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Effect of *Petiveria alliacea* leaf extract and its active components on heart muscle cell apoptosis induced by hyperglycemia

Nurmawati Fatimah^{1,2} , Sri Agus Sudjarwo³ , Arifa Mustika^{2*} , Suharjo Suharjo⁴ , Jusak Nugraha⁵ , Hari Basuki Notobroto⁶ , Rochmah Kurnijasanti³ , Reny I'tishom⁷ , Wibi Riawan⁸ , and Alphaia Rahniayu⁹ 

¹Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

²Department of Anatomy, Histology and Pharmacology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

³Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia

⁴Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

⁵Department of Clinical Pathology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

⁶Department Biostatistics, Faculty of Public Health Airlangga University, Surabaya, Indonesia

⁷Department of Medical Biology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

⁸Department of Biochemistry and Biology Molecular, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia

⁹Department of Anatomical Pathology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

ABSTRACT

Background: Hyperglycemia is a condition in which blood sugar levels increase excessively due to a variety of factors, one of which is the body's inability to regulate insulin properly. Diabetes closely relates to this condition, which significantly contributes to premature death and disability. Long-term diabetes treatment accompanied by a strict diet provides real results in controlling blood glucose levels but can cause side effects. Therefore, it is necessary to conduct research on the potential for new drug sources with minimal side effects. Traditional medicine empirically uses the plant *Petiveria alliacea*.

Aim: This study was conducted to determine the effect of *P. alliacea* on hyperglycemic model rat and to further determine the mechanism of its active compounds *in silico*.

Methods: The experimental animals were divided into 5 groups: 1 normal group, 1 group induced with Streptozotocin (STZ) 50 mg/kgBW to treat hyperglycemia, and 3 other groups induced with STZ 50 mg/kgBW and given 70% ethanol extract of *P. alliacea* leaves (EPPa) in different doses. Each group was measured for B-cell lymphoma 2 (Bcl2) expression and apoptosis. We also carried out *in silico* identification of the isoarborinol acetate and myricitrin compounds contained in EPPa against alpha-glucosidase and caspase-3.

Results: It was found that giving STZ to rat can cause hyperglycemia. This was shown by measuring fasting blood glucose in rat and then measuring Bcl2 as an antiapoptotic agent. Bcl2 levels rose compared to the hyperglycemic control group and can lower apoptosis expression in heart cells of hyperglycemic model rat with an optimal dose of 90 mg/kgBW. In addition, the results showed that isoarborinol acetate and myricitrin compounds have inhibition of alpha-glucosidase and caspase-3.

Conclusion: It can be concluded that EPPa is one of the alternative choices to overcome hyperglycemia.

Keywords: Apoptosis, Bcl2, Diabetes, Hyperglycemia, *Petiveria alliacea*.

Introduction

Hyperglycemia is a condition characterized by blood glucose levels greater than 100 mg/dl when fasting (8–12 hours) or 200 mg/dl at random. This condition occurs when the body has little or cannot regulate insulin properly. Hyperglycemia is associated with diabetes (Tedjamartono *et al.*, 2023). The International Diabetes Federation estimated that in 2013, the number

of adults with diabetes reached around 382 million people in the world, which means a global proportion of 8.3%, with around 80% of these people living in low and middle-income countries (Guariguata *et al.*, 2014; Amegan *et al.*, 2022). This disease is a major cause of premature death and disability. Globally, almost half of deaths from high blood glucose occur before the age of 70 (Islam *et al.*, 2019).

*Corresponding Author: Arifa Mustika. Department of Anatomy, Histology and Pharmacology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. Email: arifa-m@fk.unair.ac.id



Significant and persistent hyperglycemia can cause dysfunction in various types of cells (Wang *et al.*, 2020), which ultimately triggers complications such as nephropathy, retinopathy, angiocardopathy, cerebrovascular disease, and neuropathy (Cusi *et al.*, 2017). Diabetes treatment, combined with a strict diet and exercise control, provides real results in controlling blood glucose levels. However, it can cause side effects such as hypoglycemia, gastrointestinal reactions, liver damage, and lactic acidosis (Srivali *et al.*, 2015). This impacts the level of patient compliance and causes less than satisfactory management of complications (Bai *et al.*, 2022).

These side effects encourage the need for various studies on the potential sources of new drugs with minimal side effects (Ma'arif *et al.*, 2022). Identification of bioactive compounds derived from natural products could be a promising approach to solving this problem (Gondokesumo *et al.*, 2023a,b). Indonesians widely use the perennial plant *Petiveria alliacea* as a traditional medicine to treat a variety of diseases, including diabetes, bloody coughing, anti-inflammatory, and immunomodulator (Mustika *et al.*, 2021).

A study shows that myricitrin and isoarborinol acetate compounds are one of the active compounds contained in a 70% ethanol extract of *P. alliacea* (EEPa) leaves and are predicted to have anti-inflammatory activity against IL1 and TNFA receptors *in silico* (Fatimah *et al.*, 2024). *In vivo* studies show that this plant can also lower blood sugar levels in T2DM rat models and raise the expression of 5'-adenosine monophosphate-activated protein kinase in the liver (Gunawan *et al.*, 2020). Based on the background that has been mentioned, this study was conducted to determine the effect of *P. alliacea* on hyperglycemic rat models and to further determine the potential mechanisms of its active compounds *in silico*.

