

# Purple sweet potato extract and vitamin C increase the proliferation of endothelial progenitor cells from stable coronary artery disease patients



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## ABSTRACT

**Introduction:** Endothelial progenitor cell (EPC) numbers are reduced in stable coronary artery disease (CAD), partly due to oxidative stress. This study aimed to evaluate the effect of different antioxidants, purple sweet potato (PSP) extract and vitamin C, on EPC proliferation in stable CAD patients.

**Methods:** Peripheral blood mononuclear cells were isolated and cultivated on fibronectin-coated plates with the colony-forming unit (CFU)-Hill medium for three days. Non-adherent cells were divided into control, PSP extract (1, 5, 25 µg/ml), and vitamin C (10, 50, 250 µg/ml) groups; then cultured for two days. EPC proliferation was assessed with MTT Cell Proliferation Assay Kit. EPCs were identified by detecting the expression of CD34. Resulted CFU-Hill colonies were counted under an inverted light microscope.

**Results:** EPC proliferation was increased in low, moderate, and high doses of PSP extract and vitamin C compared to control (all groups vs. control,  $p < 0.001$ ). The high dose of PSP extract increased EPC proliferation non significantly compared to the moderate dose ( $p = 0.289$ ). Vitamin C increased EPC proliferation better than PSP extract in moderate and high dose groups ( $p = 0.042$  and  $p < 0.01$ , respectively). Meanwhile, the low dose of both treatments increased the EPC proliferation equally ( $p = 0.353$ ). CFU numbers, representing EPC differentiation capability, were highest in the groups given PSP extract compared to control and vitamin C groups.

**Conclusions:** PSP extract and vitamin C increased EPC proliferation dose-dependently. Vitamin C induced EPC proliferation better than PSP extract. Furthermore, PSP extract was presumably a better EPC differentiation inducer.

**Keywords:** Antioxidant effect; Progenitor cells; Coronary artery disease.

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## INTRODUCTION

Endothelial progenitor cells (EPCs) are peripheral blood- or bone marrow-derived mononuclear cells (MNCs) believed to play an important role in promoting the repair of blood vessels and improving reperfusion of the ischemic area.<sup>1,2</sup> Patients with stable coronary artery disease (CAD) exhibited reduced number and function of circulating EPCs which inversely correlate with the number of CAD risk factors.<sup>3</sup> Increased oxidative stress and excessive inflammation in CAD contributed to functional impairment and decreased the number of circulating EPCs.

Oxidative stress increases the generation of reactive oxygen species (ROS), which has direct and indirect cytotoxic effects on EPCs.<sup>4</sup> Oxidative stress is also involved in telomere-shortening and regulation of cellular lifespan. Several evidences suggest that decreasing oxidative stress by natural compounds with antioxidant properties may improve EPCs bioactivity.<sup>5</sup>

Anthocyanin is one among others in the list of natural compounds known to work as powerful antioxidants.<sup>6</sup> Studies revealed that purple sweet potato (PSP) is a strong *in vitro* and *in vivo* antioxidant due to the high content of anthocyanin.<sup>7</sup> Purple sweet potato extract has been proven to

increase the expression of superoxide dismutase and decrease malondialdehyde level.<sup>8,9</sup> vitamin C or ascorbic acid is well known to prevent low-density lipoprotein (LDL) oxidation and ROS scavenging and has a protective effect against endothelial dysfunction induced by peroxide lipid.<sup>10</sup> vitamin C can also increase EPC proliferation through the MEK-ERK1/2 pathway.<sup>11</sup> But *in vivo* studies showed that vitamin C has no beneficial effects in cardiovascular disease prevention.<sup>12</sup> The purpose of this study was to evaluate the effect of PSP extract and vitamin C on the proliferation of EPC from peripheral blood of stable CAD patients.

