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Characterization of *Rhodiola heterodonta* (Crassulaceae): Phytocomposition, Antioxidant and Antihyperglycemic Activities

Zaripova MR[1], Gayibova SN[1], Makhmudov RR[1], Mamadrahimov AA[1], Vypova NL[1], Gayibov UG[1], Miralimova SM[2], and Aripov TF[1]

[1]Institute of Bioorganic Chemistry, Academy of Science of Uzbekistan, Tashkent 100125, Uzbekistan
[2]Institute of Microbiology, Academy of Science of Uzbekistan, Tashkent 100128, Uzbekistan

ABSTRACT: Plant extracts have been widely used in traditional medicine to prevent diabetes. The present study aimed to examine the antihyperglycemic properties of an ethanolic extract from *Rhodiola heterodonta* roots. *In vitro* evaluation revealed that treatment with the *R. heterodonta* extract resulted in significant reactive oxygen species inhibition, glucose binding, glucose transporter activation, and suppression of α-amylase and α-glucosidase. Moreover, the treatment with 100 mg/kg of *R. heterodonta* extract dramatically decreased glucose levels in glucose-, alloxan-, or adrenaline-induced diabetic rats. The information gathered in this study bridges the knowledge gap between traditional healers in Uzbekistan who utilize *R. heterodonta* and its potential for future medication development.

Keywords: alpha-amylase, alpha-glucosidase, antioxidants, hypoglycemic agents, qualitative/quantitative determination of compounds

INTRODUCTION

Rhodiola heterodonta (Hook. f. et Thomson) Boriss. is a semi-succulent perennial herb that is generally distributed in the subalpine or subarctic biome of Central Asia (Pamir-Alai) and the Himalayan region of India, Nepal, Bhutan, Central Mongolia, Tibet, Kashgar, and Afghanistan (Khodzhimatov, 2021). *Rhodiola rosea* was the earliest known species, so the genus was named *Rhodiola* ("Rhodiola" is Greek for "rose-like") by Linnaeus (1753). However, in 1772, the species was renamed to *Sedum roseum/rosea* by Italian naturalist Giovanni Antonio Scopoli. Thus, the genus was named as *Sedum* until the early 1930s. In 1855, Hooker and Thomson (1855) described *R. heterodonta* in detail. In the *Journal of the Proceedings of the Linnean Society: Botany* (1857), *R. heterodonta* is named *Sedum heterodontum* ["Sedum" is from the Latin word "sedo" (to sit) and refers to the fact that some *Sedum* species attach themselves to stone] but referred as Rhodiola again in 1859 (Hooker and Thomson, 1859). The term *Sedum* is still used as a synonym for *Rhodiola*, although very rarely. The search with the keyword "*Rhodiola heterodonta*" revealed 219 results in Google Scholar, 3 results in PubMed, and 7 results in the Scopus database, whereas the search with the keyword "*Sedum heterodontum*" revealed 41 results in Google Scholar and 1 result in the Scopus database. Most studies that searched "*Rhodiola heterodonta*" in electron databases are dedicated to the phytochemical profile of *R. heterodonta* extracts and their comparison to other species. Thus, *R. heterodonta* was found to contain a unique and specific phenylethanoid glycoside (heterodontoside) and share compounds, including tyrosol, viridoside, salidroside, and rhodiocyanoside A, with other *Rhodiola* species (Yousef et al., 2006). Wang et al. (2005) used heterodontoside as a unique biochemical marker for *R. heterodonta* species identification through reversed-phase high-performance liquid chromatography (HPLC). The other phytoconstituents of *R. heterodonta* include phenolic (tyrosol methyl ether, mongrhoside) and cyanogenic glycosides (glucoside A) (Bhardwaj et al., 2018), phenylpropanoids (rosarin and rosavin), proanthocyanidins (epigallocatechin gallate, epigallocatechin-epigallocatechin-3-O-gallate, and 3-O-galloylepigallocatechin-epigallocatechin-3-O-gallate) (Yunuskhodjaev and Iskandarova, 2015). Compared with other *Rhodiola* species, the element profile of *R. heterodonta* rhizomes and roots was distinguished by a high content of calcium, potassium, magnesium, copper, iron, zinc, and calcium (Terninko et al., 2022).

Although *R. heterodonta* has a long history, data regard-

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Correspondence to Gayibova SN, E-mail: gayibova.sabina@gmail.com

ing the effect of its therapeutic activity are limited. *Rhodiola* is widely used as an adaptogen in Ayurveda and traditional medicine of most Asian countries. Rinchen and Pant (2014) reported that *R. heterodonta* decoctions are used to cure cough and lung infection in the Indian Himalayan region. Pullaiah et al. (2017) reported that the leaves and tender shoots of *R. heterodonta* are used to prepare a recipe called tantur among tribal communities living in the cold arid regions of Indian Himalaya.

Among the reviews concerning the biological activity of *R. heterodonta*, Bhardwaj et al. (2018) provide comprehensive and up-to-date information.

Aside from them, the *in vivo* studies of Yunuskhodjaev et al. (2015) showed that the oral administration of 100 mg/kg of *R. heterodonta* diminished pain sensitivity by up to 153% using Haffner's method for testing analgesic drugs. The intraperitoneal administration of 40 mg/kg of dry *R. heterodonta* increased the antihypoxic effect by up to 6 min (~34%). Moreover, the presence of salidroside was proposed to be responsible behind the main mechanism of action of dry *R. heterodonta*. Kurmukov et al. (2011) showed that dry *R. heterodonta* extract restored bile secretion in rats with chronic toxic hepatitis. Approximately 75%~80% *R. heterodonta* ethanol extract demonstrated strong antihypoxic (up to 192%) (Grace et al., 2009), hypothermic (−2.1°C in 30 min), and analgesic effects in mice, suggesting the presence of adaptogenic properties (Yunuskhodjaev et al., 2014). The long-term study (up to 3 months) of oral *R. heterodonta* extract administration (up to 200 mg/kg) did not reveal any toxicity symptoms in experimental animals (Yunuskhodjaev et al., 2014). However, there still might be more biological activities of *R. heterodonta* that have not been fully studied, and there might be many more potential therapeutic properties waiting to be discovered. Thus, the present study aimed to observe the potential antidiabetic effects of ethanolic root extract of *R. heterodonta* grown in Uzbekistan.

