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Original Article
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Antidiabetic Effect of Standardized *Chrysanthemum rubellum* Hydroethanolic Extract by Targeting α-Glucosidase and the PTP-1B Signaling Pathway for Alleviating Diabetes in Experimental Model

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Objectives: The study's goal was to find out whether *Chrysanthemum rubellum* extract has anti-diabetic properties by concentrating on α-glucosidase and the PTP-1B signaling pathway. *C. rubellum* flowers were used for extraction using Methanol/water (80/20) as solvent.
Methods: LC-MS techniques was used to check the presence of phytoconstituents present in *C. rubellum* extract. In vitro antidiabetic activity was evaluated using α-glucosidase inhibitory activity and PTP-1B signaling pathway. On Streptozotocin (STZ)-induced rats with diabetes, the in vivo antidiabetic efficacy was assessed using a test for oral glucose tolerance.
Results: The phytoconstituents identified in the extract of *C. rubellum* were apigenin, diosmin, myricetin, luteolin, luteolin-7-glucoside, and Quercitrin as compound 1-6, respectively. Results showed that diosmin exhibited highest α-glucosidase inhibitory activity i.e. 90.39%. The protein level of PTP-1B was lowered and the insulin signalling activity was directly increased by compounds 1-6. The maximum blood glucose levels were seen in all groups' OGTT findings at 30 minutes following glucose delivery, followed by gradual drops. In comparison to the control group, the extract's glucose levels were 141 mg/dL at 30 minutes before falling to 104 mg/dL after 120 minutes. The current study has demonstrated, in summary, that extract with phytoconstituents reduce blood sugar levels in rats.
Conclusion: This finding suggests that extract may reduce the chance of insulin resistance and shield against disorders like hyperglycemia.

Keywords: *Chrysanthemum*, diosmin, diabetes, insulin, Streptozotocin

INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disease that can have major health repercussions due to a disturbance in the body's control of glucose and lipid metabolism. DM is characterized by decreased production of insulin, resistance to insulin, and pancreatic beta cell loss [1]. Medication, dietary therapy, and exercise therapy are all viable options for managing diabetes [2]. Notably, sulfonylureas are used to increase insulin secretion by the pancreas, while α-glucosidase inhibitors are used to block intestinal uptake of glucose. Additionally, biguanides are used to reduce glucose synthesis in the liver. Fatal adverse effects such as hypoglycemia and lactic acidosis have been linked to drug therapy [3, 4]. Consequently, there has been a concerted effort to explore safer agents from herbal or natural sources in recent years.

The chrysanthemums, perennial members of the Asteraceae family, are quite simple to cultivate. They form open, circular mounds that are 2-3 feet in height and are native to the regions spanning from Europe to India and from the subarctic of North

America to the north of Canada. The word chrysanthemum originated from a combination of the Greek words for gold and flower. *Chrysanthemum rubellum* is known as rock garden and dwarf. The rubellum and Korean chrysanthemum species share many similarities in growth habits and robustness [5]. However, *C. rubellum* has a wider rootstock compared to the Korean chrysanthemum and a stronger aroma that is thought to be inherited from its ancestral species, *C. indicum*.

It shares some similarities with *C. zawadskii*. However, it is significantly superior due to its greater height, more abundant growth, rougher leaves, and larger flowers. In traditional medicine, *C. zawadskii* has been used to treat inflammatory diseases, gastrointestinal issues, high blood pressure, bladder problems, irregular periods, and loss of fertility [6]. Researchers have demonstrated its hepatoprotective, antibacterial, and antioxidant properties. *C. zawadskii* is rich in flavonoids, essential oils, and polysaccharides. Flavonoids, of which linarin is the best representative chemical, have been demonstrated to exert neuroprotective, hepatoprotective, and osteogenic differentiation effects [7, 8].

