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In Vitro and *in Vivo* Anti-Diabetic Activity of *Rhododendron Griffithianum* for Insulin Dependent (Type I) Diabetes Mellitus

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KEYWORDS

Anti-Diabetic, Rhododendron Griffithianum

ABSTRACT:

Diabetes mellitus, a chronic metabolic disorder, poses a significant global health challenge, with the insulin-dependent (Type I) form requiring continuous therapeutic interventions. In this study, we investigate the potential anti-diabetic properties of Rhododendron griffithianum through both in vitro and in vivo assessments, targeting the specific needs of individuals with Type I diabetes. The in vitro phase involves the exploration of the inhibitory effects of Rhododendron griffithianum extracts on key enzymes involved in glucose metabolism, such as α-amylase and α-glucosidase. Additionally, the impact on glucose uptake in insulin-sensitive cells will be assessed to elucidate potential mechanisms of action at the cellular level. The in vivo component of the study employs an animal model of Type I diabetes to evaluate the efficacy of Rhododendron griffithianum in ameliorating hyperglycemia and associated complications. Diabetic animals will be treated with different doses of Rhododendron griffithianum extracts, and parameters including blood glucose levels, insulin sensitivity, lipid profile, and histopathological changes in pancreatic tissue will be examined. The findings from this research may provide valuable insights into the development of novel therapeutic agents for managing Type I diabetes. Moreover, the dual in vitro and in vivo approach enhances the translational relevance of the study, bridging the gap between laboratory discoveries and clinical applications. The results could potentially pave the way for the development of Rhododendron griffithianum-derived pharmaceuticals or nutraceuticals as adjunctive therapies for individuals with insulin-dependent diabetes.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies distinguished by a failure of glucose homeostasis with disturbances of carbohydrate, fat and protein metabolism as a result of defects in insulin secretion and/or insulin action **(Tafesse et al., 2017).** Diabetes-related chronic hyperglycemia is linked to long-term damage, dysfunction, and failure of various organs, particularly the eyes, kidneys, nerves, heart, and blood vessels. Diabetes is caused by several pathogenic mechanisms. These range from autoimmune destruction of pancreatic -cells with resulting insulin shortage to anomalies that result in insulin resistance. Diabetes is characterized by anomalies in glucose, lipid, and protein metabolism due to insulin's ineffective action on target tissues. Insulin secretion dysfunction and insulin action dysfunction frequently occur in the same patient, and it is frequently unclear which abnormality, if either alone, is the major cause of hyperglycemia **(Giri et al., 2018).** Blurred vision, polyuria, polydipsia, weight loss, and occasionally polyphagia are signs of severe hyperglycemia. Chronic hyperglycemia may also be accompanied by growth impairment and increased

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susceptibility to certain illnesses. Uncontrolled diabetes can lead to acute, potentially fatal effects such as hyperglycemia with ketoacidosis or nonketotic hyperosmolar syndrome **(Poznyak et al., 2020).** Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes **(American Diabetes Association, 2010).** Diabetes mellitus is one of the major causes for early death, worldwide **(World Health Organization, 2008).** Rhododendron is one of the naturally occurring plants which possess various health benefits, such as prevention and treatment of diseases associated with heart, dysentery, diarrhea, detoxification, inflammation, fever, constipation, bronchitis and asthma (**Nisar et al., 2013**). The leaves exhibit potent antioxidant properties. Young leaves are used as a headache remedy. This plant's wood is useful for crafting gift boxes, packsaddles, gunstocks, khukri handles, and posts (**Sakalani et al., 2012**). Rhododendron contains essential minerals like manganese, iron, zinc, copper, and cofactors, which are crucial for life processes. Phenolic acids from its leaves and twigs have anti-HIV, anti-inflammatory, and anti-nociceptive properties, and are used for treating various illnesses.