MATERIALS AND METHODS

Plant extraction

Dried and ground *R. heterodonta* roots were provided by BIOTON Ltd. The plants were collected in August 2020 in a mountainous region (locality Angren and Brichmulla, Uzbekistan, 1,200~1,400 m.a.s.l.). 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%.

Animal experiments

All animal experiments complied with the European Directive 2010/63/EU on protecting animals used for scientific purposes (European Union, 2010). The study protocol was approved by the Animal Ethical Committee based on the Institute of Bioorganic Chemistry, AS RUz (Protocol Number: 133/1a/h, dated August 4, 2014).

Phytochemical analysis

The crude ethanol extract of *R. heterodonta* L. roots was diluted in distilled water and then tested for the presence of flavonoids (Hossain et al., 2013), carbohydrates (Sorescu et al., 2018), saponins (Gul et al., 2017), phenols (Apostică et al., 2018), protein (Pant et al., 2017), terpenoids (steroids) (Das et al., 2014), alkaloids (Kancherla et al., 2019), tannins (Yadav et al., 2017), and cardiac glycosides (Gul et al., 2017). The qualitative results are expressed as positive and negative for the presence and absence of phytochemicals.

Quantitative spectrophotometric phytochemical analysis

The quantity of secondary metabolites including total flavonoids (Fattahi et al., 2014), total saponins (Senguttuvan et al., 2014), total terpenoids (Panchal and Charuben, 2021), total phenols (Aryal et al., 2019), total tannins (Batool et al., 2019), and total reducing sugars (TRS) was measured. TRS was assessed using the glucose oxidase/peroxidase (GOD/POD) method.

HPLC analysis

Five grams of *R. heterodonta* extract sample was carefully weighed and dissolved in 300 mL of distilled water. Then, 50 mL of 70% ethanol was added. The mixture was stirred for 1 h at 40°C~50°C and then for 2 h at room temperature, and centrifuged at 3,500 *g* for 20 min. The supernatant was collected, and the extraction was repeated twice. The supernatants were filtered, combined, volumetrically increased to 100 mL with 70% ethanol, and subjected to HPLC. Approximately 0.1% trifluoroacetic acid, acetate buffer, and acetonitrile were used in the analysis.

The chromatographic conditions for separation are as follows: Agilent HPLC 1260 Infinity autosampler; HPLC Column Eclipse, XDB-C18, 80, 5 μm, 4.6×250 mm; DAD, 247, 254, 276 nm; flow rate 1 mL/min; eluent phosphate buffer:acetonitrile: 0~5 min 95:5, 6~12 min 70:30, 12~13 min 50:50, 13~15 min 95:5; thermostat 30°C; volume of sample 10 μL; column temperature 30°C.

Liquid chromatography-mass spectrometry (LC/MS) analysis

The mass spectra of HPLC fractions were obtained by electrospray ionization mass spectrometry (electrospray) using a 6420 Triple Quad LC/MS mass spectrometer (Agilent Technologies). The mass spectra were recorded

using negative ionization. The following parameters of the mass spectrometer were selected: scanning range 30～1,100 m/z, desiccant gas flow rate 4 L/min, gas temperature 350°C, gas pressure at the nebulizer needle 20 psi, evaporator temperature 350°C, voltage to the coronary needles 4 microamps, voltage on capillary 4,500 V.

Antioxidant activity

The antioxidant activity was determined using the following assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Gayibova et al., 2019), lipid peroxy radical (LOO·) inhibition (Upadhyay et al., 2014), hydroxyl radical (·OH) scavenging (Wei et al., 2020), superoxide anion radical ($O_2^{\cdot-}$) scavenging activity (Zargar et al., 2014), nitric oxide (·NO) scavenging activity (Govindarajan et al., 2003), and hydrogen peroxide (H_2O_2) scavenging activity (Al-Amiery et al., 2015). The percent inhibition was calculated using the following formula (1):

$$\% \text{ inhibition} = [(1 - \text{absorbance of sample}) / \text{absorbance of control}] \times 100\% \quad (1)$$

A control was prepared without adding extract, and the half maximal inhibitory concentration (IC_{50}) was calculated through GraphPad Prism software (n=10) ($P<0.001$).

Acute toxicity test

An acute toxicity test was performed on 30 mature white outbred rats (male, 200±20 g) in accordance with OECD guidelines for the testing of chemicals (OECD. 2001) and following the method of Ugwah-Oguejiofor et al. (2019) with some modifications. The animals were randomly divided into five groups, with six animals in each group. The extract was orally administered to the animals in doses of 4,000, 5,000, 6,000, 8,000, and 10,000 mg/kg. The animals were monitored for toxicity signs (e.g., general health status, possible convulsions, death) hourly during the first day of the experiment in the laboratory and then daily for 2 weeks under vivarium conditions.

Hypoglycemic activity

Hyperglycemia was induced by glucose, adrenaline, and alloxan. Fifty-four mature white outbred rats (male, 200± 20 g) were divided into three groups for the glucose, adrenaline, and alloxan tests, with 18 animals in each group. Every group was subdivided into three (six animals in each subgroup): one subgroup for intact animals and two subgroups for experiments. All animals were weighed before the investigation, and the initial blood sugar level of all animals in each group was averaged and determined as a control. Blood was drawn from the tail of rats under conditions that prevented excessive animal excitement. Sixty minutes before hyperglycemia induction, *R. heterodonta* extract was orally introduced to experimental animals using a gastric catheter in doses of 100 and 200 mg/kg. The glucose group received a single intragastric administration of a hypertonic glucose solution in a dose of 5,000 mg/kg. The adrenaline group received an intraperitoneal injection of adrenaline in a single dose of 50 mg/kg. The alloxan group received intraperitoneal injections of alloxan in a dose of 100 mg/kg. In each group, intact animals received distilled water only and were not subjected to hyperglycemia. The blood glucose level (mM) was determined using a Cypress Diagnostics test kit.

