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Antihyperglycemic and hypoglycemic activities of *Castanopsis costata*: An experimental study with *in vitro*, *in vivo*, and histopathological evaluation

Maulana Yusuf Alkandahri[1*], Asman Sadino[2], Wilda Fhitriany Usman[3], Eva Feriadi[4], Zulpakor Oktoba[5], Atri Sri Ulandari[5], Ana Yulyana[6], Annis Fathurrohmah[7], Shofa Khairunnisa[7], Indah Sari Amanatun Nisa[7], Annisa Rizqya Salmaduri[7]

[1]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Buana Perjuangan Karawang, Karawang, Indonesia.
[2]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Garut, Garut, Indonesia.
[3]Pharmacist Professional Education Study Program, Faculty of Pharmacy, Universitas Islam Sultan Agung, Semarang, Indonesia.
[4]Department of Pharmacy, Faculty of Science and Technology, Universitas Sembilanbelas November Kolaka, Kolaka, Indonesia.
[5]Department of Pharmacy, Faculty of Medicine, Universitas Lampung, Bandar Lampung, Indonesia.
[6]Faculty of Pharmacy, Institut Sains dan Teknologi Nasional, Jakarta, Indonesia.
[7]Faculty of Pharmacy, Universitas Buana Perjuangan Karawang, Karawang, Indonesia.

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ABSTRACT

Several currently available antihyperglycemic drugs for diabetes mellitus have many weaknesses and adverse side effects, underscoring the need to find alternative therapies derived from medicinal plants, such as *Castanopsis costata*. Therefore, the objective of this study was to investigate antihyperglycemic and hypoglycemic effects of ethanol extract in *C. costata* (EECC) with *in vitro* and *in vivo* experimental models as well as identify the chemical compounds. The inhibitory activity of α-amylase and α-glucosidase by EECC was assessed using the DNSA and p-nitrophenyl-α-D-glucoside methods. The activity of lowering blood glucose level by EECC was examined in three test models in mice, namely normoglycemia, oral glucose tolerance test, and alloxan-induced hyperglycemia mice models, as well as histopathological studies of pancreatic tissue. The chemical compounds contained in EECC were identified using liquid chromatography tandem mass spectrometry. The results showed that EECC had inhibitory activity against α-amylase and α-glucosidase with respective IC_{50} values of 454.72 ± 0.86 and 575.08 ± 0.93 µg/ml. The administration of EECC caused a significant decrease in BGL in the three test models and a significant improvement from histopathological changes in pancreatic tissue. Meanwhile, chemical compound identification results showed that there were 14 compounds contained in EECC. This study showed that EECC has hypoglycemic and antihyperglycemic effects and was able to restore histopathological changes.

1. INTRODUCTION

After cancer and cardiovascular/cerebrovascular disease, diabetes mellitus (DM) is the third leading cause of death [1]. Currently, DM accounts for about 5% of all deaths worldwide, but that number is predicted to rise to 50% in the next 10 years [2]. It is an endocrine disease attributed to abnormalities in insulin secretion (deficiency or insufficient insulin synthesis from the pancreas), action (insulin resistance and hyperinsulinemia), or both [3–5], resulting in hyperglycemia and severe irreversible micro-and macrovascular complications [6]. These complications include diabetic-(neuropathy, retinopathy, nephropathy, and foot), atherosclerosis, and cardiovascular disease [7]. A previous study reported that in DM patients, approximately 70% of the total mass of host pancreatic β cells was destroyed due to immune-mediated

*Corresponding Author
Maulana Yusuf Alkandahri, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Buana Perjuangan Karawang, Karawang, Indonesia. E-mail: alkandahri @ gmail.com

processes detected during clinical diagnosis [8]. Therefore, management of hyperglycemia is very important to reduce or prevent severe complications [6]. Conventional DM treatment comprises insulin injections and some antidiabetic drugs, including biguanides [9], sulfonylureas [10], glinides [11], thiazolidinediones [12], alpha-glucosidase inhibitors [13], dipeptidyl peptidase-4 inhibitors [14], amylin analogues [15], and glucagon-like peptide 1 [16]. Although these drugs are successful in reducing and controlling blood glucose level (BGL), the majority hold unfavorable side effects, such as digestive disorders, hypoglycemia, anemia, weight gain, and kidney failure. This shows the need for new natural medicines with safer and effective properties that are capable of protecting and regenerating pancreatic β cells as a focus for the finding of new antidiabetic drugs [17–19].

Medicinal plants are already utilized for the treatment and prevention of diverse diseases, such as DM [19], malaria [20,21], hepatoprotective [22], antipyretic [23], skin protection from UV rays [24], and wound healing [25]. Furthermore, more than 800 species of medicinal plants were reported to have antihyperglycemic effects [26]. The content of active compounds is known to affect blood glucose through several different mechanisms, such as increasing insulin secretion [27] and β-cells in the pancreas by activating regeneration [28,29]. Some compounds also function as antioxidants by lessening oxidative stress caused by free radicals in the pancreas [30,31]. *Castanopsis costata* known as Cep-cepan is among the medicinal plants widely distributed in Thailand, Peninsular Malaysia, and Indonesia (Kalimantan and Sumatra). It is a woody plant thriving at an altitude of 1,200–1,421 masl to a height of 12–15 m. *Castanopsis costata* has a round, hard, and strong stem with a diameter of approximately 80 cm and branches. The leaf stalks are in the proper shape, featuring pinnate veins, reddish-white flowers, and green fruit measuring 3 cm, with 2 cm cone-shaped brown seeds [32]. Previous studies have reported that ethanol extract from *C. costata* (EECC) possesses various pharmacological activities, including antimalarial [33], antioxidant [34], antipyretic [34], antihyperlipidemic [35], antidiarrheal [36], and anti-inflammatory [37]. Other studies have also confirmed the anti-diabetic effects of leaf extracts and fractions in alloxan-induced mice [38]. The antidiabetic effects reported are due to the high content of flavonoids and phenolic compounds found in *C. costata* [35], which can affect glucose metabolism in the body [39].

