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Dose-dependent effects of *Stevia rebaudiana* leaf extract on malondialdehyde and catalase activity in alloxan-induced hyperglycemic rats

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ABSTRACT

Background: Diabetes mellitus-induced hyperglycemia triggers oxidative stress, characterized by elevated malondialdehyde (MDA) and impaired catalase (CAT) activity. *Stevia rebaudiana*, rich in steviol glycosides and polyphenols, demonstrates promising antioxidant properties, yet systematic dose-response data on oxidative stress biomarkers remain limited.

Objectives: To evaluate the dose-dependent effects of *stevia* leaf extract on serum MDA levels and CAT activity in alloxan-induced hyperglycemic rats.

Methods: Twenty-five male Wistar rats were allocated into normal control, diabetic control (alloxan 120 mg/kg), and three treatment groups receiving alloxan plus stevia extract at 100, 200, or 400 mg/kg body weight orally for 14 days (n=5/group). Serum MDA and CAT were measured spectrophotometrically.

Results: Diabetic control showed significantly elevated MDA (2.68±0.62 mg/dL) versus normal control (1.78±0.30 mg/dL). Stevia extract dose-dependently reduced MDA: 1.70±0.19, 1.54±0.20, and 1.38±0.09 mg/dL at 100, 200, and 400 mg/kg, respectively, representing 36.6%, 42.5%, and 48.5% reduction. The 400 mg/kg dose achieved MDA levels comparable to normal control. CAT activity showed dose-dependent restoration trend (7.92±0.76 to 8.58±0.52 mg/dL).

Conclusion: Stevia leaf extract (400 mg/kg BW) effectively reduces oxidative stress in hyperglycemic rats through significant dose-dependent MDA reduction, with potential catalase benefits requiring further investigation.

Keywords: Stevia rebaudiana extract, lipid peroxidation biomarkers, antioxidant enzymes, alloxan-induced diabetes, oxidative stress modulation

Introduction

Diabetes mellitus (DM) represents a major global public health challenge characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The worldwide prevalence of diabetes continues to escalate at an alarming rate, with projections from the International Diabetes Federation (IDF) Atlas 2021 estimating that the global diabetic population will reach 783 million by 2045. Indonesia ranks fifth globally in diabetes burden, with prevalence expected to increase from 19.5 million to 28.5 million cases over the same period [1]. Persistent hyperglycemia drives the development of severe long-term complications, including diabetic retinopathy, nephropathy, neuropathy, and cardiovascular disease, which arise from progressive dysfunction of multiple organ systems [2].

The pathophysiological mechanisms linking chronic hyperglycemia to tissue damage are complex and multifactorial. In both Type 1 DM, characterized by absolute insulin deficiency due to autoimmune

pancreatic beta-cell destruction, and Type 2 DM, characterized by insulin resistance and relative insulin deficiency, sustained elevation of blood glucose triggers multiple biochemical pathways. These include non-enzymatic glucose autooxidation, advanced glycation end-product (AGE) formation, activation of the polyol pathway, and increased flux through the hexosamine pathway. Collectively, these processes accelerate the generation of reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals. When ROS production exceeds the neutralizing capacity of endogenous antioxidant defense systems, oxidative stress ensues, resulting in widespread damage to cellular macromolecules such as lipids, proteins, and nucleic acids [3].

Malondialdehyde (MDA) serves as a widely recognized and reliable biomarker of oxidative stress, representing a stable end product of lipid peroxidation. During oxidative stress, ROS attack polyunsaturated fatty acids within cellular membranes, initiating a self-propagating chain reaction that generates lipid peroxides and ultimately produces MDA. Elevated MDA concentrations in biological fluids reflect the extent of oxidative damage and are strongly associated with diabetic complications [4]. Conversely, endogenous antioxidant defense mechanisms play crucial roles in maintaining cellular redox homeostasis. Catalase (CAT), a key enzymatic antioxidant localized primarily in peroxisomes, catalyzes the rapid decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2), thereby preventing the formation of highly reactive hydroxyl radicals through Fenton chemistry. However, during prolonged hyperglycemia and severe oxidative stress, catalase activity may become compromised due to enzyme inactivation, depletion, or impaired synthesis, further exacerbating cellular damage [5].