## METHODS

### Purple sweet potato extract and vitamin C preparation

Purple sweet potato extract was obtained from Balinese purple sweet potato tubers grown on the farm in Marga, Tabanan, Bali, Indonesia. The aqueous extract was made using the following protocol: PSP tubers obtained from farmers were washed with clean water then peeled off. After peeled off, the PSP tubers were cut crosswise with a thickness of 2-2.5 cm. The PSP slices were mixed with clean water using a ratio of 1:1 (1 kg of PSP plus 1 liter of water), then crushed with a blender and filtered with three layers of gauze. The liquid obtained from the filter is boiled for 30 minutes for sterilization. This PSP extract contained 146 µg/mL of anthocyanin. In this study, the PSP aqueous extract was diluted with a culture medium to achieve the concentration of anthocyanin of 1 µg/ml (low dose), 5 µg/ml (moderate dose), and 25 µg/ml (high dose).

Vitamin C used in this study was in the form of L-ascorbic acid powder (Sigma-Aldrich, USA), which was suspended in double-distilled water and diluted with culture medium to achieve the concentration of 10 µg/mL (low dose), 50 µg/ml (moderate dose) and 250 µg/mL (high dose).

### Peripheral blood collection and MNCs isolation

Peripheral blood was drawn from subjects with stable CAD. All subjects were male, aged 40–55 years old, and had significant stenosis (>50% of LMCA or >70% of other coronary arteries) based on coronary angiography. A lipid profile test was performed for baseline data. To isolate peripheral blood mononuclear cells (PBMNCs), peripheral blood was diluted with an equal amount of phosphate buffer saline (PBS) plus 2% fetal bovine serum (FBS), then layered on top of Ficoll Histopaque®-1007 (Sigma Aldrich, USA). PBMNCs layer was formed and collected after centrifugation at 800 g for 20 minutes.

### Endothelial progenitor cells culture

The collected PBMNCs were rinsed in PBS plus 2% FBS and resuspended in CFU-Hill Liquid Medium Kit (Stemcell Technologies,

Canada) to achieve the concentration of  $5 \times 10^6$  cells/ml. In each well of the 6-well plate, 1 ml of PBMNCs suspension was plated and incubated for 48 hours in humidified 37°C 5%CO<sub>2</sub> incubator. After 48 hours, non-adherent cells were harvested on 96-wells fibronectin-coated plate for cell proliferation assay and on 24-wells fibronectin-coated plate for colony-forming unit (CFU) formation. Cells were incubated for 24 hours in CFU-Hill basal medium with the addition of the supplement included in the kit as much as 1/5 of CFU-Hill basal medium volume. After 24 hours, the cells from each subject were divided into seven groups: untreated or control group (C), groups with the addition of purple sweet potato extract with an anthocyanin content of 1 µg/mL (E1), 5 µg/mL (E2), and 25 µg/mL (E3), and groups with the addition of vitamin C with the concentration of 10 µg/mL (E4), 50 µg/mL (E5), and 250 µg/mL (E6). All groups of cells were cultured for another 48 hours and followed by EPC proliferation assay, CFU quantification, and immunofluorescence assay.

### Endothelial progenitor cells proliferation assay

Endothelial progenitor cell proliferation assay was performed using MTT Cell Proliferation Assay Kit (Sigma Aldrich, USA). The assay is based on the extracellular reduction of MTT by nicotinamide adenine dinucleotide (NADH) produced in the mitochondria via transplasmic membrane electron transport and an electron mediator. MTT mixture was prepared and added into each well. Cells were then incubated in a 37°C 5% CO<sub>2</sub> incubator for 4 hours. The absorbance value which expressed the EPC proliferation was measured in optical density using a microplate reader with 450 nm wavelength.

### CFU-Hill colonies quantification

CFU-Hill colony numbers expressed the differentiation rate of EPCs. The more mature EPCs will form more colonies. CFU-Hill colony quantification was performed in low and high doses of PSP extract, low and high doses of vitamin C, and control groups on the sixth day. The CFU-Hill colonies formed in the 24-well

plate wells were documented and counted using a hemocytometer under an inverted light microscope. A single CFU-Hill colony consisted of  $\geq 15$  EPCs in a round, spindle, or cobblestone shape.

