


The Anti-hypercholesterolemic Effects of *Cnidosculus aconitifolius* Leaves Ethanol Extract on the Formation of Coronary Artery Atherosclerotic Lesions in Atherogenic Diet-Fed Male Wistar Rat (*Rattus norvegicus*)

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ABSTRACT

Introduction: Foods high in cholesterol increase the production of free radicals and inflammation, and may also trigger the formation of atherosclerotic lesions. *Cnidosculus aconitifolius* or tree spinach leaves were known to have antioxidant and anti-inflammatory activities. This study was to determine the effect of *Cnidosculus aconitifolius* on blood cholesterol level and coronary artery atherosclerotic lesions formation using a rat model. **Methods**: A randomized post-test-only control group of 30 male Wistar rats (*Rattus norvegicus*), aged 2-3 months, randomly divided into 3 groups: standard control was given regular food; negative control was given an atherogenic diet; and the treatment group was given an atherogenic diet + *Cnidosculus aconitifolius* leaf ethanol extracts. The treatment lasted 44 days. Data was analyzed with One-Way ANOVA and Kruskal-Wallis test. **Results**: The total cholesterol and LDL levels were significantly lower in the treatment group compared to the control group ($p<0.001$). A decrease of atherosclerotic lesion scores in the treatment group compared to the control ($p=0.010$) was also observed. **Conclusion**: *Cnidosculus aconitifolius* leaf ethanol extracts have an anti-hypercholesterolemic effect, hence the potential to prevent the formation of atherosclerotic lesions in the coronary arteries of hypercholesterolemic Wistar rats.

Keywords: Atherosclerotic lesions, cholesterol, *Cnidosculus aconitifolius*, hypercholesterolemia, LDL.

ABSTRAK

Pendahuluan: Makanan tinggi kolesterol meningkatkan produksi radikal bebas dan peradangan dalam tubuh, serta akan memicu terbentuknya lesi aterosklerotik. Daun pepaya Jepang (*Cnidosculus aconitifolius)* diketahui memiliki aktivitas antioksidan dan antiinflamasi. Penelitian ini bertujuan untuk mengetahui pengaruh *Cnidosculus aconitifolius* terhadap kadar kolesterol dan lesi aterosklerotik arteri koroner pada model tikus. **Metode:** Penelitian *randomized, post-test-only control group,* menggunakan 30 ekor tikus Wistar jantan (*Rattus norvegicus*), berumur 2-3 bulan, secara acak dibagi menjadi 3 kelompok: kontrol standar diberi makanan biasa, kontrol negatif diberi diet aterogenik, dan kelompok perlakuan diberi diet aterogenik disertai ekstrak etanol daun *Cnidosculus aconitifolius*. Perlakuan dilakukan selama 44 hari. Data dianalisis menggunakan *One-Way ANOVA* dan uji Kruskal-Wallis. **Hasil:** Kadar kolesterol total dan LDL secara signifikan lebih rendah pada kelompok perlakuan dibandingkan kelompok kontrol ($p<0,001$). Terdapat penurunan skor pembentukan lesi aterosklerotik pada kelompok perlakuan dibandingkan kontrol ($p=0,010$). **Simpulan:** Ekstrak etanol daun *Cnidosculus aconitifolius* memiliki efek antihiperkolesterolemia dan berpotensi mencegah terbentuknya lesi aterosklerotik pada arteri koroner tikus Wistar dengan hiperkolesterolemia. **Ni Ketut Rake Putri Saraswati, Ida Sri Iswari, I Wayan Juli Sumadi. Efek Anti-hiperkholesterolemik Ekstrak Etanol Daun *Cnidosculus aconitifolius* pada Pembentukan Lesi Aterosklerotik di Arteri Koronaria Tikus Wistar Jantan (*Rattus norvegicus*) yang Diberi Diet Aterogenik.**

Kata Kunci: Lesi aterosklerotik, kolesterol, *Cnidosculus aconitifolius*, hiperkolesterolemia, LDL.