Determination of the inhibitory activity of samples against α-amylase *in vitro*

The inhibitory activity of *R. heterodonta* extract on α-amylase was determined in homogenates derived from the pancreas and small intestine of mature rats in accordance with the method of Zaripova et al. (2022). *R. heterodonta* extract was used in doses of 2～24 μg/mL. The results were expressed as milligram of starch broken down by 1 mg of protein per 1 min (mg/min/mg protein).

***In vitro* α-glucosidase inhibition study**

Rat small intestinal tissue homogenate was employed as an enzyme source. A section of the small intestine was removed, properly cleaned, dried on blotting paper, weighed, and then homogenized in a glass Teflon homogenizer at 1,400 *g* for 30 min with precooled phosphate buffer saline. The final volume of the supernatant was kept at 20% (w/v). The spectrophotometric assay method was used in accordance with the method of Kajaria et al. (2013) with slight modifications. Here, 40 μL of tissue homogenate was mixed with *R. heterodonta* extract in doses 0.01～5.00 mg/mL and incubated for 15 min at 37°C. Then, 280 μL of maltose (37 mM) was added, and the mixture was again incubated for 30 min. Finally, the tubes were submerged in hot water for 10 min to halt the process. The tubes were centrifuged, and a Cypress Diagnostics test kit (Germany) was used to determine the glucose concentration in the supernatant. Percent inhibition was calculated using Eq. (1), where untreated tissue homogenate was used as an absorbance control.

Evaluation of yeast cell glucose absorption *in vitro*

A 10% (v/v) suspension of commercial baker's yeast was made by repeatedly centrifuging the yeast (3,000 *g*, 5 min) in distilled water until the supernatant fluids were clear. Next, various extract concentrations (1～5 mg/mL) were added to 1 mL of glucose solution (5, 10, and 25 mM), and the mixture was then incubated for an additional 10 min at 37°C. The reaction was initiated by adding 100 μL of yeast suspension, vortexing, and further incubating at 37°C for 60 min. After 60 min, the tubes were

centrifuged (2,500 *g*, 5 min), and glucose was estimated in the supernatant (Pitchaipillai and Ponniah, 2016). The percentage increase in glucose uptake by yeast cells was calculated using Eq. (1), where the absorbance of the control contained all reagents except the test sample.

Evaluation of glucose absorption of the *R. heterodonta* extract *in vitro*

The glucose adsorption capacity was determined in accordance with the method of Rehman et al. (2018) with slight modifications. Briefly, 2 mL of extract in varied concentrations (10, 8, 6, 4, and 2 mg/mL) were mixed with varied glucose concentrations (2, 25, and 55 mM) and incubated at 37°C in the High Precision Constant Temperature Incubator (FAITHFUL Instrument (Hebei) Co., Ltd.) for 6 h. Then, the mixtures were centrifuged (1,400 *g*, 20 min), and the glucose content in the supernatant was measured using the GOD/POD method. The bound glucose content was calculated using the following formula (2):

$$Gb=(G1-G6)/\text{sample weight} \quad (2)$$

where Gb is the amount of bound glucose, G1 is the initial glucose concentration, and G6 is the glucose concentration after incubation.

Statistical analysis

GraphPad Prism 8.0.1 (ANOVA and unpaired *t*-test) was used for statistical analysis. The result was considered significant if the *P*-value was 0.05 or lower ($P \leq 0.05$).

RESULTS

Preliminary phytochemical screening

Our preliminary phytochemical analysis of crude ethanol extract of *R. heterodonta* root samples revealed the presence of secondary metabolites, including flavonoids, saponins, phenols, tannins, and terpenoids, and reducing sugars. No proteins, alkaloids, or cardiac glycosides were detected.

Quantitative analysis

The *R. heterodonta* extract was examined for its total flavonoid, saponin, tannin, phenol, and terpenoid content (Table 1). The total tannin content was estimated using epigallocatechin and expressed as 0.308±0.015 μg/eq epigallocatechin at 100 mg/DW. The results were derived from a calibration curve of epigallocatechin (y=0.3629x +0.2201; R^2=0.982). The total phenolic content was estimated using gallic acid and expressed as 255.00±10.75 mg/eq gallic acid at 100 mg/DW. The results were derived from a calibration curve of gallic acid (y=0.00081x +0.0151; R^2=0.985). The total saponin content was estimated using glycyrrhizic acid and expressed as 0.44± 0.02 μg/eq glycyrrhizic acid at 100 mg/DW. The results were derived from a calibration curve of glycyrrhizic acid (y=1.1807x+0.2489; R^2=0.989). The total flavonoid content was estimated using rutin and expressed as 192.00 ±9.40 mg/eq rutin at 100 mg/DW. The results were derived from a calibration curve of rutin (y=0.0826x+ 0.001; R^2=0.9776). The total terpenoid content was estimated using ecdysterone and expressed as 111.00±6.55 μg/eq ecdysterone at 100 mg/DW. The results were derived from a calibration curve of ecdysterone (y= 0.00081x+0.0151; R^2=0.985). The TRS content was estimated using glucose and expressed as 0.016±0.001 μg/eq glucose at 100 mg/DW.

HPLC analysis

On the basis of the analysis of actual *R. heterodonta* extract chromatograms, the chromatographic profile was complex (Fig. 1). According to the obtained results, the extract contained two main flavonoid fractions: 5.01% of fraction-1 (3.265 min) and 86.955% of fraction-2 (16.872 min).

