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Research Article

Effectiveness of Earthworm Extract on the Lipid Profile of Diabetic Wistar Rats

Efektivitas Ekstrak Cacing Tanah terhadap Profil Lipid Tikus Wistar Diabetes

Suandy Suandy[1], Linda Chiuman[2], Angel Jonathane Halim[3]

[1]Department of Biochemistry Faculty of Medicine Dentistry and Health Sciences Universitas Prima Indonesia Medan

[2]Department of Physiology Faculty of Medicine Dentistry and Health Sciences Universitas Prima Indonesia Medan

[3]Medical Study Program Faculty of Medicine Dentistry and Health Sciences Universitas Prima Indonesia Medan

ABSTRACT

Various metabolic diseases, such as diabetes mellitus, are characterized by hyperglycemia, stemming from decreased insulin action, secretion, or both. The use of chemical medicines for diabetes mellitus, especially acarbose, involves regulating the digestion and absorption of complex carbohydrates. However, acarbose may disrupt liver function, prompting exploration into alternative sources for treatment, such as earthworm extract. The purpose of this study was to identify the effectiveness of ethanol extract and coelomic fluid from earthworms (E. eugeniae) on the lipid profile of male diabetic wistar rats (R. norvegicus) in vivo. The research hypothesis is that earthworm extracts could effectively lower the lipid profile of diabetic Wistar rats. This research is experimental research with pre-test and post-test control study group design, using an in vivo method. The results of the one-way ANOVA test of HDL were p=0.441; p=0.441; p=0.000; for LDL were p=0.691; p=0.101; p=0.049; for total cholesterol were p=0.107; p=0.347; p=0.486; and for triglycerides were p=0.028; p=0.926; p=0.553 on days 0, 7, and 14, respectively. Based on the research data, both ethanol extract and a combination of ethanol extract and coelomic fluid were able to reduce lipid profile levels on the 7th and 14th days. Earthworm extract has the potential to promote repair of β cells and is anti-inflammatory which can be used to reduce lipid profile levels.

Keywords: *Coelomic fluid, diabetic, earthworm, ethanol extract, wistar rats*

ABSTRAK

Berbagai penyakit metabolik seperti diabetes mellitus ditandai dengan hiperglikemia yang disebabkan oleh penurunan kerja atau sekresi insulin, atau keduanya. Penggunaan obat-obatan kimia Diabetes Melitus khususnya Acarbose, bekerja dengan menunda pencernaan dan penyerapan karbohidrat kompleks. Acarbose memiliki efek berupa gangguan fungsi hati, sehingga dilakukan terobosan pengobatan dari sumber alternatif yaitu ekstrak cacing tanah. Penelitian ini bertujuan untuk mengetahui efektivitas ekstrak etanol dan cairan *coelomic* dari cacing tanah (*E. eugenie*) terhadap profil lipid tikus wistar (*R. norvegicus*) jantan diabetes secara in vivo. Hipotesis dari penelitian ini berupa ekstrak cacing tanah memiliki efektivitas dalam menurunkan profil lipid pada tikus yang diabetes. Penelitian ini adalah penelitian eksperimental dengan rancangan *pre-test and post-test control study group* metode *in vivo*. Hasil dari uji ANOVA satu arah pada HDL *p*=0,441*; p*=0,441; *p*=0,000*;* LDL *p*=0,691*; p*=0,101*; p*=0,049; Total kolesterol *p*=0,107*; p*=0,347*; p*=0,486; Trigliserida *p*=0,028*; p*=0,926; *p*=0,553 pada hari ke-0, 7, dan 14 secara berurutan. Berdasarkan data penelitian, didapatkan bahwa ekstrak etanol dan kombinasi ekstrak etanol dengan cairan *coelomic* mampu menurunkan kadar profil lipid pada hari ke-7 dan ke-14. Ekstrak cacing tanah memiliki potensi mendorong perbaikkan pada sel β dan anti-inflamasi yang dapat digunakan untuk menurunkan kadar profil lipid.

