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Sargassum aquifolium Extract Prevents Elevated Cholesterol Levels and Blood Vessel Thickening in Rats Fed an Atherogenic Diet

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ABSTRACT

Hypercholesterolemia is a health disorder in which cholesterol in the blood increases beyond normal limits. This disorder chronically can cause atherosclerosis. *Sargassum aquifolium* contains bioactive compounds that can prevent increased blood cholesterol levels. This study aimed to prove the increasing cholesterol levels and changes in the blood vessel profile of rats fed an atherogenic diet by *S. aquifolium* extract. *S. aquifolium* was obtained from Ekas Bay, Province of West Nusa Tenggara. Male *Rattus norvegicus* 2-3 months ages used in this study. Normal rats were fed an atherogenic diet for four months to produce a hypercholesterolemia animal model. The phytochemical profile was screened based on the Harborne method. Identity of bioactive compounds determined by HPLC-HRMS. The parameters observed were cholesterol levels and blood vessel profiles. The juice of *S. aquifolium* contained steroids, saponins and tannins, and their bioactive showed anticholesterolemic. Rats given an atherogenic diet had increased cholesterol levels. Administration of *S. aquifolium* juice twice daily can reduce cholesterol levels and prevent the aorta's and arteries' thickening. In conclusion, *S. aquifolium* juice contains bioactive compounds and administration of *S. aquifolium* juice twice a day prevents an increase in cholesterol and damage to the aortic and arterial profiles.

Keywords: atherogenic-diet, blood-vessel-profiles, hypercholesterolemia, *Rattus-norvegicus*, *Sargassum-aquifolium*

INTRODUCTION

Hypercholesterolemia is a disorder of cholesterol metabolism caused by blood cholesterol levels exceeding normal limits [1]. Hypercholesterolemia is also associated with metabolic disorders in the blood [2]. Eating high-calorie foods is one of the triggers of high cholesterol levels [3].

Hypercholesterolemia can cause damage to the lining and walls of blood vessels [4]. Damage to blood vessels causes different types of disorders. Atherosclerosis is a type of damage that occurs in the lining of the arteries. Plaque in the blood vessels leads to narrowing or blockage [5]. The presence of blockages in the blood vessels causes the lumen of the blood vessels to narrow and lose elasticity [6]. It is related to the intake of fat and carbohydrates in excessive amounts. In addition, prolonged consumption of high cholesterol can cause hypercholesterolemia [7]. Previous studies have shown thatcholesterol levels in rats fed an atherogenic diet were higher than in those fed a normal diet [8].

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S. aquifolium is a species of brown algae in the class of Phaeophyceae. Several Sargassum species found in Indonesia and have been identified include *S. polycystum, S. echinocarpum, S. binderi, S. plagyophyllum, S. cinereum, S. duplicatum, S. gracillimum, S. mollerii, S. hystrix, S. siliquosum, S. filipindula, S. fluitan, S. polyseratium, S. vulgare,* and *S. fenitan* [9]. *S. aquifolium* contains bioactive compounds such as tannins and saponins. These compounds of this seaweed can be obtained by juicing [10]. The slow juicer method is preferred in obtaining the extract because this method can prevent a decrease in bioactivity [11]. In a previous study, the administration of 1 mL and 1.5 mL of juice and decoction of *S. polycystum* in DM rats could reduce blood sugar levels by inhibiting the absorption of blood sugar in the small intestine [10; 12]. Based on previous research, the juice of *S. polycystum* and *S. olygocystum* has shown its ability to lower blood glucose levels. *S. aquifolium* contains several bioactive which are generally known to function as antibacterial, antiviral, antioxidant, and antifungal agents. Therefore, this study evaluates the effect of *S. aquifolium* juice on increasing blood cholesterol and the thickening of blood vessels in rats fed an atherogenic diet.

