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Original Article

Antidiabetic and pancreato-protective effect of clove leaf essential oil in diabetic rats: *in vivo* and silico study

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Abstract

Type 1 diabetes mellitus is an autoimmune disease that causes pancreas defects and insulin deficiency, which can lead to several complications. Clove leaf essential oil was found to control glycemic levels and protect the pancreatic tissue. Therefore, this study aims to investigate the antidiabetic and pancreatic-protective effects of clove leaf essential oil in alloxan-induced diabetic rats. A true experimental post-test was conducted only to evaluate the effect of clove leaf essential oil administration on alloxan-induced diabetic rats. The phytochemical content was determined by the Gas Chromatography–Mass Spectrometry method. Blood glucose levels were measured after two weeks of clove leaf essential oil administration. Computational and histopathology analyses were also performed. Clove leaf essential oil contained 66.5% eugenol and significantly reduced fasting glucose levels of diabetic rats in the treatment group (p=0.013). In silico results showed that eugenol had higher energy binding to albumin compared to glucose (-Δ2.75 *vs.* -Δ1.27 kcal/mol), thus reducing glucose-albumin-induced oxidative stress and protecting the pancreatic cell. The histopathological changes of the pancreas showed a better morphology of islet cells in the treatment group. Clove leaf essential oil has antidiabetic and pancreatic-protective effects in diabetic rats.

Keywords: antidiabetic, clove leaf, diabetes mellitus, essential oil, eugenol.

Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by an inappropriate increase in blood glucose levels or hyperglycemia due to abnormalities in insulin secretion and action. DM can be classified into two main subtypes, i.e., type 1 diabetes mellitus (T1DM) due to impaired insulin secretion and type 2 diabetes mellitus (T2DM) due to impaired insulin action [1, 2]. DM has become a global problem with a prevalence of up to 9.8%, which can cause various cardiovascular complications and contribute to 11.3% of deaths worldwide [3]. In general, T1DM occurs in children or adolescents, whereas T2DM occurs in middle-aged and older adults due to poor lifestyles and diets [2]. If diabetes is not controlled or managed properly, complications of diabetes can occur according to the degree and duration of diabetes that is not well controlled. Complications of DM include the occurrence of nephropathy, retinopathy, neuropathy, and cardiovascular disease, especially associated with other comorbidities [2, 4].

The main treatment of T1DM is the administration of insulin by daily injection or an insulin pump, whereas, in T2DM, diet and exercise may be adequate therapy initially. Other pharmacological therapies include metformin, sulfonylureas, thiazolidinediones, and sodium-glucose transporter-2 (SGLT-2) inhibitors, where metformin is the first line of diabetes medication prescribed to patients [2]. However, these current therapies that are used in DM have various side effects. The most common side effect of insulin is that it can cause hypoglycemia. Metformin can cause lactic acidosis and should be used with caution in patients with kidney disease. Sulfonylureas can cause hypoglycemia

and cardiovascular death in DM patients [5]. Thiazolidinediones can cause fluid retention and worsen heart failure [6, 7]. SGLT-2 inhibitors may cause an increase in urinary tract infections due to increased urinary glucose excretion [8].

Thus, the development of herbal medicines can be an alternative solution. One promising alternative is clove leaf essential oil. Clove (*Syzygium aromaticum* L.) is an aromatic flower belonging to the *Myrtaceae* family, which is widely cultivated in tropical and subtropical countries, one of which is Indonesia [9–11]. In cloves, there are at least thirty compounds have been identified, and the main compound contained in cloves is eugenol, which reaches 50% [10]. The processes involved in many pathophysiological conditions, such as DM, are oxidative stress and inflammation [12]. The anti-inflammatory effects of clove essential oil and its eugenol were found to reduce inflammation by 40% after 3 hours [13, 14]. By alpha-amylase inhibition assay, clove essential oil exhibited maximum antidiabetic activity with 95.30% inhibition of α-amylase [15]. However, essential oil is a substance that is volatile, lipophilic, unstable, and sensitive to oxygen, light, humidity, and heat. Improving the stability, good appearance, and drug solubility can be done by using microemulsion technology [16, 17]. Therefore, with the potential antidiabetic effect of clove essential oil, this study aims to observe more about the effect of clove leaf essential oil in diabetic rats.

