing atherosclerosis: non-pharmacological and pharmacological. There is still a need for an alternate method of preventing progressive atherosclerosis because statins, which were once the first line of defense in the prevention of atherosclerosis, have side effects with long-term use, including myalgia, rhabdomyolysis, and an increase in liver enzymes [9]. Research on herbal medicines as the prevention of atherosclerosis is still being studied [10,11]. *Camellia sinensis (L.)*, the plant that produces tea, is one of the most widely used herbs in the area. The type of tea leaf with the highest catechin content is white tea [12]. After harvest, white tea leaf are quickly evaporated to stop the enzyme polyphenol oxidase, which can eliminate catechins [13].

Various bioactive substances, including polyphenols, caffeine, theogallin, gallic acid, theaflavin, flavanol glycosides, and catechins, particularly epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG), are present in white tea [14]. Previous studies demonstrated that the administration of EGCG, a Nrf2 activator, significantly decreased the development of atherosclerotic lesions, the regulation of inflammatory cytokines IL-6 and TNF-α, and total LDL cholesterol levels in ApoE KO mice model fed a high-fat diet [15].

Several studies have been conducted to determine the effect of white tea extract on lipid profiles, but to date, no studies have explicitly linked the levels of IL-6, foam cell count, and the ratio of intimal tunica thickness to media abdominal aorta thickness in atherosclerosis. The purpose of this research was to examine the anti-inflammatory effects of white tea in rats exposed to cigarette smoke and high-fat diets.

MATERIALS AND METHODS

This research was an experimental study with a post-test only group design. The study was conducted at the Animal Experiment Laboratory, Traditional Medicine Laboratory, and Basic Biomedical Laboratory at Faculty of Medicine, Diponegoro University.

Experimental animals

This investigation utilized thirty male *Rattus Novergicus* rats, weighing 180–220 grams and aged 2-3 months, from the Faculty of Animal and Agricultural Sciences at Diponegoro University. For seven days, rats adjusted to their environment, were fed and drank as necessary. To induce atherosclerosis, rats were exposed to cigarettes and given a high-fat diet. Rats divided into 5 groups with 6 rats each, namely healthy control (HC), normal rats received cigarette smoke exposure and high-fat diet as a negative control (NC) for 1 week, and then atherosclerosis-induced rats groups (WT100, WT200 and WT400) received white tea leaf extract at 100, 200, and 400 mg/kgBW/day, respectively for 56 days [16]. A digital scale was used to weigh each group. Rats were sacrificed in order to obtain blood and abdominal aorta tissue. The activities of this investigation were carried out in facilities that met the standards for human and animal health research. The subject's ethics review panel approved using animals in experiments (No. 124/EC/H/FK-UNDIP/XI/2022).

Administration of high-fat diets and cigarette smoke

To induce atherosclerosis, rats were exposed to cigarettes and given a high-fat diet. Each group of six rats was housed in an identical cage. The cages are kept in an animal room under controlled condition of stable humidity (50%), temperature (23-26^{o}C) with a 12/12 h light and dark cycle. Each group is exposed to the cigarettes one by one in a different room. On the side, there is a hole for inserting a conventional cigarette, and on the top of the smoking box, there are openings for smoke to escape. When one end of the cigarette burns, the other end is sprayed and the syringe is repeatedly drawn out, allowing cigarette smoke to be injected repeatedly into the box until one of the cigarettes burns out. In this investigation, the treatment consisted of daily exposure to cigarette smoke from two cigarettes. The amount is equivalent to 1.38 mg of nicotine and 23.7 mg of tar for inducing an inflammatory response when quantities exceed the permissible levels of nicotine and tar [17]. After the exposure was done, the cage was placed back in the animal laboratory.

The high-fat diet contains standard foods in addition to egg yolk. The human dose of egg yolk was 110 mg per day. The daily dosage of yellow eggs for Wistar rats is 2 g/200 gBW after conversion. The egg yolk is separated from the egg white, taken using oral gavage and then given directly to the rats. Rats are also provided with water and libitum.

