ORIGINAL ARTICLE

Anti-diabetic potential of chloroform extract of flowers of *Calotropis gigantea*: An *in vitro* and *in vivo* study

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The chloroform extract of *Calotropis gigantea* flowers was evaluated for anti-diabetic activity in alloxan-induced hyperglycemia *in vivo* and inhibition of α-amylase and α-glucosidase *in vitro*. It was also intended to establish correlation between the serum marker antioxidant enzymes and diabetes. Diabetes was induced by a single intraperitoneal injection of alloxan monohydrate freshly prepared in a dose of 150 mg/kg. Chloroform extract showing presence of flavonoids was administered orally at the doses of 100 and 200 mg/kg for 21 consecutive days. Fasting blood glucose level, glycosylated haemoglobin, blood glutathione, serum creatinine kinase, serum lactate dehydrogenase levels as well as final change in body weight were evaluated. *In vitro* inhibition of carbohydrate digestive enzymes (α-amylase and α-glucosidase) was also determined. Experimental findings showed moderately significant anti-diabetic potential of extract in terms of reduction of fasting glucose level in diabetic rats. The extract was found statistically significant in maintaining the level of serum marker antioxidant enzymes. Overall, the effect of chloroform extract particularly 200 mg/kg was moderate as compared to that of standard drug glibenclamide.

Key words: Anti-hyperglycemic, *calotropis gigantean*, glibenclamide, α-amylase, α-glucosidase

INTRODUCTION

The pharmacotherapy of diabetes has recently undergone unprecedented expansion. Diabetes mellitus is a disease in which homeostasis of carbohydrate, protein and lipid metabolism is improperly regulated by hormone insulin resulting in elevation of fasting and postprandial blood glucose level.[1] The major complications associated with diabetes include retinopathy, neuropathy, nephropathy, atherosclerotic coronary artery disease and peripheral atherosclerotic vascular disease. Besides hyperglycemia, several other factors like hyperlipidemia and enhanced oxidative stress play a major role in diabetic pathogenesis.[2] However, the challenge is to optimize glycaemic control with minimum number of medication while taking into consideration the cost of the therapy, adverse effect profiles, ease of administration, and the urgency for blood sugar normalization. Insulin, Insulin sensitizers and insulin secretogogues, as well as enzyme inhibitors such as α-glucosidase and α-amylase inhibitors contribute as an important therapeutic option in the treatment of diabetes.[3]

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Recent awareness of therapeutic potential of several traditionally used plants has opened a new dimension for the study and research of medicinal plants. In traditional medicine, several Indian medicinal plants or their extracts have been used to treat diabetes.[4] Release of the glucose from food sources by key gastrointestinal enzymes (α-amylase and α-glucosidase) is the main factor for the postprandial rise in diabetic glucose level. α-amylase inhibitors can play an important role to reduce the rise in glucose level after meals. α-glucosidase catalyses the final digestion of carbohydrates. α-glucosidase inhibitors (acarbos, miglitol and voglibose) are known to reduce postprandial rise in diabetic glucose level.[5,6]

Calotropis gigantea (family, *Asclepidaceae*) known commonly as *Akda* is widely distributed throughout India. The whole plant when dried and consumed is a good tonic, antihelmintic and an expectorant. Leaves and arial parts of the plant were reported for anti-diarrhoeal activity, anti-candida activity and anti-bacterial activity.[7-9]

Traditionally, the dried root is powdered and effectively used to cure bronchitis, asthma, leprosy, eczema and

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elephantiasis. The ark latex is processed and finds use in treating vertigo, baldness, hair fall, tooth aches, intermittent fevers, rheumatoid /joint swellings and paralysis.[10]

The literature survey reveals that the flower of plant contains flavonoids, triterpenoids and volatile long chain fatty acids.[11] Flavonoids are reported to have antioxidant and anti-hyperglycemic activity.[12,13] This prompted us to explore the scientific basis of *C. gigantea* in treating alloxan-induced hyperglycemia *in vivo* and inhibition of α-amylase and α-glucosidase *in vitro*.

MATERIALS AND METHODS

Collection and Authentification

Dried *C. gigantea* flowers, were collected locally from Neemuch (in Madhya Pradesh, India, it is commonly available) and authenticated by Department of Botany, Rajasthan University, Jaipur (Raj.). A voucher specimen was retained in the department (Voucher Specimen No. RUBL 20686).

