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RESEARCH ARTICLE

# Antioxidant Effects of *Rhodiola Heterodonta*, *Silybum Marianum*, and *Morus. Nigra* Extracts on Oxidative Stress Biomarkers in Alloxan-induced Diabetic Rats

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**Abstract**

Oxidative stress critically contributes to the pathogenesis of diabetes mellitus. This study evaluated the effects of three antioxidant plant extracts *Rhodiola heterodonta* (Hook.f. & Thomson) Boriss. (Crassulaceae), *Silybum marianum* (L.) Gaertn. (Asteraceae), and *Morus nigra* L. (Moraceae) on oxidative stress markers in alloxan-induced diabetic rats. Male outbread rats (180 - 220 g) were injected intraperitoneally with alloxan monohydrate (140 mg/kg) to induce diabetes over 14 days. Blood glucose levels and urine biochemical parameters were monitored during this period. Rats were then orally treated with the plant extracts at dose 75 mg/kg for 14 days. On day 29, animals were sacrificed, and oxidative stress biomarkers including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and mitochondrial lipid peroxidation were measured. *In vitro* DPPH assay confirmed the extracts' radical-scavenging activity. Treatment with all three extracts significantly reduced oxidative stress in a dose-dependent manner by enhancing antioxidant enzyme activities and lowering lipid peroxidation. Among the extracts, *Rhodiola heterodonta* showed the strongest protective effect. These results suggest that *Rhodiola heterodonta*, *Silybum marianum*, and *Morus nigra* extracts have notable antioxidant potential and may improve oxidative stress parameters in diabetes.

**Keywords:** Diabetes mellitus, Alloxan, Oxidative stress, Antioxidant plant extracts, MDA, SOD, Catalase, Mitochondrial lipid peroxidation, DPPH

## Introduction

One of the central mechanisms implicated in the pathogenesis and progression of diabetes is oxidative stress, defined as an imbalance between excessive reactive oxygen species (ROS) production and insufficient antioxidant defense mechanisms. Hyperglycemia induced oxidative stress directly damages cellular components through lipid peroxidation, protein oxidation, and DNA damage, ultimately contributing to β cell dysfunction and the onset of diabetic complications. Experimental and clinical studies consistently report elevated oxidative stress and impaired antioxidant enzyme activity in diabetic models and patients, underscoring its critical role in disease progression [1].

Such oxidative damage not only affects cytosolic components but also impairs mitochondrial homeostasis, which serves as a major intracellular source of ROS. Mitochondrial dysfunction under hyperglycemic conditions further increases ROS production, leading to enhanced lipid peroxidation and diminished endogenous antioxidant defenses [2]. Consequently, elevated levels of malondialdehyde

(MDA), a marker of lipid peroxidation, and suppressed activities of key antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) have been documented in experimental diabetic models [3].

Natural plant extracts rich in polyphenols, flavonoids, and other bioactive phytochemicals have shown promise as modulators of oxidative stress due to their free radical scavenging and antioxidant properties. Preliminary studies indicate that *R. heterodonta* extracts may inhibit reactive oxygen species and exert hypoglycemic activity. Nevertheless, comprehensive evaluation of their effects on oxidative stress parameters and metabolic disturbances in experimental diabetes remains insufficient. Previous studies have reported that root extracts of *R. heterodonta* exhibit notable ROS-scavenging activity and glucose-lowering effects in alloxan- or glucose-induced diabetic rats, suggesting potential antioxidant and antihyperglycemic properties. [4].

Similarly, extracts from *S. marianum* (milk thistle) have been associated with antioxidant and antidiabetic effects. The major flavonoid component silibinin has been shown to reduce reactive oxygen species and improve metabolic parameters in various experimental and animal models of diabetes, suggesting that *S. marianum* extracts can mitigate oxidative stress and support glucose metabolism [2].

*M. nigra* leaf extracts have also been reported to improve oxidative stress markers and glycemic response in diabetic rat models. In alloxan induced diabetic rats, treatment with *M. nigra* leaf extract decreased the superoxide dismutase/catalase ratio and other indicators of oxidative imbalance while attenuating hyperglycemia, indicating a protective effect against ROS associated damage [5].

Despite these promising findings, comparative evaluations of multiple antioxidant plant extracts within a standardized diabetic model are limited. The present study aimed to assess the effects of *R. heterodonta*, *S. marianum*, and *M. nigra* extracts on oxidative stress biomarkers including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and mitochondrial lipid peroxidation as well as metabolic parameters in an alloxan induced diabetic rat model. We hypothesized that oral administration of these extracts would reduce oxidative stress and enhance endogenous antioxidant defenses in diabetic animals.

## Materials and methods

### Plant material and extraction

Dried and ground *R. heterodonta* roots, *S. marianum* blossom, *M. nigra* leafs were provided by BIOTON Ltd. Dried samples were ground into a fine powder and kept in desiccators until extracted. The extraction was conducted in a Soxhlet extractor for 24 h using 40% ethanol. Then, the solvent was evaporated using a rotary evaporator at +50 °C and concentrated in a vacuum until the moisture content percentage was less than 15% [4].

### Animal experiments

All animal experiments complied with the European Directive 2010/63/EU on the protection of animals used for scientific purposes [6]. All experimental procedures involving animals were carried out in accordance with Protocol No. 1, approved on January 4, 2025, by the Ethics Committee for the Use of Laboratory Animals in *in vivo* Research at the Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan.

Adult male outbred rats weighing 180 - 220 g were used in the study and housed under standard laboratory conditions (22 ± 2 °C, 12 h light/dark cycle) with free access to food and water. Animals were randomly assigned to five experimental groups (n = 6 per group), including the intact control group, alloxan-induced diabetic control group, and three treatment groups receiving the respective plant extracts. Rats were orally administered the plant extracts via oral gavage at a dose of 75 mg/kg body weight once daily for 14 consecutive days (days 15 - 28). On day 29, following overnight fasting, the animals were sacrificed, and blood and tissue samples were collected for biochemical analyses.

### Determination of acute toxicity of plant extract

The acute toxicity parameters of the extracts were determined using the Litchfield and Wilcoxon method [14]. The study was conducted on male white mice weighing 20 ± 2.0 g, with six animals per group; a total of 24 mice were used.

All pharmacological studies were performed on healthy, sexually mature animals that had undergone a quarantine period of at least 10 - 14 days. The extracts were administered orally at doses of 2,000 and 5,000 mg/kg body weight.

Animals were observed hourly during the first day of the experiment under laboratory conditions. Functional status indicators included survival throughout the study, general condition, occurrence of convulsions, and mortality. Subsequently, animals in all groups were monitored daily for two weeks under vivarium conditions for general condition and activity, behavioral characteristics, respiratory rate and depth, condition of fur and skin, tail position, quantity and consistency of feces, frequency of urination, changes in body weight, and other relevant parameters.

All experimental animals were maintained under identical conditions and fed a standard diet with free access to food and water. At the end of the experiment, the median lethal dose ($LD_{50}$) was calculated and the toxicity class was determined.