Current therapeutic approaches to diabetes management rely predominantly on synthetic pharmacological agents, including oral hypoglycemic drugs and injectable insulin formulations. While these medications effectively control blood glucose levels, they are associated with various adverse effects, accessibility limitations, and high costs, particularly in resource-limited settings. Consequently, there has been growing interest in complementary and alternative therapies derived from medicinal plants, which offer potential advantages including improved safety profiles, affordability, and cultural acceptability. The emerging field of nutraceuticals has increasingly recognized the therapeutic potential of plant-based bioactive compounds in managing metabolic disorders [6].

Stevia rebaudiana Bertoni, commonly known as stevia, is a perennial herbaceous plant native to South America that has gained international recognition primarily as a natural non-caloric sweetener. Beyond its sweetening properties, which are attributed to steviol glycosides such as stevioside and rebaudioside A, stevia leaves contain a rich array of bioactive phytochemicals, including polyphenols, flavonoids, tannins, and phenolic acids. These compounds have demonstrated substantial antioxidant capacity through multiple mechanisms, including direct free radical scavenging via hydrogen atom or electron donation, metal chelation, and modulation of endogenous antioxidant enzyme expression. Previous studies have reported that stevia extract exhibits potent antioxidant activity comparable to synthetic antioxidants and can ameliorate oxidative stress-induced cellular damage. Additionally, steviol glycosides have been shown to exert antihyperglycemic effects by stimulating insulin secretion from pancreatic beta cells, enhancing insulin sensitivity in peripheral tissues, and inhibiting hepatic gluconeogenesis [7,3].

Despite accumulating evidence regarding the beneficial effects of stevia in glucose metabolism and oxidative stress modulation, systematic dose-response investigations evaluating its effects on specific oxidative stress biomarkers in experimental diabetes models remain limited. Understanding the relationship between stevia extract dosage and its capacity to modulate oxidative stress markers is essential for establishing optimal therapeutic regimens and elucidating mechanisms of action. Furthermore, comprehensive evaluation of both oxidative damage markers (MDA) and endogenous antioxidant defense capacity (catalase activity)

provides a more complete assessment of stevia's antioxidant efficacy.

Given the central role of oxidative stress in diabetic pathophysiology and the promising antioxidant and antihyperglycemic properties of stevia leaves, the present study was designed to evaluate the dose-dependent effects of stevia leaf extract on oxidative stress parameters in an alloxan-induced hyperglycemic rat model. Specifically, this investigation aimed to determine the effects of three graded doses of stevia extract (100, 200, and 400 mg/kgBW) on serum malondialdehyde levels, as a marker of lipid peroxidation and oxidative damage, and catalase enzyme activity, as an indicator of endogenous antioxidant defense capacity. The findings of this study may contribute to the scientific foundation for developing stevia-based therapeutic interventions for managing oxidative stress in diabetes mellitus.

Methods

Ethical approval

This study was conducted in accordance with the principles of laboratory animal care and approved by the Animal Ethics Committee of Faculty of Medicine, Universitas Andalas (Ethical Clearance No: 565/UN.16.2/KEP-FK/2025). All procedures followed the ARRIVE guidelines for reporting animal research.

Experimental design and animal subjects

A post-test only control group design with complete randomization was employed. Twenty-five healthy male Wistar rats (*Rattus norvegicus*, 8-12 weeks old, 200-250 g). Animals were housed in polycarbonate cages (maximum 5 rats/cage) under controlled conditions: 12-hour light/dark cycle, temperature 22±2°C, humidity 50-60%, with ad libitum access to standard pellet diet and water. Following a 7-day acclimatization period, rats were randomly allocated using computer-generated random numbers into five groups (n=5/group): (i) normal control (NC): received intraperitoneal (i.p.) saline injection and oral distilled water; (ii) diabetic Control (DC): received alloxan monohydrate (120 mg/kg BW i.p.) and oral distilled water; (iii) treatment 100 (T1): received alloxan (120 mg/kg BW i.p.) plus stevia extract 100 mg/kg BW orally; (iii) treatment 200 (T2): received alloxan (120 mg/kg BW i.p.) plus stevia extract 200 mg/kg orally; (v) treatment 400 (T3): Received alloxan (120 mg/kg BW i.p.) plus stevia extract 400 mg/kg BW orally.