Therefore, this study aimed to test the chemical compounds contained in EECC using liquid chromatography tandem mass spectrometry (LC-MS/MS). The hypoglycemic effects were also determined, including normoglycemic mice and oral glucose tolerance test (OGTT), antihyperglycemic effects *in vitro* (α-glucosidase and α-amylase enzyme inhibition models) and *in vivo* (alloxan-induced hyperglycemic mice model), as well as histopathological examination of pancreatic tissue.

2. MATERIALS AND METHODS

2.1. Chemicals and drugs

The chemicals used included glibenclamide (PT. Kimia Farma Tbk, Indonesia), alloxan monohydrate (Sigma Chemical Company, USA), diethyl ether (PT. Brataco, Indonesia), 0.9% NaCl (PT. Widatra Bhakti, Indonesia), paraffin, 10% formalin solution, xylene, acarbose, α-amylase solution, α-glucosidase solution, p-nitrophenyl-α-D-glucoside, and 3,5-dinitrosalicylic acid (DNSA). Others included potassium sodium tartrate tetrahydrate, starch solution, glucose (Sigma-Aldrich, Merck, Germany), hematoxylin-eosin stains (HiMedia, USA), 70% ethanol, acetonitrile, aquadest, sodium hydroxide, disodium hydrogen phosphate anhydrous, sodium bicarbonate, and methanol (EMSURE® ACS Merck, Darmstadt, Germany) with analytical grade.

2.2. Plant materials and determination

Fresh leaf samples used in this study were obtained in February 2022 from Pancur Batu Traditional Market, North Sumatra, Indonesia. The plant was determined at Herbarium Medanense, Universitas Sumatera Utara (No. 183/MEDA/2022) by Dr. Etti Sartina Siregar, and identified as *Castanopsis costata* species of the Fagaceae family.

2.3. Preparation of *C. costata* extract

Castanopsis costata leaf was collected, cleaned with water, cut into small pieces, dried, and blended to obtain powder form. Furthermore, 2 kg of the powder was macerated for 3 × 24 hours with 70% ethanol. A rotary evaporator (Eyela OSB-2100) was employed to collect and concentrate the liquid extract at 50°C [40].

2.4. Analysis of EECC using LC-MS/MS

As much as 50 mg of EECC was dissolved in acetonitrile and filtered utilizing a Millipore 0.54 µ filter unit. Subsequently, a Quadrupole Time-of-Flight (QTOF) LC-MS/MS apparatus system was filled with 5 µl of sample filtrate. LC-MS/MS analysis was provided with a binary pump and QTOF mass spectrometer linked to liquid chromatography (LC) with an electrospray ionization (ESI) source. The QTOF system with negative and positive ionization modes was employed in the mass spectrometry process. ESI parameters utilized were atomic gas of 500 L/hour, a voltage source of 3 kV, and a capillary temperature of 120°C. The source temperature was set to 110°C for the whole scan mode from m/z 100–5,000. Furthermore, LC column used was C18 (2.1 × 150 mm, 1.8 µm) (Acquity UPLC® HSS), with eluents comprising H_2O (A) and acetonitrile p.a (B) at a total flow rate of 0.3 ml/minute. The isocratic elution system was performed at 0-1 minute with a 95:5 ratio, 0 minute linear gradient elution of solvent A from 95% to 5%, and 6–7 minute isocratic elution with a 0:100 ratio. Other conditions included 6–7 minute linear gradient elution of solvent A from 0% to 100%, 7.5–9 minute isocratic elution with a 95:5 ratio, 7.5–9 minute linear gradient elution of solvent A from 95% to 5%. LC-MS/MS data interpretation referred to the personal compound database and library [41].

2.5. α-Amylase inhibition assay

In this assay, EECC was initially diluted in phosphate buffer (pH 6.9) to get diverse test concentrations of 250, 500, 750, and 1,000 µg/ml. Subsequently, a test concentration of 200 µl was intermixed with α-amylase solution of 200 µl (2 units/ml) into each tube and subjected to incubation at 30°C for 10

minutes. Each tube acquired around 200 µl of starch solution (1% in water w/v), which was then subjected to incubation for 3 minutes. The reaction was halted by providing 200 µl of DNSA reagent (12 g of $KNaC_4H_4O_6 \cdot 4H_2O$ in 8 ml of 2 M NaOH and 20 ml of 96 mM 3,5-DNSA solution) and simmered in a water bath for 10 minutes at 85 °C–90°C. After cooling to room temperature, the liquid was diluted with distilled water of 5 ml, followed by measuring the absorbance at 540 nm with a UV-visible spectrophotometer. Blanks with 100% enzyme activity were formulated by substituting EECC with 200 µl of buffer. Moreover, EECC at any concentration without the use of an enzyme solution was employed to create blank reactions. Positive control samples were prepared using acarbose (250, 500, 750, and 1,000 µg/ml), and the reactions were carried out similarly to EECC [42]. The α-amylase inhibition was represented as % inhibition and computed using the equation below:

$$\% \text{ Inhibition} = \left(\frac{AzC - AzS}{AzC}\right) \times 100$$

where AzC and AzS indicate the absorbance of the control and sample.

The percentage of α-amylase inhibition was plotted against EECC concentration and IC_{50} value was acquired from the graph.

