

# Anthocyanin-rich Buni-berry (*Antidesma bunius*) Extract Increases Paraoxonase 1 Gene Expression in BALB/c Mice Fed with a High-fat Diet

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## ABSTRACT

**Objective:** The aim of this study was to determine the effectiveness of buni-berry (*Antidesma bunius*) extract to increase PON1 mRNA expression in BALB/c mice fed with a high-fat diet. **Methods:** Twenty, thirty-weeks old male BALB/c mice were randomly allocated into four intervention groups; buni berry extract, simvastatin, control-sick and control-normal groups. All groups received high fat diet, except control-normal group which received control normal-diet. The diets were provided for 12 weeks. Treatments were started in all groups at the same time with the administration of the diets. Mice in buni-berry group were treated with oral buni-berry 300 mg/kgBW/day. In simvastatin group the mice were provided with oral simvastatin 6 mg/kgBW/day. The mice in control-sick and control-normal group were not received additional treatment. The intervention was conducted in 12 weeks. Mice body weight was measured weekly. Blood samples from tails were examined at the baseline and after 12 weeks intervention for PON1 mRNA expression. Expression of PON1 mRNA was analysed by *quantitative Real Time Polymerase Chain Reaction (qRT-PCR)*.

**Results:** The changes mice body weights during interventions in all groups were not significantly different. PON1 mRNA expression was significantly increase in the buni berry extract group and simvastatin group, while in control-sick group the expression was significantly reduced. **Conclusion:** Oral administration of 300 mg/kgBW/day of buni-berry extract is effectively increases PON1 expression in BALB/c mice fed with a high fat diet.

**Key words:** Buni-berry extract, *Antidesma bunius*, PON1 mRNA, Atherosclerosis, High fat diet.

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## INTRODUCTION

Berries are known as fruits with high anthocyanins content and other polyphenolic compounds that is associated with cardiovascular disease prevention.<sup>1</sup> Phenolic compounds in berries particularly anthocyanins were found to be effective in the reduction of atherosclerosis process that causes coronary heart disease through inhibition of LDL oxidation and increases HDL efflux.<sup>2,3</sup> Buni - berries (*Antidesma bunius*) is a berry-fruit that is native to Southeast Asia and Northern Australia. The fruit in Indonesia is known as *buni*, *whoonee*, *bunne* and many other traditional names. The dark-purple ripe fruits are rich in phenolic compounds particularly flavonoid anthocyanins and high antioxidant capacity.<sup>4,5</sup> Paraoxonase 1 (PON1) is an HDL associated enzyme, that has recently drawn researchers attention on its roles on the prevention of the development and process of atherosclerosis.<sup>6</sup> Studies by Litvinov *et al.*<sup>7</sup> and Lusis *et al.*<sup>8</sup> have revealed that PON1 as protein and hydrolyzing enzyme responsible for the most of the antioxidant properties of HDL which prevent LDL and HDL from oxidation. Martini *et al.*<sup>9</sup> reported in their review that polyphenolic compounds from fruits and other part of plants can increase expression of PON1 mRNA and concentration of PON1 enzyme. The aim of our study was to demonstrate whether buni-berry

(*Antidesma bunius*) extract can increase mRNA expression of PON1 in balb/c mice fed with a high-fat diet.

## MATERIALS AND METHODS

### Animals

Male BALB/c mice aged 6 weeks (15-20 gr) were obtained from Unit Maintenance and Development of Animal Trial Molecular Biology and Immunology Laboratory Faculty of Medicine, Hasanuddin University, Makassar. All the mice were caged in groups of six under environmentally controlled room with 12 h light/dark cycle and provide standard rodent diet and water ad libitum until further 30 weeks of age. The 30 weeks old mice were then randomly allocated into the four experiment groups that matched for body weight (Table 3).

### Diets

Standard diet (control diet) D12102 and high fat diet Clinton/Cybulsky D12109 used, were purchased from Research Diet Inc. USA. The control diet D12102 contained 10% calory from fat. The high fat diet contained 40% calory from fat mainly from cocoa butter with 1.25% cholesterol (w/w)

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**Table 1: The composition of Clinton/ Cybulsky rodent diet D12102C and D12109C.**

