The Increase of HDL-C Levels using Ethanol Extract of Vernonia amygdalina Leaves in Wistar Rats Models of Metabolic Syndrome

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ABSTRACT

Introduction: Metabolic syndrome (MetS) is a cluster of metabolic factors, including low high-density lipoprotein cholesterol (HDL-C) levels. Vernonia amygdalina (VA) leaves have been documented for their anti-cholesterol properties. The purpose of this study to evaluate ethanol extract of VA leaves in rising HDL-C levels in the MetS model.

Methods: An experimental study using pre-and post-test design was conducted using 30 male Wistar rats divided into five groups randomly. KN group served as the control group. KP group was the MetS model without ethanol extract administration. Meanwhile, P1, P2, and P3 were MetS models and were administered ethanol extract of VA leaves administration (50mg/kg, 100mg/kg, and 150 mg/kg) for 28 days. The data were analysed using paired t-test and one-way ANOVA (α=0.05).

Results: This study discovered that administering the extract in P1, P2, and P3 significantly improved HDL levels. Furthermore, increasing the dose resulted in significantly greater HDL levels. VA leaves contain phytochemicals that have impacts on cholesterol levels in the blood. Flavonoids and tannin inhibit ester cholesterol formation. Terpenoid and flavonoid hinder HMG-CoA. Saponin contributes to free cholesterol transporter, reducing oxidative stress and increasing LDL receptors. Lastly, vitamin C raises catabolism levels and clearance LDL.

Conclusion: There are increases in HDL levels in the MetS model group receiving ethanol extract VA leaves.

Keywords: metabolic syndrome, HDL; Vernonia amygdalina

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INTRODUCTION

Metabolic syndrome (MetS) is a group of metabolic abnormalities, among them hypertension, central obesity, insulin resistance, and atherogenic dyslipidaemia. MetS is still a global health problem and increases the risk of cardiovascular disease, type 2 diabetes mellitus, and stroke. However, the prevalence of MetS is challenging to ascertain due to differences in criteria for diagnosis. The most frequently used criteria is the National Cholesterol Educational Program Adult Treatment Program III (NCEP ATP III), which diagnoses MetS when three of the five conditions are present, including hypertension, hyperglycaemia, central obesity, hypertriglyceridemia, and low high-density lipoprotein cholesterol (HDL-C) levels. Dyslipidaemia in MetS occurs due to impaired fat and lipoprotein metabolism, which causes a decrease in HDL-C and increases low-density lipoproteins (LDL). Low levels of HDL-C can increase the risk of cardiovascular disease and type 2 diabetes mellitus. Meanwhile, increasing plasma HDL-C levels by one mg/L may reduce the chance of coronary heart disease by 2% in men and 3% in women.

Vernonia amygdalina (VA) leaves are known to have anti-cholesterol effects. These leaves contain tannins, saponins, flavonoids, terpenoids, and vitamin C. Saponins, tannins, and flavonoids lower LDL levels by various mechanisms. All three compounds increase the expression of LDL receptors, thereby accelerating the clearance of LDL. In addition, tannins have an antioxidant mechanism that will prevent oxidative stress so that it can inhibit LDL oxidation. Flavonoids contain isoflavones, flavones, and flavanones that can also inhibit cholesterol synthesis. Vitamin C acts as an antioxidant that can prevent the process of lipid oxidation by capturing free radicals, thereby increasing catabolism and clearance of LDL. Therefore, it is expected that a decrease in LDL will trigger a rise in HDL-C in the blood. In addition, VA leaves are thought to have an antiatherogenic role by inhibiting lipid oxidation, which also increases HDL-C.

There is only a little research on the effect of VA ethanol extract leaves on HDL-C levels in the MetS model. At the same time, increased HDL-C levels are expected to benefit metabolic syndrome. Hence, the purpose of this study is to analyse the effect of ethanol extract from VA leaves in increasing HDL-C levels of Wistar Rats Models of Metabolic Syndrome.