2.6. α-Glucosidase inhibition assay

EECC was diluted in phosphate buffer (pH 6.9) to acquire diverse test concentrations of 250, 500, 750, and 1,000 µg/ml. This was followed by mixing EECC of 20 µl from each test concentration with α-glucosidase solution of 10 µl (1 unit/ml) into each tube and incubating at 37°C for 5 minutes. After adding around 20 µl of a 1 mM p-nitrophenyl-α-D-glucoside solution, the mixture was subjected to incubation at 37°C for 30 minutes. The reaction was halted by providing 50 µl of 0.1 M Na_2CO_3 and absorbance was gauged at 405 nm employing a UV-Visible spectrophotometer. Blank with 100% enzyme activity was formulated by substituting EECC with buffer of 50 µl. Moreover, blank reactions were formulated utilizing EECC at any concentration without the use of enzyme solution. Positive control samples were prepared using acarbose (250, 500, 750, and 1,000 µg/ml), and the reaction was carried out similarly to EECC [43]. The α-glucosidase inhibition was represented as % inhibition and computed using the equation:

$$\% \text{ Inhibition} = \left(\frac{AzC - AzS}{AzC}\right) \times 100$$

where AzC and AzS indicate the absorbance of the control and sample.

The percentage inhibition of α-glucosidase was plotted against EECC concentration and IC_{50} value was acquired from the graph.

2.7. Experimental animals

As many as 80 male Swiss albino mice with a weight of 25–30 g and 8–12 weeks old were acquired from the animal house of CV. Mitra Putra Animal, Cicalengka, Indonesia. The animals obtained were reared properly in the Pharmacology Laboratory, Universitas Buana Perjuangan Karawang. *Ad libitum* access to water and standard pellets was provided to mice housed in plastic cages with softwood shavings on a 12-hour light/dark cycle.

2.8. Hypoglycemic test on normal mice

Despite a 6-hour fast, all male Swiss albino mice were allowed unlimited access to water *ad libitum*. Subsequently, mice were diverged randomly into five groups (n = 5 in each group). Group I (normal control) obtained 10 ml/kg distilled water, group II was provided glibenclamide 5 mg/kg, while groups III, IV, and V were administered EECC at 50, 100, and 200 mg/kg, respectively. Blood samples were aseptically taken from the mice's tail vein and BGL was measured at 0, 1, 2, 3, and 4 hours after treatment utilizing a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Mannheim, Germany) [44].

2.9. Oral glucose tolerance test in mice

Mice were randomly diverged to five groups (n = 5 mice per group) and allowed unlimited access to water *ad libitum* despite fasting for 6 hours prior to treatment. Mice in group I (normal control) obtained distilled water (10 ml/kg), group II was provided 5 mg/kg standard drug glibenclamide, while groups III, IV, and V received 50, 100, and 200 mg/kg EECC, respectively. This was followed by administering glucose solution of 2 g/kg 30 minutes after treatment. Blood samples were taken from the mice's tail vein and glucose levels were defined at 0 minutes (before treatment), 30, 60, 120, and 180 minutes after, using a digital glucometer [45].

2.10. Induction of hyperglycemia in mice

After the acclimatization period was completed, all mice were prepared for hyperglycemia induction with alloxan monohydrate after fasting for 18 hours. The body weight and BGL were recorded before and after induction. In this experiment, mice were allocated randomly into six groups (n = 5 mice in each group). Group I served as a normal control (NC), while II to VI were injected intraperitoneally with alloxan in saline solution (0.9% NaCl) at 150 mg/kg. After 1 hour of alloxan administration, all mice were fed *ad libitum* [7].

2.11. Antihyperglycemic activity

After 72 hours of alloxan induction, mice with BGL >200 mg/dl were deemed hyperglycemic and incorporated in this study (day 0). Treatment was given for 14 days followed by BGL measurement on days 0, 5, 10, and 15, which was acquired from the tail vein utilizing a digital glucometer [46]. Subsequently, treatment was given once a day in the morning.

Group I: noninduced (normal control) obtained distilled water (10 ml/kg) orally.

Group II: alloxan-induced nontreated mice (hyperglycemic control) obtained distilled water (10 ml/kg) orally.

Group III: alloxan-induced and treated with 5 mg/kg of glibenclamide orally.

Group IV: alloxan-induced and treated with 50 mg/kg of EECC orally.

Group V: alloxan-induced and treated with 100 mg/kg of EECC orally.

Group VI: alloxan-induced and treated with 200 mg/kg of EECC orally.

The percentage DBGL (decline in blood glucose level) was computed using the formula:

$$\% \text{ DBGL} = \left(\frac{\text{BGL (Day 0)} - \text{BGL (Day 15)}}{\text{BGL (Day 0)}} \right) \times 100$$

2.12. Pancreatic tissue sampling

Diethyl ether was used to anesthetize the mice in each treatment group on the 15th day of the trial. All mice were sacrificed by cervical dislocation, where an incision was made in the midline of the abdomen approximately ≈ 1.5 cm long to remove the pancreas, and wrapped immediately in filter paper. Subsequently, the pancreas was rinsed with normal saline, dried, as well as placed into a sample bottle for histological examination [19].

2.13. Histological examination

For histological examination, pancreatic tissue samples taken during the autopsy were directly processed at the Histology Laboratory, Universitas Jenderal Achmad Yani, Indonesia. Pancreatic tissue samples were processed via alcohol dehydration, heeded by xylene cleaning and paraffin infiltration. The preparations were deparaffinized in xylene and stained with H&E (hematoxylin and eosin) after paraffin blocks were sliced to a thickness of 7 µm [47]. These preparations were viewed at 100x magnification employing a light microscope provided with a camera linked to a computer for histopathological examination. Histopathological changes were recorded using a standard nonlinear semiquantitative scoring system and a scale from 0 to 3 adapted from Dyson *et al.* [48]. Significant findings were scored 0 (normal architecture), 1 for mild changes (0%–25% of cell), 2 for moderate changes (25%–50% of cell), and 3 for severe changes (>50% of cell) that could be observed by light microscopy.

2.14. Statistical analysis

All of the experiment's data are displayed as the mean ± SEM of five replicates for each group. Differences between means of the parameters measured were compared utilizing one-way analysis of variance continued with Tukey's post hoc test employing GraphPad Prism version 8. A $p < 0.05$ value was deemed significant.