Materials and Methods

The materials used in this study were *P. alliacea* leaves (in Indonesia called "singawalang") obtained and determined from Balai Materia Medika, Batu, with letter number 074/349/102.7-A/2021. We obtained the Streptozotocin (STZ) and B-cell lymphoma 2 (Bcl2) examination kit from [®]santacruz, the 70% ethanol from Merck, and the *in situ* apoptosis kit from Elabscience. The tools used include micropipettes, pipette tips, stirring rods, polypropylene microcentrifuge tubes, syringes, microscopes, cover slips, object slips, spectrophotometers, vortexes, magnetic stirrers, water baths, and minor surgery.

Extraction

A 70% EEPa was obtained from the extraction results using the maceration method with 70% ethanol solvent (1:10) for 3 × 24 hours, with remaceration carried out every day (Gondokesumo *et al.*, 2023a,b). The maceration filtrate was evaporated using a rotary

evaporator and then dried in an oven until a thick extract was obtained.

Treatment of experimental animals and analysis fasting blood glucose (FBG), Bcl2, and apoptosis

Adult male Wistar rats (*Rattus norvegicus*) weighing 160–180 g and aged 16–18 weeks were chosen to minimize hormonal influences. We divided the experimental animals into five groups (@12 rats each group): negative control with CMC Na 0.5%/day (C1), positive control with STZ induction and CMC Na0.5%/day (C2), STZ induction and EEPa 90 mg/kg/day (T1), STZ induction and EEPa 180 mg/kg/day (T2), and induction of STZ and EEPa 360 mg/kg/day (T3). We intraperitoneally injected the rats with STZ at a dose of 50 mg/kgBW after a 2-week acclimatization period. To prevent post-injection hypoglycemia, the rats were provided with a 10% dekstroze solution overnight. Blood glucose levels were evaluated before and after treatment, while Bcl2 and apoptosis measurements were evaluated after treatment. Blood sugar levels were monitored by collecting tail blood samples using the @Nesco-glucose stick. On the last day of treatment, apoptosis and Bcl2 testing were performed by dehydrating and clearing heart tissue using graded concentrations of alcohol and xylol solution. This was followed by infiltration and block preparation using paraffin, and then thin sectioning using a microtome to obtain slides. Deparaffinization was performed using xylol and graded concentrations of alcohol, followed by immunohistochemistry (IHC) staining (Bioenzy[®] kit), for Bcl2 (monoclonal AB, Santacruz) and apoptosis (E-CK-A331, Elabscience). Counterstaining was then conducted using methyl green, followed by the mounting process. Observations were made using a Nikon F600L light microscope equipped with a DS F12 digital camera (300 megapixels) at 400x magnification, along with Nikon Image System software (Novak *et al.*, 2007). Data obtained from *in vivo* testing were analyzed using Statistical Package for the Social Sciences software to assess the relationships between variables and differences between groups.

Collection of compound and protein data

Isoarborinol acetate and myricitrin data were acquired from the PubChem database, and the 3D structure of each compound was downloaded in "sdf" format. Furthermore, the 3D structures of alpha-glucosidase (PDB ID 3L4Y) and caspase-3 (PDB ID 3KJF) were obtained from the PDB database, with each protein structure downloaded in PDB format.

Analysis of potential biological activity, toxicity, and druglikeness of active compounds

The potential biological activity of isoarborinol acetate and myricitrin compounds was predicted using the PASS Online webserver. The values of probability of activity (Pa) and probability of inactivity (Pi) were used as parameters in various biological functions related to hyperglycemia. Meanwhile, the toxicity analysis of the compounds was predicted using the

Protox-3 webserver, which aims to determine the toxicity classification of each compound. The drug feasibility analysis of isoarborinol acetate and myricitrin compounds was analyzed using Lipinski's Rule of 5, that is using the parameters of molecular size (MW), hydrogen donor, hydrogen acceptor, and LogP. All analyses were performed using the SWISS ADME webserver.

Molecular Docking analysis

Identification of binding activity between isoarborinol acetate and myricitrin compounds with target proteins was carried out through molecular docking simulations using the VinaWizard plugin in PyRx 1.1 software. The results of the analysis were then visualized using PyMOL software.

Target protein prediction and interaction analysis

Target protein prediction analysis of each compound was performed using the SWISS TARGET webserver, aiming to determine the default protein of each compound. Furthermore, the identification of interacting proteins of each compound was performed using predicted-protein results from target protein analysis. The STRING database facilitated the analysis of the pathway mechanism.

Ethical approval

Ethical approval was obtained from the Health Research Ethics Committee, Faculty of Medicine, Airlangga University (letter number: 257/EC/KEPK/FKUA/2023).