LC/MS analysis

Preliminary identification of the peaks in LC/MS chromatograms was conducted with the help of available mass spectra m/z databases (including MassBank of Europe & National Library of Medicine) and related literature reviews (Han et al., 2016; Zakharenko et al., 2021) (Fig. 2). The identified peaks were as follows:

(1) Epicatechin 289.9 [M-H], M=290.268 (fraction 2)
(2) (-)-Epigallocatechin-3-O-gallate-(4β-S) benzylthioether 581 [M-H], M=580 (fraction 2)
(3) Salidroside 281.9 [M-H], M=300.4 (fraction 1)

Table 1. Quantitative spectrophotometric phytochemical analysis of *Rhodiola heterodonta* extract

Total flavonoids content (mg/eq rutin 100 mg/DW)	Total saponins content (μg/eq glycyrrhizic acid acid 100 mg/DW)	Total tannins content (μg/eq epigallocatechin 100 mg/DW)	Total phenolics content (mg/eq gallic acid at 100 mg/DW)	Total terpenoids content (μg/eq ecdysterone 100 mg/DW)
192.00±9.40	0.44±0.02	0.308±0.015	255.00±10.75	111.00±6.55

Values are presented as mean±SE.
n=10, $P \leq 0.001$.

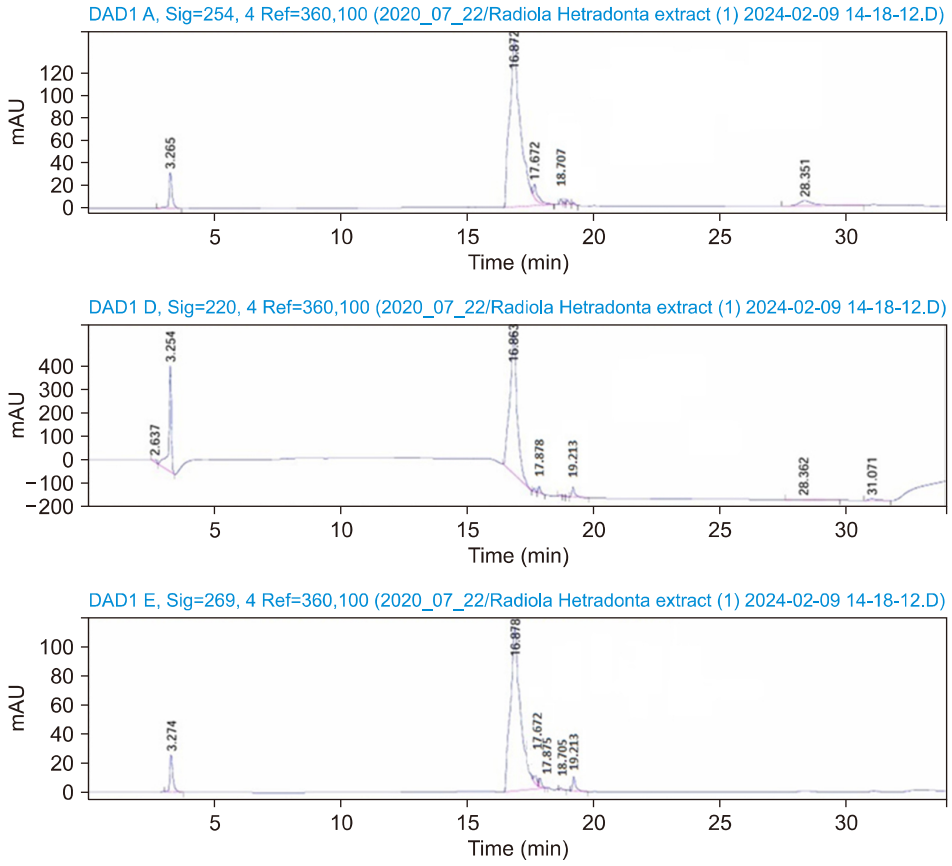

Fig. 1. High-performance liquid chromatography chromatograms of the *Rhodiola heterodonta* extract.

Antioxidant activity

Radical scavenging activity was observed using several *in vitro* protocols. Table 2 summarizes the *in vitro* antioxidant capacity of toothed Rhodiola extract expressed as IC_{50} mg/mL.

The antiradical and antioxidant activities of *R. heterodonta* extract were studied *in vitro* using DPPH radical, LOO·, ·OH, $O_2^{\bullet-}$, ·NO, and hydrogen peroxide (H_2O_2). The scavenging effect was expressed as IC_{50}. *R. heterodonta* exhibited the strongest scavenging effect against DPPH radical (0.01 mg/mL), followed by LOO· (0.09 mg/mL), ·OH (0.77 mg/mL), and superoxide anion (0.85 mg/mL). The least inhibited radicals were ·NO and H_2O_2.

Oral acute toxicity test

Several hours (1～4 h) after the administration of *R. heterodonta* extract, the animals experienced tachycardia. Mice also demonstrated some signs of stress, including squinted eyes and tendency to bunch. Mortality was observed after 24～48 h, and the mortality rate and acute toxicity of *R. heterodonta* extract increased as the dose increased from 5,000 to 10,000 mg/kg. The mortality rate was used to calculate the lethal doses (LD_{16}, LD_{50}, and LD_{84}) of *R. heterodonta* extract, which were 5.2, 7.0, and 9.8 g/kg, respectively. Thus, the *R. heterodonta* extract was categorized as a category V chemical, which is of relatively low acute toxicity according to the guideline for testing of chemicals (OECD, 2001).

In vivo hypoglycemic activity

As a result of oral glucose administration in animals, there was a significant increase in serum blood glucose levels, which was a sign of hyperglycemia (Fig. 3). *Rhodiola* reduced the blood sugar levels of experimental animals to 5.6±0.4 and 5.1±0.4 mmol/L at doses of 100 and 200 mg/kg; its hypoglycemic effect was 35.6% and 41.4%, respectively.

