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Flaxseed extract: A potential therapeutic intervention for type-2 diabetes mellitus and cardiovascular risk reduction in Wistar rats

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ABSTRACT

Introduction: Type 2 diabetes mellitus (T2DM) affects millions of people worldwide, highlighting the importance of dietary interventions. Flaxseed (*Linum usitatissimum* L.) has shown promise in treating chronic metabolic diseases. This study investigates the therapeutic potential of flaxseed as an antidiabetic agent and its protective effect on cardiovascular diseases.

Methods: Rats were randomly divided into five groups: normal fed with drug carrier group (N), diabetes with drug carrier group (D), diabetes with metformin (M) group (D+M), and diabetes with flaxseed extract doses of 100 and 200 mg/kg (D+F1 and D+F2) groups. The diabetic rat model was established by administration of drinking water containing 25% fructose and a single intraperitoneal injection of 100 mg/kg alloxan. After a six-week intervention, the rats were sacrificed to assess the antidiabetic and cardiovascular effects.

Results: The doses of 100 and 200 mg/kg flaxseed extract significantly reduced body weight and fasting glucose levels and increased insulin sensitivity index (K_{ITT}) compared to the diabetes group ($P<0.05$). In addition, histological analysis showed a significant improvement and increase in pancreatic beta cells in the treatment group compared to the diabetes group ($P<0.05$). Flaxseed extract doses of 100 and 200 mg/kg significantly reduced weight gain and insulin resistance, contributing to decreased arterial stiffness and atherogenic index compared to the diabetic group ($P<0.05$). The hypoglycemic effect suggested a dose-dependent response.

Conclusion: Flaxseed extract regulates blood glucose levels probably by enhancing insulin sensitivity and revitalizing damaged pancreatic beta cells. It mitigates cardiovascular risk, by reducing arterial stiffness and atherogenicity.

Implication for health policy/practice/research/medical education:
This study indicates that flaxseed extract has the potential to improve insulin sensitivity, restore pancreatic beta cells, and reduce arterial stiffness. This therapeutic modality may serve as an adjunctive therapy for patients diagnosed with type 2 diabetes mellitus and at risk for cardiovascular diseases.

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Introduction

Diabetes mellitus, a complex metabolic disorder affecting millions of people worldwide, has emerged as a global health problem. Its multifaceted nature, defined by hyperglycemia caused by defective insulin secretion or action, requires novel therapeutic approaches (1).

Insulin resistance, often associated with hyperglycemia, can affect lipid metabolism, resulting in a higher atherogenic index by increasing triglycerides and decreasing high density lipoprotein (HDL) cholesterol (2,3). In addition, elevated glucose levels can cause oxidative stress and inflammation, which promote endothelial dysfunction and vascular damage. This damage may contribute to atherogenesis by facilitating the retention and modification of lipoproteins (4).

In diabetic patients, the combination of hyperglycemia,

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arterial stiffness, and an unfavorable atherogenic index significantly increases the risk of developing cardiovascular complications. In addition to elevated blood pressure (5), increased arterial stiffness predicts adverse cardiovascular events such as myocardial infarction and stroke independently of traditional risk factors (6).

Diabetes-related hyperglycemia promotes the accumulation of advanced glycation end products, which can cross-link with proteins in the arterial walls and increase arterial stiffness (7). Arterial stiffness is characterized by a diminished elasticity of the arterial walls, impeding their capacity to undergo movements of expansion and contraction in reaction to blood flow. Additionally, inflammation, endothelial dysfunction, and oxidative stress are all consequences of elevated glucose levels that contribute to the development of atherosclerosis. It is a marker of vascular health and a predictor of cardiovascular events (8). Inflammation, oxidative stress, and endothelial dysfunction are all consequences of elevated glucose levels, and they all contribute to the development of atherosclerosis. The combination of hyperglycemia, arterial stiffness, and an unfavorable atherogenic index increases the risk of developing cardiovascular complications in diabetic patients (9). In brief, addressing hyperglycemia, arterial stiffness, and adverse lipid profiles (as indicated by the atherogenic index) through a multifaceted approach that includes glycemic control, lipid management, and lifestyle changes is critical in reducing the risk of cardiovascular complications in diabetic patients (10).

