ANALYSIS OF APOLIPOPROTEIN-B (APO-B) GENE IN ATHEROSCLEROSIS MICE GIVEN CURCUMINOID EXTRACT OF ZANTHORRIZA IN ORAL

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ABSTRACT

The aim of this study was to investigate the apolipoprotein-B (apo-B) gene in atherosclerosis mice which were orally given curcuminoid extract of *Curcuma xanthorriza*. A total number of 30 white mice were split into 6 groups, the first group considered as control (without any treatment), second group as atherogenic feed control, the third group as extract control, while the fourth, fifth and sixth groups as atherogenic feed and curcuminoid *Curcuma xanthorriza* extract group treated with 5 mg/mouse, 10 mg/mouse and 15 mg/ mouse, respectively for three months. The blood samples were taken from all six groups for the deoxyribonucleic acid (DNA) analysis using total DNA isolation, DNA amplification with polymerase chain reaction (PCR), and DNA sequencing. The data analysis showed that 374 bp nucleotide sequence gen of apo-B from *Rattus norvegicus* in groups B, C, D, E, and F did not cause any changes in genes. The analysis showed the sequence of apo-B *Rattus norvegicus* gene in the treatment group was apparently identical with that of *Rattus norvegicus* group A as the control group without treatment. As conclusion, the administration of curcuminoid zanthorrizza to atherosclerosis mice did not change the gene structure of apo-B 100.

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Key words: atherogenic feed, curcuminoid zanthorriza extract apo-B gene, sequencing

ABSTRAK

Tujuan dari penelitian ini untuk mengkaji gambaran gen apolipoprotein-B (apo-B) pada tikus penderita arterosklerosis yang diberi ekstrak kurkuminoid. Sebanyak 30 ekor tikus putih dibagi menjadi 6 kelompok, kelompok pertama sebagai kontrol tanpa perlakuan apapun, kelompok 2 sebagai kontrol pakan aterogenik, kelompok 3 kontrol ekstrak, dan 4, 5, dan 6 masing-masing diberi pakan aterogenik dan diberi kurkumonoid yang berbeda kosentrasinya (5 mg/ekor, 10 mg/ekor dan 15 mg/ekor) selama 3 bulan. Sampel darah diambil dari 6 kelompok untuk dianalisis deoxyribonucleic acid (DNA) *yang terdiri dari isolasi total DNA, amplifikasi DNA dengan* polymerase chain reaction (PCR)*, dan pengurutan DNA. Hasil penelitian menunjukkan bahwa gen apo-B pada urutan nukleotida 374 bp dari Rattus norvegicus kelompok B, C, D, E, dan F tidak menyebabkan perubahan pada gen tersebut. Hal ini didukung analisis yang menunjukkan bahawa sekuen gen apo-B Rattus norvegicus pada perlakuan identik dengan Rattus norvegicus kelompok A sebagai kontrol tanpa perlakuan.*

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Kata kunci: pakan aterogenik, ekstrak temulawak gen apo-B, pengurutan

INTRODUCTION

Fatty acids enter the blood vessels in the body in the form of lipoprotein particles, especially, low-density lipoprotein (LDL). Apolipoprotein-B (apo-B) 100 plays an important role in atherosclerosis. The interaction between apo-B 100 and the artery walls causes the LDL particles to be retained inside the interior of artery walls. These retained apo-B lipoprotein particles increase the risk of atherosclerosis when the lipoprotein particles are converted into harmful particles that increase the risk of coronary heart disease (CHD).

Currently, herbal medicines have been massively developed from the herb plants that easily grow in Indonesia. Indonesia is rich with its biodiversity worth exploiting. Turmeric (*Curcuma longa*) has been grown as an ingredient of traditional medicine for centuries. Its drug potency is due to its proven anti-inflammatory and anti-germ substances (Tonnesen *et al.*, 1987) and anti-rheumatism benefits (Kiso *et al.*, 1983). Even turmeric is believed to have anti-tumor and antioxidant potency with its active substances called curcuminoid and flavonoids. Based on this condition, the researchers intended to study how much the effect of curcuminoid extract of Javanese turmeric can inhibit (suppress) the formation of atherosclerosis plaque in mice fed with atherogenic feed.

