Full Length Research Article

Antihyperglycemic Potency of Tulsi (Ocimum tenuiflorum) Ethanolic Extracts in Diabetic Mice Induced by Multiple Low Dose Streptozotocin

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ABSTRACT

Background : Diabetes Mellitus (DM) is one of the most challenging health problems today. Diabetes mellitus is a chronic metabolic disease characterized by high blood glucose concentrations due to impaired insulin production, function, or both. The standard antidiabetic treatment is unable to control blood sugar and stop the oxidative stress process in diabetics. This research aims to investigate the antihyperglycemic potency of Tulsi, the sacred plants of Ayurveda, in diabetic mice induced by multiple low dose streptozotocin.

Methods : We used eighteen male, healthy, white Swiss mice, aged 2-3 months, with 20-30 grams body weight to be included in this procedure, then, induced by low dose streptozotocin 40 mg/kg intraperitoneally for 5 days to make them become diabetic mice (fasting blood glucose level more than 150 mg/dL). Eighteen diabetic mice divided into three groups as follows: control group didn't receive any treatment, two treatment groups that received Tulsi extract macerated with 70% ethanol at doses: 56 mg/kg and 112 mg/kg orally for 14 days. Paired t-test were used to analyze the difference of blood glucose level before and after treatment and One-way Anova was used to analyze the difference of blood glucose level between groups.

Results: Multiple low dose streptozotocin were successfully increased blood glucose level of all mice without any death during the procedure. Administration of tulsi extract at doses of 56 mg/ kg and 112 mg/ kg succeeded in significantly reducing blood glucose levels in diabetic mice (P value <0.05). Among the 3 groups, the lowest blood glucose level after treatment was in the group of mice that received Tulsi ethanolic extract at a dose of 112 mg/kg. The difference in blood glucose level values after this treatment was significant according to the One Way Anova test (P value <0.05).

Conclusion: This study demonstrates that the ethanolic extract of Tulsi (*Ocimum tenuiflorum*), when administered at doses of 56 mg/kg and 112 mg/kg, significantly lowers blood glucose levels in mice with STZ- induced diabetes. This antihyperglycemic activity is likely due to the antioxidant properties of the various active compounds in Tulsi. These findings support the development of Tulsi as a natural alternative pharmacotherapy in managing diabetes mellitus.

INTRODUCTION

Diabetes Mellitus (DM) constitutes a major health concern in modern society. It is recognized as the fourth leading cause of mortality in numerous high-income and developing nations. The World Health Organization has issued a cautionary statement indicating that the prevalence of diabetes is expected to rise sharply each year. Currently, it is estimated that around 382 million individuals globally, accounting for 8.3% of the adult population, are living with diabetes [1]. If this trend continues, it is anticipated that by 2035, around 592 million people, or one in ten adults, will be impacted by diabetes [2]. Indonesia ranks 7th in the world with 10.7 million DM cases. By 2045, the estimated number of those with DM in Indonesia may reach 16.6 million, with potential healthcare costs around 760.3 billion USD [3].

Diabetes Mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from inadequate insulin secretion, impaired insulin action, or a combination of both [4]. This sustained elevation in blood glucose leads to progressive damage to multiple organ systems, including the heart, blood vessels, eyes, kidneys, liver, and nerves [5]. Cellular damage related to DM occurs via multiple mechanisms, among which enhanced oxidative stress that targets glycolytic enzymes is dominant. Moreover, the buildup of AGEs promotes activation of the NF-KB signaling pathway. This activation not only reduces diacylglycerol-protein kinase C pathway but also decreases nitric oxide, which also increases cellular dysfunction [6]. DM condition-induced oxidative stress also causes lipid peroxidation resulting in elevated vascular permeability and inflammation [7]. Oxidative stress is a condition characterized by high levels of Reactive Oxygen Species (ROS) that may arise from either excessive ROS production or incapacity of endogenous antioxidants to detoxify them. To safeguard cells from oxidative stress, the activity of endogenous antioxidants is essential [8].

Metformin continues to be regarded as the primary treatment option for managing type 2 diabetes [9]. However, with adequate administration of metformin, it turns out that it is unable to control blood sugar and stop the oxidative stress process, so the process of forming micro and macrovascular complications in DM continues to occur. For this reason, the problem-solving approach needed is through the discovery of new therapies or adjuvant combinations made from natural ingredients that are able to work alone or synergistically in lowering blood sugar levels and reducing DM complications that arise due to oxidative stress.

