

## Effect of purified gambir leaves extract to prevent atherosclerosis in rats

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Received: September 7, 2015; Revised: September 20, 2015; Accepted: September 25, 2015

### Abstrak

**Latar belakang:** Aterosklerosis merupakan faktor risiko timbulnya penyakit jantung koroner (PJK). Senyawa katekin memiliki aktivitas antioksidan tinggi yang dapat menghambat terjadinya aterosklerosis. Ekstrak daun gambir (*Uncaria gambir*; Roxb.) mengandung katekin tinggi sehingga berpotensi menghambat terjadinya aterosklerosis. Penelitian ini bertujuan untuk menguji efek ekstrak terpurifikasi daun gambir dalam menghambat aterosklerosis pada tikus.

**Metode:** Desain penelitian ini adalah eksperimental laboratorium yang dilakukan di Laboratorium Farmasi dan Laboratorium Hewan, Badan Litbangkes, Kemenkes RI pada tahun 2014. Ekstrak gambir dipurifikasi untuk diperoleh kandungan katekin yang optimal, selanjutnya dilakukan uji aktivitas antioksidan menggunakan pereaksi 2,2-difenil-1-pikrilhidrazil (DPPH) dengan pembandingan asam askorbat. Penelitian ini menggunakan 36 ekor tikus putih jantan galur Sprague Dawley berusia 2,5 bulan yang dibagi secara acak ke dalam enam kelompok yaitu kelompok normal, kontrol negatif (akuades), kontrol positif (atorvastatin 2 mg/200 g bb), ekstrak dosis I (20 mg/200 g bb), dosis II (40 mg/200 g bb) dan dosis III (80 mg/200 g bb). Tikus diinduksi dengan makanan yang mengandung lemak tinggi dan perlakuan pengobatan sesuai kelompoknya selama 60 hari, kecuali kontrol normal.

**Hasil:** Kadar katekin dalam ekstrak gambir terpurifikasi diperoleh sebesar 92,69%. Dari uji aktivitas antioksidan diperoleh  $IC_{50}$  11,76  $\mu$ g/mL. Hasil pengukuran aktivitas antiaterosklerosis menunjukkan bahwa jika dibandingkan dengan kontrol negatif, ketiga dosis ekstrak gambir terpurifikasi mampu mencegah terjadinya aterosklerosis dengan menghambat penebalan dinding aorta dan pembentukan sel busa ( $p < 0,05$ ). Aktivitas antiaterosklerosis meningkat dengan bertambahnya dosis.

**Kesimpulan:** Ekstrak gambir terpurifikasi mempunyai efek mencegah penebalan dinding dan pembentukan sel busa aorta tikus. (*Health Science Journal of Indonesia 2015;6:105-10*)

**Kata kunci:** gambir, katekin, antiaterosklerosis

### Abstract

**Background:** Atherosclerosis is a risk factor for coronary heart disease (CHD). Catechin have high antioxidant activity that can prevent atherosclerosis. Gambir (*Uncaria gambir*, Roxb.) leaves extract have high catechin content thereby potentially inhibiting atherosclerosis. This research was aimed to examine effect of purified gambir leaves extract to prevent atherosclerosis in rats.

**Methods:** The experimental laboratory study was conducted in Pharmacy Laboratory and Animal Laboratory, National Institute of Health Research and Development, Ministry of Health, Republic of Indonesia in 2014. Gambir leaves extract were purified to gain optimum catechin. Afterwards, antioxidant activity was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, with ascorbic acid as positive control. Thirty six white male Sprague Dawley rats aged 2.5 months were randomly divided into six groups, i.e. normal control group, negative control group (aquadest), positive control group (atorvastatin 2 mg/200 g bw), extract dose I (20 mg/200 g bw), dose II (40 mg/200 g bw) and dose III (80 mg/200 g bw). The rats were given high fat diet and treatment according to their group for 60 days, except for normal control group.