**Determination of antiradical activity of plant extracts**

The radical scavenging activity of the extracts was determined using the standard method by monitoring the kinetics of the optical density of an ethanolic DPPH (1,1-diphenyl-2-picrylhydrazyl) solution. The concentration of DPPH was 0.1 mM. Extract solutions were tested at concentrations of 6.7, 13.3, 20.0, 26.7, and 33.3 µg/mL. Changes in the optical density of the DPPH solution were measured in 1 cm path-length cuvettes with a total reaction volume of 2 mL using a METASH spectrophotometer [7].

**Modeling Alloxan-induced oxidative stress**

To induce experimental diabetes and oxidative stress, alloxan monohydrate (Sigma, 140 mg/kg body weight) was used [8]. Rats were fasted overnight prior to alloxan administration, with free access to water. Alloxan was freshly dissolved in physiological saline and administered via intraperitoneal injection. Successful induction of diabetes was confirmed by marked elevation of blood glucose levels together with urinary biochemical abnormalities, including glucosuria, in the untreated diabetic group. No significant mortality was observed following alloxan administration. Biochemical characteristcs of urine Biochemical parameters of urine were analyzed using the Genrui BA600 urine analyzer (GENRUI Biotech, China). The analytical method is based on reflectance photometry, which provides high sensitivity and reproducibility in color determination. Genrui BA11 test strips were used for the analysis, minimizing measurement errors and variability. These test strips are designed to measure 8 parameters: Urobilinogen, bilirubin, ketones, erythrocytes, protein, nitrites, leukocytes, glucose, specific gravity, pH, and ascorbic acid. All data were automatically recorded and displayed on the analyzer's touchscreen.

**Determination of plasma glucose level**

Plasma glucose levels in rats were determined using the Cypress Diagnostics GOD-POD Glucose (liquid) assay kit. All reagents and samples were equilibrated to room temperature prior to analysis. Spectrophotometric measurements were performed at a wavelength of 510 nm at room temperature using cuvettes with a 1 cm optical path length.

**Determination of malondialdehyde (MDA) content in rat tissues**

Prior to MDA determination, tissue samples were weighed and homogenized in ice-cold Krebs buffer at a tissue-to-buffer ratio of 1:9 (w/v) using a laboratory homogenizer.

The level of thiobarbituric acid–reactive substances (TBARS), reflecting malondialdehyde (MDA) content and the intensity of lipid peroxidation (LPO), was determined in tissue samples using the method described by Heath and Packer [11]. For analysis, 1 mL of tissue supernatant was mixed with 4 mL of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA). The resulting mixture was incubated in a water bath at 95 °C for 30 min and then cooled to room temperature. The optical density of the formed MDA-TBA complex was measured at 532 nm using a METASH spectrophotometer.

**Isolation of mitochondria**

Mitochondria were isolated from the livers of rats weighing 180 - 220 g by differential centrifugation according to a previously described method [9]. Animals were decapitated, the liver was rapidly excised, and immediately placed in an ice-cold solution containing 250 mM sucrose and 10 mM Tris buffer (pH 7.4). After weighing, the liver tissue was gently blotted between filter paper sheets and passed through a mechanical press.

The tissue was then homogenized in a Teflon homogenizer using a six-fold volume of isolation medium (IM) consisting of 250 mM sucrose, 0.5 mM EDTA, and 10 mM Tris buffer (pH 7.4). Nuclei and cellular debris were removed by centrifugation at 1,500 rpm for 7 min at 0 - 1 °C using a PC-6MI centrifuge equipped with an angle rotor.

Mitochondria were pelleted and washed by centrifugation at 6,000 rpm for 15 min at the same temperature. For washing, an isolation medium without EDTA was used. After washing, the mitochondrial pellet was resuspended in EDTA-free isolation medium to obtain a suspension containing 50 - 70 mg of mitochondrial protein per mL. The resulting dense mitochondrial suspension was stored on ice throughout the experiment. All procedures for mitochondrial isolation were carried out at 0 - 2 °C. The mitochondrial samples were maintained on ice throughout the experimental procedures, and no freezing or long-term storage was applied.

**Protein determination**

Protein content was determined using the biuret reaction according to a standard method [10]. Briefly, 0.1 mL of the mitochondrial suspension was mixed with 0.9 mL of 2 N KOH containing 10 mg of deoxycholate to disrupt mitochondrial membranes. After complete protein solubilization, 4 mL of biuret reagent was added, and the mixture was incubated at room temperature for 30 min.

A control sample containing 1 mL of 2 N KOH with 10 mg of deoxycholate and 4 mL of biuret reagent was prepared in parallel. Absorbance was measured at 540 nm using cuvettes with a 10 mm optical path length. Protein concentration was calculated from a calibration curve generated using bovine serum albumin standards.

**Determination of lipid peroxidation**

Non-enzymatic $Fe^{2+}$/ascorbate-dependent lipid peroxidation was induced by adding $10^{-5}$ M $FeSO_4$ and $2\times10^{-4}$ M ascorbate to the incubation medium containing 125 mM KCl, 10 mM Tris-HCl (pH 7.5), and a mitochondrial suspension at a final protein concentration of 8 mg per mL of incubation medium. Incubation was carried out at 37 °C in a water bath with constant stirring.

Lipid peroxidation was initiated by the addition of the $Fe^{2+}$/ascorbate system. The reaction was terminated by adding 0.2 mL of 70% trichloroacetic acid (TCA). The samples were then centrifuged at 3,000 rpm for 15 min. After centrifugation, 2 mL of the supernatant was collected and mixed with 1 mL of 80% thiobarbituric acid (TBA). For the control sample, 2 mL of distilled water and 1 mL of TBA were used.

The mixtures were heated in a boiling water bath for 15 min, cooled to room temperature, and the optical density was measured at 540 nm. The amount of malondialdehyde (MDA) formed was calculated using the molar extinction coefficient ($\varepsilon = 1.56\times10^5$ $M^{-1}\cdot cm^{-1}$) according to the following formula nmol MDA/mg protein = D / 1.56×30, where D represents the absorbance at 540 nm.

**Determination of superoxide dismutase (SOD) activity**

Superoxide dismutase (SOD) activity was determined according to the method described by Bagul *et al*. [12], with minor modifications. Enzyme activity was assessed based on the ability of the sample to inhibit the formation of blue formazan by scavenging superoxide radicals generated in the riboflavin–light–nitroblue tetrazolium (NBT) system. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.6), 0.1 mg/mL riboflavin, 12 mM EDTA, and 0.1 mg/3 mL NBT, added sequentially. The reaction was initiated by illuminating the mixture in the presence of various concentrations of the sample extract for 120 s. After illumination, the absorbance of the solution was measured at 560 nm. Methanol was used as the blank control, while ascorbic acid served as the positive

control. All experiments were performed at 24 - 26 °C using a spectrophotometer. The sample used for the SOD assay were freshly isolated and analyzed immediately after preparation.