Javanese turmeric (*Curcuma xanthorrhiza* Roxb) is now massively grown as medicinal plants. Curcumin is the active substance contained in this plant type of Zingiberacea. Chemically, curcuminoids are diferuloylmethane derivatives comprising demethoxy diferuloylmethane (curcumin) and monodesmethoxy diferuloylmethane (desmethoxy-curcumin). Curcumin has a molecular formula of $C_{21}H_{20}O_6$ with a molecular weight of 368 bp. Currcumin is yellow and easily turns brownish because of sunlight (Quiles *et al.*, 2002; Sreejayan and Rao, 1997). Curcumin is stable below pH 6.5 and will change its structure when it is above pH 6.5. Other types of curcumin are bisdesmethoxy-curcumin and desmethoxy-curcumin.

Based on the curcuminoid structure, the methoxy group presented in bis-desmetoxy-curcumin is replaced by a hydrogen atom. The phenolic group is thought to function as antibacterial. Accordingly, the curcumin has the ability to eliminate the oxygen radical derivatives presented in the media and is responsible for lipid peroxidation in the cells. This phenolic group is essential for superoxide scavenger and the presence of ortho methoxy group in the phenolic molecules will increase the activity of curcumin (Rao 1995; Sreejayan and Rao, 1997).

The oxidized LDL molecules stimulate the scavenger receptors from the macrophages to capture the LDL continuously to form the foam cells. According to the theory found by Steinberg (1993), atherosclerotic lesions are initiated by the oxidation of LDL resulting in endothelial expression of monocyte

attachment and resulting in monocyte chemotactic protein (MCP), macrophage colony stimulating factor (M-CSF). Such induction causes monocytes to turn into macrophages and attach to the endothelium. Furthermore, the macrophages phagocytosis the oxidized LDL which accumulates in the blood vessel walls, forming foam cells and finally forming an early lesion known as fatty streak. The oxidized LDL also stimulates growth factors such as platelet ground factor derivate (PGDF) resulting in the proliferation and migration of smooth muscle cells of the blood vessels. The oxidized LDL also stimulates the formation of free radicals, thus aggravating the process of atherosclerosis.

High cholesterol in blood, such as total serum cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), apolipoprotein (apo-B), or low levels of high-density lipoprotein (HDL-C) and apo-A1, is a sign of the risk factors for coronary heart diseases. There are two main forms of apo-B, namely, apo-B 48 and apo-B 100. Apo-B 48 is synthesized mainly by the small intestine. Apo-B 100 is an apolipoprotein found in lipoproteins synthesized by the liver (Law *et al.*, 1985). Therefore, based on the viewpoint of atherosclerosis and cardiovascular risks, apo-B 100 is important. Apo-B 48 molecules are usually found in the same forms as apo-B 100, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL and lipoprotein-A particles and all of these particles are atherogenic. Each of these particles contains a single apo-B molecule. Therefore, the analysis of apo-B genes, which are the major lipoprotein particles involved directly in the atherosclerosis process, is highly beneficial. Typically, 85-90% apo-B is presented in LDL particles. Thus, apo-B reflects the particle concentrations similar to LDL (Tabas *et al.*, 2007; McQueen *et al.*, 2008).

Atherosclerosis is a degenerative disorder of large and medium vessels characterized by thickening of blood vessels (Munro and Cotrans, 1988). The thickening of blood vessels is due to the lipid accumulation and fibrous elements in the medium and large arteries. Ultimately, the obstruction occurs in the lumen of the artery. Atherosclerosis generally attacks the coronary arteries, aorta, iliac, femoral, and cerebral arteries (Ross and Glomset, 1976). The thickening of these blood vessels is characterized by the presence of foam cells, namely, macrophage cells that contain cholesterol and ester cholesterol. The formation of foam cells is caused by the excessive macrophages taking oxidized LDL. In addition to cholesterol or ester cholesterol, in atherosclerotic lesions, there are also proteins, carbohydrates, and cellular components including smooth muscle cells, macrophages, and lymphocytes (Kaplan and Aviram, 2001).

