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THE ANTI-ATHEROSCLEROSIS ACTIVITY OF FUCOIDAN CRUDE EXTRACT FROM SARGASSUM CRASSIFOLIUM

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Abstract
This purpose of this research is to examine the anti-atherosclerosis activity of *Sargassum crassifolium* brown algae in mouse, which was administered high-fat diet, and further examining the molecular mechanism of action, through the anti-inflammation test at RAW cell 264.7, inducted by lipopolysaccharide. *Sargassum crassifolium* was collected from *Garut* water, extracted by acid solvent, condensed with ethanol, and freeze-dried till the fucoidan *crude extract* was obtained. However, the mouse was dosed at 50, 100, and 200 mg/kg, after a high-fat diet, therefore, the blood lipid profile and abdominals aortae artery insulation were measured to calculate the amount of foam cell and the aortae thickness respectively. Furthermore, fucoidan *crude extract* was tested at RAW cell 264.7, which was inducted by LPS, at doses of 25 and 50 µg/ml, in order to observe its activity, toward the TNF-α, IL-1β, IL-6, VCAM-1, and ICAM-1, measured using ELISA. Hence, the isolate was proven to have atherosclerosis protective effect, through the improvement of blood lipid profile and the reduction of the foam cells, as well as the thickness of abdominal aortae. Furthermore, fucoidan *crude extract* obstructed the redemption of TNF-α, IL-1β, IL-6, VCAM-1, and ICAM-1 *in vitro*, therefore demonstrating that its activity was through the inflammation redemption response from *Sargassum crassifolium*.
Keywords: Atherosclerosis; Fucoidan *crude extract*; Inflammation; *Sargassum crassifolium*

Introduction

In the world today, atherosclerosis is the primary cause of cardiovascular disease with an elevated level of mortality. It is a chronic, progressive, and dynamic abnormality, which occurs at the artery, due to the high degree of *low-density lipoprotein* (LDL) in the blood. Furthermore, the progression of this condition causes thrombosis, which triggers arterial obstruction, which is an antecedent to myocardia infarct and stroke.[1,2,3]

The formation of plaque is initiated by endotel disfunction, which triggers the disappearance of the physiological cell function of regulating blood fluidity, arterial cell permeability, and the interaction with circulatory leucocyte. Furthermore, the accumulation and LDL modification have been reported to activate the endotel cell, therefore initiating the obstruction of several pro-inflammation mediators which play an important role in each step of atherosclerosis formation.[1,2,4]

LDL has been attributed to the obstruction of various cytokine, which causes leucocyte infiltration to an inflammation point. However, improving the expression of cell adhesion molecule causes the linkage of endotel cell to the monocyte, which differentiates and becomes a macrophage. Furthermore, this obstructs proatherogenic cytokine, such as TNF-α, IL-1β, and IL-6, which play a role in LDL diffusion to the formation of lipid core, endotel and yeast cells, before the atherosclerosis plaque stabilization.[1,2,4,5]

Fucoidan is a complex polysaccharide found in brown algae, which is generally composed of *sulfated fucose* as its main component and the other carbohydrate monomer in lesser amount. Furthermore, it is obtained from different species of brown algae, which possess different composition. However, the characteristic differentiation used is influenced by the extraction method, which causes various pharmacological activities, e.g. Anti-inflammation.[6,7,8,9]Fucoidan from *Laminaria japonica* possesses *in vivo* anti-atherosclerosis activity, which was shown through the obstruction to plaque forming, reduction of blood lipid degree, the impediment of mac-2 and SM-22, and also the decreased expression of ROS, LOX-1, TNF-α, IL-1β, IL-6, ICAM-1, and VCAM-1 on the $LDLR^{-/-}$ model. Furthermore, the research carried out on the cell of RAW 264.7 further illustrated the obstruction of LOX-1, TNF-α, IL-1β, IL-6, ICAM-1, and VCAM-1.[10] inducted by LPS.

The aim of this research therefore, was to investigate the anti-atherosclerosis activity of fucoidan *crude extract* from *Sargassum crassifolium* brown algae, collected from *Garut* water (West Java), and further analyze the molecular mechanism of action. However, this was carried out through the observation of the risk factors of the disease, which include blood lipid profile and atherosclerosis plaque formation, observed in mouse

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abdominal aortae, which were administered high-fat diet. The mechanism of action was examined *in vitro*, at the cell of RAW 264.7, inducted by lipopolysaccharide (LPS).