Administration of white tea leaf extract

The main ingredients used were white tea from the Research Institute for Tea and Cinchona (RITC), Gambung, West Java, Indonesia. Using a processor, dry white tea leaves are ground into a powder. Simplicity powder is immersed in a 96% ethanol solution containing a 1:1 ratio of simplisia powder and solvent for three cycles of 24h with six-hourly mixing. The maseration product is then filtered. Using a rotary evaporator, the filter produced by maseration is separated from its solvent until it thickens. The white tea leaf maseration extract is then diluted with 1 mL of aquades, squirted into the mouth using a 1 mL syringe, and administered orally with a gavage needle.

Blood levels of IL-6 analysis

On the 57th day [18], blood samples are taken through the medial canthus sinus orbitalis using a syringe. A blood sample is inserted into a tube containing the anticoagulant EDTA. The sample is centrifugated for 10 minutes at a speed of 3000 rpm at a temperature of 40 C, and then the serum is taken. Enzyme-linked immunosorbent assay (ELISA) kits (Elabscience®, Texas) are used to test IL-6 levels according to the manufacturer's recommendations. The ELISA reader is from Agilent Technologies, Inc (ELx800) United States.

Foam cells and intima-media thickness ratio analysis

Using 10% buffered formalin, tissue samples from the abdominal aorta were fixed. After the material was fixed it was cleaned with running water to create 5 m-thick paraffin slabs. A section of abdominal aorta was stained with Hematoxylin-Eosin (HE). The foam cell count and intima-media thickness ratio were quantified utilizing an Olympus CH20 microscope (Evident Scientific, Japan) with a 400x magnification. A certified pathologist carried out the preparation and histological examination.

Statistical analysis

The primary data obtained was tested for data normality with the Saphiro-Wilk test to see the data distribution spread and the Levene's test for data homogeneity. When the data is normally distributed, a one-way ANOVA test is performed to analyze the differences between groups. If the data is not distributed normally, a Kruskal Wallis test will be performed. If there are meaningful differences, it will be continued with the Tukey's post-hoc test for the test of the differences of 2 groups. A difference test is considered significant when the p value is < 0.05 with a 95% confidence interval.

RESULTS

Effect of white tea leaf extract on body weight

The Shapiro-Wilk normality test for body weight revealed significance in all group ($p>0.05$), indicating that the data is of normal distribution (Figure 1). The one-way ANOVA test showed a $p = 0.072$ ($p>0.05$) and the Levene's test result is significance with $p=0.47$ ($p>0.05$), indicating that the weight measurement findings do not differ significantly, indicating that the data variants are homogeneous.

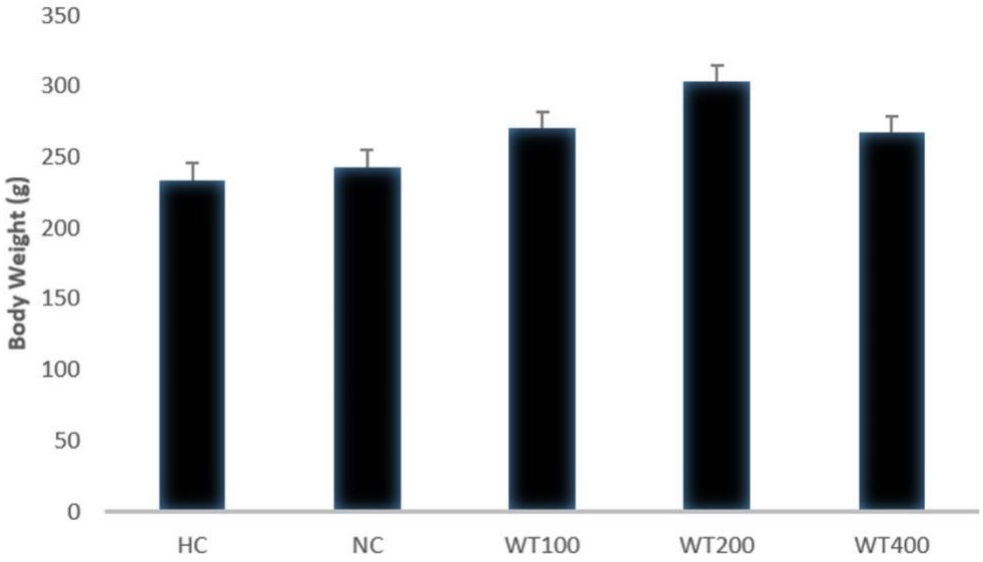

Figure 1. Effect of white tea leaf extract on body weight in rats. According to the results of a one-way ANOVA test, there was no significant difference in body weight between groups. HC (healthy control), NC (negative control), WT100, WT200, and WT400 (white tea leaf extract 100/200/400 mg/kgBW/day), *n*=6.