Sample size calculation

Sample size was determined using the formula for comparing multiple groups with continuous outcomes, assuming α=0.05, power=80%, effect size f=0.60 based on preliminary data, yielding a minimum of 4 rats/group. To account for potential dropouts, 5 rats/group were used (total n=25).

Diabetes induction

After 12-hour overnight fasting with free access to water, hyperglycemia was induced by a single i.p. injection of freshly prepared alloxan monohydrate (Sigma-Aldrich, USA) dissolved in sterile 0.9% saline at 120 mg/kg body weight. To prevent fatal hypoglycemia, rats received 5% glucose solution for 24 hours post-injection. Fasting blood glucose (FBG) was measured 72 hours post-induction via tail vein puncture using a calibrated glucometer (Accu-Chek® Active, Roche, Germany). Rats with FBG ≥135 mg/dL were confirmed as hyperglycemic and included in the study. Animals failing to achieve hyperglycemia or showing signs of severe distress were excluded and replaced.

Preparation of stevia leaf extract

Fresh *Stevia rebaudiana* Bertoni leaves (40-60 days maturity) were harvested from Sungkai Ecotourism, Padang, Indonesia. Three kilograms of fresh leaves were washed with distilled water, air-dried at room temperature (avoiding direct sunlight) until 1 kg dry weight was obtained. Dried leaves were pulverized using a mechanical grinder (40-mesh sieve) and stored in airtight containers.

Extraction was performed using cold maceration method: 1 kg leaf powder was macerated in 10 L of

96% ethanol (1:10 w/v ratio) at room temperature for 48 hours with periodic shaking every 8 hours. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated using a rotary evaporator (Heidolph Laborota 4000, Germany) at 40°C under reduced pressure until a viscous extract was obtained (yield: approximately 12% w/w). The concentrated extract was stored at 4°C in dark glass bottles until use.

Extract administration

Stock solutions were prepared daily by dissolving the extract in distilled water to achieve concentrations of 10, 20, and 40 mg/mL for the 100, 200, and 400 mg/kg doses, respectively. Administration volume was standardized at 1 mL per 100 g body weight. Extract or vehicle (distilled water) was administered once daily via oral gavage using a stainless steel feeding needle for 14 consecutive days, beginning immediately after hyperglycemia confirmation. Body weight was monitored every 3 days, and extract doses were adjusted accordingly.

Blood glucose monitoring

FBG was measured at three time points: baseline (day 0, before alloxan), day 3 post-alloxan (diabetes confirmation), and day 14 (end of treatment). After 12-hour fasting, blood samples (~10 µL) were collected from the lateral tail vein and analyzed immediately using a glucometer. Glucose levels are expressed in mg/dL.

Blood collection and serum preparation

On day 15 (24 hours after the final extract administration), rats were fasted for 12 hours and anesthetized using ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Blood samples (3 mL) were collected via retro-orbital venous plexus puncture using heparinized capillary tubes into non-anticoagulant tubes. Blood was allowed to clot at room temperature for 30 minutes, then centrifuged at 3,500 rpm (1,225 × g) for 10 minutes at 4°C. Serum was separated using sterile micropipettes, aliquoted into cryogenic vials, and immediately stored at -80°C until biochemical analysis. Following blood collection, animals were euthanized by cervical dislocation under deep anesthesia.

Determination of malondialdehyde (MDA) levels

Serum MDA concentration was quantified using the thiobarbituric acid reactive substances (TBARS) assay, which measures lipid peroxidation products. The assay was performed as follows: blank, standard, and sample tubes were prepared in triplicate. Each tube received 500 µL of distilled water (blank), MDA standard solution (standard), or serum sample (sample), respectively. Subsequently, 2.5 mL of 5% trichloroacetic acid (TCA) reagent was added to each tube, followed by vigorous mixing using a vortex mixer. The mixture was centrifuged at 10,000 rpm for 15 minutes at room temperature to obtain a clear supernatant.

From each tube, 1 mL of supernatant was transferred to a new tube, and 1 mL of 0.67% thiobarbituric acid (TBA) reagent was added. The reaction mixture was incubated in a boiling water bath at 100°C for 30 minutes to allow the formation of the MDA-TBA adduct. Following incubation, tubes were rapidly cooled to room temperature in an ice bath. The absorbance of the pink chromogen was measured spectrophotometrically at a wavelength of 530 nm using a digital spectrophotometer. MDA concentrations were calculated by comparing sample absorbance values to a standard curve prepared using known concentrations of MDA equivalents, and results were expressed in mg/dL.