Results

Table 1 shows random blood sugar measurements in hyperglycemic model rat before and after EEPa administration. The results showed that rat's FBG changed after induction. Administration of STZ resulted in a significant increase in random sugar levels in rat in group C2, exceeding 200 mg/dl, indicating a diagnosis of hyperglycemia based on internationally recognized criteria (Fajardo *et al.*, 2014). Furthermore, we considered the administration of EEPa to the treatment group (T1-T3) to have an effect on the FBG of rat, as the results demonstrated a significant difference compared to group C2, which did not receive EEPa treatment.

Table 2 and Figure 1 show the results of Bcl2 expression in rat in each treatment group. The results in Table 2 reveal that Bcl2 levels in group C2 are significantly lower than those in group C1, with a significance value of $p = 0.000$. This is followed by a group of rat given EEPa at a dose of 90 mg/KgBB/day (T1), and finally,

Table 1. FBG levels in hyperglycemic model rat before and after administration of EEPa.

Group	Fasting blood sugar levels		p
	PRE	POST	
	Mean $\pm$ SD	Mean $\pm$ SD	
C1	99.50 $\pm$ 20.81 ^b	85.10 $\pm$ 17.94 ^a	0.048***
C2	74.57 $\pm$ 13.04 ^a	326.43 $\pm$ 176.93 ^{ab}	0.010***
T1	90.18 $\pm$ 14.30 ^{ab}	183.91 $\pm$ 84.47 ^b	0.003***
T2	103.83 $\pm$ 16.95 ^b	268.33 $\pm$ 85.77 ^b	<0.001***
T3	93.33 $\pm$ 12.27 ^{ab}	308.17 $\pm$ 113.99 ^b	<0.001***
p	0.005*	0.001**	

(*): significant at $\alpha=0.05$ (Oneway Anova). (**): significant at $\alpha=0.05$ (Brown-Forsythe test). (***): significant at $\alpha=0.05$ (Paired t test). (ab) the same superscript in one column indicates no difference between groups (Tukey HSD/Games Howell test).

Table 2. Bcl2 expression in hyperglycemic rat models after EEPa administration.

Group	Median $\pm$ IQD			
	Bcl-2 expression	p	Apoptosis expression	p
C1	19.00 $\pm$ 4.28 ^c		0.17 $\pm$ 0.01 ^c	
C2	2.20 $\pm$ 2.25 ^{ab}		0.45 $\pm$ 0.04 ^d	
T1	9.80 $\pm$ 3.30 ^b	<0.001*	0.10 $\pm$ 0.04 ^{ab}	<0.001*
T2	2.90 $\pm$ 3.25 ^a		0.11 $\pm$ 0.02 ^{bc}	
T3	1.20 $\pm$ 2.13 ^a		0.07 $\pm$ 0.02 ^a	

(*): significant at $\alpha=0.05$ (Kruskal-Wallis test). (^{abc}): the same superscript indicates no difference between groups (Mann-Whitney test).