1. Materials and methods

1.1. Material

The fucoidan *crude extract* was obtained with techniques from previous research, involving the extraction of brown algae *Sargassum crassifolium*, collected from *Cicalobak* beach, *Karang Wangi* village, *Garut*, West Java, in October 2017. However, this process required the use of acid solvent, subsequently followed by alcoholic precipitation and freeze-drying. The isolates obtained yielded about 1,12%, with 9,44% fucoidan, 31,31% sulphate, and the total of carbohydrate of about 21,41%.

1.2. In vivo Anti-atherosclerosis

1.2.1. The testing animal

The animal used in this research were 8 weeks old white male groove Sprague Dawley mouse, weighing about 100 – 150 g, allowed to acclimatize for 7 days, then fed with the high-fat diet for 35 days. Furthermore, the fucoidan *crude extract* was administered from the fifteenth to the thirty-fifth day, at doses of 50 mg/kgBB, 100 mg/kgBB, or 200 mg/kgBB per oral and atorvastatin (10 mg/kgbw) was given per oral to the positive group. Furthermore, the examination of lipid profile (total cholesterol, LDL, HDL, and triglyceride) was carried out in the first, fourteenth and thirty-sixth day. However, the estimation of the amount of foam celland abdominal aortae thickness were conducted only on the last day.

1.2.2. The measure of lipid profile and atherogenic index

On the first and fourteenth day, the blood sample was taken through the orbitalis sinus and subsequently extracted on the thirty-sixth day from the heart organ. Furthermore, it was patched in Eppendorf tube and centrifuged at the rate of 3000 rpm for about 10 minutes, and the total level of cholesterol, LDL, HDL, and triglyceride in serum were measured enzymatically, using Respond® 901. Hence, the atherogenic index was counted., using the formula.[11]

$$\text{Atherogenic Index(IA)} = \frac{Total\ Cholesterol - \text{HDL}}{\text{HDL}}$$

1.2.3. The amount of foam cell and abdominal aortae thickness

The amount of foam cell was counted in the intima and media tunica, at the aortae transverse section. However, the aortae thickness was measured from the intima to the adventitia tunica, whose estimation was carried out through a pigmentation system, using *Hematoxylin Eosin (HE).*

1.3. *In vitro* testing toward the inflammation mediator at the culture cell of RAW 264.7

RAW cell 264.7 was grown in RPMI, with supplementation of 10% FBS and 1% streptomycin penicillin. Furthermore, the culture cell was incubated at 37°C, in a conditioned humidified atmosphere and CO_2 of about 5% until the confluent cell, which was therefore cropped with trypsin-EDTA.

1.3.1. Viability Test

To decide the test doses that have no toxic characteristic, a cell viability test, using the MTT method was conducted. Furthermore, the cell of RAW 264.7 about 5 x 10^3 per *well* in 96-*well plate* was incubated for about 24 hours, which was then left with 0 concentration of the test material up to 1000 µg/ml for 24 hours, therefore, left again, then the cell was washed with PBS. Subsequently the solvent (about 10 µL and 5 mg/ml), MTT was added in each *well*, then incubated for 4 hours, and the formazan crystal formed was dissolved in 100 µL DMSO. Furthermore, the micro*plate* reader was used to measure absorption at a wavelength of about 570 nm. Stating the cells' present viability was conducted by comparing the absorption of the group tested with the material with the control (0 µg/ml).

1.3.2. Inflammation activity and measuring the degree of TNF-α, IL-1β, IL-6, VCAM-1, and ICAM-1

The cell of RAW 264.7 was cultivated at about 5 x 10^5 cell per *well* in 96-*well plate*, which was incubated for 24 hours. Therefore, the medium was discarded, and the test compound solvent was added to the cell till a concentration of 25 and 50 µg/ml of the compound was obtained, which was further incubated for 2 hours. Furthermore, the LPS solvent was introduced in each *well* until a concentration of about 1 µg/ml medium was obtained, which was incubated back for another 24 hours. Afterwards, this was obtained and centrifuged at 2000xg for 20 minutes, at 2°C to 8°C temperature. However, the supernatant was taken and saved at -80°C, to measure the degree of TNF-α, IL-1β, IL-6, VCAM-1, and ICAM-1, and the cytokine degree was evaluated using the ELISA kit *Elabscience*.