**Results:** Catechin content in the purified gambir leaves extract was 92,69%. From antioxidant activity test,  $IC_{50}$  was found to be 11,76  $\mu$ g/mL. Anti-atherosclerotic activity study shown that compared to negative control, all three doses of purified gambir leaves extract were able to prevent atherosclerosis through inhibition of aortic wall thickening and foam cell formation due to high fat diet ( $p < 0.05$ ). Anti-atherosclerotic activity increased with increasing dose.

**Conclusion:** Gambir leaves purified extract had the effect of preventing the thickening of the walls and foam cell formation rat aorta. (*Health Science Journal of Indonesia 2015;6:105-10*)

**Keywords:** gambir, catechin, antiatherosclerosis

In the last decade, cardiovascular disease caused by atherosclerosis develop into a major killer in Indonesia. It has become a national health problem, thus we need an effective prevention efforts in overcoming atherosclerosis. Management therapy often used nowadays, especially in the acute phase, is administration of fibrinolytic or management of intervention, followed by drug therapy. However, such management should be supported by preventive measures in order to prevent the incidence of new patients or the emergence of similar attacks in older patients in the future.<sup>1-2</sup>

Atherosclerosis is accumulation of fat in the matrix of the tunica intima, followed by the formation of connective tissue in blood vessel walls. High level of total cholesterol, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in the blood will intensify the process of atherosclerosis.<sup>3</sup> Synthetic drugs that are usually used to prevent the atherosclerosis are cholesterol-lowering drugs such as simvastatin and atorvastatin. However, these drugs have side effects such as myopathy, hepatotoxicity, peripheral neuropathy, dizziness, diarrhea, allergies and increase diabetes mellitus risk. Today, several research investigate potential medicinal plants that have similar effect with synthetic drugs, but with more tolerable side effects. Catechins are flavonoid derivatives secondary metabolites that have antioxidant and anti-atherosclerotic activity. Catechin compounds have cholesterol-lowering activity through inhibition of pancreas cholesterol esterase, bile acid binding, and reduction of cholesterol solubility in micelles that can delay the absorption of cholesterol. Administration of catechins for six weeks can reduce the average area of atherosclerotic lesions by 32% in rat aorta.<sup>4-6</sup>

One of the native plants in Indonesia which contains large amount of catechins is Gambir (*Uncaria gambir* Roxb.). Gambir leaf extract contains catechins as a major component as well as some other components such as catechutannic acid, quercetin, red catechu, gambir fluorescent, fats and waxes.<sup>7</sup> The presence of a high content of catechins in Gambir leaves makes it a potential plant to be used as traditional medicine. This study aimed to examine effect of purified gambier leaves extract to prevent atherosclerosis in rats.

## METHODS

This study was an experimental laboratory design and was conducted in Pharmacy laboratory and Animal Laboratory, Center for Biomedic and Basic

Technology of Health, National Institute of Health Research and Development, Ministry of Health Republic of Indonesia in 2014.

### Preparation and purification of extract

The leaves of gambir was procured from Herbal Plant, Lima Puluh Kota District in West Sumatra and it was authenticated by Herbarium Laboratory, Andalas University, Padang. Gambir extract was prepared by boiling the leaves and pressing them. The liquid extract was filtered and then concentrated using vaccum oven at 80°C to get dried extract. Extract purification process is done through following steps. The gambir extract was grinded into powder, suspended in *n*-hexane and homogenized using sonicator for 10 minutes. The suspension was filtered using filter paper. The residue is then dissolved in ethyl acetate and homogenized using sonicator for 10 minutes. After that, the solution was partitioned by adding distilled water, then shaken in a separator funnel and allowed to stand for 30-60 minutes until two layers were formed (layer of ethyl acetate on top and a layer of distilled water at the bottom). Both layer formed is then separated. Ethyl acetate layer is evaporated using rotary evaporator (Buchi) until viscous extract is gained. Furthermore, viscous extract is evaporated in a fumehood, and then dried using vacuum oven at 40-50 °C until a fixed weight is obtained. Finally, characterization of the purified extract is conducted. This include organoleptic inspection, moisture content, loss on drying, and ash content.<sup>8,9</sup>

