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Manuscript Title: **GREEN TEA EXTRACT PROTECTS ENDOTHELIAL PROGENITOR CELLS FROM OXIDATIVE INSULT THROUGH REDUCTION OF INTRACELLULAR REACTIVE OXYGEN SPECIES ACTIVITY**

Authors: Authors: Wahyu Widowati, Rachma Micho Widyanto, Winsa Husin, Hana Ratnawati, Bambang Setiawan, Dian Nugrahenny, Indra Bachtiar

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Dear Dr. Wahyu Widowati

Your manuscript entitled: **GREEN TEA EXTRACT PROTECTS ENDOTHELIAL PROGENITOR CELLS FROM OXIDATIVE INSULT THROUGH REDUCTION OF INTRACELLULAR REACTIVE OXYGEN SPECIES ACTIVITY**

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Reviewer Note For Author:

- 1-The effect of green tea consumption on circulating endothelial progenitor cells (EPC) has been shown previously. The authors have to explain the novelty of their study?
- 2- Why did the authors use maceration method for extraction?
- 3- It is recommended to determine the level of phenolic compounds in the extract.
- 4- Please update the references. Most of them are too old.

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Green tea extract protects endothelial progenitor cells from oxidative insult through reduction of intracellular reactive oxygen species activity

Wahyu Widowati ^{1*}, Rahma Micho Widyanto ², Winsa Husin ¹, Hana Ratnawati ¹, Dian Ratih Laksmiawati ³, Bambang Setiawan ⁴, Dian Nugrahenny ⁵, Indra Bachtiar ⁶

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ARTICLE INFO	ABSTRACT
<p>Article type: Original article</p>	<p>Objective(s): Many studies have reported that tea consumption decreases cardiovascular risk, but the mechanisms remain unclear. Green tea is <i>known to have</i> potent antioxidant and <i>free radical scavenging</i> activities. This study aimed to investigate whether green tea extract (GTE) can protect endothelial progenitors cells (EPCs) against oxidative stress through antioxidant mechanisms.</p> <p>Materials and Methods: Mononuclear cells (MNCs) were isolated from peripheral blood by density gradient centrifugation with Ficoll. The cells were then plated on fibronectin-coated culture dishes. After 7 days of culture, EPCs were characterized as adherent cells double positive for DiI-ac-LDL uptake and lectin binding. EPCs were <i>further identified</i> by assessing the expression of CD34/45, CD133, and KDR. EPCs were then treated with hydrogen peroxide (H₂O₂) at doses of 50, 100, 200 μM and incubated with or without GTE (25 μg/ml). The intracellular reactive oxygen species (ROS) levels were detected by <i>flow cytometry using</i> a 2',7'-dichlorofluorescein diacetate (DCF-DA) <i>fluorescent</i> probe.</p> <p>Results: GTE ameliorated the cell viability of EPCs induced by H₂O₂ at doses of 50, 100, 200 μM for about 25.47, 22.52, and 11.96% higher than controls, respectively. GTE also decreased the intracellular ROS levels of EPCs induced by H₂O₂ at doses of 50, 100, 200 μM for about 84.24, 92.27, and 93.72% compared to controls, respectively.</p> <p>Conclusion: GTE improves cell viability by reducing the intracellular ROS accumulation in H₂O₂-induced EPCs.</p>
<p>Article history: Received: Accepted:</p>	
<p>Keywords: Antioxidant Endothelial progenitor cells Green tea Oxidative stress Reactive oxygen species</p>	

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Introduction

Endothelial dysfunction plays an important role in pathogenesis of atherosclerosis caused by cardiovascular risk factors. It has been observed in patients with established coronary artery disease or coronary risk factors (1). Endothelial progenitor cells (EPCs), a kind of stem cells forming a new vessel, play critical roles in maintaining the vessel tone and repairing the endothelial cells injury (2-5). Recent studies indicated that the number of circulating EPCs reduces in patients with atherosclerosis risk factors leading to the development of atherosclerosis lesions (6, 7).

Endothelial dysfunction is closely related with increased oxidative stress and may be reversed by antioxidant treatment (2, 8). Previous studies suggested that flavonoids may improve endothelial

function (8, 9). Flavonoids have powerful antioxidant properties and they exert their effects by scavenging free radicals (10, 11). Previous studies also suggested that tea flavonoid prevents LDL (low density lipoprotein) oxidation, inflammatory, endothelial dysfunction that are crucial to the development of atherosclerotic plaque (8, 12).