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS analysis of EECC

Based on the chemical compound identification results using LC-MS/MS, a total of 14 compounds were found in EECC (Table 1). These compounds were described through the chromatogram results showing peaks with various molecular weights, as presented in the supplementary data.

3.2. In vitro α-amylase inhibition activity of EECC

In this study, α-amylase enzyme inhibitory activity test was carried out *in vitro*. EECC and acarbose indicated concentration-dependent inhibitory activity against the α-amylase enzyme (Fig. 1) with respective IC_{50} values of 454.72 ± 0.86 µg/ml and 115.45 ± 0.75 µg/ml (Table 2).

3.3. In vitro α-glucosidase inhibition activity of EECC

Activity study on α-glucosidase inhibition *in vitro* showed that EECC had a concentration-dependent α-glucosidase inhibitory effect, as presented in Figure 2. EECC and acarbose showed α-glucosidase inhibitory activity with respective IC_{50} of 575.08 ± 0.93 µg/ml and 438.01 ± 0.78 µg/ml (Table 3).

3.4. Hypoglycemic activity of EECC in normoglycemic mice

This study evaluated the hypoglycemic effect after EECC administration in normoglycemic mice. The results indicated that the group given EECC at 200 mg/kg experienced a substantial decline in BGL after 2 ($p < 0.05$), 3 ($p < 0.01$), and 4 ($p < 0.0001$) hours of treatment. EECC group at 100 mg/kg indicated a substantial decline after 3 ($p < 0.05$) and 4 ($p < 0.001$) hours. Meanwhile, 50 mg/kg groups had a substantial decrease after 4 ($p < 0.01$) hours of treatment in comparison with mice in the NC. The glibenclamide group at 5 mg/kg indicated a substantial decline in BGL after 1 ($p < 0.05$), 2, 3, and 4 ($p < 0.0001$) hours of treatment. The effect of EECC administration on BGL of normoglycemic mice is outlined in Figure 3.

3.5. Effect of EECC on OGTT in normal mice

The results showed no substantial difference in BGL across all groups before glucose administration ($p > 0.05$). However, all groups indicated a significant increase in BGL 30 minutes after glucose administration, verifying the hyperglycemia induction. Further analysis indicated a substantial decline in BGL in the EECC group at 50 mg/kg ($p < 0.05$), 100 mg/kg ($p < 0.05$), and 200 mg/kg ($p < 0.01$) only after 60 minutes of glucose administration in comparison with mice in the NC group. Meanwhile, the glibenclamide group at 5 mg/kg indicated a substantial decline in BGL after 30 ($p < 0.001$), 60 ($p < 0.0001$), 120 ($p < 0.001$), and 180 ($p < 0.0001$) minutes. The effect of EECC administration on BGL of mice in the OGTT is presented in Figure 4.

3.6.Effect of EECC on BGL of alloxan-induced hyperglycemic mice

Based on the results, all EECC and the glibenclamide groups showed significant BGL-lowering effects in comparison with the hyperglycemia control (HC) group. EECC administration at 50, 100, and 200 mg/kg significantly ($p < 0.001 - p < 0.0001$) lowered BGL from day 5 to 15. Meanwhile, a substantial decline in BGL ($p < 0.0001$) was found in the glibenclamide group at 5 mg/kg from day 5 to 15. The effect of EECC administration on the BGL of alloxan-induced hyperglycemic mice is summarized in Figure 5. After 15 days of treatment with EECC at 50, 100, and 200 mg/kg, the percentage DBGL was 50.19%, 59.23%, and 64.95%, respectively, and 51.48% for glibenclamide at 5 mg/kg (Table 4).

Table 1. Chemical compounds identified from EECC using LC-MS/MS.

No	Category	RT (minutes)	Molecular mass (*m/z*)	Ionization (ESI^-/ESI^+)	Mass error (ppm)	Molecular formula	Identified compounds
1	Flavonoids	9.78	317.06,487	$[M + H]^+$	−2.2	$C_{16}H_{12}O_7$	3- Methoxyherbacetin
2		17.4	553.28,059	$[M + H]^+$	1.8	$C_{32}H_{40}O_8$	6,17-Epoxylathyrol-5,15-diacetate-3-phenylacetate
3		8.76	303.04,948	$[M + H]^+$	−1.5	$C_{15}H_{10}O_7$	Isoetin
4		16.58	403.13,775	$[M + H]^+$	−2.1	$C_{21}H_{22}O_8$	Nobiletin
5		10.02	317.06,498	$[M + H]^+$	−1.9	$C_{16}H_{12}O_7$	Rhamnetin
6		9.67	303.04,944	$[M + H]^+$	−1.6	$C_{15}H_{10}O_7$	Robinetin
7		12.91	593.12,941	$[M-H]^-$	−1.1	$C_{30}H_{26}O_{13}$	Buddlenoid A
8		9.99	623.16,327	$[M-H]^-$	2.4	$C_{28}H_{32}O_{16}$	Isorhamnetin-3-O-β-D-robinobioside
9		11.16	431.09,917	$[M-H]^-$	1.9	$C_{21}H_{20}O_{10}$	Kaempferol-3-O-rhamnoside
10		10.29	417.08,346	$[M-H]^-$	1.8	$C_{20}H_{18}O_{10}$	Kaempferol-3-O-α-L-arabinoside
11		9.78	593.15,211	$[M-H]^-$	1.5	$C_{27}H_{30}O_{15}$	Kaempferol-3-O-β-rutinoside
12		11.13	563.14,296	$[M-H]^-$	4.1	$C_{26}H_{28}O_{14}$	Patuletin-7-O-[6″- (2-methylbutyryl)]-glucoside
13	Triterpenoids	17.99	441.37,161	$[M + H]^+$	−2.5	$C_{30}H_{48}O_2$	Kansenol
14		16.95	617.38,401	$[M-H]^-$	−1.2	$C_{39}H_{54}O_6$	3β-O-trans-p-Coumaroyl alphitolic acid