### Immunofluorescence

The cells were fixed with 3% paraformaldehyde, rinsed with PBS, and dried. Samples were incubated in 0.1% bovine serum albumin in PBS to block non-specific binding. Cells were rinsed with PBS again. FITC anti-human CD34 antibody (BioLegend, San Diego, CA) was used for antibody detection. Cells were incubated in a 37°C 5% CO<sub>2</sub> incubator for 45 minutes and then rinsed with PBS. Cells expression by CD34 was conducted under an inverted fluorescence microscope.

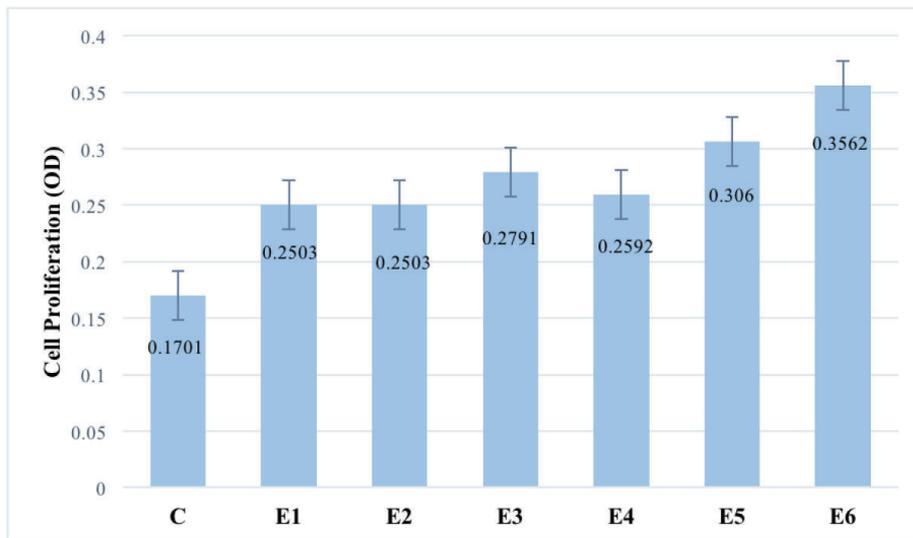
### Statistical analysis and Ethical Statement

ANOVA test followed by Least Significant Difference test was performed using SPSS for Windows version 20.0 (IBM Corp., Armonk, NY). A probability value <0.05 was considered to be statistically significant. The study protocol has been approved by Ethic Committee of RSUD Dr. Soetomo Surabaya (no. 292/Panke. KKE/IV/2016).

## RESULTS

### Endothelial Progenitor Cells Proliferation

The mean EPC proliferation (absorbance value) in control group, low (1 µg/mL), moderate (5 µg/mL) and high dose (25 µg/mL) of PSP extract is  $0.1701 \pm 0.075$ ,  $0.2503 \pm 0.053$ ,  $0.287 \pm 0.0427$  and  $0.2971 \pm 0.097$ , respectively. EPC proliferation was increased significantly and dose-dependently in low, moderate, and high doses of PSP extract compared to control (each group vs. control,  $p < 0.001$ ). Moderate and high doses of PSP extract increased EPC proliferation significantly compared to the low dose group (both  $p < 0.001$ ). However, the high dose of PSP extract increased EPC proliferation insignificantly compared to a moderate dose ( $p = 0.289$ ). The mean EPC proliferation (absorbance value) in control group, low (10 µg/mL), moderate (50 µg/mL) and high dose (250 µg/mL) of vitamin C is  $0.1701 \pm 0.075$ ,  $0.2592 \pm 0.016$ ,  $0.306 \pm$