CONTENT	D12102C (CONTROL DIET)		D12109C (HIGH FAT DIET)	
	Gram	Cal (%)	gram	Cal (%)
Protein	19	20	23	20
Carbohydrate	67	70	45	40
Fat	4	10	20	40
Total		100		100
Kcal/gram	3.85		4.5	
Ingredients	Gram	Kcal	Gram	Kcal
Casein, Lactic	200	800	200	800
L-cystein	3	12	3	12
Corn starch	375	1500	212	848
Maltodextrin 10	125	500	71	284
Sucrose	200	800	113	452
Cellulose,BW200	50	0	50	0
Soybean oil	25	225	25	225
Cocoa butter	20	180	155	1395
Mineral mix S10021	10	0	10	0
Dicalcium phosphate	13	0	13	0
Calcium carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
Cholesterol	0	0	11.25	0
Sodium cholate	0	0	4.5	0
Red Dye, FD&C#40	0	0	0.05	0
Blue Dye,FD&C#1	0	0	0.05	0
Yellow Dye, FD&C#5	0.1	0	0	0
TOTAL	1055.1	4057	901.85	4056

Source: Research Diet Inc. USA, 2017

and 0.5% cholic acid (w/w).<sup>10</sup> The detail composition of the diets used is presented in Table 1.

#### Plant material

Overripped, black purple *A. bunius* were obtained from local farm in District of Malino, South Sulawesi, Indonesia. The berries were then cleaned, washed and packaged in plastic bags and store in freezer at -20°C until used.

#### Chemicals and Drugs

Deionized-water was purchased from OneMed Laboratories Inc. Indonesia. Ascorbic, citric and gallic acid, Folin-Ciocalteu reagent, methanol, ethanol, acetone, sodium carbonate, potassium chloride, sodium acetate, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were procured from Merck, Germany. Sodium carboxymethyl cellulose, ethylene-diamine-tetra-acetic acid (EDTA) and Tris-HCl were obtained from Sigma Aldrich, Switzerland. Guanidium thiocyanate was from Fluka Chemie, Switzerland. TritonX-100 was from Roche, Germany. Celite was purchased from Jansen Chimica, Belgium. Enzyme Superscript II reverse transcriptase was purchased from Invitrogen, USA. SYBR Green qRT-PCR Supermix

was purchased from Bio-Rad, USA. Simvastatin was obtained from Kimia Farma, Pharmaceuticals, Indonesia. All other chemicals were of analytical grade.

#### Preparation of buni-berry (*Antidesma bunius*) extract

The frozen berries were then extracted with a 70% ethanol acidified with 0.01% citric acid at pH of 4.9 and put in a room with temperature of 26°C for about 6 hrs.<sup>11</sup> The extracts were then filtered on Whatman 415 paper and concentrated using vacuum rotary evaporator (Buchi, Switzerland) until all alcoholic residues were removed.

#### Buni-berry extract analysis

##### Total phenols

The extract then was analysed for total phenolic content using Folin-Ciocalteu method.<sup>12</sup> Briefly, A volume of 0.5 mL of the extract (100 µg/mL) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min. The absorbance was measured at 765 nm. The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of extract.

##### Total anthocyanins

Total anthocyanins content (TAC) of the buni-berry extract was measured using pH differential method.<sup>13</sup> Two samples of extract were weighed to 0.05 g each. The first sample was mixed with 0.025 M potassium chloride buffer (pH 1.0) and the other sample with 0.4 M sodium acetate buffer pH 4.5. Following 2 hrs of incubation at room temperature (~25°C), the absorbance for each sample was then read versus the buffer solution pH 1.0 and buffer solution pH 4.5 as the blank at  $\lambda = 510$  nm (for the cyanidin 3-glucoside) and  $\lambda = 700$  nm (for correction factor). The absorbance values were calculated using the equation:

$$A = \{(A_{516-A700})_{\text{pH 1}} - (A_{516-A700})_{\text{pH 4.5}}\}$$

TAC was expressed as cyanidin-3-glucoside (% w/w) equivalents, as follows:

$$\text{TAC} \left( \% \frac{W}{W} \right) = \frac{A}{\epsilon_l} \times MW \times DF \times \frac{W}{Wt} \times 100\%$$

where, A= absorbance value; MW (molecular weight) = 449.2 g.mol<sup>-1</sup> for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor; V = final volume after dilution; W = sample weight (mg); l = width of the cuvette (cm);  $\epsilon = 26,900$  M extinction coefficient in L mol<sup>-1</sup> cm<sup>-1</sup> for cyd-3-glu.

##### Antioxidant capacity

The antioxidant capacity of buni-berry extract and of the standard solution (ascorbic acid) were measured using 1,1,-diphenyl-2-picrylhydrazyl (DPPH) method.<sup>12,14</sup> The mixture of 2 mL of 1.0 mmol/L DPPH solution in methanol and 1 mL of standard solution of extract solution with different concentration (10-500µg/mL). The mixture solution incubated in dark at 37°C for 20 min. The decrease in absorbance of each solution was measured at 517 nm. Ascorbic acid used as positive control while DPPH radical solution was taken as blank. The percentage of radical scavenging activity was measured using formula:

$$\% \text{ free radical scavenging activity} = \frac{\text{blank absorbancy} - \text{sample absorbancy}}{\text{blank absorbancy}} \times 100\%$$

The concentration of sample required to neutralize 50% of DPPH (IC<sub>50</sub>) was determined using the curve of percent inhibitions plotted against the respective concentration.