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RESULT

Lipid profile and testing of index animal atherogenic

Table 1 showed that administering a high-fat diet for 35 days improves the LDL, triglyceride, and total cholesterol lever. However, it also significantly decreases the HDL degree, when compared with animals on a normal regime *(p < 0,05)*. Upon the administration of fucoidan *crude extract*, there was an observed reduction in LDL, triglyceride, and total cholesterol levels, and also the HDL degree improvement. Furthermore, at a dose of 50, 100, and 200 mg/kgBB, the total cholesterol further decreased significantly normally *(p < 0,05)*, which is compatible, as a group which was administered atorvastatin (10 mg/kgBB), also reported proportionally with group normal dietary.

The degree of triglyceride reduction at fucoidan doses of 100 and 200 mg/kgBB was compatible with the group treated with the drug, although not well-suited with the group on a normal diet. Furthermore, the LDL degree, with the extract being dosed 200 mg/kgBB, reduced significantly and normal ($p < 0,05$), till it was proportional with atorvastatin, though this was higher than the normal dietary group. Fucoidan *crude extract* at the experimental doses significantly increased the HDL of the normal dietary fat ($p < 0,05$) in comparison to the group of an elevated fatty diet, till it was compatible with the test group.

Based on the data of blood lipid profile, the atherogenic index was calculated. Therefore, a low value reduced the risk factor of atherosclerosis. However, based on the calculation, this declined comparatively against the group of high fat dietary. Hence, at a dose of 200 mg/kgBB, the atherogenic index obtained was normal, while the lowest value was achieved by the atorvastatin group.

Table 1. Lipid profile and atherogenic index

Group	LDL (mg/dl)	HDL (mg/dl)	Triglyceride (mg/dl)	Total Cholesterol (mg/dl)	Atherogenic Index
Normal dietary	12.8 ± 1.30^{a}	44 ± 4.35^{a}	75.6 ± 2.60^{a}	68.2 ± 1.30^{a}	0.34
High fat dietary	37.2 ± 1.92^{b}	37.4 ± 4.21^{b}	102.2 ± 6.49^{b}	84.6 ± 3.05^{b}	1.23
Fucoidan *crude extract* 50 mg/kgBB	28.6 ± 2.07^{c}	$42 \pm 4.94^{a,b,c}$	89.2 ± 2.68^{c}	68.4 ± 2.19^{a}	0.56
Fucoidan *crude extract* 100 mg/kgBB	24.4 ± 2.19^{d}	$46.4 \pm 3.70^{a,c,d}$	$86.8 \pm 4.32^{c,d}$	67.6 ± 3.13^{a}	0.43
Fucoidan *crude extract* 200 mg/kgBB	21.8 ± 1.64^{e}	$47.8 \pm 3.11^{a,c,d}$	$84.20 \pm 0.83^{c,d}$	65.8 ± 4.20^{a}	0.30
Atorvastatin 10 mg/kgBB	17.8 ± 0.83^{e}	51.2 ± 6.90^{d}	82.4 ± 3.84^{d}	60.4 ± 2.30^{a}	0.17

The amount of foam cell and the abdominal thickness aortae on animal testing

Figure 1 showed the amount of foam cell and the thickness of abdominal aortae on the thirty-sixth day.

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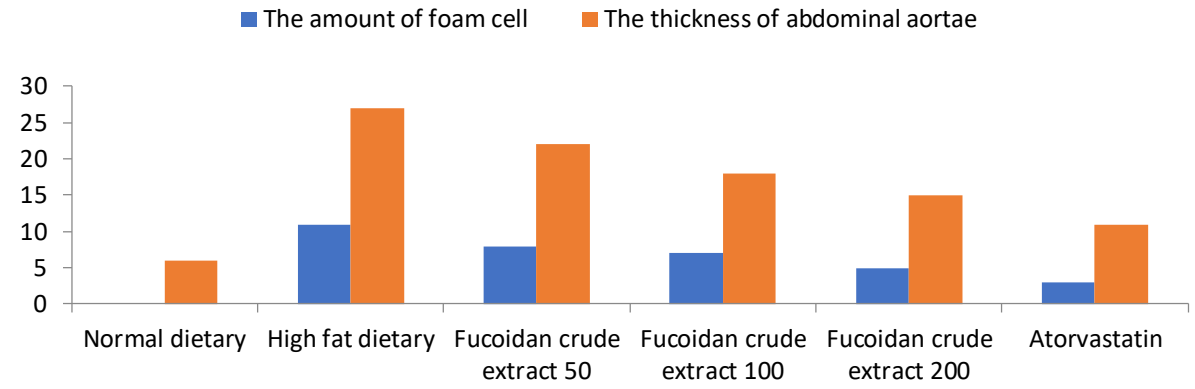