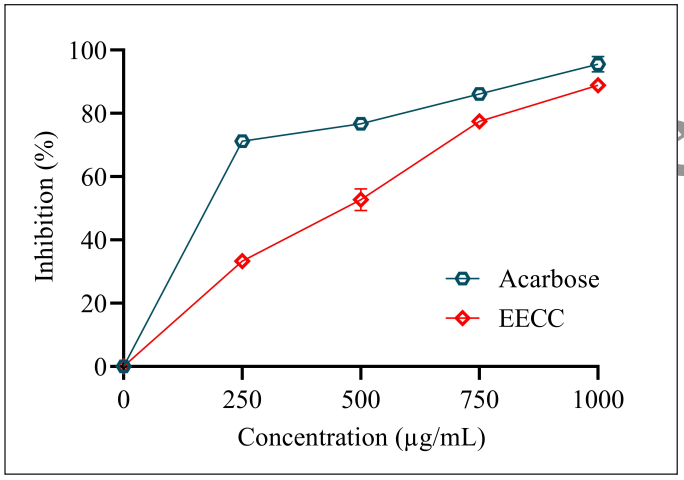

Figure 1. α-Amylase inhibitory activity of EECC.

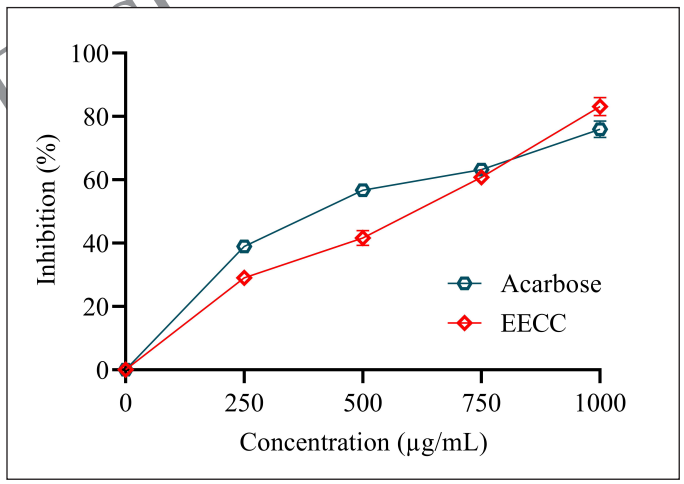

Figure 2. α-Glucosidase inhibitory activity of EECC.

Table 2. IC_{50} of EECC on α-amylase inhibitory activity.

α-Amylase inhibitory	
Samples	**IC_{50} (µg/ml)**
EECC	454.72 ± 0.86
Acarbose	115.45 ± 0.75

The data are presented as the mean ± SEM of five replicates for each group.

Table 3. IC_{50} of EECC on α-glucosidase inhibitory activity.

α-Glucosidase inhibitory	
Samples	**IC_{50} (µg/ml)**
EECC	575.08 ± 0.93
Acarbose	438.01 ± 0.78

The data are presented as the mean ± SEM of five replicates for each group.

3.7. Effect of EECC administration on the histological appearance of pancreatic tissues

The histological examination of hyperglycemic mice induced by alloxan is depicted in Table 5 and Figure 6. Alloxan-induced hyperglycemia in mice causes significant histopathological damage to the pancreas, specifically targeting the islet cells and leading to acinar cell vacuolization. This damage is a consequence of alloxan's toxic effects on the pancreatic beta cells [49]. The administration of EECC significantly reduced the pathology caused by alloxan induction

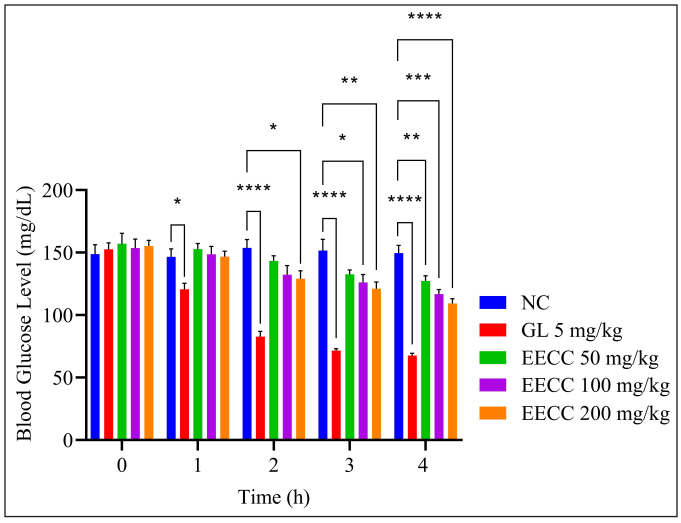

Figure 3. Effect of EECC administration on BGL of normoglycemic mice. The data are displayed as the mean ± SEM of five replicates for each group. NC: Normal control group; GL 5 mg/kg: Glibenclamide dose 5 mg/kg. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus NC group.

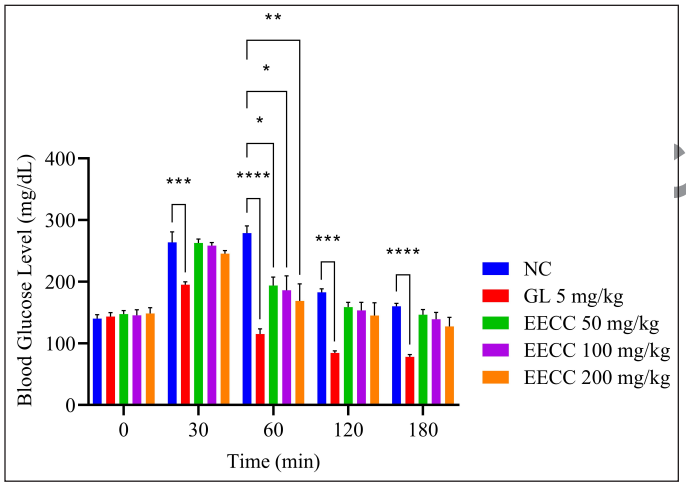

Figure 4. Effect of EECC administration on BGL of mice loaded with oral glucose. The data are displayed as the mean ± SEM of five replicates for each group. NC: Normal control group; GL 5 mg/kg: Glibenclamide dose 5 mg/kg. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus NC group.

compared to the hyperglycemic mice group, but was not significantly different from glibenclamide (Table 5).