Flavonoids, which are frequently present in plant-based foods and herbal medicines, exert their beneficial effects on diabetes management via diverse mechanisms, including antioxidant (11) and anti-inflammatory properties (12), improved insulin sensitivity, cardiovascular health, lipid modulation (13), beta-cell protection, and glucose metabolism regulation (14). Integrating various flavonoid-rich foods or herbal medicines containing these compounds into a well-balanced diet may yield advantageous outcomes in managing diabetes (15).

Flaxseed, a well-known functional food, has long been valued for its nutritional and therapeutic properties (16). Flaxseed, which is high in omega-3 fatty acids, lignans, and phytoestrogens (17), offers an intriguing avenue for further research into its potential in treating diabetes (18). There is evidence that flaxseed possesses antidiabetic properties, including improvement in fasting blood sugar, HbA1C, insulin concentrations, and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) in patients with prediabetes and type 2 diabetes mellitus (T2DM) (19). Flaxseed has been found to have significant potential in preventing the onset of type 2 diabetes in humans (20). Although the evidence is encouraging, a further comprehensive study is required to ascertain the potential of flaxseed as a therapeutic agent in diabetes management definitively (21). The objective of this study was to evaluate the effects of flaxseed extract on diabetes management and its potential to mitigate cardiovascular risk in animal models of type 2 diabetes by assessing the glycemic control, arterial stiffness, and lipid profiles in Wistar rats. Wistar rats are widely used in biomedical research and offer advantages such as genetic stability and physiological similarity to humans, enhancing the relevance and applicability of the study's outcomes.

Materials and Methods

Material

The following substances were utilized in the experiment: Sodium carboxymethyl cellulose (Bratachem, Indonesia), metformin (Sanbe Farma, Indonesia), alloxan (Sigma Aldrich, Indonesia), distilled water (Bratachem, Indonesia), 96% ethanol (Bratachem, Indonesia), rat chow C551 (purchased from a local pet food store), liquid fructose (Bratachem, Indonesia), drinking water (Amidis, Indonesia), physiological NaCl 0.9% (B Braun, Indonesia), buffered neutral formalin (BNF). A proline reagent kit for the analysis of plasma glucose, total cholesterol, triglyceride, LDL-cholesterol, and HDL-cholesterol was purchased from Prodia Diagnostic Line, Indonesia. Experimental animals were anesthetized with carbon dioxide gas for non-invasive pulse wave velocity (PWV) assessment. An insulin pen was purchased from a local pharmacy under the brand name NovoRapid.

Plant material

An extract of flaxseed (*Linum usitatissimum* L.) was procured from the Research Center for Spice and Medicinal Plants, BALITTRO (*Balai Penelitian Tanaman Rempah dan Obat*) in Bogor, Indonesia. The flaxseed was identified at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, West Java, Indonesia, with herbarium letter number 23/HB/03/2023. Furthermore, a certificate of analysis issued by BALITTRO under the number 91/T/LAB/V/23 validated the results of the phytochemical screening conducted on flaxseed extract.

Determination of total flavonoids

Total flavonoids were calculated according to the Ordon method (22). A 0.5 mL sample was mixed with 0.5 mL of 2% AlCl3 ethanol solution. Absorbance at 420 nm was measured after 1 hour at room temperature. A calibration curve was used to calculate the amount of total flavonoids equivalent to quercetin.

Qualitative analysis of herbacetin contained in flaxseed extract

Qualitative analysis of the active metabolite herbacetin from flaxseed extract was carried out by PT MarkHerb (Institut Teknologi Bandung, ITB), Bandung, West Java, Indonesia, as evidenced by a certificate of analysis no. 23-06/MH/Rep/LHA/005. In this test, optimization of HPLC

analytical conditions and determination of maximum UV were carried out using the herbacetin standard compound. The analysis conditions used an ArcTM HPLC instrument with a PDA Detector, Poroshell 120 EC-C18® 2.7 µm column (4.6 × 150 mm), and a column temperature 40 °C. Other conditions were as follows: Solvents A (water) and B (acetonitrile); flow rate: 0.5 mL/min; wavelength: 276 nm; injection volume: 5 µL.