METHOD

This study was an experimental laboratory study using post-test and control group design. The research was performed at the Pusat Studi Pangan dan Gizi Universitas Gadjah Mada (PSPG UGM). The subjects were male white rats (Rattus norvegicus) Wistar albino strain aged eight weeks with a body weight of 150-200 grams. The Federer formula calculated the sample size, and to anticipate drop-out, six rats for each group were involved. Hence, for five groups, the sample size was 30 mice. Sampling was carried out by purposive sampling.

The KN group, which is the normal group, got standard feed pellets BR-2. The KP, P1, P2, and P3 groups received metabolic syndrome-induced treatment. These four groups received standard BR-2 pellets ad libitum, and High Fructose Fat Diet (HFFD) feed with a composition of coconut oil 2 mL, cholic acid 1%, cholesterol 2%, and fructose 0.36 mg/200gBB during days 8 to 60. Then, the rats received nicotinamide (Na) at 110 mg/kg intraperitoneally on day 29. After 15 minutes, a single dose of streptozotocin (STZ) 45 mg/kg was injected peritoneally. Induction of metabolic syndrome is successful when characterized by HDL-C <35mg/dL, triglycerides (TG) >150mg/dL, total cholesterol >110mg/dL, fasting blood glucose (FBG) >100mg/dL, and weight change of 8% from baseline body weight. KP was a positive control with no treatment. P1, P2, and P3 groups received VA leaves ethanol extract treatment for 28 days.
The dependent variable of this study was HDL-C levels. HDL-C levels were defined as blood serum HDL-C levels measured before and after Wistar rats received treatment (administration of ethanol extract for 28 days). Blood sampling was taken from the retroorbital vein on day 26 (pre-test) and day 54 of the rats (post-test). Blood serum HDL-C levels were measured by the LDL precipitation method with KitDiaSys (Diagnostisc System Holzheim Germany) and expressed in mg/dL. In comparison, the independent variable was the ethanol extract of VA leaves. Ethanol extract of VA leaves was defined as VA leaves extract made by maceration method using 96% ethanol solvent. VA leaves were obtained in dry conditions from Bulurejo, Karangpandan Village, Karangpandan District, Karangnyar Regency, Central Java 57791. The dose of these extracts was based on the research of Wu et al.\textsuperscript{17}, namely doses of 50mg/kg/day, 100mg/kg/day, and 150 mg/kg/day for 28 days. The extract was made as a suspension solution with a mixture of 0.5% CMC-Na and given at 2 mL orally with a gastric sonde.

Then, the data was analysed using SPSS software. Before analysing the data, the distribution of data obtained was tested using Shapiro-Wilk. One-way ANOVA was conducted to determine the difference in HDL-C levels between all groups. If there is a significant difference between the two groups, then, the post hoc test using the Tukey test was done. In addition, a paired T-test was performed to look for differences in HDL-C levels between pre-test and post-test in each treatment group. The level of significance was $\alpha=0.05$. This study was approved with ethical clearance no 2549/A.2/KEPK-FKUMS/XI/2019.