**Figure 1.** The effect of purple sweet potato (PSP) extract and vitamin C on EPC proliferation. Cell proliferation is expressed as absorbance value in optical density (OD). C= control group (untreated); E1= low dose (1  $\mu\text{g}/\text{mL}$ ), E2= moderate dose (5  $\mu\text{g}/\text{mL}$ ), E3= high dose PSP extract (25  $\mu\text{g}/\text{mL}$ ); E4= low dose (10  $\mu\text{g}/\text{mL}$ ), E5= moderate dose (50  $\mu\text{g}/\text{mL}$ ), E6= high dose vitamin C (250  $\mu\text{g}/\text{mL}$ ). C vs. E<sub>1</sub> ( $p<0.001$ ), C vs. E<sub>2</sub> ( $p<0.001$ ), C vs. E<sub>3</sub> ( $p<0.001$ ), E<sub>1</sub> vs. E<sub>2</sub> ( $p<0.001$ ), E<sub>1</sub> vs. E<sub>3</sub> ( $p<0.001$ ), E<sub>2</sub> vs. E<sub>3</sub> ( $p=0.289$ ), C vs. E<sub>1</sub> ( $p<0.001$ ), C vs. E<sub>2</sub> ( $p<0.001$ ), C vs. E<sub>3</sub> ( $p<0.001$ ), E<sub>1</sub> vs. E<sub>2</sub> ( $p<0.001$ ), E<sub>1</sub> vs. E<sub>3</sub> ( $p<0.001$ ), E<sub>2</sub> vs. E<sub>3</sub> ( $p<0.001$ ), E1 vs. E4 ( $p=0.353$ ), E2 vs. E5 ( $p=0.042$ ), and E3 vs. E6 ( $p<0.001$ ).

0.223 and  $0.3562 \pm 0.023$ , respectively. EPC proliferation was increased significantly and dose-dependently in low, moderate, and high doses of vitamin C compared to control (each group vs. control,  $p<0.001$ ). EPC proliferation was also increased significantly in low doses compared to moderate and high doses and moderate to high dose of vitamin C (all  $p<0.01$ ). Comparing mean EPC proliferation in low, moderate, or high dose of PSP extract vs. vitamin C revealed that both low dose groups exhibited similar cell proliferation ( $0.250$  vs.  $0.259$ ;  $p=0.353$ ). In comparison, moderate and high doses of vitamin C increased the EPC proliferation more significantly compared to moderate and high doses of PSP extract ( $0.287$  vs.  $0.307$ ;  $p=0.042$  and  $0.2971$  vs.  $0.3526$ ;  $p<0.001$ , respectively) (Fig. 1).

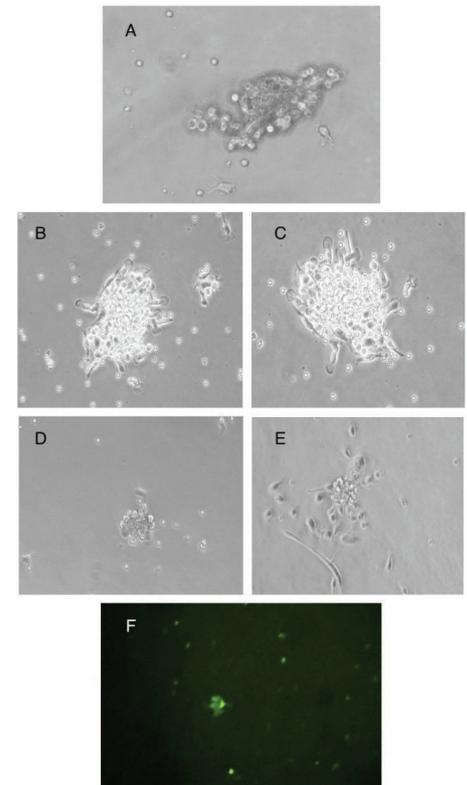
#### CFU-Hill Colonies Quantification

CFU-Hill colony number represents the EPC differentiation capability as a part of functional confirmation. CFU-Hill colony number ( $/10^6$  EPCs) for the control group, low dose (E1) and high dose of

PSP extract (E3), low dose (E4), and high dose of vitamin C (E6) are respectively 34, 82, 140, 10, and 99 colonies. Figure 2A-E showed the microscopic images of CFU-Hill colonies in each group on the sixth day of observation. Immunofluorescence assay with EPCs membrane markers of CD34 (Alexa Fluor 488) was conducted to confirm the EPCs. EPCs were demonstrated as colonies under a light microscope (Fig. 2F). CFU-Hill colony numbers/ $10^6$  EPCs on a low and high dose of PSP extract and vitamin C groups were seen in Figure 3.