Measurement of catalase enzyme activity

Catalase enzyme activity was determined using a spectrophotometric method based on the decomposition of hydrogen peroxide (H_2O_2). The assay consisted of two phases: generation of a standard curve and analysis of serum samples.

For the standard curve, varying concentrations of H_2O_2 (10-160 µmol) were reacted with 2 mL of catalase dye reagent (potassium dichromate in glacial acetic acid). The reaction mixture was heated

in a boiling water bath for 10 minutes, during which the yellow dichromate was reduced to green chromic acetate in proportion to the residual H_2O_2. After cooling to room temperature, the absorbance was measured at 570 nm. A calibration curve was constructed by plotting absorbance values against corresponding H_2O_2 concentrations.

For sample analysis, a reaction mixture was prepared containing 4 mL of H_2O_2 substrate, 5 mL of phosphate buffer (pH 7.0), and 1 mL of serum sample, mixed thoroughly but gently. At predetermined time intervals (0, 60, 120 seconds), 1 mL aliquots were withdrawn and immediately added to 2 mL of dye reagent to stop the enzymatic reaction. Each sample tube was then heated in a boiling water bath for 10 minutes, cooled to room temperature, and measured for absorbance at 570 nm. The amount of H_2O_2 consumed by catalase enzyme over time was calculated using the standard curve, and enzyme activity was expressed in units milligrams per deciliter (mg/dL). Results were reported in mg/dL for consistency with the original study protocol.

Data analysis

All experimental data from 25 rats were analyzed using IBM SPSS Statistics software. Prior to inferential testing, data normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was evaluated using Levene's test. A significance level of $\alpha = 0.05$ was applied for all statistical tests.

For MDA levels, the Shapiro-Wilk test indicated that data were normally distributed ($p>0.05$ for all groups); however, Levene's test revealed heterogeneity of variance ($p=0.000$, $p<0.05$). Due to violation of the homogeneity assumption, a non-parametric Kruskal-Wallis test was employed to compare MDA levels across all five groups. Following detection of significant differences, pairwise comparisons were conducted using the Mann-Whitney U test with Bonferroni correction to identify specific group differences.

For catalase enzyme activity, both normality (Shapiro-Wilk test, $p>0.05$) and homogeneity (Levene's test, $p = 0.182$, $p>0.05$) assumptions were satisfied. Therefore, a one-way analysis of variance (ANOVA) was performed to compare catalase activity among groups, with a 95% confidence interval. Although post hoc testing was planned, it was not conducted due to the absence of statistically significant differences in the ANOVA ($p = 0.089$, $p>0.05$).

All data are presented as mean ± standard deviation (SD). Statistical significance was defined as $p<0.05$ for all analyses.

Results

Serum malondialdehyde (MDA) levels

Alloxan-induced hyperglycemia significantly elevated serum MDA concentrations compared to normal controls (Figure 1A). The diabetic control exhibited the highest mean MDA level at 2.68±0.62 mg/dL, representing a 50.6% increase compared to normal control (1.78±0.30 mg/dL).

Administration of stevia leaf extract produced a dose-dependent reduction in MDA levels across all treatment groups. The T1 group (100 mg/kg BW) showed MDA of 1.70±0.19 mg/dL, T2 (200 mg/kg BW) demonstrated 1.54±0.20 mg/dL, and T3 (400 mg/kg BW) achieved the lowest level at 1.38±0.09 mg/dL. These values represent 36.6%, 42.5%, and 48.5% reduction from diabetic control, respectively. Notably, the T3 group's MDA level approached that of the normal group, indicating significant restoration of oxidative balance.

Statistical analysis revealed significant differences in MDA levels among groups (Kruskal-Wallis test, $p=0.003$). Post hoc pairwise comparisons indicated that all stevia-treated groups showed significantly lower MDA compared to diabetic control, with the reduction being most pronounced in the T3 group (Mann-Whitney U test, $p<0.05$ for all comparisons vs. diabetic control).

Serum catalase enzyme activity

Alloxan induction reduced catalase activity in the diabetic group (7.92±0.76 mg/dL) compared to NC (9.37±1.04 mg/dL), representing a 15.5% decrease in antioxidant enzyme capacity (Figure 1B).