EXPERIMENT

Chemicals and instrumentation

S. aquifolium collected from the waters of Ekabuana Bay, East Lombok Islands, West Nusa Tenggara (8°52'M.5" S 116°27'03.9"E). The samples were morphologically identified at the National Research and Innovation Agency (BRIN), Jakarta, Oceanographic Research Center. Male Wistar rats (*Rattus novergicus*) 2-3 months old with a weight range of 150 - 200 g was used as experimental animals. The calory content of normal and atherogenic diets was 3771.68 and 4270.50 kCal/kg. A cholesterol kit (ABX Pentra Cholesterol CP, Horiba) was used to determine the cholesterol level. The staining on rats' aorta and artery tissue used Haematoxylin and Eosin.

Instrumentations used in this study were a slow juicer (Phillips, Model HR1889), tissue processor (Tissue Tec Xpress), slide Stainer (Tissue-Tek DRS), spectrophotometer (ABX Pentra 400, Horiba), and microscope (Olympus). HPLC – HRMS (Thermo Scientific).

Phytochemical screening

S. aquifolium juice was prepared by washing the sample from the research location with running water. Then 100 g of the sample was placed in a slow juicer to separate the juice and residue. Next, sieving of the liquid portion was performed to maximize separating the liquid portion or juice from the residue [13]. The obtained *S. aquifolium* juice was then used in research and phytochemical tests.

A total of 0.1 mL of *S. aquifolium* juice extract was placed in a test tube, and then 5 mL of distilled water was added. The liquid mixture was boiled on a hot plate for 5 minutes, then five drops of $FeCl_3$ were added, and the colour change of the solution was observed; when the solution turned black-blue, this indicated the presence of polyphenols.

A 0.1 mL of *S. aquifolium* juice extract was mixed with 5 mL of ethanol. The mixture was shaken, heated, and shaken again. Then, 0.2 g Mg and three drops of HCl were added. The colour change of the solution to red indicates the presence of flavonoids.

The 0.5 mL sample was mixed with 5 mL distilled water. The solution was boiled for 5 minutes, and then five drops of $FeCl_3$ were added. The presence of tannins was indicated by the formation of dark blue and black-green colors.

S. aquifolium juice was mixed with 10 mL of hot water and shaken vigorously. One drop of HCl was added to the solution, and the appearance of foam or foam 1-10 cm high was observed over ± 10 minutes. If foam forms within this time, the sample contains saponins.

Up to 0.05 mg of *S. aquifolium* juice was added to 2 mL chloroform. Then, ten drops of acetic acid solution and three drops of concentrated sulfuric acid (H_2SO_4) were added. The sample contains steroids if a blue or green colour appears.

HPLC-HRMS analysis

The juice was diluted in distilled water, vortexed and spindown. The extract was filtered with a syringe filter measuring 0.22 µm. The HPLC used was the Thermo Scientific Dionex Ultimate 3000 RSLCnano. The supernatant obtained was placed in an autosampler and injected into a column (Hypersil GOLD aQ 50 x 1 mm x 1.9 m) from LC at 30°C. The two solvents used in this analysis were 0.1% Formic acid in Water (solvent A) and 0.1% Formic acid in Acetonitrile (solvent B). The solvent flow rate during the test was 40 L per minute. The flow model was a gradient, where at 0-2 minutes, the solvent system was 95% solvent A, and at 2-15 minutes, the proportion of solvent A was 40%, then at 15-25 minutes, the proportion of solvent A was 5 % and finally delivered at 25-30 minutes, the proportion of solvent A became 96 %. The High-Resolution Mass Spectrometer (Thermo Scientific Q Exactive) was run in Full scan mode at a resolution of 70,000 and data dependent MS2 at 17,500. Data were analyzed using Compound Discoverer (MS/MS Library) software [14].

PassOnline

Prediction of bioactive anti-hypercholesterolemia activity of *S. aquifolium* juice as anti-hypercholesterolemia was carried out by in-silico analysis. The Canonical SMILE of the bioactive was obtained from the PubChem website. Canonical SMILE code for bioactive compounds identified anti-hypercholesterolemia activity using the online software PASS (Prediction of Activity Spectra for Substances) (http://pharmaexpert.ru/passonline/org). The probability of activity (Pa) value of the bioactive *S. aquifolium* juice was used as an estimator of the ability of these compounds to anti-hypercholesterolemia [15].

