

Effect Essential Oils of Soursop Leaf (*Annona muricata* Linn) on LDL and HDL in Male Rats Induced by Rifampicin

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ABSTRACT

Introduction: Rifampicin is among the drug that causes liver damage through an increased level of Reactive Oxygen Species (ROS). Liver damage that occurs will disrupt lipoprotein metabolism, resulting in an increase of LDL and a decrease of HDL. Terpenoid compounds contained in essential oils of *Annona muricata* Linn were thought to have antioxidant activity. The purpose of this study is to demonstrate the effect of essential oils from *Annona muricata* Linn on LDL and HDL serum level induced with rifampicin. **Methods:** This study was an experimental study with a control group post-test only design using 25 male rats which was divided into 5 groups. A negative control (KN) group was induced with corn oils and a positive control (KP) was induced with rifampicin + corn oils. Treatment group (P1, P2, and P3) were similar with KP but were added with essential oils with a dose of 1,5%, 3%, and 6%, respectively. This study was conducted for 6 weeks. At the end of study, the animals were sacrifice to examined LDL and HDL levels. Results were analysed using One Way ANOVA followed with a post hoc test using the Least Significant Difference (LSD). **Result:** LDL levels between KP vs KN were significantly different. However, HDL levels between KP vs KN were not. These findings may be influenced by rifampicin dose, duration of induction, corn oils use, and serum hemolysis. Treatment of essential oils can reduce LDL levels significantly but, it wasn't significant for HDL levels. Terpenoid and steroid compounds acts as antioxidant and anti-inflammatory agents, and was able to inhibit the formation of free radicals, therefore minimizing liver damage. Variations of doses of essential oils in this study was not shown to have a different effect significantly. **Conclusion:** Essential oils of *Annona muricata* Linn can reduce LDL, but was not effective in increasing HDL levels in male rats induced with rifampicin.

Keywords: Rifampicin; Essential oils of *Annona muricata* Linn; LDL; HDL

INTRODUCTION

Rifampicin is one of the drugs used to inhibit the growth of *Mycobacteria tuberculosis* and *Mycobacteria leprosy*.^{1,2} Rifampicin is inducer of the CYP-450 system in the liver which has the potential to increase the production of free radicals, resulting in conditions of lipid peroxidation which can result in damage to liver cells.^{4,5} A survey conducted in India in 2007 found that the incidence of death related to rifampicin hepatotoxicity was 16 out of 500,000 patients.

The liver is an organ that has many interconnected functions. One of the liver functions that can be disrupted in conditions of liver cell damage is the function of lipoprotein metabolism. Liver damage causes an increase in fatty acids thereby increasing the formation of triglycerides which will stimulate the formation of LDL lipoproteins. In addition, liver damage will also reduce APO-A1 protein synthesis, which is one of the building blocks of HDL.

These conditions cause an increase in LDL and a decrease in HDL.^{6,7}

There is a trend back to nature in maintaining health, thus encouraging the search for medicines originating from nature.⁸

Plants that are currently being developed as alternative therapies for several diseases are soursop leaves.

Compounds contained in soursop leaves include steroids/terpenoids, flavonoids, coumarins, alkaloids, and tannins.⁹ Based on GC-MS (*Gas chromatography – mass spectrometry*) analysis on soursop leaf essential oil (*Annona muricata* Linn), it was identified that the largest component is sesquiterpene hydrocarbons consisting of caryophyllene 38.9%; δ -cadinene 6.0%; α -humulene 4.3% and phenylpropanoid eugenol 30.2% and sesquiterpenoid caryophyllene oxide 5.0%.¹⁰ Terpenoid compounds are thought to act as antioxidants and steroid compounds are thought to have anti-inflammatory effects.¹¹

Empirically *Annona muricata* Linn can be used as anti-arthritic, anticancer, anticonvulsant, antidiabetic, anti-inflammatory, antioxidant, antihypertensive, antiparasitic, insecticide, and wound healing for 8 weeks can improve liver function.

So far, research on the use of soursop leaf essential oil has never been done. Therefore, researchers wanted to prove the effect of giving soursop (*Annona muricata* Linn.) leaf essential oil on serum HDL and LDL levels of rifampicin-induced male Wistar rats.

