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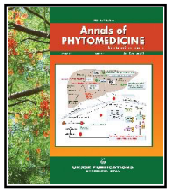

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Antidiabetic potential of *Bunchosia glandulifera* (Jacq.) Kunth leaf extract in diabetic rats: *In vivo*, *in vitro* and *in silico* analysis

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Abstract

The study aimed to evaluate the antidiabetic activity of ethanolic leaf extract of *Bunchosia glandulifera* (Jacq.) Kunth in streptozotocin (STZ: 35 mg/kg) induced diabetic rats. *B. glandulifera* is an exotic plant that is now being cultivated around the world due to its high nutritional value. Carbohydrates, flavonoids, alkaloids and tannins were identified by phytochemical tests in the extracts. The research makes use of an *in vivo* streptozotocin induced diabetic model, an *in vitro* rat hemidiaphragm model, as well as an *in silico* analysis to examine the antidiabetic activity as well as binding capacity. Ethanolic leaf extract of *B. glandulifera* was subjected to acute toxicity test, which demonstrated the drugs safety at a dose of 2000 mg/kg body weight. Effect of various doses (100, 200 and 400 mg/kg p.o) of extract was evaluated. Glimepiride (2 mg/kg) and insulin (0.25 IU/ml) were used as the standard drugs for *in vivo* and *in vitro* studies, respectively. The reduction in the blood glucose level and increased liver and muscle glycogen content in streptozotocin induced diabetic rats and the increased glucose uptake and glycogen content observed in *in vitro* rat hemidiaphragm technique proved that *B. glandulifera* leaf extract has a significant antidiabetic activity. The *in silico* study showed highest binding affinity of quercetin and rutin to the target protein AKT1, indicating that these compounds are responsible for antidiabetic activity of *B. glandulifera* leaf extract.

1. Introduction

Type 2 diabetes mellitus is a chronic condition of impaired insulin secretion, resistance or combination of both which leads to increased glucose level in the blood which further causes metabolic disruption of carbohydrate, lipid and protein (Defronzo *et al*., 2015; Duraisami *et al*., 2021; Venkatachalam *et al.*, 2021). Diabetes mellitus causes difficulties in the microvascular and macrovascular systems, including diabetic nephropathy, diabetic retinopathy, and diabetic kidney disease, which is the primary cause of death in these individuals (Cole and Florez, 2020; Bhatt *et al.*, 2019). Diabetes is a major health problem across the world. International Diabetes Federation has predicted that 451 million adults have diabetes worldwide and projected it will reach up to 693 million by 2045 (Cho *et al*., 2018). Given the numerous adverse effects associated with currently available antihyperglycemic agents, there is a growing interest in exploring herbal products for their therapeutic potential. Plants have demonstrated promising therapeutic activity, paving the way for the discovery of new therapeutic agents with minimal adverse effects (Divya and Sanjeev, 2021). Therefore, it is imperative to delve into herbal products to identify novel antihyperglycemic treatments that offer a more favourable safety profile (Nabi *et al.*, 2013; Kumar *et al.*, 2009).

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B. glandulifera is an exotic plant popularly known as *Falso guaraná* belonging to the family Malphiguiaceae. *B. glandulifera* is primarily native to northern America and also found in some parts of southern America which is grown around the world due to its high nutritional value (Silva *et al*., 2016). *B. glandulifera* is a natural source of antioxidants due to presence of bioactive compounds that has protective activity against oxidative stress mediated disease (Blank *et al.*, 2018). Leaf extract of *B. glandulifera* has shown very high antioxidant activity and the extract has high concentration of phenolic compounds and carotenoids (Peixoto *et al.*, 2017). Oxidative stress, operating *via* distinct molecular pathways, exerts a substantial influence on the pathological development of both diabetes mellitus and insulin resistance, providing an explanatory framework for various physiological processes contributing to diabetes and its associated complications (Saiharini and Padmaja, 2022). Antioxidants prevent the effects of oxidative stress by preventing the oxidation of free radicals as well as safeguarding and improving the function of β-cells (Rajendiran *et al*., 2018).