According to Hansson (2009), atherosclerosis is an inflammatory disease. The process of atherosclerosis begins when LDL accumulates in the intima, activating the endothelium, increasing the retrieval of monocytes and T cells. Monocytes undergo the differentiation stages to form macrophages, converting lipoproteins into foam cells. Meanwhile, T cells in the lesions will recognize local antigens that contribute to plaque formation. The initial change in the process of atherosclerosis involves the inside of the blood vessels. The atherosclerosis begins since the childhood characterized by the development of fatty streak in the blood vessels (Rackley, 2007). The continuous fatty streaks form fatty plaques that can be examined biochemically and microscopically (Small, 1988; Stary et *al.*, 1990). Fatty streaks can be found in human arteries since their teens. An autopsy study of 2,876 men and women aged 15-34 years showed the fatty streaks in the aorta (Rackley, 2007). Fatty streaks are precursors to atherosclerotic plaques of further stages. It turns out that genetic factors are a major factor affecting the acceleration of fatty streaks into atherosclerotic plaques (McGill, 1968). This study was aimed at assessing the mechanism of the Javanese turmeric's curcumonoid in the early process of atherosclerosis by analyzing the gene DNA encoding apo-B, the causal factor of atherosclerosis at the cellular level in vivo, and to determine the appropriate dose of Javanese turmeric extract.

MATERIALS AND METHODS

A total of 30 white rats were divided into 6 groups, group 1 functioned as the control group without any treatment, group 2 as control group with atherogenic feed, group 3 as control group with turmeric extract, and groups 4, 5 and 6 as the treatment groups, which were fed with atherogenic feed and given curcumonoid with different concentrations (5 mg/each rat, 10 mg/each rat and 20 mg/each rat, respectively) for 3 months.

Total DNA Isolation

The total DNA was obtained from the extraction of blood. The blood was taken from blood vessels and added with 10% ethylenediaminetetraacetic acid (EDTA) solution as anticoagulant. The preparation of blood samples followed Duryadi method (1993). As many as 50-100 µL blood was added with 1x lysis solution. The blood was then supplemented with digestion buffer (500 mg of STES + 0.5 mg/mL Proteinase K) solution. Then it was incubated at the water with temperature of 55° C for 16 hours or overnight. The total DNA purification followed the procedure in DNA isolation kit.

DNA Fragment Amplification with PCR

The polymerase chain reaction (PCR) amplication for the apo-B region was carried out under the following conditions: The initial pre-denaturation for 5 minutes at 94° C followed by a denaturation temperature of 94° C for 30 seconds, annealing at 53° C for 45 seconds, elongation at 72° C for 1 minute; for 35 cycles, then ended with the 5 minute extension reaction at 72° C using apo-B primer (Forwards 5'AATGTCTGACTGTGGGGACAC3'; Reverse 5'GC CCAAATCCTTTCCTCC TAGA3')

Gel Agarose Electrophoresis

The PCR product was transferred to 2% agarose gel using a 1x TBE buffer in Submirine Electrophoresis (Hoefer, USA). Band observations were carried out with the help of UV rays ($\lambda = 300$ nm). A DNA marker of 100 bp was used as a molecular weight marker.