Figure 1. The foam cell amount and the abdominal aortae thickness of testing animal on the thirty-sixth day

The median value of the foam cell was elevated in the high dietary fat control group to about 11 parts of broad visibility. However, the unit to which fucoidan *crude extract* was given at doses of 200 mg, obtained a median value of about 5 broad visibility of white mouse yeast cell. Furthermore, the set treated at a dosage of 100 mg and 200 mg exhibited about 7 and 8 parts, respectively. Therefore, the least median of foam cellwas reported in atorvastatin group treated with about 10 mg/kgBB, which revealed about 3 parts of broad visibility.

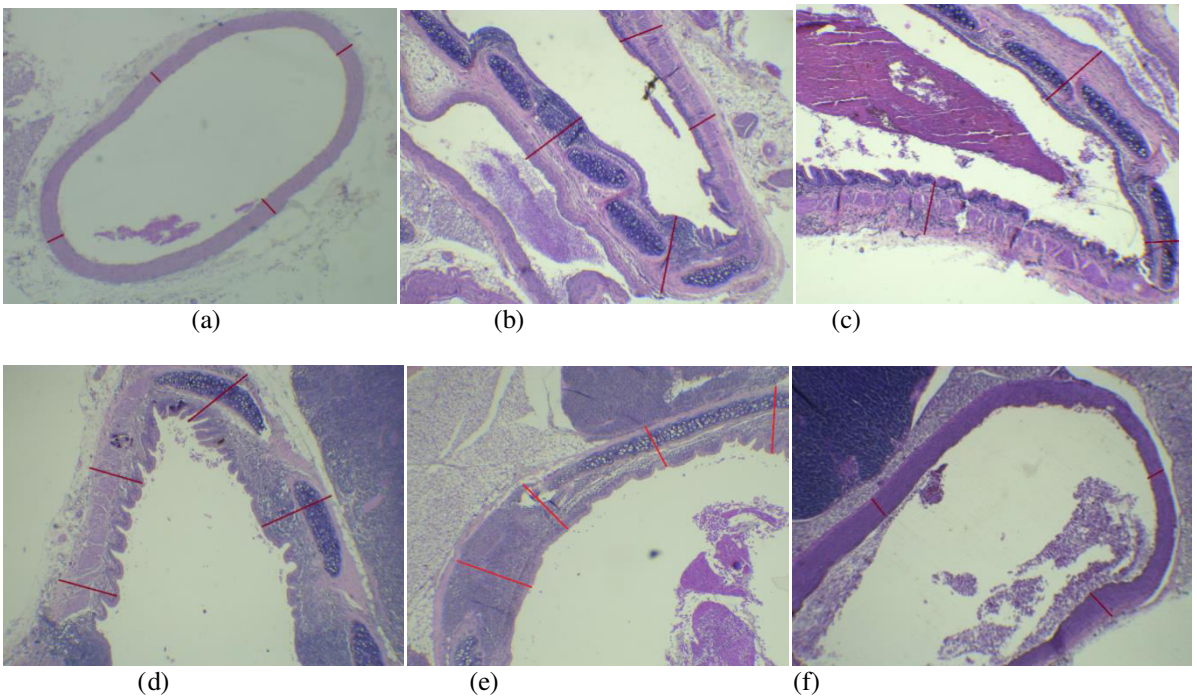

Figure 2. The transverse cut of animal group test abdominal aortae (a) normal diet; (b) high fat diet; (c) fucoidan *crude extract* 50 mg/kgBB; (d) fucoidan *crude extract* 100 mg/kgBB; (e) fucoidan *crude extract* 200 mg/kgBB; (f) atorvastatin 10 mg/kgBB

As shown in Figure 2, the control group with high dietary fat possessed the thickest median value of about 26 mm. However, the abdominal aortae thickness, on the unit treated with 200, 100, and 50 mg/kgBB of the isolates, recorded 15 mm, 18 mm, and 22 mm, respectively, while on the group to which atorvastatin 10 mg/kgBB was administered, the value obtained was 11 mm.

The activity toward the inflammation mediator on the cell of RAW 264.7

Cell viability test of RAW 264.7 on the administration of the test sample showed a reduction in the amount of corpuscle, in line with the elevated dose, as shown in Figure 3. Furthermore, based on the linear regression analysis, the concentration of the extract, which produced the percentage of RAW 264.7 cell viability of 80%, was 86 µg/mL. Hence this was selected as the maximum dose, which is not toxic toward cell and it can be used for anti-inflammation tests.