Tulsi or Tulasi (*Ocimum tenuiflorum*), belonging to the Lamiaceae family, is a sacred plant in India that is often used in Ayurveda medicine to treat colds, stomach aches, skin aches, smallpox, or diarrhea. A GC-MS analysis of Tulsi extract, using ethanol as a solvent, revealed the presence of several phytochemical compounds, including ursolic acid, carvacrol, and eugenol. These bioactive components are known for their therapeutic properties, contributing to the medicinal efficacy of the plant [10]. Previous study revealed that Tulsi ethanolic extract had very strong antioxidant activity with an IC_{50} of 12.28 ppm that measured by DPPH method [11]. Tulsi extract, given at a dosage of 400 mg/kg of body weight, exhibited a significant decrease in blood glucose levels in rats induced with Alloxan [12]. The impact of Tulsi ethanolic extract on diabetic mice induced by streptozotocin (STZ) has not yet been investigated. Taking into consideration all above, a study was undertaken to evaluate antihyperglycemic activity of ethanolic extract of Tulsi (*Ocimum tenuiflorum*) using mice as different animal model and repeated low-dose streptozotocin as different diabetic induction method.

METHODS

Preparation of Tulsi Ethanolic Extract

The specimen used was Tulsi (*Ocimum tenuiflorum*) which was taken from the herbal garden in the central area of Denpasar city that had been established in previous studies [13]. That extraction process was done at the Agricultural Laboratory, Warmadewa University. The materials were leaves, stems, and flowers. The dried weight measured 1000 grams, and the samples were dried in an oven at a temperature of 50-60°C until the weight became stable. This mass was then ground with a blender and macerated with ethanol 70% in a 1:5 ratio, covered with aluminum foil. Three maceration (3x24 hours) steps followed by another remaceration at 24 hours intervals until a clear colour was obtained in the filtrate. Then, the filtrate was concentrated to its boiling point using a vacuum rotary evaporator at 40 °C or higher, which produced a paste-like crude extract [11,13].

Experimental Animals

The experimental animals in this study were white mice (*Mus musculus* L) Swiss strain, with inclusion criteria as follows: male mice, 2-3 months old, body weight 20-30 grams, healthy (mice move actively), and fasting blood glucose levels after STZ induction are 150 mg/ dl. Mice exhibiting anatomical abnormalities and those that perished during the acclimatization process were excluded. The sample size of the mice utilized in this research was determined using the degrees of freedom (DF) formula due to insufficient data on effect size or variance for a reliable power analysis. This approach ensures that the degrees of freedom (E- value) fall within an acceptable range (10-20) for valid ANOVA-based statistical analysis [14]. Because the number of research groups was set at 3, the results obtained were that the minimum number of mice used was 5 per group for a total of 15 mice. Considering a drop out of 20%, the total number of mice used was 18.

Preparation of Experimental Animals

Prior to receiving treatment, the experimental subjects, specifically male mice aged 2 to 3 months, underwent a 7- day acclimatization period in the Animal Laboratory at Warmadewa University. All mice were housed in containers or cages covered with wire on top and covered with husks, which were replaced every 3 days. The animals were also given standard food and drink at will. The animal cages were in a room that was free from noise. Cleanliness was maintained with humidity (± 50%), 25oC temperature, adequate ventilation, and 12- hour light/ dark cycle [15]. Following the adaptation phase, the mice underwent a fasting period of 10 hours, during which they were permitted to have access to drinking water. Afterward, their body weight was recorded, and their baseline blood glucose levels were assessed.

Animals Grouping

Eighteen mice that satisfied the inclusion and exclusion criteria were randomly divided into three groups, each consisting of six mice. The three groups were as follows:

Control group (C): mice induced by STZ that received distilled water (aquadest) as the solvent used for dissolving the Tulsi extract, administered orally once daily for 14 days.

Treatment group 1 (T1): mice induced by STZ that were administered 56 mg/kg of Tulsi extract for 14 days

Treatment 2 group (T2): STZ-induced mice received 112 mg/kg of Tulsi extract for 14 days

Dose determination: The study by Parasuraman et al. (2015), where it was mentioned that the dosage of Tulsi extract was 200 mg/ kg and 400 mg/ kg for showing antidiabetic potential in rats, with higher dose (400 mg/ kg) producing a more significant reduction in blood glucose levels [16]. We did not use body surface area-based interspecies scaling. The doses of 56 mg/ kg and 112 mg/kg were selected based on preliminary study consideration.