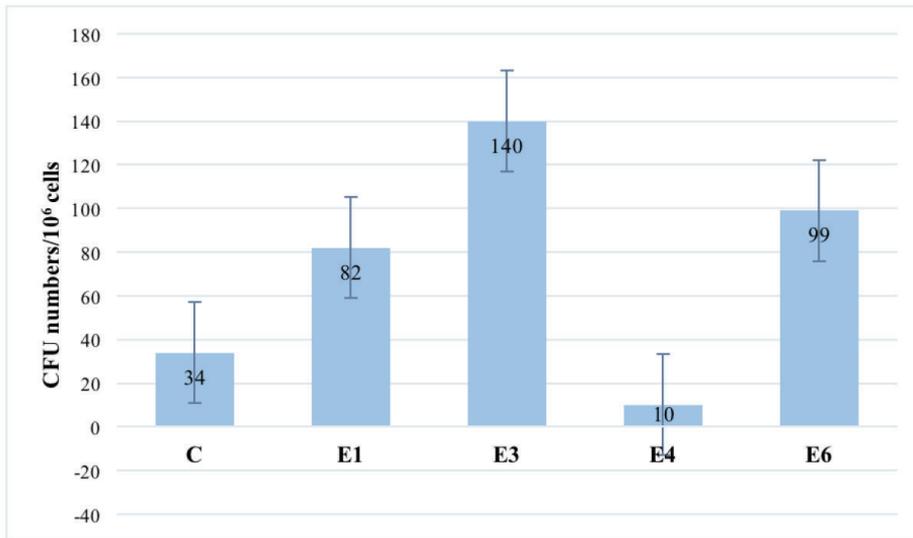
#### DISCUSSION

The present study demonstrated that PSP extract and vitamin C increased EPC proliferation from stable CAD patients' peripheral blood compared to the control. Both antioxidants increased cell proliferation in a dose-dependent manner. A moderate dose of PSP extract showed significantly increased proliferation compared to a low dose. Although the high dose of PSP extract (25  $\mu\text{g}/\text{mL}$ )



**Figure 2.** CFU-Hill colonies. CFU-Hill colonies in the control group on the 6th-day observation (A). CFU-Hill colonies in low dose (B) and high dose (C) purple sweet potato extract groups on the 6th-day observation. CFU-Hill colonies in low dose (D) and high dose (E) vitamin C groups on the 6th-day observation. EPCs were marked by the expression of CD34 (green) (F).

increased cell proliferation more than the moderate dose, the difference was not significant. Thus, it appears that only a small dose of PSP extract is needed to affect EPCs number, while a higher dose has less effect on EPCs number. A biphasic action on EPCs where low doses promote antioxidant effects while higher doses showed pro-oxidant role followed by cell damage and apoptosis had been reported for several compounds including statins, thiazolidinediones, insulin, and resveratrol.<sup>13-15</sup> Moderate dose used in this study was 5  $\mu\text{g}/\text{mL}$ , which is the standard dose in the previous in vitro study.<sup>9</sup> This dose is possibly the optimal dose for EPCs



**Figure 3.** CFU numbers/10<sup>6</sup> EPCs on low and high dose of purple sweet potato (PSP) extract and vitamin C groups. C= control group (untreated); E1= low dose (1 µg/mL) and E3= high dose (25 µg/mL) PSP extract; E4= low dose (10 µg/mL) and E6= high dose (250 µg/mL) vitamin C. CFU = colony forming unit.

proliferation. This is the first study of the Balinese PSP extract effect on EPCs proliferation. This variety of purple sweet potato has a high content of anthocyanin (110-210 mg/100 g potato) compared to other variants of PSP (0.7-74.3 mg/100 g potato).<sup>16</sup> Previous studies demonstrated that anthocyanin from various food compounds or fruits is a potent antioxidant both tested *in vitro* and *in vivo*.<sup>8,9</sup> Chokeberry extracts improve EPCs proliferation by reducing intracellular ROS level, improving telomerase activity, and preventing cell senescence.<sup>17</sup> Grape juice activates PI3K/Akt/eNOS pathway, which prevents cell apoptosis.<sup>18</sup> Based on the study results, anthocyanin in PSP extract could improve EPCs proliferation from stable CAD patients, possibly by protecting the EPCs against oxidative stress and preventing cell senescence and apoptosis.