The introduction of alloxan in the control group caused an increase in blood glucose in experimental animals by 135% after 1 h (from 4.0±0.4 mmol/L to 9.4±0.7 mmol/L). After 5 days, the glucose content increased by 76% (7.1±0.6 mmol/L).

The introduction of adrenaline in the control group after 1 h caused an increase in blood sugar level by 188.6% (from 3.5±0.3 mmol/L to 10.1±0.7 mmol/L). *R. heterodonta* reduced the blood sugar level of experimental animals to 5.5±0.4 and 6.3±0.5 mmol/L at doses of 100 and 200 mg/kg; its hypoglycemic effect was 35.6% and 41.4%, respectively.

α-Amylase and α-glucosidase inhibition study

The specific *in vitro* activity of α-amylase in the homogenate derived from the pancreas and small intestine of rats decreased linearly with the addition of *R. heterodonta* extract compared with the control (Fig. 4). The enzyme activity in the homogenates showed the greatest decrease (～29%) at a dose of 100 mg/mL compared with untreat-

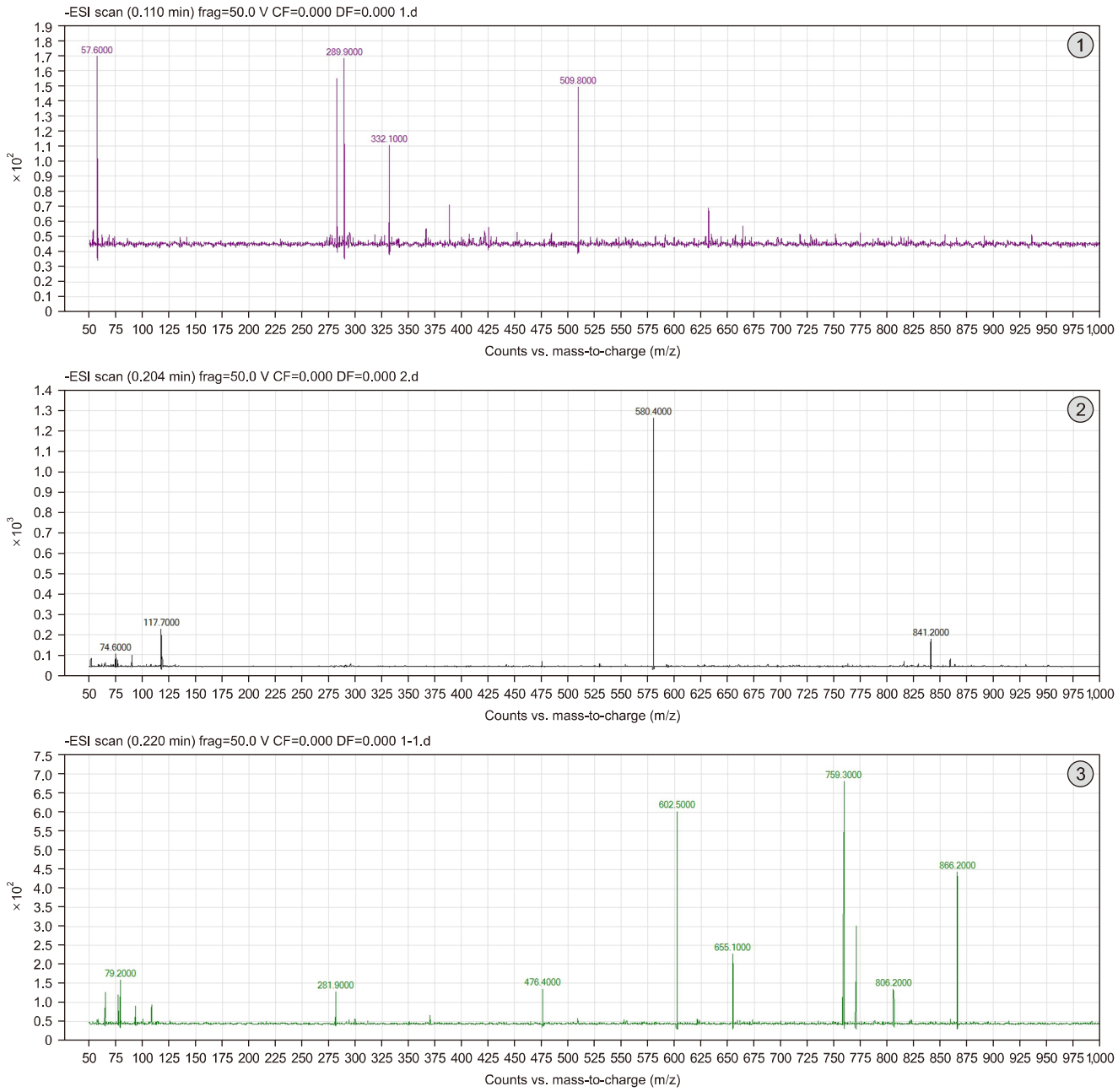

Fig. 2. Liquid chromatography-mass spectrometry chromatograms of fractions 1 and 2 from the *Rhodiola heterodonta* extract.

Table 2. *In vitro* radical scavenging activity of *Rhodiola heterodonta* extract

IC_{50} (mg/mL)					
DPPH	LOO·	·OH	$O_2^{\bullet -}$	·NO	H_2O_2
0.01±0.00	0.09±0.00	0.77±0.04	0.85±0.04	4.34±0.22	33.00±1.65

Values are presented as mean±SE.
n=10, P<0.001.
IC_{50}, half maximal inhibitory concentration; DPPH, 2,2-diphenyl-1-picrylhydrazyl; LOO·, lipid peroxy radical; ·OH, hydroxyl radical; $O_2^{\bullet -}$, superoxide radical; ·NO, nitric oxide; H_2O_2, hydrogen peroxide.

ed homogenates.