Several processes in cells, including the breakdown of carbohydrates, lysosomal catabolism of glycoconjugates, as well as modification after translation of cellular glycoproteins, depend on glycosidases, enzymes that hydrolyze the glycosidic bonds found in polysaccharides and glycoconjugates. Due to the prolonged breakdown of carbohydrates in the small intestines and the decreased postprandial blood glucose excursion, inhibiting α-glycosidases has a significant effect on polysaccharide digestion, glycoprotein processing, and cell-based interactions [9] and provides the basis for the identification and development of medicinal products for treating illnesses such as obesity, type 2 DM, metastasized cancer, and viral infections.

Protein tyrosine phosphatase 1B (PTP-1B) is a negative regulator of insulin and leptin signaling, potentially regulating glucose and energy homeostasis [10]. PTP-1B functions as an insulin signaling pathway downregulator and is involved in pancreatic β-cell death. In addition, it is linked to the regulation of various pathways associated with DM [11]. Thus, the present study was designed to evaluate the potential of *C. rubellum* in treating diabetes by inhibiting α-glucosidase and the PTP-1B signaling pathway. Methanol/water (80/20, %v/v) extracts of *C. rubellum* flowers were obtained. The liquid chromatography-mass spectrometry (LC-MS) technique was used to identify the phytoconstituents. In vitro antidiabetic activity was evaluated, focusing on α-glucosidase inhibitory activity and the PTP-1B signaling pathway. Additionally, an in vivo assay was performed using streptozotocin (STZ)-induced diabetic rats. The rats were assessed using the oral glucose tolerance test (OGTT).

MATERIALS AND METHODS

1. Extraction of *C. rubellum* flowers

Dried *C. rubellum* flowers were purchased from the Paramount Garden Center in K0A, Ontario, Canada. The *C. rubellum* flowers were cut into pieces and macerated in a methanol/water (80/20, %v/v) solution for 24-48 hours. The solvent was then replaced, and the procedure was repeated three more times. A syrupy residue was obtained after concentrating the extract at a temperature ≤ 35℃. To remove chlorophyll via precipitation, an amount of 600 mL of distilled water was added per 1 kilogram of dry matter. Following filtration, the aqueous phase was sequentially extracted in a separatory funnel by liquid-liquid extraction using water-immiscible solvents of high polarity (chloroform, ethyl acetate, and n-butanol). The resulting organic phases were concentrated to dryness under decreased pressure, weighed, and dried with anhydrous sodium sulfate to eliminate all traces of water.

2. LC-MS analysis

The LC-MS analysis was performed using DGU-20A3R high-performance LC, equipped with a mass spectrometer and a diode array detector (Shimadzu Corporation, Kyoto, Japan). The flow rate was set to 1.0 mL/min, and a Symmetry C18 column (250 mm × 4.6 mm, 5 mm) with a Sentry guard column (20 mm × 3.9 mm, 5 mm) was employed. The column temperature was set at 25℃. The mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The gradient was varied linearly from 10% to 26% B (v/v) in 40 min, to 65% B at 70 min, and 100% B at 71 min. Subsequently, the gradient was maintained at 100% B for 75 min. For real-time peak intensity monitoring, the diode array sensing was set at 350, 310, 270, and 520 nm, and complete spectra (190-650 nm) were constantly documented for plant component identification. Electrospray ionization was used to concurrently capture mass spectra in both positive and negative ionization modes spanning the m/z range of 100-1,000 at high and low fragmentation voltages (–3.5 and 4.5 kV).

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3. Phytochemical screening

1) Test for glycosides

(1) Keller-Killiani test

Two milliliters of acetic acid, one drop of 5% $FeCl_3$, and concentrated H_2SO_4 were added to 2 mL of the extract. The presence of glycosides was indicated by a reddish-brown layer at the junction of the two liquid layers, with the upper layer appearing bluish-green.

(2) Glycoside test

Two drops of the extract were taken and shaken properly with 1 mL of water. NaOH solution was then added to the mixture. Glycosides were detected by the appearance of a yellow color.

(3) Concentrated H_2SO_4 test

One drop of 5% $FeCl_3$ and one drop of concentration H_2SO_4 were added to 5 mL of the extract. Glycosides were indicated by the presence of a brown ring.