Effect of white tea leaf extract on blood IL-6 levels

The results of Shapiro-Wilk test for blood IL-6 levels demonstrated significance of $p> 0.05$ in all groups, so normal distribution of data can be concluded. The Levene test result was $p=0.477$ ($p>0.05$) and the one-way ANOVA test result was $p<0.05$, indicating that the data variants were homogeneous, and the measurement findings of IL-6 levels differed significantly. A Tukey's post-hoc test was used to determine which groups were statistically different. The results in Figure 2 showed significant differences in IL-6 levels in the WT100, WT200 and WT400 groups against the NC group.

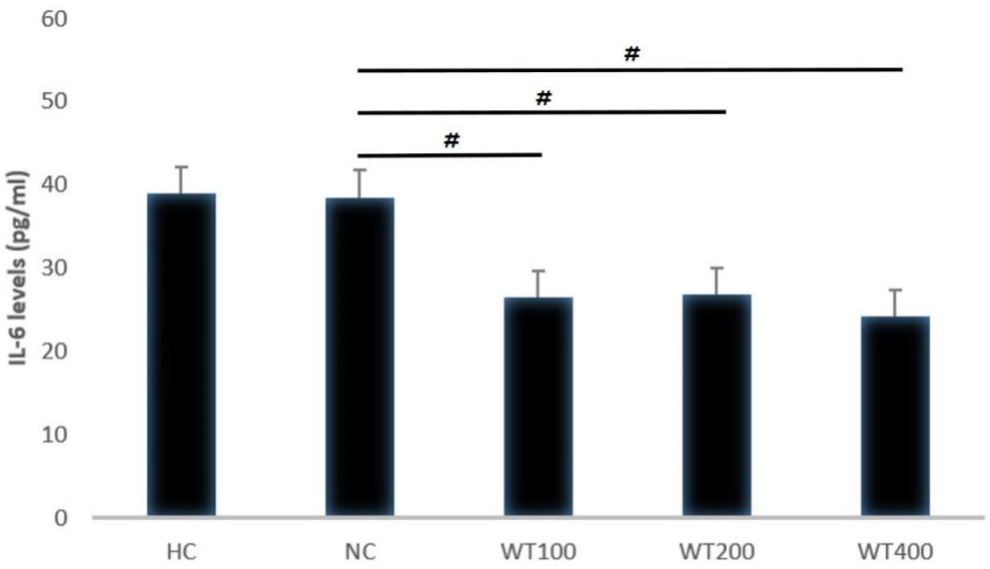

Figure 2. Effect of white tea leaf extract on blood IL-6 levels in rats. The chart shows the results of the post-hoc test, which indicated significant differences in IL-6 levels between groups. HC (healthy control), NC (negative control), WT100, WT200, and WT400 (white tea leaf extract 100/200/400 mg/kgBW/day), *n*=6. # = significant ($p < 0,05$).

Effect of white tea leaf extract on foam cell count

According to Shapiro Wilk test for the foam cell count, the outcomes for all groups were significant ($p>0.05$). This demonstrates that the data had a normal distribution. The one-way ANOVA test showed $p=0.001$ ($p<0.05$), and the Levene's test result was $p=0.477$ ($p>0.05$), indicating that the measurement results of IL-6 levels differed significantly, and data variants were homogeneous. Post-hoc analysis in Figure 3 revealed significant differences in IL-6 levels between the WT400 group and the NC group. Figure 4 showed the foam cell on tunica intima based on histological assessment in the experimental rats.

