ORIGINAL ARTICLE

Effect of green tea extract and epigallocatechin-3-gallate potency on lipid profile and coronary artery morphology of dyslipidemic rats

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Abstract
Objective: Dyslipidemia is a major risk factor in atherosclerosis and cardiovascular disease. Green tea and catechins have anti-dyslipidemic potency. This research was carried out to evaluate the effects of green tea extract (GTE) and epigallocatechin-3-gallate (EGCG) on lipid profile, antioxidant activities and histopathology of coronary artery in dyslipidemic rats.

Methods: Six weeks old male Sprague-Dawley rats were fed high cholesterol diet for 2 month. Three groups were treated with green tea extract (450, 300 and 150 mg/kg body weight/daily), another 3 groups were treated with EGCG (15, 10 and 5 mg/kg BW/daily), and a final group remained as untreated positive control. The treatment duration was 21 and 42 days. Lipid profile including total cholesterol (TC), triglyceride (TG), low and high density lipoprotein (LDL-HDL) levels, superoxide dismutase (SOD) activity, malondialdehyde (MDA) and coronary artery histopathology were evaluated. Data was analyzed using ANOVA and Duncan post hoc test.

Results: GTE and EGCG decreased TC, TG, LDL and MDA; and increased HDL, SOD levels significantly in both 21 and 42 days treatment groups. Furthermore, GTE and EGCG improved the coronary arteries histology.

Conclusion: GTE and EGCG improved lipid profile and coronary arteries histological structure in dyslipidemic rats.

INTRODUCTION
Cardiovascular disease (CVD) is the leading cause of death worldwide [1]. According to the family health survey in Indonesia, CVD caused 16% of death in 1992 and increased to 26.4% in 2001 [2]. Oxidative stress, abnormalities in lipid metabolism, vascular tone disturbance, platelet aggregation, inflammation, and proliferation of vascular cells are the major causes of CVD [3-8]. Dyslipidemia is a main risk factor in atherosclerosis and CVD; reducing cholesterol level can prevent and minimalize CVD risk [9, 10].

Several experimental and clinical studies exhibited that increasing level of reactive oxygen species (ROS) such as superoxide anion (O2•−) and hydroxyl radicals (OH•) in heart failure, caused cell membrane damages and destruct antioxidative defense system [11]. Oxidative stress can be induced by free radicals, which lead lipids, proteins and nucieic acids to damage, linking to many chronic diseases including CVD [12].

Green tea polyphenols have been studied as chemopreventive agents in CVD [13, 14]. The fresh tea leaves contain 17.2% protein, 27% crude powder, 22.2% polyphenols, 4.3% caffeine, 3.5% reduced sugar, 6.5% pectins, 0.5% starch, 5.6% ash and 2% ether extract [15]. Polyphenolic catechins are the major compounds in green tea [16]. The levels of the main catechins are 45.5% (-)epigallocatechin gallate (EGCG), 18.2% (-)epicatechin gallate (ECG), 10.1% (-)epicatechin (EC) and 4.2% (-)epigallocatechin (EG) [17].

Catechins have antioxidant and cellular signaling pathways modulating effects that lead to reduction of inflammation, platelet aggregation and elevation of vascular reactivity [18]. Several epidemiological studies showed an inverse correlation between tea consumption or polyphenol rich diets and the incidence of CVD [19]. The incidence of CVD in Japanese and Asia are lower than in Western countries. In Japan, as well as in other Asian countries, most people consume tea regularly [20].

Based on the above mentioned facts, the present research was carried out to evaluate the effects of green tea extract (GTE) and EGCG on lipid profile, antioxidant activities and histopathology of coronary artery in dyslipidemic rats.
MATERIAL AND METHODS

Extract preparation
Dried green tea leaves were obtained from Walini Tea Manufacturer (PTPN VIII, Bandung, Indonesia); tea plantation is located in Cigaruni, Garut, West Java, Indonesia. Green tea leaves were extracted by maceration method. One kilogram of green tea leaves were soaked in 70% ethanol. The filtrates were collected and then evaporated with a rotatory evaporator at 40°C [21, 22]. The green tea extract produced was 143 g (14.3%); it was stored at 4°C.