Animal model and treatment

Animal modelling was performed on 2-3-month-old male Wistar rats weighing 150-200 g. Ethical clearance of animal use in this study was approved by the ethical committee of Universitas Brawijaya (096-KEP-UB-2021). The rats were acclimated for seven days before treatment by feeding the rats 40 g/day and drinking water. After acclimation, the rats were divided into normal and treated rats. Hypercholesterolemia rats' groups were produced by administering the atherogenic diet for four months. In this study, the calorie content of normal and atherogenic diets was 3771 kcal and 4270 kcal. Each group consisted of 5 rats. This treatment group includes:

A = rats fed a normal diet (negative control)
B = rats fed an atherogenic diet (positive control)
C = rats fed an atherogenic diet and treated with 1 mL *S. aquifolium* juice once daily
D = rats fed an atherogenic diet and treated with 1 mL *S. aquifolium* juice twice daily
E = rats fed an atherogenic diet and treated with 1 mL *S. aquifolium* juice three times daily

Cholesterol determination

Cholesterol level was analyzed using the CHOD-PAP method (cholesterol oxidase - peroxidase- amino antipyrine). Free cholesterol was obtained after hydrolysis and oxidation. First, it becomes cholesterol-4-en-3one, which simultaneously produces H_2O_2. After this, a peroxidase catalyst (H_2O_2) reacts with 4-amino antipyrine and phenol to form a quinone imine (red color). Blood samples were taken from the hearts of rats. 2 mL of blood was placed in a vacutainer and centrifuged for 10 minutes at a speed of 5000 rpm: 500 µL blood serum plus 1000 µL cholesterol reagentin a test tube. The cholesterol level is read on the ABX Pentra 400

Profile of aorta and artery analysis

Histopathological analysis was initiated by dissecting the aorta and arteries from rats' tails. The tissue was washed with normal saline and then fixed with 10% formalin buffer. The fixed tissues were dehydrated with graded alcohol 70%, 80%, 90%, or absolute alcohol for 3 minutes. Immersion of xylene twice for 2 hours each time and then embed with liquid paraffin at 56 ºC. Next, the tissue forms were frozen and cut into slices with a thickness of 3-5 µm using a rotary microtome. Then the slices were placed on a slide and incubated for 30 minutes at a temperature of 70-80 °C. The procedure was continued by staining with hematoxylin-eosin for approximately 10-15 minutes. Finally, the slide or slides were covered with a coverslip. The tissue damage was observed under a microscope at 400X magnification for five fields of view. The thickness of the aorta and arterial lining were evaluated by a micrometer.

Data analysis

The data obtained in this study were expressed as the mean and standard deviation. The data obtained were analyzed using analysis of variance and the least square difference test with a 5% confidence interval.

RESULT AND DISCUSSION

Phytochemical

The juice of *S. aquifolium* obtained by the juicing method was then tested to determine the phytochemical content in the juice. The results of the phytochemical test of *S. aquifolium* juice can be seen in Table 1.

Table 1. Phytochemical of *S. aquifolium* juice.

Phytochemicals reagent test	Results
Polyphenol	-
Flavonoid	-
Tannin	+
Saponin	+
Steroid	+

Note: (+) indicate that juice contains this bioactive, and (-) indicate juice does not contain the bioactives.

The results of the phytochemical test of S. *aquifolium* juice showed that *S. aquifolium* juice contained three bioactive compounds, namely saponins, tannins and steroids and did not contain polyphenols and flavonoid. Previous research found that the *S. duplicatum* that is

macerated using ethanol, methanol, and acetone contains alkaloids, saponins, steroids, glycosides, tannins, phenolics and flavonoids [16]. However, another study found that *S. crassifolium* which was soaked using ethanol, contained flavonoids, triterpenoids, phenolics, and saponins as active compounds [17]. It is possible because the extraction method used juicing. Bioactive components such as flavonoids and polyphenols will be damaged at temperatures above 50°C because they can undergo structural changes and produce low bioactivity [18].A slow juicer has the principle of a slow screw system. The extraction process is carried out by pressing (pressing) the fruit using a particular fiber thread at a low speed of 32 rpm, so it does not produce heat, and more liquid is extracted [11].