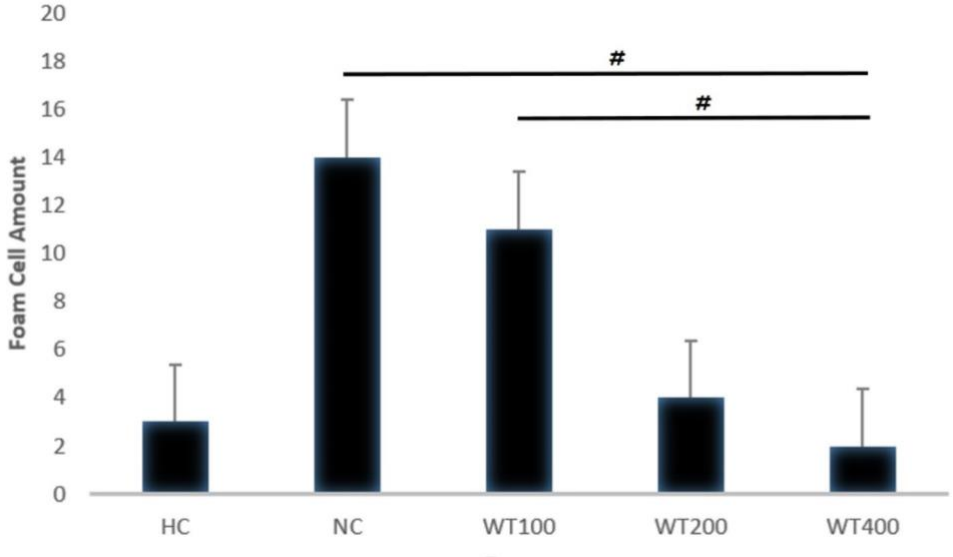

Figure 3. The effect of white tea leaf extract on foam cell count in rats. The chart shows the results of the post-hoc test, which indicated significant differences in foam cell count between groups. HC (healthy control), NC (negative control), WT100, WT200, and WT400 (white tea leaf extract 100/200/400 mg/kgBW/day), *n*=6. # = significant ($p < 0.05$).

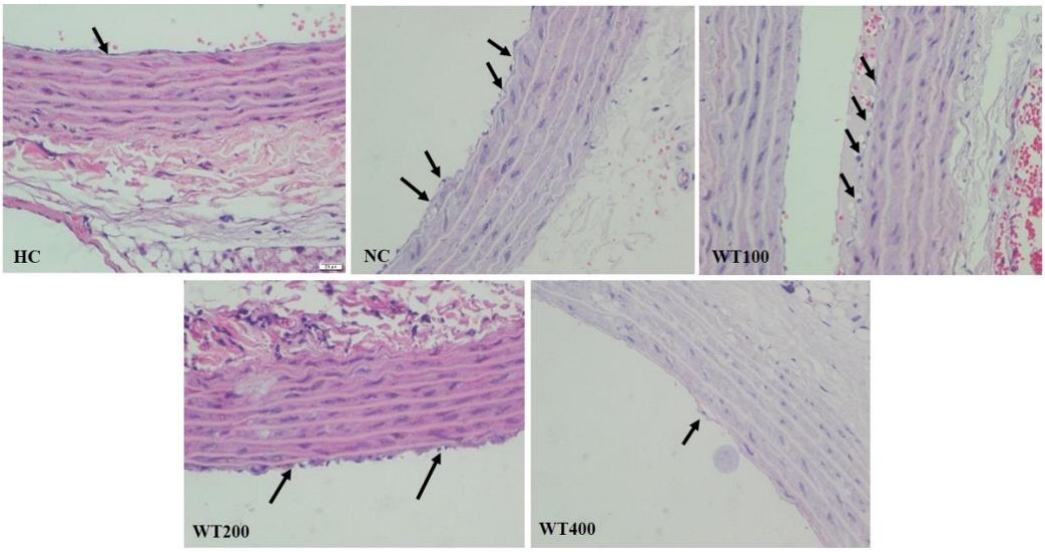

Figure 4. Effect of white tea leaf extract on abdominal aorta (400x magnification). Abdominal aorta cross slice displaying tunica intima foam cells (black arrow). HC (healthy control), NC (negative control), WT100, WT200, and WT400 (white tea leaf extract 100, 200, 400 mg/kgBW/day), *n*=6.

Effect of white tea leaf extract on intima-media thickness ratio

In one of the groups, the Shapiro-Wilk test showed $p<0.05$, indicating an abnormal distribution of data. The Kruskal-Wallis test for the intima-media thickness ratio resulted in a $p = 0.001$ ($p<0.05$), indicating that the intima-media thickness ratio was significantly different (Figure 5). The intima-media thickness ratio was markedly different in WT200 and WT400 groups against the NC group, as determined by the Tukey's post-hoc test in Figure 5.