### Catechin assay in purified extract

Catechin standard calibration curve was done by plotting six concentrations, i.e. 25; 50; 100; 150; 200 and 300 ppm. Standards and purified extract samples were analyzed utilizing High Performance Liquid Chromatography/HPLC (Waters), with C18 column sized 4.6 × 150 mm, 0.45 mL/min flow rate, 1.0 mL injection volume and UV detection at 280 nm using PDA detector (Waters). The mobile phase gradient is used with the mobile phase A consist of 0.03% acetic acid in a mixture of acetonitrile: water (5:95) and mobile phase B consist of 0.1% trifluoroaset acid in acetonitrile. Mobile phase gradient conditions was 100% A at minutes 0 to 4, 71.5 A and 28.5 B at minutes 4 through 20, and 100% B until minutes 30.<sup>10</sup>

### Antioxidant activity test

The antioxidant activity test was done using DPPH procedure. The sample with concentration of 0.2 mg/mL was dissolved in 100 mL ethanol. The solution was added with 5 mL of 0.1 mmol/L ethanol

solution of DPPH. The solution was then pipetted to spectrophotometer cuvette and incubated at 27°C for 20 minutes. Control blanks were made from ethanol, meanwhile positive control used was ascorbic acid. Absorbance was measured using a UV-VIS spectrophotometer (Hitachi) at 515 nm.<sup>11</sup>

### Aortic thickness measurement and foam cell observation

The protocol of animal study has received the ethical clearance from Health Research Ethics Committee Faculty of Medicine University of Indonesia number 365/H2.F1/ETIK/2014.

Thirty six male Sprague Dawley strain rats aged 2,5 months old were randomly divided into six groups: normal group, negative group, positive group (atorvastatin), dose I (20 mg/200 g bw), II (40 mg/200 g bw) and dose III (20 mg/200 g bw) groups. Rats were induced with high cholesterol and saturated fat feeds for 60 days, except for normal group. After that, test animals were decapitated. For histopathological treatment using hematoxylin and eosin stain (HE), 5 cm abdominal aortic tissue were taken. Observation of the aorta thickness was conducted using microscope equipped (Nikon E 2000) with a micrometer at 100 times magnification. The thickness of the aorta were measured on 8 view zone clockwise: 12:00, 13:30, 15:00, 16:30, 18:00, 19:30, 21:00 and 22:30. The thickness of the aorta was then calculated using the following formula:

$$\text{aortic thickness} = \frac{\text{number of micrometer scale}}{\text{magnification}} \times 1000 \mu\text{m}$$

Observations of foam cell formation in histopathology preparations of rat aorta is performed using a microscope at 400 times magnification.<sup>12</sup>

### Data analysis

Data from the animal experiments were expressed as mean  $\pm$  SD. The statistical significance of differences between the groups were analyzed with one-way ANOVA, followed by LSD post-hoc test analysis using SPSS software 17.0 version, p values of less than 0.05 were considered to indicate significant differences.

## RESULTS

Extract purification process was done twice. Each purification process used 200 g gambir leaves

extract. Purified extract obtained respectively were 129.48 g and 128.72 g. The average yield of purified extract obtained is 64.55%. This indicates a more optimal purification process using ethyl acetate as the solvent. The characterization results showed in Table 1 proved that the purified extract met all parameters required in The Indonesian Herbal Pharmacopoeia.<sup>13</sup>

Table 1. Several characteristics of purified extract

Characteristics	Results	Requirements
Appearance	Solid, powder	Solid, powder
Color	Yellowish brown	Yellowish brown
Odor	Specific gambier	Specific gambier
Taste	Slightly astringent	Slightly astringent
Yield of extract	64.88 %	$\geq 50$ %
Water content	5.44%	$\leq 14$ %
Loss on drying	7.56 %	$\leq 14$ %
Total ash content	0.36 %	$\leq 0.5$ %
Residual solvent	0 %	$\leq 0.5$ %
Level of catechin	92.69 %	$\geq 90$ %

Antioxidant activity test of purified extract using DPPH method came with IC<sub>50</sub> value of 11.56 mg/mL. This value is smaller than ascorbic acid used as comparator (IC<sub>50</sub> of 24.72 mg/mL). The smaller the IC<sub>50</sub> value means the higher the antioxidant activity. These data show the antioxidant activity of purified extract of gambir leaves is very strong.