MATERIAL AND METHOD

Collection and identification of plant material

The leaves of *Rhododendron Griffithianum* belonging to family Ericaceae obtained from Kanatal, Uttrakhand state. The leaves of plant are authenticated by Mr. Sandeep Kumar, Botanist, Deptt. of Botany and Microbiology, Gurukul Kangri Vishwvidhyalay, Haridwar, Uttrakhand.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study was of analytical grade.

Extraction of plant by soxhlet extraction method

Coarsely powered leaves of *Rhododendron griffithianum* was then extracted by successive extraction using different organic solvents, defatted with petroleum ether and successively extracted with methanol for 36 hrs using soxhlet apparatus **(Alara et al., 2019)**.

Animals and treatment

Albino wistar rats of weighing 190±10g were selected and procured from PBRI animal house. The animals were maintained under standard conditions of humidity, temperature (25±2 ◦C) and light (12 h light/dark). They were fed with standard rat pellet diet and water *ad libitum*.

IN VITRO ANTIDIABETIC ACTIVITY

α-Amylase Inhibitory Assay

The assay was carried out following the standard protocol with slight modifications **(Iniyan G. Tamil et al., 2010**). Starch azure (2 mg) was suspended in 0.2 mL of 0.5M Tris–HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ (substrate solution). The tubes containing substrate solution were boiled for 5 min and then preincubated at 37°C for 5 min. *Rhododendron griffithianum*, hydroalcoholic extract was dissolved in DMSO in order to obtain concentrations of 10, to 50 µg/mL. Then, 0.2 mL of sample of particular concentration was added to the tube containing the substrate solution. In addition, 0.1 mL of porcine pancreatic amylase in Tris–HCl buffer (2 units/mL) was added to the tube containing the plant extract and substrate solution. The reaction was carried out at 37°C for 10 min. The reaction was stopped by adding 0.5 mL of 50% acetic acid in each tube. The reaction mixture was centrifuged at 3000 rpm for 5 min at 4°C. The absorbance of resulting supernatant was measured at 595 nm using spectrophotometer (Systronic India 2202 UV–VIS spectrophotometer). The experiments were repeated thrice. The α-amylase inhibitory activity was calculated by using following formula:

%Inhibition = (Control – Sample/ Control) *100

α-Glucosidase Activity

The inhibition of α-glucosidase activity was determined using the modified published method (**Dewi et al., 2007).** One mg of α-glucosidase (*Saccharomyces cerevisiae*, Sigma-Aldrich, Germany) was dissolved in 100mL of phosphate buffer (pH 6.8) containing 200 mg

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of bovine serum albumin (Himedia). The reaction mixture consisting 10 µL of *Rhododendron griffithianum*, hydroalcoholic extract at varying concentrations (10 to 50 µg/mL) was premixed with 490 µL phosphate buffer pH 6.8 and 250 µL of 5mM p-nitrophenyl α-D-glucopyranoside (Sigma-Aldrich, Germany). After preincubating at 37◦C for 5min, 250 µL α-glucosidase (0.15 unit/mL) was added and incubated at 37◦C for 15 min. The reaction was terminated by the addition of 2000 µL Na_2CO_3 200 mM. α-glucosidase activity was determined spectrophotometrically at 400nm on spectrophotometer UV-Vis (2202 Systronic, India) by measuring the quantity of p-nitrophenol released from p-NPG. (**Elya et al., 2012).**

Fractionation of *Rhododendron griffithianum* hydroalcoholic extract

Fractionation is a process of separation of plant extracts into various fractions. When several solvents are required for the fractionation, they should be added according to the order of increasing polarity.