Diabetes Mellitus Induction

STZ was administered by intraperitoneal injection at a low dosage of 40 mg/kg to each mouse in the above groups. This mixture was made using a sodium citrate buffer (pH 4.5), and the final volume of 0.5 ml was delivered on five consecutive days [17]. Fasting blood glucose level were determined after multiple administration of STZ. Mice that displayed fasting blood glucose levels below 150 mg/ dL following the repeated STZ administrations were excluded from the study.

Blood Glucose Measurement

Blood glucose levels in the mice were assessed following five days of STZ induction and subsequent administration of Tulsi ethanolic extract for 14 days [18]. The measurements of blood glucose were performed utilizing a Nesco Glucose Test, with blood samples obtained from the tail vein.

Data Analysis

Blood glucose levels were treated as a quantitative variable and presented as the mean ± standard deviation. In instances where the data deviated from a normal distribution, median values (minimum-maximum) were provided instead. Given that the sample size in this study was ≤50, the Shapiro-Wilk test was employed to assess normality, utilizing 95% confidence interval, α = 5%. Data were considered to follow a normal distribution when the significance value (p) > 0.05. To assess the changes in blood glucose levels before and after the administration of Tulsi ethanolic extract within each group, a paired t-test was conducted for statistical evaluation. Furthermore, a one-way ANOVA was applied to compare the treatment effects among various groups, with a significance threshold established at $p < 0.05$.

Ethical Clearance

This study was conducted after obtaining ethical approval from the Ethics Committee of the Faculty of Medicine and Health Sciences, University of Warmadewa, approval number 67/Unwar/FKIK/EC-KEPK/VII/2024.

RESULTS

Eighteen healthy male white Swiss mice, aged 2 to 3 months and weighing between 20 to 30 grams, received an intraperitoneal injection of streptozotocin (STZ) at a dosage of 40 mg/kg for five consecutive days. On the sixth day, their fasting blood glucose levels were measured. After the administration of streptozotocin, all mice displayed fasting blood glucose levels of 150 mg/dL or greater, as shown in Figure 1. The DM induction technique employing repeated low doses of streptozotocin successfully increased blood glucose levels in all mice without causing any deaths. Following this, eighteen diabetic mice were divided into three groups: the control group (C), treatment group 1 (T1), and treatment group 2 (T2), with each group comprising six mice housed collectively in one cage.

The study involved the administration of an ethanolic extract of Tulsi, with treatment group 1 receiving a dosage of 56 mg/kg and treatment group 2 receiving 112 mg/kg. In contrast, the control group did not receive Tulsi extract, but only administered with aquadest. The extract was administered orally through oral gavage each morning for 14 days. Following this treatment period, blood glucose levels were evaluated by collecting blood samples from the tail vein of all groups. The findings are illustrated in Figure 2.

As indicated in Table 1, the control group, demonstrated an increase in average blood glucose levels, rising by 20.33 mg/dL from 214.33 mg/dL to 234.66 mg/dL, with a p-value of 0.02 as determined by the paired t-test. In contrast, treatment group 1, which received 56 mg/kg of Tulsi ethanolic extract for 14 days, demonstrated a significant reduction in average blood glucose levels, decreasing by 47.83 mg/dL from 208.16 mg/dL to 160.33 mg/dL. Similarly, treatment group 2, which was given 112 mg/kg of Tulsi ethanolic extract over the same duration, exhibited a marked decline in average blood glucose levels, falling by 82.33 mg/dL from an initial measurement of 212.67 mg/dL to a concluding level of 130.33 mg/dL. The decrease in average blood glucose levels observed in both treatment groups was statistically significant, with a p-value of less than 0.01, as established by the paired t-test. Among the three groups compared, treatment group 2 demonstrated the lowest final blood glucose level, measured at 130.33 mg/dL, when compared to the other groups. A statistical analysis of the blood glucose levels among the three groups was conducted using one-way ANOVA, given that the data followed a normal distribution. The findings from the ANOVA test indicated a p-value of less than 0.01, highlighting a statistically significant difference in the final blood glucose levels among the groups.