The results for the normal group showed a normal pancreatic structure, where pancreatic lobules were encircled by connective tissue capsules comprising nerves, blood vessels, excretory ducts, and lymphatics, along with regular islets and clustered β-cells located in the center. The secretory acini has masses of spherical and tubular cells, while islets comprise anastomotic cords of polygonal endocrine cells (Fig. 6A). On the other hand, pancreatic tissues from the hyperglycemic mice group showed histological damage, such as fatty changes in the parenchyma. There was also an indication of extreme necrosis and shrinkage of the pancreatic islets, with severe vascular

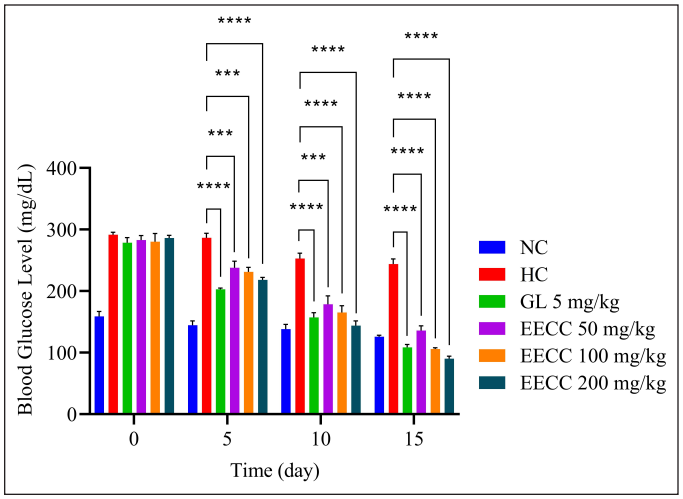

Figure 5. Effect of EECC administration on BGL of alloxan-induced hyperglycemic mice. The data are displayed as the mean ± SEM of five replicates for each group. NC: Normal control group; HC: hyperglycemic control group; GL 5 mg/kg: Glibenclamide dose 5 mg/kg. ***$p < 0.001$, ****$p < 0.0001$ versus HC group.

Table 4. Effect of administration of EECC on DBGL of alloxan-induced hyperglycemic mice.

Groups	DBGL (%)
HC	18.88 ± 1.04
GL 5 mg/kg	51.48 ± 2.39***
EECC 50 mg/kg	50.19 ± 4.11***
EECC 100 mg/kg	59.23 ± 2.56****
EECC 200 mg/kg	64.95 ± 1.52****

The data are shown as the mean ± SEM of five replicates for each group. HC: hyperglycemic control group; GL 5 mg/kg: Glibenclamide dose 5 mg/kg. ***$p < 0.001$, ****$p < 0.0001$ versus HC group.

Table 5. Semiquantitative analysis of pancreatic histopathology.

	Histopathological parameters	
Groups	Islet cell damage	Acinar cells vacuolization
NC	0.00 ± 0.00	0.00 ± 0.00
HC	3.00 ± 0.00	3.00 ± 0.00
GL 5 mg/kg	1.00 ± 0.00	1.00 ± 0.00
EECC 50 mg/kg	1.40 ± 0.24	1.60 ± 0.24
EECC 100 mg/kg	1.20 ± 0.20	1.00 ± 0.00
EECC 200 mg/kg	1.00 ± 0.00	1.00 ± 0.00

The data are presented as the mean ± SEM of five replicates for each group. NC: Normal control group; HC: hyperglycemic control group; GL 5 mg/kg: Glibenclamide dose 5 mg/kg.

obstruction and vacuolization of the acini, as shown in Figure 6B. The histological results of the EECC groups at 50 mg/kg (Fig. 6D), 100 mg/kg (Fig. 6E), and 200 mg/kg (Fig. 6F) showed significant recovery in the histopathological changes