The *R. heterodonta* extract was effective against inhibiting α-glucosidase activity. Fig. 5 shows the result of α-glucosidase inhibition by different *R. heterodonta* concentrations (0.03~2.20 mg/mL). The reference drug acarbose was more effective in inhibiting α-glucosidase, and a dose of 0.45 mg/mL caused 86% α-glucosidase inhibition.

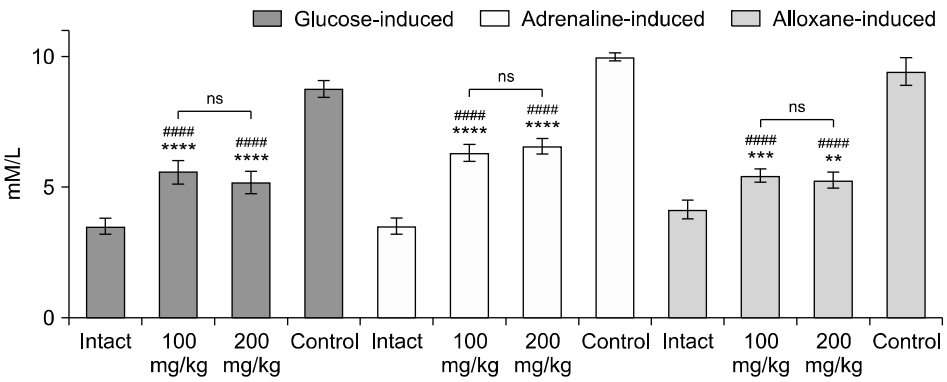

Fig. 3. Improvement of glucose tolerance of diabetic rats treated with glucose, adrenaline, and alloxan (n=6, error bars, mean±SE). One-way ANOVA was used for the test ($P \leq 0.05$). Statistical differences are indicated with asterisks for comparisons between intact and experimental animals and hash tags for comparisons between control and experimental groups. Asterisk is used to compare with the intact group, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$. Hashtag is used to compare with the control group, $^{\#\#\#\#}P<0.0001$. ns, no significant difference.

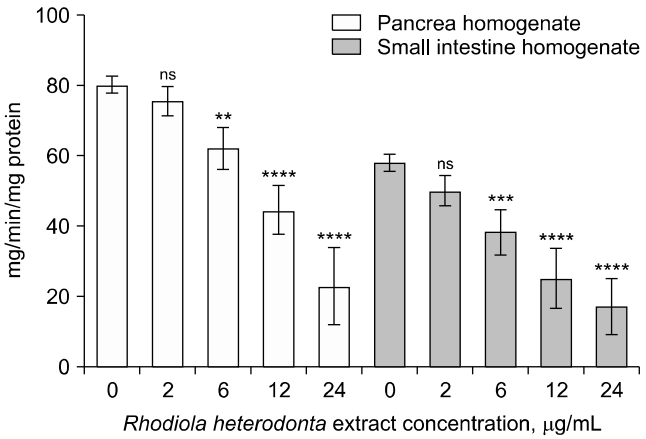

Fig. 4. α-Amylase activity of *Rhodiola heterodonta* extract in pancreas and small intestine homogenates (n=6, error bars, mean±SE). Unpaired Student's *t*-test was used ($P \leq 0.05$). Statistical differences are indicated with asterisks for comparisons of samples that were not treated with *R. heterodonta* extract. $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$. ns, no significant difference.

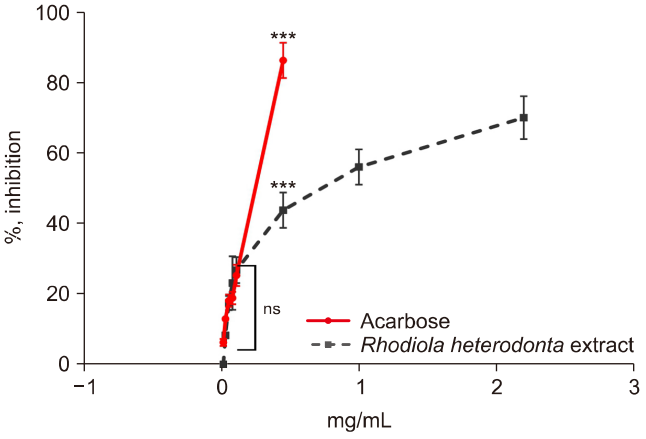

Fig. 5. α-Glucosidase inhibitory activity of *Rhodiola heterodonta* extract and standard drug acarbose (n=6, mean±SE). Unpaired Student's *t*-test was used ($P \leq 0.05$). Statistical differences between acarbose and *R. heterodonta* extract are indicated with asterisks. $^{***}P<0.001$. ns, no significant difference.

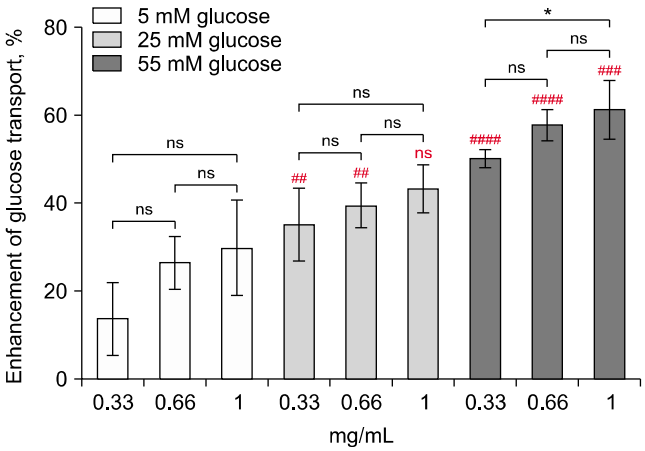

Fig. 6. Effect of *Rhodiola heterodonta* extract on glucose uptake by yeast cells at different glucose concentrations (n=10). The x-axis represents the dose of *R. heterodonta* extract (n=10, error bars, mean±SE). One-way ANOVA was used for the test ($P \leq 0.05$). Statistical differences are indicated within each group (black color) and between the same concentrations of groups (red color). $^{*}P<0.1$. Hashtag is used to compare to 5 mM Glucose; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, and $^{\#\#\#\#}P<0.0001$. ns, no significant difference.

