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Extraction, Purification and Testing of Anti Diabetic Activity of Secondary Metabolites from Calotropis

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Abstract
Diabetes being a life threatening disease of the 19th and 20th century affecting 2 out of every 5 individuals needs an attention for the development of promising treatment protocols. Being a hereditary disease makes the count far more than the genetic and external factors for the disease. The worst part of diabetes is the lack of cure and the fact that it can only be managed by life style and diet changes. Thus adapting a healthy lifestyle and use of anti diabetic food and herbs improves the condition of the victim. The current work involves the study of anti diabetic activity of Calotropis leaf and flower extracts. Solvents like ethanol, water and chloroform were used to prepare the extracts from plant based samples. These extracts were tested for their efficacy against blood glucose by measuring their inhibition against the vital enzymes in glucose absorption alpha amylase and glucosidase. The parameter used to check the anti diabetic activity was inhibition of alpha amylase and alpha glucosidase by the extracts. They act as references for the anti diabetic activity of the samples.

Keywords
Calotropis, Anti diabetic, Alpha glucosidase, Alpha amylase, Phytochemical characterization

Introduction
India is long been known for its culture and heritage. It is well known to be an archive of medicinal plats and herbs [1]. This forms the basis of unani and ayurvedic therapy in the country. Calotropis is one such medicinal plants which is abundantly available [4]. Though known to be poisonous in some parts this plant is a very good medical substitute being used in several health disturbances [7]. These plants can be used as potential resources for the extraction of several important therapeutics [9]. This plant originated form the family apocynaceae [13].
The plant is also available in countries like Indonesia, China, Pakistan, Cambodia, Sri Lanka, Malaysia etc. Thus its a wild plant with no specific restriction in climatic conditions. This plant is commonly named as giant milk weed in view of the latex that it secretes from the leaf tips upon tearing from the plant. It is known for its plethora of medicinal activities.

There are several herbal and alternative consumables coming up against diabetic treatment. The better and economic method for the detection of their therapeutic role is to test them for their inhibitory activity against alpha amylase and alpha glucosidase. Inhibition of these two enzyme would directly delay the digestion of carbohydrates causing a decline in the glucose absorption rate [5]. This causes a reduction in the postprandial glucose levels. Finally reducing the risk of diabetic complications.

Calotropis procera is a flowing plant which belongs to the family apocynaceae and available widely in south Asia and North Africa. It is well known for its white milky and poisonous sap. Further several research studies have reveled the antidote activity of calotropis against snake bite [14]. The plant is a large bushy shrub with auriculate leaves and extraaxillry umbellate inflorescene [15].

The plant is identified to posses high medicinal values and used in treating conditions like diarrhoea, Sinus fistula, Stomach issues and skin irritations. Additionally the leaf is known as a best medicine in treating Jaundice [16, 17]. Studies show that this plant extract is widely and traditionally used in the treatment of diabetes since generations [20].

Several researches have used the two enzymes, alpha amylase and alpha glucosidase inhibition as an indication for the detection of antidiabetic agents. Divya Kajaria *et al.,* in their research on antidiabetic activity of antiasthmatic drug — Shirishadi [8] studied the inhibitory activity against alpha amylase and alpha glucosidase as an index for anti diabetic effect. Their study targeted the identification of antidiabetic activity of selected anti asthmatic drug, shirishadi. Major reason for selecting anti asthmic drugs is the co-occurrence and interrelation of Diabetes and Asthma. The aim of their study was to identify and develop a new drug that can treat both the conditions together. Study revealed that the ethanolic extract of the compound exhibited an inhibition of 76.40% for alpha amylase activity and 63.85% inhibition of alpha glucosidase activity. The IC 50 values in both the cases were calculated to be 0.68 mg/ml and 2.89 mg/ml, respectively.

Another study by Nguyen Phuong Thao et.al included the detection of antidiabetic activity of Wedelia chinensis extracts, a folk medicine [2]. Study included the administration of ethanolic extracts of herb to Swiss albino mice and tested for their efficacy.The antidiabetc effect was tested considering the inhibition of alpha amylase and alpha glucosidase as reference standards. Results revealed a reduction in the activity of alpha amylase by 48.39% and 39.37% inhibition of glucosidase at a dose rate of 50mg/ml and 10mg/ml respectively.