Material and methods

Preparation and isolation of essential oil from clove leaves

The tools and materials needed include clove leaves (*Syzygium aromaticum*), test animals in the form of 24 male Wistar rats aged 70–90 days with a body weight of 200–250 g, distilled kettle, condenser, Na2SO4 solution, Chromatography-Mass Spectrometry, alloxan solution, 0.1 M sodium-citrate buffer, 5% glucose, glucometer, camera, ethanol, tissue paper, citicoline, liquid paraffin, Eppendorf tube, plate reader, and aquadest. The clove leaves that have been washed and then dried are left in an open place and not exposed to sunlight and then put into a distilled kettle with pressure for 7 hours. The liquid from the condenser is allowed to stand for 2 hours to separate the water from the oil. For the process of refining clove leaf essential oil, Na2SO4 was added and stirred for 1 hour.

Analysis of compound composition in clove leaf essential oil

Analysis of compound composition in clove leaf essential oil was carried out using the Gas Chromatography-Mass Spectrometry method. The volatile oil obtained was observed for its composition, and the percentage yield was calculated using GC-MS QP2010SE SHIMADZU with the Rastek Rxi-5MS column. GC-MS operating conditions with ionizing type EI (Electron Impact), column type Cp sit 5 CB, 30 meters long, column temperature 70 C to 270 C, Helium carrier gas 10 kPa, Injector Mode: Split 1: 80 temperature 300 C and the detector temperature is 300 C.

Test animal preparation and glucose level measurement

The experimental animals used were 24 male Wistar rats that were divided into four groups, including the negative control group (healthy rats/C-), the positive control group (induced by alloxan without essential oil/C+), treatment group 1 (induced by alloxan and received essential oil of 250 mg/kg/T1), and treatment group 2 (induced alloxan and received essential oil of 500 mg/kg/T2). The essential oil was given to rats in the treatment group that had experienced increased fasting blood glucose. The modalities were given to two different treatment groups, as much as 250 mg/kg and 500 mg/kg. The modality was given daily using an oral probe every morning before the feed was given after 2 weeks post-induction for 2 weeks. Measuring rat blood glucose levels was measured using a glucometer with blood samples taken from veins in the rats' tail after the rats were fasted for 16 hours.

Histopathological evaluation of rat pancreas

Rats were generally anesthetized using ketamine at a dose of 35 mg/kg BW and euthanized. The pancreas was collected and fixed in a 10% Neutral Formalin Buffer (BNF) solution for at least 48 hours. The pancreatic histology preparation was made using Mayer's Hematoxylin-Eosin stain. The preparation was then observed using a microscope with 400x magnification.

Computerized analysis

The Computerized/In silico method further supported our findings by simulating how eugenol will interact with the proteins responsible for important

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cascades. To achieve this, Autodock Vina and Biovia Discovery Studios were used to aid us in simulating ligand and receptor docking. We first determine the active site of glucose on albumin by conducting Blind docking using Autodock Vina to determine the highest binding affinity of said substrate with its respective receptor, which would be human serum albumin. The glucose conformation with the highest affinity will be used as the active site. Then, eugenol is docked near the residues of the glucose conformation with the highest affinity. The results are then analyzed using Biovia Discovery Studio to determine what kind of interactions are involved between the ligand and the receptor.

Data collection and analysis

The data will be analyzed using SPSS 25.0 for Windows. The normality test was performed using the Shapiro-Wilk test. The data that met the requirements were then processed and analyzed quantitatively using bivariate one-way ANOVA analysis followed by posthoc analysis.

Results

GC-MS analysis result

In our study, from the GC-MS test, we found that clove leaf essential oil contains some compounds, such as n-Tridecan-1-ol, dimethyl ester, eugenol, ethoxy methyl, cryophilic acid, and phenol acetate. We found that the most abundant compound in clove leaf essential oil is eugenol, also known as caryophillic acid, with a rate of 66.5% of the total (Figure 1 and Table 1).