This study showed similar results to other *in vitro* studies regarding the effect of vitamin C on EPCs. The EPCs proliferation was increased significantly in cells treated with vitamin C in a dose-dependent manner. The low, moderate, and high doses of vitamin C has a significant difference in EPCs proliferation. Thus, we can assume that vitamin C has a broader dosage range compared to purple sweet potato extract. There has been a controversy of vitamin

C between *in vitro* and clinical studies. *In vitro* studies showed that vitamin C prevented LDL oxidation and enhanced eNOS coupling, and when combined with vitamin E, could improve TNF-α induced EPC reduction.<sup>19,20</sup> On the other hand, clinical studies revealed that vitamin C has no beneficial effect in reducing long-term cardiovascular disease risk or events.<sup>21,22</sup>

The EPCs proliferation was insignificantly different between low doses of PSP extract and vitamin C, while moderate and high doses of vitamin C increased EPC proliferation significantly more than the moderate and high doses of PSP extract. This shows that both antioxidants have a similar ability to improve EPC numbers in low doses. Still, as the dose is increased, vitamin C improves EPCs proliferation more than PSP extract does. This result showed that vitamin C is a better EPCs proliferation inducer compared to PSP extract.

It is interesting to observe the effect of both antioxidants on CFU-Hill colony counts that were inversely related to EPCs proliferation in this study. CFU-Hill colony number represents the capability of EPCs to differentiate into mature endothelial cells. In this study, a low dose of vitamin C produced lower CFU-Hill colony numbers than control, while high dose vitamin C increased the CFU-Hill

colony numbers steeply more than low dose and control. Although exhibiting lower EPCs proliferation compared to vitamin C, both low and high dose of PSP extract groups had higher CFU-Hill colony numbers. This particular result might be due to different antioxidant mechanisms between anthocyanin in PSP and vitamin C. It has been suggested that anthocyanin activates signaling transduction pathways that regulate cell proliferation, migration, differentiation, and survival.<sup>6</sup> Meanwhile, vitamin C is mainly an electron donor that exerts ROS scavenging action and prevents cell apoptosis instead of increasing cell proliferation.<sup>23</sup> Although the low vitamin C (0-100 µg/mL) had proven to prevent apoptosis induced by oxidized LDL, a higher physiological intracellular concentration of vitamin C (230-440 µg/mL) is needed to prevent the binding between superoxide ion and nitric oxide.<sup>24,25</sup> In this study, low dose vitamin C (10 µg/mL) has exerted an anti-apoptosis effect, thus increase EPCs proliferation. Still, a higher dose (more than 250 µg/mL) might be needed in order to produce functional EPCs that formed the CFU-Hill colonies. We can assume that PSP extract is a better cell differentiation inducer with a similar cell proliferation rate compared to vitamin C.

## CONCLUSION

Purple sweet potato extract and vitamin C increased the proliferation of EPCs from peripheral blood of stable CAD patients dose-dependently. Both low doses of PSP extract and vitamin C show a similar level of EPC proliferation. However, the moderate and high doses of vitamin C increased EPC proliferation significantly more than moderate and high doses of PSP extract. Thus, vitamin C induced EPC proliferation better than PSP extract. Furthermore, PSP extract is presumably a better EPC differentiation inducer due to higher CFU numbers in low and high dose of PSP extract compared to vitamin C and control groups. However, the underlying mechanism or pathway in which both antioxidants increased EPC proliferation should be further investigated. An experiment with broader dosage range is also needed to determine the optimal and toxic dose of each antioxidant.

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## CONFLICT OF INTEREST

The authors declared there is no conflict of interest.

## AUTHORS CONTRIBUTION

All authors contribute equally in the study.

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