Figures

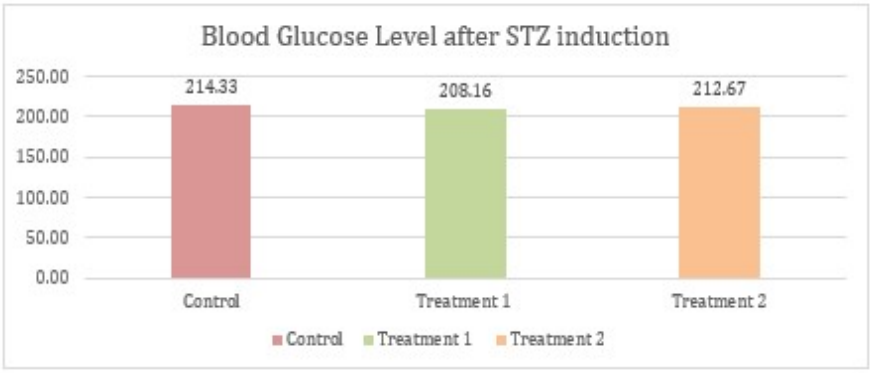

Figure 1: Blood Glucose Level After STZ induction (Initial Blood Glucose). The average blood glucose levels after STZ induction (initial blood glucose levels) were as follows: control group (214.33 mg/dl), treatment 1 group (208.16 mg/dl), and treatment 2 group (212.67 mg/dl)

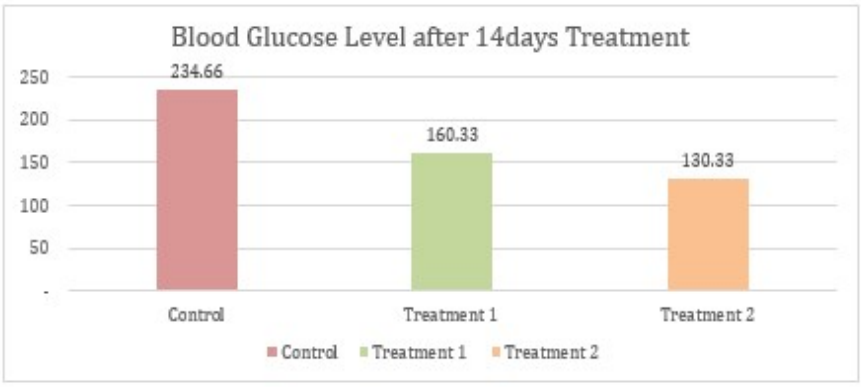

Figure 2: Blood Glucose Level After 14 Days Treatment (Final Blood Glucose). The average blood glucose levels after 14 days treatment (Final Blood Glucose) were as follows: control group (234.66 mg/dl), treatment 1 group (160.33 mg/dl), and treatment 2 group (130.33 mg/dl).

Tables

No	Animals Group	Initial Blood Glucose	Final Blood Glucose	P value*
1	Control	214.33 ± 10.46	234.66 ± 12.80	0.02
2	Treatment 1 (56 mg/kg)	208.16 ± 7.78	160.33 ± 7.78	<0.01
3	Treatment 2 (112 mg/kg)	212.67 ± 12.83	130.33 ± 4.84	<0.01

Table 1: Comparison of Initial Blood Glucose and Final Blood Glucose. Values are presented as mean ± SD (mg/dL). Within-group comparisons were analyzed using paired t-test. p < 0.01 indicates statistical significance.

DISCUSSION

Multiple Low Dose Streptozotocin: A Reliable Approach for Diabetes Induction in Animal Models

Streptozotocin (STZ) and alloxan are widely utilized compounds for the induction of Diabetes Mellitus (DM) in laboratory animals. STZ, a natural substance derived from the *bacterium Streptomyces achromogenes*, possesses extensive antibacterial characteristics. Originally, STZ was employed as a chemotherapeutic agent for the treatment of metastatic pancreatic cancer and other types of cancer. However, in 1963, its diabetogenic effects were identified, which subsequently led to its application as a means to induce DM in experimental subjects. STZ is effective in inducing DM across a range of animal models, including rats, mice, monkeys, hamsters, rabbits, and guinea pigs. The cytotoxic impact of STZ on pancreatic beta cells is observable within 72 hours following administration, depending on the dosage. The toxicity begins when STZ is taken up by cells via the low-affinity glucose transporter 2 (GLUT2), which is present in the plasma membranes of beta cells, hepatocytes, and renal tubular cells. Research involving insulin-secreting cells that lack GLUT2 expression has demonstrated a resistance to the induction of STZ [19-21]. The toxicity of STZ exhibits a greater selectivity for pancreatic beta cells, attributed to its glucose component, which enhances its uptake by these cells that absorb glucose more efficiently than other cell types [21,22].