Comparison of blood sugar level (left) and histopathological result (right) between the sample groups. A: negative control (C-); B: positive control (C+); C: treatment 1 (T1); D: treatment 2 (T2). In our study, from the One-Way ANOVA test, it was found that clove leaf oil can significantly reduce fasting glucose levels (mg/dL) of diabetic rats in the treatment group (450.3±34.5 in the C+ group *vs*. 87.0±6.2 at T2 group; p=0.013). From the Post Hoc LSD test, we also found that there was a significant mean difference between the T1 and T2 group and the diabetic group (p<0.001).

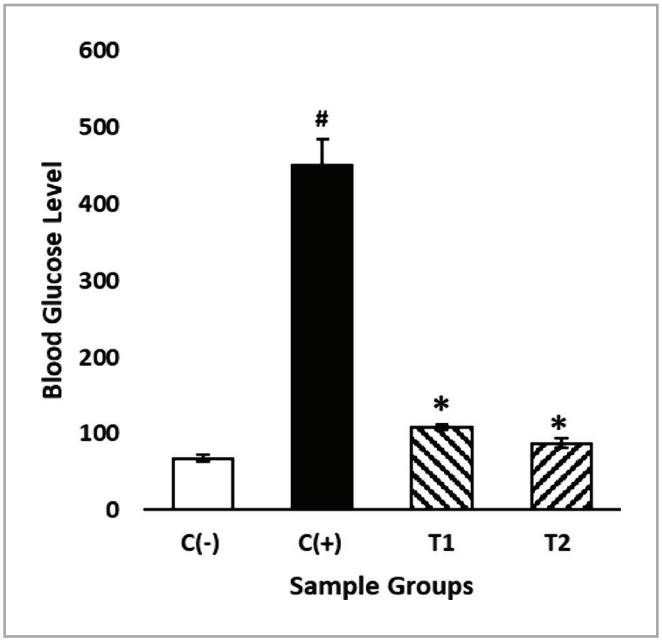
Computerized analysis result

Results of blind docking indicate glucose has a high tendency to dock with histidine146 and aspartate108 residues (-6.7 kcal/mol) through conventional hydrogen and carbon-hydrogen bond interactions (Figure 2 AB and Table 2).

Eugenol was then docked near the residues involved in glucose and albumin interactions. The resulting docking of eugenol and albumin showed π-cation interactions such as π-alkyl interactions between amino acid residues and the aromatic ring of eugenol, and some docking confirmations showed conventional

Table 1: GC-MS analysis result of clove leaves essential oil.

No	Name of compound	R time	m/z	Area	Percentage of total (%)
1	n-Tridecan-1-ol	11.671	200.00	527376	3.2
2	Dimetil Ester	13.759	272.00	524356	3.2
3	Dimetil Ester	14.035	272.00	466638	2.8
4	Eugenol	16.182	164.00	3278183	19.8
5	Ethoxy methyl	17.831	166.00	665960	4.0
6	Ethoxy methyl	17.902	166.00	675964	4.1
7	Ethoxy methyl	18.132	166.00	666960	4.0
8	Caryophilic acid	19.643	164.00	2318220	14.0
9	Caryophilic acid	20.623	164.00	2348819	14.2
10	Caryophilic acid	20.858	164.00	3070142	18.5
11	Phenol Acetate	21.100	206.00	1542300	9.3
12	Phenol Acetate	21.425	206.00	219662	1.3
13	Phenol Acetate	21.948	206.00	253428	1.5