The goal of the present research is to determine how well the leaf extract of *B. glandulifera* reduces blood sugar levels in streptozotocin induced diabetic rats for this, we integrated three approaches *in silico*, *in vitro*, and *in vivo*. Initially, we assessed the potential of *B. glandulifera* against diabetes which was further supported with *in silico* studies.

2. Materials and Methods

2.1 Animals

Albino wistar rats of both the sex weighing about 170- 260 g were used for the current study under the approval from the Institutional

Animal Ethical Committee (NGSMIPS/IAEC/AUG-2022/313). The animals are provided with standard food and clean water. The animals are maintained in 12 h light/dark cycle.

2.2 Plant material

The *B. glandulifera* leaves were collected from the local farm in Mangalore, Karnataka, India. The plant was authenticated by Botanist, Dr. Krishnakumar, Department of Botany, Mangalore University, Konaje, Karnataka, India and the Herbarium Number: 21PY004R was deposited at NGSM Institute of Pharmaceutical Sciences, Paneer, Karnataka, India.

2.3 Acute toxicity study

The ethanolic leaf extract of *B. glandulifera* was found safe up to 2000 mg/kg body weight by oral route. Animals were found to be well tolerated 24 h later. As no death was observed, three dose levels 100 mg/kg, 200 mg/kg and 400 mg/kg body weight were chosen for the research.

2.4 Induction of diabetes

Six groups of animals were taken wherein each group contained six animals each weighing 170-250 g. In order to induce diabetes in overnight fasted rats, a single intraperitoneal injection of freshly prepared streptozotocin 35 mg/kg body weight in 0.1M cold citrate buffer at pH 4.5 was employed. To combat drug induced hypoglycaemia, the animals were given a 5% solution of glucose to drink. The rats in the control group were given only injection of citrate buffer as a placebo. Treatment was started on confirmation of diabetes induction by assessing the blood glucose levels. The grouping of animals has been given in Table 1. The blood was withdrawn from tail vein for the determination of blood glucose level (Vogel, 2002; Anand *et al.*, 2007).

Table 1: The grouping of animals

Group No.	Group name	Treatment
I.	Normal control	Normal food and water ad libitum
II.	Disease control	Streptozotocin (35 mg/kg i.p.)
III.	Standard control	Glimepiride (2 mg/kg)
IV.	Low dose	Ethanolic leaf extract of *B. glandulifera* (100 mg/kg, *p.o.*)
V.	Mid dose	Ethanolic leaf extract of *B. glandulifera* (200 mg/kg, *p.o.*)
VI.	High dose	Ethanolic leaf extract of *B. glandulifera* (400 mg/kg, *p.o.*)

2.5 Determination of blood glucose level

Blood glucose levels were assessed by obtaining blood samples from the tail vein region of the animals using clot accelerator vacutainers, and 2 ml of blood was collected. Subsequently, the blood samples were centrifuged at 10,000 rpm at a temperature of 4°C, resulting in the separation of serum. This collected serum was then utilized for further analysis. Specifically, 10 µl of the collected serum was mixed with 1 ml of the GOD-POD kit and incubated at 37°C for a duration of 10 min. After incubation, the absorbance of the mixture was measured at a wavelength of 505 nm, following the methods described in the studies by Noor *et al*. (2008); Trinder (1969).

2.6 Estimation of the glycogen content

Minced tissue was extracted by boiling in 3 ml of 30% potassium hydroxide for 20-30 min or until the tissue has dissolved. The glycogen was precipitated by adding 0.5 ml of saturated sodium sulfate and 3.5 ml of ethanol, heating again until the mixture boils. After cooling, the mixture was centrifuged in cold at 3000 rpm and the solution was discarded. The sediment was dissolved in 2 ml water and reprecipitated with 2.5 ml of 95% ethanol. The collected sediment was hydrolyzed for 2 to 2.5 h in 6 ml of 0.6 M hydrochloric acid in a bath of boiling water. The hydrolysate was chilled and one drop of phenol red indicator was added and neutralized carefully with sodium hydroxide until the indicator changes from pink through orange to a yellow colour. It was the diluted to 5 ml with distilled water and the glucose content was determined by glucose oxidase method (Goel *et al*., 2004).