Tea from *Camellia sinensis* is the second most widely consumed beverage in the world after water (13-15). Many studies have reported the relation between tea consumption and cardiovascular risk (15-17), and suggested that the risk reduction is due to flavonoid compounds in tea (8, 9, 18, 19). Other studies also indicated that dietary flavonoid from tea and other sources (such as red wine, apples, onions, chocolate, blueberries, and strawberries) is related

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with reduced cardiovascular risk (20-23). Green tea has abundant flavonoids, including catechins (30-36% of dry weight), and epigallocatechin-3-gallate (EGCG) constitutes up to 63% of total catechins in tea (24). The antioxidant activity of EGCG has been shown to be 25-100 times more potent than vitamins C and E (25). We hypothesized that green tea extract (GTE) is able to protect EPCs from oxidative stress through antioxidant mechanism, thereby contributes to the protective effect on endothelial cells. To test this hypothesis, we assessed the protective effects and ROS-inhibiting effects of GTE on H₂O₂-induced oxidative damage in human EPCs.

Materials and Methods

Preparation and extraction of green tea

Dried green tea leaves was obtained from PT. Perkebunan Nusantara (PTPN) VIII, Bandung, west Java Indonesia. Green tea was planted and harvested from Cisaruni plantation, Garut, West Java. The dried green tea leaves contained water level 7.15%; protein 22.00%; fiber 14.33%; ash 5.13%, crude lipid 1.33%; carbohydrate 57.31%. The green tea plant were identified by staff of herbarium, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The green tea plant was identified as *Camellia sinensis* L. Kuntze or *Thea sinensis* (L.), *Camellia theifera* Griff. The preparation and extraction of green tea were performed according maceration extraction method (12, 26, 27). One kilogram of dried green tea leaves was extracted with distilled methanol 96% by maceration method for 5 days filtered and collected until the colorless methanol filtrate. The collected methanol filtrate was evaporated using rotatory evaporator to produce methanol extract of green tea 173.9 g or 17.39%. The methanol extract of green tea was stored at 4°C.

Superoxide Dismutase (SOD) assay

The SOD assay was done using a SOD assay kit (Cayman) comprising assay buffer, sample buffer, radical detector, SOD standard, and xanthine oxidase. SOD standards were prepared by introducing 200 µl diluted radical detector and 10 µl SOD standard (7-level standard) per well. Green tea extract was dissolved in DMSO in concentrations of 500, 125, and 31.25 µg/ml (27). The sample well contained 200 µl diluted radical detector and 10 µl sample. All wells were added 20 µl diluted xanthine oxidase. The mixtures were shaken carefully for few seconds, incubated for 20 min at room temperature, SOD activity was measured on a microplate reader at 450 nm (Cayman). The SOD value was calculated using the equation from the linear regression of standard curve substituting linear rate (LR) for each sample.

$$\text{SOD (U/ml)} = \left[\frac{\text{Sample LR-y intercept}}{\text{Slope}} \right] \times 0,23\text{ml}/0,01\text{ml} \times \text{Sample dilution}$$

Total phenol content

Total phenol content was assayed according to the Folin-Ciocalteu method. Samples (15 µl) were introduced into microplate; 75 µl of Folin-Ciocalteu's reagent (2.0 M) and 60 µl of sodium carbonate (7.5%) were added. The samples were mixed and incubated at 45°C for 15 min (28). Subsequently, absorbance value was measured at 760 nm. The total phenolic content expressed as Epigallocatechin Gallate equivalent (EGCGE) and Galocatechin equivalent (GCE) was calculated by the following formula:

$$C = \frac{c * V}{m}$$

where C : total content of phenolic compounds, µg/mg green tea extract, in EGCGE and in GCE;

c : the concentration of EGCG or GC established from the calibration curve, µg/ml;

V: the volume of extract (ml);

m : the weight of green tea extract (mg).

Total phenol value was obtained from the regression equation for EGCG: Y=0.0048X+0.036, with R²=0.99 and for GC: Y=0.02X+0.057 with R²=0.97.