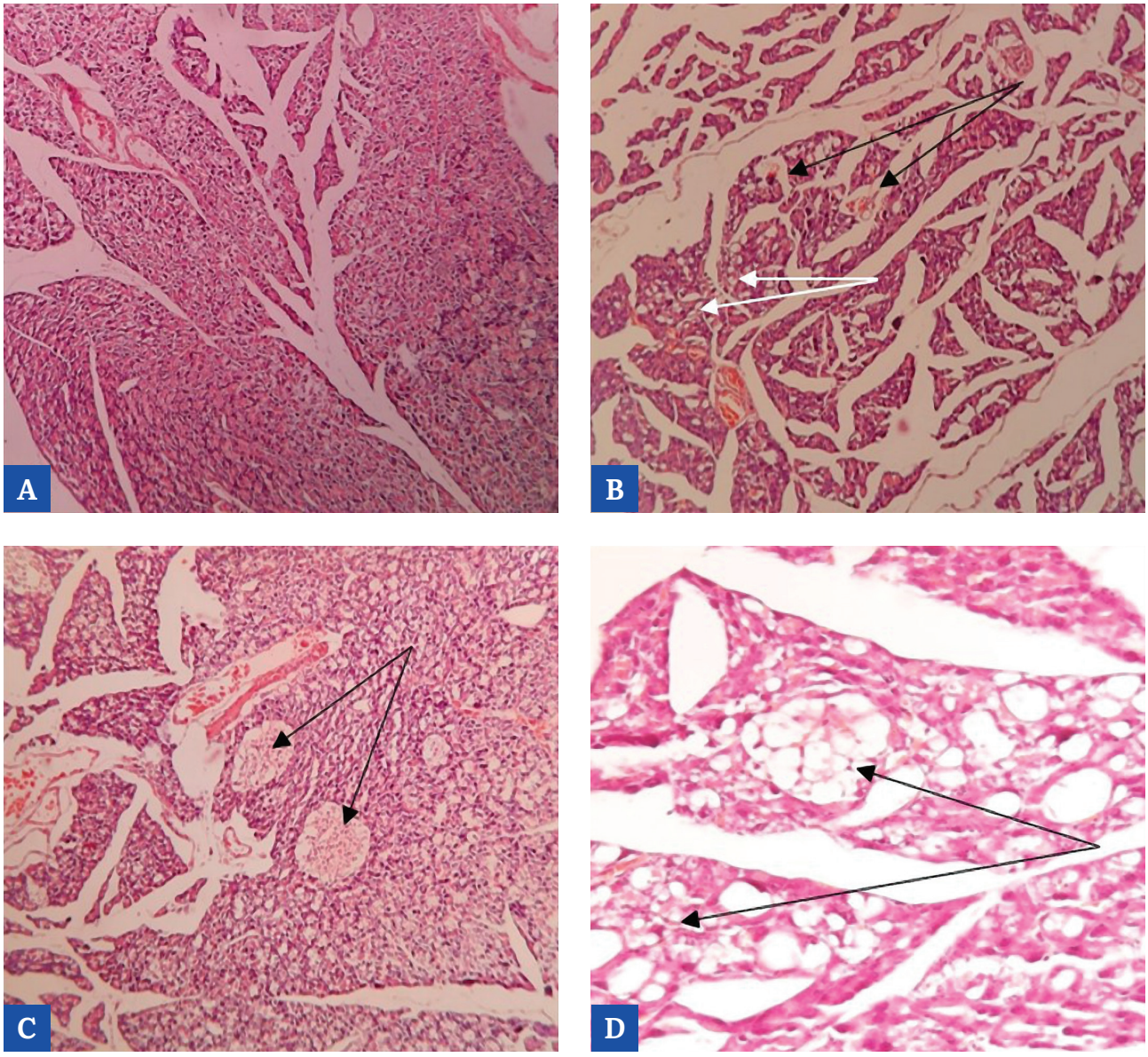


Figure 1: Blood glucose level and histopathological analysis.