Induction of diabetes

Fructose was used to create animal models of metabolic disorders, including hyperglycemia (23) along with alloxan, which is a diabetogenic compound that produces reactive oxygen species (ROS) (24). Animal models of diabetes are generated in two ways: exogenously and endogenously. Exogenously is induced by drinking water containing 25% fructose and endogenously is induced by intraperitoneal injection of alloxan, which damages pancreatic beta cells.

Experimental animal

Twenty healthy, adult female Wistar rats weighing 200-300 g were provided from Laboratory Animal (West Java, Indonesia). The animals had free access to food and water and were maintained under standard laboratory conditions for one week before the study. The laboratory temperature was 18-23 °C with 60-70% humidity, and the light/dark cycle was 12/12 hours. The experimental protocol was approved by the Research Ethics Committee of Padjadjaran University, West Java, Indonesia (Ethical Approval Number: 739/UN6.KEP/EC/2023). The rats were randomly divided into five groups, including normal control group (N), diabetic control group (D), metformin drug group (D+M), treatment group 1 (D+F1) received 100 mg/kg/day, and treatment group 2 (D+F2) received 200 mg/kg/day.

For four weeks, the experimental rats were administered drinking water containing 25% fructose to induce diabetes. The rats were then fasted for 12 hours, and alloxan was administered intraperitoneally at a dose of 100 mg/kg. Alloxan was administered as a single injection to induce diabetes mellitus. The rats were then monitored for glucose levels for three days while receiving standard chow and drinking water ad libitum. The presence of hyperglycemia was verified by analyzing blood glucose monitoring data. All rats were administered test drugs for two weeks (weeks 5 and 6).

Measurement of PWV and blood glucose levels

A previous publication described the technique for determining arterial elasticity (25). Non-invasive PWV measurements were performed with ECG (electrocardiography) and PPG (photoplethysmography) sensors, producing data in less than a minute. Animals were trained several times to ensure that measurements were accurate and stress-free. During measurement sessions, the animal was standing on gel patches with the PPG sensor attached to the base of its tail. ECG and PPG signals were monitored on a computer until they stabilized, at which the point data was captured for about 10 seconds. This method was created to help accelerate the study and development of anti-atherosclerotic medications. PWV measurements were obtained at weeks 0, 4, and 6.

Fasting blood glucose was measured at weeks 0, 4, and 6. Blood was collected from the orbital sinus. Plasma glucose levels were determined at a wavelength of 546 nm using a Photometer Microlab 300 equipped with a glucose reagent kit.

Measurement of lipid profile and atherogenic index

Total cholesterol was measured using the CHOD-PAP method, whereas triglycerides were assessed using the GPO (glycerol-3-phosphate-oxidase) method at a wavelength of 500 nm. A *Sekisui* reagent kit was used to determine HDL and LDL levels. HDL was measured at 700 nm, and LDL at 660 nm.

The atherogenic index based on lipid profile measurements was derived using the following formula (26): Atherogenic Index = log (Triglyceride/HDL)

Measurement of insulin sensitivity index by insulin tolerance test (K_{ITT})

At the end of the study, an insulin-resistant rat model was established by intraperitoneal insulin injection at a dose of 0.0125 U/kg BW. Following insulin administration, fasting blood glucose levels were assessed using a glucometer every 15 minutes for 60 minutes. The K_{ITT} index was obtained by calculating r (slope) multiplied by 100 (27). The regression coefficient (r) or slope was determined by linear regression. The effect of the extract on the insulin sensitivity index was assessed by comparing the K_{ITT} of the treatment group with that of the control group. A low insulin sensitivity index indicated the presence of insulin resistance. A reduced glucose elimination rate suggested insulin resistance during an insulin test tolerance (ITT).