2.7 Rat hemidiaphragm technique

Albino wistar rats weighing 100-150 g that have been fasted overnight. The rats were decapitated, and the diaphragm was quickly removed to avoid damage and separated into halves. Before being placed in a tiny conical flask with 2 ml physiological tyrode's solution and 2% glucose and incubated, the hemidiaphragm was rinsed in cool physiological tyrode's solution in order to dissolve any clots in the blood (Sabu and Subbaraju, 2002; Ghosh *et al.*, 2004; Dwivedi *et al.*, 2023) as follows: For glucose uptake: The hemidiaphragms were incubated for 30 min at 37°C in an incubator. A total of eight experiments were carried out. The hemidiaphragms were exposed to the following conditions:

Physiological tyrode solution in 2% glucose – control

Physiological tyrode solution in 2% glucose + insulin (0.25 IU/ml)

Physiological tyrode solution in 2% glucose + leaf extract (100 mg/ml)

Physiological tyrode solution in 2% glucose + leaf extract (200 mg/ml)

Physiological tyrode solution in 2% glucose + leaf extract (400 mg/ml)

Physiological tyrode solution in 2% glucose + insulin + leaf extract (100 mg/ml)

Physiological tyrode solution in 2% glucose + insulin + leaf extract (200 mg/ml)

Physiological tyrode solution in 2% glucose + insulin + leaf extract (400 mg/ml)

The hemidiaphragm was removed, and the incubated medium's glucose content was determined using the glucose-peroxidase (GOD/POD) process. The difference between the initial and final glucose content in the incubation medium is used to measure glucose uptake.

2.8 Statistical analysis

ANOVA test was used to carry out statistical analysis. Mean ± standard error of mean (SEM) will be calculated and compared using ANOVA. When *p* value is less than 0.05 then it will be considered as statistically significant. Statistical treatment of data was conducted using GraphPad Prism software version 9.5 using multiple comparison Dunnett's test. Values are given as mean ± SEM, n=6 in each group with statistical significance ($p<0.05$) .

2.9 *In silico* analysis

2.9.1 Protein selection and preparation

Initially, the proteins regulated by the phytoconstituents were predicted which were further matched with proteins involved in the pathogenesis of diabetes mellitus (Ternikar *et al.,* 2021). The proteins involved in disease progression were identified from DisGeNET database (https://www.disgenet.org/). Whereas, protein targets were predicted using the Swiss target prediction (http://www.swisstargetprediction.ch/). Further, the common targets was taken from the Venny 2.1.0 online tool (Kulkarni *et al.*, 2021; Madiwalar *et al.,* 2022; Khanal *et al.,* 2023).

2.9.2 Docking

Docking was performed with autodock vina on pyrx platform. The protein AKT1 (PDB ID: 7FCV) was prepared by removing hetero atoms followed by energy minimization. Further, the grid size was kept to maximum and docking was performed with the ligands. On completion of molecular docking discovery studio was utilized to perform analysis of interaction (Dwivedi and Shastry, 2023; Dwivedi *et al.,* 2021).

3. Results

3.1 Effect of *B. glandulifera* leaf extracts on body weight in STZ induced diabetic rats

The animals that were untreated for 28 days after the induction of diabetes by streptozotocin when noted down has showed 21.72% decrease in the body weight as compared to the initial weight of the animal where the animals that were treated with ethanolic extract of *B. glandulifera* in low dose test, mid dose test and high dose test groups has shown a gradual increase in body weight with 11.06%, 17.20% and 19.67%, respectively, as represented in Table 2.