ABTS-reducing activity

The antioxidant capacity green tea extract was measured using the 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) diammonium salt free radical assay (29, 30, 31). ABTS^{•+} was produced by reacting 14 mM ABTS and equal volume of 4.9 mM potassium persulfate achieved final concentration of 7 mM ABTS in 2.45 mM potassium persulfate. The mixture was incubated in the dark room temperature for 16 h. The ABTS^{•+} solution was diluted with 5.5 mM PBS (pH 7.4) and measured with microplate reader at 745 nm, resulting the absorbance of 0.70±0.02. Briefly 2 µl sample was added to 198 µl of ABTS^{•+} solution, incubated for 6 min at 30°C, and the absorbance was measured at 745 nm. The percentage inhibition of ABTS radical (%) was expressed by the ratio of the reducing of ABTS^{•+} absorbance in the presence of the test sample relative to the ABTS^{•+} absorbance in the absence of the test sample (negative control). ABTS-reducing activity (%) was then continued to be calculated as median Inhibitory Concentration (IC₅₀).

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay (FRAP) of each standard solution and sample were measured according to a modified protocol developed by Mishra *et al* (2006) (32). The FRAP reagent was prepared by adding 2,4,6-tripyridyl-s-triazine (TPTZ) and ferric chloride, forming the Fe³⁺-TPTZ complex. Antioxidant reduced to Fe²⁺-TPTZ at low pH was measured at 595 nm. The standard curve was linear between 0.019 and 95 µg/ml FeSO₄. Results were expressed in µM Fe (II)/µg extract and compared with EGCG (33).

Table 1. The mean of SOD activity (U/ml) of GTE. SOD activity was measured in triplicate for each concentration of GTE. Linear equation, coefficient of regression (R^2) of SOD standard, and SOD activity of GTE were calculated. The data are presented as mean±standard deviation

Sample	Concentrations of green tea extract ($\mu\text{g/ml}$)		
	500	125	31.25
GTE	1.24±0.06 (U/ml)	1.22±0.04 (U/ml)	1.09±0.03 (U/ml)

Isolation and cultivation of EPCs

EPCs were cultured according to the previously described method of Chen *et al* (2004) (34). Mononuclear cells (MNCs) were isolated from peripheral blood of healthy young human volunteers by Ficoll-Paque plus (GE Healthcare) using density gradient centrifugation. MNCs were then plated on culture dishes coated with human fibronectin (Roche) and cultured in VascGrow™ medium (Stem Cell and Cancer Institute) at 37°C in a 5% CO₂ incubator. After 4 days of culture, medium were changed and the culture was maintained through day 7. Informed consent was obtained from all volunteers and all procedures performed in this study were approved by Research Ethics Committee of Faculty of Medicine, Maranatha Christian University and Immanuel Hospital, Bandung, Indonesia.

EPCs characterization

EPCs were characterized as adherent cells after 7 days of culture. Direct fluorescent staining was used to detect dual binding of Fluorescein isothiocyanate-conjugated *Ulex europaeus* agglutinin I (FITC-UEA-I; Sigma) and 1,1-dioctadecyl-3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (DiI-ac-LDL; Invitrogen). To detect the uptake of DiI-ac-LDL, adherent cells were incubated with DiI-ac-LDL (1 mg/ml) at 37°C for 4 hr. Cells were then fixed with 3% paraformaldehyde for 10 min. After washing, cells were incubated with FITC-UEA-I (1 mg/ml) at 37°C for 1 hr. Cell nuclei were also stained with 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen). Finally, cells were observed using an inverted fluorescence microscope (Axiovert 40 CFL, Zeiss). Cells that were double positive for DiI-ac-LDL and lectin were defined as EPCs (35).

Further identification of EPCs was performed using fluorescence-activated cell sorting (FACS) analysis. Adherent cells were detached using trypsin-ethylenediamine tetra-acetic acid (EDTA). Cells (1×10^5) were pre-incubated with FcR blocking reagent (Miltenyi Biotech) for 15 min at room temperature. Cells were then incubated at 4°C with FITC-conjugated anti-CD45/phycoerythrin (PE)-conjugated anti-CD34 (BD Biosciences) and PE-conjugated anti-

Table 2. The mean of phenolic content of GTE and GC was expressed as EGCGE and GCE. The phenolic content was measured in triplicate for each sample. Linear equation, coefficient of regression (R^2) of EGCG and GC were calculated. The data are presented as mean±standard deviation

Sample	Phenolic content	
	$\mu\text{g EGCGE/mg GTE}$	$\mu\text{g GCE/mg GTE}$
GTE	139.17±0.833	29.20±0.200

CD133 (Miltenyi Biotech) for 15 min, and PE-conjugated vascular endothelial growth factor receptor 2 (VEGFR2)/KDR (R&D System) for another 40 min. Isotype-identical antibodies served as negative controls. Quantitative FACS was performed on a FACSCalibur Flow Cytometer (BD Biosciences).