INTRODUCTION

A rise in plasma cholesterol levels >200 mg/dL and an increase in LDL cholesterol >130 mg/dL are indicators of hypercholesterolemia.[1] Among the primary causes are obesity and a diet rich in saturated fat and cholesterol.[2] The accumulation of LDL in the lumen of blood vessels, especially in coronary arteries, will trigger an increase in ROS (Reactive Oxygen Species) levels in the body, causing inflammation and endothelial dysfunction. Oxidized LDL will produce pro-inflammatory, pro-atherosclerosis particles, which are atherosclerosis-causing substances.[3] Atherosclerosis is a condition characterized by the accumulation of fat, cholesterol, calcium, and other substances in the arterial wall, resulting in thickening of the blood vessel wall

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as well as the development of plaques called atherosclerotic lesions.[4] Hypercholesterolemia is the main cause of atherosclerosis as well as atherosclerosis-related diseases such as coronary heart disease (CHD), ischemic cerebrovascular disease, and peripheral vascular disease (PVD).[2]

Antioxidants play a major role in eliminating free radicals and preventing additional oxidation events to halt the lipid peroxidation chain cycle. As intrinsic antioxidant mechanisms may not be sufficient to neutralize all free radicals, antioxidant intake is crucial for maintaining health and preventing disease. *Cnidoscolus aconitifolius,* also known as the Japanese papaya plant, has long been used in Indonesia as a vegetable and also used as traditional medicine. Bioactivity examination showed that *Cnidoscolus aconitifolius* leaves have benefits such as antimicrobial, antidiabetes mellitus, hepatoprotection, antioxidant, antianemia, increased testosterone levels, treatment of gastric ulcers, analgesic, anticancer, and protective effects on the kidneys.[5] *Cnidosculus aconitifolius* leaves have two to three times more nutrients than other plants such as cabbage (*Brassica oleracea*), spinach (*Amaranthus* sp.), and lettuce (*Lactuca* sp.).[5]

This study is to explore the anti-hypercholesterolemic potential of *Cnidosculus aconitifolius* leaves ethanol extract and their capacity to inhibit the formation of atherosclerotic lesions in rats' coronary arteries. This research proposal has received approval from the Research Ethics Committee of the Faculty of Medicine, Udayana University (Number: B/187UNI14.2.9/PT.01.04/2022).

METHODS

Research Design

This study was an experimental randomized post-test-only control group design method, carried out at the Faculty of Medicine, Integrated Biomedical Laboratory, Udayana University, Bali, Indonesia. Rats (*Rattus norvegicus*) were randomly selected and then divided into 3 groups: normal control group, negative control, and treatment group. Rats were given standard feed twice a day (40 g/day) and water *ad libitum*. On day eight, the negative control group was given an atherogenic diet, and the treatment group was given an atherogenic diet - high-cholesterol feed consisting of feed 594 (50%), wheat flour (25%), cholic acid (0.5%), pork oil (9.5%), goat oil (10%), and duck eggs (5%) + *Cnidosculus aconitifolius* leaves ethanol extract. *Cnidosculus aconitifolius* leaves ethanol extract were administered by oral gavages once daily at 600 mg/kg body weight, while the atherogenic diet was given twice daily at doses of up to 40 g/day. The experiments lasted for 44 days.

Preparation of *Cnidosculus aconitifolius* Leaves Ethanol Extract

Cnidosculus aconitifolius, or Japanese papaya leaves, were obtained from Tibubeneng Village, North Kuta, Badung. This plant can live in various climates and grows faster in tropical areas with high temperatures.[5] Dark green leaves were selected from the 5th to the 15th internode. The total weight for this study was 5.5 kg. Fresh *Cnidosculus aconitifolius* leaves were washed and dried at room temperature, then blended to produce dry powder and sieved with a 40-mesh sieve; a total of 480 grams of fine powder was obtained. *Cnidosculus aconitifolius* leaves powder was macerated with 96% ethanol solvent and allowed to stand for 48 hours at room temperature, then filtered to obtain liquid extract. The liquid extract was evaporated with a vacuum rotary evaporator at 40°C to separate the solvent from the pure extract. The end product was an extract with a total weight of 54 grams. The whole process was carried out at the Laboratory of Food and Science Technology, Udayana University. The dose of 600 mg/kg BW/day used in this experiment is based on previous research.[6]