Table 2. Aortic thickness

Groups	Aortic thickness ( $\mu\text{m} \pm \text{SD}$ )
Normal	96.26 $\pm$ 11.40
Negative	228.72 $\pm$ 17.27
Positive	142.06 $\pm$ 10.35 <sup>#</sup>
Dose I	144.62 $\pm$ 8.83 <sup>#</sup>
Dose II	127.38 $\pm$ 8.16 <sup>#</sup>
Dose III	109.54 $\pm$ 9.53 <sup>#</sup>

Remarks: n = 6 rats.

Significantly different: # p<0.05 compared with normal group; \*p<0.05 compared with negative group, one way Anova

Aortic thickness shown that compared to negative control, all three doses of purified gambir leaves extract were able to prevent atherosclerosis through inhibition of aortic wall thickening. (p<0.05).

After being observed in microscope at 400 times x magnification, the formation of foam cells in negative group shown the foam cells are seen in tunica intima and tunica media seemed more than other group.

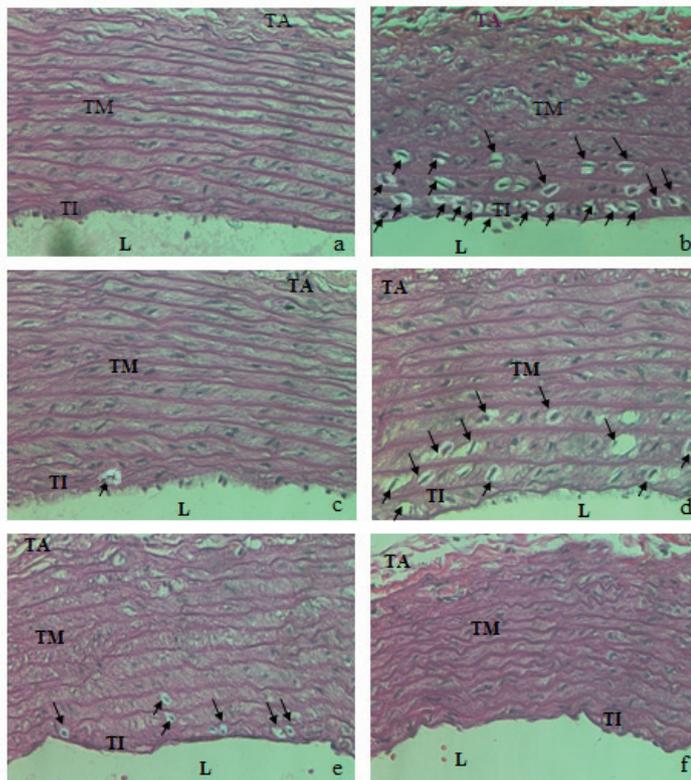


Figure 1. Formation of foam cells in aortic rats

Remarks: a = normal group, b= negative group, c = positive group, d = dose I, e = dose II, f= dosis III, TI = tunica intima, TM =tunica media, TA = tunica adventitia