Measurement of total flavonoid content of flaxseed extract

The colorimetric aluminum chloride method was employed to ascertain flavonoids (28). In brief, 0.5 mL of extract was added to 1.5 mL of methanol in a separate volume, followed by 2% aluminum chloride (1:1), and the solution was left at room temperature for an hour. A UV/Vis spectrophotometer was employed to determine the absorbance of the reaction mixture at 420 nm. The total flavonoid content was determined using quercetin with three sets of measurements and a calibration curve. Quercetin solutions ranging in concentration from 10 to 60 µg/mL in methanol were prepared to generate the calibration curve.

Pancreatic histology analysis

A histologic evaluation was conducted to assess the

impact of flaxseed extract on beta cells in the pancreas. After the study, the pancreatic organs of sacrificed rats were obtained. Phloxine staining was utilized to prepare pancreatic organs for histopathological analysis after rinsing with 0.9 percent physiological NaCl and fixation with 10% BNF (28). Histopathological preparations were examined under a microscope at 400× magnification. A total of five histologic fields were photographed at a magnification of 400× for each histologic section. The mean score of pancreatic beta cells for each treatment group was calculated by taking the average of beta cells in each field. In addition to observing the number of beta cells, histopathologic changes, including necrosis, were also observed.

Statistical analysis

The data obtained were tested for normality using the Shapiro-Wilk test, and the data homogeneity was tested with the Levene test. Analysis of variance (ANOVA) test with 95% confidence interval (CI) ($\alpha = 0.05$) was then conducted. To identify differences between treatment groups, the appropriate post hoc test (LSD) was conducted after the ANOVA analysis.

Data were provided as mean ± SD. Using SPSS software version 26, IBM Corp, USA, data were subjected to one-way ANOVA, followed by LSD multiple comparison as a post hoc test. *P* values < 0.05 were considered statistically significant.

Results

All treatment groups showed comparable body weight data at week 0 before treatment ($P > 0.05$). At week 4, after induction of diabetes by oral administration of 25% fructose in drinking water, there was a significant increase in body weight compared to the normal group ($P < 0.05$). Oral administration of flaxseed extract doses of 100 and 200 mg/kg BW for two weeks showed a decrease in body weight at week 6, which was significantly different from the diabetic group ($P < 0.05$) (Figure 1).

Antidiabetic activity

After two weeks of flaxseed extract supplementation, the groups receiving doses of 100 and 200 mg/kg BW showed a significant decrease in glucose levels compared to the diabetic control group ($P < 0.05$), indicating the hypoglycemic effect of flaxseed extract. The group receiving a dose of 200 mg/kg BW exhibited a significant decrease in blood glucose levels ($P < 0.05$) compared to the group administered a dose of 100 mg/kg BW. This suggests that increasing the extract dosage correlates with a greater hypoglycemic effect, indicating a dose-dependent response. Furthermore, compared to the diabetic control group, the group that received the extract exhibited a statistically significant ($P < 0.05$) increase in insulin sensitivity, as measured by a high K_{ITT} value (insulin sensitivity index). In contrast to the diabetic control group, histological analysis revealed a notable increase in the quantity of pancreatic beta cells, indicating a significant improvement in pancreatic beta cell function ($P < 0.05$) (Figure 2).

Antidiabetic impact of flaxseed extract on atherogenic index and arterial stiffness

The groups that received flaxseed extract following the induction of diabetes mellitus and insulin resistance demonstrated improved lipid profiles (total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels) (Figure 3). Furthermore, the atherogenic index calculation indicated a reduction in the ratio of triglycerides to HDL ($P < 0.05$). Compared to the normal group, the diabetic