EPCs cytotoxicity assay

EPCs cytotoxicity was performed to determine the maximum tolerance concentration of GTE on EPCs culture and to determine the optimal oxidative damage concentration of H₂O₂ for the following experiments. Cytotoxicity of EPCs was determined by CellTiter® (Promega) based on quantitative colorimetric assay. After 7 days of culture, cells were then digested with trypsin-EDTA and cultured at a density of 5×10^4 cells/ml on 96-well tissue culture plates using serum-free medium for 24 hr before treatment (28, 36, 37). Cells were then treated with various concentrations of GTE (3.13-100 $\mu\text{g/ml}$) and H₂O₂ (12.5-400 μM) for 24 hr. EPCs were supplemented with 20 μL of CellTiter® (Promega) each well and incubated for another 4 hr. Optical density values were measured at 490 nm using microplate reader (Bio-Rad).

Assessment protective effect of GTE on oxidative damage in EPCs

After 7 days of culture, cells were digested with trypsin-EDTA and 5×10^4 cells were cultured on 96-well tissue culture plates using serum-free medium for 24 hr before treatment. Cells were then treated with GTE (25 mg/l) for 1 hr before treatment with various concentrations of H₂O₂ (50, 100, 200 μM) for a subsequent 24 hr (38). Cell viability was measured by CellTiter® assay (Promega). Cells treated without H₂O₂ served as controls. Optical density values were measured at 490 nm using microplate reader (Bio-Rad). The values were expressed as a percentage of control.

Measurement of intracellular reactive oxygen species

The intracellular ROS levels were detected by flow cytometry using a DCF-DA fluorescent probe (Invitrogen) according to the method of Jie *et al* (38)

Table 3. The IC₅₀ of ABTS-reducing activity of GTE and EGCG. ABTS-reducing activity (%) was calculated in triplicate for each concentration of GTE and EGCG. Seven concentrations of sample included: 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.038 $\mu\text{g/ml}$. Linear equation, coefficient of regression (R^2) of GTE and EGCG were calculated

Sample	The highest activity of ABTS reducing activity (%)	Linear equation	R^2	IC ₅₀ ($\mu\text{g/ml}$)
GTE	97.30	Y=17.248X+12.636	0.9197	2.17
EGCG	97.83	Y=9.950X+36.452	0.9597	2.14

Table 4. The FRAP activity. The standard curve was linear between 0.019 and 95 $\mu\text{g/ml}$ FeSO_4 . [The FRAP activity was calculated in triplicate for each concentration of GTE and EGCG. Seven concentrations of sample included 25.000, 12.500, 6.250, 3.125, 1.563, 0.782, 0.391 $\mu\text{g/ml}$. Results of FRAP activity were expressed in $\mu\text{M Fe (II)/}\mu\text{g}$ sample. Linear equation of FeSO_4 was $Y=0.0064X+0.0445$]. The data are presented as mean \pm standard deviation

Concentration ($\mu\text{g/ml}$)	FRAP activity ($\mu\text{M Fe (II)/}\mu\text{g}$)	
	GTE	EGCG
25.000	98.35 \pm 1.73	186.16 \pm 14.64
12.500	60.76 \pm 0.56	163.08 \pm 19.40
6.250	34.85 \pm 0.86	83.77 \pm 3.48
3.125	15.74 \pm 0.48	46.19 \pm 1.45
1.563	14.42 \pm 0.40	31.72 \pm 0.41
0.782	11.36 \pm 0.71	14.14 \pm 0.52
0.391	10.38 \pm 0.47	11.92 \pm 0.15

Measurement of intracellular reactive oxygen species

The intracellular ROS levels were detected by flow cytometry using a DCF-DA fluorescent probe (Invitrogen) according to the method of Jie *et al* (38) and Stolzing & Scutt (39) with modification. After 7 days of culture, EPCs were digested with trypsin-EDTA and 1×10^5 cells were incubated with 10 μM DCF-DA at 37°C for 30 min. Next, the excess probes were washed out with phosphate-buffered saline (PBS)+KCl, and then incubated with GTE (25 $\mu\text{g/ml}$) for 30 min. Cells were then incubated with H_2O_2 at final concentrations of 50, 100, and 200 μM for another hour. Finally, the intracellular ROS levels were measured using FACSCalibur Flow Cytometer (BD Biosciences). Cells treated with H_2O_2 without GTE pre-treatment served as controls. The measured fluorescence values were expressed as a percentage of control.