DNA Sequencing

The amplified PCR product was purified by using the GFX Column Purification Kit (Amersham, USA), The result was used as a DNA template for DNA sequencing reaction. The conditions for the sequencing reaction were as follows: the initial denaturation lasted for 2 minutes at 94° C followed by denaturation at 55° C for 45 seconds, and at 72° C for 1 minute (35 cycles), then terminated with a 5 minute extension at 72° C. The sequencing reaction product was purified using Column outoseq G-50, then the DNA was concentrated with the addition of absolute alcohol followed by 70% alcohol washing. When the concentrated DNA was drying, the 6 μL stop solution was added. The solution was incubated at 72° C for 5 minutes and was inserted into the ice. For DNA sequencing, ALFexpres II DNA sequencing automatic device (Amersam Pharmacia, Biotech) was used at 1500 V, 60 mA electric current, 25 W power, and 55° C for 700 minutes.

The reaction for the determination of apo-B gene was done by using the solution reactor Thermo Sequentase Cy5 Dye Terminator Cycle Sequencer Kit (Amersam, USA) with GeneAmpRPCR System machine (Perkin Elmer). The conditions for the trace determination reaction were as follows: The product of the determination reaction of trace was purified by using Column outoseq G-50, and then the DNA was concentrated with the addition of absolute alcohol followed by 70% alcohol washing. After the DNA was dried, 6 μL stop solution was added to it. The solution was incubated at 72° C for 5 minutes. Then the solution was inserted into the ice. The nucleotide was traced by using a DNA tracer ALFexpres II (Amersam Pharmacia, Biotech) at 1500 V, electric current 60 mA, 25 W power, 55° C for 700 min.

The total DNA was isolated from 6 samples of white rat (*Rattus norvegicus*) with codes A1 and A2 for rats with normal feeding, B1 and B2 for rats with normal feeding plus 5% extract, C1 and C2 for rats treated with 10% cholesterol without extract, D1 and D2 for rats treated with 10% cholesterol feed plus 5% extract, E1 and E2 for rats treated 10% cholesterol feed plus 10% extract, F1 and F2 for rats treated with 10% cholesterol feeding plus 20% extract.

RESULTS AND DISCUSSION

Apo-B Amplification of Genes by PCR Technique

Amplification of apo-B *Rattus norvegicus* genes with primary apo-B F and apo-B R produced a 497 bp PCR product. The sequence of apo-B genes in chromosome number 6 was located between the spacer base (on the left or front) and the spacer (to the right or the back) (Gibbs *et al.*, 2013). The electrophoresis of apo-B gene amplification results was shown in Figure 1, 2, and 3.

The amplification results of primary apo-B F and apo-B R based on chromosome genome sequence number 6 (Gibbs *et al.*, 2013) consisted of 497 bp of partial apo-B gene. The result of this amplification was located in the apo-B gene from the sequence of 27.323 bp to that of 27.696 bp. The target gene was an exon of 374 bp apo-B. The schemes of the apo-B gene and the amplification product are shown in Figure 4.

The primary apo-B F and apo-B R had 21 and 22 nucleotides. The primary attachment of apo-B F and apo-B R was located at apo-B genes. The primary attachment schemes and amplification area are shown in Figure 5.

### Determination of Nucleotide Sequences with MEGA Program 6.06

The DNA sequence analysis of *Rattus norvegicus* was done by multiple alignments with DNA sequences of the apo-B *Rattus norvegicus* genes from Genbank. The nucleotide of apo-B *Rattus norvegicus* gene from Genbank was used as a long nucleotide standard to be analyzed. The fragment of sequenced DNA with apo-B
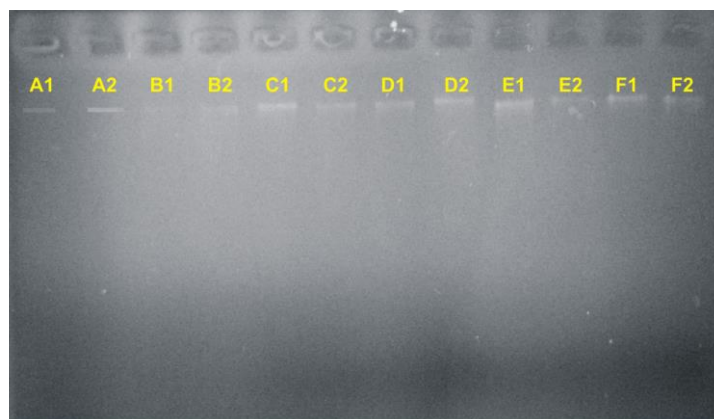