Yeast glucose absorption

After the yeast cells were treated with *R. heterodonta* extract, no dose-dependent glucose uptake was observed. The percentage of glucose uptake by yeast cells increased with glucose concentrations (5, 25, and 55 mM) in the presence of 0.33, 0.66, and 1 mg/mL of *R. heterodonta* extract. The highest concentration of *R. heterodonta* extract (1 mg/mL) exhibited the highest activity at all glucose concentrations and showed the maximum increase (60%) in the presence of 55 mM glucose (Fig. 6).

R. heterodonta glucose absorption

The *R. heterodonta* extract did not show activity in binding glucose. At the same time, there was a slight increase in the sugar content in the mixture with an increase in *R. heterodonta* concentration (Fig. 7), which may be be-

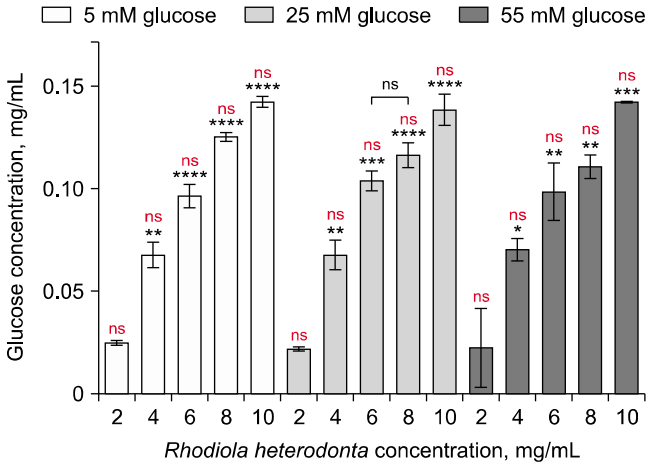

Fig. 7. Glucose binding ability of the *Rhodiola heterodonta* extract (n=10, error bars, mean±SE). One-way ANOVA was used for the test ($P \leq 0.05$). Statistical differences are indicated within the concentrations of each group (black color) and the same concentrations of different groups (red color). ns, no significant difference. Asterisk is used to compare with the minimal concentration (2 mg/mL); $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$.

cause of the presence of a certain amount of sugars in the extract, as presented in Table 1. However, these results do not adversely affect the hypoglycemic activity of *R. heterodonta* mediated by other mechanisms.

DISCUSSION

A persistent hyperglycemic state has long been recognized as the root cause of the chronic abnormalities found in diabetes. Postprandial hyperglycemia is a well-known risk factor related to the development of macrovascular and microvascular complications of type 2 diabetes mellitus. Several studies showed that managing postprandial hyperglycemia is critical for establishing glycemic control. Some evidence suggests that some *Rhodiola* species may help decrease postprandial (after-meal) glucose levels (Kim et al., 2006; Yue et al., 2022). In this study, the ethanol root extract of *R. heterodonta* displayed a blood-glucose-lowering effect at a dose of 100 mg/kg. After treatment with 200 mg/kg, the postprandial glucose levels remained almost unchanged, suggesting that the dose of 100 mg/kg is the maximum concentration. Inhibiting carbohydrate-hydrolyzing enzymes, including the major enzyme responsible for the conversion of starch into simple sugars that can be absorbed by the small intestine, is one of the therapeutic options for decreasing postprandial hyperglycemia. Recent studies demonstrated that plant polyphenols alleviate postprandial blood glucose levels by blocking relevant carbohydrate-hydrolyzing enzymes (α-amylase and α-glucosidase) (Ćorković et al., 2022), increasing glucose uptake by the muscles (Zhao et al., 2020), and protecting body cells from free radical damage (Gao et al., 2009). Thus, the ability of *R. heterodonta* to inhibit enzymes is crucial in regulating postprandial glucose. In the present study, the *R. heterodonta* extract inhibited α-amylase activity in a dose-dependent manner (up to 70% at 24 μg/mL). We also observed 70% inhibition of α-glucosidase activity (*in vitro*) with the minimum dose of 2.2 mg/mL, suggesting that the polyphenolic chemicals in the extracts contributed to α-amylase inhibition rather than α-glucosidase inhibition. Morales et al. (2018) reported that ethyl gallate and gallic acid are potential α-glucosidase inhibitors. The potent antidiabetic effect of this extract might be related to the high content of phytochemical compounds, including tannins (0.308 μg in terms of 100 g epigallotechingallte). In a subacute study, potential plant extracts showed antihyperglycemic activity after 12 h (Ayele et al., 2021). The *R. heterodonta* extract did not show the ability to bind glucose effectively but could control hyperglycemia in the short term, which offers alternative options for first-line dietary therapy. Our *in vitro* results showed that the *R. heterodonta* extract is involved in the stimulation of glucose uptake through glucose transporters.