In addition, a Kruskal Wallis test was conducted to determine the effect of white tea extract on the tunica intima and media thickness separately (Figure 6). The results of the test showed a $p = 0{,}001$ ($p<0.05$), so it could be concluded that the thickness of the tunica intima differs significantly, while there was no significant difference in the tunica media thickness with $p = 0{,}605$ ($p>0.05$). The results of the post-hoc trial in Figure 6 showed that the thickness of the tunica intima was significantly different in WT400 group against NC group and WT100 group, also WT200 group against NC group.

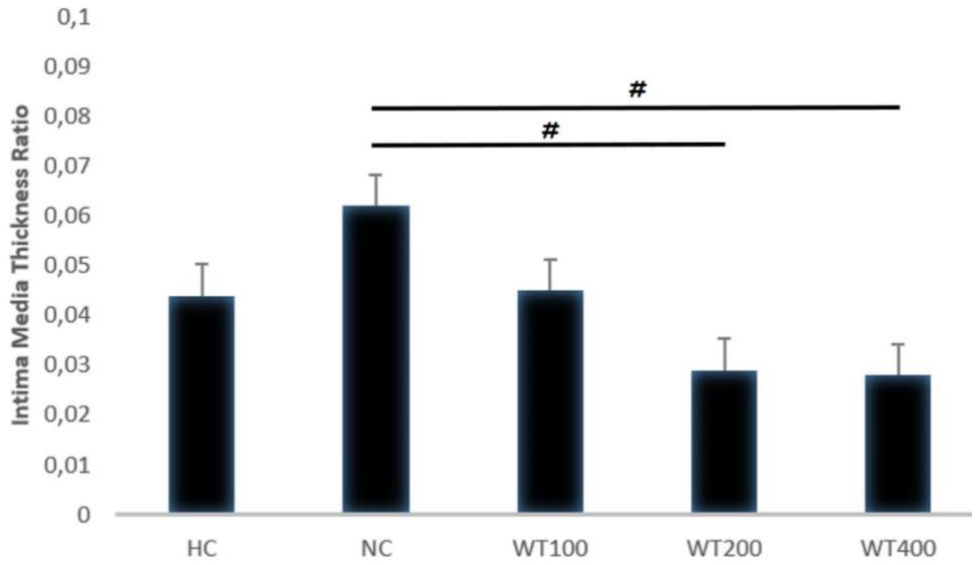

Figure 5. Effect of white tea leaf extract on the intima-media thickness ratio in rats. The chart shows the results of the post-hoc test, which indicated significant differences in intima-media thickness ratio between groups. HC (healthy control), NC (negative control), WT100, WT200, and WT400 (white tea leaf extract 100/200/400 mg/kgBW/day), *n*=6. # = significant ($p < 0.05$).