**RESULT**

**Induction of Metabolic Syndrome**

Induction of metabolic syndrome is successful if marked at least 4 out of 5 criteria, namely FBG levels $>100\text{mg/dL}$, total cholesterol $>110\text{mg/dL}$, triglyceride $>150\text{mg/dL}$, HDL-C $<35\text{mg/dL}$, and weight change 8% of initial body weight\textsuperscript{16}. Table 1 shows a normal result of FBG levels, total cholesterol levels, triglyceride levels, and HDL-C levels in the group that did not receive MetS induction (KN group). Meanwhile, the KP, P1, P2, and P3 groups displayed FBG levels $>100\text{mg/dL}$, total cholesterol $>110\text{mg/dL}$, and HDL-C $<35\text{mg/dL}$. As for triglyceride levels in the four groups were still below 150 mg/dL. However, the triglyceride still increased compared to the KN group. Lastly, there were increases of the weight for KP, P1, P2, and P3 group more than 8% of the initial weight. In this study, these criteria fulfilled the requirement for the MetS animal model. Thus, it can be concluded that the induction of HFFD and STZ-Na succeeded in developing a model of metabolic syndrome in Wistar Rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean FBG (mg/dL)</th>
<th>Mean Total Cholesterol (mg/dL)</th>
<th>Mean Triglyceride (mg/dL)</th>
<th>Mean HDL-C (mg/dL)</th>
<th>Weight changes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN</td>
<td>63.39</td>
<td>82.88</td>
<td>64.66</td>
<td>80.28</td>
<td>26.62</td>
</tr>
<tr>
<td>KP</td>
<td>263.62</td>
<td>189.84</td>
<td>129.21</td>
<td>25.84</td>
<td>8.62</td>
</tr>
<tr>
<td>P1</td>
<td>267.51</td>
<td>188.24</td>
<td>127.68</td>
<td>29.07</td>
<td>20.90</td>
</tr>
<tr>
<td>P2</td>
<td>260.40</td>
<td>187.67</td>
<td>125.91</td>
<td>25.84</td>
<td>24.29</td>
</tr>
<tr>
<td>P3</td>
<td>260.17</td>
<td>190.98</td>
<td>128.39</td>
<td>24.45</td>
<td>25.14</td>
</tr>
</tbody>
</table>

**Increasing HDL-C Levels with Ethanol Extract of VA Leaves in Wistar Rats MetS model**

Table 2 displays that the KN group (no MetS model and no treatment) had normal HDL-C levels ($>35\text{mg/dL}$) in both the pre-test and post-test. In contrast, the KP group (MetS model without treatment) experienced a decline in HDL-C levels, which were below $<35\text{mg/dL}$ for both measurements. As for the treatment group (P1, P2, P3), HDL-C levels decreased during the pre-test but
increased after treatment with ethanol extract of VA leaves. The highest levels were shown in the P3 group, receiving the highest extract dose, 150mg/kg.

Table 2. Mean HDL-C Levels during pre-test and post-test

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean HDL-C levels (mg/dl) ± SD</th>
<th>Mean Difference HDL-C Post and Pre-test (mg/dl) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN</td>
<td>80.27±0.50</td>
<td>1.59±0.68</td>
</tr>
<tr>
<td>KP</td>
<td>25.83±0.72</td>
<td>-1.71±1.44</td>
</tr>
<tr>
<td>P1</td>
<td>29.06±0.50</td>
<td>9.15±3.11</td>
</tr>
<tr>
<td>P2</td>
<td>25.83±0.58</td>
<td>25.07±1.14</td>
</tr>
<tr>
<td>P3</td>
<td>24.45±0.75</td>
<td>39.70±3.10</td>
</tr>
</tbody>
</table>

Table 3 displays the results of the One-Way ANOVA test, which reveals five groups during the pre-test and post-test were significantly different (p-value <0.000).

Table 3. One-Way Anova Result

<table>
<thead>
<tr>
<th>Time of Measuring HDL-C</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-test</td>
<td>0.000</td>
</tr>
<tr>
<td>Post-test</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 4. Post-Hoc Tukey HSD Result

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pre-test</th>
<th>Post-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN – KP</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>KN – P1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>KN – P2</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>KN – P3</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>KP – P1</td>
<td>0.010</td>
<td>0.000</td>
</tr>
<tr>
<td>KP – P2</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>KP – P3</td>
<td>0.532</td>
<td>0.000</td>
</tr>
<tr>
<td>P1 – P2</td>
<td>0.010</td>
<td>0.000</td>
</tr>
<tr>
<td>P1 – P3</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>P2 – P3</td>
<td>0.532</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Based on the results of the post-hoc Tukey HSD test (table 4) during the pre-test, there was a significant difference in HDL-C levels in the group without MetS induction (KN) and with MetS induction (KP, P1, P2, P3). The difference in HDL-C levels also appeared significantly in the KP group with P1, P1 with P2, and P1 and P3. On the other hand, the difference in HDL-C levels between KP and P2, KP and P3, as well as P2 and P3 showed insignificant results. This indicates that the three MetS induction groups (KP, P2, and P3) have uniform HDL-C levels, but this is not the case for P1. For post-test results, differences in HDL-C levels in all group pairs showed significant results. The P1, P2, and P3 groups had higher HDL-C levels than the untreated MetS induction group (KP). However, HDL-C levels in all three treatment groups were still lower compared to the negative control group (KN).