**Determination of catalase (CAT) activity**

Catalase activity was measured using the method proposed by Hadwan (2016), with minor modifications [13]. In this method, dichromate dissolved in acetic acid is reduced to chromium (III) acetate upon heating in the presence of hydrogen peroxide ($H_2O_2$), forming an intermediate unstable compound, perchromic acid. The concentration of hydrogen peroxide is directly proportional to the amount of chromium acetate formed. Quantitative determination of chromium acetate was performed colorimetrically at a wavelength of 570 nm, reflecting the level of catalase activity. The sample used for the catalase assay were freshly isolated and analyzed immediately after preparation.

**Quantitive analysis of secondary metabolites**

Total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method using quercetin as the standard, with absorbance measured at 510 nm. Total phenolic content (TPC) was quantified by the Folin–Ciocalteu method using gallic acid as the reference standard and absorbance recorded at 765 nm [15].

Total tannin content (TTC) was evaluated using the ferric chloride–potassium ferrocyanide colorimetric method with epigallocatechin as the calibration standard and absorbance measured at 492 nm [16].

Total saponin content (TSC) was assessed by the vanillin–sulfuric acid colorimetric assay and expressed as diosgenin equivalents, with absorbance measured at 544 nm [17]. Total terpenoid content was determined spectrophotometrically at 780 nm using cholesterol as the standard [18].

**Statistical analysis**

The results are presented as mean ± standard deviation (SD). Each assay was independently performed at least 3 times, and statistical analysis was conducted using the Student's t-test. Data for each treatment represent 3 independent replicates (n = 3 - 4 and n = 5 - 6; $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). Figures were created using OriginPro Lab 8.5 software.

## Results and discussion

The evaluation of medicinal plant toxicity is a crucial step in assessing their safety and potential therapeutic applicability [19]. In the present study, acute and chronic toxicity of *R. heterodonta*, *M. nigra*, and *S. marianum* extracts, as well as their combinations, was investigated using experimental animal models. Acute toxicity was assessed based on behavioral, physiological, and survival parameters following administration of various doses of the extracts. The results demonstrated that none of the tested extracts produced mortality or caused signs of severe intoxication at the studied concentrations, indicating a favorable safety profile.

To evaluate the toxicity of *R.heterodonta*, *S.marianum*, and *M.nigra* extracts, as well as their combinations, studies were conducted using experimental animal models (**Table 1**).

**Table 1** Assessment of acute toxicity of plant sxtracts.

| Groups | Animal species and sex | Dose mg/kg | Mortality, Total animals | $LD_{50}$ (with confidence interval) |
|---|---|---|---|---|
| *R.heterodonta* | Mouse, male | 2,000 | 0/5 | >2,000 mg/kg, V class |
| *S.marianum* | | 5,000 | 0/5 | >5,000 mg/kg, VI class |
| *M.nigra* | | 5,000 | 0/5 | >5,000 mg/kg, VI class |

Note: $LD_{50}$ > 2,000 mg/kg corresponds to Class V, considered practically non-toxic substances. $LD_{50}$ > 5,000 mg/kg corresponds to Class VI, considered relatively harmless substances. *$p < 0.05$ indicates statistically significant differences compared with the intact group. A total of 24 mice were used in the acute toxicity experiment.

As shown in **Table 1**, all investigated plant extracts exhibited low acute toxicity. No mortality was observed in mice following oral administration of the extracts at the tested doses. According to the obtained $LD_{50}$ values, *R. heterodonta* was classified as toxicity class V, whereas *S. marianum* and *M. nigra* belonged to toxicity class VI, indicating their relative safety under the experimental conditions.

Following the assessment of acute toxicity, the antioxidant potential of the plant extracts was evaluated using the DPPH radical scavenging assay.

The DPPH method allows the evaluation of the antioxidant activity (AOA) of substances *in vitro*, which is important at the early stages of screening plant extracts for their biological activity. Studies indicate that plant extracts rich in flavonoids, polyphenols, and other biologically active compounds can exert strong antiradical effects by neutralizing free radicals [21].

Our experimental results clearly demonstrate that increasing the extract volume leads to a noticeable decrease in the optical density of the solution. This decrease reflects the effective interaction of antioxidants present in the extract with DPPH radicals, resulting in the neutralization of free radicals. The higher the extract concentration, the more pronounced this effect, particularly at doses of 26.7 and 33.3 µg/mL (**Figure 1**).

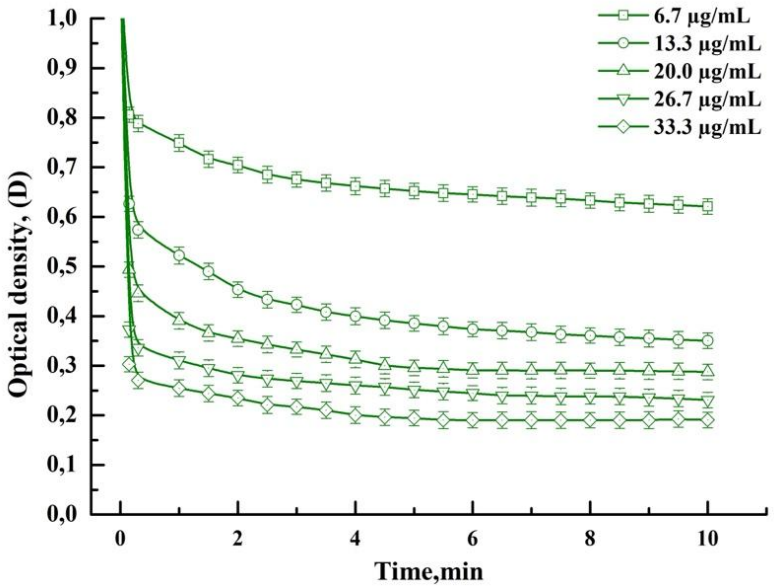



**Figure 1** Dynamics of changes in the relative optical density of the DPPH solution in ethanol upon addition of *R. heterodonta* extract at different concentrations. The DPPH concentration was 0.1 mM.

**Figure 1** illustrates the dynamics of changes in the relative optical density of the DPPH solution in ethanol upon the addition of different volumes of *R.heterodonta* extract. This figure clearly demonstrates the evaluation

of the antiradical activity of the plant extract at a fixed DPPH concentration (0.1 mM) depending on its dosage. The $IC_{50}$ value of the extract was 9.4 μg/mL, indicating high antiradical activity.

The experimental results for *S.marianum* clearly show that an increase in extract volume leads to a noticeable decrease in the optical density of the DPPH solution. This indicates an effective interaction between the antioxidants present in the extract and DPPH radicals, contributing to the neutralization of free radicals. The highest activity was observed at doses of 26.7 and 33.3 μg/mL (**Figure 2**).