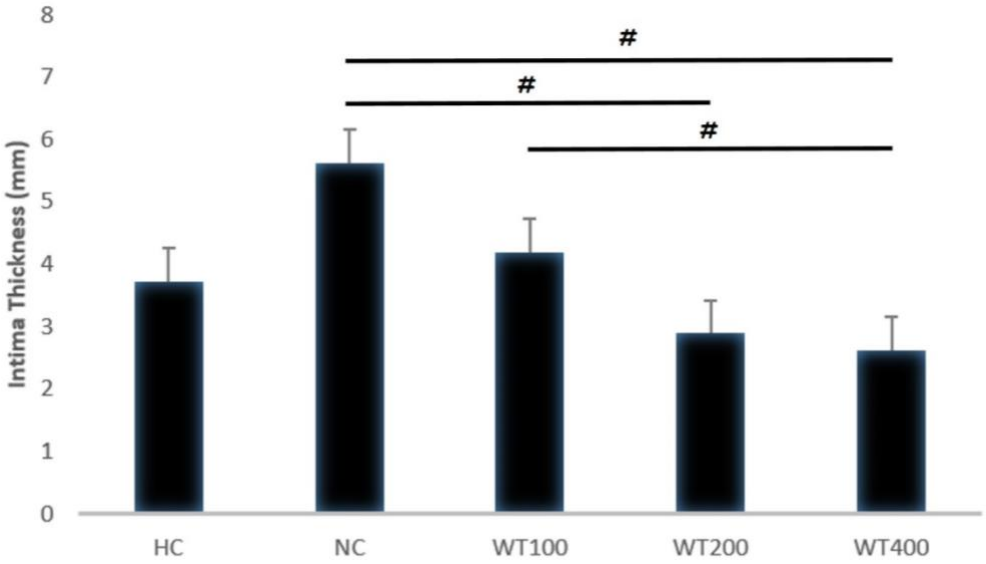

Figure 6. Effect of white tea leaf extract on the thickness of tunica intima in rats. The chart shows the results of the post-hoc test, which indicated significant differences in the thickness of tunica intima between groups. HC (healthy control), NC (negative control), WT100, WT200, and WT400 (white tea leaf extract 100/200/400 mg/kgBW/day), and *n*=6. # = significant ($p < 0.05$).

Correlation between IL-6 levels, foam cell count, and intima-media thickness ratio

There was a significant relationship ($p<0.0001$) between foam cells count and intima-media thickness ratio in atherosclerosis-induced rats according to Pearson correlation test (Figure 7C). The Pearson correlation test showed $p=0.618$, indicating a strong positive relationship. While there was no significant relationship between foam cells count and IL-6 levels, and relationship between intima-media thickness and IL-6 levels (Figure 7A-B).

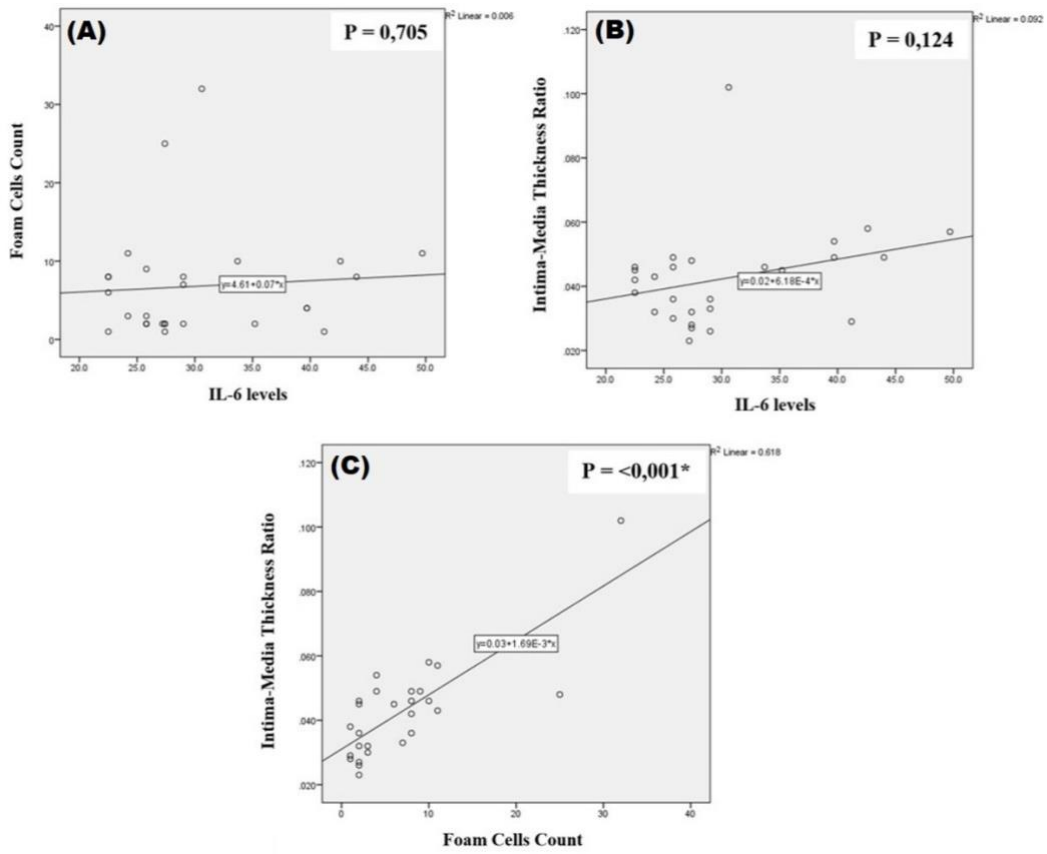

Figure 7. Charts show the correlation between variables (A) IL-6 levels and foam cell count, (B) IL-6 levels and intima-media thickness ratio, (C) foam cells count and intima-media thickness ratio. *=significant.