Phytochemical Screening and Quantification

Phytochemical properties of *Cnidosculus aconitifolius* leaves ethanol extract, including flavonoids, alkaloids, saponins, tannins, and vitamin C, were examined with a variety of techniques. Saponin was screened using the Forth method.[7] Alkaloid components were quantified using Meyer reaction techniques.[8] Flavonoids, tannin, and vitamin C were measured for quantification. Flavonoid levels were determined using the UV-Vis spectrophotometry method. Tannin content was measured by using Folin-Ciocalteu method.[9] Vitamin C levels were determined using the redox iodometric titration method.[10]

Rat Models and Treatment

Thirty male Wistar strain rats (*Rattus norvegicus*), aged 2–3 months and healthy with a body weight of 150–180 grams, were used in this experiment. The rats were adapted for environmental adjustment for 7 days before the study. During the acclimatization phase, rats were given a standard diet using feed 594 containing 17.5%-19.5% protein, 3.0% fat, 8.0% fiber, 0.9% calcium, and 0.6% phosphorus, given at 40 grams/day and drinks given ad libitum. The atherogenic diet consists of feed 594 (50%), wheat flour (25%), cholic acid (0.5%), pork oil (9.5%), goat oil (10%), and duck eggs (5%), given at doses of 40 grams per day, twice per day.

The negative control group received the atherogenic diet and distilled water. The treatment group received an atherogenic diet and 600 mg/kg BW/day *Cnidosculus aconitifolius* leaves ethanol extract. The dose of extract was 96 mg in 1 mL distilled water orally per day for 44 days. After 44 days, the rats were starved for 12 hours, and their LDL and total cholesterol levels were measured. Blood was obtained from the retro-orbital plexus. Subsequently, the heart was subjected to a histological analysis of its coronary arteries.

Total Cholesterol Level

Total cholesterol levels in rats were analyzed using the Cholesterol Oxidase Diaminase Peroxidase Aminoantypirin (CHOD PAP) assay technique and a spectrophotometer with the cholesterol FS kit number 113009983021, DiaSys Diagnostic Systems GmbH, Holzheim, Germany. A total of 2.5 mL of blood was drawn from the retro-orbital plexus vein of rats, then inserted into a vacutainer tube and centrifuged to separate the blood and serum. Blood serum was used to measure total cholesterol levels; 1 mL of total cholesterol reagent is pipetted into a labeled microtube, then 10 µL of the sample (or blood serum) is pipetted. After five minutes of incubation at 37°C or ten minutes in a temperature-controlled room, the results can be read within sixty minutes using spectrophotometry at a wavelength of 500 nm.[11]

LDL Level

LDL is measured indirectly by dividing total cholesterol-HDL minus triglycerides/5.[12]

The methods used to measure HDL levels


are the precipitation method and enzymatic colorimetry. The precipitation method is performed with the addition of a precipitate reagent. After centrifugation, HDL in the supernatant was measured using the same kit reagent as the total cholesterol measurement. The kits used were Triglycerides FS (number 157109910021, DiaSys Diagnostic Systems Gmbh, Holzheim, Germany) and HDL Precipitant (number 135409990885, DiaSys Diagnostic Systems Gmbh, Holzheim, Germany).[13]

Histopathological Examination

Preparation

Specimen preparation begins by washing the rat's heart in physiological sodium chloride (NaCl). The heart is then cut into two parts to visualize the coronary arteries, and one part is placed into a tissue cassette. Next, the fixation was carried out with 10% neutral phosphate formalin for 24 hours. Then the tissue is processed in a tissue processor for 22 hours in stages: (1) dehydration using 70%, 80%, and 96% alcohol, absolute alcohol I and II, each for two hours; (2) clearing process using xylol I, II, and III, each for 2 hours; and (3) infiltration process using liquid paraffin I, I, and III, for 2 hours each. The next stage is the embedding process using paraffin blocks. After the block has finished and hardened, the organ is cut using a rotary microtome with a thickness of 3-5 μm. The final process is staining with Hematoxylin Eosin (HE).[14]