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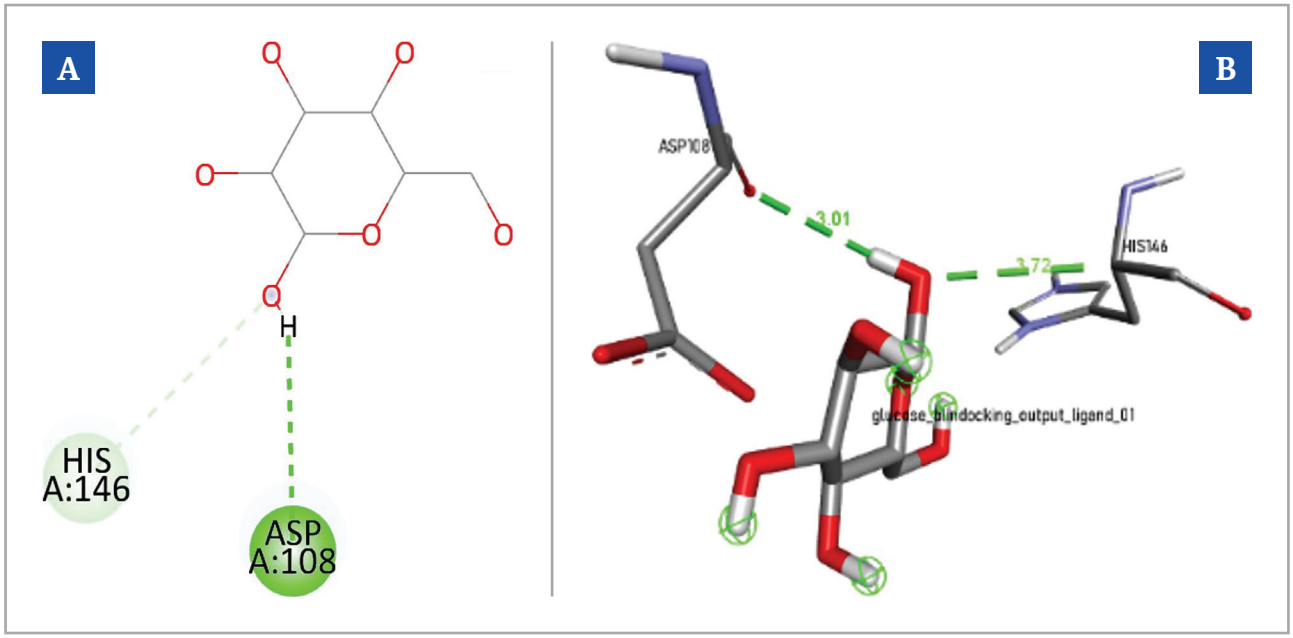

Figure 2: A – 2D; B – 3D (models representing hydrogen bond interactions between glucose and albumin).

Table 2: Glucose blind docking on human serum albumin results.

Mode	Affinity (kcal/mol)	Distance from the best mode	
		RMSD L.B.	RMSD U.B.
1	-6.7	0.000	0.000
2	-6.6	1.794	2.668
3	-6.4	26.957	27.982
4	-6.4	26.826	27.847
5	-6.3	10.391	11.252
6	-6.3	10.385	11.986
7	-6.3	26.748	27.621
8	-6.3	2.204	4.012
9	-6.2	19.674	21.488
10	-6.2	25.906	27.308
11	-6.0	18.479	19.597
12	-6.0	1.682	3.905
13	-6.0	18.686	19.679
14	-6.0	9.216	10.759
15	-5.9	11.034	12.708
16	-5.9	1.787	3.579
17	-5.9	17.772	19.002
18	-5.9	9.694	10.914
19	-5.9	10.658	11.863
20	-5.8	10.304	12.202

hydrogen bonds and carbon-hydrogen bonds accompanied with previously mentioned π-alkyl interaction (Figure 3 A–D and Figure 4 A–D). When compared to glucose, eugenol's affinity briefly showed a slightly lower affinity towards albumin near the same residues (Table 3). However, since affinity is measured in bonding energy divided by molecules involved within the interaction, this creates a less ideal representation of the real interaction. Glucose itself only interacts with 2 residues compared to eugenol, which interacts with 4 residues, due to the disparity in bonding energies between hydrogen bond and π-alkyl interactions along with fewer residues involved within the glucose and albumin interaction creates a higher affinity compared to eugenol and albumin interactions. Nevertheless, the overall interaction of eugenol results in a more stable docking compared to glucose.