Preparation of the Extract

The *C. gigantea* were successively extracted with petroleum ether, chloroform and 90% ethanol by continuous hot percolation method using soxhlet apparatus. The solvent was removed under reduced pressure to give a dried extract, and the percentage yield calculated in terms of dried basis was 3.9, 2.4 and 2.1% w/w with respect to the crude material.

Phytochemical Screening

The preliminary phytochemical studies were performed for testing different chemical groups in Petroleum ether, Chloroform and 90% ethanol extracts.[14]

In vitro Anti-diabetic Activity

Inhibition of α–amylase

Starch solution (0.5% w/v) was prepared in Tris HCl buffer with 6.7 mM sodium chloride (pH 6.9) in boiling water for 5 min and preincubated at 37°C for 5 min. Chloroform extract of *C. gigantea* was dissolved in DMSO to obtain concentration of 10, 20, 40, 60, 80 and 100 µg/ml. Then 0.2 ml of plant extract was added to the tubes containing starch solution. A total of 0.1 ml pancreatic amylase solution prepared in Tris HCl buffer (2 units/ml) was added to the tube containing plant extract and starch solution. The reaction was carried out at 37°C for 10 min. The reaction was stopped by adding 0.5 ml 50% acetic acid. The reaction mixture was centrifuged at 3000 rpm for 5 min 4°C. The absorbance of supernatant was measured at 595 nm.[15,16]

$$\text{Percentage inhibition} = \frac{\text{Control-Test}}{\text{Control}} \times 100$$

Inhibition of α – glucosidase

Enzyme solution prepared in Tris buffer (pH 8) was added to the tubes containing increasing concentration of Chloroform extract of *C. gigantea* (5, 10, 20, 40, 80 and 100 µg/ml) at 37°C for 60 min. Then the reaction mixture was heated for 2 min in boiling water to stop reaction. The absorbance was measured at 540 nm. Percentage inhibition was calculated by using the following equation:[17]

$$\text{Percentage inhibition} = \frac{\text{Control-Test}}{\text{Control}} \times 100$$

In-vivo Anti-diabetic Activity

Animals

Healthy adult Wistar albino rats of either sex (150 to 200 g) were obtained from the central animal house facility of Hamdard University, New Delhi. They were maintained under standard laboratory conditions at 25 ± 2°C, relative humidity (50 ± 15%) and normal photoperiod (12-hour light-dark cycle) were used for the experiment. Commercial pellet diet (MFD, by Nav Maharastra Chakan Oil Mills Ltd., New Delhi, India) and water were provided ad libitum throughout the course of study. The Institutional animal Ethical Committee (IAEC) for animal experimentation of the institute approved the study protocol (Registration no: PBRI/173/CPCSEA/009/002).

Acute Toxicity Study

Acute oral toxicity test was carried out according to the OECD guideline No. 423. Wistar Albino Rats were kept for overnight fasting prior to drug administration. A total of three animals were used, which received a single oral dose in 2000 mg/kg, body weight of CG chloroform extract. The animals were observed for a period of 24 hr for the changes in behaviour, hypersensitivity reactions etc. Mortality, if any, was determined over a period of 2 weeks.[18]

Induction of Diabetes

Animals were fasted for 24 hr and then a single intra peritoneal injection of 150 mg/kg of alloxan monohydrate in freshly prepared 0.9% NaCl was injected by IP route. The diabetes was confirmed by estimating the blood glucose level after 2 days by glucometer based on glucose oxidation method. Rats having blood glucose level >250 mg/dl were selected for further study.[19,20]

Experimental Design

All the procedures for antidiabetic activity were performed in accordance with the Institutional Animal Ethical Committee constituted as per the norms of CPCSEA, under the Ministry of Animal Welfare, Govt. of India, New Delhi, India. In order to assess the anti-diabetic activity, the animals were divided in five groups of six animals in each group.
Group 1: Normal control, 0.9% NaCl-treated animals

Group 2: Diabetic control, Alloxan-treated rats (150 mg/kg body weight)
Group 3: Test drug treated, *Calotropis gigantea* extract, CG (100 mg/kg body weight)
Group 4: Test drug treated, *Calotropis gigantea* extract, CG (200 mg/kg body weight)
Group 5: Standard drug, Glibenclamide-treated rats (5 mg/kg body weight)

The extract was administered orally at two dose level for a period of 21 days from starting day of diabetes. The blood samples were withdrawn from the rats by tail vein puncturing with hypodermic needle on hours 0, 1, 2, 4, 6 (short term study after a single administration of doses) and then on days 1st, 7th, 14th and 21st (long term study). The blood glucose level was determined by using the glucometer based on glucose oxidation methods. For other serum profiles, the blood was collected from the retro-orbital plexus of the rats using capillary tubes in to eppendorf tubes containing heparin. The plasma separated by centrifugation, was analysed for serum cholesterol, serum urea and serum creatinine.[19,21]

Statistical Analysis

All the data are expressed as mean ± SEM and analysed statistically using ANOVA followed Dunnets test and compare with respective control group. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Phytochemical Studies

The preliminary phytochemical analysis of chloroform extract of *C. gigantea* flowers showed the presence of flavonoids and triterpenoids.