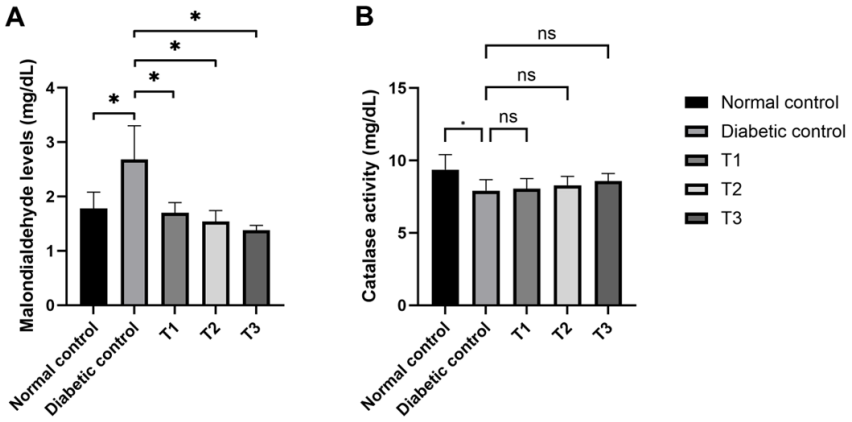

Figure 1. Dose-dependent effects of stevia leaf extract on oxidative stress biomarkers in alloxan-induced hyperglycemic rats. (A) Serum malondialdehyde (MDA) levels. **(B)** Serum catalase enzyme activity. Data presented as mean ± SD (n=5/group). Statistical significance determined by Kruskal-Wallis test followed by Mann-Whitney U test for MDA (panel A), and one-way ANOVA for catalase (panel B). Horizontal lines with asterisks indicate significant pairwise comparisons.

Stevia extract treatment demonstrated a dose-dependent trend toward catalase restoration. Catalase activity progressively increased across treatment groups: T1 (8.05±0.70 mg/dL), T2 (8.29±0.61 mg/dL), and T3 (8.58±0.52 mg/dL), representing 1.6%, 4.7%, and 8.3% increases from diabetic group, respectively. Although the dose-response pattern was evident, the differences did not achieve statistical significance (one-way ANOVA, p=0.089).

Discussion

This study demonstrates that *Stevia rebaudiana* leaf extract significantly reduces oxidative stress in alloxan-induced hyperglycemic rats through dose-dependent modulation of serum malondialdehyde levels. The highest dose tested (400 mg/kgBW) produced the most substantial antioxidant effects, nearly restoring MDA levels to those observed in non-hyperglycemic controls.

Alloxan selectively destroys pancreatic beta-cells through its structural similarity to glucose, facilitating cellular uptake via GLUT2 transporters [9]. Once internalized, alloxan undergoes redox cycling with dialuric acid, generating excessive reactive oxygen species (ROS) including superoxide radicals, hydrogen peroxide, and hydroxyl radicals [9]. This oxidative burst overwhelms endogenous antioxidant defenses, triggering lipid peroxidation in cell membranes rich in polyunsaturated fatty acids, ultimately producing malondialdehyde as a stable end product [10]. Our findings confirm this mechanism, with the untreated diabetic group showing 50.6% elevation in MDA compared to normal controls, consistent with previous reports of alloxan-mediated oxidative damage [8,10].

The dose-dependent MDA reduction observed in our study (36.6% to 48.5% across treatment groups, Kruskal-Wallis p=0.003) aligns with previous investigations of plant-based antioxidants in hyperglycemic models. Afrianti et al. [8] reported similar dose-dependent MDA reduction with *Isotoma longiflora* leaf extract at 200-400 mg/kgBW in hyperglycemic rats, demonstrating that polyphenol-rich extracts effectively suppress lipid peroxidation at higher doses.

Stevia's antioxidant efficacy derives from dual mechanisms. First, its bioactive constituents—steviol glycosides (stevioside and rebaudioside A) and polyphenolic compounds including flavonoids, phenolic acids, and tannins—function as potent free radical scavengers through hydrogen donation and electron transfer mechanisms [11,12]. These compounds directly neutralize ROS before lipid peroxidation initiation. Second, stevia demonstrates antihyperglycemic properties by stimulating insulin secretion, enhancing peripheral insulin sensitivity, and facilitating glucose uptake, thereby reducing glucose autooxidation and advanced glycation end-

product formation—major ROS sources in diabetic conditions [12]. This dual action creates synergistic effects that substantially reduce oxidative burden, as evidenced by decreased MDA formation in our dose-response analysis.