Figure 1. Electrophoresis of DNA isolation of total *Rattus norvegicus* using gel agarose 1%. A1= *Rattus norvegicus* A1; A2= *Rattus norvegicus* A2; B1= *Rattus norvegicus* B1; B2= *Rattus norvegicus* B2; C1= *Rattus norvegicus* C1; C2= *Rattus norvegicus* C2; D1= *Rattus norvegicus* D1; D= *Rattus norvegicus* D2; E1= *Rattus norvegicus* E1; E2 = *Rattus norvegicus* E2; F1= *Rattus norvegicus* F1; F2= *Rattus norvegicus* F2; DNA ladder 100 bp (Genaid)
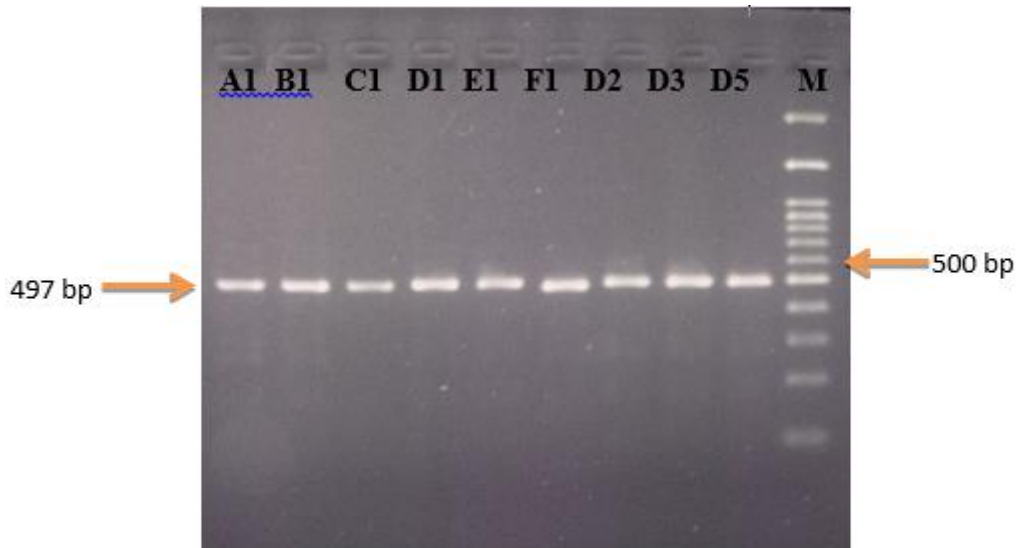
Figure 2. Electrophorosis of apo-B gene amplification of sample *Rattus norvegicus* using gel agarose 2%. A1= *Rattus norvegicus* A1; B1= *Rattus norvegicus* B1; C1= *Rattus norvegicus* C1; D1= *Rattus norvegicus* D1; E1= *Rattus norvegicus* E1; F1= *Rattus norvegicus* F1; D2= *Rattus norvegicus* D2; D3= *Rattus norvegicus* D3; D5= *Rattus norvegicus* D5; DNA ladder 100 bp (Genaid)