SEPARATION FUNNEL METHOD

When five different solvents (Petroleum ether, chloroform, ethyl acetate, methanol and water) are selected, fractionation begins by moistening or complete dissolution of crude extract with 250mL of water. This is followed by transfer into a separating funnel, shaken, and allowed to settle. Furthermore, to 250mL of petroleum ether the least polar solvent was added and shaken. The content can settle, and the bottom of the separating funnel opened to remove the aqueous layer. The remaining content in the separating funnel was poured into a clean container to get *petroleum ether* fraction. Equal volume of *petroleum ether* was added again, shaken, and separated. The addition continued until after adding *petroleum ether* and shaken no reasonable quantity of extract appeared to move into the *petroleum ether* portion. Similar cycle was performed for chloroform, *ethyl acetate, methanol* to get chloroform, *ethyl acetate, methanol* fractions. The remaining portion left after the fractionation is termed as residual aqueous fraction (RAF) as the crude extract was first dissolved in water.

***IN VIVO* ACTIVITY**

Acute oral toxicity study was checked as per the flow diagram of OECD 423 Guideline

It was done as per Organization for Economic Co-operation and Development (OECD) guidelines 423 for acute toxicity studies. For this study male Wistar rats were used.

The acute toxic class method set out in this Guideline is a stepwise procedure with the use of 3 animals. The substance is administered orally to a group of experimental animals.

The acute toxicity study was according to OECD (423) guidelines. The test group include four treatment groups with dosages at 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg body weight. The test substance was administered in a single dose by oral gavage.

***IN VIVO* ANTIDIABETIC STUDY**

Experimental induction of diabetes

Male Wistar rats were allowed to acclimatize to the laboratory environment for 12 Days. Six rats were randomly separated as the normal control group (NC) and fed on a normal diet. The other treatment group of animals was then fed on normal diet for 21 days with sufficient food and water. The animals were fasted for 12 h and then received an intra-peritoneal injection of streptozotocin (STZ) (Sigma-Aldrich, USA), dissolved in 0.1 M citric acid/sodium citrate buffer, pH 4.5 at a dose of 65 mg/kg. Standard were treated with (Glibenclamide,10mg/kg). Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 h after STZ administration. Rats with fasting blood sugar levels around 160 to 300 mg/dl were selected for the study.

Wistar rats were divided into six groups with at least six animals each.

Group I: Normal control, rats received vehicle, Group II: Diabetic control (DC) (STZ), Group III: Diabetic (STZ) rats treated with Standard Glibenclamide (10 mg/kg), Group IV-VI: Rats treated with test samples F1-F3 (200 mg/kg) body weight for 21 days.

HISTOPATHOLOGY

The liver, kidney and pancreas specimens were immersed in 10% formalin solution for histopathological examination. These tissues were

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processed, dehydrated in different grades of alcohol, cleared in toluene, and impregnated in molten paraffin wax for specified periods. Sections were at 3 µ and dried on a hot plate for 15 min and stained with hematoxylin and 1% aqueous eosin to demonstrate general tissue structure. Stained slides were dehydrated in various ascending grades of alcohol, cleared in xylene, and mounted in Canada balsam. Sections were viewed microscopically using ×10 objective lenses.

STATISTICAL ANALYSIS

Results are provided as Mean ± SD (n=6). Results were analysed statistically using one-way analysis of variance (ANOVA) followed by Bonferroni t-test. $P < 0.05$ was considered as level of significance while comparison between groups.

RESULTS AND DISCUSSION

IN VITRO ANTI DIABETIC ACTIVITY

α-amylase activity of hydroalcoholic extracts of *Rhododendron griffithianum.*

Table 1 α-amylase activity of Acarbose and *Rhododendron griffithianum*

Sample	Acarbose	*Rhododendron griffithianum*
IC50 Value	13.67±0.765	44.14±0.638

Glucosidase activity of *Rhododendron griffithianum*

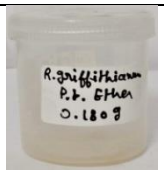
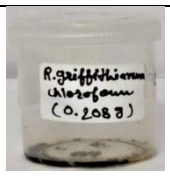
Table 2 α-glucosidase activity of Acarbose and *Rhododendron griffithianum*

Sample	Acarbose	*Rhododendron griffithianum*
IC50 Value	**37.33±0.098**	**50.83±0.076**

Percentage yield by fractionation in various solvents

Plant material is the most explored source of natural compounds due to its accessibility. In this study, fractionation was done for methanolic extract of *Rhododendron griffithianum* as it is showing best *invitro* results, thus this sample is further fractionated in five solvents and obtained yield will be found maximum in methanolic and aqueous solvent. Thus, the resulted yield obtained in methanolic fraction employed in further study.