Catalase plays a critical role in cellular antioxidant defense by catalyzing hydrogen peroxide decomposition into water and oxygen, preventing hydroxyl radical formation via Fenton reactions [5]. Our results showed 15.5% catalase reduction in untreated diabetic rats, reflecting enzyme inactivation and depletion under severe oxidative stress [9,10]. While stevia treatment demonstrated a dose-dependent restoration trend (1.6% to 8.3% increases), these changes did not achieve statistical significance (one-way ANOVA $p=0.089$).

This contrasts with Erang et al. [13], who reported significant catalase increases with *Uncaria gambir* extract (50-100 mg/kgBW) in streptozotocin-induced diabetic rats. Several factors may explain this discrepancy. First, streptozotocin and alloxan induce diabetes through different mechanisms, potentially affecting enzyme responses differently. Second, catalase upregulation requires transcriptional activation, translation, and protein stabilization—processes potentially requiring longer than our 14-day intervention period [12]. Third, the proximity of our p-value to significance ($p=0.089$) with moderate effect size suggests insufficient statistical power, indicating that larger sample sizes might reveal significant effects.

Polyphenolic compounds in stevia can upregulate antioxidant enzyme expression through nuclear factor erythroid 2-related factor 2 (Nrf2) pathway activation, controlling transcription of antioxidant response elements [12]. Additionally, by reducing overall oxidative stress through direct ROS scavenging, stevia may preserve existing catalase from oxidative inactivation while creating favorable conditions for enzyme synthesis [11]. The consistent dose-dependent trend observed, despite statistical non-significance, suggests biological relevance warranting further investigation.

The 400 mg/kgBW dose, which produced optimal antioxidant effects in rats, translates to approximately 64.8 mg/kg for humans using standard human equivalent dose calculations [FDA conversion factor: rat dose × 0.162]. For a 70 kg adult, this corresponds to approximately 4.5 g daily—a dose within the safe range established for stevia consumption [7,12]. This suggests potential feasibility for clinical translation, though human studies are needed to confirm efficacy and safety.

Our findings support stevia's therapeutic potential as a complementary intervention for managing oxidative stress in diabetes mellitus. The significant MDA reduction at 400 mg/kgBW indicates that stevia extract possesses antioxidant capacity, primarily through direct ROS neutralization leading to reduced lipid peroxidation. While a dose-dependent trend toward catalase restoration was observed, this effect did not reach statistical significance and requires further investigation to confirm potential enhancement of endogenous antioxidant enzyme systems.

Several limitations should be acknowledged. The 14-day treatment duration, while sufficient for demonstrating MDA reduction, may not allow adequate time for maximal antioxidant enzyme induction. We did not measure other antioxidant defense components such as superoxide dismutase or glutathione peroxidase, which would provide more comprehensive understanding of stevia's effects on the antioxidant system. Finally, blood glucose levels post-treatment were not included as study endpoints, precluding direct correlation between glycemic control and oxidative stress reduction.

Future investigations should employ extended treatment durations (≥28 days), dose-escalation studies beyond 400 mg/kgBW, and comprehensive antioxidant enzyme profiling including superoxide dismutase and glutathione peroxidase. Phytochemical standardization using HPLC analysis and evaluation of specific bioactive compounds would strengthen mechanistic understanding.

Conclusion

Stevia rebaudiana leaf extract effectively reduces oxidative stress in alloxan-induced hyperglycemic rats through significant, dose-dependent reduction of

serum malondialdehyde levels, with 400 mg/kgBW representing the optimal dose in this experimental model. While catalase activity showed favorable dose-dependent trends toward restoration, these changes require validation in studies with larger sample sizes and extended durations. These findings support stevia's potential as a complementary therapeutic agent for managing oxidative stress in diabetes mellitus, meriting further translational research.

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Author Contributions

ZSA: Investigation, data collection, formal analysis, writing, and preparation of the original draft. EY, DA: Conceptualization, methodology, supervision, writing, revision and editing, project administration. RA, F, WPW: Methodology, supervision, validation, writing, revision, and editing. HY: writing, revision, and editing. All authors have read and approved the final version of the manuscript.

Declaration of interest

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