Table 5 demonstrates that all groups had significant differences in HDL-C levels before and after treatment. This showed that the P1, P2, and P3 groups significantly increased HDL-C levels after treatment with VA leaves ethanol extract. The incremental increase in VA leaf extract doses - 50 mg/kg, 100 mg/kg, and 150 mg/kg - significantly increased HDL-C levels in the Wistar Rats MetS model. This is also supported by the post hoc Tukey HSD test results during the post-test comparing groups P1 and
P2, P1 and P3, and P2 and P3 showed significant differences. Hence, the higher the dose, the higher the chance of higher HDL-C levels.

Table 5. Paired t-test result

<table>
<thead>
<tr>
<th>Group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN</td>
<td>0.002</td>
</tr>
<tr>
<td>KP</td>
<td>0.034</td>
</tr>
<tr>
<td>P1</td>
<td>0.001</td>
</tr>
<tr>
<td>P2</td>
<td>0.000</td>
</tr>
<tr>
<td>P3</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**DISCUSSION**

This study showed that the induction of metabolic syndrome was successful in Wistar Rats. The MetS induction in this study was done by feeding HFFD and injecting STZ-Na. HFFD feeding to male Wistar rats can induce MetS conditions within a span of 3-4 weeks, marked by obesity, hyperglycaemia, and dyslipidaemia. STZ-Na injection can also maintain insulin resistance due to cytotoxicity to pancreatic beta cells. This may trigger persistent hyperglycaemic conditions that might contribute to the increase of oxidative stress degree. Increased oxidative stress can preserve insulin resistance. Therefore, the STZ-Na injection can amplify the induction of HFFD to develop a model of metabolic syndrome in Wistar rats.

MetS is characterised by obesity, insulin resistance, and dyslipidaemia. The pathophysiology of insulin resistance can be attributed to increased free fatty acids (FFA). This is due to hypertrophy and hyperplasia of fat cells, which impact decreasing vascular supply and induce hypoxia and inflammation. Additionally, insulin resistance can be caused by proinflammatory cytokines, such as Tumour Necrosis Factor alpha (TNFα) and Interleukin-6 (IL-6), where TNFα can trigger apoptosis and reduce adiposity sensitivity to insulin. Elevated C-reactive protein (CRP) levels are also associated with increases in waist circumference, body mass index (BMI), and insulin resistance. Insulin resistance can contribute to elevated blood glucose levels in metabolic syndrome due to inadequate compensation by pancreas beta cells. Moreover, insulin resistance can trigger an increase in lipolysis that causes FFA to increase. This FFA will be converted back into triglycerides, part of the lipoprotein very low-density lipoprotein (VLDL). Triglycerides in VLDL will exchange with cholesterol esters in LDL lipoproteins, triggering the formation of triglyceride-rich LDL. Triglycerides in LDL will be hydrolysed, forming atherogenic LDL. Triglycerides in VLDL will also exchange with cholesterol esters in HDL lipoproteins, resulting in HDL-C formation, which is poor in cholesterol ester and rich of triglycerid. This HDL-C is easily catabolised by the kidneys, reducing HDL-C levels in the blood. Therefore, dyslipidaemia in a metabolic syndrome is characterised as high triglyceride levels, low HDL-C levels, and increased atherogenic LDL subfraction.