Alloxan is a common drug used to mimic diabetes. An experimental study of the antihyperglycemic effects of crude extracts in alloxan-induced diabetic animals was conducted to establish scientific proof for the utility of this extract for treating diabetes mellitus. In this investigation, the *R. heterodonta* extract (dose: 100 mg/kg body weight) effectively lowered glucose levels in diabetic rats. The antidiabetic effect in alloxan-induced diabetic rats was not dose-dependent and not statistically significant at a dose of 200 mg/kg, indicating that a new equilibrium between bioactive phytochemical compounds and their molecular targets of action was reached at 100 mg/kg when the active centers of regulatory enzymes are saturated; therefore diffusion of bioavailable forms of phytochemical compounds and their distribution in tissues of experimental animals reached the effective level. The short treatment duration of the experimental animals with *R. heterodonta* extract may represent another shortcoming for this study. On the whole, the oral administration of potential antidiabetic extracts was manifested in the long term. Thus, the *R. heterodonta* extract can be considered as an excellent candidate to immediately bring down blood sugar levels. Furthermore, the diabetogenic activity of alloxan is characterized by an increase in reactive oxygen species (ROS). In all studies, the crude extract of *R. heterodonta* roots displayed the best DPPH scavenging activity (0.01 mg/mL IC_{50}) compared with other radicals. The ability of antioxidants to transfer hydrogen is related to their DPPH activity. The donation of a hydrogen atom is critical in demonstrating antioxidant activity by breaking the free radical chain. In this study, the *R. heterodonta* extract demonstrated substantial LOO·

inhibitory action, indicating that *R. heterodonta* has a cell-protective effect. The drop in glucose levels suggests that *R. heterodonta* may exert a particular effect on pancreatic cells. In alloxan-induced diabetic rats, ROS including ·NOs and ·OHs, as well as pro-oxidants including hydrogen peroxides, are produced. The results of *in vitro* superoxide anion and ·OH, NO, and hydrogen peroxide scavenging potentials showed that *R. heterodonta* is a strong OH and $O_2^{\bullet -}$ scavenger. Controlling postprandial hyperglycemia and ROS inhibition have often been suggested as important measures in the treatment of diabetes mellitus. The generation of lipid peroxides by free radical derivatives is one of the primary mechanisms of diabetes-induced damage (Ben Salem et al., 2017). Consequently, antioxidant activity or the prevention of free radical formation is vital in the protection against diabetes-induced organ damage.

The results of our study are in line with recently published data, which suggest that polyphenol-enriched plant extracts preserve and protect the pancreas through their strong antioxidative capacity (Habtemariam and Varghese, 2017). This could ultimately lead to enhanced pancreatic function and improved insulin secretion.

Persistent hyperglycemia in individuals with diabetes can also be influenced by stress. Stress does not cause diabetes, but it can affect blood sugar levels by disturbing glucose hemostasis, misregulating gluconeogenesis, and disrupting GLUT-4 functioning (Sharma et al., 2022). These findings are in line with the study of Li et al. (2019), which showed the ability of epigallocatechin gallate to regulate glucose homeostasis by repressing gluconeogenesis and glycogenolysis. Even though tannins were not a predominant part of secondary metabolites in the extract studied in our work, Grace et al. (2009) found that tannins are a part of ethanol extract.

The phytochemical composition showed that herbal ethanolic extracts contain bioactive molecules, including polyphenols, flavonoids, tannins, and terpenoids, that contribute to antioxidant activity. Among the major classes of phytochemicals found in plants, flavonoids are naturally occurring phenolic compounds with strong antioxidant properties because of the presence of aromatic hydroxyl groups. Some flavonoid compounds were identified in the *R. heterodonta* extract by HPLC and LC/MS. Epicatechin and its derivatives, which have the ability to reduce fasting blood glucose, improve glucose tolerance, protect against oxidative damage, and preserve β-cell integrity, have been reported to be abundant in *Rhodiola* species (Han et al., 2016). No reports were found regarding the effect of salidroside, a major component and the most potent constituent, on α-glucosidase and α-amylase and inhibition and GLUT activation. Our preliminary in silico data showed that salidroside does not conformationally suit the enzyme active sites (data not published). However, salidroside can regulate glucose metabolism through AMPK-related signaling pathways (Ju et al., 2017). Thus, the synergistic biological activity of different *R. heterodonta* extract compounds could attract considerable attention. These results explained the ability of ethanol extract from *R. heterodonta* roots to reduce blood glucose levels, which could be attributed to (i) an inhibition of digestive enzymes, (ii) activation of glucose transport, and (iii) ROS scavenging. This provided evidence in favor of the view that *R. heterodonta* ethanol extract could contribute to nutritional strategies for preventing and managing type 2 diabetes mellitus.

With a consumer-driven "return to nature," scientific validation and valuation of herbal medicines, including their efficacy and safety, are critical. Our findings reveal the antihyperglycemic activities of endemic *R. heterodonta* and enrich ethnomedicinal knowledge that surely benefits further research.

Conclusion

This study investigated the antihyperglycemic and antioxidant effects of an ethanolic extract from *R. heterodonta* roots. *In vitro* analyses demonstrated that the extract can inhibit ROS, bind glucose, activate glucose transporters, and suppress α-amylase and α-glucosidase enzymes. Phytochemical screening revealed that the extract contains flavonoids, saponins, phenols, tannins, and terpenoids, which may contribute to its antidiabetic properties. Further HPLC analysis identified specific compounds within the extract. Animal studies using diabetic rats showed that the *R. heterodonta* extract significantly reduces blood glucose levels, supporting its potential role in diabetes management. The extract also exhibits effective antioxidant activities, which are crucial in preventing diabetes-induced organ damage. The results of *in vivo* and *in vitro* evaluation suggest *R. heterodonta*'s potential for developing future diabetes medications, underscoring the therapeutic promise of natural antioxidants and plant-derived compounds in disease management. This research underscores the importance of scientifically validating the efficacy and safety of herbal remedies. The findings indicate that the *R. heterodonta* extract is a promising agent for further investigation and potential therapeutic application.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: ZMR. Analysis and interpretation: GSN, MRR, MAA, VNL. Data collection: MRR, MAA, GUG. Writing the article: ZMR. Critical revision of the article: ATF, MSM. Final approval of the article: all authors. Statistical analysis: ZMR. Obtained funding: ATF, MSM. Overall responsibility: ZMR.

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