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The cholesterol-lowering activity of *Averrhoa bilimbi* L. leaves ethyl acetate fraction in hypercholesterolemic model

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ABSTRACT

Averrhoa bilimbi L. is used widely as spices and traditional medicine, which contained several chemical compounds such as alkaloid, flavonoid, saponin, and tannin. The aim of this study is to discover the best dose of ethyl acetate fraction from *A.bilimbi* leaves, which have pharmacology activity on total cholesterol and triglycerides in the hypercholesterolemic model. Thirty adult male white mice were divided into six groups; each group was containing five animals. Five groups were given high-fat diet foods and PTU induced the animals to obtain hypercholesterolemia with a frequency of 1 time a day orally for 14 days, while the normal group was given vehicle (Na CMC 5%) and standard feed. On the 15th day, the normal group was given vehicle (Na CMC 5%), negative control group was given high-fat feed, a positive control group was given atorvastatin dose 0.26 mg/kg BW, and another group was given ethyl acetate fraction of *A.bilimbi* leaves dose 200, 400, and 800 mg/kg BW orally. All animals were treated until the 28th day. On day 29, the blood were taken to determine the total cholesterol andtriglyceride levels using clinical photometer 5010 v5+. The data were statistically analyzed using a one-way ANOVA followed by the Duncan Multiple Range Test.. This study indicates that administration of ethyl acetate fraction from *A.bilimbi* leaves can affect the total cholesterol levels and triglycerides in hypercholesterolemic mice ($P<0.05$). Ethyl acetate fraction dose 200 mg/kg BW shows the best activity in decreasing total cholesterol levels and triglycerides on mice hypercholesterolemic model ($P<0.05$).

Keywords: *Averrhoa bilimbi* L., total cholesterol, triglycerides

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INTRODUCTION

Cholesterol is one of the plasma lipids; two primary cholesterol sources in the blood are obtained from food (exogenous) and the liver's synthesis (endogenous). (Price & Wilson, 2006). Blood cholesterol levels should not exceed 240 mg/dL; the condition of blood cholesterol levels past normal values is called hypercholesterolemia (Guyton & Hall, 2007). The management of hypercholesterolemia can be done through non-pharmacological efforts by changing a healthy lifestyle and dietary regulation by limiting cholesterol and saturated fat intake and increasing the consumption of fruits and vegetables (Anwar, 2009).

Nearly all lipoproteins are formed in the liver, where cholesterol, phospholipids, and triglycerides are synthesized except chylomicrons absorbed from the intestine. During the absorption of fatty acids from the gut, small quantities of high-density lipoproteins are also produced in the intestinal epithelium. Lipoproteins' principal role is to transport lipid components in the blood. Very low-density lipoproteins primarily transport triglycerides produced in the liver to adipose tissue, whereas other lipoproteins, including cholesterol and other phospholipids, move from the liver to peripheral tissues or from peripheral tissues back to the liver (Katzung, 2002).

Empirically, *A.bilimbi* leaves can be used to treat joint pain, diabetes, high blood pressure, fever, colds, inflammation of the colon, ulcers, and rheumatism. *A.bilimbi* leaves contain potassium citrate, saponins, flavonoids, sulfur, tannins, peroxidase, formic acid, calcium oxalate, and coumarin. The overall flavonoid content of star fruit leaves is not less than 0.7% (Badan Pengawas Obat dan Makanan Republik Indonesia, 2006). The ethanol extract of *A.bilimbi* leaves contains alkaloids, glycosides, tannins, saponins, and steroids (Karon et al., 2011).

The ethyl acetate fraction of *A.bilimbi* fruit shows antihypercholesterolemic activity in mice with doses low 0.8 mg/kg (Ambili et al., 2009), while the semi-polar fraction of *A.bilimbi* extract can reduce blood glucose at a dose of 125 mg/kg body weight of diabetic rats compared to metformin (Pushparaj et al., 2001). Other studies have also shown that the ethanol extract of at 400 mg/kg BW, A.bilimbi leaves had a substantial influence on cholesterol and low-density lipoprotein (LDL) levels than atorvastatin in diabetic rats (Azeem & Vrushabendraswami, 2015). Study by Fauziah et al. (2018) showed that ethanol extract of *A.bilimbi* leaves a dose of 800 mg/kg BW can reduce total cholesterol and LDL levels in hypercholesterolemic mice. Meanwhile, in vitro research states that *A.bilimbi* leaves extract promoted adipogenesis in much the same way as a PPAR gamma agonist did (Lau et al., 2019). This study will continue the research of ethanol extract *A.bilimbi* leaves conducted by Fauziah et al., (2018), the ethyl acetate fraction of *A.bilimbi* leaves by Fauziah et al., (2020) and to determine the best dose of ethyl acetate fraction from *A.bilimbi* leaves, which have pharmacology activity on total cholesterol and triglycerides. The ethyl acetate fraction was used in this study because ethyl acetate was able to dissolve certain semi-polar alkaloids and flavonoid compounds