Table 5. The EPCs functional characterization of Dil-ac-LDL uptake and lectin binding (adherent cells were stained with DAPI and percentage of the double-stained cells was calculated)

No	Number of cells	Number of double positive cells (Dil-ac-LDL uptake and lectin binding)	% of double-stained cells
1.	30	22	73.33
2.	30	25	83.33
3.	31	23	74.19
4.	30	26	86.67
5.	24	22	91.67
6.	29	22	75.86
7.	26	13	50.00
8.	40	20	50.00
9.	31	16	51.56
Mean \pm SD			70.74 \pm 16.28

Statistical analysis

Data are presented as mean, the differences between groups were analyzed using one-way analysis of variance (ANOVA) with SPSS 20.0 statistical package. Only probability values of $P<0.05$ were considered statistically significant and later subjected to Duncan's *post hoc* test.

Results

Antioxidant activity of GTE

The antioxidant activities of GTE were examined including SOD activity, total phenolic content, ABTS-reducing activity, and FRAP activity. SOD activity was measured in concentrations of 500, 125, and 31.25 $\mu\text{g/ml}$. GTE showed high antioxidant activity and similar activity among concentrations (Table 1).

Total phenolic content of GTE was measured using the EGCG and GC standard, the phenolic major in green tea. It was demonstrated that compared to the GC, the content of EGCG was higher in GTE (Table 2).

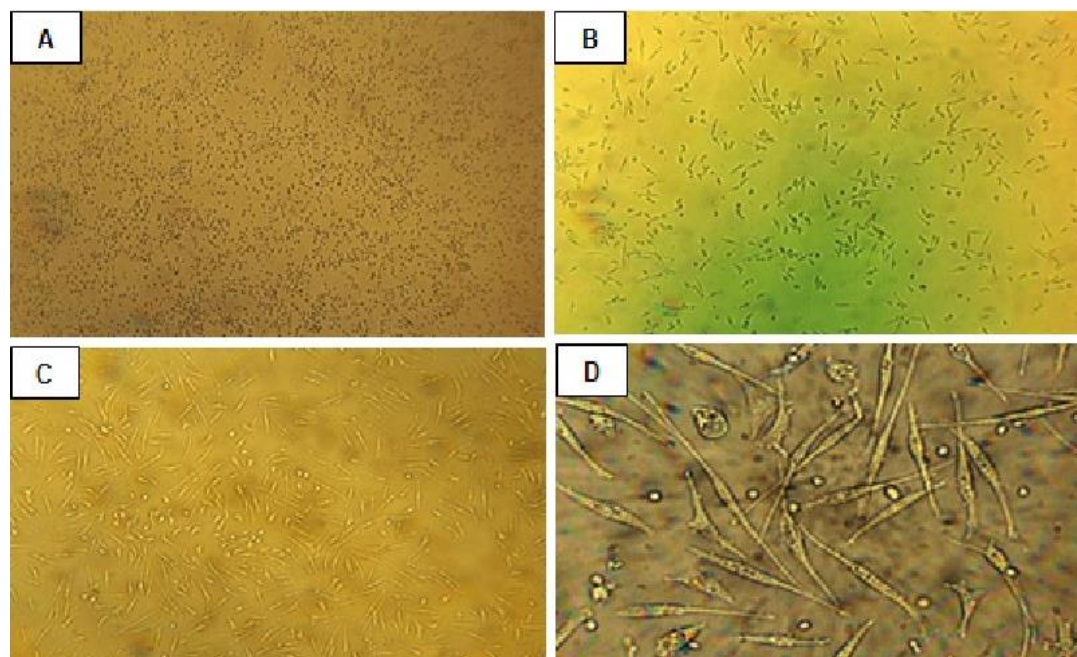


Figure 1. The EPCs morphology characterization. MNCs (A, 400x magnification) were cultured on VascGrow™ medium. Cells exhibited a spindle-shaped on day 4 (B, 400x magnification) and were being sharper in pattern on day 7 (C, 400x magnification; D, 800x magnification)