Kata Kunci: Cairan coelomic, diabetes, ekstrak etanol, cacing tanah, tikus wistar

Correspondence: Angel Jonathane Halim. Medical Study Program Faculty of Medicine Dentistry and Health Sciences Universitas Prima Indonesia, Jl. Sampul No.3, Sei Putih Bar., Kec. Medan Petisah, Kota Medan, Sumatera Utara 20118 Tel. +628116088898 Email: angeljonathane@icloud.com

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INTRODUCTION

Various metabolic diseases, such as diabetes mellitus, are characterized by hyperglycemia resulting from decreased insulin action, secretion, or both (1). Chronic hyperglycemia increases the risk of cardiovascular disease (CVD) and is associated with several long-term microvascular problems that can affect the nervous system, kidneys, and eyes. The glycemic threshold associated with microvascular disease forms the basis of the diagnostic criteria for diabetes. Over the last three decades, the number of diabetes mellitus cases worldwide has quadrupled and is currently estimated to be the ninth largest cause of death (2).

A diabetes diagnosis is established if the fasting blood glucose level is 126mg/dl, blood glucose 2 hours postprandial is 200mg/dl, HbA1c is 6.5%, or their random blood glucose reading is 200mg/dl with symptoms. Retesting, ideally with the same assay, is recommended as early as possible the next day to confirm the diagnosis if better results are seen in asymptomatic individuals. In type 2 diabetes mellitus, lipogenesis decreases, but lipolysis is higher due to decreased insulin action in adipose tissue. LDL (Low-Density Lipoprotein) cholesterol levels increase due to the stimulation of glucotoxicity and lipotoxicity. When hyperglycemia occurs due to persistently high blood glucose levels, LDL oxidation occurs more quickly. Insulin resistance in Type 2 DM patients causes increased lipolysis in adipose tissue, thereby increasing blood fat levels, including triglycerides and cholesterol (3). Treating people with high lipid levels can lower their chances of developing cardiovascular disease, while the prevalence of diabetes is increasing globally (4). The average normal person has HDL-C levels between 40 and 60mg/dL, LDL levels must be less than 100mg/dL, and triglycerides must be less than 150 mg/dl (5,6).

Chemical medicines for diabetes mellitus, especially acarbose, work by delaying the digestion and absorption of complex carbohydrates (7). However, acarbose may disrupt liver function, so a breakthrough treatment has been made using alternative sources, such as earthworm extract (8). Indonesia, as a tropical country, offers easy access to earthworms. Since ancient times, earthworms have been widely used in traditional Chinese medicine as an important component, and recent research on the therapeutic effects of earthworms has been conducted, coinciding with advances in biochemical technology (9).

Earthworms demonstrate pharmacological effects through proteins and active substances, including lisenin, antitumor protein, lumbrokinase, collagenase, superoxide dismutase, cholinesterase, catalase, glycosidase, metallothionein, calmodulin-binding protein, and proteins with increased proliferative activity. The extraction of earthworms involved the use of the ethanol extract method and coelomic fluid extraction. Coelomic fluid has no or minimal absorbance, indicating no functional groups in the chemical compound, while ethanol extract shows several functional groups, including aliphatic groups, ethanol, and amide A, suggesting the presence of amino acids as protein monomers (10). Earthworm extract has antioxidant, fibrinolytic, antibacterial, and antipyretic properties. Furthermore, *Eudrilus eugeniae* coelomic fluid exhibits anti-inflammatory potential comparable to aspirin, with effectiveness similar to indomethacin (11).

In diabetes mellitus, pancreatic β cells from ROS (Reactive Oxygen Species) caused by oxidative stress can be blocked and maintained by *Eudrilus eugeniae* extract, which contains antioxidants (12). To determine the antioxidant content of earthworm extracts, an examination can be carried out using the DPPH (diphenylpicrylhydrazil) method, a test used to measure antioxidant activity based on its capacity to neutralize free radicals (13). DPPH, a stable free radical compound, serves as a reagent in free radical scavenging tests (14). Compound classification is based on the IC50 value, with less than 50 classified as very strong, 50-100 as strong, 100-150 as medium, and 151-200 as weak. A lower IC50 value indicates higher antioxidant activity (15).