MATERIALS AND METHODS

Materials

Materials used in this study were fresh *A.bilimbi* leaves approximately 3 kg obtained from Kurao Pagang Padang, 70% ethanol (PT Brataco), aqua dest (PT Brataco), *n*-hexane (PT Brataco), ethyl acetate (Merck), high-fat foods consisting of beef fat, quail egg yolk, propylthiouracil (PT Dexa Medica), atorvastatin 10 mg (PT Kimia Farma), *Natrium Carboxymethyl Cellulose* (Na CMC) (PT Brataco), butanol (Merck), toluene (PT Brataco), hydrochloric acid (PT Brataco), acetic acid (Merck), cholesterol reagent (Diasys®), triglyceride reagent (Diasys®).

Plant Determination

Determination and identification were carried out in ANDA Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang.

Extract preparation

The extract was made from the dry powder of *A.bilimbi* leaves by maceration using 70% ethanol as a solvent. 300 grams of dried *A.bilimbi* leaves powder were put into the macerator plus 3L of 70% ethanol. The maceration process is carried out for 18 hours. The macerate is separated from the pulp using a flannel cloth. The waste obtained is macerated again twice with the same type and amount of solvent. All macerate was collected and evaporated under a vacuum evaporator until a thick extract was obtained. The yield obtained is weighed. Based on the literature, the yield obtained is not less than 31.6% (National Food and Drug Administration Agency of the Republic of Indonesia, 2006).

Fraction preparation

The condensed extract of *A.bilimbi* leaves dissolved in hot water, then add n-hexane and water (1: 1) in a separating funnel and beaten for 15 minutes. After that, two layers were formed, the n-hexane layer and the water layer. The fractionation process was carried out again in the ethanol and ethyl acetate phases. From these results, two layers will be obtained: the ethanol fraction and the ethyl acetate fraction. This fractionation aims to attract the chemical components contained in ethanol extract based on polarity differences. The resulting ethyl acetate fraction was then concentrated with a vacuum evaporator to obtain a thick fraction. The viscous fraction is then oven-dried at 50 ºC.

Phytochemical screening test

Alkaloid identification

500 mg of extract was weighed, then 1 ml of 2N hydrochloric acid and 9 mL of water were added. Then heated in a water bath for 2 minutes, cooled and separated the filtrate. Three drops of filtrate were transferred into the watch glass, add two drops of *Bouchard at* LP. If with *Mayer* LP, a white or yellow condensed precipitate is formed that dissolves in methanol P. And with *Bouchard at* LP, a brown to black precipitate is formed. There is a possibility that an alkaloid is present (Zulkffle et al, 2014).

Glycosides identification

0.1 mL of the extract solution were evaporated in water bath, the remainder extract dissolve in 5 mL of anhydrous acetic acid. Ten drops of sulfuric acid were added, blue or green color occurs, indicating glycosides' presence (Bogoriani., 2008).

Flavonoids identification

0.5 grams of extract were dissolved in 1 ml to 2 mL ethanol (95%), 0.1 g magnesium powder and ten drops hydrochloric acid were added; the colour ranges from red-orange to red-purple, indicating the presence of flavonoids. The presence of an orange-yellow hue indicates the presence of flavones, and halcones. (Koirewoa et al, 2012).

Saponins identification

0.5 grams of extract were dissolved into the tube reaction, 10 mL of boiling water was added, cooled, and the extract was firmly shaken for 10 seconds. Forming a stable foam for at least 10 minutes at a height of 1-10 cm yields positive results. The foam does not dissipate when 1 drop of 2N hydrochloric acid is added. (Soeharto., et al, 2012).

Making high-fat diet foods

Cholesterol induction in animals, referring to the modified method by Rismawati et al., (2012). Each High-fat diet food consists of 4 kg of standard food, 1 kg of beef fat, 50 g quail egg yolk,. High-fat diet foods are made by heating beef fat, then adding quail egg yolks, stirring until evenly distributed. These ingredients then mixed with 4 kg of standard food.