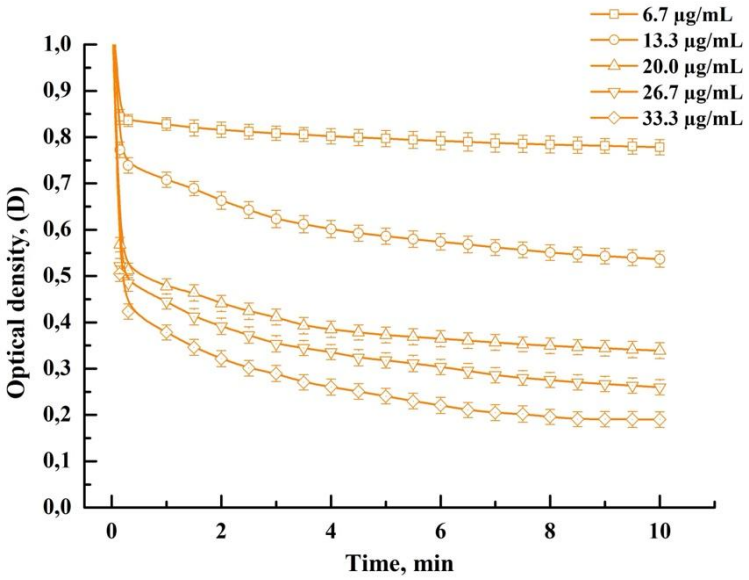



**Figure 2** Dynamics of changes in the relative optical density of the DPPH solution in ethanol upon addition of S.*marianum* extract at different concentrations. The DPPH concentration was 0.1 mM

**Figure 2** demonstrates that the antioxidant activity of *S.marianum* increases with rising extract concentration, as evidenced by changes in the optical density of the DPPH solution at a fixed concentration (0.1 mM). These results support the antioxidant properties of *S.marianum* and highlight its potential role in the neutralization of free radicals. This is particularly important in the context of diseases associated with oxidative stress. The IC50 value of the extract was 14.4 μg/mL, indicating high antiradical activity. Accordingly, the results for *M.nigra* confirm that increasing the extract volume leads to a noticeable decrease in the optical density of the DPPH solution. This indicates effective interaction of the extract's antioxidants with DPPH radicals, contributing to the neutralization of free radicals. The highest activity is observed at doses of 26.7 and 33.3 μg/mL (**Figure 3**). The figure demonstrates that the antioxidant activity of *M.nigra* increases with the extract concentration, as evidenced by changes in the optical density of the DPPH solution at a fixed concentration (0.1 mM).

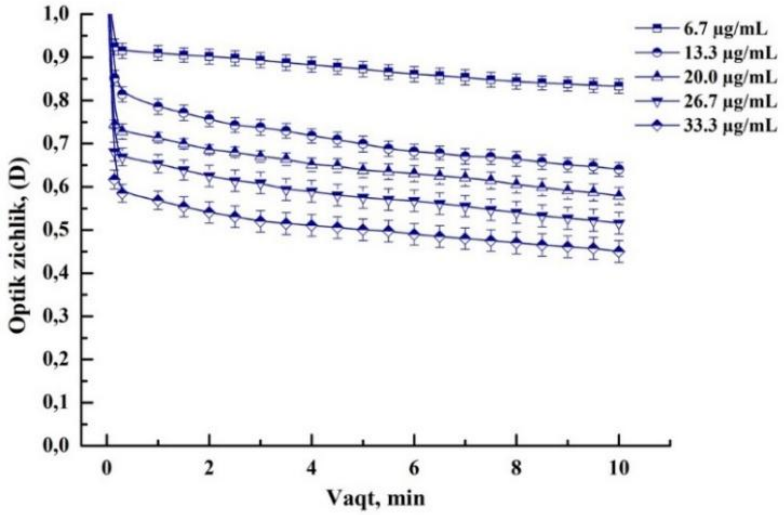



**Figure 3** Dynamics of changes in the relative optical density of the DPPH solution in ethanol upon addition of *M.nigra* extract at different concentrations. The DPPH concentration was 0.1 mM.

**Figure 3** illustrates the dose-dependent radical-scavenging activity of *M.nigra* extract. At a fixed DPPH concentration (0.1 mM), the $IC_{50}$ was 28.2 µg/mL. Maximum activity was observed at 26.7 and 33.3 µg/mL, confirming the extract's effectiveness in neutralizing free radicals and its potential against oxidative stress.

To assess the protective efficacy of the studied extracts *in vivo*, an alloxan-induced oxidative stress model in rats was employed. Alloxan monohydrate reliably induces diabetes by selectively damaging pancreatic β cells, leading to sustained hyperglycemia and excessive generation of reactive oxygen species (ROS), thereby reproducing the biochemical features of oxidative stress associated with diabetic pathology [22]. The successful establishment of the alloxan model was confirmed by multiple biochemical and physiological parameters, including significantly elevated blood glucose levels and disturbances in urine biochemistry, which reflect metabolic derangements characteristic of alloxan-induced diabetes. These alterations validate the induction of oxidative stress and provide a foundation for evaluating the antioxidant potential of the test extracts under pathological conditions [23].

Biochemical parameters of urine reflecting the extent of metabolic disturbances and the level of oxidative stress were analyzed (**Table 2**).

**Table 2** Parameters and results of urine analysis in rats on day 25 after treatment with the extracts at various doses.

| Gropus | Bilirubin (mmol/) | Ketones (mmol/l) | Blood (cells/ µL) | Protein (g/l) | Nitrites | Leukocytes (cells/ µL) | Glucose (mmol/l) | Specific gravity |
|---|---|---|---|---|---|---|---|---|
| Intact | negative | negative | negative | negative | positive | negative | negative | 1.015 ± 0.015 |
| Model (Alloxan) | 8.6 | ± 0.5 | 200 | 0.3 | positive | negative | 55 | 1.030 ± 0.03 |
| *R.heterodonta* 75 mg/kg | negative | negative | 80 | negative | negative | negative | 14 | 1.010 ± 0.01 |
| *S.marianum* 75 mg/kg | negative | ± 0.5 | 25 | negative | negative | negative | 14 | 1.020 ± 0.02 |
| *M.nigra* 75 mg/kg | negative | ± 0.5 | 80 | negative | negative | negative | 14 | 1.015 ± 0.01 |

Note: Data are presented as mean ± standard deviation (SD). *$p < 0.05$ indicates statistically significant differences compared with the intact group. n = 3 animals per group.

**Table 2** shows urine biochemical changes in rats depending on the treatment. In intact rats, bilirubin, ketones, blood, and protein were absent, glucose was undetectable, and specific gravity was 1.015 ± 0.015. Alloxan (140 mg/kg) caused a marked increase in bilirubin (8.6 mmol/L), ketones (±0.5 mmol/L), erythrocytes (200 cells/μL), protein (0.3 g/L), glucose (55 mmol/L), and urine specific gravity (1.030 ± 0.03), indicating nephropathy due to hyperglycemia and oxidative stress.

Alloxan-induced diabetes caused in significant urinary biochemical disturbances, including increased glucose, ketones, bilirubin, protein, erythrocytes, and urine specific gravity, indicating severe metabolic dysfunction and renal impairment. Alloxan is known to selectively damage pancreatic β-cells through the generation of reactive oxygen species, leading to insulin deficiency, persistent hyperglycemia, and oxidative stress-mediated tissue injury [24].