For this reason, there is a need for research to evaluate the effectiveness of earthworm extract on the lipid profile of male diabetic *Rattus norvegicus* Wistar rats induced by alloxan. Alloxan-induced diabetes mellitus has the advantage of cost-effective per gram compared to streptozotocin. Pancreatic β cells are toxically affected by alloxan, a urea derivative with glucose-like characteristics. Alloxan enters the cytosol via GLUT2 (Glucose Transporter 2) and passes through the plasma membrane due to its hydrophilic properties and a molecular structure resembling glucose. Following Mostafavinia's findings, groups of experimental animals induced with DM with different doses did not show the same survival rate as those induced by Alloxan 120 mg/kg BW subcutaneously and 140 mg/kg BW intraperitoneally (16).

The research aims to demonstrate the effectiveness of ethanol extract and coelomic fluid from *Eudrilus eugeniae* in reducing the lipid profile of diabetic male *Rattus norvegicus* Wistar rats.

METHOD

Ethical approval for this study was obtained from the Health Research Ethics Committee, Prima Indonesia University No 057/KEPK/UNPRI/V/2023.

Participant Characteristics and Research Design

The first samples used were 60 male Wistar strain rats, *Rattus norvegicus*, weighing 300 grams and aged 2-2.5 months. The rats had unrestricted access to food of a special rodent diet and drink under controlled conditions: a temperature of 25°±2°C, humidity at 50%, and a 12-hour light-dark cycle.

The second sample used were earthworms, *Eudrilus eugeniae*, an invertebrate (no backbone) strain belonging to *Kingdom Animalia, Phylum Annelida, Class Clitellata, Subclass Oligochaeta, Ordo Haplotaxida, Family Eudrilidae, Genus Eudrillus, and Species Eudrilus eugeniae.* A total of 2000 grams of *Eudrilus eugeniae* were collected and acclimated in soil with a diet of vegetables and fruit for a week.

The research design used was an experimental method, including *in vivo* experimental (*Rattus norvegicus* wistar). *In vivo*, sampling and processing of samples were carried out, including making ethanol extract and coelomic fluid, as well as the measurement of Wistar rats' body weight, measurement of HDL (High-Density Lipoprotein), LDL (Low-Density Lipoprotein), triglycerides, and total cholesterol.

Sampling Procedures

The total sample size for this research was 60 diabetic Wistar rats. To prevent the animals from aggression, five Wistar rats were placed in each cage.

$$(n-1)(t-1)\geq 15 \quad n=6/\text{group}$$

The experimental animals were divided into four treatment groups: the positive control group, the negative control group, treatment 1, and treatment 2.

The data were collected at two places: a.) Integrated Laboratory, Faculty of Medicine, Dentistry, and Health Sciences, Prima Indonesia University, for ethanol extract preparation and collecting coelomic fluid from *Eudrilus eugeniae* worms; b.) Animal House Eka Rasmi Gg Eka Perjuangan No. 15 Medan Johor, for measurement of body weight of experimental animals, blood sampling from the tail vein (Vena coccygeus), and measurement of HDL, LDL, triglyceride, and total cholesterol levels.

Preparation of Eudrilus Eugeniae Ethanol Extract

Extraction was carried out using the maceration method with a 96% ethanol solvent. Ethanol (CH3CH2OH) is an alcohol, a class of chemical substances with a hydroxyl group, -OH, attached to a carbon atom. Ethanol is often used in making extracts due to its characteristics of being food-grade and medicinal-grade (17). Ethanol is able to precipitate proteins even though it has gone through processes such as crystallization and purification. Therefore, earthworms are extracted using the ethanol maceration method so that their protein potential is good. The initial step involved washing 1000 grams of *Eudrilus eugeniae* with distilled water and blending until achieving a smooth consistency. The refined *Eudrilus eugeniae* was then immersed and macerated with 5000ml of 96% ethanol in a dark jar for 6 days, accompanied by bastosonics for 30 minutes daily over 4 days. Subsequently, the macerated results underwent rotary evaporation at a temperature of 50°C for 7 days. Samples that did not reach complete thickening underwent a water bath process, allowing all ethanol to evaporate at a temperature of 50°C for 1 month to achieve an extract concentration of 100%. The evaporation process that the researchers carried out was quite long because, in one day, they only carried out the evaporation process for 7 hours a day, so it took up to 1 month in total for the extract concentration to reach 100%. The final results of this procedure were stored in a refrigerator at 2°C for subsequent research and examination.