DISCUSSION

The health benefits of tea are numerous [19,20]. Flavonoids, theaflavin, thehanine, alkaloids, and polysaccharides are only a few of the compounds linked to tea's pharmacological advantages [21]. How the tea leaves are processed is a key factor in determining the content of tea. White tea has more polyphenols than other forms of tea, according to prior studies. White tea contains more polyphenols than green and black tea, including gallic acid [22], some catechins [23], some flavanol glycosides, and flavone glycosides [24]. White tea contains a large class of polyphenols known as catechins, which have been linked to antioxidant effects in numerous studies [25,26]. White tea has larger quantities of the catechins EGC and EGCG than other forms of tea [27].

Increased LDL cholesterol and blood glucose levels promote endothelial dysfunction, which leads to atherosclerosis [28,29]. Endothelial cell injury induces macrophages to secrete inflammatory cytokines such as TNF-α, IL-1, and IL-6 that promote local inflammation [29]. CRP has been proven to release inflammatory mediators when cholesterol levels rise [30]. CRP may exacerbate vascular dysfunction by blocking eNOS and increasing reactive oxygen species (ROS) generation via IL-6 [31]. Cigarette smoke also produces a considerable amount of ROS, which leads to the rapid inactivation of nitric oxide (NO) and the creation of peroxynitrites, lowering NO's biological activity [32]. Through ROS, cigarette smoke triggers the NLRP3 inflammasome, causing the release of IL-6, IL-1b, IL-18, and other inflammatory mediators that cause endothelial cell death [33].

This study revealed that gradual administration of white tea extract to rats with atherosclerosis did not result in a significant weight difference. The weight of post-intervention rats in HC group through group WT200 increased, except in WT300 group which received the highest dose of white tea extract. These findings suggest that white tea can promote weight loss at specific dosages. White tea's mechanism of weight loss can be ascribed to a variety of variables. Tea inhibits catechol-o-methyltransferase activity as a result of increased oxygen intake caused by sympathetic nerve thermogenesis [34,35]. Catechins reduce serum lipid levels and glucose absorption by inhibiting the formation of intestinal muscles in the small intestine and the activity of alpha-glucosidase in animals [36]. Diets containing catechins inhibit the accumulation and synthesis of triglycerides in 3T3-L1 cells [37].

Transcription factor such as NF-κB is activated during the progression of atherosclerosis [38]. NF-κB activation has been documented in rats with high-fat or high-cholesterol diet-induced atherosclerosis [39,40]. In our study, the progressive administration of 100, 200, and 400 mg/kgBW/day of white tea extract led to a decrease in the levels of IL-6. The levels of IL-6 were significantly lower in WT100, WT200, and WT400 groups compared to NC group. The anti-inflammatory mechanism of polyphenols involves by disabling the NF-κB pathway and a decrease in neutrophil and macrophage infiltration [41]. Decreased macrophage infiltration leads to a decrease in the amount of inflammatory cytokines secreted. A previous study indicates that white tea extract inhibits the secretion of IL-6, IL-8, and CCL-5 by oral epithelial cells stimulated with 10 lg/mL of *P. gingivalis* extract [42]. Studies in vivo and in vitro [43,44] have demonstrated that catechins, theaflavin, and thearubigine are anti-inflammatory components of tea [45].

In the presence of hypercholesterolemia and cigarette smoke, monocytes infiltrate the subendothelial space and differentiate into macrophages that oxidize LDL and cholesterol, resulting in foam cell formation and atherogenesis [46]. The progressive administration of white tea extract resulted in a significant reduction of foam cell count in Wistar rats with atherosclerosis. The ability of catechins to prevent foam cell formation is by decreasing levels of free radicals and total cholesterol [44]. Catechin act as free radical scavenger by rapid donation of a hydrogen atom to radicals. Decreased total cholesterol levels by catechin is demonstrated by reducing the process of cholesterol biosynthesis, specifically by decreasing the activity of the key enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) [47,48]. Flavonoids have the capacity to alter lipoprotein metabolism by increasing LDL absorption via LDL receptor upregulation [47]. In addition, the activity of lecethin-choleterol acyltransferase (LCAT) increases, which can ameliorate hypercholesterolemia. Flavonoids can reduce the requirement for NADPH in cholesterol and fatty acid synthesis [48].