Animals and experimental design

The Research Ethics Committee, Faculty of Medicine, Universits Andalas, approved this study for ethical consideration No. 124/KEP/FK/2019. The experimental animals used were male BALB/c white mice. A total of thirty mice aged 2-3 months with a bodyweight of 20-30 grams were used in this study. Before being treated, the mice were acclimatized to standard laboratory conditions with 12:12 light/dark cycles for one week and provided standard food and water ad libitum. Thirty adult male white mice were divided into six groups; each group was containing five animals. Five groups were given high-fat diet foods and PTU induced the animals to obtain hypercholesterolemia with a frequency of 1 time a day orally for 14 days, while the normal group was given vehicle (Na CMC 5%) and standard feed. The administration of high-fat diet foods was given 1% of body weight and PTU dose 0.5 mg/kg BW 0.01% of body weight. After that the animals were given standard chow and water ad libitum. On the 15^{th} day the research was carried out until the 28^{th} day. The normal group received a vehicle (5% Na CMC), the negative control group received high-fat feed, the positive control group received atorvastatin at a dosage of 0.26 mg/kg BW, and another group received an ethyl acetate fraction of A.bilimbi leaves dose 200, 400, and 800 mg/kg BW orally until the 28^{th} days.

Determination of total cholesterol levels

Blood was taken from the orbital sinus of the mice eye. At first, the mice were anesthetized by inhalation using ether. Blood was collected carefully into a microtube, then centrifuged for 20 minutes at 3000 rpm. The blood serum was piped with a 10 µL micropipette and was put in a test tube, then 1000 µL of cholesterol reagent solution was added and then mixed and left for 10 minutes at 37^0 C. The uptake was measured using clinical photometer 5010 V5+ (Riele) at a wavelength of 546 nm. As a blank used 1000 µL cholesterol reagent and distilled water.

Determination of triglyceride levels

The blood serum was piped with a 10 µL micropipette and was put in a test tube, then 1000 µL of triglyceride reagent solution was added, then mixed and left for 10 minutes 37^0 C. The absorption was measured using a 5010 photometer V5+ (Riele) at a wavelength of 546 nm. As a blank used 1000 µL triglyceride reagent and distilled water.

Data Analysis

The data were statistically analyzed using a one-way analysis of variance (ANOVA) test, followed by a post hoc Duncan's Multiple Range Test. This data was analyzed using statistical software SPSS program version 22.

RESULT AND DISCUSSION

Plant determination

Based on analysis of the plant determination, it implies that the plant identified as A.bilimbi of the Oxalidaceae family.

Results of phytochemical screening

The phytochemical screening analysis indicated that the leaves of *A.bilimbi* contained flavonoids, alkaloids, and saponins (Setyawan et al., 2021). Table 1 presents the data of the phytochemical screening test.

Table 1. Phytochemistry screening test result of *A.bilimbi* leaves

Phytochemical compound	Result
Alkaloid	+
Flavonoid	+
Saponin	+

Note: (+): Positive

Ethyl acetate fraction *A.bilimbi* leaves prevent an increase in total blood cholesterol levels

Table 2. Mean of total cholesterol levels (mg/dL) of mice after 15 day administration of ethyl acetate fraction *A.bilimbi* leaves (EAF)

Groups	Mean ± SD (mg/dL)
Normal	100.00 ± 11.00^{b}
High-fat diet	140.00 ± 16.00^{a}
Atorvastatin	95.33 ± 3.05^{b}
EAF 200	94.67 ± 2.51^{b}
EAF 400	54.00 ± 1.00^{ab}
EAF 800	37.00 ± 2.64^{ab}

Note: a= significant difference compared to normal group ($p<0.05$).
b=significant difference compared to High-fat diet group ($p<0.05$)

Table 2. shows that in comparison to the normal group, high-fat diet meals produce a substantial rise in total cholesterol levels in the high-fat diet group ($p <0.05$). High-fat diet foods elevate the concentration of chylomicrons in the blood, which promotes the production of lipoprotein. This condition promotes a rise in total cholesterol levels in the high-fat diet foods group due to increased accumulation of exogenous cholesterol in peripheral tissue (Giralt & Villarroya, 2013;Murray, 2009; Sherwood, 2010). Propylthiouracil (PTU) is added to high-fat diet foods in order to elevate cholesterol levels as well as raise body weight. PTU is an antithyroid agent that can prevent the production of thyroid hormones. Thyroid hormone increased can lower the level of cholesterol, phospholipids, and triglycerides in the blood by stimulating cholesterol production, resulting in a rise in cholesterol level. (Guyton & Hall, 2007).