## DISCUSSION

The purified extract gained has a moisture content of 5.44%, which complies to Indonesia Herbal Pharmacopoeia (IHP), i.e. below 14%. The less water content in the material ingredients can reduce the risk of microbial and fungal growth, or damage caused by insects. Loss on drying (LOD) examination aims to determine water and volatile components lost when heated at 105°C. The purified extract was found to have LOD of 7.56%. LOD value that is bigger than water content explains that in addition to water, there are also volatile components. Determination of total ash content was conducted to determine the amount of material remains after burning at 700°C. The total ash content obtained was 0.36%, which also met Herbal Pharmacopoeia requirements i.e. less than 0.5%. Small ash content value indicates that the material is left slightly. The remaining materials include physiological ash, derived from plant tissue itself as well as non-physiological ash which is a residue of foreign material on the surface of plant, such as sand and soil. It can be said that smaller value of ash content means smaller impurity in the purified extract.<sup>9-13</sup>

Total catechin content in the purified extract derived from HPLC analysis was 92.69%. The catechin content which is more than 90% met the requirement of IHP. These result was consistent with Kurniatri (2015) that purification process can improve the purity and levels of catechin.<sup>14</sup>

Catechins are flavonoid of polyphenol groups that function as a free radical scavenger to provide the hydrogen atom. The structure that allows the radical scavenging activity of polyphenols is the presence of 3,4-dihydroxyl (catechol structure) in the B ring, which acts as an electron donor and became the target of radical. The 3-OH structure of ring C is also beneficial to the activity of antioxidant polyphenols. Conjugation bond at C2-C3 with 4-keto group plays a role for the electron delocalization of the B ring, which in turn increases free radical scavenging capacity. Besides that, the 3-OH group and 5-OH, in combination with 4-carbonyl function and C2-C3 double bond, also raise antioxidant activity. In the absence of o-dihydroxy structure in the B ring, the hydroxyl substituents on the catechol ring A can be compensated and the ability antiradikal activity of polyphenols is increased.<sup>15,16</sup>

The third dose of purified extract was able to reduce the aortic thickness of rats under the mechanism of inhibition of LDL oxidation. Catechin as the largest component in the purified extract has antioxidant activity that is able to reduce LDL oxidation which can induce the formation of several cytokines that can activate pathways stimulated by mitogen-activated protein kinase (MAPK) leading to high expression of the enzyme matrix metalloproteinase-9 (MMP 9). Antioxidants also reduce the toxicity of oxidized LDL on endothelial cells, smooth muscle cells and macrophages as well as reduce degradation oksidatif.<sup>17</sup> Catechins increase HDL levels. HDL has a role to transport cholesterol away from the arteries and back to the liver so it may lower the amount of cholesterol in plaque atheroma.<sup>17-18</sup>

The observation of microscopic atherosclerosis can be seen from the formation of foam cells in the aorta. Foam cells was formed of many macrophages and dendritic cells such as membrane-bound lipids in the cytoplasm. Cells containing the lipid looks like a bubble. The cells' formation begin when phagocytic cells ingest Apolipoprotein-B. The observations of foam cell would appear more clearly with microscope at 400× magnification. Purified extract was able to prevent the formation of foam cells on rats aorta. From microscopic observations, it can be seen that higher dose of purified extract can reduce the formation of foam cells better. Hartayo's *in vitro* study showed that catechin phenolic group, either in extract or single-isomer form, can protect LDL from oxidation triggered by free radical.<sup>19</sup> Furthermore, according to Yang and Koo, catechins have an activity to lower the number of lipid peroxidation products and is able to inhibit LDL oxidation in the endothelium. MMP 9 enzyme contribute a role in stabilizing the atherosclerotic plaque so those plaques would not rupture which in turn may flow through bloodstream and cause blood clots. Decreased expression of MMP 9 shows that catechins can be used as an agent to prevent plaque rupture in the foam cells of aorta.<sup>20</sup> These results are consistent with research from Auclair et al which explain that oral administration of catechins for six weeks can reduce the average area of atherosclerotic lesions in the rat aorta.<sup>6</sup>

In conclusion, gambir leaves purified extract had the effect of preventing the thickening of the walls and foam cell formation rat aorta.

#### Acknowledgment

The authors would like to thank Prof. Bastaman Basuki, Prof. Agus Suwandono, and Dr. M. Wien Winarno for technical assistance in preparing this article.

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