Table 6. The expression of EPCs markers (CD34/45, CD133, KDR) identified by flow cytometry. The data are presented as mean±standard deviation

No.	CD34/45 (%)	CD133 (%)	KDR (%)
1.	0.12	0.10	0.24
2.	0.10	0.16	0.26
3.	0.18	0.16	0.20
Mean	0.13±0.041	0.14±0.035	0.23±0.031

As a representative of antioxidant activity, ABTS-reducing activity of GTE was measured using EGCG as the control. The IC₅₀ is the concentration of GTE or EGCG to scavenge 50% of the ABTS radical. ABTS-reducing activity of GTE was comparable with EGCG (Table 3).

FRAP activity of GTE and EGCG was measured using FeSO₄, TPTZ, and ferric chloride forming the Fe³⁺-TPTZ complex. The presence of antioxidant will reduce Fe³⁺-TPTZ to Fe²⁺-TPTZ. Based on FRAP assay, EGCG showed more active antioxidant compared to GTE (Table 4).

EPCs characterization

The attached EPCs proliferated rapidly forming spindle-shaped cells within 4-7 days of culture (40). Beside cell morphology, functional assay was also used to demonstrate that putative progenitors have endothelial cell potential, including uptake of DiI-ac-LDL (41) and binding of FITC-UEA-1 plant lectin (42). Cell surface markers analysis by flow cytometry has also been used to identify the EPCs.

In the present study, MNCs isolated and cultured for 7 days resulted in attached spindle-shaped endothelial cell-like morphology (Figure 1). EPCs were then characterized as adherent cells double positive for DiI-ac-LDL uptake and lectin binding (70.74±16.28%) (Table 5). Surface markers identification of EPCs demonstrated the expression of CD34/45 (0.13±0.041%), CD133 (0.14±0.035%), and KDR (0.23±0.031%) (Table 6).

EPCs cytotoxicity assay

The cell viability was measured using colorimetric method for determining the cytotoxicity assay. CellTiter® solution (Promega) was used in this study. The solution contains a novel tetrazolium compound

Table 8. The effect of GTE pretreatment (25 µg/ml) on H₂O₂-induced EPCs

(The cells viability was estimated by CellTiter® assay after treatment with 50, 100, 200 µM H₂O₂ for 24 hr. The data are presented as mean±standard deviation)

Samples	Cell viability (%) over negative control (Mean±standard deviation)
H ₂ O ₂ 0 µM (Control)	100±3.31 ^d
H ₂ O ₂ 50 µM	63.21±9.31 ^d
H ₂ O ₂ 100 µM	42.66±4.98 ^b
H ₂ O ₂ 200 µM	32.29±1.51 ^e
H ₂ O ₂ 50 µM + GTE 25 µg/ml	88.69±2.61 ^d
H ₂ O ₂ 100 µM + GTE 25 µg/ml	65.16±3.38 ^c
H ₂ O ₂ 200 µM + GTE 25 µg/ml	44.25±6.86 ^b

The data are presented as mean±standard deviation. Different superscripts in the same column (^{a,b,c,d}) indicate significant differences among the means of groups (concentrations of H₂O₂ or H₂O₂ + GTE) based on Duncan's post-hoc comparisons (*P* < 0.05)

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS]. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product due to conversion by dehydrogenase enzymes in metabolically active cells (27, 43).

The result of cytotoxicity effect from GTE and H₂O₂ is shown in Table 7. After treatment with GTE at concentrations of 50 and 100 µg/ml for 24 hr, the viability of cells decreased about 40 and 80%, respectively, relative to the negative controls. Treatment with GTE at concentration of 3.13-25 µg/ml had relatively no effect on cells cytotoxicity.

The cells treated with H₂O₂ at concentrations of 12.5-25 µM demonstrated no toxicity. The viability of EPCs decreased for about 21, 55, 75, and 84% relative to the negative controls at the concentrations of 50, 100, 200, and 400 µM, respectively. GTE concentration of 25 µg/ml and H₂O₂ concentrations of 50, 100, and 200 µM had been chosen for independent and dependent concentrations of the following experiments.