### Intervention

After acclimatization for the new environment (individual cage and new diets), the 30 weeks male BALB/c mice were randomly allocated to four intervention groups with matched body weight. (1) Buni-berry extract (treatment), (2) Simvastatin (control positif), (3) Control sick and (4) Control normal. Each intervention group consisted of five mice.

The mice in the first three intervention groups were fed with a high-fat diet (D12109C), while the mice in control-normal group were fed with control diet (D12102C).

The intervention was conducted for 12 weeks as follows: Every mouse was put in a different individual cage and had free access to diet provided and drinking water. Each mouse in the buni-berry extract group and the simvastatin group respectively received buni-berry extract 300 mg/kgBW/day<sup>15</sup> and simvastatin 6 mg/kgBW/day.<sup>16</sup> Each, the extract and simvastatin was separately dissolved in sodium carboxymethyl cellulose 5% solution and delivered orally using ball tip needle to avoid damage in the esophagus or passing through the respiratory tract. In the control-sick group and control-normal group, the mice did not receive additional intervention except the high-fat diet and control-normal diet, respectively.

All mice were weighted weekly, provided and leftover food weighted daily. Blood samples were withdrawn from mouse's tail at baseline (before the intervention was started) and from cardiac puncture at the end of the intervention.

### Nucleic acid extraction

Extraction of nucleic acid was extracted according to the protocol of previous study.<sup>17</sup> Briefly, lysing, binding and washing were three main steps of this method. One hundred µl of blood sample was dissolved into "L6" solution which is developed from 120 g guanidinium thiocyanate in 100 mL 0.1 M Tris-HCl pH 6.4, 22 mL EDTA pH 8.0 and 2.6 g Triton X-100. After lysing process the nucleic acid was bound to celitein 50 mL H<sub>2</sub>O and 500 µL of 32% (w/v). The solution then was vortexed and centrifuged in effendorf tube 1.5 ml at 13,000 for 15 sec. The supernatant was discarded and the sediment left then was washed with 1 ml "L2" solution (guanidinium thiocyanate and Tris HCl pH 6.4). The mixture then was vortexed and centrifuged at 13,000 rpm for 15 sec, this washing process was repeated twice and continued with 1 mL ethanol 70% twice and the last with 1 mL acetone. The result then was incubated in a water-bath at 56°C for 10 minutes. After incubation the sample was added with 60 µL "TE" solution (1mM EDTA in 10 mM Tris-HCl pH 8.0) then vortexed and centrifuged at 13,000 rpm for 2 min and incubated in oven for 10 minutes at 56°C. Following the incubation, the sample was again vortexed and centrifuged at 13,000 rpm for 30 sec. The supernatant of this process was obtained and stored at -80°C before RNA analysis.

### Analysis of Paraoxonase 1 (PON1) mRNA expression

PON1 mRNA expression was analysed with Real Time-Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) using SYBR Green qRT-PCR Supermix (Bio-Rad, USA) and read using RT-PCR machine (BR004129USA, Bio-Rad Laboratories, USA). The following forward and reverse primer were used for PON1: TTCCTTTGTACACAGCAGCG and TGCTGGCTCACAGATTC respectively<sup>18</sup> For housekeeping gene β-actin forward primer was CTCTGGCTCCTAGCACCATGAAGA and reverse primer was GTAA AACGCAGCTCAGTAACAGTCCG.<sup>19-20</sup> cDNA templates for use in real time PCR were synthesized from 5 µg of total RNA by *in vitro* transcription in 20 µl reaction containing 0.5 µg Oligo (dT), 10 µM dNTPs and

1 µl of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 42°C for 50 min. Typical real time PCR reaction mixture included the same amount of cDNA templates from RT, 10 pmol of each primers, 25 µl iQ SYBR Green Supermix and sterile water in a reaction volume of 50 µl. The PCR conditions were: 3 min at 95°C followed by 40 cycles at 95°C for 30 sec, 55°C for 30 seconds and 68°C for 1 min. Relative PON1 gene expression levels were calculated by subtracting the threshold cycle number (Ct) of the β-Actin gene from the Ct of PON1 and raising 2 to the power of this difference. Ct values are defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold.<sup>18</sup>

### Statistical Analysis

The results are presented as mean ± SD (standard deviation). The differences between groups are analysed using paired t-test or ANOVA with p-value less than 0.05 is considered significant

### Ethics Statement

The animals were treated following the principles and protocols of The Declaration of Helsinki. The trial was approved by The Committee on the Ethics of Medical Research of University Hasanuddin (Recommendation number: 597/H4.8.4.5.31/PP36-KOMETIK/2017) dated August, 23, 2017.