Bioactive identity

The juice of *S. aquifolium* obtained by the juicing method was then bioactive identity determined by the HPLC-HRMS method. The results of the phytochemical test of *S. aquifolium* juice can be seen in Table 2.

Table 2. Bioactive identity of *S. aquifolium* juice.

Compounds	Retention time (min.)	PassOnline (*Pa*)
Betaine	0.966	0.329
DL-Carnitine	0.974	0.449
Choline	1.179	-
Nicotinic acid	1.392	0.589
Caprolactam	3.987	-
Triethyl phosphate	8.655	0.348
DEET	12.398	0.139
2,2,6,6-Tetramethyl-1-piperidinol	13.094	0.242
D-(+)-Camphor	14.823	-
1-Tetradecylamine	16.669	0.337
3,5-di-tert-Butyl-4-hydroxybenzaldehyde	17.548	0.536
Tetranor-12-(S)-HETE	17.576	0.663
Dibutyl phthalate	18.587	0.657
4-Methoxycinnamic acid	21.122	0.573
Erucamide	22.376	0.495
Bis(2-ethylhexyl) phthalate	23.524	0.587
Triethanolamine	26.429	0.357
2-Aminonicotinic acid	26.440	0.306

Table 2 shows that *S. aquifolium* juice contains several polar and non-polar bioactive. *Ascophyllum nodosum* was reported to contain polar compounds, such as phlorotannin [19], while *Undaria pinnatifida* was found to contain non-polar compounds, such as fucoxanthin [20]. The variability of the results is possible due to the differences in the solvents used. Polar solvents tend to dissolve polar compounds, while non-polar solvents will get non-polar compounds [21]. This study found both groups of compounds because the juice technique can dissolve all groups of compounds. This technique can dissolve all these compounds through the destruction of the material matrix so that all the compounds attached to or in the material will be released [18].

Table 2 shows that the potential activity value (Pa) as anti-hypercholesterol of the identified compounds is less than 70 per cent. It means that the bioactive in *S. aquifolium* juice has low anti-hypercholesterol activity as a single compound but will show strong activity when it combines (synergistic effect). Flavonoids and polyphenols of *Sargassum* sp. showed its activity as an antihyperglycemic and anti-cardiovascular [15,22]. Improvement of both disorders by the bioactive Sargassum was associated with improved fat metabolism [23]. Preliminary studies suggest that the bioactive in Sargassum can increase the expression of Uncoupling Protein 3 (UCP3), thereby lowering cholesterol levels to prevent the risk of atherosclerosis [24].

Cholesterol level

Blood cholesterol in the body of experimental rats between treatments at the end of the study was significantly different ($P<0.05$). Blood cholesterol levels of rats in various treatments at the end of the study period can be seen in Figure 1.

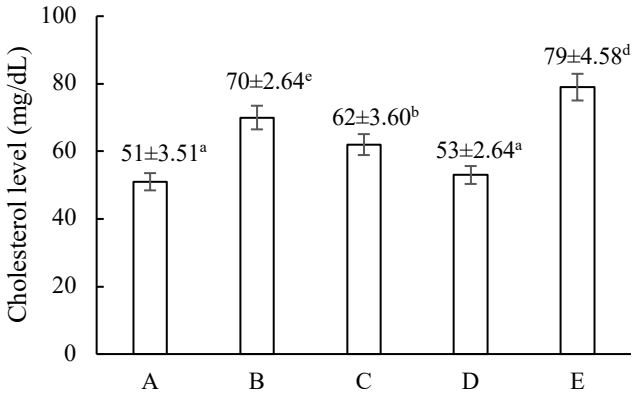

Figure 1. The cholesterol level of normal and rats fed an atherogenic diet treated with several times of *S. aquifolium* juice.