Examination

Examination of coronary artery histology uses an Olympus BX 41 electric binocular microscope at 400x magnification to score the atherosclerotic lesions. **Score 0** for normal blood vessels: flat and polygonal endothelial cells aligned with blood flow, tunica adventitia, tunica media, and tunica intima layers arranged in order and neat, and regular smooth muscle cells inside. **Score 1** for elastin fiber dilation with a few foam cells; **score 2** with fragmentation of elastic lamellae and many foam cells and calcification or fibrosis, intracellular lipid infiltration, and proliferation of vascular smooth muscle cells (VCMCs); **score 3** for ulcerated thrombus or plaque with some modifications. Score 0 is normal, score 1 indicates the initiation phase, score 2 indicates the proliferation phase, and score 3 indicates the complication phase, with some modifications.[15]

Histopathological features of coronary artery atherosclerosis lesions with with hematoxylin & eosin staining are shown in **Figure 3**.

Data Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software for Windows v.25.0 (IBM Corporation, Armonk, NY, USA). Normality testing of total cholesterol and LDL levels used the Shapiro-Wilk test, and the homogeneity test used Levene's test. Comparative analysis of cholesterol and LDL levels between groups used the One-Way ANOVA test with the Games-Howell post-hoc test. A comparative analysis of atherosclerotic lesion scores between groups was performed using the Kruskal-Wallis test and Dunn's post-hoc test. The findings were displayed as figures using GraphPad Prism 9.0.1 for Windows (GraphPad Software, San Diego, CA, USA) and Tables.

RESULTS

Phytochemical Compounds

The ethanol extract of *Cnidosculus aconitifolius* leaves contains a variety of antioxidants, including flavonoids, saponins, tannins, alkaloids, and vitamin C. The quantitative results showed that the concentration of flavonoid is 89330.151 mg QE/100 mL, tannin 5388.85 mg TAE/100 mL, vitamin C 1510.21 mg/100 g, and a positive result for alkaloid and saponin.

The Effect of *Cnidosculus aconitifolius* Leaves Ethanol Extract on Total Cholesterol and LDL

Total cholesterol levels showed a significant difference ($p<0.001$) between the treatment group and the negative control group. The cholesterol levels in the treatment group were 206.84 ± 3.76 mg/dL, while the values in the negative control group were 241.93 ± 11.32 mg/dL (**Figure 1**). Similarly, the LDL parameter showed substantial results ($p<0.001$). The average LDL level in the treatment group was 108.80 ± 7.37 mg/dL, lower than the negative control - 136.69 ± 7.46 mg/dL (**Figure 2**).

The Effect of *Cnidosculus aconitifolius* Leaves Ethanol Extract on The Formation of Coronary Artery Atherosclerotic Lesions

The treatment group assigned to the ethanol extracts of *Cnidosculus aconitifolius* leaves showed a mode value of 0, meaning that the majority of the coronary arteries in the ten samples were normal, as compared to the

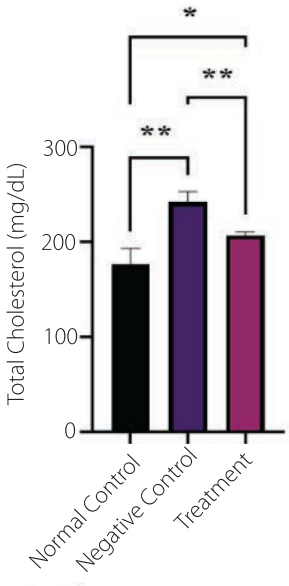

Figure 1. Total cholesterol levels of Wistar rats (n=10). Expressed in mean±SD; *significant difference ($p<0.05$); **significant difference ($p<0.001$) between the two groups based on statistical tests with One-Way ANOVA test.