Oral treatment with *R.heterodonta*, *S.marianum*, and *M.nigra* extracts (75 mg/kg) reduced these pathological changes. In *R.heterodonta* -treated rats, bilirubin and ketones disappeared, erythrocytes decreased to 80 cells/μL, and protein normalized. Similar improvements were observed with *S.marianum*, and *M.nigra*, showing their potential to protect against alloxan-induced kidney damage.

In addition to urinary biochemical alterations, diabetes mellitus is primarily characterized by disturbances in systemic glucose homeostasis. Therefore, the effect of the studied extracts on plasma glucose levels was further evaluated to determine their potential antihyperglycemic activity (**Table 3**).

On day 25 of the experiment, the plant extracts exhibited more pronounced hypoglycemic activity. As shown in **Table 3**, intact animals exhibited normal blood glucose levels (4.5 ± 1.5 mmol/L). In the alloxan-induced diabetic model, treatment with plant extracts at a dose of 75 mg/kg resulted in a marked reduction in blood glucose concentration.

**Table 3** Effect of plant extract treatment on blood glucose levels on day 25 after alloxan induction.

| Groups | Dose (mg/kg body weight) | Blood glucose level (mmol/l) |
|---|---|---|
| Intact | healthy control | 4,5 ± 1,5 |
| Model (Alloxan) | 140 | 12.65 ± 0,54 |
| *R.heterodonta* | 75 | 6.35 ± 0.85 |
| *S.marianum* | 75 | 9.56 ± 0.68 |
| *M.nigra* | 75 | 7.26 ± 0.61 |

Note: Data are presented as mean ± standard deviation (SD), n = 3 animals per group. $*p < 0.05$ indicates statistically significant differences between the compared extract groups.

The lowest glucose level was observed in the *R.heterodonta* group (6.35 ± 0.85 mmol/L), followed by *M.nigra* (7.26 ± 0.61 mmol/L) and *S.marianum* (9.56 ± 0.68 mmol/L). These results indicate that the tested extract exhibited the most pronounced hypoglycemic effect among all evaluated extracts at this dose. The observed reductions in blood glucose following treatment with the plant extracts suggest an improvement in metabolic control. Since hyperglycemia is closely linked to oxidative stress [24], the next step was to assess lipid peroxidation by measuring malondialdehyde (MDA) levels, a key marker of oxidative damage in tissues (**Table 4**).

**Table 4** Malondialdehyde (MDA) levels (nmol/g) in rat blood on day 25 after correction with various plant extracts at a dose 75 mg/kg.

| Organs | Brain | Heart | Kidneys | Small intestine | Pancreas | Testes | Liver | Lungs |
|---|---|---|---|---|---|---|---|---|
| **Intact** | 10.94 ± 0.42 | 11.65 ± 0.15 | 9.19± 0.36 | 12.71 ± 0.36 | 8.85 ± 0.41 | 8.44 ± 0.15 | 11.65 ± 0.15 | 12.06 ± 0.31 |
| **Alloxan model** | 18.22 ± 0.21 | 17.43 ± 1.02 | 18.45 ± 0.69 | 19.65 ± 0.36 | 17.33 ± 0.88 | 17.23 ± 0.71 | 17.84 ± 1.08 | 18.18 ± 0.42 |
| ***RH*** | 11.28 ± 1.10 | 14.29 ± 0.78 | 12.68 ± 1.46 | 13.75 ± 0.17 | 12.64 ± 0.42 | 11.82 ± 0.87 | 12.44 ± 0.79 | 12.68 ± 1.39 |
| ***SM*** | 15.79 ± 0.41 | 16.30 ± 0.67 | 15.89 ± 0.10 | 15.00 ± 0.15 | 15.76 ± 0.31 | 15.55 ± 0.15 | 13.88 ± 0.15 | 16.92 ± 0.20 |
| ***MN*** | 14.15 ± 0.57 | 14.25 ± 0.21 | 14.90 ± 0.46 | 14.94 ± 0.61 | 14.70 ± 0.72 | 14.49 ± 0.72 | 14.39 ± 0.51 | 14.12 ± 0.46 |

Note: Data are presented as mean ± standard deviation (SD). Each experimental group consisted of 3 animals. Statistical significance was considered at $*p < 0.05$ compared with the intact group.

As shown in **Table 4**, alloxan administration caused a pronounced increase in malondialdehyde (MDA) levels in all examined organs compared with intact animals, indicating intensified lipid peroxidation. For example, brain MDA increased from 10.94 ± 0.42 nmol/g in intact rats to 18.22 ± 0.21 nmol/g in the alloxan model, while kidney MDA rose from 9.19 ± 0.36 nmol/g to 18.45 ± 0.69 nmol/g.

Treatment with plant extracts significantly reduced MDA concentrations relative to the model group. The most marked effect was observed with *R. heterodonta*, which decreased brain and liver MDA levels to 11.28 ± 1.10 nmol/g and 12.44 ± 0.79 nmol/g, respectively. *M.nigra* also produced notable reductions (brain: 14.15 ± 0.57 nmol/g; liver: 14.39 ± 0.51 nmol/g), whereas *S.marianum* showed a moderate decrease (brain: 15.79 ± 0.41 nmol/g; liver: 13.88 ± 0.15 nmol/g).

While tissue MDA levels reflect overall lipid peroxidation, mitochondria represent a primary intracellular source and major target of oxidative damage under diabetic conditions. Therefore, the next stage of the study focused on evaluating lipid peroxidation in isolated liver mitochondria to further characterize mitochondrial oxidative stress.

Mitochondrial swelling is one of the key markers of oxidative stress and mitochondrial dysfunction, playing a significant role in the development of pathophysiological processes. Under oxidative stress, excessive formation of reactive oxygen species (ROS) leads to damage of lipids, proteins, and DNA, as well as disruption of mitochondrial membrane integrity. This can trigger the opening of the mitochondrial permeability transition pore (mPTP), allowing uncontrolled influx of water and ions into mitochondria, resulting in their swelling [25].

As a result, the mitochondrial membrane potential decreases, impairing ATP synthesis and activating cell death cascades, including apoptosis and necrosis. This process plays a central role in the pathogenesis of various oxidative stress-related diseases, such as neurodegenerative disorders, cardiomyopathies, and diabetes.

Thus, in subsequent series of experiments, a detailed investigation of mitochondrial swelling under conditions of oxidative stress was conducted. Particular attention was paid to evaluating the effects of the extracts on this process, as well as their ability to inhibit lipid peroxidation (LPO), as illustrated in **Figure 4**.