Inhibition of α–amylase

Chloroform extract of *C. gigantea* as a test drug and Acarbose as reference standard were analysed for α-amylase inhibitory activity at concentration of 10, 20, 40, 60, 80 and100 µg/ml. A dose dependent, gradual rise in inhibition of α-amylase was observed for both drugs as shown in Figure 1. The IC_{50} value for test drug and Standard drug were found to be 52.3 µg/ml and 47.1 µg/ml, respectively.

Inhibition of α–glucosidase

A gradual rise in inhibitory activity of α-glucosidase was observed for chlorofrom extract of *C. gigantea* (Test Drug) and acarbose (Standard reference) as shown in Figure 2. The concentration of both drugs varies from 5–100 µg/ml. The IC_{50} value for test drug and standard drug were found to be 18.2 µg/ml and 71.9 µg/ml, respectively. The test drug shows poor α-glucosidase inhibitory activity compared to acarbose.

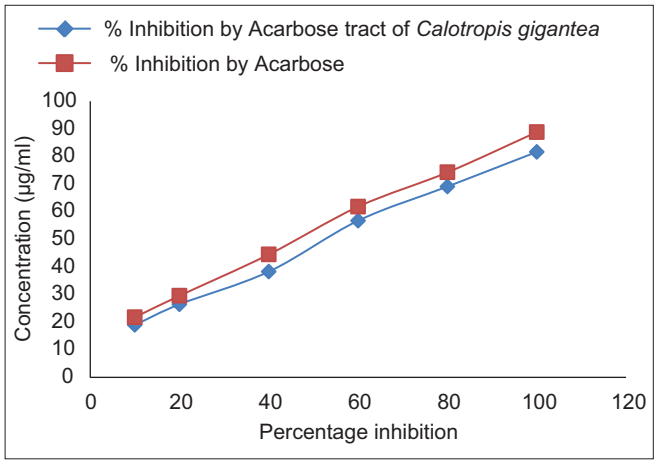

Figure 1: α-amylase inhibitory activity

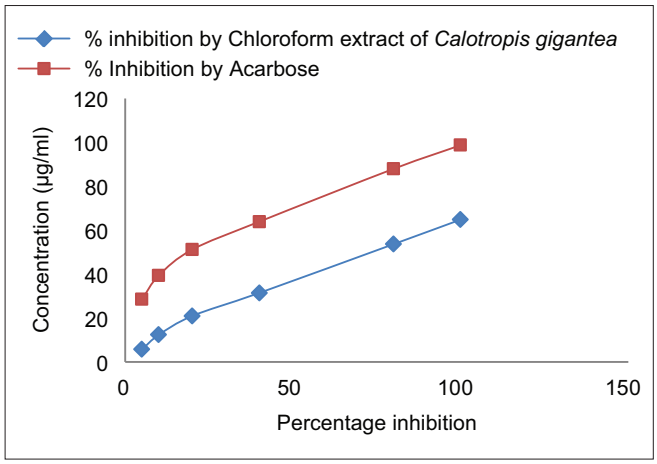

Figure 2: α-glucosidase inhibitory activity

Acute Toxicity Study

Acute toxicity study revealed the non-toxic nature of the drug. Chloroform extract of *C. gigantea* showed no mortality or any toxic reaction at the maximum tested dose level of 2000 mg/kg.

Effect on Blood Glucose level

The anti-hyperglycemic effect of chloroform extract of *C. gigantea* (100 and 200 mg/kg) on the fasting blood sugar level of normal and diabetic rats are shown in Figure 3 (Short term study) and Figure 4 (Long term study). The chloroform extract of *C. gigantea* showed moderately significant ($P<0.01$) decrease in the fasting blood glucose level as compared to highly significant ($P<0.001$) diabetic control group. On chronic administration, significant difference was observed between experimental and diabetic control rats in lowering fasting blood glucose level. At a dose of 200 mg/kg, the extract significantly lowered blood glucose level and showed maximum reduction of 53.19% ($P<0.01$) on day 21, whereas inhibition of 60.83% ($P<0.001$) was found for glibenclamide on day 21.