## RESULTS

### The Extract Profiles

The concentration of total phenolic compounds is 3.42 % and total anthocyanins is 0.83% (Table 2). It means, the extract contained not only anthocyanin, but also other polyphenolic compounds that made up to 3.42%.

The extract showed the ability to neutralize free radicals with antioxidant capacity of 56.46 ppm in term of IC<sub>50</sub> (Table 2). Inhibition concentration 50 (IC<sub>50</sub>) means that the minimal concentration of the extract that can neutralize at least 50% of the free radicals of 1, 1-diphenyl-2-picrylhydrazyl (DPPH). IC<sub>50</sub> of less than 50 ppm is considered as very strong antioxidant capacity, while between 50 to 100 ppm is considered strong.

### The mice body weight

During 12 weeks intervention the mortality rate of the BALB/c mice was 2/28. The average weight of mice at the initial intervention and the change of body weight during intervention in four different groups were not significantly different (Table 3).

### Expression of PON1 mRNA

The mRNA expression of PON1 enzyme significantly increased in buni berries extract group and simvastatin group (Table 4). On the other hand the expression of PON1 mRNA reduced in the group that was fed with a high fat diet without any intervention (control-sick). In the normal diet (control-normal) group, there was not any significant change in the mRNA expression of paraoxonase 1 (PON1) enzyme.

**Table 2: Buni Fruit (*Antidesma bunius*) extract profiles.**

Buni Berry Extract (BBE) profile	Result	Unit	Measurement method
Total phenols	3.42	%	Folin-Ciocalteu
Total anthocyanins	0.83	%	pH differential
Antioxidant capacity (Radical scavenging activity)	56.46*	ppm	DPPH

\*IC<sub>50</sub> (Concentration of the extract required to scavenge 50% of the free radical DPPH)

**Table 3: Body weight of mice at the baseline and after 12 weeks intervention.**

Groups*(Each group n = 5)	Body weight (g)		p-value
	Baseline	12 weeks	
Buni berry extract	36.40 ± 2.99	36.14 ± 3.80	0.5 <sup>c</sup>
Simvastatin	37.82 ± 2.99	37.14 ± 2.71	0.5 <sup>d</sup>
Control-sick	38.02 ± 1.09	37.36 ± 2.75	0.6 <sup>f</sup>
Control-normal	37.86 ± 2.75	41.66 ± 3.93	0.4 <sup>g</sup>
p-value	0.8 <sup>a</sup>	0.08 <sup>b</sup>	

\*Values are means ±SD n = 5, p-value < 0.05 is considered significant; a=p-value of the different of means body weight at baseline across four intervention groups using ANOVA; b= p-value of the different of means body weight at 12 weeks intervention using ANOVA; c,d,f,g = p-value of the different of means body weight between baseline and 12 weeks intervention using paired t test.

**Table 4: Changes of PON1 mRNA expression at baseline and after 12 weeks intervention in the four different intervention groups.**

Groups (each group n = 5)	PON1 mRNA expression				p-value
	Baseline	12 Weeks	Mean difference	95% Confidence Interval	
Buni berry extract	6.24 ± 0.16	6.72 ± 0.27	0.48	0.07 ; 0.90	< 0.05
Simvastatin	6.33 ± 0.19	6.83 ± 0.25	0.5	0.05 ; 0.92	< 0.05
Control-sick	6.24 ± 0.13	4.81 ± 0.30	-1.43	-1.73 ; -1.13	< 0.001
Control-normal	6.25 ± 0.14	6.27 ± 0.15	0.02	-0.11 ; 0.14	0.7