Protective effect of GTE on oxidative damage in EPCs

Pre-treatment with 25 µg/ml of GTE on H₂O₂-induced EPCs at concentrations of 50, 100, and 200 µM increased the cells viability of EPCs induced by H₂O₂ at doses of 50, 100, 200 µM for about 25.47%

Table 7. The effect of GTE and H₂O₂ on cytotoxicity of EPCs

(EPCs were cultured on 96-well tissue culture plates (5 x 10⁴ cells/well) and treated with GTE or H₂O₂ at a wide range of doses for 24 hr. After treatment, cells were prepared for CellTiter[®] analysis of cells cytotoxicity)

Samples	Cell viability (%) over negative control (Mean±standard deviation)	Samples	Cell viability (%) over negative control (Mean±standard deviation)
Green tea extract samples		H ₂ O ₂ samples	
GTE 0 µg/ml	100.00±4.48 ^d	H ₂ O ₂ 0 µM	100.00±4.26 ^d
GTE 3.13 µg/ml	95.70±11.03 ^{cd}	H ₂ O ₂ 12.5 µM	104.49±9.20 ^d
GTE 6.25 µg/ml	101.75±6.49 ^d	H ₂ O ₂ 25 µM	103.82±5.34 ^d
GTE 12.5 µg/ml	84.51±16.38 ^c	H ₂ O ₂ 50 µM	79.06±8.35 ^c
GTE 25 µg/ml	101.19±6.28 ^d	H ₂ O ₂ 100 µM	49.67±3.95 ^b
GTE 50 µg/ml	60.15±5.50 ^b	H ₂ O ₂ 200 µM	25.44±0.99 ^a
GTE 100 µg/ml	20.06±1.07 ^a	H ₂ O ₂ 400 µM	15.87±2.83 ^a
DMSO 0.5%	105.29±3.71 ^d		

The data are presented as mean±standard deviation. Different superscripts in the same column (^{a,b,c,d}) indicate significant differences among the means of groups (concentrations of GTE/DMSO for the first column and H₂O₂ for the second column) based on Duncan's post-hoc comparisons (P < 0.05).

Table 9. The fluorescence intensity of ROS in H₂O₂-induced EPCs

(The cells were incubated with 10 µM DCF-DA for 30 min and exposed to several doses of H₂O₂ (50, 100, and 200 µM). The basal level of ROS with no exposure to H₂O₂ was obtained by gating with the ROS level of control unstained-DCF-DA cells. High concentrations of H₂O₂ increased ROS levels in EPCs but not statistically significant. Parallel samples were treated with 25 µg/ml of GTE and the ROS level in cells decreased relative to the controls (H₂O₂-induced cells) at the concentrations of 50, 100, and 200 µM. The measured ROS levels were expressed as a percentage of negative controls (cells not induced by H₂O₂). H₂O₂ exhibited an increase in ROS level)

Samples (H ₂ O ₂)	ROS level (%) over negative control	Samples (H ₂ O ₂ +GTE)	ROS level (%) over negative control
H ₂ O ₂ 0 µM (Control)	100.00±0.00 ^a	H ₂ O ₂ 500/100/200 µM	100.00±0.00 ^c
H ₂ O ₂ 50 µM	61.75±11.17 ^b	H ₂ O ₂ 50 µM + GTE 25 µg/ml	15.76±1.92 ^b
H ₂ O ₂ 100 µM	77.38±11.79 ^b	H ₂ O ₂ 100 µM + GTE 25 µg/ml	7.73±1.07 ^a
H ₂ O ₂ 200 µM	80.59±7.88 ^b	H ₂ O ₂ 200 µM + GTE 25 µg/ml	6.28±0.36 ^a

The data are presented as mean±standard deviation. Different superscripts in the same column (^{a,b,c}) indicate significant differences among the means of groups (concentrations of H₂O₂ for the first column and H₂O₂ + GTE for the second column) based on Duncan's post-hoc comparisons (P<0.05).

(88.69-63.21%), 22.52% (65.16-42.66%), and 11.96% (44.25-32.295) relative to the positive controls (H₂O₂-induced EPCs), respectively (Table 8). These data showed that GTE protects the cells from oxidative damage and ameliorates the H₂O₂-induced loss of EPCs viability.