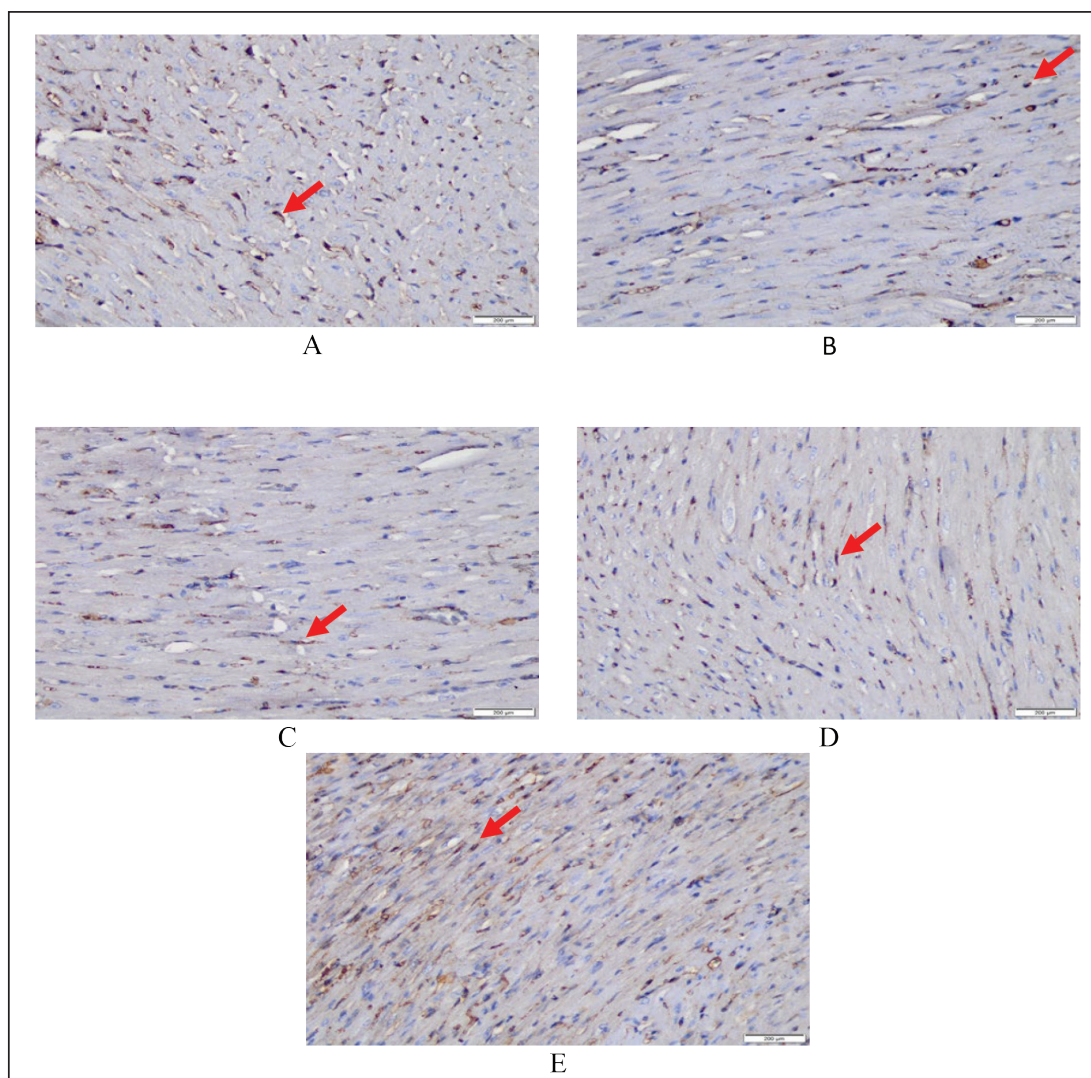


Fig. 1. Immunohistochemical examination results of Bcl2 in the heart tissue; A. (C1), B. (C2), C. (T1), D (T2), E (T3).

the T2-T3 treatment group. Since Bcl2 functions as an antiapoptosis, these results indicate that group C2 underwent apoptosis, while group C1 did not. Table 2 and Figure 2, also display the results of apoptosis testing. Based on the results of FBG, Bcl2, and apoptosis, it can be seen that the optimal dose of EEPa in hyperglycemia therapy is 90 mg/KgBW because a small dose already provides optimal results compared to multiple doses.

Further research was conducted to determine the mechanism pathway of EEPa as an antihyperglycemic using *in silico* methods. We confirmed the interactions between the compounds isoarborinol acetate and myricitrin with alpha-glucosidase and caspase-3. Previous research predicted both compounds in EEPa to have anti-inflammatory activity against IL1 and TNFA receptors *in silico* (Fatimah et al., 2024).

Table 3 displays the findings from the biological activity analysis. The results show that all compounds have various activities related to hyperglycemia and cardiac fibrosis, including antidiabetic, anti-inflammatory, antioxidant, and alpha-glucosidase inhibitors. This is shown based on the Pa score of each compound, a Pa value approaching 1 indicates that the biological activity of the compound has been validated based on *in vitro* research to *in vivo* scale (Pramely and Raj, 2012).

Table 4 also displays the predicted toxicity level results. The results show that the 2 compounds have a safe toxicity level based on the LD₅₀ dose. This indicates that the safe dose threshold allows for the oral consumption of all these compounds.

Drug feasibility analysis was conducted based on the principle of Lipinski's rule of 5. The results of the