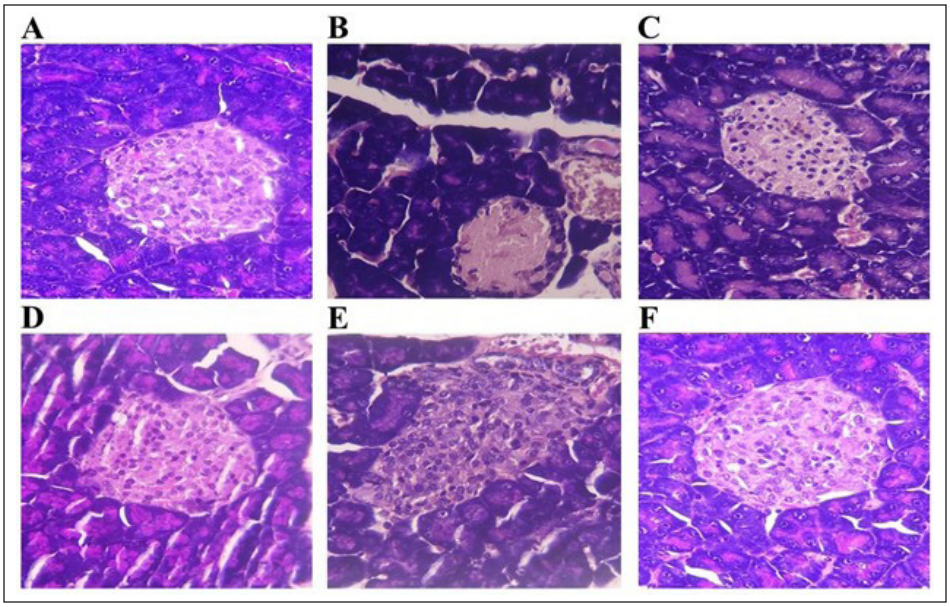

Figure 6. Effect of EECC administration on the pancreatic histopathological of alloxan-induced hyperglycemic mice. (A) Pancreatic tissue in normal control mice showed the normal histological structure of parenchyma, lobules encircled by connective tissue capsules, excretory ducts, islets, and acini that appeared normal. (B) Pancreatic tissue in hyperglycemic control mice showed histopathological changes, including irregular pancreatic acini tissue, severe shrinkage and necrosis of islets with fat infiltration in pancreatic parenchyma, severe blockage of blood vessels, and vacuolation of pancreatic acini, as well as disruption of islet outline, and dilation of intralobular ducts. (C) Pancreatic tissue in hyperglycemic mice treated with glibenclamide at 5 mg/kg indicated mild histopathological changes. (D-F) Pancreatic tissue in hyperglycemic mice treated with EECC at 50, 100, and 200 mg/kg, everyday for 14 days, showed significant recovery from histopathological changes, where normal pancreatic acini and islets were observed, similar to the normal control group. Haematoxylin-eosin stain: 100x magnification.

of the pancreas. Based on observation, normal acini and pancreatic islets were found similar to the normal control. The standard drug glibenclamide at 5 mg/kg also caused restoration of histopathological changes in pancreatic tissue, as observed in intact islets (Fig. 6C).

In fasting conditions, low insulin levels can increase glucose production by stimulating gluconeogenesis and glycogenolysis reactions in the liver [50]. Glucagon is also known to stimulate these reactions in the liver and renal medulla [51]. After eating, the glucose produced triggers an increase in insulin secretion and a decline in glucagon. Most of this postprandial glucose will be used by skeletal muscle, which is the effect of insulin-stimulated glucose absorption [52]. However, increased glucose production in the liver and impaired uptake in peripheral tissues, as well as decreased insulin secretion and excess counter-regulatory hormones (glucagon, cortisol, catecholamines, and growth hormone) that can facilitate lipolysis and protein breakdown (proteolysis), may cause hyperglycemia [53]. A good antihyperglycemia drug does not reduce BGL below normal limits in normoglycemic patients, so it does not cause hypoglycemia and has a good BGL-reducing effect in hyperglycemic patients [54].

In this study, EECC administration at 50, 100, and 200 mg/kg in normoglycemic mice showed DBGL effect after 1–4 hours, but the value was still in the normal range. Based on the results, there was a progressive decrease from the first to the fourth hour in glibenclamide group at 5 mg/kg. DBGL effect of EECC in normoglycemic mice did not cause severe hypoglycemia as shown by the glibenclamide group. Meanwhile, oral administration of EECC at 50, 100, and 200 mg/kg caused a significant decline in BGL ($p < 0.05$—$p < 0.01$) at 60 minutes after glucose administration in comparison with normal group given glucose without treatment. This suggested that EECC could increase glucose tolerance in mice, showing the potential to reduce DM complications related to postprandial hyperglycemia (PPHG). Controlling PPHG is an essential strategy for managing type 2 DM. The body's ability to use glucose is measured by OGTT, which can be used to control the amount of sugar that DM patients can consume [44].

To investigate antihyperglycemic effect of EECC, *in vitro* and *in vivo* antihyperglycemic experiments were conducted. For *in vitro*, antihyperglycemic experiments were conducted using α-amylase and α-glucosidase enzyme inhibition models. These enzymes regulate postprandial blood glucose, which can hydrolyze carbohydrates into glucose [55]. Specifically, α-amylase enzyme initiates the carbohydrate digestion process by hydrolyzing the 1,4-glycosidic bond of polysaccharides (starch) into disaccharides (maltose). α-Glucosidase catalyzes disaccharides into monosaccharides (glucose), causing PPHG [56]. The administration of agents that have an inhibitory effect on the activity of α-amylase and α-glucosidase enzymes is effective in controlling hyperglycemia to postpone carbohydrate

digestion, thereby reducing postprandial plasma glucose levels [57]. The results indicated that EECC administration at 250, 500, 750, and 1,000 µg/ml had an inhibitory effect on the α-amylase and α-glucosidase enzymes *in vitro* with respective IC_{50} of 454.72 and 575.08 µg/ml. The inhibitory potency of the EECC on α-amylase and α-glucosidase (IC_{50} > 450 µg/ml) is moderate compared to acarbose, a known α-amylase and α-glucosidase inhibitor. This indicates that a higher concentration of EECC is needed to achieve the same level of inhibition that a concentration of acarbose would produce. In addition, further investigation into the specific mechanisms of action of the EECC bioactive compounds is needed to understand why it is less potent and to potentially identify ways to improve its efficacy.

Regarding *in vivo*, antihyperglycemia experiments were conducted using an alloxan-induced hyperglycemic mice model. Alloxan can induce glucose accumulation in pancreatic β-cells through GLUT2 and selectively inhibit insulin secretion by specifically hindering glucokinase and glucose sensors in pancreatic β-cells [58]. This was confirmed by an increase in fasting BGL in mice measured 72 hours after alloxan injection. For 14 days, EECC was administered orally to mice that had experienced hyperglycemia. EECC administration at 50, 100, and 200 mg/kg significantly ($p < 0.001$—$p < 0.0001$) lowered BGL of alloxan-induced hyperglycemic mice from day 5 to day 15. It was also observed that the higher the dose of EECC given, the greater the decrease in BGL of mice. The results indicated that EECC administration is able to reduce BGL of alloxan-induced hyperglycemic mice in a time and dose-dependent manner. According to previous studies, the administration of alloxan caused ROS formation leading to selective necrosis of pancreatic islets which were important target organs for insulin. Generally, pancreatic islets can produce insulin to maintain glucose homeostasis and damage to the organ potentially affects β-cell function [55]. Based on the results, administration of EECC with various doses can repair pancreatic islet damage due to alloxan induction. This was confirmed by the histological observation results of pancreatic tissue in hyperglycemic mice. Significant improvements were observed in the pancreatic islets of EECC group compared to the hyperglycemic control group. Additionally, the histological appearance of EECC group was similar to that found in the normal control. The results of this study are consistent with previous studies, which stated that other members of the Fagaceae family, namely *Quercus coccifera* extract, have antihyperglycemic activity *in vitro* and *in vivo*. *Quercus coccifera* extract can inhibit α-amylase and α-glucosidase activity with IC_{50} values > 100,000 µg/ml, respectively. Meanwhile, with the same dose as EECC (200 mg/kg), *Q. coccifera* extract was able to significantly reduce BGL in diabetic mice, but not as strongly as EECC [59].