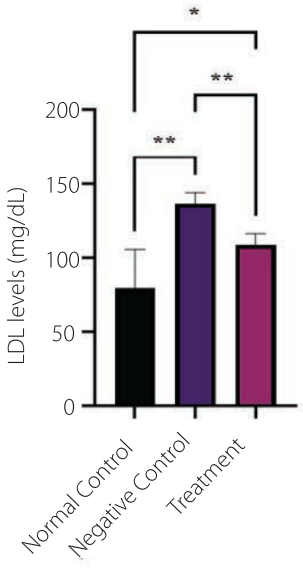

Figure 2. LDL levels of Wistar rats (n=10). Expressed in mean±SD; *significant difference ($p<0.05$); **significant difference ($p<0.001$) between the two groups based on statistical tests with One-Way ANOVA test.

significant differences compared to the negative control group (p=0.010) **(Table 2).**

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majority of the coronary arteries in the ten samples were normal, as compared to the negative control group, which had a mode value of 2 (proliferative phase) **(Table 1)**. The mode value of the treatment group revealed significant differences compared to the negative control group (p=0.010) **(Table 2).**

DISCUSSION

The phytochemical analysis results showed that among the many antioxidant compounds in the *Cnidosculus aconitifolius* leaves ethanol extract used in this study, flavonoids constituted the largest percentage. Tannin is the second most prevalent ingredient in our extract. The results of the phytochemical tests in this study reinforce the results of research conducted by Panghal, *et al*, showing that *Cnidosculus aconitifolius* leaves are rich in antioxidants such as phenolic acids, alkaloids, saponins, flavonoids, and tannins. *Cnidosculus aconitifolius* leaves also contain essential amino acids and vitamins such as vitamin A, vitamin C, niacin, riboflavin, and thiamine.[16]

The *Cnidosculus aconitifolius* leaves ethanol extract used in this study were effective in inhibiting increases in total cholesterol and LDL levels in Wistar rats fed on an atherogenic diet. High cholesterol diet in rat can cause increased total cholesterol and LDL levels.[3] Furthermore, it can raise the body's ROS levels, which raises lipid peroxidation.[17]

Antioxidants play a major role in stopping the chain reaction of lipid peroxidation by removing free radicals and inhibiting other oxidation reactions so that intracellular damage can be prevented, limited, and inhibited. Flavonol subclasses such as kaemferol, trans-resveratrol, and quercetin have the highest percentage in *Cnidosculus aconitifolius* leaves.[18]

Flavonoids work similarly to statin medications in lowering cholesterol levels when compared to pharmaceutical treatment for hypercholesterolemia. Statins work as HMG-CoA reductase inhibitors, which reduce cholesterol formation in the liver by competitively inhibiting the action of the HMG-CoA reductase enzyme. Reduction of intracellular cholesterol concentration leads to increased expression of LDL receptors on the surface of hepatocytes, this condition will increase the release of LDL and other apo-B lipoproteins.[3] *Cnidosculus aconitifolius* leaves also contain vitamin C. Vitamin C can lower cholesterol levels by inducing hydroxylation reactions in the formation of bile fluid, which increases cholesterol removal. Vitamin C also plays a role in helping lipid absorption in the body and maintaining fat balance in the body. As an antioxidant, vitamin C functions to bind oxygen so that it does not support oxidation reactions.[16]

Atherosclerosis is a chronic inflammatory disease of the arteries that affects the structure and function of all three layers of the coronary artery wall. ROS plays a significant role in the development of atherosclerotic lesions. Oxidized low-density lipoprotein or oxidised LDL (ox-LDL) and advanced glycation end-products (AGE) can bind to their receptors (LOX-1 and RAGE, respectively) and induce endothelial cell dysfunction by increasing the expression of vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule (ICAM-1) and inducing the secretion of monocyte chemotactic protein-1 (MCP-1).[19]