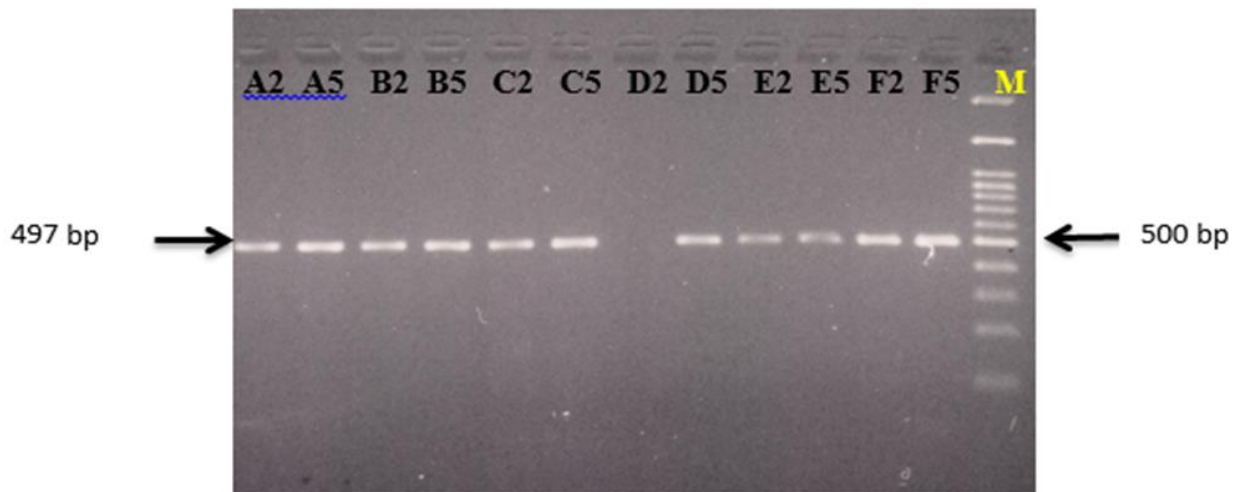
Figure 3. Electrophorosis of apo-B gen amplification of sample *R. norvegicus* using gel agarosa 1.2 %. A1= *Rattus norvegicus* A1; B1= *Rattus norvegicus* B1; C1= *Rattus norvegicus* C1; D1= *Rattus norvegicus* D1; E1= *Rattus norvegicus* E1; F1= *Rattus norvegicus* F1; D2= *Rattus norvegicus* D2; D3= *Rattus norvegicus* D3; D5= *Rattus norvegicus* D5; DNA ladder 100 bp (Genaid)
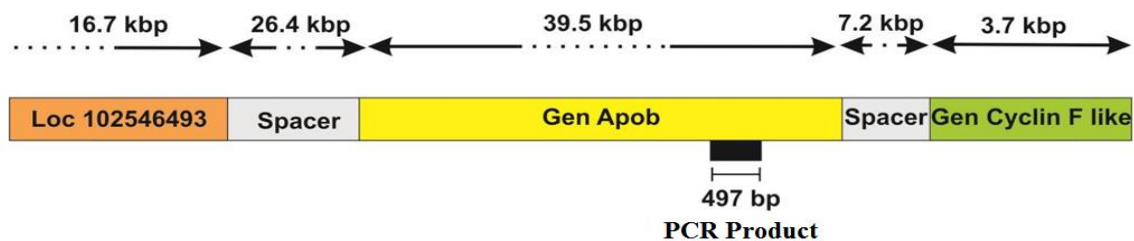
Figure 4. Schematic layout of apo-B genes and partial apo-B gene amplification products
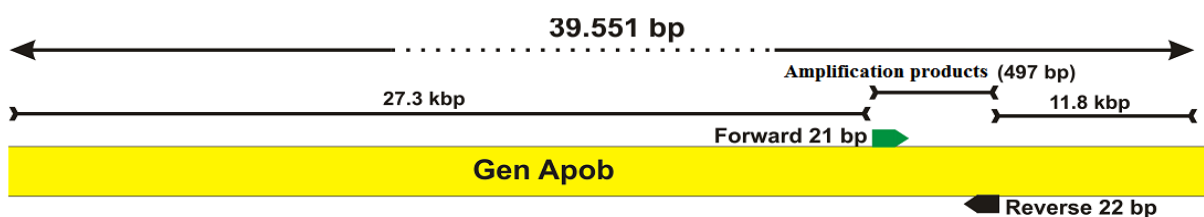
Figure 5. Schematic of forward and reverse primary attachment in apo-B Genes of *Rattus norvegicus*