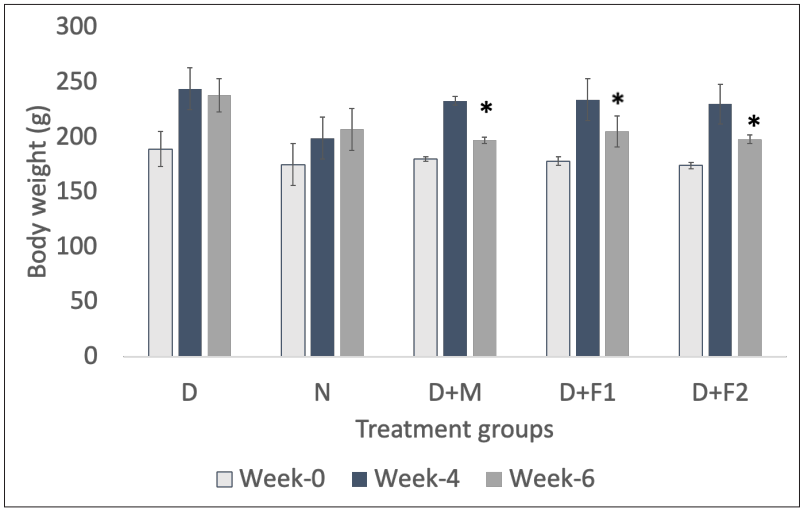

Figure 1. Effect of oral administration of flaxseed extract on the body weight of rats. *$P < 0.05$ compared to the diabetes group. D (diabetes group), N (normal group), D+M (diabetes and metformin), D+F1 (diabetes and flaxseed 100 mg/kg), and D+F2 (diabetes and flaxseed 200 mg/kg). Week 0 was before treatment, week 4 was after induction of diabetes, and week 6 was after oral administration of flaxseed extract doses of 100 and 200 mg/kg BW for 2 weeks.

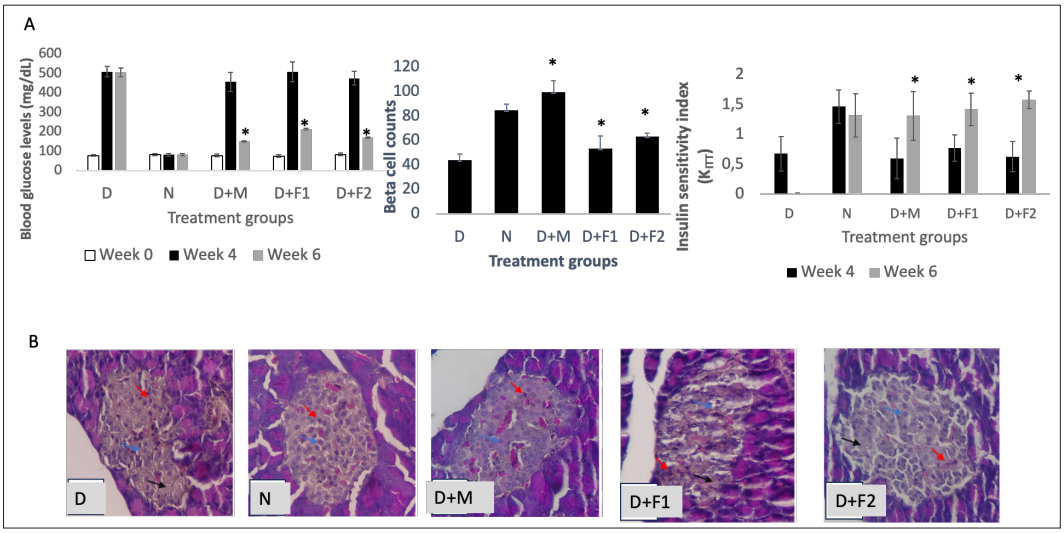

Figure 2. The impact of flaxseed extract supplementation on blood glucose level, index of insulin sensitivity (KITT), beta cell counts (A) and pancreas histopathology in an rat model of diabetes (B). Histological analysis shows reduced necrosis (black arrows) and increased beta cells (blue arrows) in the flaxseed-treated groups (F1 and F2) compared to the diabetic control (D). The alpha cells (red arrows) remain prominent, but the flaxseed groups exhibit less tissue damage. The groups were as follows: D (diabetes group), N (normal group), D+M (diabetes and metformin), D+F1 (diabetes and flaxseed 100 mg/kg BW), and D+F2 (diabetes and flaxseed 200 mg/kg BW). * indicates significant difference compared to the diabetes group ($P < 0.05$).