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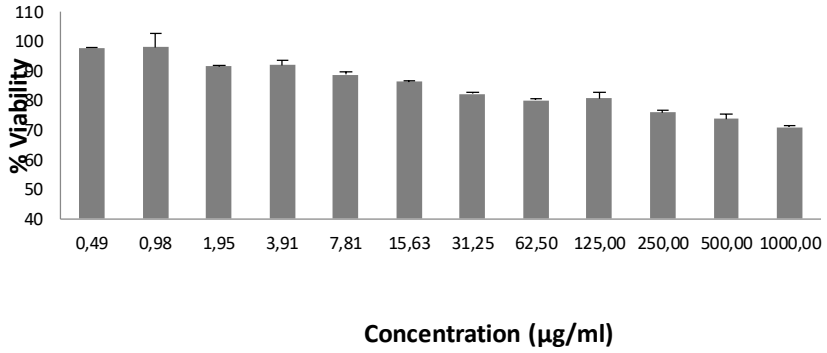

Figure 3. RAW 264.7 viability percentage cell after giving testing material.

This investigation proves that LPS significantly increases the percentage of TNF-α, IL-1β, IL-6, VCAM-1, and ICAM-1, in comparison with normal cell RAW 264.7 ($p < 0,05$), as shown in Table 2. Hence, the amount of TNF-α observed in the group to which fucoidan *crude extract* was given, was significantly lower than the value recorded in the control ($p < 0,05$). The highest obstruction percentage obtained at the dose of 25 µg/ml was about 85,57 ± 4,05%; while the TNF-α percentage difficulty at a dose of 50 µg/ml was 76,94 ± 5,21%.

Table 2. Inflammation mediator degree on the cell RAW 264.7 inducted by LPS

Treatment	Degree of TNF-α (pg/mL)	Degree of IL-1β (pg/mL)	Degree of IL-6 (pg/mL)	Degree of VCAM-1 (ng/mL)	Degree of ICAM-1 (ng/mL)
Normal control	494.20 ± 105.99[a]	21.24 ± 8.67[a]	60.48 ± 5.40[a]	2.24 ± 0.54[a]	4.97 ± 0.66[a]
Inflammation control	985.49 ± 132.81[b]	49.69 ± 10.17[b]	1992.45 ± 21.11[b]	2.98 ± 0.42[b]	6.32 ± 0.49[b]
Fucoidan *crude extract* 50 µg/mL	227.27 ± 51.32[c]	40.97 ± 9.10[b]	2048.00 ± 33.74[b]	0.75 ± 0.20[c]	2.46 ± 0.56[c]
Fucoidan *crude extract* 25 µg/mL	142.17 ± 39.95[c]	4.93 ± 5.99[c]	1779.29 ± 38.15[c]	0.60 ± 0.37[c]	1.63 ± 1.23[c]

The provision of test dose extract 50 µg/ml on cell RAW 264.7, did not initiate a significant reduction in the amount of IL-1β. However, at 25 µg/ml, the value decreased significantly in comparison with the negative control ($p < 0,05$), at about 4,93 ± 5,99 pg/ml, with the percentage difficulty of 90,08 ± 12,05%. Furthermore, at a test material dose of 50 µg/ml, IL-6 does not show any differences, in comparison with the control. However, a concentration of 25 µg/ml, resulted in IL-6 of about 1779.29 ± 38.15 pg/ml, with an obstruction percentage of 10,70 ± 1,91%, which was statistically significant ($p < 0,05$). Furthermore, the degree of VCAM-1 and ICAM-1 in the normal diet group was decreased significantly ($p < 0,05$), while the most significant reduction was obtained at a dose of 25 µg/ml. However, the sequent amount of VCAM-1 and ICAM-1, were 0.60 ± 0.37 ng/ml and 1.63 ± 1.23 ng/ml, with the percentage of 79.94 ± 12.53% and 74.26 ± 19.40% respectively.

DISCUSSION

This research showed that fucoidan *crude extract* improves the blood lipid profile of the inducted mouse with high fat dietary - hence, the atherogenic index value is reduced (Table1). However, observation of the abdominal aortae illustrated the formation of obstructive atherosclerosis plaque in the group treated with the test material, marked with foam cellreduction and abdominal aortae thickness (Figure 1 and 2). Furthermore, this indicated the existence of anti-atherosclerosis activity on fucoidan *crude extract* administration, which resulted in a dose-dependent autoprotective effect.