Animals and diets
The research has been approved by the Research Ethics Committee, Faculty of Medicine, Maranatha Christian University and Immanuel Hospital, Bandung, Indonesia. Forty adult male Sprague-Dawley rats (6 weeks old) from National Agency of Drug and Food Control, Jakarta, were used in this study. Their body weight (BW) ranged between 150-170 g. The rats were kept in cages with controlled temperature (25-27°C), humidity (60%) and 12/12 h light/darkness cycle. Rats were fed food and drank water ad libitum.

The Rats were adapted for 14 days until their body weight reached 170-190 g. After acclimatization, the rats were randomly classified into 7 groups: one of the groups was served as negative control (n = 5) and fed basal diet; the other groups were fed high cholesterol diet, i.e. basal diet supplemented with 1.25% cholesterol (Sigma-Aldrich) and 0.5% cholic acid (Sigma-Aldrich). The rats were fed for two months.

After dyslipidemia was detected, cholesterol diet was stopped and the subgroups were set as 450, 300 and 150 mg/kg BW/day GTE, or 15, 10 and 5 mg/kg BW/day EGCG-treated groups (n = 5 for all) (Chengdu Biopurify Phytochemicals, Sichuan, PR China). The rats were treated for 21 and 42 days. After 21 days (experiment 1), the blood was collected from orbital vein for lipid profile, superoxide dismutase (SOD) and malondialdehyde (MDA) assays, and after 42 days (experiment 2), rats were anesthetized using ketamin (10% (50 mg/kg BW) and ilium-xylazil-20 (12 mg/kg BW) for the same biochemical assays plus histopathological analysis [22].

Sample preparation for biochemical assays
One and a half milliliter of blood was collected in the tube, the blood centrifuged at 3000 rpm for 10 min and the blood serum were used for measuring total cholesterol (TC, 10 µl), triglyceride (TG, 10 µl) low density lipoprotein (LDL, 100 µl) and high density lipoprotein (HDL, 200 µl). The assays were performed with commercial kits from DiaSys Diagnostic System (Holzheim, Germany). TC and HDL assays are based on the enzymatic CHOD-PAP photometric test, LDL assay on the reaction of releasing LDL and LDL-cholesterol selectively determined by a color produced, and TG assay is based on the colorimetric test using glycerol-3-phosphate-oxidase.

SOD activity was measured using a kit supplied by Randox Laboratory: 0.5 ml whole blood was centrifuged for 10 min at 3000 rpm and then washed four times with 0.9% NaCl; the blood samples were re-centrifuged after each wash. Then the sample was made up to 2 ml with cold redistilled water, mixed and left to stand at 4°C for 15 min. The lysate was then 50-fold diluted with RANSOD diluting buffer for samples (final dilution factor; 200). SOD activities were calculated as Units/gram-hemoglobin (U/g Hb). The reaction was measured at 505 nm; Hb was calculated according to the guidelines of the International Committee for Standardization in Hematology [23]:

\[ \text{Hb concentration} = A_{\text{sample}} \times 36.77 \text{(g/dl)} \]

MDA, as an index of lipid peroxidation, was formed from polyunsaturated fatty acids breakdown which reacted with thiobarbituric acid (TBA): 0.2 ml of 8.1% sodium dodecyl sulfate (SDS) was added in to 0.5 µl sample plasma, then 1.5 ml of 20% acetic acid solution was added; using NaOH the pH was adjusted to 3.5, and then 1.5 ml of 0.8% TBA aqueous solution was mixed. The mixture was made up to 4 ml with distilled water, and then it was boiled at 95°C for 1 h. After cooling in ice box, sample was rinsed with methanol and aqua des, showed red species absorbance at 535 nm [24, 25].