Effect on Body Weight

Figure 5 shows the significant (P<0.001) effect of alloxan drug on the weight of group II animals. Chloroform extract of *C. gigantea* shows moderate significant (P<0.01) increase in weight of the animals in Group III and IV. However, glibenclmide shows highly significant (P<0.001) rise in the weight of Group V animals as compared to alloxan-treated group.

Effect on Different Serum Parameters

Further, Figure 6 shows the level of glycosylated haemoglobin. Glibenclamide shows highly significant (P<0.001) reduction in glycosylated haemoglobin comparable to normal animals.

Furthermore, the level of serum marker antioxidant enzymes, i.e. blood glutathione (GSH), serum creatine kinase (CK), serum lactate dehydrogenase (LDH) levels were also altered significantly (P<0.001) in group II rats as shown in Figures 7-9. GSH level decreases significantly in group II rats, which was significantly restored by *C. gigantea* (200mg/*kg*) as shown in Figure 3. CK and LDH levels were significantly (P<0.001) increased in alloxan-treated group II. However, treatment with chloroform extract of *C. gigantea* reduced it.

DISCUSSION

The number of people suffering from diabetes all over the world increases gradually. It is possibly world's largest growing metabolic disease. The basic fundamental lying behind hyperglycemia involved overproduction and decreased utilization of glucose.[22] Alloxan, destroys β cells of islet of langerhens of pancreas resulting in decrease in the insulin secretion and leads to decreased use of glucose by tissues.[23] Expression of elevated fasting blood glucose level confirmed the induction of diabetes. The present study was focused on exploring the flowers of *C. gigantea* for anti-hyperglycemia. The result of present study revealed that the decrease in final fasting blood glucose levels of different groups in both short as well as long term studies indicates the effectiveness of extract in alloxan-treated diabetic animals.

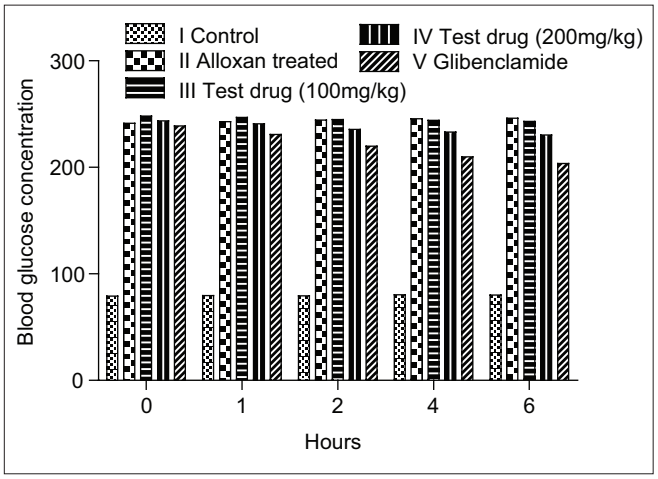

Figure 3: Effect of chloroform extract of *Calotropis gigantea* (Short term study)

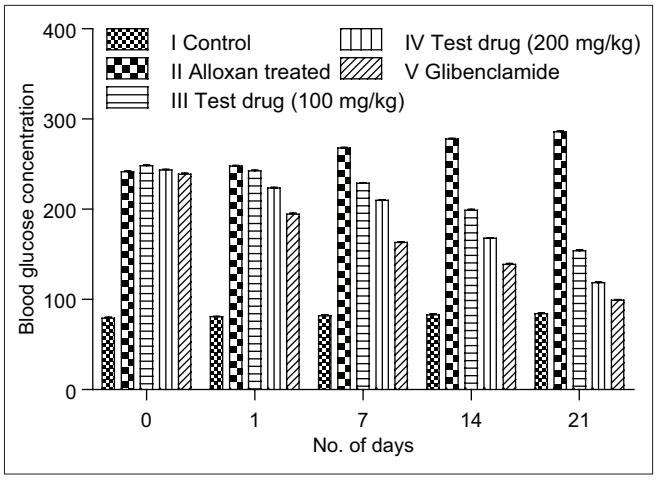

Figure 4: Effect of chloroform extract of *Calotropis gigantea* (Long term study)