Atherosclerosis is a chronic inflammatory disease associated with elevated oxidative stress on the endothelium of the blood vessels [49]. VCAM-1 was found to be elevated in the serum of patients with premature atherosclerosis and was expressed in vascular lesions associated with early atherosclerosis. VCAM-1 is the primary mediator of mononuclear cell adhesion to arterial walls. White tea can prevent ROS production which are involved in regulation of adhesive molecular expression [50]. A reduction in adhesive molecular expression will reduce the recruitment of monocyte cells, resulting in a decrease in foam cell formation.

As the thickness of the intima-media tunica correlates with risk factors, it can serve as a standard for identifying atherosclerosis. The nicotine in cigarette smoke directly induces the transformation of vascular smooth muscle cells (VSMC) [51]. This promotes

VSMC migration from the media tunica to the atheromatous plaque in the vascular intima tunica. As a result of degeneration and atherosclerosis development, the intimal layer thickens and the media thins [52]. Consistently, patients with hypertension or hyperlipidemia showed larger intima layers and a higher intima/media thickness ratio than patients in the control group ($P < 0.00005$ and $P < 0.05$, respectively) [53].

This study revealed that progressive administration of white tea extract doses significantly decreased the intima-media thickness ratio of abdominal aorta in Wistar rats with atherosclerosis ($p = 0.001$). The correlation test revealed a robust positive relationship between foam cell count and intima-media thickness ratio (p 0.0001; $r = 0.786$). White tea extract can substantially reduce the thickness of the intima tunica ($p = 0.001$) when measuring the intima and medium tunica separately. The mechanism by which white tea extract reduces intima thickness is that EGCG in white tea can induce the release of NO and decrease endothelin-1 secretion in endothelial cells [48]. This results in decreased in VCAM-1 and ICAM-1 expression and reduced monocyte infiltration in the intima [54]. EGCG increases the bioavailability of NO by decreasing the levels of asymmetric dimethylarginine, an endogenous NO inhibitor [7]. EGCG can reduce the intima thickness by activating Nrf2/Caspase-3 signaling, resulting in inhibition of angiotensin II (Ang II) [54]. Ang II induces the phenotypic transformation of VSMC from contractile to synthetic proliferative cells, which causes the proliferation and migration of VSMC [55]. Inhibition of Ang II, thereby preventing the proliferation and migration of VSMC toward the tunica intima.

This study determines that the administration of white tea leaf extract inhibits atherosclerosis progression, as assessed by blood IL-6 levels, the number of foam cells, and the intima-media thickness ratio. In comparison to the other extract-treated groups, the atherosclerosis-induced rats' group that received the highest dose of white tea extract (400 mg/kg BW/day) produced the lowest levels of blood IL-6, the fewest number of foam cells, and the lowest intima-media thickness ratio. However, this study didn't include the group of atherosclerosis-induced rats that receive statin therapy, so we couldn't determine the effective dose of white tea leaf extract for atherosclerosis prevention.

CONCLUSION

The administration of white tea leaf extract has potential as an adjuvant therapy to inhibit the progression of atherosclerosis by reducing blood IL-6 levels, the number of foam cells, and the intima-media thickness ratio in cigarette smoke exposure and high-fat diet-induced Wistar rats. Further research is needed to compare the group of atherosclerosis-induced rats receiving statin therapy with the group that receives white tea extract, in order to know effective dose of white tea in the prevention of atherosclerosis.

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AUTHOR CONTRIBUTIONS

JF, the study's principal investigator, conceptualized and designed it, wrote the manuscript, and oversaw data collection; BR evaluated the text and advised on its interpretation and data analysis; HI analyze and interpret abdominal aorta preparation. The following people reviewed the manuscript: NS, MM, and EM. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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