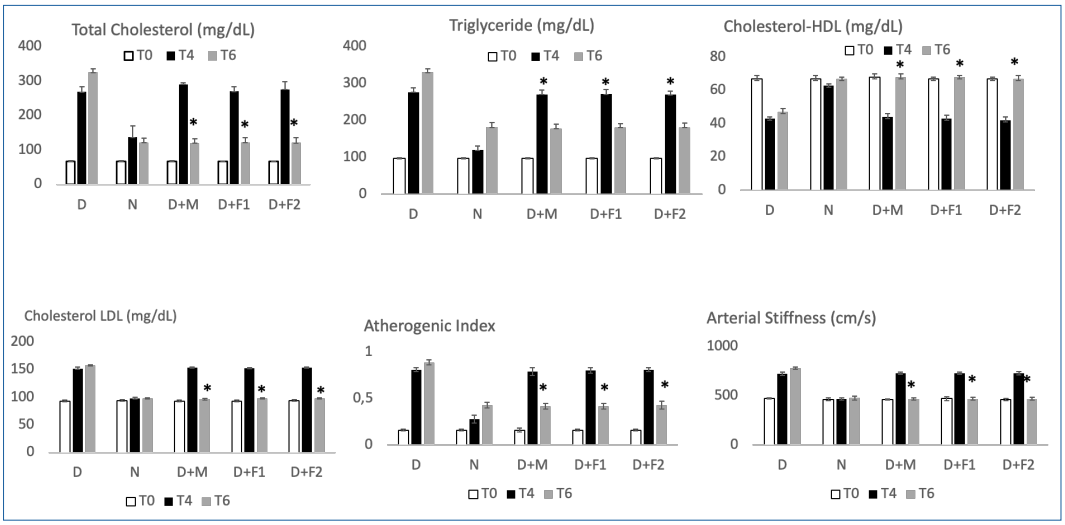

Figure 3. The impact of flaxseed extract supplementation on arterial stiffness, lipid profile (total cholesterol, triglyceride, cholesterol HDL, and cholesterol LDL), and atherogenic index in a diabetic rat model. Diabetes was induced by four weeks of drinking water containing 25 percent fructose and alloxan as a single injection at 100 mg/kg BW at week 4 (T4). The groups were as follows: D (diabetes group), N (normal group), D+M (diabetes and metformin), D+F1 (diabetes and flaxseed 100 mg/kg BW), and D+F2 (diabetes and flaxseed 200 mg/kg BW). T0= week 0 before treatment. *$P < 0.05$ compared to the diabetes group.

control group had substantially increased arterial stiffness ($P < 0.05$). In conjunction with a reduced atherogenic index, flaxseed extract decreased the arterial stiffness ($P < 0.05$).

Total flavonoid content of flaxseed extract

Flaxseed extract had an extract yield of 4.26% with a water content of 5.58% v/w. Flaxseed extract contained alkaloids, tannins, phenolics, flavonoids, glycosides, triterpenoids, and saponins. The total flavonoid content was 44.2 ± 0.012 mg quercetin equivalent (QE)/g of the extract using the standard quercetin curve (y = 0.0046x + 0.0934, R^2 = 0.993).

Analysis of herbacetin in flaxseed extract

Analysis of the active flavonoid components in flaxseed extract revealed the presence of the compound herbacetin. A peak (A) resembled the common chemical herbacetin (B) with a retention time of 12.638 minutes (Figure 4).