The formation of atherosclerosis plaque involved several inflammation mediators, and the foam cellis formed as a result of cholesterol metabolism hindrance. Therefore, the cytokines, such as TNF-α, IL-4, and IL-13, take part in LDL oxidation, through related enzyme activation, which forms *ox*LDLthat further inducts the expression of TNF-α, IL-1β, and IL-6, through the PPAR- γ receptor. Macrophages caught more *ox*LDL, as it transforms into a yeast cell, which can be modulated by various cytokines, through the hindrance toward homeostatic cholesterol, such as TNF-α, TNF-*like protein 1A* (TL-1A), and IFN-γ.[1,2,12]

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To observe the mechanism of action of fucoidan *crude extract* on the inflammation mediators involved continuous *in vitro* investigation, utilizing macrophage cell RAW 264.7, which produces the inflammation effect, when stimulated by LPS. RAW 264.7 are cells derived from murine macrophages and are widely used to study anti-inflammatory mechanisms. As macrophages, they secrete various cytokines such as TNF-α, IL-1, IL-6 and others. In this research, we used 50 and 25 μg/ml of crude fucoidan, according to viability test to RAW 264.7 cell (Figure 3). Generally, administration of crude fucoidan obstructs the release of TNF-α, IL-1β, IL-6, VCAM-1, and ICAM-1, especially at dose 25 μg/ml. Hence, this research proved that fucoidan activity pushes the inflammation route, through the release of obstructive pro-inflammation cytokine and cell adhesion molecule (Table 2).

LPS incited the irritation response, through the interaction with TLR4, which activated the line of MAPK, followed by phosphorylase and complex solution of I-κB, and the subsequent activation of NF-κB. Furthermore, this NF-κB translocates to the nucleus and activates the various genetic transcription, such as COX-2, VCAM-1, ICAM-1, TNF-α, iNOS, lipoxygenase, IL-6, IL-1, chemokine, as well as other pro-inflammation mediator.[13,14]

NF-κB and TNF-α possess a positive feedback relationship, which is the key to chronic inflammatory conditions as the TNF-α interaction with its receptor, activated the MAPK inflammation line, therefore explaining its ability to induct the other cytokines. IL-6 was inducted by its interaction with the IL-1 receptor, which is also capable of activating the MAPK line.[15]Based on the previous research, the reduction of IL-1β, IL-6, and TNF-α was in line with the obstruction of MAPK activity and NF-κB.[16,17,18,19] However, the fucoidan *crude extract* sample also obstructed these activities - hence, the amount of inflammatory mediators measured is lower than in a typical condition.

The results agree with the study, which tested the activity of fucoidan antiatherosclerosis from *Laminaria japonica*, which was capable of obstructing atherosclerosis forming plaque, decrease the blood lipid degree, obstruct Mac-2 and SM-22. Furthermore, reduced expression of ROS, LOX-1, TNF-α, IL-1β, IL-6, ICAM-1, and VCAM-1 on $LDLR^{-/-}$ aortae model was also reported. However, at the RAW 264.7 macrophage cell, inducted by LPS, it also exhibited anti-inflammation activity, through the obstruction of LOX-1, TNF-α, IL-1β, IL-6, ICAM-1, and VCAM-1.[10]

At the dose 50 μg/ml, the test material does not show the percentage reduction of mediators, which was higher than the value recorded at 25 μg/ml (Table 2). Therefore, it is not agree with the previous study, which portrayed an improvement of anti-inflammation activity, at an exponent of the dose. Furthermore, this happened due to the prediction of dual effect from the fucoidan compound, which stipulates an elevated anti-inflammation activity at a low dose and immunostimulant capability at a high concentration as observed in previous research.[20] However, they also showed immunostimulant activity from several species of brown algae.[21,22,23] Hence, further research is needed to confirm the existence of this effect on the fucoidan *crude extract*.

Conclusion:

The fucoidan *crude extract* obtained from *Garut* water *Sargassum crassifolium* was isolated with acid solvent. It was further observed to possess anti-atherosclerosis activity, through LDL, total cholesterol, and triglyceride reduction, improving HDL. However, the amount of foam cellreduced and a decline in the abdominal aortae thickness on the mouse administered high-fat diet was recorded. Furthermore, these anti-atherosclerosis activities are related to its ability to limit the release of TNF-α, IL-1β, IL-6, VCAM-1, and ICAM-1, which was proven by RAW 264.7 cell, inducted by LPS.

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