Preparation of Eudrilus Eugeniae Coelomic Fluid

The coelomic fluid was produced and collected using the cold shock method (10). Initially, 2000 grams of Eudrilus eugeniae underwent cleaning with distilled water. Subsequently, the clean *Eudrilus eugeniae* was wrapped in filter paper, placed in a glass funnel, and covered with plastic wrap to prevent the worms from escaping. A glass funnel was then placed atop a glass beaker, serving as a receptacle for collecting the ensuing coelomic fluid. The glass funnel and glass beaker were then refrigerated for 6-7 hours. Following this process, 45 ml of coelomic fluid was obtained from the 2000 grams of Eudrilus eugeniae worms. Means that 1000 grams of earthworms can produce approximately 22–23 ml of coelemic fluid. The coelomic fluid underwent centrifugation for 30 minutes at a speed of 3000 rpm and was subsequently stored in the freezer at a temperature of -25°C (18).

Induction of Diabetes Mellitus in Experimental Animals

After an 18-hour fasting period, *Rattus norvegicus* Wistar rats were given a single injection of a 10% alloxan monohydrate solution dissolved in a 0.1M sodium citrate buffer solution. Alloxan, an unstable hydrophilic molecule, is toxic to the liver and kidneys and also destroys pancreatic beta cells at certain doses. This compound induces a multiphasic blood glucose response in experimental animals, which is accompanied by variations in plasma insulin levels and, ultimately, necrotic cell death (19). The Wistar rats were injected with alloxan at a dose of 130mg/kg BW via the intraperitoneal method. Fasting blood sugar levels were examined in *Rattus norvegicus* Wistar rats three days post-injection, revealing an absence of diabetes mellitus. Consequently, the same dose and procedure were replicated for the second injection. An examination conducted three days later confirmed the manifestation of diabetes mellitus in the test animals.

Testing Earthworm Extract on Experimental Animals

The dosage for earthworm ethanol extract and coelomic fluid was determined based on previous research references. Eudrilus eugenie extract in powder form at a dose of 42.128mg/kgBW was able to inhibit alpha-glucosidase by phenolic compounds and reduce apoptosis in pancreatic Langerhans B cells. Meanwhile, coelomic fluid with a dose of 4.6 micrograms per milliliter has anticancer activity with an IC50 of 50. In this study, the dose of coelomic fluid was reduced to 5 microliters because the extract was combined with the administration of ethanol extract to obtain maximum therapeutic effect.

The positive control group was administered acarbose, given three times a day at mealtime at a dose of 4.5mg/kg BW to Wistar rats with diabetes. The negative control group received distilled water, given three times a day at mealtime to Wistar rats with diabetes. Treatment group I received ethanol maceration extraction dissolved in water at a ratio of 1:2, administered to Wistar rats with diabetes at a dose of 42.128mg/kg body weight, three times a day at mealtime. Treatment group 2 received a combination of ethanol maceration extraction with a dose of 42.128mg/kgBW dissolved in water with a ratio of 1:2 and 1 microdrop (5µl) of coelomic fluid given personally three times a day at mealtime. Wistar rats' lipid profiles were examined on days 0, 7, and 14. The tools used in this research were cages, places to eat and drink, water, syringes, oral probes, dropper pipettes, scissors, cotton, ether, analytical scales, rat scales, filter paper, micropipettes, tissue, Petri dishes, beaker glass, glass funnel, blender, rotary evaporator, BactoSonic, centrifuge, EDTA tube, glucometer, lipid prometer. As for the treatment stage: a.) Measurement of blood sugar levels during fasting and 2 hours after eating using the AutoCheck® device. The lancing device was used to extract a small drop of blood, which was then applied to the target location of the test strip in the blood glucose meter. After a few seconds, the results appeared on the meter. In case of an insufficient blood sample, an error warning would be displayed, and the test needed to be repeated; b.) Measurement of HDL, LDL, and triglycerides using the Lipid Pro® device. Lipid-Pro is a user-friendly analytical tool for analyzing complex MS(E) lipidomics data, including a module for constructing user-specific lipid datasets (20). A small drop of blood, which was then applied to the target location of the test strip in the lipid pro meter. After a few minutes, the results appeared on the meter.