Figure 1 shows that rats' blood cholesterol levels were significantly different group A and B, where B had significant higher cholesterol levels than rats in group A. Previous studies showed that rats' average total cholesterol levels fed a standard diet was 96.63. mg/dL, lower than the group given the atherogenic diet, 239.53 mg/dL [25]. Another study also showed it in rats fed an atherogenic diet for eight weeks [26]. The administration of an atherogenic diet results in an excessive increase in energy reserves. Giving a high-fat diet will affect fat absorption in the intestine, and lipid metabolism and increase blood cholesterol levels [27]. In addition, Figure 1 also shows that the cholesterol levels of groups B, C, and D rats were significantly different. Groups C and D showed decreased cholesterol levels, but treatment E contained the highest. In a previous study, the administration of *S. polycystum* extract could reduce total blood cholesterol levels in rats [28]. The presence of bioactive compounds found in *S. aquifolium* juice reduces cholesterol blood levels. Bioactive compounds such as saponins in *Sargassum* sp. can lower blood plasma cholesterol because saponins will bind to bile acids and increase the excretion of bile acids in the feces and neutral sterols [29]. In addition, tannins reduce cholesterol levels by inhibiting fat absorption in the intestine by reacting with mucosal proteins of intestinal epithelial cells [30]. Steroids, in this case, are phytosterols in the body that function to lower cholesterol levels by inhibiting the absorption

of cholesterol through competition with cholesterol in the absorption process in the intestine, thereby helping to reduce the amount of cholesterol entering the bloodstream and accelerating the excretion of cholesterol [30].

Figure 1 shows that group E had the highest cholesterol levels. It is not following previous studies regarding the administration of *S. polycystum* can reduce total cholesterol, LDL, and triglycerides and increase HDL serum levels of male rats fed a high-fat diet [31]. There may be a disturbance due to the excessive presence of these bioactive compounds and the frequency of administration of *S. aquifolium* juice. Steroids affect increasing cholesterol levels because cholesterol is one of the steroids that is a common component and a precursor in animal cell membranes where this steroid will synthesize other steroids. Active compounds such as steroids and tannins are antinutrients. In small amounts, these compounds are needed for the body. However, in excessive amounts, these compounds will be detrimental to the body [30].

Aorta profile

Profile of aorta rats was used to describe the extent of tissue damage caused by the treatment. Photomicrographs of each group are shown in Figures 2 and Table 2.

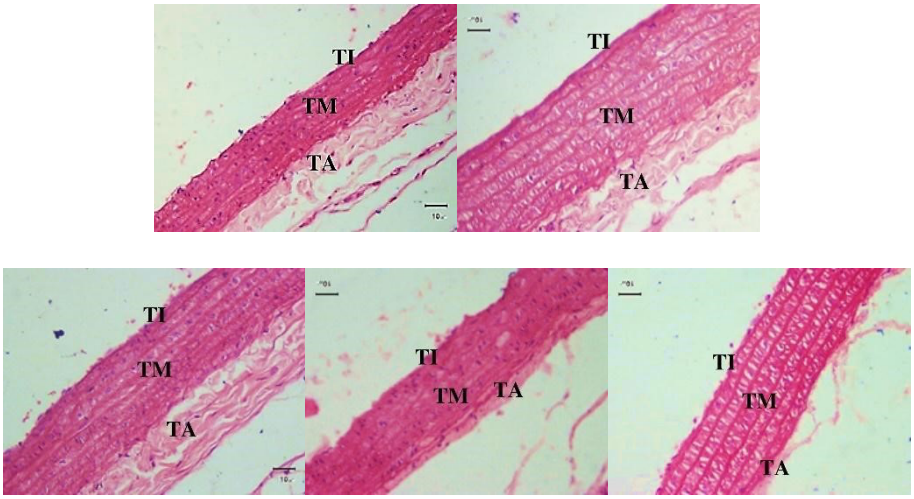

Figure 2. Photomicrograph of aorta rats fed a normal and atherogenic diet treated with *S. aquifolium* juice**.** (Magnification 400x) (HE). (Note: TI = Tunica intima, TM = Tunica media, and TA = Tunica adventitia)