METHODS

Design

The study was carried out in a true experimental laboratory using a control group posttest only study design in vivo using experimental male Wistar rats induced by a therapeutic dose of rifampicin for 6 weeks.¹⁴ This design is an adaptation of a study conducted by Nahdhiyah (2012) who conducted preliminary research. This research was conducted at the Laboratory of Physiology and Clinical Pathology, Faculty of Medicine, University of Brawijaya, Putra Indonesia Laboratory and Biomedical Herbal Laboratory, Faculty of Medicine, Unisma.

Ethical Clearance

Information on the ethical feasibility of this study was obtained and approved by the Health Research Ethics Commission, Faculty of Medicine, University of Brawijaya.

Research procedure

The experimental animals used white wistar rats (*Rattus norvegicus*) with inclusion criteria 1) male, 2) around 12 weeks of age, 3) 150-200 gram body weight, and 4) healthy condition. This study used male wistar rats, because they were not influenced by hormonal cycles which could affect the results of the study.

The sample used was 25 tails which were divided into 5 groups by random sampling (Table 1).

Rifampicin Induction

The rifampicin used was a therapeutic dose of 10 mg/KgBB converted to the body weight of the rats.³

Then it was dissolved in 1 ml of corn oil. Rifampin was induced with a sonde every day for 6 weeks.¹² Rifampicin induction was given to KP, P1, P2, and P3.

Essential Oil of *Annona muricata* Linn

Essential oil of *Annona muricata* Linn using water vapor distillation method. Simplicia of soursop leaves obtained from Balai Materia Medika Batu, sliced and put in a distillation kettle. The steam boiler is then heated to a pressure of 1 atm. The steam produced is channeled through a hose that is connected to a distilled kettle. Water flows into the condenser and is kept flowing so that all the evaporated oil condenses and does not escape into the air. The result of distillation is essential oil that is not yet pure which is then separated in a separatory funnel by adding N-hexane as an oil binder. Then evaporated at a temperature of 45°C. The result is pure essential oil.

Preparation of Essential Oil Suspension of *Annona muricata* Linn

Annona muricata Linn essential oil with a concentration of 1.5%, 3%, and 6% was obtained by the formula V1M1 = V2M2.¹⁵ The results obtained were then dissolved in corn oil up to 1 ml.

Annona muricata Linn essential oil was administered personally to P1, P2, and P3 after 2 hours of being induced by rifampicin.

TABLE 1: Experimental Animals.

Control	1. Negative control (NK) n = 5 tails	• Corn oil
	2. Positive contro (KP) n = 5 tails	• Corn oil • Rifampicin 10mg/kgBB

Treatment	3. Treatment group 1 (P1) n = 5 tails	• Corn oil • Rifampicin 10mg/kgBB • Essential oils 1,5%
	4. Treatment group 2 (P2) n = 5 tails	• Corn oil • Rifampicin 10mg/kgBB • Essential oils 3%
	5. Treatment group 3 (P3) n = 5 tails	• Corn oil • Rifampicin 10mg/kgBB • Essential oils 6%

Experimental Animals

Dissection of the experimental animals was initiated by injecting the rats with Ketamine 50 mg/kgBB, then the rats were dissected vertically following the median line from the abdomen to the thorax with scissors until all of them were opened. Then, 5 ml of blood is taken from the heart by aspiration using a syringe slowly and placed in a non-EDTA blood specimen tube for further examination.

Examination of Serum LDL Levels

LDL levels were measured by taking 10µl of serum and adding 150µl of R1 reagent as a standard solution and incubating for 5 minutes at 37oC. Then, 50µl of Reagent 2 was added and homogenized. Then, incubated at 37oC for 5 minutes. Then the results of the absorbance of the samples and standards were measured with a spectrophotometer at a wavelength of λ 550 nm (cobas c system 2012).