3.2 Effect of *B. glandulifera* leaf extract on blood glucose level

On the first day before treatment, the animals exhibited a noticeably elevated ($p<0.05$) blood glucose level when compared to the normal control group. However, upon treatment with the standard drug glimepiride and various doses of the ethanolic extract of *B. glandulifera*, a significant reduction ($p<0.05$) in blood glucose levels was observed in comparison to the untreated disease control group. This data is presented in the Table 2.

3.3 Effect of leaf extract of *B. glandulifera* on glycogen

On the 28th day of the study, the glycogen levels in the tissue were assessed. The diabetic untreated animals displayed notably lower glycogen levels ($p<0.05$) in comparison to both the normal control animals and the treated animals receiving standard and ethanolic extracts. Conversely, the treated animals showed glycogen levels similar to those of the normal control group and significantly higher glycogen levels ($p<0.05$) when contrasted with the diabetic control group. This information is detailed in Table 2.

Table 2: Effect of *B. glandulifera* leaf extracts on blood glucose level, glycogen concentration in tissue and change in body weight in streptozotocin induced diabetic rats

Groups	Blood glucose level		Glycogen concentration		
	On induction of diabetes (Day 0)	28th day	Liver	Muscle	% Change in body weight
Normal control	99.83 ± 2.937^{b}	102.3 ± 3.480^{b}	45.27 ± 1.328^{b}	7.350 ± 0.2825^{b}	27.98%
Disease control	225.7 ± 8.143^{a}	330.3 ± 6.800	10.32 ± 0.577^{a}	1.767 ± 0.2362^{a}	-21.72%
Standard group	232.7 ± 7.074^{a}	95.50 ± 2.997^{b}	41.60 ± 0.975^{b}	6.533 ± 0.473^{b}	25.03%
Low dose test extract	230.3 ± 8.842^{a}	116.8 ± 2.414^{b}	33.20 ± 0.901^{ab}	3.350 ± 0.349^{ab}	11.06%
Mid dose test extract	249.8 ± 8.344^{a}	92.33 ± 3.792^{b}	34.73 ± 1.165^{ab}	4.833 ± 0.2552^{ab}	17.20%
High dose test extract	253.2 ± 7.245^{a}	87.33 ± 4.688^{b}	38.57 ± 0.759^{b}	5.600 ± 0.2671^{b}	19.67%

Values are given as mean ± SEM, n=6 in each grou.

where, a represents statistical significance ($p<0.05$) vs normal control and b represents statistical significance ($p<0.05$) vs disease control.

3.4 Effect of *B. glandulifera* leaf extract on glucose uptake by isolated rat hemidiaphragm

The *in vitro* study of glucose uptake by rat hemidiaphragm incubated in tyrode solution containing 2% glucose when treated with different dose of ethanolic leaf extract has shown significantly increased uptake of glucose from the incubated solution. Uptake of glucose by rat hemidiaphragm incubated in physiological tyrode solution containing 2% glucose when treated with insulin and ethanolic extract in combination with insulin has shown significantly higher amount of glucose uptake than that of treated with ethanolic extract alone that is mentioned in Table 3.

Table 3: Effect of *B. glandulifera* leaf extract on glucose uptake by isolated rat hemidiaphragm

Group	Groups	Glucose uptake
I (Control)	Physiological tyrode solution with 2% glucose	4.652 ± 0.1432[b]
II (Standard)	Physiological tyrode solution with 2% glucose + insulin	9.810 ± 0.075[a]
III (Low dose)	Physiological tyrode solution with 2% glucose + leaf extract (100 mg/ml)	5.277 ± 0.1326[ab]
IV (Mid dose)	Physiological tyrode solution with 2% glucose + leaf extract (200 mg/ml)	6.093 ± 0.0727[ab]
V (High dose)	Physiological tyrode solution with 2% glucose + leaf extract (400 mg/ml)	6.475 ± 0.0630[ab]
VI (Standard + Low dose)	Physiological tyrode solution with 2% glucose + insulin + leaf extract (100 mg/ml)	10.33 ± 0.0432[ab]
VII (Standard + Mid dose)	Physiological tyrode solution with 2% glucose + insulin + leaf extract (200 mg/ml)	11.43 ± 0.0445[ab]
VIII (Standard + High dose)	Physiological tyrode solution with 2% glucose + insulin + leaf extract (400 mg/ml)	13.21 ± 0.1096[ab]

Values are given as mean ± SEM, n=6 in each group

where, a represents statistical significance ($p<0.05$) vs normal control and b represents statistical significance ($p<0.05$) vs disease control.