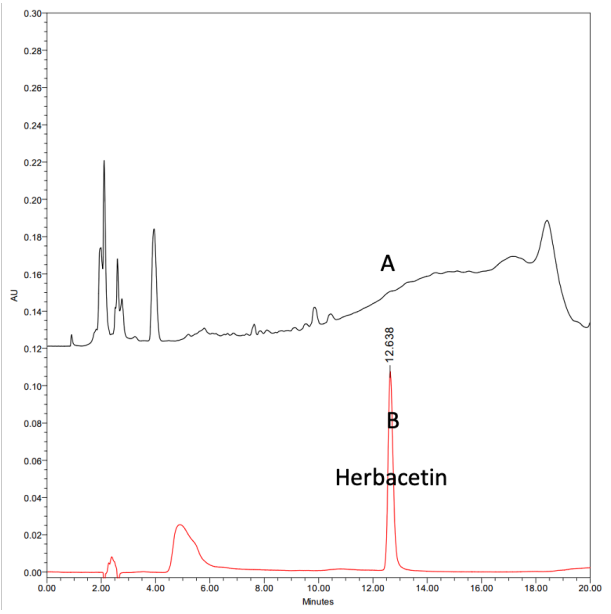

Figure 4. Overlay the chromatograms of the flaxseed extract (A) and the herbacetin standard compound (B) to identify the active compound herbacetin in the flaxseed extract.

Discussion

Type 2 diabetes is a complex metabolic disease characterized by insulin resistance, hyperglycemia (29), and an increased risk of cardiovascular disease (30). Cardiovascular disease continues to be the major cause of death among diabetes patients. Diabetes is a complicated disorder and its control results in a significant decrease in cardiovascular events (31). As the global prevalence of type 2 diabetes is rising, discovering new pharmaceutical options to address its complex etiology remains crucial (32).

This study examined the potential antidiabetic benefits of flaxseed extract in an alloxan-induced rat model of type 2 diabetes. This animal model of diabetes provided an excellent platform to study the effects of flaxseed extract on various aspects of diabetic pathophysiology. For 4 weeks, the fructose-induced diabetes group, followed by a single injection of alloxan, showed weight gain that appeared in the fourth week and continued until the sixth week (Figure 1). Moreover, the diabetic group exhibited insulin resistance, pancreatic beta cell dysfunction, dyslipidemia, increased atherogenic index, and arterial stiffness. These findings are in line with previous studies that created an animal model of T2DM with a combination of a high-fructose diet and diabetogenic substances. The model was able to create a unique, non-genetic preclinical T2DM model that is in line with current diets and lifestyles. This model shows how the disease progresses, from weight gain to insulin resistance, pancreatic β-cell dysfunction, hypertriglyceridemia, and cardiomyopathy; it closely resembles the damage that happens to target organs in people with advanced type 2 diabetes (30).

In this study, the diabetic rats showed insulin resistance, hyperglycemia, and damage to the pancreatic organs (Figure 2). Further impairment occurred in the vasculature, as evidenced by arterial stiffness in the form of an increase in PWV (Figure 3).

Flaxseed extract doses of 100 and 200 mg/kg BW could help lower body weight, enhance insulin sensitivity (by raising the K-ITT index), manage blood sugar levels, and revitalize damaged pancreatic function in diabetic animal models (Figure 2). Moreover, it mitigated cardiovascular risk factors by reducing arterial stiffness and the atherogenic index. Notably, the group receiving a dose of 200 mg/kg, in week 6 exhibited a significant decrease in blood glucose levels ($P<0.05$) compared to the group receiving a dose of 100 mg/kg. This indicates that the extract has a dose-dependent hypoglycemic effect, which is consistent with the increased dosage.

Type 2 diabetes has severe metabolic consequences. It is well-established that weight loss improves glucose management and cures the underlying metabolic abnormalities of type 2 diabetes; individuals with type 2 diabetes may experience a disease-modifying effect from weight loss (31).

The comprehensive assessment of physiological markers associated with type 2 diabetes pathogenesis is central to our work. Monitoring blood glucose levels is the foundation for assessing the efficacy of flaxseed extract in alleviating hyperglycemia, a defining feature of type 2 diabetes (32). A significant reduction in blood glucose levels ($P<0.05$) could be observed with flaxseed extract supplementation compared to the diabetic control group. Our study rigorously investigated insulin sensitivity measures, which were critical in determining the impact of flaxseed extract on the body's responsiveness to insulin-an essential aspect of regulating glucose homeostasis in type 2 diabetes (33).

Furthermore, considering the complex relationship between dyslipidemia and increased cardiovascular risk in diabetes (34), the measurement of the atherogenic index, which reflects lipid abnormalities, assumes relevance. Incorporating this evaluation provides insights into the flaxseed extract's possible significance in reducing atherogenic consequences (35).