In this study, atherosclerotic lesions in the normal control group were not significantly different with the treatment group; both had a mode score of 0. This indicates that after the administration of *Cnidosculus aconitifolius* leaves ethanol extract in the treatment group, there was an inhibition of the formation of atherosclerotic lesions, so most of them were still in normal condition. Antioxidants protect cells from oxidative damage and free radicals but do not stop redox reactions necessary for metabolism, energy synthesis, signaling, and other cellular functions.[20] The flavonoid concentration of *Cnidosculus aconitifolius* leaves ethanol extract is high, which might function as antioxidants and encourage the repair of damaged cells caused by free radicals. Flavonoids can reduce the thickening of the epicardium layer in the coronary arteries of metabolic syndrome animal models.[21]

Saponins have antioxidant effects, inhibit platelet aggregation, induce vasodilation, and lowering lipid profiles, so they can prevent and treat cardiovascular diseases.[22] Tannins work to inhibit the oxidation process of LDL that attaches to the endothelium of blood vessels, thereby reducing the accumulation

Table 1. Distribution of atherosclerotic lesion formation scores in 3 groups.

	Modus	Median	Minimum	Maksimum
Normal Control	0	0	0	1
Negative Control	2	2	0	2
Treatment	0	1	0	2

Table 2. Comparison analysis of atherosclerotic lesion in 3 groups.

Group	*p Value*
Normal Control vs Negative Control	0.001*
Normal Control vs Treatment	0.407
Negative Control vs Treatment	0.010*

*p-value was tested with Kruskal-Wallis test with significance of $p<0.05$

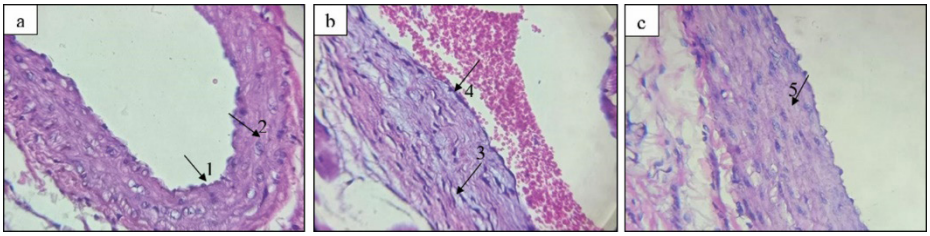

Figure 3. Histopathological features of coronary artery atherosclerosis lesions with hematoxylin & eosin staining (400x magnification). a. Normal control, b. Negative control, c. Treatment, 1. Flat and polygonal endothelial cells aligned with blood flow; 2. Tunica media arranged regularly; 3. Proliferation of vascular smooth muscle cells (VCMCs); 4. Foam cells; 5. Regular smooth muscle cells


of foam cells.[23] Vitamin C has a role in increasing nitric oxide production from the endothelium, reducing monocyte adhesion to the endothelium, lowering blood pressure, and increasing vasodilation.[24]

According to the parameters examined, there were differences in the values among the groups, indicating that the administration of Japanese papaya leaf extract was effective in preventing the rise in LDL and total cholesterol and the development of coronary artery atherosclerotic lesions associated with the antioxidant compounds in the extract. To confirm the reported combination of phytochemical compounds against potential side effects and enhance their pharmacological potential as novel phytomedical substances, further clinical investigations and research are required to understand the synergistic mechanisms of the herbal ingredients we use.

CONCLUSION

Cnidosculus aconitifolius leaves ethanol extract have a significant effect as an antihypercholesterolemic and contain antioxidants. The phytochemical compounds obtained from *Cnidosculus aconitifolius* leaves ethanol extract are effective in preventing the increase in total cholesterol, LDL, and the formation of atherosclerotic lesions in hypercholesterolemic Wistar male rats (*Rattus norvegicus*). The research on the effectiveness of ethanol leaf extracts from *Cnidosculus aconitifolius* on total cholesterol, LDL levels, and atherosclerotic lesions has to be further investigated in humans.

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Disclosure statement

The authors reported no potential conflict of interest

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