This study found that the P1, P2, and P3 groups showed significant increases in HDL-C levels after administration of VA leaves ethanol extract, in which the higher the dose given will increase the chance of higher HDL-C levels (p-value < 0.05). Previous studies showed significant HDL-C levels increase in diabetic rat models. A study by Tekou et al. displayed different effects of VA after various culinary treatments in lowering HDL-C levels. The aqueous extract and powders -unwashed and washed- elevated the HDL-C levels in diabetic rats, with the highest increase of HDL-C levels found in rats receiving unwashed forms of VA leaves. The washed form was obtained by multiple trituration with water to eliminate the bitterness. Meanwhile, the form of juice of VA leaves failed to display any increase of HDL-C in diabetic rat models. Hence, this previous study suggests that washing and cooking the VA leaves might reduce the activity of its compounds. Similarly, there were increases
in HDL-C levels in STZ-induced diabetic rats after the administration of ethanolic VA leaves, both young leaf (YL) and old leaf (OL), in which the highest elevation of HDL-C was displayed in rats obtaining 300 mg/kg YL and OL. In addition, an elevated HDL-C was also seen significantly in atherosclerotic rabbits compared to control, although no significant results were found between treated and untreated atherogenic diet rabbits.

VA leaves contain several phytochemicals, among them flavonoids, glycosides, saponins (vernonisides and steroid saponins), tannins, triterpenoids/stereoids, sesquiterpene lactones (vernolide, vernoladin, vernolepin, and vernomydin), and vitamin C. Flavonoids reduce cholesterol synthesis by inhibiting ACAT (Acyl-CoA:cholesterol acyltransferase) and HMG-CoA enzymes. Tannins also inhibit the ACAT enzyme, inhibiting cholesterol esters in forming chylomicrons and VLDL. Moreover, the role of antioxidants possessed by tannins can obstruct LDL oxidation and increase LDL receptors through the hindrance of oxidative stress.

Saponins prevent cholesterol absorption by binding to bile acids that act as free cholesterol transporters. Additionally, saponins may lower oxidative stress and increase LDL receptors. Terpenoids can inhibit HMG-CoA reductase, which decreases cholesterol levels. Lastly, vitamin C will accelerate LDL catabolism and clearance of LDL cholesterol with its antioxidant properties, thereby reducing the formation of LDL oxidation.

A study by Adaramoye et al. displayed that VA increased plasma HDL-C in rats fed on a high-cholesterol diet by 41% and 59% with a dose of 100 and 200 mg/kg, respectively, suggesting a dose-dependent relationship. It is thought that VA may act as an anti-atherogenic by inhibiting lipid oxidation and elevating HDL cholesterol. Similarly, a dose-dependent relationship was seen in a study using various doses of aqueous leaf VA extract in rats, in which HDL-C increased and LDL-C decreased. Excluding group with the dose of 800mg/kg, all groups had significant increases in HDL-C levels after 21 days of treatment, with the highest being in group receiving 400 mg/kg and the lowest in group receiving 50 mg/kg with a concentration of 42.3±0.01 and 33.6±0.09, respectively.

The study had several limitations. Firstly, this study did not conduct phytochemical testing. As it has been previously mentioned, various phytochemicals are contained in VA leaves, in which each has different role in lipid metabolism. Thus, it might be difficult to determine the exact compound contributing to the increase of HDL-C levels. A further study should be done to assess the phytochemical in order to provide a depth understanding of their mechanism in increasing HDL-C levels. In addition, further research is needed to analyse the effect of VA leaves ethanol extract on various parameters in MetS. Furthermore, this study has not determine the optimum dose of VA leaves ethanol extract in increasing HDL-C levels, as well as analyse the lethal dose and observe the toxicity of VA leaves ethanol extract. Hence, a further study is required to determine the dose to comprehend the dose-effect relationship.

CONCLUSION

This study shows a significant increase in HDL-C levels in the MetS induction group treated with ethanol extract from VA leaves. Hence, this might suggest that ethanol extract of VA leaves might contribute to the increase of HDL-C levels in this study. Moreover, the greater the dose indicated the higher the HDL-C level.

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CONFLICT OF INTEREST

In this study, the author discloses no conflicts of interest.

REFERENCES