All treatment groups differed significantly compared to the high-fat diet group ($p<0.05$). The ethyl acetate fraction dose 200 mg/kg BW group and atorvastatin group did not differ significantly from the normal group ($p>0.05$). It shows that all treatment groups have effects on total cholesterol levels. The administration of ethyl acetate fraction of *A.bilimbi* leaves dose 200, 400, and 800 mg/kg BW prevented an increase in cholesterol level in mice with hypercholesterolemic conditions. *A.bilimbi* leaves contain flavonoid compounds such as apigenin 7-cellobioside, kaempferol 3,4′-dixyloside, maysin, 3,5,6-Trimethoxy-3′4’-methylene-dioxyfurano, syringaresinol O-beta-D-glucoside, vitexin 4″-O-rhamnoside, retusin 7-O-neohesperidoside (Alhassan & Ahmed, 2016; Giralt & Villarroya, 2013; Pushparaj et al., 2001). It is presumed that flavonoid compounds positively affect lowering total serum cholesterol levels through increased excretion of bile acids with feces (Ranti et al., 2013). Flavonoids also act as a reducer of LDL in the body (Radhika et al., 2011). In addition to lowering LDL, flavonoids enhance the density of LDL receptors in the liver and bind to apolipoprotein B (Baum et al., 1998). In research by Azhari et al. (2017), by inhibiting the enzyme 3-hydroxyl-3-metalhlutarilkoenzim-A reductase, the water extract of A.bilimbi shown antihypercholesterolemic efficacy. This enzyme acts to decrease blood cholesterol levels by limiting fatty acid absorption in the digestive system by inhibiting pancreatic lipase.

Ethyl acetate fraction *A.bilimbi* leaves prevent an increase in triglyceride levels

Table 3 shows that a high-fat diet foods causes an increase in triglycerides in the high-fat diet group different from other groups ($p < 0.05$). Triglyceride levels rise significantly of high-fat diet food administration. During the digesting phase, meals with a high lipid content will be transformed into monoglycerides and free fatty acids. Epithelial cells will convert monoglycerides and fatty acids back into triacylglycerol molecules. All of this material will enter the lymph as chylomicrons. Increased triglyceride levels as a result of high chylomicron concentrations (Murray, 2009; Sherwood, 2010).

Table 3. Mean of triglyceride levels (mg/dL) of mice after 15 day administration of ethyl acetate fraction *A.bilimbi* leaves (EAF)

Groups	Mean ± SD (mg/dL)
Normal	114,67±3,51[b]
High-fat diet	129,00±1,00[a]
Atorvastatin	87,67±7,50 [b]
EAF 200	81,33±2,51[b]
EAF 400	67,33±1,52[ab]
EAF 800	43,33±0,57[ab]

Note: a= significant difference compared to normal group ($p<0.05$).
b=significant difference compared to High-fat diet group ($p<0.05$)

As compared to the high-fat diet group, all treatment groups showed significant difference ($p<0.05$). The atorvastatin and ethyl acetate fraction doses 200 mg/kg BW did not differ statistically from the control group ($p>0.05$). It indicates that all treatment groups have a significant effect on triglyceride levels. The administration of an A.bilimbi leaves ethyl acetate fraction dose 200, 400, and 800 mg/kg BW prevented an increase in triglyceride level in mice with hypercholesterolemic conditions. An interesting point that emerges from the study is the importance of dose-response in pharmacology activity; there is a linear relationship between dose to total cholesterol and triglycerides lowering activity. EAF, at dosages of 400 mg/kg and 800 mg/kg, was able to decrease cholesterol and triglyceride levels below normal boundaries. Hypocholesterolemia is also an indication of impaired liver, spleen and thyroid function (Moutzouri, et al, 2011).

CONCLUSION

The ethyl acetate fraction of A.bilimbi leaves can decrease hypercholesterolemic mice's cholesterol and serum triglyceride levels ($P < 0.05$). The best dose of the ethyl acetate fraction on decreasing total cholesterol and triglycerides is 200 mg/kg BW because it is almost similar to the normal group response.

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