Table 1. The number of the difference of nucleotides on partial *Rattus norvegicus* apo-B gene with the MEGA program version 6.06

	1	2	3	4	5	6	7	8	9	10	11	12
Rattus norvegicus Genbank												
Rattus norvegicus A1	0											
Rattus norvegicus A2	0	0										
Rattus norvegicus B1	0	0	0									
Rattus norvegicus B2	0	0	0	0								
Rattus norvegicus C1	0	0	0	0	0							
Rattus norvegicus C2	0	0	0	0	0	0						
Rattus norvegicus D1	0	0	0	0	0	0	0					
Rattus norvegicus D2	0	0	0	0	0	0	0	0				
Rattus norvegicus E1	0	0	0	0	0	0	0	0	0			
Rattus norvegicus E2	0	0	0	0	0	0	0	0	0	0		
Rattus norvegicus F1	0	0	0	0	0	0	0	0	0	0	0	
Rattus norvegicus F2	0	0	0	0	0	0	0	0	0	0	0	0

1= *Rattus norvegicus* Genbank; 2= *Rattus norvegicus* A1; 3= *Rattus norvegicus* A2; 4= *Rattus norvegicus* B1; 5= *Rattus norvegicus* B2; 6= *Rattus norvegicus* C1; 7= *Rattus norvegicus* C2; 8= *Rattus norvegicus* D1; 9= *Rattus norvegicus* D2; 10= *Rattus norvegicus* E1; 11= *Rattus norvegicus* E2; 12= *Rattus norvegicus* F1

F and apo-B R primers along 497 bp produced 374-nt apo-B encoding genes. The alignment based on *Rattus norvegicus* (Genbank) DNA fragment produced the segment as along as 374 bp. The alignment results between 374 bp apo-B genes in this research and *Rattus norvegicus* Genbank as a comparison produced an identical nucleotide. The nucleotide differences in the *Rattus norvegicus* of the study are shown in Table 1.

The multiple alignment results between *Rattus norvegicus* samples, and *Rattus norvegicus* (Genbank) were found 0. The number 0 indicated that there was no nucleotide difference from the compared samples. The apo-B gene sequences of *Rattus norvegicus* samples compared were identical. There were no differences in rats A1 and A2 as controls compared with the treated B1, B2, C1, C2, D1, D2, E1, E2, F1, and F2 rats. The different treatments did not cause any changes to the apo-B gene.

The results of this study showed that the administration of curcuminoid extracts of 5%, 10%, and 20% to the rats fed with10% atherogenic feeding did not change the structure of the apo-B 100 gene associated with cholesterol. This result means the administration of 5-20% curcuminoid extracts neither prevented nor reduced atherosclerosis. Some other researches showed contradictory findings with the results of the present study. Tian *et al.* (2014) reported that the application of curcumin on rats reduced the level of apo-B 100 which could prevent the atherosclerosis in long run. Hussein *et al.* (2018) reported similarly that the administration of curcumin to the atherosclerosis rats reduced the apo-B concentration.

From the present study the authors noted that the different results of the study could be caused by the small amount of curcuminoid extract (5-20 mg/a mouse) administered to the rats compared to the 200 mg/kg, body weight as reported by Hussein *et al.* (2018).

CONCLUSION

Based on 374 bp of the apo-B gene nucleotide sequence from *Rattus norvegicus* groups of B, C, D, E, and F analyzed using MEGA program version 6.06, curcuminoid extract of Javanese turmeric (*Curcuma xanthorrhiza* Roxb given in the diet as a treatment did not cause changes in the apo B-100 gene. The present study showed that the apo-B gene sequence of *Rattus norvegicus* from the treatment groups was identical to that of *Rattus norvegicus* group A as the control without treatment.

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