Examination of Serum HDL Levels

HDL levels were measured by taking 10µl of serum and adding 0.5 ml of Reagent (Cholesterol Kit) mixed evenly for 10 minutes, then centrifuged at 4000 rpm for 10 minutes, then the supernatant was taken. The supernatant or standard is taken 100µl, then put into a test tube. 1 mL of reagent (cholesterol kit) was added, mixed until homogeneous, then allowed to stand for 30 minutes at room temperature or 10 minutes at 37oC. The results of the absorbance of samples and standards were measured with a spectrophotometer at a wavelength of λ 500 nm (BioSystem 2013).

Data Analysis

The data obtained were tested for normality and homogeneity. After being normally distributed, the data were analyzed using the One-Way ANOVA (*Analysis of Variances*) test method to test the existing hypotheses. If there is a significant difference, it is followed by a post hoc test using the *Least Significant Difference* (LSD) to find out the differences between treatments. Results are said to be significant if p <0.05. The statistical test uses the computerized SPSS program.

RESULT

Effects Essential Oils of *Annona muricata* Linn on Rifampicin-Induced Serum LDL Levels

Serum LDL levels induced by rifampicin 10 mg/kg, and essential oil of *Annona muricata* Linn given for 6 weeks showed that the mean serum LDL in KP vs KN increased by 28.9% (p<0.05).

The average results of serum LDL P1, P2, and P3 vs KP showed a decrease of 40%, 30%, and 40% respectively (p<0.05). The dose variations at P1, P2, and P3 did not show any significant differences. This can be seen in Figure 1.

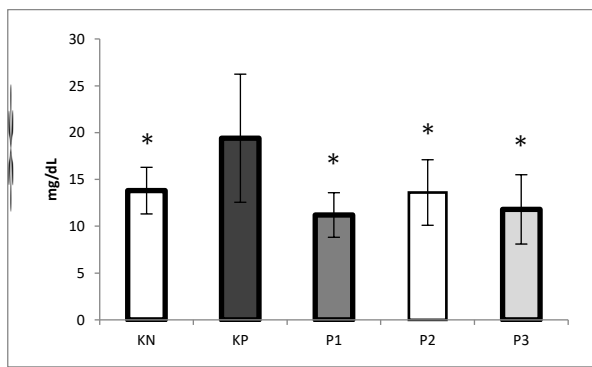


FIGURE 1: Results of serum LDL levels of male Wistar rats induced by rifampicin.

* = $p < 0.05$ vs KP

Effects Essential Oils of *Annona muricata* Linn on Rifampicin-Induced Serum HDL Levels

Serum HDL levels induced by 10 mg/kg BW rifampicin, corn oil, and essential oil of *Annona muricata* Linn administered for 6 weeks showed that the mean serum HDL levels of KP vs KN rats ($p > 0.05$).

The average results of P1, P2, and P3 vs KP ($p > 0.05$) although there is a tendency that the higher the dose of essential oil given, the HDL value will increase. This can be seen in Figure 2.

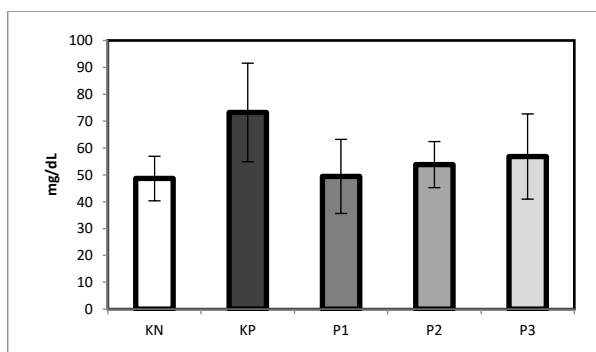


FIGURE 2: Results of mean serum LDL levels of rifampicin-induced male Wistar rats.

DISCUSSION

Effect of Rifampicin Induction on Serum LDL Levels in Male Wistar Rats

Induction of rifampicin in experimental animals for 6 weeks showed that serum LDL levels KP vs KN increased ($p < 0.05$).

Rifampicin can cause liver damage by inducing hepatic CYP-450 which will increase the formation of free radicals. Hepatic damage occurs through the process of damage to cell membranes, mitochondria and endoplasmic reticulum which will cause disruption of calcium homeostasis. The formation of triglycerides in the circulation will stimulate the formation of LDL lipoproteins. These conditions cause an increase in LDL.