(4) Molisch's test

Two drops of Molisch's reagent and 2 mL of concentrated H_2SO_4 were mixed in a test tube with a little bend. Following that, 1 mL of the extract was added. The development of a violet-colored ring at the junction of the two layers indicated the presence of glycosides.

2) Test for tannins

(1) $FeCl_3$ test

An $FeCl_3$ test was performed by mixing a few drops of 5% $FeCl_3$ solution with 2 mL of test solution. The formation of a blue hue indicated the presence of hydrolyzable tannins.

(2) Lead acetate test

A volume of 2 mL of the extract and a few drops of 10% lead acetate solution were added. A yellow or scarlet precipitate indicated the presence of tannins.

(3) Acetic acid solution

A volume of 2 mL of the extract and a few drops of acetic acid were added. The formation of a red-colored solution indicated the presence of tannins.

(4) Potassium dichromate test

A volume of 2 mL of the extract and a few drops of potassium dichromate solution were mixed. The display of a red PowerPoint slide on the computer's screen.

(5) Dilute iodine solution

A volume of 2 mL of the extract was mixed with a few drops of weak iodine solution. The appearance of a crimson hue indicated the presence of tannins.

(6) Dilute HNO_3

A volume of 2 mL of the extract was mixed with a few drops of dilute HNO_3. A reddish-yellow hue indicated the presence of tannins.

3) Test for flavonoids

An amount of lead acetate solution was added to a small amount of the extract. A yellow color was produced. NaOH when added in increasing amounts, causes the residue to become colored, which fades when acid is added.

4) Test for carbohydrates

(1) Benedict's test

An amount of 5 mg of the extract was mixed with a few drops of Benedict's solution, followed by heating. The formation of a reddish-brown precipitate indicated the presence of carbohydrates.

(2) Molisch's test

An amount of 2 mg of the extract and 1 mL of Molisch's solution were mixed in a test tube. After that, 2 mL of concentrated H_2SO_4 was cautiously added to the test tube. The formation of a violet interface indicated the presence of carbohydrates.

5) Test for alkaloids

(1) Mayer's test

A volume of 2 mL of the extract was added to 1% w/v HCl, and the resulting solution was gently warmed to dissolve the extract. The formation of a red color indicated the presence of alkaloids.

(2) Wagner's test

A volume of 0.5 mL of Wagner's reagent was added to about 2 mL of the extract, and the resulting mixture was thoroughly shaken. The appearance of a reddish-brown color indicated the presence of alkaloids.

6) Test for saponins

A foam test was performed using 1 mL of the aqueous extract and 5 mL of distilled water to identify the existence of saponins in the sample. After adding the distilled water, the resulting mixture was vigorously shaken until the formation of a foamy layer, which is indicative of the presence of saponins. The mixture was shaken vigorously with the addition of a few foams and 2 drops of olive oil. Saponin emulsion was created.

7) Test for steroids

Two milligrams of the *C. rubellum* extract were dissolved in chloroform, and the resulting solution was mixed with H_2SO_4 and acetic acid. The presence of steroids was indicated by the appearance of a greenish hue. Salkowski's test was also conducted. With this test, 3 drops of concentrated H_2SO_4 were added to 2 mg of the extract, and the presence of steroids was indicated by the appearance of a red color.

4. In vitro α-glucosidase activity

Fifty microliters of the sample solution (extract or acarbose) and 50 μL of 5 mM p-nitrophenyl α-d-glucopyranoside solution (in phosphate buffer) were mixed and kept at 37℃ for 5 min. Then, 100 μL of 0.1 U/mL Baker's yeast α-glucosidase (in phosphate buffer) was added. Employing a microplate reader with the temperature set to 37℃ and a blank, the absorbance at 405 nm was measured after 30 min. The following formula was used to determine the α-glucosidase inhibitory activity:

% α-glucosidase inhibitory activity =

Absorbance of sample/Absorbance of control × 100

5. PTP-1B inhibitory activity

The PTP-1B inhibitory activity was measured using p-nitrophenyl phosphate (pNPP) as a substrate. Dimethyl sulfoxide (DMSO) was used to dissolve compounds 1-6. Assay buffer (pH 6.0), which includes 150 mM NaCl, 50 mM 2-(N-morpholino) ethanesulfonic acid, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 0.05% NP-40, was used to dissolve PTP-1B, pNPP, and $NaVO_4$. PTP-1B (20 mg/mL, 5 mL), the test compounds (10 mL), and buffer (75 mL) were combined and maintained at 30℃ for 5 min. The reactions were then started by adding pNPP (100 mM, 10 mL) and allowed to proceed at 30℃ for 10 min. Spectrophotometry at 405 nm was used to identify enzyme activity [12].

6. In vivo antidiabetic activity of *C. rubellum* flower extracts

1) Experimental animals

Wistar rats weighing 175-200 g were purchased from the Royal College of Pharmacy in Orissa's Central Animal House. The IAEC committee of the institute approved the study protocol. Using a 12-hour light/dark cycle, our animals were housed in a setting with a fixed temperature (25 ± 2℃) and relative humidity (60 ± 5%) for one week. They were kept in polypropylene cages and provided with an unlimited supply of water while being given standard laboratory food (Lipton India Ltd.).

2) Acute toxicity studies

The "up-and-down" approach was used in healthy adult albino mice of any gender. The mice were acclimated in their cages for 5 days before experimentation. Additionally, the mice/rats fasted the night before dosing. During the fasting period, the mice/rats were weighed and divided into four groups of five. Single doses of 55, 175, 550, and 2,000 mg/kg of the extract were administered via gastric gavage. The behavioral, neurological, and autonomic profiles of the mice/rats were carefully assessed. At this early stage, preliminary pharmacological investigations were conducted to evaluate the acute effects and LD_{50} of the root extracts of *C. rubellum* extract. Despite the maximum dose of 2,000 mg/kg, no mouse/rat died. However, specific autonomic responses increased in magnitude in a dose-dependent manner. An increase in irritability and a decrease in pain response was observed [13].

3) Induction of diabetes

A solution of STZ in 0.1 M citrate buffer (50 mg/kg) was administered via the intraperitoneal route at a dose of 1 mL/kg. Within two days of receiving STZ, the rats developed hyperglycemia. Diabetes was confirmed by assessing the fasting blood glucose levels 48 hours following the injection of STZ. For further research purposes, rats in the STZ group with a blood glucose level exceeding 200 mg/dL were categorized into distinct diabetes groups [14].

4) OGTT

For the OGTT, normal rats that had fasted for 18 hours were used. Eight groups of rats were established (n = 6). Groups II and III received glibenclamide (10 mg/kg), Group IV received the extract (200 mg/kg), and Group V received apigenin, diosmin, myricetin, luteolin, luteolin-7-glucoside, and quercitrin. Group X also received the extract (200 mg/kg). Group I was the control group, which received distilled water. After 30, 60, and 120 min of glucose administration, blood samples were collected from the retroorbital sinus under ether inhalation anesthesia, and glucose levels were determined using glucose oxidase-peroxidase reactive strips and a glucometer [6].

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7. Statistical analysis

Data are represented as mean ± standard error of the mean (SEM). The GraphPad Prism 5 software (San Diego, CA, USA) was used for data analyses. A one-way analysis of variance (ANOVA) was performed, followed by Dunnett's multiple comparison test. The threshold for statistical significance was set at $p < 0.05$.

RESULTS

1. LC-MS identification of *C. rubellum* phytoconstituents

By analyzing the retention times and high-resolution mass spectra for pure standards, the phytoconstituents in *C. rubellum* flowers were discovered (Lin and Harnly, 2010). The phytoconstituents responsible for peaks 1-6 were apigenin, diosmin, myricetin, luteolin, and luteolin-7-glucoside. Fig. 1 presents the chromatograms of the *C. rubellum* extract. Table 1 presents the retention times, molecular ions, and main fragment ions of the principal peaks.