Table 3: Fraction yield

Sr. No.	Fraction	Extract	Sr. No.	Fraction	Extract
1	Water		4	Ethyl Acetate	
2	Methanol		5	Pet. Ether	
3	Chloroform				

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ACUTE ORAL TOXICITY

Body weight changes

Results shows the effects of three fractions (chloroform, ethyl acetate and methanolic) of *Rhododendron griffithianum* on wistar rat body weight in an acute toxicity investigation. The body weight of animals in the treatment groups was measured during the fasting period, i.e. Day 1, as well as on Day 7 and Day 14 following fractions of *Rhododendron griffithianum* administration. No significant variations in weight gain were found in the experimental animals' body weight. The body weight of experimental animals was increased normally and progressively during the 14 days. Acute toxicity studies revealed that the administration of fractions of *Rhododendron griffithianum* at various dose (5, 50, 300 and 2000) did not cause significant changes to the behaviour of the animals as observed by parameters such as body weight, urinations, convulsion, tremor, changes in skin colours etc.

ANTIDIABETIC ACTIVITY

Effect of Test samples F1, F2 and F3 on body weight

Table 4: Variations in body weight in normal control, inducer, standard and Test samples F1, F2 and F3

Group No.	Treatment	Days				
		0	3	7	14	21
I.	Normal Control (Vehicle treated)	193.56±5.053	198.41±5.646	201.71±6.478	205.42±6.551	209.60±6.145
II.	STZ induced only	195.59±4.193	191.75±4.396	188.20±5.574	183.77±5.413	172.72±5.891
III.	STZ+ Standard Glibenclamide (10 mg/kg)	196.18±1.834^{NS}	197.59±3.515^{NS}	200.57±5.534^{NS}	205.02±5.070^{NS}	210.22±4.496**
IV.	Fraction F1	190.13±2.784^{NS}	188.96±3.277^{NS}	187.54±3.504^{NS}	192.16±3.618^{NS}	200.28±3.512*
V.	Fraction F2	186.46±3.184^{NS}	188.29±3.248^{NS}	189.47±3.165^{NS}	195.55±3.340^{NS}	200.42±3.784*
VI.	Fraction F3	191.00±3.193^{NS}	192.18±3.610^{NS}	193.03±5.432^{NS}	198.85±5.689^{NS}	204.38±5.149*

Values are expressed as MEAN±SD at n=6, One-way ANOVA followed by Bonferroni test, *P<0.050, **P<0.001 and ^{NS}P>0.001 compared to the Diabetic control group STZ treated

At the start of the trial, or starting body weights, the normal control rats (Group I) had normal body weights with little change as the animals grew older until the end of the study, which was 21 days. When compared to the healthy control group (Group I), diabetic rats (Group II) had a lower body weight (p 0.05) at the end of the trial. Treatment for 21 days with (Group IV-VI and Group III) Glibenclamide (10 mgkg1) significantly (p<0.05) preserved the changes in bodyweight to normal body weight.