Some supporters of this include the results of another study which proved that induction of 10 mg/kg BW rifampicin was proven to increase cholesterol levels ($p < 0.05$) and had no effect on serum triglyceride levels of Wistar rats ($p > 0.05$) (Unpublished data). The results of a study conducted by Shabana (2012) proved that giving a dose of 200 mg/kg BW of rifampicin for 30 days caused an increase in total cholesterol, triglyceride, and LDL levels.

Effect of Rifampicin Induction on Serum HDL Levels of Wistar Male Rats

Rifampicin induction in experimental animals for 6 weeks showed that serum HDL levels were KP vs KN ($p > 0.05$). Giving rifampicin can cause liver damage by free radicals so that it will cause a decrease in APO-A1 protein synthesis which is one of the building blocks of HDL. These conditions cause a decrease in HDL.^{1,7,23}

In a study conducted by Shabana (2012) proved that giving a dose of 200 mg/kg BW of rifampicin for 30 days caused a decrease in HDL levels compared to the control group. In another study conducted by Peter (2013) regarding the effect of 600 mg/kg BW rifampicin on the lipid profile of albino rats, it showed an increase in average triglycerides and cholesterol and a decrease in HDL levels after 20, 40, and 60 days of administration compared to the control group which was induced by distilled water.

The difference in results between previous studies and the results of research conducted by researchers is thought to be caused by several factors, namely the dose of rifampicin given, the duration of rifampicin induction, the addition of corn oil as a rifampicin solvent, and serum hemolysis.

In this study, the therapeutic dose of rifampicin was 10 mg/kg per day which is usually given to Tuberculosis patients and converted to a dose of experimental animals. From previous studies, the hepatotoxic effect of using rifampicin for 6 months only caused side effects on the serum AST and ALP levels of patients.¹⁹ Other studies stated that the hepatotoxic effect of rifampicin was lower than that of INH. Thus, it is suspected that the liver damage that occurred was still minimal. This was also supported by the results of Elvandasari's study (2016) which showed no significant difference in SGOT/SGPT levels between the negative control group and the positive control group.

The second factor is the length of induction of rifampicin. In this study, rifampicin induction was given for 6 weeks every day. This is converted to the duration of therapy in humans and it is found that the duration of induction is classified as chronic (2 years). To show a state of hepatotoxicity at HDL levels requires longer exposure. This is due to the influence of corn oil which can play a role in reducing the production of free radicals due to rifampicin.

The third factor that influenced the results of this study was the administration of corn oil as a rifampicin solvent. Corn oil contains more unsaturated fatty acids.²¹ The results of the antioxidant activity test of corn oil using the 2,2 diphenyl-1-picrylhydrazil (DPPH) method showed a radical inhibition value of 50% (LC50) of 183.85 ppm. This shows the potential for antioxidants contained in corn oil. Corn oil is thought to be able to reduce liver damage caused by rifampicin so that it can reduce the increase in cholesterol, triglyceride, LDL levels and increase HDL levels.²¹ To make a rifampicin solution, dimethylformamide (DMF) or dimethylsulfoxide is added with an aqueous buffer such as Phosphate buffered saline (PBS.) pH 7.4 for solubility and maintain stability of rifampicin.

The fourth factor is the condition of the serum that is not good (hemolysis occurs). There are several factors that can interfere with the process of examining serum HDL levels, one of which is the level of bilirubin (10mg/dL) and hemoglobin (5mg/dL). This factor affects the results because the method used to measure HDL levels is absorbance. If the serum undergoes lysis, it will become a disturbing factor for the examination due to the unfavorable condition of the serum.

Effects Essential Oils of *Annona muricata* Linn on Serum LDL Levels of Rifampicin-Induced Wistar Male Rats

The results showed a decrease in serum LDL levels of Wistar rats at P1, P2, and P3 vs KP ($p < 0.05$). Each of 42.3%, 29.9% and 39.2%.