The impairment of pancreatic beta cells due to STZ is a result of the initiation of oxidative stress and subsequent DNA damage. Upon entering beta cells, STZ induces DNA damage. This occurs due to the activation of STZ alkylation, which targets DNA and alters nucleotide bases, particularly guanine, thereby compromising the structure and functionality of DNA. In addition to causing direct harm to DNA, STZ promotes the production of free radicals, specifically reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide. This results in oxidative stress within beta cells. The oxidative stress further intensifies cellular damage through mechanisms such as lipid peroxidation, protein impairment, and mitochondrial dysfunction. Furthermore, STZ activates poly ADP-ribose polymerase (PARP) in response to the DNA damage incurred. Activation of PARP causes rapid consumption of NAD+, a molecule essential for cellular respiration and cell energy. NAD+ depletion causes a decrease in ATP which leads to cellular dysfunction and cell death [21,23]. Significant DNA damage and oxidative stress resulting from STZ lead to apoptosis in pancreatic beta cells. This process triggers the intrinsic apoptotic pathway, and cytochrome c then leaks out of the damaged mitochondria into the cytosol. This release then activates caspase-9 and caspase-3, leading to DNA degradation and cell death [23,24].

STZ is often used in two basic protocols to induce diabetes in animal models. First, low doses of STZ (<60 mg/ kg) are administered as repeated injections on a daily basis for five consecutive days. While the second protocol is a more intensive protocol, where a single high dose of STZ (200 mg/kg) is given [25]. In this study, the protocol used was repeated injection of low dose STZ 40 mg/kg for 5 days. Multiple low doses of STZ specifically and slowly destroy pancreatic beta cells, allowing the body of the mice a chance to respond to the damage caused by STZ. High doses of STZ (200 mg/kg) leads in general to the rapid and massive destruction of beta cells, and the fast evolution of acute type 1 diabetes. Moreover, higher doses of STZ can lead to increased mortality in mice, partly due to nocturnal hypoglycemia. This hypoglycemia can happen due to extensive and significant destruction of pancreatic beta cells. These effects include the massive release of insulin (from damaged Beta cells) after the first few hours of STZ administration leading to considerable hypoglycemia. Apart from that, in mice, which are nocturnal animals, metabolic activity at night tends to be higher. When this happens, there is sudden decrease in insulin secretion due to pancreatic β-cell injury, resulting in unregulated glucose levels in the body which ultimately leads to hypoglycemia [25,26]. In this study, to induce the diabetes model, STZ was administered at a low dose concentration of 40 mg/kg for 5 times. This approach successfully resulted in elevation of blood glucose in all mice (n = 18 mice) without causing mortality.

Antihyperglycemic Potency of Tulsi Ethanolic Extract

In this study, the plant investigated was Tulsi (*Ocimum tenuiflorum*) or Tulasi and taxonomically synonymous with *Ocimum sanctum*. Tulsi (*Ocimum tenuiflorum*) is an aromatic shrub in the Lamiaceae family. *Ocimum tenuiflorum* is not the only type of Tulsi: there are about 160 species of tulsi found throughout the tropics. Other types of Tulsi having medical benefits are *Ocimum sanctum, Ocimum gratissimum, Ocimum canum, Ocimum basilicum, Ocimum americanum, Ocimum camphora,* and *Ocimum miranthum* [27]. This plant is highly revered in Indian Hinduism or Ayurveda belief and is extensively used for health purpose, such as bronchial, gastrointestinal and chronic diseases [28].

This study was able to show that ethanolic extract of Tulsi (*Ocimum tenuiflorum*) has antihyperglycemic action. It is able to decrease blood glucose levels at the doses of 56 mg/kg and 112 mg/kg in multiple low dose STZ-induced diabetic mice. In contrast, the untreated diabetic control group showed a modest increase in blood glucose levels over the 14-day observation period, that may reflect the progressive nature of STZ-induced hyperglycemia. This finding is in agreement with several past studies. Previous study by Rao et al (2013) stated that ethanol extract of Tulsi at a dose of 400 mg/kg can show an antidiabetic effect on Alloxan-induced diabetic rats [12]. Other study from Parasuraman et al (2015) also suggested that hydroalcoholic Tulsi extract showed significant antidiabetic effects at doses of 200 mg/kg and 400 mg/kg in streptozotocin and nicotinamide-induced diabetic rats. Those research also noted that hydroalcoholic Tulsi extract did not result in mortality up to a dose of 2000 mg/kg when administered as a single dose [16].