Histological analysis
Coronary artery samples were immersed in neutral buffer 10% formalin, followed by dehydration and embedding in paraffin wax using standard procedures. Hematoxylin and eosin-stained sections of coronary arteries were examined for signs of atherosclerosis [22].

Statistical analysis
To verify the statistical significance of all parameters, the data were calculated as means and standard deviation (SD) and 95% confidence interval (CI) of means. This research used completely randomized design; to compare differences among treatment groups analysis of variance (ANOVA) was used. P values less than 0.05 were considered statistically significant. Furthermore, to know the best treatment, Duncan post hoc test at 95% confidence interval was used. Statistical analysis was done using SPSS version 20.0.

RESULTS
After two month of receiving high cholesterol diet, the dyslipidemic rats were found. The negative control rats had normal lipid profile, high SOD, low MDA levels and normal features of coronary artery histopathology.
GTE and EGCG were able to significantly decrease TC, TG and LDL levels, and increase HDL and SOD levels at both 21 (Table 1) and 42 (Table 2) days of treatment.

The variance analysis showed that the data for 21 days treatment were significantly different (P < 0.01) to each other. Based on Duncan post hoc test (Table 1), EGCG with 15 mg/kg BW was the most active to decrease TC, TG, LDL and MDA as well as the most active to increase SOD. Similarly, GTE at dosage of 450 mg/kg BW had the highest effect on lipid profile and acted as the most active antioxidant.

Table 2 shows that positive control for 42 days treatment exhibited significantly higher TC, TG, LDL, MDA, and lower HDL and SOD levels compared to treatment groups. EGCG at 15 mg/kg BW was the most active hypolipidemic dosage to decrease TC and TG, and the most active to decrease MDA. On the other hand, the highest HDL increasing effect of EGCG was seen at the dose of 5 mg/kg BW. GTE at 300 mg/kg BW showed a higher effect to decrease LDL, and at 150 mg/kg BW the highest effect to increase HDL and SOD levels. Moreover, these results showed that the longer the treatment the higher the hypolipidemic and antioxidant activities for both GTE and EGCG.

Table 1. Effect of GTE and EGCG treatment for 21 days on lipid profile, antioxidant (SOD) and lipid peroxidation (MDA) levels

<table>
<thead>
<tr>
<th>Sample</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>SOD (U/g Hb)</th>
<th>MDA (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTE 450 mg/kg</td>
<td>165.69±4.53^b</td>
<td>98.21±3.72^ab</td>
<td>64.18±1.84^b</td>
<td>49.43±1.85^b</td>
<td>620.34±21.1^a</td>
<td>5.76±0.39^b</td>
</tr>
<tr>
<td>GTE 300 mg/kg</td>
<td>189.88±14.95^c</td>
<td>96.73±4.31^ab</td>
<td>57.36±2.46^a</td>
<td>55.54±2.93^c</td>
<td>510.17±33.58^d</td>
<td>6.89±0.38^c</td>
</tr>
<tr>
<td>GTE 150 mg/kg</td>
<td>205.22±6.09^d</td>
<td>112.69±5.35^e</td>
<td>64.46±1.59^c</td>
<td>435.59±20.41^b</td>
<td>7.26±0.15^d</td>
<td></td>
</tr>
<tr>
<td>EGCG 15 mg/kg</td>
<td>146.41±7.32^a</td>
<td>92.84±1.8^a</td>
<td>64.31±3.86^c</td>
<td>46.11±1.72^c</td>
<td>618.64±15.85^a</td>
<td>4.4±0.5^a</td>
</tr>
<tr>
<td>EGCG 10 mg/kg</td>
<td>172.17±3.18^b</td>
<td>99.55±4.71^b</td>
<td>61.22±0.95^b</td>
<td>56.31±1.46^a</td>
<td>535.59±18.37^c</td>
<td>6.26±0.34^b</td>
</tr>
<tr>
<td>EGCG 5 mg/kg</td>
<td>190.99±4.89^a</td>
<td>107.91±4.3^b</td>
<td>57.11±2.63^b</td>
<td>59.11±4.16^d</td>
<td>398.31±24.71^a</td>
<td>7.4±0.45^a</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Different letters in the same column indicate significance at P < 0.05 (Duncan post hoc test).