Measures and Covariates

The independent variables in this research were ethanol extract and a combination of ethanol extract and coelomic fluid with *Eudrilus eugeniae*. The dependent variables in this study were the levels of HDL, LDL, triglycerides, and total cholesterol in *Rattus norvegicus* Wistar rats with diabetes induced by alloxan. Wistar rats with diabetes were given therapy consisting of ethanol extract and a combination of ethanol extract and coelomic fluid. The administration of this therapy was then compared with the administration of acarbose and distilled water therapy as positive and negative control groups. To assess the lipid profile, 5 µl of blood was extracted from the tail vein blood of Wistar rats and placed on a strip. The tool subsequently measured and presented the results of the lipid profile on the screen. The lipid profiles of the rats were examined on days 0, 7, and 14.

Data Analysis

The data obtained were quantitative in the form of HDL, LDL, triglyceride, and total cholesterol levels. The initial step involved testing the data for normality using the Shapiro-Wilk test. Subsequently, a homogeneity test was conducted using Levine's test. In cases where the data demonstrated homogeneity, a one-way ANOVA test was carried out. If the data were not homogeneous, the Kruskal-Wallis test was carried out. Following these primary tests, post-hoc analysis was conducted using the Bonferroni test for homogeneous data and the Games-Howell test for non-homogeneous data.

RESULTS

Lipid profile analyses were conducted on diabetic rats induced by alloxan at a dose of 130mg/kg body weight. These rats received therapy three times a day during meals, containing ethanol extraction of earthworms at a dose of 42.128mg/kg body weight and a combination of the same dose with 5µl of coelomic fluid. The outcomes indicate a reduction in lipid profile levels. The following histograms show the research results.

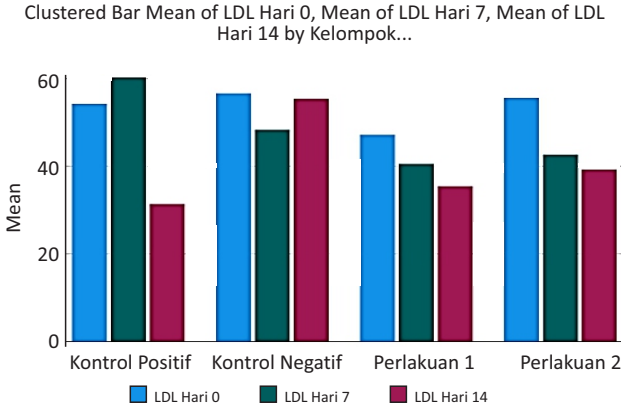

Figure 1. Histogram of LDL

According to the diagram, the LDL value in treatment group 2 experienced a drastic decrease on day 7. Meanwhile, treatment group 1 experienced a stable decline on days 7 and 14 (Figure 1).

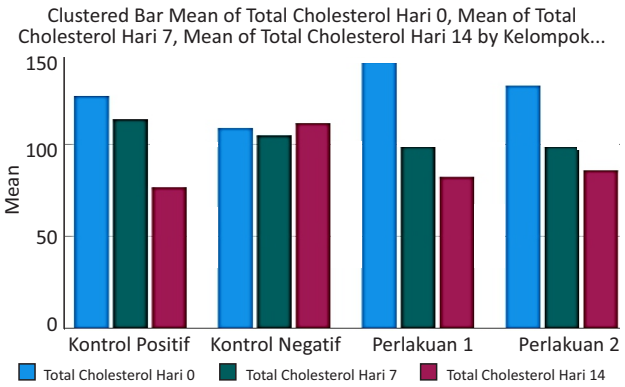

Figure 2. Histogram of total cholesterol

According to the diagram, the total cholesterol value in treatment group 1 and 2 experienced a drastic decrease on day 7. Meanwhile, the positive control group experienced a drastic decrease on day 7 (Figure 2).