Tulsi has various health effects, including antidiabetic, that arise from plant's metabolite content of the Ocimum genus, such as camphor, methyleugenol, eugenol, limonene, thymol, myrcene, cineole, and caryophyllene [28]. A GC-MS test in 2023 succeeded in identifying 6 types of secondary metabolites in Tulsi ethanolic extract with peaks above 60%. Meanwhile with chloroform solvent, 12 types of secondary metabolites were obtained with different concentrations [13]. Research by Eswar et al (2016) stated that *Ocimum tenuiflorum* leaf extract contains mostly eugenol (71%) and the remaining 20% consists of methyl eugenol, carvacrol, and sequisterpene hydrocarbon caryophyllene [29]. Tulsi ethanolic extract is also known to have very strong antioxidant activity according to the Blois classification with an IC_{50} value of 12.28 ppm [11], so that the ethanolic extract of Tulsi is suitable for development as a pharmacotherapeutic agent for diseases that arise due to oxidative stress, such as diabetes.

Eugenol is recognized as the most potent antihyperglycemic compound among the antioxidants present in Tulsi. Chemically identified as 1-hydroxy-2-methoxy-4-allylbenzene, eugenol is a phenolic compound found in the essential oils of various Ocimum species. The greatest concentration of eugenol is found in *Ocimum tenuiflorum* , which makes up 84%, followed by *Ocimum gratissimum* , which has a concentration of 74.80% [30]. In vitro research has demonstrated that eugenol possesses the ability to inhibit the formation of advanced glycation end products (AGEs). It is noted that eugenol exhibits a significant affinity for the ε-amine group on lysine, which contributes to its protective effects against glycation processes [31]. Research conducted in vivo has shown that eugenol can reduce blood glucose levels by 38% by inhibiting α-glucosidase activity [30].

In-silico testing of bioactive compounds in Tulsi leaves, such as kaempferol, apigenin, and luteolin, suggests that their antidiabetic pathways resemble those of metformin, particularly the PI3K/AKT pathway. These compounds protect cells from lipotoxicity by inhibiting the PI3K/AKT pathway. PI3K/ AKT activation also promotes insulin secretion from pancreatic cells. Overexpression and activation of AKT in pancreatic cells lead to increased cell mass, proliferation, and size, mediated by AKT signaling intermediaries such as Fox01, GSK3, and mTOR1. Fox01 is highly expressed in pancreatic cells and promotes apoptosis induced by fatty acids [32,33]. Apigenin found in Tulsi leaves also engages with insulin receptor 2, a mechanism that is indirectly supported by IGF-1 (Insulin-like growth factor 1). IGF-1 regulates various biological functions through tyrosine kinase homologs that phosphorylate insulin receptor substrate (IRS) proteins. Upon ligand binding, activated IGF-1 recruits IRS proteins, phosphorylating several tyrosine residues and activating the IP3-kinase and MAP kinase pathways. IRS-2 plays a crucial role in normal nutrient homeostasis by mediating peripheral insulin action and IGF-1 effects on cell growth [34].

This study demonstrates that Tulsi (*Ocimum tenuiflorum*) ethanolic extract at doses of 56 mg/kg and 112 mg/kg effectively lowers blood glucose levels in mice with STZ-induced diabetes. This antihyperglycemic activity is likely due to the antioxidant properties of the various active compounds in Tulsi. The results of this study endorse the advancement of Tulsi as a natural alternative treatment for the management of Diabetes Mellitus. Additional research is required to assess its effectiveness and safety in various animal models of diabetes, as well as in clinical trials involving human subjects. This development has the potential to offer a more affordable and safer treatment option for diabetes patients.

CONFLICT OF INTEREST

The authors affirm that there are no conflicts of interest associated with the publication of this

paper.

AUTHOR CONTRIBUTIONS

Pande Ayu Naya Kasih Permatananda conceived, designed, and conducted the study, including experimental procedures, data collection, statistical analysis, and manuscript preparation. I Wayan Sutirta Yasa, I Wayan Sumardika, and Made Ratna Saraswati contributed to development of research concept, study design, and critical analysis of the data. I Gde Suranaya Pandit and Sri Masyeni provided technical support during the laboratory process and contributed to data acquisition. All authors discussed the result, reviewed the manuscript, and approved the final version for submission.

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Generative AI Statement

The authors declare that Generative AI tools were used to enhance the language clarity of this work. We take full responsibility for the accuracy and integrity of the content.

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