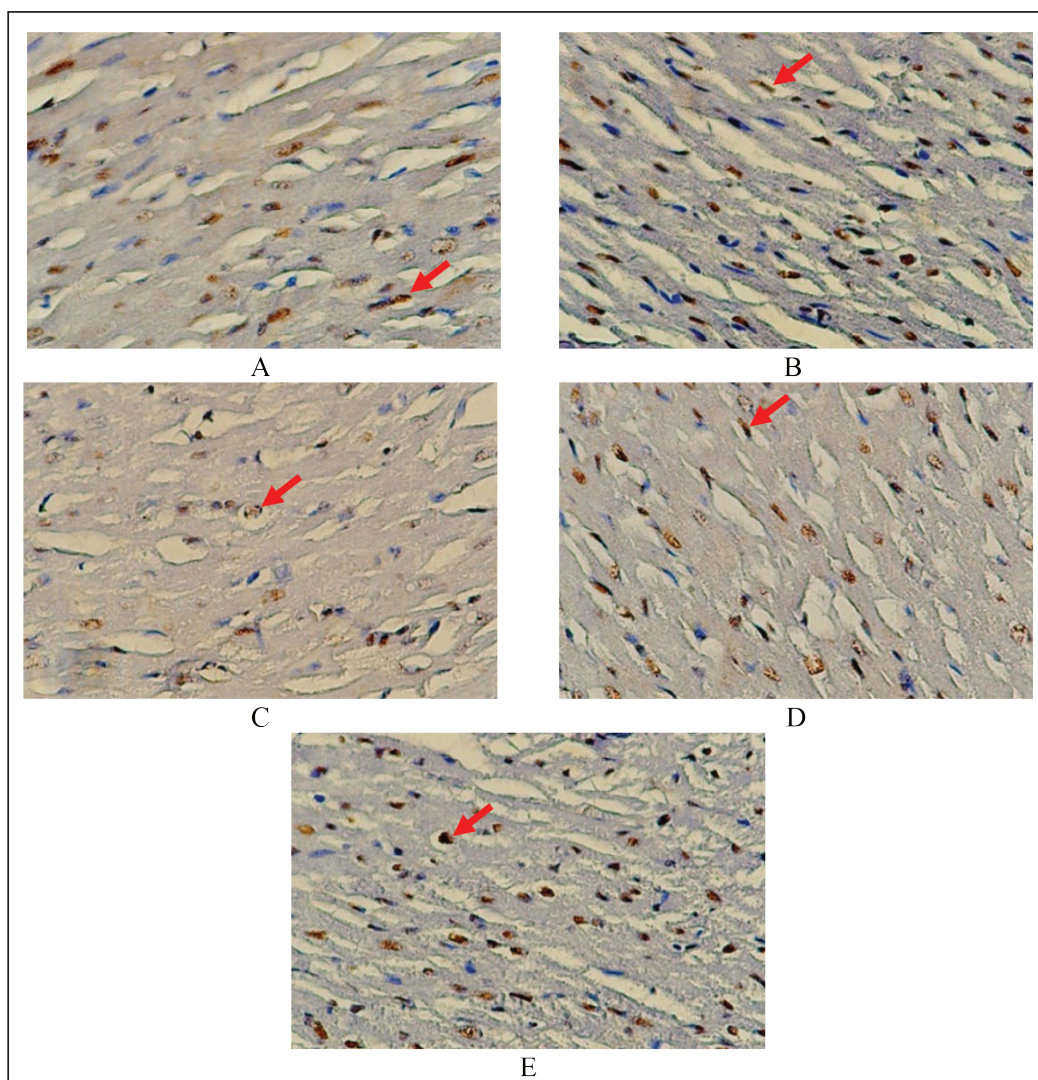


Fig. 2. Immunohistochemical examination results of apoptosis in the heart organ; A. (C1), B. (C2), C. (T1), D (T2), E (T3).

Table 3. Pa and Pi values for the active compound EEPa.

Compound	Pa score	Pi score	Biological activity
Isoarborinol acetate	0.773	0.009	Antiinflammatory
	0.242	0.038	Antioxidant
	0.162	0.008	Alpha glucosidase inhibitor
Myricitrin	0.938	0.002	Antioxidant
	0.782	0.001	Alpha glucosidase inhibitor
	0.762	0.009	Antiinflammatory
	0.508	0.022	Antidiabetic

analysis are shown in Table 5. The analysis reveals that myricitrin, one of the sample compounds, exhibited two violations of the Lipinski rule of five parameters. This indicates that the myricitrin compound may not

be easily absorbed by cells as a candidate for oral drugs.

The initial interaction analysis is a simulation of the interaction between the compound of interest and the

Table 4. Results of the toxicity level prediction of compound.

Compound	Toxicity class	LD ₅₀	Accuracy
Isoarborinol acetate	5	4,300 mg/kg	72.90%
Myricitrin	5	5,000 mg/kg	72.90%

Table 5. The results of predicting the drug suitability of compounds.

Compound	Molecular weight (≤500 g/mol)	Hydrogen donor (≤5)	Hydrogen acceptor (≤5)	MLogP (≤5)	Violation
Isoarborinol acetate	468.75	0	2	7.08	0
Myricitrin	464.38	8	12	-2.32	2

Table 6. Molecular docking simulation results for interest compounds and controls against alpha-glucosidase.

Compound	Binding affinity score (Kcal/mol)
NR4 ligand (Control)	-5.1
Isoarborinol acetate	-7.0
Myricitrin	-7.4

control alpha-glucosidase protein (PDB ID: 3L4Y). The native ligand NR4 is a ligand/small molecule that attaches to the active site of the alpha-glucosidase protein, so it is called the native control. The results indicate that both isoarborinol acetate and myricitrin exhibit stronger inhibitory binding interactions with the target protein compared to the native ligand. This is indicated by the compound binding affinity score indicator, that is -7.0 kcal/mol and -6.6 kcal/mol. The results can be seen in Table 6 and Figure 3.

The next molecular docking interaction analysis is a simulation between the compound of interest and the control against Caspase 3 (PDB ID: 3KJF). It was found that the compounds isoarborinol acetate and myricitrin bind to the target protein more strongly than the B62 ligand control. This is indicated by the compound's binding affinity score indicator, which is more negative than the control. The results can be seen in Table 7 and Figure 4.

Furthermore, the two compounds of interest are predicted for their pathway mechanisms in hyperglycemia pathology. The analysis results predict a profitable opportunity for the isoarborinol acetate compound to interact with various proteins. This is shown by the probability score listed in Table 8.