3.5 *In silico* studies

The results in Table 4 indicate the binding affinities of various ligands to AKT1. Binding affinity refers to the strength of the interaction between a ligand and its target, typically a protein or receptor. It is often measured using equilibrium binding constants (Kd) or association rate constants (Ka). Kaempferol exhibits a binding affinity of -7.8, it is 3D and 2D interaction is shown in (Figure 1). This indicates a relatively strong interaction between kaempferol and the AKT1. The carotenoid compound shows a binding affinity of -8.4, it is 3D and 2D interaction is shown in (Figure 1). A negative value suggests a favourable binding interaction, and the magnitude of -8.4 indicates a relatively high affinity. Citric acid demonstrates a binding affinity of -5.4. The negative value suggests binding, but the lower magnitude (-5.4) compared to the other ligands indicates a weaker interaction. Malic acid exhibits a binding affinity of -4.8, which is lower than the other ligands mentioned. This suggests a weaker binding interaction with the target compared to the other compounds. Quercetin displays a binding affinity of -8.6, it is 3D and 2D interaction is shown in Figure 1, respectively. Similar to carotenoid, this indicates a strong interaction between quercetin and the target. Rutin also demonstrates a binding affinity of -8.6, and it is 3D and 2D interaction is shown in Figure 1, respectively. This suggests a strong binding interaction similar to quercetin. Spirolaurenone exhibits a binding affinity of -7.1. Although, slightly lower than the affinities of quercetin and rutin, it still indicates a relatively strong binding interaction.