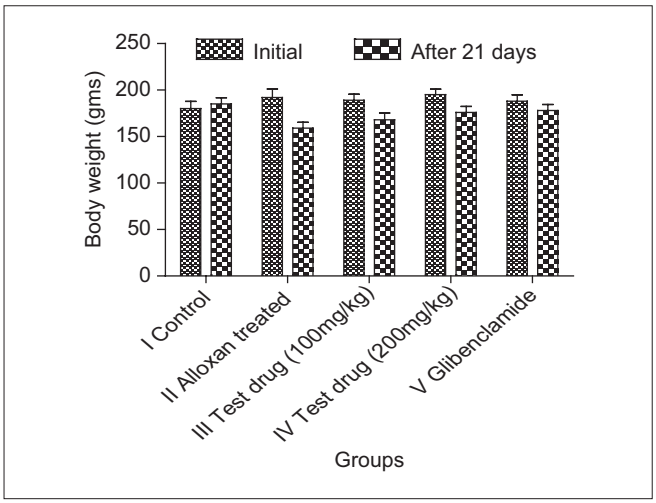

Figure 5: Effect of chloroform extract of *Calotropis gigantea* on body weight

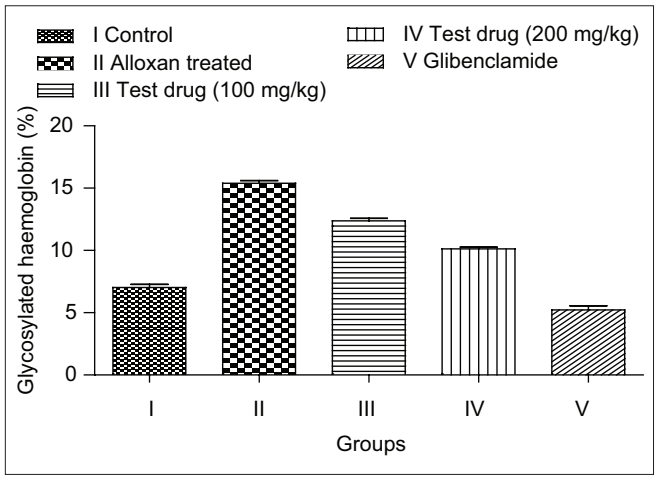

Figure 6: Effect of chloroform extract of *Calotropis gigantea* glycosylated haemoglobin

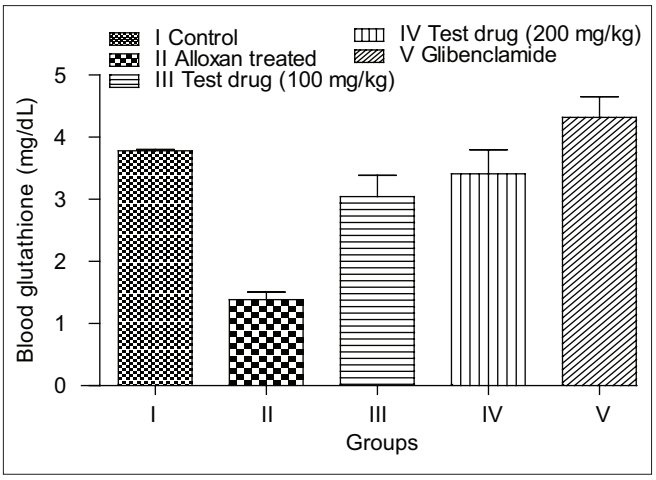

Figure 7: Effect of chloroform extract of *Calotropis gigantea* on blood glutathione level

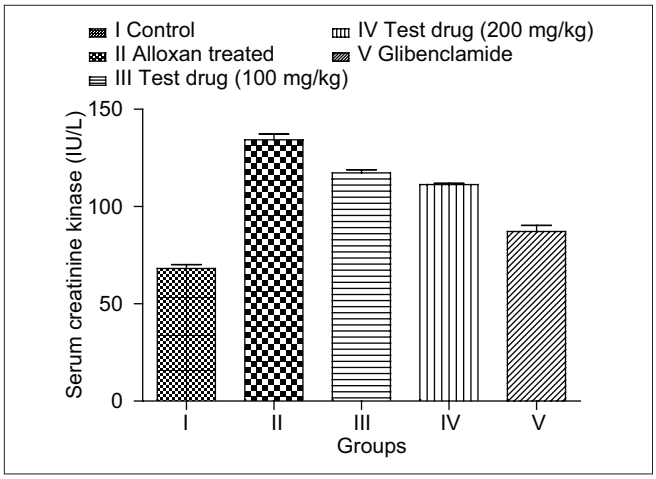

Figure 8: Effect of chloroform extract of *Calotropis gigantea* on serum creatinine kinase