The target proteins were further analyzed to determine the protein network formed. The results revealed that the protein network formed from the target protein compound isoarborinol acetate consisted of 50 nodes and 627 edges, or interactions (Fig. 5).

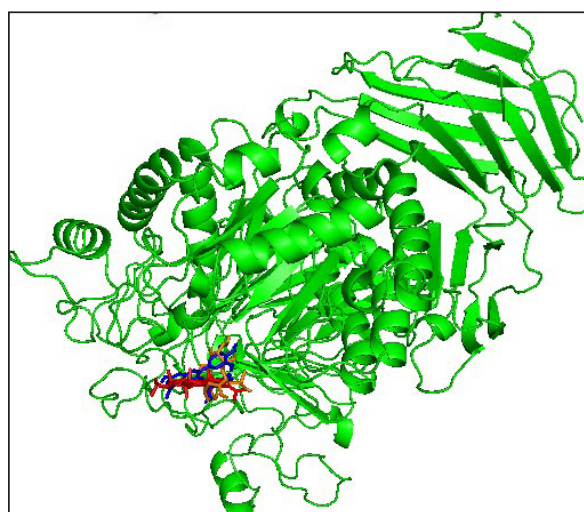


Fig. 3. Alpha-glucosidase protein complex (green) with isoarborinol acetate (red), myricitrin (blue), and NR4 ligand control (oranges).

Table 7. Molecular docking simulation results for interest compounds and controls against caspase 3.

Compound	Binding affinity score (Kcal/mol)
B62 ligand (control)	-8.3
Isoarborinol acetate	-8.6
Myricitrin	-7.8

In particular, several pathways are involved in the steroid catabolic process and the estrogen biosynthesis process. Different colors mark the associated proteins according to their respective pathways (Fig. 6). The False Discovery Rate (FDR) value indicates the validity of the analysis results on each involved pathway, with a lower FDR value indicating greater validity. This has a similar concept to the *p*-value (Table 9).

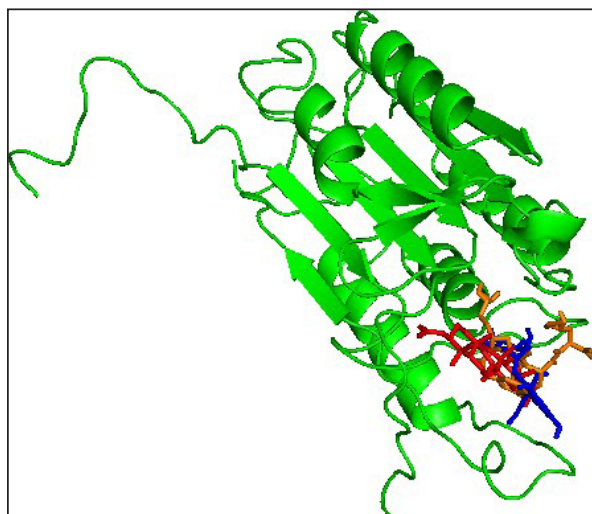


Fig. 4. Caspase-3 protein complex (green) with isoarborinol acetate (red), myricitrin (blue), and B62 ligand control (orange).

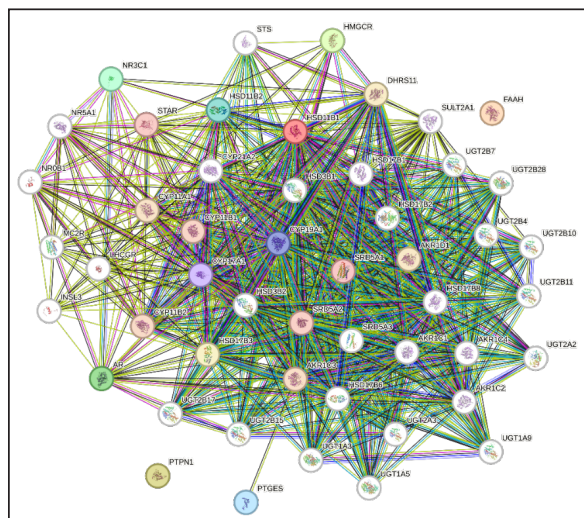


Fig. 5. Protein-protein interaction network on the target protein of isobornyl acetate compound.

Table 8. Prediction of target proteins from isoarborinol acetate compounds.

Compound	Target protein	Probability
Isoarborinol acetate	PTGES	0.16
	HSD11B1	0.12
	AR	0.12
	CYP17A1	0.12
	HSD11B2	0.12
	CYP19A1	0.12
	HMGCR	0.12
	FAAH	0.12
	PTPN1	0.12
	NR3C1	0.12

In addition to the isoarborinol acetate compound, we also performed target protein prediction on the myricitrin compound. The results of the analysis showed that the myricitrin compound has the opportunity to interact with various types of proteins. This is shown by the probability score listed in Table 10.

The target proteins of the myricitrin compound were further analyzed to determine the protein network formed. The results showed that the protein network consisted of 60 nodes with 397 edges (interactions) (Fig. 7).