This is in accordance with research conducted by Miguel (2010) which suggests that essential oils have antioxidant and anti-inflammatory activity. The results of research conducted by Damayanti (2016) which measured the antioxidant activity of soursop leaf essential oil using the 2,2 diphenyl-1-picrylhydrazil (DPPH) method showed that 50% radical inhibition (IC₅₀) was 318.45 ppm. This indicates the presence of antioxidant potential contained in the essential oils of *Annona muricata* Linn.

The antioxidant mechanism elicited is thought to be due to the role of terpenoid derivatives, including β -caryophyllene, eugenol, germacrene, δ -cadinene which are capable of inhibiting lipid peroxidation and as oxygen scavenging.¹¹ Under these conditions, liver damage can be minimized. This condition is reflected by low LDL levels.

In addition, the compounds β -caryophyllen, trans-caryophyllen, sesuiterpene, and α -pinene can act as anti-inflammatories through the 5-lipoxygenase inhibitor pathway. Another compound that acts as an anti-inflammatory through a pro-inflammatory IL-1 inhibitor is caryophyllen oxid. Meanwhile, the compounds caryophyllen and germacrene D inhibit the TNF- α pathway.¹¹ This is supported by research that proves giving essential oils of soursop leaf can reduce TNF- α levels ($p < 0.05$) (Unpublished data).

The content contained in corn oil has the effect of reducing the increase in cholesterol, triglyceride, LDL levels and increasing HDL levels. This is due to the ability to replace saturated fatty acids with polyunsaturated fatty acids. The results of other studies state that giving corn oil at a dose of 12.5 ml can reduce blood cholesterol levels and reduce LDL in rabbits.²¹

Based on the factors above, it is suspected that they work synergistically to inhibit free radicals produced by rifampicin so that the liver damage that occurs can be minimized. Thus, LDL levels in the blood decrease.

The dose variations used in this study, namely 1.5%, 3%, and 6%, showed results that were not significantly different between treatments. This is because at a dose of 1.5% essential oil it has a maximum therapeutic effect so that at higher doses there is no therapeutic response. This is because at low doses it has maximum binding to the receptor.

Effect Essential Oils of *Annona muricata* Linn on Serum HDL Levels of Rifampicin-Induced Wistar Male Rats

The results showed the serum HDL levels of Wistar rats P1, P2, and P3 vs KP ($p > 0.05$). This shows that essential oils of soursop leaf have no effect on increasing serum HDL levels. The factors that can affect the formation of HDL include 1) high levels of triglycerides in the blood, 2) high activity of Hepatic lipase (HL) and 3) Cholesteryl-Ester Transfer Protein (CETP).²⁶

The antioxidant and anti-inflammatory effects of the compounds contained in soursop leaf essential oil have the ability to prevent liver damage due to ROS caused by rifampicin which has been described previously. The content of soursop leaf essential oil which has potential as an antioxidant has similarities to other essential oil studies.

Rashid, et al (2013) Artemisia indica essential oil containing the same active ingredients as soursop leaf essential oil, including β -caryophyllen, germacrene D, sesquiterpene, δ -cadinene has antioxidant potential in the DPPH test and hydroxyl radical scavenging test. Another study regarding terpenoid compounds conducted by Goto, et al (2010) reported that terpenoid compounds are ligands for PPAR (*peroxisome proliferator-activated receptor*). Most of the synthesis of Apo A1 which is the precursor for the formation of HDL is regulated by PPAR so that direct PPAR activation can contribute to increasing HDL levels in the blood.

CONCLUSION

Based on the results in this study, it can be concluded that:

1. Essential oils of *Annona muricata* Linn was able to reduce serum LDL levels of rats induced by rifampicin.
2. Essential oils of *Annona muricata* Linn did not affect the increase in serum HDL levels of rats induced by rifampicin.

SUGGESTION

Researchers suggest to conduct an experiment using toxic doses of rifampicin and lengthen the duration of induction to induce liver damage as a whole. It is necessary to test the toxicity of essential oils *Annona muricata* Linn on normal rats to determine the safety of use and maximum therapeutic dose. Using a solvent other than corn oil as a rifampicin solvent.

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