Effect of Test samples F1, F2 and F3 on fasting blood glucose level

Table 5: Variations in BGL after 0, 3, 7, 14 and 21 week of treatment period

Group No.	Treatment	Days				
		0	3	7	14	21
I.	Normal Control (Vehicle treated)	87.66±3.011	89.66±3.204	91.16±4.119	93.00±4.604	94.25±5.251
II.	STZ induced only	270.16±5.707	285.83±8.232	299.16±10.284	328.66±16.256	354.33±18.457

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III.	STZ+ Standard Glibenclamide (10 mg/kg)	290.50±6.565^{NS}	249.16±8.841*	176.50±8.826**	135.33±9.223**	105.66±6.501**
IV.	Fraction F1	261.66±5.086^{NS}	240.33±4.633*	207.00±7.127**	184.33±9.288**	160.33±8.802**
V.	Fraction F2	271.66±5.317^{NS}	244.66±9.158*	203.33±7.763**	169.83±12.040**	141.33±9.585**
VI.	Fraction F3	263.33±5.680^{NS}	251.00±6.573*	214.33±24.776**	167.00±18.601**	132.33±8.454**

Values are expressed as MEAN±SD at n=6, One-way ANOVA followed by Bonferroni test, *P<0.050, **P<0.001 and ^{NS}P>0.001 compared to the Diabetic control group STZ treated

Throughout the duration of the trial, the diabetic control group's serum glucose levels significantly increased. The STZ group (Group II) displayed elevated blood sugar levels indicative of impaired glucose tolerance; however, STZ animals treated with F1, F2, and F3 and Glibenclamide (10 mg/kg) experienced a drop in blood sugar levels more in line with the group that had received vehicle treatment (Table 2).

Effect of Test samples F1, F2 and F3 on serum lipid profile

Table 6: Variations in Lipid profile in normal control, inducer, standard and Test sample F1, F2 and F3

Group No.	Treatment	TC	TG	HDL	LDL
I.	Normal Control (Vehicle treated)	102.78±1.275	90.62±4.006	43.75±7.555	40.91±7.090
II.	STZ induced only	165.78±1.917	157.51±10.737	19.00±0.606	115.26±3.093
III.	STZ+ Standard Glibenclamide (10 mg/kg)	113.80±0.693**	96.45±1.336**	47.62±2.440*	37.25±11.433**
IV.	Fraction F1	138.61±1.185**	145.05±12.276^{NS}	39.88±5.257^{NS}	69.72±4.963*
V.	Fraction F2	132.83±1.336**	129.67±9.529^{NS}	40.48±4.880^{NS}	66.42±5.744**
VI.	Fraction F3	118.90±0.525**	115.38±4.110**	42.26±7.020^{NS}	53.52±7.130**

Values are expressed as MEAN±SD at n=6 in Lipid profile, one-way ANOVA followed by Bonferroni test, *P<0.050, **P<0.001 and ^{NS}P>0.001 compared to the Diabetic control group STZ treated

TG levels were higher in the STZ inducer group (Group II) than in the vehicle-treated rats (Group I). When compared to the STZ-treated group (Group II), the serum TGs concentrations of the rats in (Group III-VI) decreased over the course of 21 days (p 0.05). Rats in Groups III–VI that had received TC treatment likewise had lower TC levels than those in Group II that had received STZ inducers (p<0.05). Additionally, rats with diabetes treated for 21 days with (Group III-VI) showed a substantial (p<0.05) decrease in LDL (p <0.05). Comparing the STZ inducer group (Group II) to the usual control group (Group I), the HDL levels decreased (19.00±0.606). In contrast, rats treated with (Group III-VI) had higher HDL levels than those treated with STZ alone.

HISTOPATHOLOGY

Histology of *Pancreas*

Group I: Normal control: rat pancreas showing normal islets of Langerhans embedded in exocrine portion of pancreas

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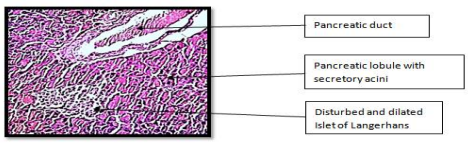
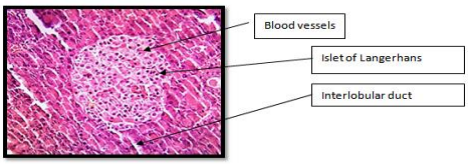
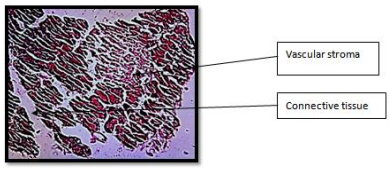