Intracellular reactive oxygen species levels in EPCs

The level of fluorescence intensity is an indicator of ROS production. The basal level of ROS with no exposure to H₂O₂ was about 7-16 % compared to that in controls (unstained-DCF-DA cells). After treatment with different doses of H₂O₂ (50, 100, 200 µM) for 1 hr, the level of ROS in EPCs increased for about 7-34 % compared to that in negative controls (untreated cells). When the cells were treated with 25 µg/ml of GTE, ROS levels decreased dose-dependently for about 84.24% (100-15.76%), 92.27% (100-7.73%), and 93.72% (100-6.28%) (Table 9). Compared to the H₂O₂-induced EPCs at concentrations of 50, 100, 200 µM, respectively. The effect of H₂O₂ concentrations on ROS levels in EPCs (high concentrations of H₂O₂ induced an increase in EPCs ROS levels) is shown in Table 9. These results indicated that GTE treatment reduces the accumulation of ROS in H₂O₂-induced cells.

Discussion

The antioxidant assay of GTE and EGCG showed that GTE had high antioxidant activities. It

was demonstrated that GTE had high SOD activity at all concentrations. This result was consistent with previous study that black tea extract supplementation is able to increase SOD activity in hypercholesterolemic rat compared to control without black tea extract (12). It was also suggested that GTE contained high level of EGCG. This result was validated with previous study that GTE contains flavonoids, including catechins (30-36% of dry weight), and EGCG constitutes up to 63% of total catechins (24). Based on ABTS-reducing activity, GTE and EGCG had high activity with similar IC₅₀. This data was validated with previous study that green tea infusion is able to inhibit ABTS radical at 71.01-78.75% (44). Meanwhile, our study result showed that GTE and EGCG exhibited higher ABTS reducing activity at 97.30-97.83%. The FRAP activity showed that EGCG had higher antioxidant activity compared to GTE. Previous study suggested that GTE has high antioxidant and it consists of EGCG. The increase in polyphenol content in green tea will increase its antioxidant activity (44).

The result demonstrated that peripheral blood MNCs were able to differentiate into EPCs during 7-d incubation. EPCs are the precursors of mature endothelial cells and initially defined by the expression of CD34 or the more immature marker protein CD133 and the endothelial marker proteins such as VEGFR2/KDR, von Willebrand factor (VWF), VE-cadherin, Dil-ac-LDL uptake, and lectin binding

(35).

Previous studies showed that intracellular steady-state concentrations of H₂O₂ above 5 μM are able to cause oxidative stress (45, 46). Median cytotoxic concentrations (EC₅₀) decreased from 500 to 30 μM with increase in incubation time from 1 to 24 hr. Twenty-four-hour treatment was proved to be sufficient to determine incipient cytotoxic concentrations of H₂O₂ inducing growth arrest and cell death (47). Cellular responses elicited by H₂O₂ depend upon the severity of the damage, which is further influenced by the cell type and the magnitude of the dose of the exposure (48, 49). Besides, the antioxidant defense mechanisms also contributed. This study showed that H₂O₂, a categorized non free radical of ROS, is cytotoxic and decrease the EPCs viability start at dose 50 μM. The toxicity mechanisms of H₂O₂ to EPCs due to intracellular ROS level, increased the phosphorylation of p38 MAPK, JNK, and NF-κB, and decreased the cellular levels of BCL-2 and AP-1 (50).

Compared with HUVECs and CAECs, EPCs exhibited ≈3- to 4-fold higher expression and activity of manganese superoxide dismutase (MnSOD) and glutathione peroxidase, but not copper zinc superoxide dismutase (CuZnSOD) or catalase (51, 52). Pretreatment with GTE (25 mg/l) was able to protect the viability of H₂O₂-induced EPCs. GTE has a potent antioxidant property, and one of the active compounds is catechin. The catechin, the most abundant component in green tea, has a potent antioxidant property and is shown to be 25 to 100 times more potent than vitamins C and E (23). The GTE (25 mg/l) was able to significantly decrease ROS levels in H₂O₂-induced EPCs. The mechanism of ROS reduction maybe due to scavenging activity or upregulated intracellular antioxidant. This study confirmed previous studies that H₂O₂ may impair EPCs proliferation and induce EPCs apoptosis. Catechin may increase the capacity of EPCs for the resistance to apoptosis induced by H₂O₂ (53).

Conclusion

H₂O₂ may induce loss of EPCs viability. GTE may increase the capacity of EPCs for the resistance to loss of EPCs viability induced by H₂O₂ through decreasing reactive oxygen species intracellular.

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Conflict of interests

The authors declare no potential conflict of

interests.

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