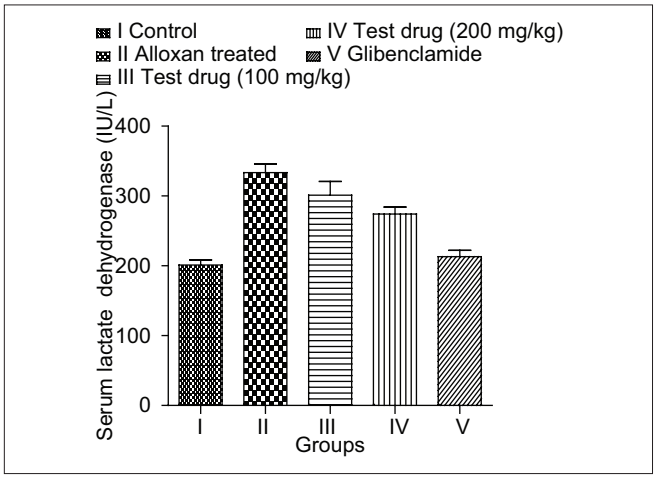

Figure 9: Effect of chloroform extract of *Calotropis gigantea* on serum lactate dehydrogenase

Induction of diabetes with alloxan is associated with the characteristic loss in body weight due to the catabolism of fats and proteins.[24] However, treatment with chloroform extract of the *C. gigantea* significantly maintains the body weight of the animals which is comparable to glibenclamide may be by the reversal of gluconeogenesis and glycogenolysis.

Diabetes is characterized by various parameters, glycosylated haemoglobin is one of the important parameter among them. During the fasting conditions, the excess glucose present in the blood reacts with haemoglobin and form the glycosylated haemoglobin. The amount of glycosylated haemoglobin is directly proportional to the blood glucose level.[25] The decrease in concentration of glycosylated haemoglobin is a good indication of anti-diabetic activity of test drug. Administration of chloroform extract of the *C. gigantea* moderately reduces the glycosylated haemoglobin level. Reduction in glycosylated haemoglobin level indicates decrease in glycation of haemoglobin and ultimately produces an anti-diabetic effect.

Alloxan produces oxygen radicals in the body, which causes pancreatic injury.[22] Glycosylated haemoglobin was found to alter the structure and function of antioxidant enzymes (GSH, CK and LDH levels) such that they are unable to fight against the free radicals, exacerbating oxidative stress in diabetes.[26] Induction of alloxan caused decrease in GSH level. In the present study, chloroform extract of *C. gigantea* (200 mg/kg) significantly restores the level as of normal group animals [Figure 3]. The level of CK and LDH decreases in hyperglycemia. Our study revealed that test drug significantly (P<0.05) increases the level as compared to highly significant effect by glibenclamide. Recovering the structure and function of antioxidant enzymes by chloroform extract of *C. gigantea* can significantly reduce the imbalance between free radical generation and its detoxication.

α- amylase is main enzyme present in pancreas responsible for the digestion of starch and absorption of glucose. Its inhibitors such as acarbose inhibit the release of glucose in the blood and thereby achieving the anti-diabetic effect.[27] Our finding revealed that the chloroform extract of *C. gigantea* efficiently inhibited the enzyme [Figure 7]. α-glucosidase is responsible for the digestion of carbohydrates to simpler carbohydrates and its absorption in small intestine.[28] Chloroform extract of *C. gigantea* significantly inhibit the enzyme and thus attributed for anti-diabetic activity.

Flavonoids are one of the most numerous and widespread groups of phenolics in plants. This phenols and flavonoids have major role in reducing oxidative stress associated with diabetes.[29] Phytochemical studies also reflect that chloroform extract of *C. gigantea* flowers also have flavonoids and triterpenoids that is capable to produce the anti-diabetic activity.

CONCLUSION

The result of this investigation revealed that the chloroform extract of *C. gigantea* flowers possesses significant antidiabetic activity in treating alloxan-induced hyperglycaemia *in vivo* and inhibition of α-amylase and α-glucosidase *in vitro*. *In vivo* activity also showed that the extract is capable of maintaining the level of serum antioxidant enzymes. The main constituent present in the extract was identified as flavonoids. From the previous literature it was reported that flavonoids and terpenoids are strong antioxidant and showed very potent antidiabetic activity. Hence in future study, fractionization is going on for the isolation of lead molecule from chloroform extract that will be responsible for potent antioxidant and anti-diabetic agents.

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