Extending our research to include PWV measurements provides a novel perspective on arterial stiffness, which plays a role in cardiovascular disease (36). Assessing arterial stiffness using PWV allows for assessing vascular abnormalities associated with diabetes and the possible role of flaxseed extract in ameliorating these modifications (37).

Our research also included a histological examination of pancreatic tissues. This study aimed to determine whether flaxseed extract had protective benefits for pancreatic architecture and beta-cell integrity, critical to insulin generation and glycaemic control in type 2 diabetes. This study showed that flaxseed extract at 100 and 200 mg/

kg BW revitalized damaged pancreatic beta cells. These results corroborate previous findings, which reported that treatment with polyphenols isolated from flaxseed extract showed improved glycosylated hemoglobin and blood glucose levels and decreased LDL cholesterol and triglycerides in treated diabetic experimental animals (38). Furthermore, our results showed that flaxseed extract might be a promising therapeutic option for diabetes management.

Our investigation of herbacetin, a flavonoid metabolite discovered in flaxseed extract, is a novel part of our research. Understanding the role of herbacetin as a possible active ingredient contributing to the purported antidiabetic effects broadens our understanding of the precise bioactive ingredients driving the therapeutic potential of flaxseed extract. The discovery of the importance of herbacetin in the purported anti-diabetic benefits of flaxseed extract sheds light on the potential mechanisms underlying its therapeutic effects. However, it is crucial to note that the reported antidiabetic benefits of flaxseed extract may be related to other bioactive constituents such as secoisolariciresinol diglucoside (39), lignans or a combination of compounds, rather than herbacetin alone (40).

In sum, our study expands insights into the therapeutic potential of flaxseed extract in reducing multidimensional diabetes dysregulation in experimental animal models of type 2 diabetes through a comprehensive approach that includes physiological, histological, and metabolite measurements. The results may contribute to a better understanding of the potential benefits of flaxseed extract in managing type 2 diabetes.

Conclusion

Flaxseed extract effectively manages blood glucose levels in diabetic rats through weight loss, enhancing insulin sensitivity, potentially revitalizing damaged pancreatic beta cells, and reducing cardiovascular risk. The reduction in the atherogenic index and arterial stiffness, as indicated by the reduced PWV, highlights the potential of flaxseed extract as a comprehensive therapeutic intervention for diabetes management, with herbacetin potentially contributing to its antidiabetic effect. However, to fully understand the interplay between cardiovascular risk factors and blood glucose regulation, further research is necessary.

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Authors' contributions

Conceptualization: Patonah Hasimun, Yani Mulyani, Herni Kusriani, and Nurasyifa Syaumi.
Data curation: Patonah Hasimun, Yani Mulyani, Herni Kusriani, and Nurasyifa Syaumi.
Formal analysis: Patonah Hasimun, Herni Kusriani, Yani Mulyani, and Nurasyifa Syaumi.
Funding acquisition: Patonah Hasimun
Investigation: Patonah Hasimun, Yani Mulyani, Herni Kusriani, and Nurasyifa Syaumi.
Methodology: Patonah Hasimun and Herni Kusriani.
Project administration: Patonah hasimun and Nurasyifa Syaumi.
Resources: Patonah Hasimun, Yani Mulyani, Herni Kusriani and Nurasyifa Syaumi.
Supervision: Patonah Hasimun, Herni Kusriani and Yani Mulyani.
Validation: Patonah Hasimun, Yani Mulyani, Herni Kusriani, and Nurasyifa Syaumi.
Visualization: Patonah Hasimun and Herni Kusriani.
Writing–original draft: Patonah Hasimun and Herni Kusriani.
Writing-review & editing: Patonah Hasimun, Yani Mulyani, Herni Kusriani, andNurasyifa Syaumi.

Conflict of interests

Regarding this work, the authors state no conflict of interest.

Ethics considerations

The study protocol was approved by the Research Ethics Committee Universitas Padjadjaran Bandung (permission number 739/UN6.KEP/EC/2023).

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