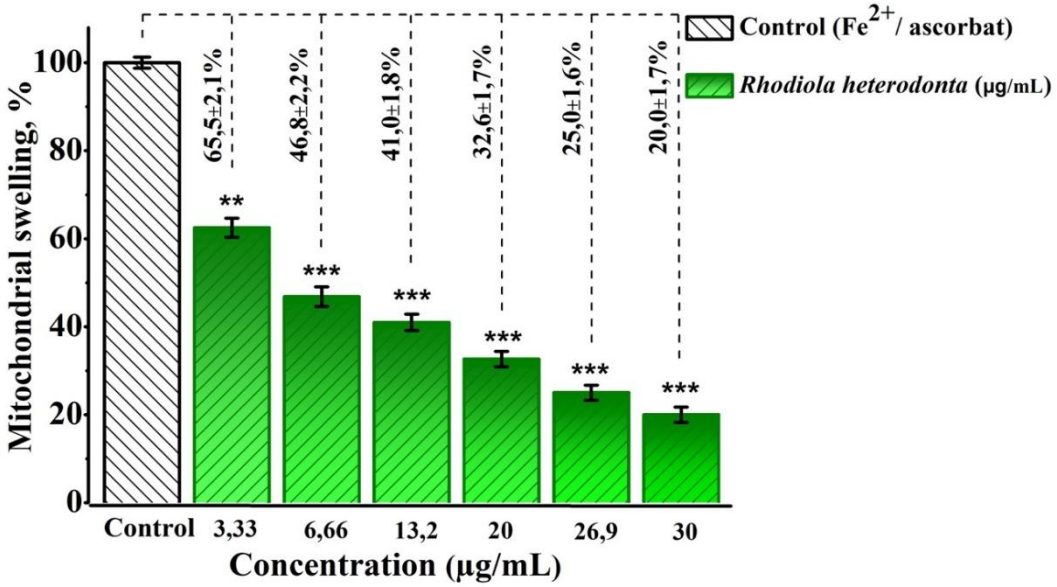



**Figure 4** Effect of the *R. heterodonta* extract on mitochondrial swelling at different extract concentrations. The Y-axis represents the percentage of mitochondrial swelling, while the X-axis indicates extract concentrations (µg/mL). Statistically significant differences between the control group and the *R. heterodonta* extract-treated groups were confirmed at $^{**}p < 0.01$ and $^{***}p < 0.001$, n = 5 - 6. Data are presented as mean ± standard deviation (SD).

The *R.heterodonta* extract dose-dependently reduced mitochondrial lipid peroxidation, with LPO decreasing from 65.5% at 3.33 µg/mL to 20% at 30 µg/mL/mL compared with control. The $IC_{50}$ value (6.04 µg/mL/mL) indicates strong antioxidant activity of the extract.

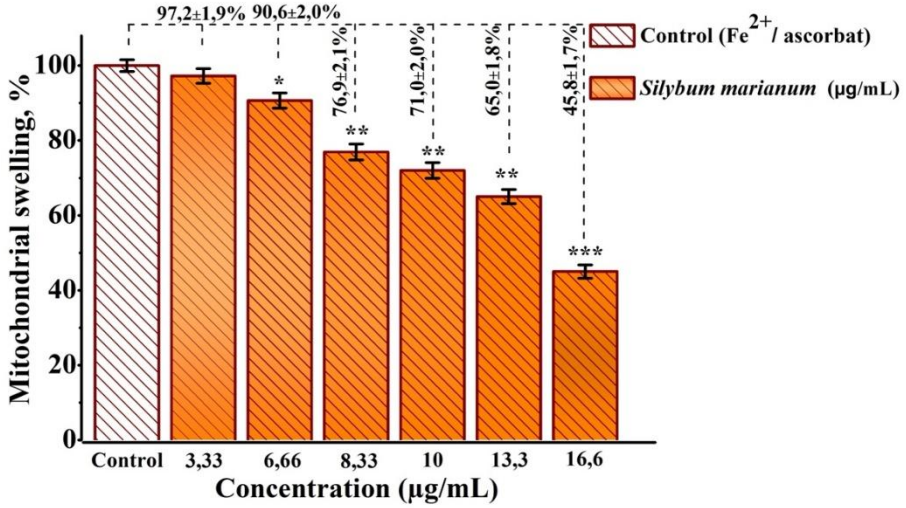



**Figure 5** Effect of the *S.marianum* extract on mitochondrial swelling at different extract concentrations. The Y-axis represents the percentage of mitochondrial swelling, while the X-axis indicates extract concentrations (µg/mL). Statistically significant differences between the control group and the *S.marianum* extract-treated groups were confirmed at $^{**}p < 0.01$ and $^{***}p < 0.001$, n = 5 - 6. Data are presented as mean ± standard deviation (SD).

The *S.marianum* extract reduced mitochondrial lipid peroxidation in a dose-dependent manner (3.33 - 16.6 µg/mL) as illustrated in **Figure 5**. Compared with the control (97%), lipid peroxidation decreased to 90 ± 2.6% at 3.33 µg/mL and reached 45 ± 2.8% at 16.6 µg/mL ($p < 0.05$ - 0.01). The $IC_{50}$ value was 29.3 µg/mL, indicating moderate antioxidant activity of the *S.marianum* extract in this model.

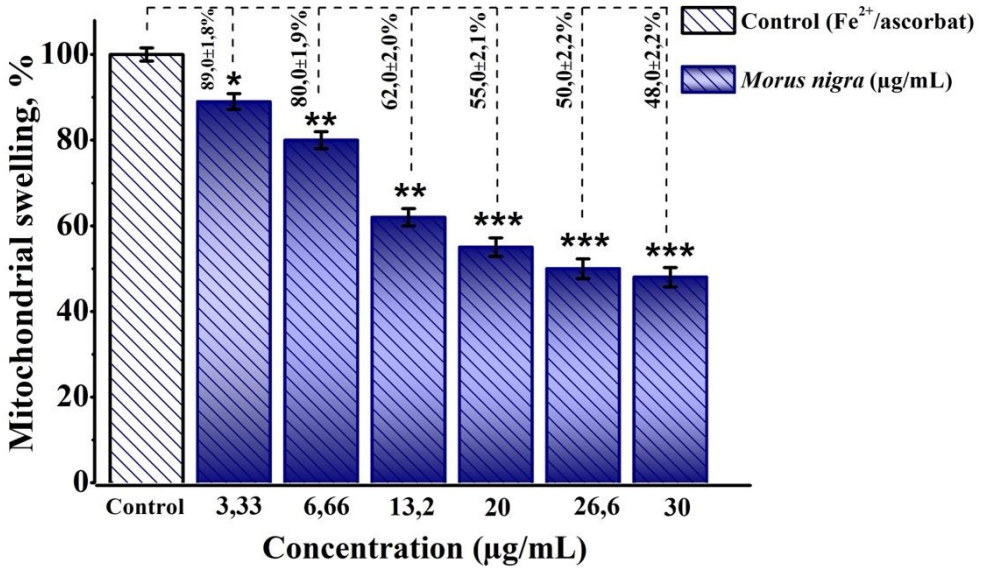



**Figure 6** Effect of the *M.nigra* extract on mitochondrial swelling at different extract concentrations. The Y-axis represents the percentage of mitochondrial swelling, while the X-axis indicates extract concentrations (µg/mL). Statistically significant differences between the control group and the *M.nigra* extract-treated groups were confirmed at $^{**}p < 0.01$ and $^{***}p < 0.001$, n = 5 - 6. Data are presented as mean ± standard deviation (SD).