Table 1. Determination of the chemical components found in *C. rubellum* extract

Peak no.	tR (min)	$[M + Na]^+/[M - H]^-$ (m/z)	Identification
1	32.45	395.2045/439.3943	Apigenin
2	36.99	694.4933	Diosmin
3	50.21	384.8493	Myricetin
4	46.23	310.8994	Luteolin
5	58.39	298.7392	Luteolin-7-glucoside
6	64.94	310.0942	Quercitrin

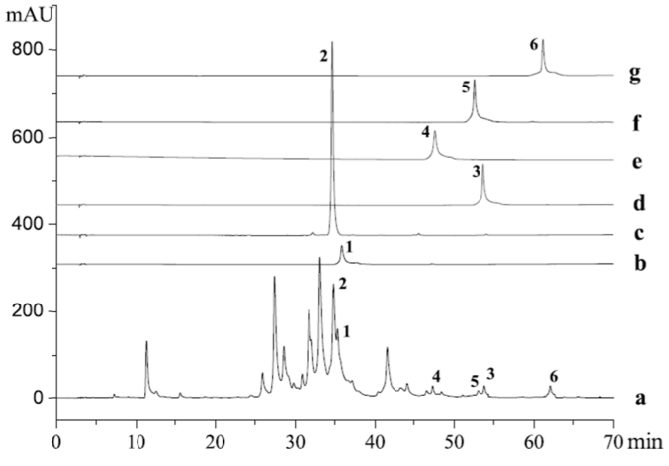

Figure 1. LC-MS chromatogram of *C. rubellum*. Chromatograms of the (a) *C. rubellum* extract, (b) Apigenin (Compound 1), (c) Diosmin (Compound 2), (d) Myricetin (Compound 3), (e) Luteolin (Compound 4), (f) Luteolin-7-glucoside (Compound 5), (g) Quercitrin (Compound 6).

2. Phytochemical screening of *C. rubellum*

The flower extract of *C. rubellum* contained several active compounds, including alkaloids, glycosides, terpenoids, flavonoids, proteins, tannins, phenolic compounds, amino acids, and starch. However, saponins and steroids were absent in the plant extract (Table 2).

3. α-Glucosidase inhibitory activity of the *C. rubellum* extract

The in vitro antidiabetic activity of the *C. rubellum* extract

Table 2. Phytochemical estimation of *C. rubellum* extract

S. no.	Chemical test	*C. rubellum* extract
1.	Alkaloids	+
2.	Glycoside	+
3.	Flavonoids	+
4.	Carbohydrate	+
5.	Saponins	–
6.	Steroids	–
7.	Proteins	+
8.	Tannins	+
9.	Phenolic compounds	+
10.	Amino acids	+
11.	Starch	+

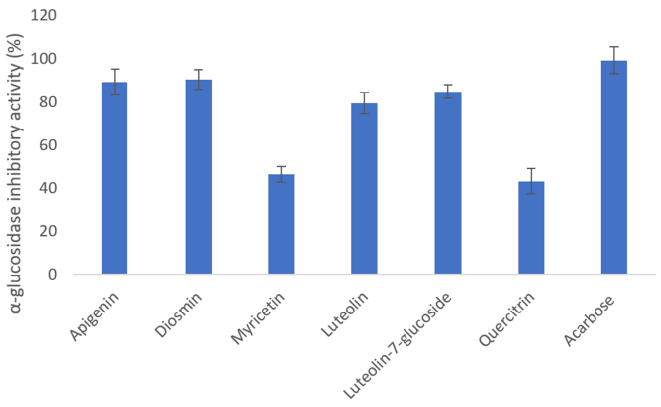

Figure 2. α-Glucosidase inhibitory activity of *C. rubellum* extract and compounds. Data are represented as mean ± SD (n = 3).

was determined using α-glucosidase inhibitory activity. Acarbose was selected as the standard α-glucosidase inhibitor. Notably, diosmin exhibited the highest inhibitory activity (90.39%). However, standard acarbose exerted 99.32% inhibition. α-Glucosidase inhibitory activity of. apigenin, myricetin, luteolin, luteolin-7-glucoside, and quercitrin were 89.23%, 46.40%, 79.40%, 84.88%, and 43.34%, respectively (Fig. 2).