The hypoglycemic and antihyperglycemic activities (*in vitro* and *in vivo*), as well as the repair effects on pancreatic tissue of mice by the EECC cannot be separated from the active compounds contained. The chemical compound identification results using LC-MS/MS showed that EECC contains 14 compounds, including three kaempferol derivatives, namely kaempferol-3-O-rhamnoside, kaempferol-3-O-α-L-arabinoside, and kaempferol-3-O-β-rutinoside. Kaempferol is known to inhibit intestinal glucose absorption by inhibiting the enzyme α-glucosidase [60], increase insulin secretion and sensitivity by activating mitochondrial calcium uptake [61], facilitate glucose utilization in peripheral tissues [62], and up-regulate blood glucose absorption into muscle cells by increasing GLUT4, AMPK, LKB1, JAK2, STAT3 translocation, as well as PI3K phosphorylation [63,64]. Additionally, kaempferol can lower BGL by increasing glucokinase levels and glycogen synthesis [65], reducing liver glucose production [66], protecting against pancreatic islet damage, and helping β-cell regeneration [67]. The isorhamnetin-3-O-β-D-robinobioside compound contained in EECC has also been reported to raise glucose absorption and reduce insulin resistance by activating PGK1 enzyme that plays an important role in the glycolysis [68]. This compound reportedly increases blood glucose absorption into muscle cells by enhancing the regulation of GLUT4 and p-AMPK-α expression [69]. It also has a protective effect on β cell integrity and regenerates islets in pancreatic cells [70]. Meanwhile, the nobiletin compound is known to increase glucose absorption in skeletal muscle [71], improve glucose tolerance by protecting pancreatic β cells from oxidative stress [72], raise glucose-induced insulin secretion, and prevent apoptosis of pancreatic β cells [73]. A previous study showed that EECC has strong antioxidant activity [34], with a significant potential to repair pancreatic β cells damage caused by exposure to ROS formed due to alloxan induction. Antioxidant compounds were also reported to have effects similar to insulin in peripheral tissues and stimulate the regeneration process or the release of insulin in pancreatic β cells. This can increase the rate of glucose removal from the circulation and enhance metabolism [74].

Based on the cited literature and the results of LC-MS/MS analysis, we can hypothesize that the main bioactive compound contained in *C. costata* extract responsible for the antihyperglycemic effect is Kaempferol. This is because the kaempferol compound dominates the results of the LC-MS/MS analysis of *C. costata* extract (3 types of kaempferol: kaempferol-3-O-rhamnoside, kaempferol-3-O-α-L-arabinoside, and kaempferol-3-O-β-rutinoside). Meanwhile, the limitations in the present study were that the sample size was too small, a lack of toxicity studies, and limited duration of *in vivo* treatment.

4. CONCLUSION

In conclusion, this study showed that EECC has hypoglycemic activity, suppressed PPHG, and demonstrated antihyperglycemic effects *in vitro* (inhibition model of α-amylase and α-glucosidase enzymes) and *in vivo* (hyperglycemic mice model induced by alloxan). EECC also repaired pancreatic tissue damage confirmed by the recovery of histopathological changes. These results provided a pharmacological basis for EECC application in DM management, particularly for traditional practitioners in North Sumatra. However, further study needed to be carried out to characterize and isolate the bioactive compounds in charge of the antihyperglycemic activity, define possible mechanisms, and test safety.

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6. LIST OF ABBREVIATIONS

AMPK, adenosine monophosphate-activated protein kinase; GLUT2, glucose transporter 2; GLUT4, glucose transporter 4; JAK2, janus kinase 2; LKB1, liver kinase B1; p-AMPK-α; phosphorylated adenosine monophosphate-activated protein kinase alpha; PI3K, phosphoinositide 3-kinases; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3.

7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study protocol was approved by the Research Ethics Commission, Universitas Padjadjaran, Indonesia, with the following numbers: 1212/UN6.KEP/EC/2023 (September 22, 2023), in compliance with ARRIVE guidelines. Universitas Buana Perjuangan Karawang has an MOU with Universitas Padjadjaran, and any Serious Adverse Events (SAE) in the research process must be immediately reported to the Research Ethics Committee of Universitas Padjadjaran, because the Research Ethics Committee of Universitas Padjadjaran has full access to all experimental access conducted.

10. DATA AVAILABILITY

The data that supports the findings of this study are available in the supplementary material of this article.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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SUPPLEMENTARY MATERIALS

This supplementary material contains a presentation of chromatograms from the results of analysis using LC-MS/MS QTOF in positive and negative ion modes. Each chromatogram contains information on the observed mass and its intensity which shows the profile of a component. The results of identifying chemical compounds using LC-MS/MS revealed that there are 14 compounds contained in EECC, which are divided into 2 groups of compounds, namely flavonoids (12 components) and triterpenoids (2 components).

The supplementary material can be accessed at the link here: [https://japsonline.com/admin/php/uploadss/4659_pdf.pdf]