\*Values are means ±SD n = 5, p-value < 0.05 is considered significant

## DISCUSSION

The anthocyanins and total phenolic content of 0.83% and 3.42% respectively, is in accordance with previous reports by Butkhuip and Samappito.<sup>4</sup> The buni-berry extract that was used in this study contains anthocyanins and other polyphenolic compounds. As what have been reported by previous studies, anthocyanins and other polyphenolic compounds from fruit demonstrated the ability to prevent atherosclerosis process through improving the expressions and concentrations of markers associated with atherosclerosis process.<sup>2,3</sup> The present study shows that buni berry extract significantly increased paraoxonase (PON1) mRNA expression. Our study result supports the finding of previous research by Kivici *et al.* using different fruit extract.<sup>20</sup> The research demonstrated that the administration of grape seed extract (GSE) at the dose of 100 mg/kgBW/day, orally, in streptozotocin-induced diabetic rat, for six weeks caused a significant increase in PON1 activity compared to the diabetic mice without GSE supplementation. Furthermore, more recent study by Estrada-Luna *et al.* also reported that another kind of fruit juice (pomegranate juice) supplementation at the dose of 300 µl/ day that contained 0.35 mmol polyphenols, orally in mice fed with a high fat diet for five months, resulted in the increase expression and activity of PON1.<sup>21</sup>

In contrast, El-Beshbishy *et al.*<sup>22</sup> failed to demonstrate a modulation on serum PON1 activity in rats supplemented with *Morus alba* (Egyptian mulberry) root bark extract at the dose of 500 mg/kg/day for 2 weeks. However, the positive result from the study is that the extract protected LDL from oxidation. This might be a direct effect of polyphenols on LDL itself and was not mediated by PON1 activity. Schrader *et al.*<sup>23</sup> also

reported that diet enriched with an isoflavone compound, genistein (2 g/ kg diet) which was provided for three weeks to male Wistar rats did not increase PON1 activity. The contrary results from the two studies may be because of the short duration of the intervention. The effect of polyphenols on PON expression and activity could effectively be detected if the duration of the intervention is at least 12 weeks.<sup>9</sup>

In our study, the effect that was resulted from the buni berry extract on PON1 expression was comparable to those with simvastatin, a typical lowering cholesterol drug. Statins modulate expression of PON1 gene through the interaction of sterol regulatory element binding protein (SREBP-2) with Sp1<sup>24</sup> and mitogen-activated protein kinase (MAPK) signaling cascade.<sup>25</sup>

Paraoxonase 1 is an HDL associated enzyme that is believed to have the major role in the prevention of atherosclerosis process.<sup>6,7</sup> Polyphenolic compounds in the buni berry extract mainly anthocyanins and other polyphenolic compounds might be responsible for the effect to increase expression of PON1 mRNA. Several study in animals and human have provided evidences that polyphenolic compounds from several different fruit and plant components have increased PON1 mRNA expression and PON1 concentration.<sup>9</sup>

In daily life, free radicals from internal body metabolisms and from outer environments can cause lipid oxidation as the initial process of atherosclerosis. PON 1 enzyme as a protein component of HDL prevents HDL and LDL from oxidation.<sup>6</sup> Through research, pathways have been postulated to explain how polyphenolic compounds can increase the expression of PON1 mRNA. Gouedard *et al.* showed that PON1 gene expression increased by an aryl hydrocarbon receptor-dependent mechanism,<sup>26</sup> whereas study by Khateeb *et al.* reported that pomegranate juice increased PON1 expression through PPAR-γ pathway.<sup>27</sup>

Another result from our study is that, mice feed with a high fat diet without any other intervention showed a very significant reduction in the expression of PON1 expression. This result is in agreement with the research conducted by Thomas-Moya *et al.* in whistar rat which showed that a high fat diet reduced the expression of PON1 mRNA and activity.<sup>28</sup> Another research also reported that pro-atherosclerotic diet in rabbit reduced PON1 activity.<sup>29</sup>

The novelty of our research is that, it was the first intervention study that utilized the potential benefit of buni-berry (*Antidesma bunius*) extract in prevention of atherosclerosis process through increasing PON1 expression as an HDL associated enzyme which has the major roles in prevention and retarding lipid oxidation process. The limitation of our study is that, the intervention was performed in animal model, therefore, further research in human is recommended to determine the actual benefit of the buni-berry (*Antidesma bunius*) extract in prevention and retarding atherosclerosis process.

## CONCLUSION

Oral administration of 300 mg buni-berry (*Antidesma bunius*) extract per kg body-weight per day in 12 consecutive weeks effectively increases PON1 expression in BALB/c mice fed with a high-fat diet. Our result supports previous evidence that fruit extract with anthocyanin content and other polyphenolic compounds might effectively be used to prevent and decelerate atherosclerosis process through promoting PON1 mRNA expression and activity as an HDL associated enzyme which contributes to the prevention of lipid oxidation as the initial process of atherosclerosis. Further trials in human are needed to strengthen the evidence.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## ABBREVIATIONS

**PON1:** Paraoxonase 1; **HDL:** High Density Lipoprotein; **LDL:** Low Density Lipoprotein; **mRNA:** messenger Ribonucleic Acid.

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