Some pathways involved in the cardiac fibrosis pathway are bicarbonate transport and pH regulation. Figure 8 marks related proteins with different colors corresponding to their respective pathways, and Table 11 displays the analysis results' validity for each pathway.

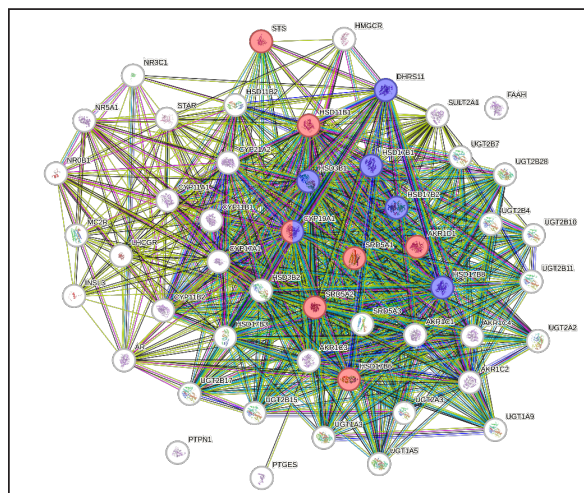


Fig. 6. Protein-protein interaction network on the target protein of the isoarborinol acetate compound: steroid catabolic process (red) and estrogen biosynthesis process (blue).

Discussion

Hyperglycemia comes from the Greek words hyper (high), glykys (sweet/sugar), and haima (blood). Hyperglycemia is blood glucose that is more prominent than 125 mg/dl when fasting and greater than 180 mg/dl 2 hours postprandial. When a patient's fasting blood glucose exceeds 125 mg/dl, it is considered diabetes (Sharma, 2021). Untreated hyperglycemia can cause many serious, dangerous complications that include damage to the eyes, kidneys, nerves, heart, and peripheral vascular system (Balaji *et al.*, 2019; Sharma, 2021).

Table 9. Protein-protein interaction network on the target protein of the isoarborinol acetate compound.

GO number	Biological process (Gene ontology)	Proteins	FDR score
GO:0006706	Steroid catabolic process	STS, HSD11B1, CYP19A1, SRD5A2, SRD5A1, AKR1D1, HSD17B6	7.22e-10
GO: 0006703	Estrogen biosynthesis process	HSD17B1, HSD17B2, DHRS11, HSD3B1, CYP19A1, HSD17B8	9.40e-10

Table 10. Prediction of target proteins from myricitrin compounds.

Compound	Protein target	Probability
Myricitrin	AKR1B1	0.95
	CA2	0.95
	CA7	0.95
	CA12	0.95
	CA4	0.95
	NOX4	0.93
	ADRA2C	0.93
	ACHE	0.80
	NQO2	0.71
	RPS6KA3	0.60

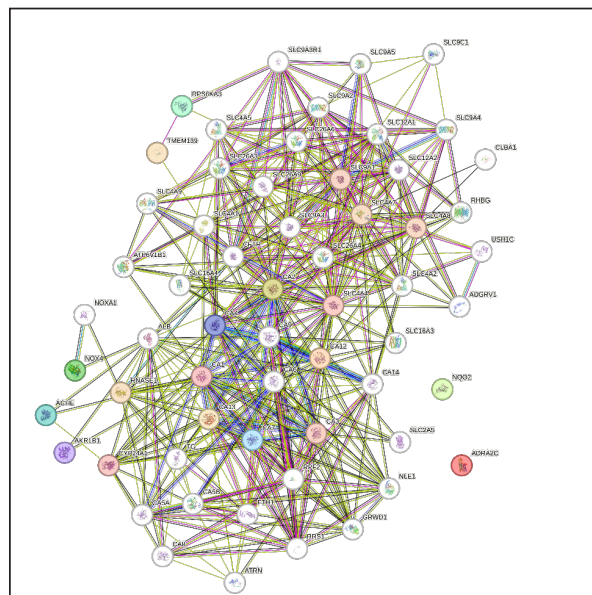


Fig. 7. Protein-protein interaction network on the target protein of myricitrin compound.

Factors that cause hyperglycemia include decreased insulin release, decreased glucose utilization, and increased glucose production. Glucose homeostasis is the balance between glucose production in the liver

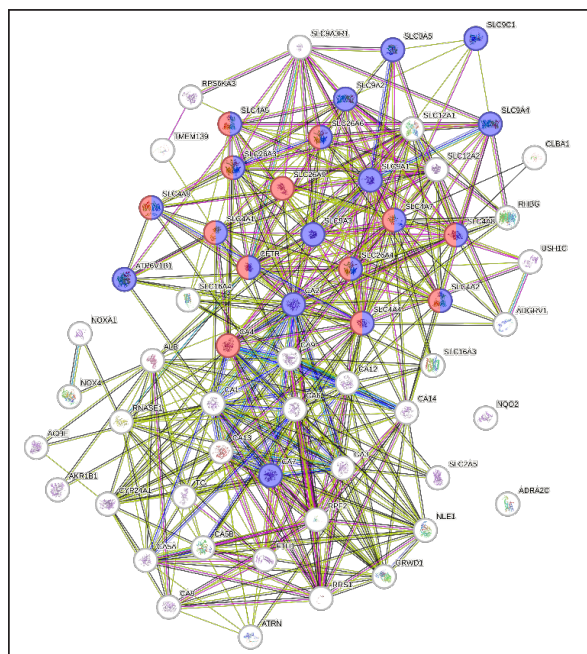


Fig. 8. Protein-protein interaction network on the target protein of the myricitrin compound: bicarbonate transport (red) and regulation of pH (blue).

and the uptake and utilization of additional glucose. Insulin is the primary controller of glucose homeostasis (Sharma, 2021).