Table 2. Effect of GTE and EGCG treatment for 42 days on lipid profile, antioxidant (SOD) and lipid peroxidation (MDA) levels

<table>
<thead>
<tr>
<th>Sample</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>SOD (U/g Hb)</th>
<th>MDA (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTE 450 mg/kg</td>
<td>131.71±5.23^b</td>
<td>85.82±3.62^b</td>
<td>98.01±2.06^b</td>
<td>46.62±3.07^b</td>
<td>650.85±31.94^d</td>
<td>1.33±0.24^b</td>
</tr>
<tr>
<td>GTE 300 mg/kg</td>
<td>154.94±15.13^c</td>
<td>84.03±4.37^ab</td>
<td>91.06±2.83^a</td>
<td>55.28±2.83^c</td>
<td>545.76±37.23^c</td>
<td>2.27±0.22^c</td>
</tr>
<tr>
<td>GTE 150 mg/kg</td>
<td>171.22±6.06^d</td>
<td>99.85±4.1^d</td>
<td>91.19±2.47^a</td>
<td>64.20±2.08^c</td>
<td>474.58±13.4^b</td>
<td>2.5±0.08^d</td>
</tr>
<tr>
<td>EGCG 10 mg/kg</td>
<td>112.09±6.36^e</td>
<td>80.15±2.02^e</td>
<td>97.75±4.24^b</td>
<td>43.57±4.64^b</td>
<td>469.15±19.51^e</td>
<td>1.01±0.11^e</td>
</tr>
<tr>
<td>EGCG 5 mg/kg</td>
<td>138.5±3.55^f</td>
<td>87.46±4.49^f</td>
<td>94.40±1.54^f</td>
<td>39.24±1.47^f</td>
<td>557.63±16.3^f</td>
<td>1.96±0.2^f</td>
</tr>
<tr>
<td>EGCG 5 mg/kg</td>
<td>157.47±4.85^g</td>
<td>96.12±4.17^g</td>
<td>91.06±2.21^g</td>
<td>36.69±1.72^g</td>
<td>415.25±13.4^f</td>
<td>2.62±0.27^f</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. Different letters in the same column indicate significance at P < 0.05 (Duncan post hoc test).
In Fig. 1, representative histopathologic pictures of coronary arteries can be seen. In brief, the coronary arteries of rats which achieved GTE or EGCG showed almost normal structural features; the intima of vascular lumen showed endothelial cells in lining layer, smooth muscle cells in medial layer, while the adventitial layer composed of collagen, elastic and fibrous tissue. In our previous research [22], the intima of coronary arteries from rats which was fed by high cholesterol diet layer becomes thicker and the elastic membrane of the intimal layer fragmented and focally lost, narrowed artery’s wall, formed foamy cells; the medial layer were thinner and the diameter of the luminalis smaller due to the atherosclerotic plaques.

**DISCUSSION**

Rats suffered dyslipidemic had oxidative stress with high MDA level and low SOD activity: This data was consistent with previous research reporting that oxidative modification of LDL plays a crucial role in the development of atherosclerosis; dyslipidemic rats were triggered by high level of circulating oxidized (ox)-LDL compared to normal rats [10, 22]. After treated with GTE and EGCG, TC value decreased. This result was consistent with previous research showing that green tea could reduce TC level [26].

Green tea has hypolipidemic effects, at least in part, due to inhibition of intestinal absorption of cholesterol and dietary fat [27]; its polyphenolic content reduces micellar solubility, so inhibits intestinal lipid absorption and increases fecal excretion of cholesterol, which lead to reduction of hepatic cholesterol level and up-regulation of hepatic LDL receptors [28].