4. PTP-1B insulin signaling

The initiation of insulin signaling relies on the stimulation of the insulin receptor, leading to the phosphorylation and recruitment of various downstream signaling molecules. Compounds 1-6 significantly reduced the protein concentrations of PTP-1B. To further elucidate their impact on PTP-1B, an inhibitory assay on human PTP-1B (hPTP-1B) was conducted. At 1, 5, and 20 mM, compounds 1-6 had mild inhibitory effects against hPTP-1B (Table 3). These findings suggest that compounds 1-6 increased insulin signaling activity, lowered PTP-1B protein levels, and inhibited PTP-1B activity.

5. Effect of extract and isolated compounds on OGTT

It was discovered that administering extract and isolated components to diabetic mice improved postprandial concentration of glucose. The maximum blood glucose levels were seen in all groups' OGTT findings at 0, 30, 60, and 120 min following glucose administration, followed by gradual drops. The level of glucose following the administration of the extract was 141 mg/dL at 30 min, which was restored to 104 mg/dL at 120 min (Fig. 3). However, the isolated compounds significantly reduced blood glucose levels in the rat models. The blood glucose levels were 92.05, 101.55, 86.23, 80.68, and 81.48 mg/dL, respectively, following the administration of apigenin, diosmin, myricetin,

Table 3. Human PTP-1B inhibition of isolated compounds

Compound no.	Name	Percentage inhibition		
		20 μM	5 μM	1 μM
1	Apigenin	29.23 ± 1.93	23.65 ± 1.77	27.65 ± 1.77
2	Diosmin	30.39 ± 1.50	31.11 ± 1.99	35.11 ± 1.99
3	Myricetin	36.40 ± 0.55	27.76 ± 0.34	24.76 ± 0.34
4	Luteolin	29.40 ± 1.93	25.22 ± 1.76	22.22 ± 1.76
5	Luteolin-7-glucoside	34.88 ± 1.21	28.76 ± 1.92	23.76 ± 1.92
6	Quercitrin	33.23 ± 0.76	30.34 ± 0.88	27.34 ± 0.88
Standard	Sodium orthovanadate	59.65 ± 1.46		

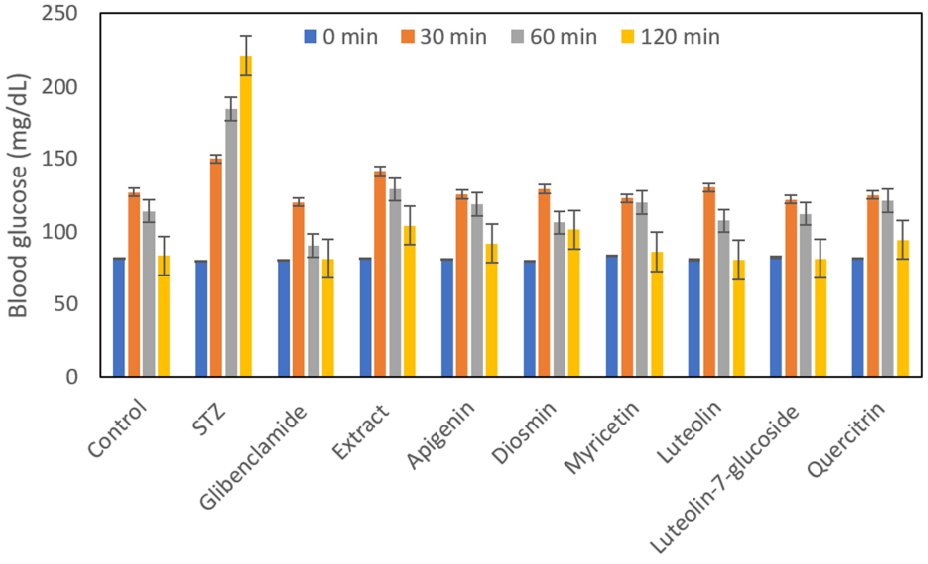

Figure 3. Effect of extract and compounds on STZ-induced diabetic rats in OGTT assay. Data are represented as mean ± SD (n = 6), significantly different at $p < 0.05$ in comparison to the control group.