Discussion

Previous studies, such as research by Al-Trad *et al.* (2019) and Srinivasan *et al.* (2014), also found similar results that the administration of 10 mg/kg eugenol could significantly reduce blood glucose levels ($p<0.05$) [18, 19]. This can occur through three mechanisms. The first mechanism, eugenol, can inhibit the activity of alpha-amylase and alpha-glucosidase enzymes that have a role in carbohydrate metabolism. In the small intestine, the alpha-amylase enzyme from the pancreas is involved in the breakdown of long-chain

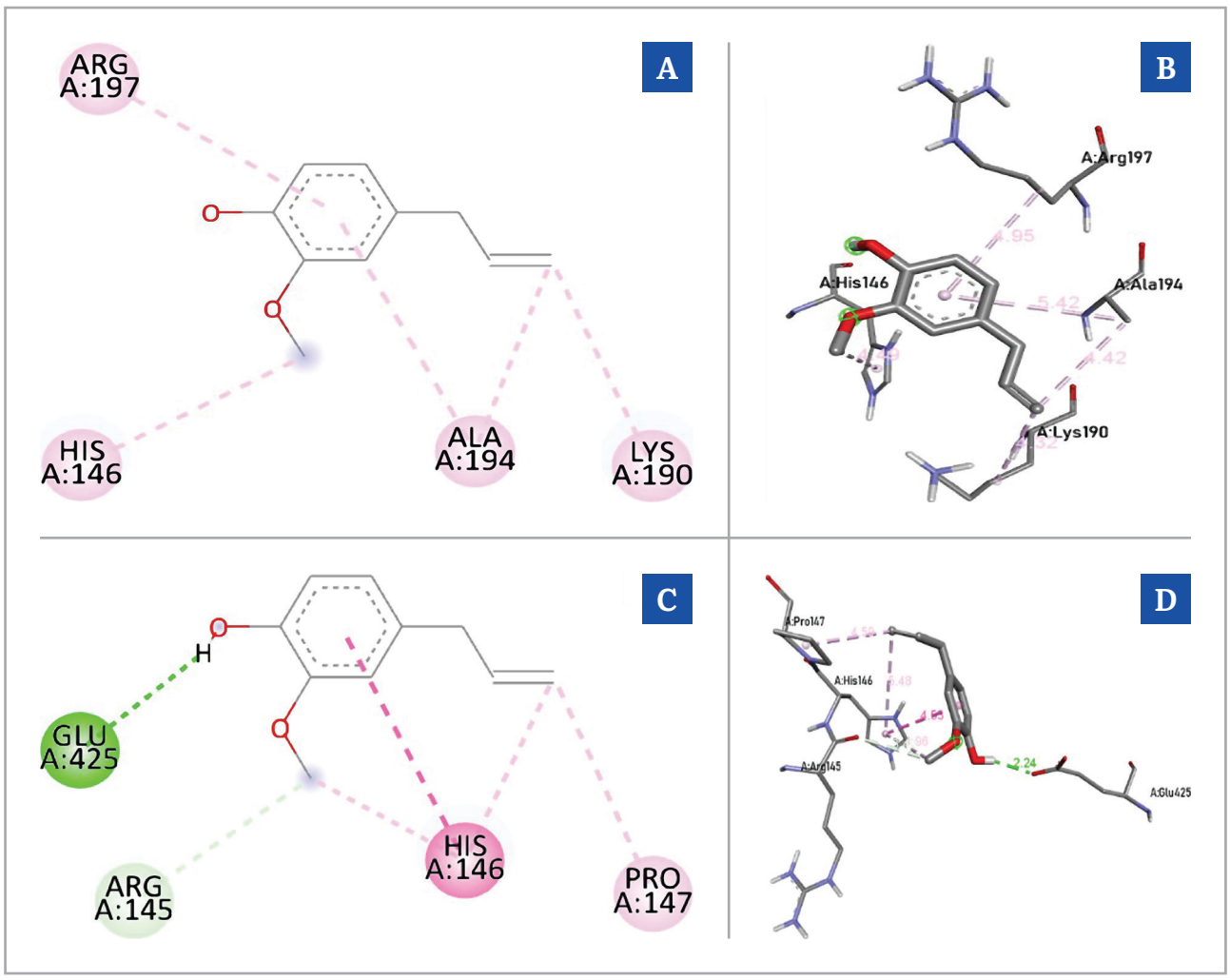

Figure 3: A – 2D; B – 3D (models representing eugenol and albumin interactions without hydrogen bonds); C – 2D; D – 3D (models representing eugenol and albumin interactions with hydrogen bonds).