GTE standardized at 25% catechins was shown to inhibit digestive lipases in an *in vitro* assay [29]. The lipid-lowering effect of green tea which is due to inhibition of hepatic lipogenesis involves activation of the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) and its responsive genes without affecting lipoprotein assembly [30]. Green tea, polyphenols and tea catechins could lower lipids in serum and liver, decrease serum total cholesterol and atherogenic index [26, 31-33]. Green tea and tea polyphenols were able to reduce serum TC and TG levels, improve the ratio of HDL to LDL and increase fecal fat, lipid and cholesterol excretions [32-39]. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) was shown to be involved in the hypcholesterolemic effect of green tea [40]. It was claimed that consuming green tea extract regularly may lower LDL level more than 15% [41].

In the present study, EGCG decreased TG, TC and LDL, and increased HDL levels of dyslipidemic rats at both 21 and 42 day treatments. This result was validated with the study by Wolfram [42] in which beverages containing low, moderate and high EGCG of green tea catechins supplemented together with fat-enriched bread, reporting that moderate and high doses reduce the postprandial TG response. Moreover, EGCG of tea could reduce the cholesterol solubility in micelles action [43, 44]. Cholesterol-lowering effect of green tea is mainly elicited by EGCG and that the effect occurs through decrease of cholesterol absorption [45]. Tea catechin supplementation increases fecal excretion of total lipids and cholesterol [46].

Focusing on oxidative stress indices, GTE and EGCG were able to increase SOD activity and decrease MDA level at both 21 and 42 days treatment. This data was verified by a previous study in which water green tea consumption decreased urinary 8-hydroxydeoxyguanosin, a marker of oxidative DNA damage, increased antioxidant capacity and decreased peroxides in plasma, and reduced oxidative damage and glutathione peroxidase activity in lymphocytes [42]. In another study, EGCG enhanced the antioxidative potential of plasma and reduced O$_2^{•−}$ production in the media of the injured artery [47]. Catechins (including EGCG) were able to induce enzymes that play crucial roles in cellular antioxidant defense mechanisms [48]. The ox-LDL plasma levels decreased after 4 weeks of green tea consumption in smokers [49]. Green tea was able to inhibit LDL oxidation in human volunteers [50]. It has ability to reduce TC and LDL, and enhance HDL and SOD levels in serum [26]. Green tea catechins inhibit the formation of ox-cholesterol and decrease linoleic acid and arachidonic acid levels [51]. Green tea polyphenols are able to reduce lipid peroxidation, particularly LDL oxidation and MDA levels [28]. Histopathologically, GTE and EGCG made wider the vascular lumen seen as the intima showing a lining layer of endothelial cells and smooth muscle cells in medial layer, while the adventitial layer is composed of collagen, elastic and fibrous tissue. This data is validated with previous research showing that green tea reduces platelet aggregation, improves lipid regulation, inhibits proliferation and migration of smooth muscle cells, and inhibits abnormal blood clots formation [42]. Thus, CVD risk could be reduced by green tea consumption [16]. Tea polyphenols could reduce free radical damage to cells and prevent ox-LDL cholesterol, consequently inhibit the formation of atherosclerotic plaques [52]. The increasing ox-LDL level and aortic lesion formation are reversed by green tea [53]. Green tea consumption in rabbits reduced atherosclerosis and
decreased vascular endothelial growth factor (VEGF) expression in atherosclerotic plaque [54]. EGCG-induced relaxation of rat aorta [55], act as an anti-inflammatory by inhibiting IL-6 and Ang II-induced C-reactive protein (CRP) secretion, Ang II-induced generation of ROS in vascular smooth muscle cells (VSMC), which contributes as antiatherosclerotic [56]. EGCG reduced the progression of an accelerated atherosclerotic carotid plaque formation induced by cuff injury and inhibited VSMC proliferation in vitro, possibly via antioxidative modulation of redox-sensitive genes expression [57].

It can be concluded that GTE and EGCG may be able to reduce CVD risk factors including TG, TC and LDL, while increase HDL levels and possess antioxidant activities. EGCG presented more potent effect than GTE; this was also confirmed by a previous work of Amani et al [58].

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REFERENCES