Table 1 shows that B significantly differed from A, where B has a thicker normal aortic layer in the tunica intima and tunica media. It was consistent with previous studies that feeding high-cholesterol rats for nine weeks can result in the thickening of the aortic wall [32]. An atherogenic diet caused the aortic wall to thicken in B. High blood cholesterol can lead to the narrowing and clogging of blood vessels. The higher the blood cholesterol, the higher the likelihood of fatty deposits and plaque formation in the blood vessels, resulting in thickening [32]. The aortic thickening in B was more significant and atherogenic than in C, D, and E. Group E aortic thickening was almost as thick as B, where the tunica intima was not clean, and the tunica media was thick. The administration of *S. aquifolium* juice in groups C and D could lower blood glucose and cholesterol levels in experimental animals. In contrast, in group B, their cholesterol levels increased because they were not given *S. aquifolium* juice, causing an aortic thickening. Giving extracts containing tannins and

saponins to hypercholesterolemic rats can reduce cholesterol levels, thereby improving the aortic profile [32].

Table 2. The thickening of tunica intima (TI), tunica media (TM), and tunica adventitia (TA) of aorta rats fed a normal and atherogenic diet administered with *S. aquifolium* juice.

Groups	Thickening (μm)		
	Tunica intima	Tunica media	Tunica adventitia
A	1.45 ± 0.19^{a}	23.03 ± 1.80^{a}	10.28 ± 0.97^{a}
B	$2.93 \pm 0{,}58^{d}$	41.15 ± 2.43^{d}	17.16 ± 1.22^{e}
C	$1.77 \pm 0{,}33^{b}$	36.00 ± 3.21^{c}	14.53 ± 1.34^{c}
D	$1.64 \pm 0{,}13^{ab}$	28.21 ± 2.48^{b}	12.23 ± 1.17^{b}
E	$2.33 \pm 0{,}21^{c}$	39.59 ± 3.58^{cd}	16.23 ± 1.43^{d}

Note: The numbers on the same column are followed by different superscripts letter indicate significantly different ($P < 0.05$).

Aortic thickening in group D rats was less than in groups C and E; meanwhile, aortic thickening in group E was almost similar to group B. Previous research showed that administration of Sargassum sp. which is not more than 600 mg/kg BW/day, does not result in marked thickening of the aorta [33]. It is consistent with research that repeated administration daily of *S. echinocarpum* extract containing tannins will cause toxic effects and tissue damage [31,34].

Artery profile

Histopathological layers of the arteries for each group can be shown in Figure 3. The analysis of the thickening data on the arteries can be seen in Table 3. Table 3 shows that the arterial thickening in groups A and B differed significantly. Previous studies have shown that a high-fat diet increases the thickening of the artery walls. Atherosclerosis is characterized by partial or complete thickening of the vessel wall due to the accumulation of lipids due to the formation of fibrous tissue and the calcification of the tunica intima [32]. High LDL cholesterol levels will trigger cholesterol accumulation in blood vessel cells, which causes plaque formation and atherosclerosis in blood vessels [27,32].

Table 3 shows that the tunica intima, tunica media, and tunica adventitia layers in groups C, D, and E are significantly different from group B and have thinner layers than those in group B. Previous studies have informed that each mL of *S. polycystum* juice contains 3.5 mg of phlorotannin per g of material [10]. Its administration is associated with the degree of intima and media thickening [33]. The presence of bioactive compounds in *S. aquifolium* juice may have a thinning effect on the degree of thickening of the tunica intima, tunica media, and tunica adventitia arteries, and their tannins may prevent atherosclerosis [35]. In addition, Table 3 shows that the layers of the tunica intima, tunica media, and tunica adventitia in group E had the largest thickness and were almost close to group B. In previous studies, *S. echinocarpum* methanol extract administration was classified as moderately toxic if consumed > 1250 mg/kg BB [36]. It is probably because the content of bioactive compounds in *S. aquifolium* juice was too much. Administration of tannins > 1500 mg/kg BW can cause liver damage (hepatotoxic). Two things allow tannins at these doses to be hepatotoxic; namely, at these doses, tannins can damage mitochondrial membranes. This

destruction will trigger the formation of reactive oxygen species so that it can have a cytotoxic effect, and the administration of huge doses can cause tissue damage [36].