The effect of the *M.nigra* extract on mitochondrial lipid peroxidation was evaluated at concentrations of 3.33 - 30 µg /mL as shown in **Figure 6**. In the control group ($Fe^{2+}$/ascorbate), lipid peroxidation was 92%. *M. nigra* extract reduced absorbance in a dose-dependent manner: 80% at 6.66 µg /mL, 62% at 13.2 µg /mL, and 48 - 50% at 26.6 - 30 µg /mL. The $IC_{50}$ value was 26.8 µg /mL, indicating moderate antioxidant activity of the *M.nigra* extract in this model.

The detected inhibition of mitochondrial lipid peroxidation suggests that the studied plant extracts may help preserve mitochondrial membrane integrity by reducing oxidative damage. Mitochondria are a major intracellular source of reactive oxygen species, and excessive mitochondrial oxidative stress is known to promote lipid peroxidation and impair cellular function [26].

Overall, *R.heterodonta, S.marianum*, and *M.nigra* extracts inhibited mitochondrial lipid peroxidation in a dose-dependent manner, with *R.heterodonta* exhibiting the strongest effect ($IC_{50}$ = 6.04 µg/mL), *S.marianum* showing moderate activity ($IC_{50}$ = 29.3 µg/mL), and *M.nigra* demonstrating noticeable antioxidant potential ($IC_{50}$ = 26.8 µg/mL), confirming their ability to reduce oxidative stress in mitochondria.

Following the assessment of mitochondrial lipid peroxidation, the study proceeded to evaluate the activity of key antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT). These enzymes constitute major components of the cellular antioxidant defense system, with SOD catalyzing the dismutation of superoxide radicals into hydrogen peroxide and catalase subsequently decomposing hydrogen peroxide into water and oxygen, thereby mitigating oxidative damage. Such enzymatic mechanisms have been demonstrated to play a pivotal role in counteracting oxidative stress in

diabetic conditions and maintaining redox homeostasis [27].

In our experiments, the activity of the antioxidant enzymes SOD and catalase was assessed in various tissues. The results demonstrated a significant decrease in the activity of these enzymes under induced oxidative stress, indicating impairment of the body's antioxidant defense system. Treatment with plant extracts *R.heterodonta, S.marianum*, and *M.nigra* showed the ability to restore the activity of SOD and CAT (**Figure 7**).

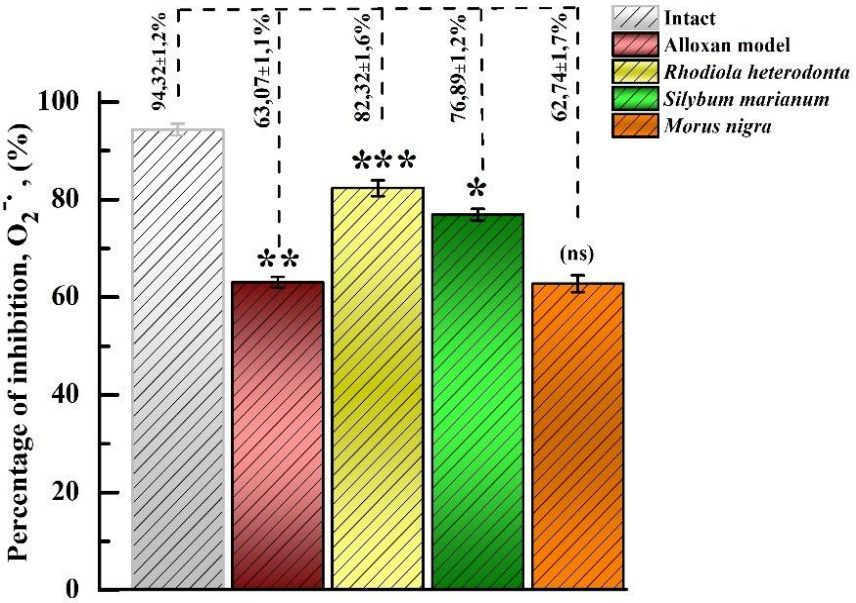



**Figure 7** Effect of plant extract treatment on superoxide dismutase (SOD) activity in liver homogenates of rats. The Y-axis represents SOD activity (% inhibition of $O_2^{\bullet-}$). The values above the bars indicate mean ± SD. The experimental groups included the intact group, alloxan model group, and extract-treated groups. Statistical significance compared with the alloxan model group is indicated as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, while ns indicates no significant difference. Data are presented as mean ± SD (n = 3 - 4).

The figure illustrates the percentage of $O_2^{\bullet-}$ inhibition across the different experimental groups. SOD activity in the intact group (94.32 ± 1.20%) was at its highest, reflecting a strong intrinsic capacity to neutralize reactive oxygen species. In contrast, the model group exhibited a marked reduction in enzyme activity (63.07 ± 1.10%), indicating functional impairment. Among the plant extracts, *R.heterodonta* (82.32 ± 1.60%) produced the most pronounced restorative effect, while *S.marianum* (76.89 ± 1.20%) also enhanced enzyme activity, albeit to a lesser degree. *M.nigra* (62.74 ± 1.70%) showed only a minimal effect, comparable to the model group. Overall, these findings indicate that *R.heterodonta* and *S.marianum* effectively restore SOD activity.

Given the differential modulation of SOD activity by the plant extracts, the study next evaluated CAT activity, another key enzymatic antioxidant. Together, SOD and CAT function synergistically to mitigate oxidative damage by first converting superoxide radicals to hydrogen peroxide and then decomposing hydrogen peroxide into water and oxygen, a mechanism shown to be critically impaired in diabetic oxidative stress models [28]. CAT activity in rat liver homogenates, expressed as percent $H_2O_2$ inhibition, was evaluated across three groups: Intact rats, alloxan-induced oxidative stress rats, and rats treated with *R.heterodonta*, *S.marinum*, and *M.nigra* extracts. CAT activity was markedly reduced in the model group compared to controls, indicating elevated oxidative

stress. Treatment with the extracts restored CAT activity to varying degrees: *R.heterodonta* showed the strongest restoration, particularly at higher concentrations, highlighting its potent antioxidant properties; *S.marinum* induced moderate recovery; *M.nigra* exhibited intermediate effects, surpassing *S.marinum* but remaining less effective than *R.heterodonta* (**Figure 8**).