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luteolin, luteolin-7-glucoside, and quercitrin.

DISCUSSION

DM is characterized by inadequate insulin production and insulin resistance in target organs. Various antidiabetic medications are used to lower blood sugar levels via several mechanisms, enhancing insulin production, lowering hepatic glucose synthesis, limiting postprandial glucose absorption, and inhibiting gluconeogenesis. Nonetheless, insulin is often prescribed when the effectiveness of these medications starts decreasing [15].

The high level of interest in tyrosine phosphorylation homeostasis can be attributed to the fact that tyrosine phosphorylation mediates insulin receptor signaling and that insulin resistance is linked to obesity and DM. PTP-1B is the most well-known of the tyrosine phosphatases that have been called into question. Since PTP-1B is involved in insulin sensitivity, blocking it may provide an effective therapeutic strategy for treating diabetes without the weight gain typically associated with thiazolidinediones [16]. Due to dose-dependent side effects and poor performance in phase II clinical studies, the use of synthetic PTP-1B inhibitors currently on the market has been restricted. The pharmacological and pharmacokinetic properties of naturally occurring compounds may also be superior to those of their synthesized counterparts. Delaying glucose absorption by inhibiting the carbohydrate hydrolyzing enzyme α-glucosidase is another mechanism of treating diabetes, especially non-insulin-dependent diabetes (postprandial hyperglycemia). The last stage of carbohydrate metabolism is mostly catalyzed by α-glucosidase. Delaying glucose absorption/reducing postprandial plasma glucose level and ultimately reducing postprandial hyperglycemia are all effects of inhibiting this enzyme. Acarbose, miglitol, and voglibose are the currently available clinical α-glucosidase inhibitors, and they all have gastrointestinal adverse effects [17]. Therefore, there is an urgent need to identify novel phytochemicals that effectively inhibit PTP-1B and α-glucosidase with few or no adverse effects.

C. rubellum is a well-known herb with a decade-long history of medicinal value. Various parts of this plant have been used for medicinal purposes. As part of our ongoing work to discover novel phytochemicals against diabetes, we evaluated the PTP-1B and α-glucosidase inhibitory potentials of compounds 1-6 isolated from this plant [18]. All the compounds exhibited potent inhibitory activities against both enzymes. These activities were similar to those of the reference drugs. α-Glucosidase catalyzes the process of digesting carbohydrates, releasing glucose. The PTP-1B signaling pathway and α-glucosidase act as dual regulators, slowing the release of glucose and increasing insulin sensitivity. This combined activity alleviates glucose and lipid metabolism dysfunctions and insulin resistance, making this mechanism a potentially effective therapeutic approach for the management of type 2 DM.

The anti-diabetic mechanisms of the test compounds were further evaluated by assessing the effect of these compounds on OGTT to investigate insulin sensitivity in normal experimental mice/rats. The extract and isolated compounds effectively reduced elevated blood glucose levels in the STZ-induced diabetic rats. Thus, the study indicates that blocking PTP-1B and α-glucosidase enzyme activity with compounds 1-6 may be a useful preventive or therapeutic approach for type II DM.

CONCLUSION

The current study demonstrated that the extract and isolated compounds can reduce blood sugar levels in rats. This finding suggests that the extract may reduce the risk of resistance to insulin and protect against disorders like hyperglycemia. The PTP-1B signaling pathway is crucial in the regulation of insulin signaling and the emergence of type 2 DM. Compounds 1-6 significantly promote the intake of glucose. Additionally, compounds 1-6 are dual regulators of the PTP-1B signaling pathway and α-glucosidase, serving as a potential avenue for exploring new oral anti-diabetic medications and possible functional food additives.

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AUTHORS' CONTRIBUTIONS

BNT – Experimental work; NS – Proofreading; SKS – designing the protocol.

CONFLICTS OF INTEREST

The authors declared no conflict of interest.

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