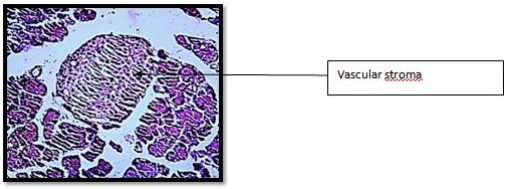
Group II: STZ induced: Rat pancreas showing shrinkage of islets of Langerhans with degeneration and necrosis with cytoplasmic vacuolation. Wider interlobular and intralobular ducts were observed.



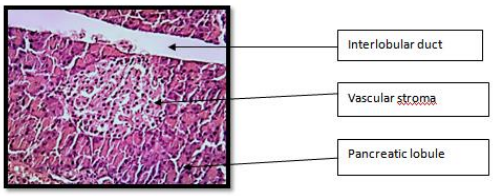
Group III: Standard drug treated: Glibenclamide (10 mg/kg) treated rats showing the islet cells interspersed between the acinar cells.



Group IV: Fraction 1 treated rats showed regeneration of islets was seen but some degeneration of the β cell in the center



Group V: Fraction 2 treated rats showed the islets appeared lightly stained than the surrounding acinar cells. Wide interlobular ducts connective tissues were observed.



Group VI: Fraction 3 treated rats showed regenerated islets of Langerhans with its normal large round shaped section containing cells that embedded in exocrine portion of pancreas.

DISCUSSION

As given in the results of α-amylase, the extract possesses concentration-dependent inhibitory activity for α-amylase. The percentage inhibition was 13.67± 0.765 for acarbose and 44.14± 0.638 for RG. Similarly, percentage inhibition in α-glucosidase was observed as 37.33±0.098 for acarbose and 50.83±0.076 for RG. A significant inhibition of 44.14 ±0.638 and 50.83±0.076 occurred in RG extract on both the invitro models.
Throughout the duration of the antidiabetic trial, the diabetic control group's serum glucose levels significantly increased. The STZ group (Group II) displayed elevated blood sugar levels indicative of impaired glucose tolerance; however, STZ animals treated with F1, F2, and F3 and glibenclamide (10 mg/kg) experienced a drop in blood sugar levels more in line with the group that had received vehicle treatment.

TG levels were higher in the STZ inducer group (Group II) than in the vehicle-treated rats (Group I). When compared to the STZ-treated group (Group II), the serum TGs concentrations of the rats in (Group III-VI) decreased over the course of 21 days (p 0.05). Rats in Groups III–VI that had received TC treatment likewise had lower TC levels than those in Group II that had received STZ inducers (p 0.05). Additionally, rats with diabetes treated for 21 days with (Group III-VI) showed a substantial (p 0.05) decrease in LDL (p 0.05). Comparing the STZ inducer group (Group II) to the usual control group (Group I), the HDL levels decreased (19.00±0.606). In contrast, rats treated with (Group III-VI) had higher HDL levels than those treated with STZ alone.

CONCLUSION

The study on *Rhododendron griffithianum*'s anti-diabetic activity in vitro and in vivo has provided valuable insights into its potential therapeutic benefits in managing Type I diabetes. The molecular mechanisms underlying these effects have been analyzed, and positive outcomes have been observed in animal models. The findings suggest that *Rhododendron griffithianum* may play a crucial role in improving insulin sensitivity and regulating glucose metabolism, making it a potential candidate for further

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exploration in developing novel anti-diabetic agents. However, more extensive research, including clinical trials involving human subjects, is needed to validate its efficacy and safety. Further interdisciplinary research and collaboration are needed to fully explore *Rhododendron griffithianum*'s potential and translate these findings into effective treatments for Type I diabetes.

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