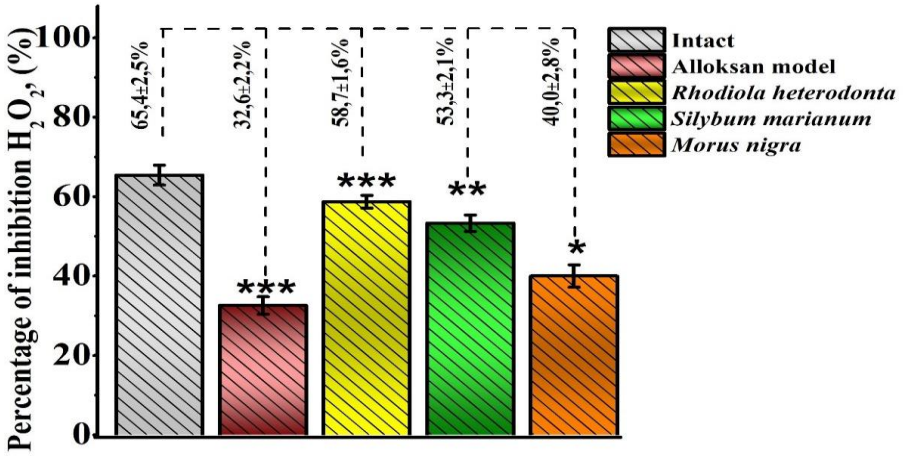



**Figure 8** Effect of plant extract treatment on catalase (CAT) activity in liver homogenates of rats. The Y-axis represents catalase activity expressed as the percentage inhibition of $H_2O_2$. The values above the bars indicate the mean percentage inhibition of $H_2O_2$ ± SD for each experimental group. The experimental groups included the intact group, alloxan model group, and plant extract-treated groups. Statistically significant differences compared with the alloxan model group are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Data are presented as mean ± standard deviation (SD), with 3 - 4 replicates per group.

The figure shows catalase activity in liver homogenates of rats under various experimental conditions. In the control (intact) group, catalase activity was 65.4 ± 2.5%, which represents the highest value among all studied groups. In the model group, a pronounced decrease in catalase activity to 32.6 ± 2.2% was observed, indicating a marked weakening of the antioxidant defense system under alloksan oxidative stress, likely as a consequence of oxidative stress.

The plant extracts produced the following effects: *R.heterodonta* induced the most pronounced increase in catalase activity to 58.7 ± 1.6%, which is close to the control values, indicating a strong antioxidant effect of this extract. *S.marianum* demonstrated catalase activity at 53.3 ± 2.1%, reflecting antioxidant effect. *M.nigra* increased catalase activity to 40.0 ± 2.8%, which was higher than the values observed in the model group.

The variation in catalase activity recovery observed among the investigated plant extracts may be explained by differeces in their phytochemical profiles, particularly the presence of biologically active secondary metabolites with established antioxidant properties. Plant-derived compounds such as flavonoids, phenolic acids, tannins, and anthocyanins have been widely reported to enhance endogenous antioxidant defense systems and reduce oxidative stress through free radical scavenging and modulation of antioxidant enzyme activity [29]. Therefore, to provide additional phytochemical context for the observed biological effects, the major secondary metabolites reported for the studied plant extracts are summarized in **Table 5**.

**Table 5** Quantitative content of biologically active compounds in plant extracts.

| Extracts | Polyphenols (mg GAE/mL) | Terpenoids (mg CE/mL) | Saponins (GlyAE/mL) | Flavonoids (mg QE/mL) | Tannins (ECE/mL) |
|---|---|---|---|---|---|
| *RH* | 0.477 ± 0.002 | 0.179 ± 0.001 | 0.163 ± 0.03 | 0.551 ± 0.04 | 0.068 ± 0.001 |
| *SM* | 0.179 ± 0.02 | — | 0.484 ± 0.08 | 0.284 ± 0.03 | 0.257 ± 0.02 |
| *MN* | 0.175 ± 0.01 | — | 0.413 ± 0.06 | 0.071 ± 0.01 | 0.086 ± 0.003 |

GAE = gallic acid equivalents; CE = cholesterol equivalents; GlyAE = glycyrrhizic acid equivalents; QE = quercetin equivalents; ECE = epigallocatechin equivalents.

Quantitative analysis showed that *R.heterodonta* contained higher concentrations of certain metabolites compared with *M. nigra* and *S. marianum*, reaching the highest value of 0.551 ± 0.04 mg/mL for flavonoids. In this time, *S. marianum* was characterized by high concentrations of saponins (0.484 ± 0.08 mg/mL) and tannins (0.257 ± 0.02 mg/mL), exceeding the corresponding values observed in *R.heterodonta* and *M. nigra*, in turn, demonstrated moderate concentrations of phenolic compounds (0.175 ± 0.01 mg/mL) and saponins (0.413 ± 0.06 mg/mL), along with a lower flavonoid content (0.071 ± 0.01 mg/mL) compared with *R.heterodonta*.

These results are consistent with previous studies reporting that *S.marianum* contains abundant bioactive secondary metabolites, particularly flavonoids, saponins, and phenolic compounds, which contribute to its pronounced antioxidant activity [30]. Similarly, *M.nigra* has been reported to contain phenolic compounds, flavonoids, and other antioxidant phytochemicals associated with free radical scavenging and protective biological effects [31]. Variation in the phytochemical composition among plant species may account for the differences in their antioxidant enzyme-modulating effects observed in the present study.

The present study has some limitations that should be acknowledged. First, the precise molecular mechanisms underlying the observed antioxidant and antihyperglycemic effects were not directly investigated. Second, the study was conducted in an experimental animal model with a relatively limited sample size, which may restrict the generalizability of the findings. In addition, the biological effects were evaluated following short-term treatment, and the individual active phytochemical constituents responsible for the observed protective effects were not isolated or examined separately. Therefore, further studies involving mechanistic investigations, long-term evaluation, and clinical validation are warranted.

**Conclusions**

The present study established that the investigated plant extracts sho low toxicity (classes V–VI), reflecting their relative safety for experimental use. Phytochemical analysis confirmed the presence of bioactive secondary metabolites, including flavonoids, tannins, saponins, and terpenoids, which may contribute to their observed antioxidant properties. The DPPH assay showed that all three extracts possess notable antiradical activity.

Biochemical analyses demonstrated a reduction in blood glucose levels and associated changes in urine parameters, suggesting a potential hypoglycemic effect in the experimental model. In addition extracts reduced malondialdehyde (MDA) levels in tissues, indicating reduction of lipid peroxidation. In addition, improvements in mitochondrial integrity markers, including reduced mitochondrial lipid peroxidation and swelling, suggest a protective effect on cellular structures in experimental conditions.

Moreover, treatment increased the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), indicating enhancement of endogenous antioxidant defense systems. Overall, these findings suggest that the studied plant extracts may exert antioxidant and metabolic protective effects in experimental models, with *R. heterodonta* showing comparatively stronger activity. However, further studies are required to confirm the underlying

mechanisms and potential translational relevance.

Overall, the findings suggest that the studied plant extracts possess notable antioxidant and metabolic protective properties in an alloxan-induced experimental model, with *R. heterodonta* showing the most pronounced activity. Nevertheless, further molecular and mechanistic studies are required to better understand the underlying pathways and to support future translational investigations.

**Declaration of generative AI in scientific writing**

Generative AI tools may be used only to improve the readability and language of a manuscript.

**CRediT Author Statement**

**Abdullaev Azizbek:** Conducted the research and wrote the manuscript. **Toirjon Saydullayev:** Contributed to conceptualization. **Nilufar Kurbanova:** Formal analysis,investigation. **Khurshidbek Mamatov:** Validation, visualization. **Ulug'bek Gayibov:** Participated in the study design, validation, visualization. **Takhir Aripov:** Data curation, project administration, supervision.

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