carbohydrates, while alpha-glucosidase converts oligosaccharides into monosaccharide glucose. Enterocyte cells then uptake glucose via sodium-glucose cotransporter 1 [20]. Eugenol inhibits glucose uptake by intestinal cells, reducing postprandial hyperglycemia [21]. This inhibition occurs due to the ability of the eugenol hydroxyl group to interact with enzymes and delay carbohydrate absorption. This mechanism of inhibition

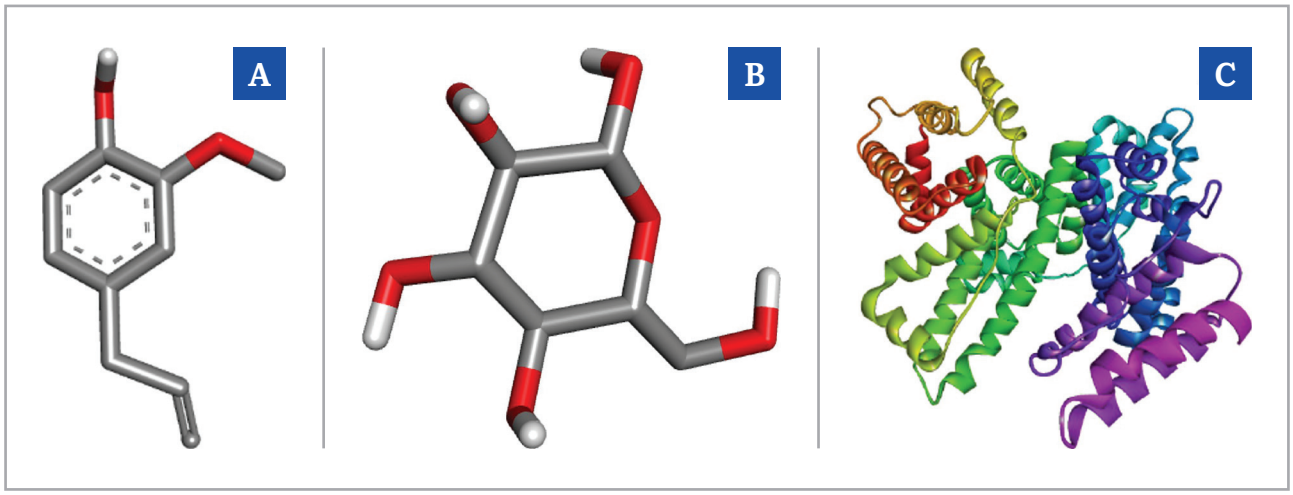

Figure 4: A – 3D model of eugenol; B – glucose; C – albumin. Used in docking simulations.

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Table 3: Eugenol docking on human serum albumin results.

Mode	Affinity (kcal/mol)	Distance from the best mode	
		RMSD L.B.	RMSD U.B.
1	-5.3	0.000	0.000
2	-5.0	2.232	4.482
3	-4.9	1.930	4.035
4	-4.8	2.145	4.083
5	-4.8	1.293	2.516
6	-4.7	1.988	4.013
7	-4.6	1.822	3.964
8	-4.6	2.307	4.407
9	-4.4	10.863	11.978
10	-4.4	2.079	3.952
11	-4.3	1.771	4.210
12	-4.2	1.569	2.246
13	-4.2	10.745	11.840
14	-4.1	2.119	3.033
15	-4.1	1.715	3.449
16	-4.0	1.670	2.556
17	-4.0	3.132	4.503
18	-4.0	2.753	4.131
19	-3.7	3.111	4.320
20	-3.7	1.507	3.368

of carbohydrate metabolism has been proven in various studies, such as research by Mnafgui *et al.* (2013), who found that eugenol could inhibit alpha-amylase enzyme up to 40% in the pancreas and 43% in the small intestine. The results of a study from Dompeipen (2017) showed that there was inhibition of the alpha-glucosidase enzyme by eugenol, and an in-silico study by Stevens & Allred (2022) also found that there was a strong binding affinity between eugenol and the active site of the alpha-glucosidase enzyme [20, 22, 23].