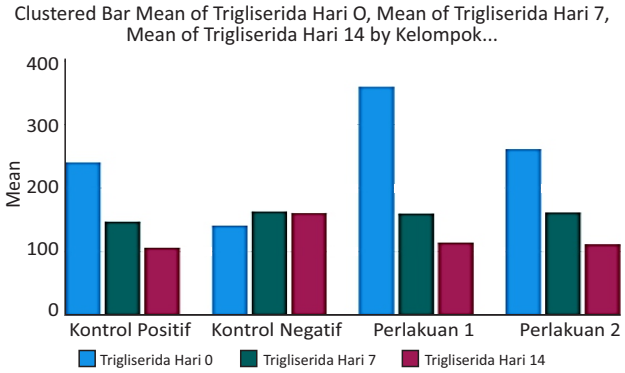

Figure 3. Histogram of triglyceride

According to the diagram, the triglyceride value in treatment group 2 experienced a drastic decrease on day 7. Meanwhile, treatment group 1 experienced a stable decline on days 7 and 14 (Figure 3).

Acarbose, ethanol extract from earthworms, and a combination of ethanol extract and coelomic fluid from earthworms demonstrated optimal reductions in LDL, total cholesterol, and triglycerides on day 14. Acarbose, known for its faster lipid profile level reduction, is recommended for more prompt results. Alternative treatments, such as those using earthworm extract, especially ethanol extract, are also able to reduce lipid profile levels but require a slightly longer time (Figure 1, 2, 3).

DISCUSSION

The efficacy of earthworm extract was evaluated using ethanol maceration and cold shock procedures. Several Wistar rats dropped out during the adaption and alloxan induction processes, leading to a repetition of the induction due to initial failures. Although the study was intended to continue until days 28 and 35, data obtained were not normally distributed, primarily due to several rats dropping out owing to factors like suboptimal habitat conditions, insufficient dosage, and resulting adverse effects. Data were collected from 36 Wistar rats in this

investigation, repeated three times in each group using different Wistar rats but the same treatment each week. Research findings indicate that both ethanol extract and a combination of ethanol extract and coelomic fluid were effective in reducing lipid profile levels on the 7th and 14th days. These outcomes align with research by Suandy that earthworm extract possesses antioxidant, fibrinolytic, antibacterial, and antipyretic capabilities, while *Eudrilus eugeniae*'s coelomic fluid has anti-inflammatory potential. The antioxidant content of earthworms can protect pancreatic cells from oxidative damage induced by ROS (Reactive Oxygen Species).

Statistical analyses, including Levene's homogeneity test, one-way ANOVA test, and post hoc examination, were conducted. The Levene's test results indicated homogeneity for LDL on day 14 and triglyceride on day 0, with $p>0.05$, suggesting consistent variances within the data. The one-way ANOVA test showed a statistically significant difference between the treatment groups, with $p<0.05$, in HDL and LDL on day 14, and triglyceride on day 0. The post-hoc test was divided into two categories: Tukey HSD for homogeneous data and Games-Howell for non-homogeneous data. In the post-hoc Tukey HSD test, LDL on day 14 showed a statistically significant difference between the positive control group and the negative control group, with $p=0.046$, meaning that the negative control group was lower than the positive control group. Triglycerides on day 0 showed a statistically significant difference between the negative control group and treatment 1, with $p=0.018$. While the one-way ANOVA test did not reveal an optimal decrease in the lipid profile. However, when viewed based on descriptive statistical data, a decrease in the lipid profile occurred every week, in treatment groups 1 and 2.

However, the statistical tests suggest that the resulting reduction may not be considered ideal. This variability could be attributed to various factors, including environmental variables, physical conditions, and potential toxicity. According to the research findings, the study's limitation was the lack of individual monitoring of Wistar rats from the initiation of alloxan induction to the injection of the extract, spanning from day 1 to day 14. Due to the treatment of Wistar rats, execution was carried out to get kidney and pancreas organs for future research. As a result of using new Wistar rats each week, the study possesses inherent flaws. Future research efforts are encouraged to investigate more extensively into the dosage and toxicity aspects of earthworm ethanol extract and earthworm coelomic fluid.

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