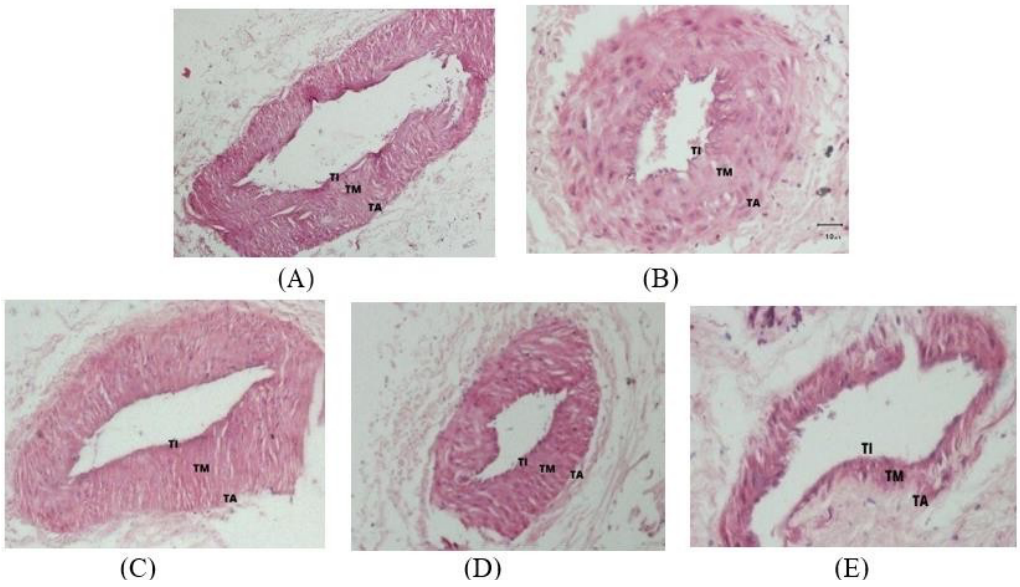

Figure 3. Photomicrograph of tunica intima, tunica media, and tunica adventitia in the artery rats fed a normal and atherogenic diet administered with *S. aquifolium* juice**.** (Magnification 400x) (Staining HE)

Table 3. The thickening of tunica intima, tunica media, and tunica adventitia artery rats fed a normal and atherogenic diet administered with *S. aquifolium* juice**.**

Groups	Thickening (µm)		
	Tunica intima	Tunica media	Tunica adventitia
A	2.4 ± 0.23^{a}	23.03 ± 1.80^{a}	12.23 ± 0.84^{a}
B	$3.4 \pm 0{,}28^{d}$	41.15 ± 2.43^{d}	21.16 ± 1.62^{e}
C	$3.0 \pm 0{,}17^{b}$	36.00 ± 3.21^{c}	18.53 ± 1.53^{c}
D	$2.6 \pm 0{,}15^{ab}$	28.21 ± 2.48^{b}	14.23 ± 1.27^{b}
E	$3.2 \pm 0{,}21^{c}$	39.59 ± 3.58^{cd}	19.43 ± 1.34^{d}

Note: The numbers on the same column are followed by different superscripts letter indicate significantly different ($P < 0.05$).

CONCLUSION

S. aquifolium juice contains bioactive compounds such as tannins, saponins, and steroids. Administration of *S. aquifolium* juice prevents increased cholesterol levels and thickening of the blood vessels in rats by dose dependent manner. *S. aquifolium* juice should not be administered more than two times a day.

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