The goals of hyperglycemia treatment include eradicating hyperglycemia manifestations and reducing long-term difficulties (Bashir *et al.*, 2019). Patients with type 1 diabetes achieve glycemic control through a varied insulin routine and an appropriate diet. Patients with type 2 diabetes are managed with dietary and lifestyle changes as well as prescriptions. Type 2 diabetes may also be monitored by a physician specializing in oral glucose-lowering. Patients with hyperglycemia should be evaluated for complications including retinopathy, nephropathy, and cardiovascular disease (Jacobsen *et al.*, 2014; Sharma, 2021).

EEPa administration is predicted to be an alternative treatment for hyperglycemic conditions. In this study, fasting blood sugar was evaluated before and after EEPa administration in hyperglycemic model rat; in addition, Bcl2 and apoptosis levels were also evaluated. The

Table 11. Protein-protein interaction network on the target protein of the mycitrin compound.

GO number	Biological process (Gene ontology)	Proteins	FDR score
GO:0015701	Bicarbonate transport	SLC4A5, SLC26A6, SLC26A3, SLC4A9, SLC4A1, SLC26A9, SLC4A7, SLC4A8, SLC4A2, SLC4A4, SLC26A4, CFTR, CA4	1.93e-20
GO: 0006885	Regulation of pH	SLC9C1, SLC9A5, SLC9A4, SLC9A2, SLC4A5, SLC26A6, SLC26A3, SLC9A1, SLC4A9, SLC4A1, SLC 9A3, SLC4A7, SLC4A8, ATP6V1B1, CFTR, CA2, SLC26A4, SLC4A2, SLC4A4	2.38e-26

results demonstrated that EEPa administration in the T1-T3 group reduced FBG values compared to the C2 group, where STZ alone induced FBG values without EEPa. Furthermore, based on the results of Bcl2 expression, EEPa administration in the treatment group can increase its expression compared to the C2 group with a significant value of <0.001. The expression of apoptosis decreases in response to this increase in Bcl2. This is in accordance with the theory that Bcl2 is an antiapoptotic agent (D'Aguanno and Bufalo, 2020). Isoarborinol acetate and myricitrin compounds are compounds that have been identified in 70% EEPa and have anti-inflammatory activity against IL1 and TNFA receptors *in silico* (Fatimah et al., 2024). In this study, the 2 compounds in EEPa were re-identified against alpha-glucosidase and caspase-3 *in silico*. The results obtained showed that these two compounds play a role in hyperglycemia and cardiac fibrosis, including antidiabetic, anti-inflammatory, antioxidant, and alpha-glucosidase inhibitors. However, isoarborinol acetate is suitable for oral medicine because it meets the requirements of Lipinski's 5 rule. Lipinski's rule This is a rule of thumb that evaluates drug-likeness and determines whether a chemical compound with a particular pharmacological activity has physical and chemical properties that would make it an orally active drug in humans. This rule is used during drug development to improve its activity and selectivity and to ensure that its drug-like physicochemical properties are maintained (Roskoski, 2019).

The overall results indicate that EEPa contains compounds that could potentially serve as an alternative therapy for hyperglycemia, as it has been shown to reduce FBG in STZ-induced hyperglycemic rat models. It is possible that administering EEPa acts as an anti-inflammatory, increasing antiapoptotic agents such as Bcl2, and inhibiting alpha-glucosidase and caspase-3, which leads to improved apoptosis in heart cells in hyperglycemic rat models.

Conclusion

Administration of EEPa can be used as one of the options to treat hyperglycemia herbally. It is known

that the compounds contained in EEPa have anti-inflammatory activity, increased antiapoptotic agents such as Bcl2, inhibition of alpha-glucosidase and caspase-3, and decreased apoptosis in heart cells in hyperglycemic rat models.

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Authors' contributions

NF: Contributed to the conceptualization, methodology, practical work, histopathological analysis and interpretation, drafting of the manuscript, and its editing and revision. SAS: Involved in the conceptualization, methodology, supervision, drafting of the manuscript, and its editing and revision. AM: Participated in the conceptualization, methodology, supervision, drafting of the manuscript, and its editing and revision. S, JN, HBN, RK, RI, WR, AR: Histopathological analysis and interpretation. All authors reviewed and approved the final manuscript for publication.

Conflict of interest

The authors declare that there is no conflict of interest.

Data availability

All the data are presented within this article.

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