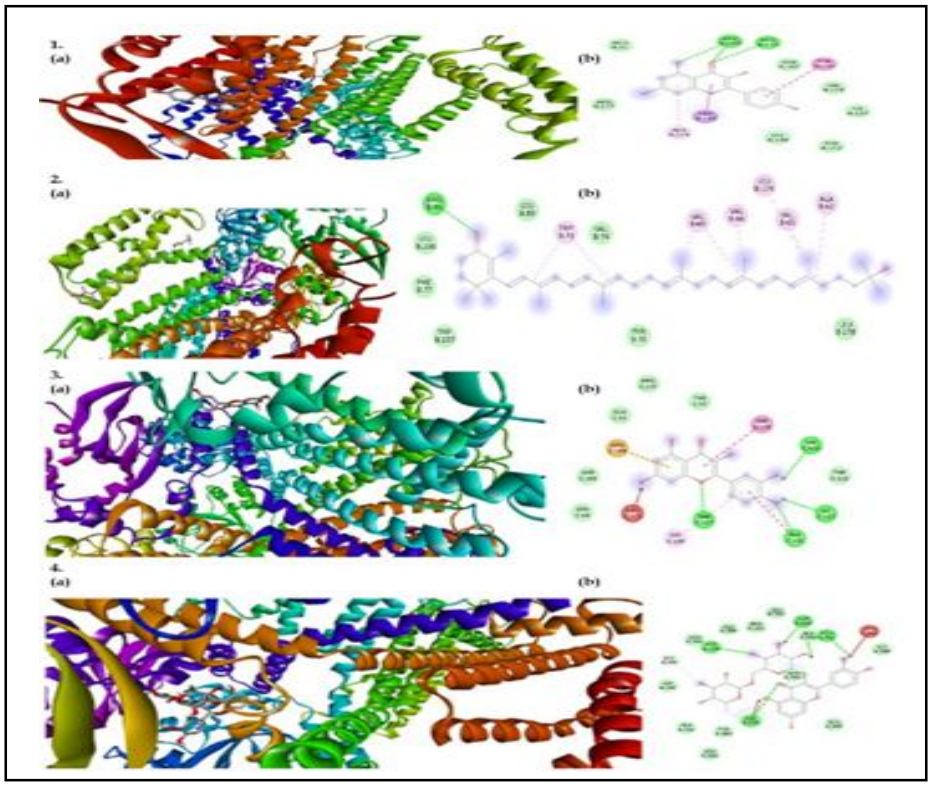

Figure 1: The (a) 3D and (b) 2D interaction of 1. Kaempferol, 2. Carotenoid, 3. Quercetin, and 4. Rutin with AKT1.

Table 4: Binding affinity with AKT1 (PDB ID: 7FCV)

Ligand	Binding affinity (kcal/mol)
Kaempferol	-7.8
Carotenoid	-8.4
Citric acid	-5.4
Malic acid	-4.8
Quercetin	-8.6
Rutin	-8.6
Spirolaurenone	-7.1

4. Discussion

The present study was designed to evaluate the antidiabetic activity of ethanolic leaf extract of *B. glandulifera*. The study was conducted using two models, *i.e., in vitro* rat hemidiaphragm model and *in vivo* streptozotocin induced diabetic model. The parameters utilized for the evaluation of antidiabetic activity were body weight, blood glucose amounts, liver as well as muscle glycogen in case of *in vivo* streptozotocin induced diabetic model, glucose uptake in case of *in vitro* rat hemidiaphragm model. In the present experiment, glimepiride was used as a standard *in vivo* studies and insulin was used as standard in *in vitro* studies (Sabu and Subbaraju, 2002).

Preliminary qualitative phytochemical analysis of ethanolic leaf extract of *B. glandulifera* showed the presence of proteins, alkaloids, carbohydrates, flavonoids and triterpenoids. Similarly, a study conducted by Blank *et al.* (2018) showed the presence of flavonoids, phenolic compounds, vitamin c, caffeine, carotenoids. Study on the acute oral toxicity of ethanolic extract of leaves of *B. glandulifera* were conducted in albino wistar rats. They displayed no harmful signs or deaths, thus the dose of 2000 mg/kg was considered safe.

A broad-spectrum antibiotic called streptozotocin damages the cells that produce insulin of the pancreas, causing diabetes in a variety of animal species. Despite the fact that the animals developed diabetes permanently, the administration of smaller dosages of streptozotocin resulted in a partial loss of pancreatic β-cells. Thus, regeneration is conceivable in these animals because they still have β-cells. In a study by Chaudhary *et al.* (2010), it was observed that there was a decrease in blood glucose levels, indicating an antidiabetic effect in mice with streptozotocin induced diabetes. Similarly, in the present study, 35 mg/kg body weight of streptozotocin (i.p) was used to induce diabetes. When compared to normal rats, the treatment of streptozotocin caused a considerable decrease in body weight. The body weights of animals in ethanolic leaf extract of *B. glandulifera* of 100 mg/kg, 200 mg/kg and 400 mg/kg were increased, respectively, and were comparable to that of diabetic animals treated with standard (glimepiride). When treated with leaf extract, the loss of body weight in streptozotocin induced diabetic rats was entirely reverted.

In *in vivo* studies, induction of diabetes with streptozotocin resulted in significant increase in blood glucose level compared to the normal. Diabetic rats on treatment with ethanolic extracts of leaf of *B. glandulifera* 100 mg/kg, 200 mg/kg and 400 mg/kg on 28th day resulted in dose-dependent reduction in blood glucose levels, respectively. The liver and muscle glycogen level in diabetic control was declined to a very low value as compared to that of normal. Application of ethanolic leaf extract of *B. glandulifera* 100 mg/kg, 200 mg/kg and 400 mg/kg resulted in restoration of liver and muscle glycogen level compared to disease control. The results indicate that the leaf extract of *B .glandulifera* reduces the blood glucose level in diabetic rats and improves glycogen storage by the tissues. The reduction in blood glucose level and improvement in liver and muscle glycogen storage produced by 400 mg/kg of leaf extract was compared favourably to the standard (glimepiride).