In the second mechanism, eugenol lowers glucose levels by activating the Ca+2calmodulin-dependent protein kinase (CAMKK) and AMP-activated protein kinase (AMPK) pathways, thereby inhibiting glucose production in the liver, suppressing the activation of the CREB-regulated protein complex. Transcription coactivator 2 – cAMP-response element-binding (CRTC2-CREB) and decreasing expression of gluconeogenic enzymes [24]. For the third mechanism, eugenol can also stimulate glucose uptake by increasing the translocation of glucose transporter-4 (GLUT4) from intracellular to the plasma membrane in skeletal muscle cells by AMPK phosphorylation. GLUT4 is a major protein involved in insulin-dependent glucose uptake. If insulin resistance occurs, glucose uptake in muscle cells will be disrupted and cause an increase in blood glucose levels [20]. According to Al-Trad *et al.* (2019), AMPK and GLUT4 protein levels in skeletal muscle tissue were significantly increased after eugenol administration compared to the diabetic group [18].

Substances that are rich in eugenol compounds, such as essential oil from clove leaves (*Syzygium aromaticum*), can repair pancreatic tissue. This has previously been observed in research by Singh *et al.* (2016). In this study, the administration of eugenol from plants of the genus *Ocimum* was able to improve the histopathological picture of several organs in rats induced with streptozotocin (STZ)-induced diabetes rats. Organs undergoing repair include the spleen, liver, heart, lung, kidney, pancreas, and brain, which were observed to have decreased necrosis and increased tissue integrity [25]. The study by Hamdin *et al.* (2019) also observed improvements in tissue histopathological results. This study used eugenol isolates from clove leaves to improve pancreatic and liver tissue in alloxan-induced diabetic rats, which was observed to increase the percentage of organ index significantly ($p<0.05$) [26].

The antioxidant activity of eugenol compounds can cause improvement of these organs. A study by Oroojan *et al.* (2020) reported the potential of eugenol in improving oxidative stress conditions in H_2O_2-induced oxidative stress and lipid peroxidation in the islets of Langerhans. This is represented by raising the level of Total Antioxidant Capacity (TAC) and catalase activity (CAT) while reducing malondialdehyde (MDA) significantly ($p<0.05$) [27]. Reducing oxidative stress will reduce necrosis and tissue atrophy in the pancreas, especially the islets of Langerhans. This will increase insulin secretion so that it reduces blood sugar and improves the condition of diabetes mellitus [28]. Moreover, the eugenol compound was found able to inhibit the interaction between glucose and albumin to form glycated albumin (GA) and Glycation End Products [29]. The high level of glucose that reacts with the site of albumin will produce fructosamine residue, reversible unstable Schiff base, irreversible conjugates (AGEs), and irreversible Amadori compound that is involved in ROS production and damage the cell, thus be able to develop into further organic and vascular complication on diabetes patients [30].

Conclusion

Our study suggests that clove leaf essential oil contains a high amount of eugenol content. The *in vivo* examination shows that clove leaf essential oil significantly decreases blood glucose levels in the treatment (T) groups compared to the positive control (C+) group. This is also supported by this essential oil's ability to repair pancreatic cell damage shown in histopathological examination. Moreover, the computerized analysis shows that the eugenol compound in clove leaf essential oil has higher energy binding with albumin than glucose with albumin, thus preventing the formation of glycated albumin. Further study is needed to determine the glycated albumin level in the sample groups.

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Conflict of interest

The authors declare no conflict of interest.

Ethics approval

The approval for this study was obtained from the Ethics and Research Committee, Faculty of Medicine, Udayana University - Prof. I G.N.G. Ngoerah, Denpasar Central Hospital (approval ID: 2021.01.1.0899).

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