In *in vitro studies*, there was a considerable rise in glucose uptake in diaphragm in tyrode solution containing 2% glucose + insulin as compared to that of diaphragm in tyrode solution containing 2% glucose only. The glucose uptake in diaphragm in tyrode solution with 2% glucose + leaf extract of 100 mg/kg, 200 mg/kg and 400 mg/kg were less than that of glucose uptake in diaphragm in tyrode solution with 2% glucose + insulin.

Insulin improved the ethanolic leaf extracts ability to influence glucose absorption which was evident in diaphragm in tyrode solution with 2% glucose + insulin + leaf extract of 100 mg/kg, 200 mg/kg and 400 mg/kg, respectively. When the tissue was treated with various doses of the extract, it showed a dose-dependent rise in the uptake of glucose by the cells in the tissue. Thus, the ethanolic leaf extract may be acting by a mechanism similar to that of the insulin. The uptake of glucose increased significantly in diaphragm in tyrode solution with 2% glucose + insulin as compared to that of glucose uptake in diaphragm in tyrode solution with 2% glucose. The glucose uptake in diaphragm in tyrode solution with 2% glucose + insulin + leaf extract of 100 mg/kg, 200 mg/kg and 400 mg/kg were less than that of diaphragm in tyrode solution with 2% glucose + insulin. The impact of the ethanolic leaf extract on glucose uptake was enhanced by insulin which was evident in diaphragm in tyrode solution with 2% glucose + insulin + leaf extract of 100 mg/kg, 200 mg/kg and 400 mg/kg. There was a dose-dependent rise in glucose uptake by the tissue when incubated with varying doses of ethanolic leaf extract of *B. glandulifera*. This increase in glucose uptake caused by the leaf extract may be due to the glycogenesis similar to insulin. Based on the results of *in slilico* studies, citric acid and malic acid exhibit lower binding affinities, and suggesting weaker interactions. On the other hand, quercetin, rutin, carotenoid, and kaempferol show the highest binding affinities, indicating strong interactions with the target which indicates the antidiabetic action of leaf extract is due to these constituents (Ain *et al*., 2022).

The outcomes of the *in vitro* studies shows two possible mechanisms by which the extract reduces the blood glucose level by increased utilization of glucose by the tissue and significant increase in the liver and muscle glycogen content. The effect of the leaf extract treated diabetic animal may be because of the reactivation of glycogen synthase system.

5. Conclusion

The following conclusion can be drawn from the results obtained from *in vivo* streptozotocin induced diabetic model and *in vitro* rat hemidiaphragm model. Diabetes leads to decrease in the body weight of the animal, current study demonstrated that administration of ethanolic leaf extract of *B. glandulifera* resulted in significant increase in body weight. Effect of the ethanolic leaf extract of *B. glandulifera* on streptozotocin induced diabetic rats showed statistically significant decrease in blood glucose level and increased glycogen storage by the liver and muscle tissue. In *in vitro* studies, the ethanolic leaf extract

of *B. glandulifera* was able to produce significant increase in the glucose uptake and glycogen storage by the tissue. The antidiabetic effect of ethanolic leaf extract of *B. glandulifera* was almost comparable to that of glimepiride in the *in vivo* model. From the *in vitro* studies we can conclude that, the ethanolic leaf extract of *B. glandulifera* may have a similar mechanism of action as that of insulin, *i.e*., facilitates glucose uptake and promotes glycogenesis. The synergistic effect of the ethanolic leaf extract of *B. glandulifera* with insulin may be due to the increased sensitivity of the tissue towards insulin. However, further investigation is required by performing various *in silico*, *in vitro* and clinical methodologies of the extract formulation to get a better understanding about the mechanism of action of the extract.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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