Nanu R Rathod et.al,. worked towards the study of hypoglycemic effect of Calotropis gigantia [18]. The team had tested both normal and streptozotocin induced diabetic mice with the flower and leaf chloroform extract from calotropis. The serum glucose levels were estimated which showed a decline in the glucose levels of mice. There was a detectable improvement in the oral glucose tolerance of diabetic mice. This indicates extracts of calotropis exhibit a positive role in the treatment of diabetes.

Rohit Sharma and team from India, conducted their research study on the therapeutic importance of calotropis procera [19]. The study states that this plant posses several medicinal values which include anti-diarrhoeal, larvicidal, anticancerous, anti-microbial, ascaricidal, insecticidal, schizonticidal,anthelmintic and anti-inflammatory and several other medicinal properties. Various metabolites of the plant which include carbonates, norditerpenic esters, falvonoids, alkaloids, the cysteine protease procerain, sterols and many cardenolides are known to highlight the importance of the plant.

Mutiu Idowu Kazeem *et.al.,* strived to understand the relation between leaf extracts of *Calotropis procera* and the two major enzymes alpha glucosidase and amylase linked to diabetes. Research was conducted on acetone, aqueous and ethanolic extracts of *Calotropis procera.* These extracts were subjected for standard enzyme inhibitory assay on porcine pancreatic α-amylase and rat intestinal α-glucosidase [10]. Results of the research revealed that the ethanolic and aqueus extarcts were very good in inhibiting the enzyme alpha amylase with an IC50 value of 7.80 mg/mL and α-glucosidase was inhibited to an extent of 3.25 mg/mL IC50. Inhibitory activity was because of the presence of phyto chemicals like flavonoids, tannins and saponins in the plant. Conclusion of the study was a well established relation of calotropis extracts against diabetes.

Materials and Methods

Collection of Samples:
Being a wild and abundantly available plant much efforts were not needed to collect the plant material. The fresh leaves and flowers of Calotropis procera were collected from a garden in Bhagayath area of Uppal, Hyderabad. The leaves and flowers were well cleaned under running tap water to remove any superficial contaminants. The material was cut into small pieces followed by shade drying at room temperature for about a week until the leaves and flowers turn complete dry and can be powdered.

Fig 1: Collected and dried leaf and flower material for the study:

Inference: Te above fig 1 shows the sample materials, leaves and flowers of Calotropis shade dried and ready to use.

Preparation of Extracts:
Air dried leaves and flowers were subjected for homogenization by motor pestle to a course powder and stored in a closed container for further processing. From among the plethora of extraction protocols the common and standard method of extracting with various solvents using high temperature is used in the current study. The major steps undertaken in the study for obtaining bioactive constituents from raw sources include maceration, digestion, superficial extraction using various selected solvents.

Aqueous, Ethanol and Chloroform extracts
Some of the bio active constituents may be soluble in polar solvents like water and ethanol, where as the other may need non polar solvents like chloroform. Thus the study involves the selection of 3 different solvents water and ethanol for polar constituents and chloroform for non polar. 3grams of finely grounded plant sample (leaves and flower) were extracted with 5ml of water, ethanol and chloroform respectively at room temperature for 4 days with continuous stirring. The extracts were filtered through a Whatman filter paper and placed in water bath.

Enzyme inhibition activity
Once all the extracts were prepared the inhibition of these extracts against alpha amylase and glucosidase were tested spectrophotometrically. The percentage inhibition of the extracts was calculated and plotted.

Testing the Antidiabetic activity of each extract using invitro technology
The enzyme inhibition potential of the extract was tested using modified protocol of McCue and Shetty [12]. The protocol is explained below.

In separately labeled test tubes 200microlitre of extract (ethanolic, chloroform and
aqueous) was added to which 200 microlitre of alpha amylase enzyme solution is added. The tubes were incubated at 25^0C for 10min. To these tubes 200 micro litre each of freshly prepared starch, buffer , NaCl solution are added. The tubes need to be incubated at room temperature for 10 min. Later 400 microlitre of 50% glacial acetic acid is to be added to the same tubes. The solutions is allowed to centrifuge at 3000 rpm for 5min. Absorbance of supernatant was measured at 595nm

Control: A control was prepared using the above procedure replacing the extract with distilled water.

Studying the alpha glucosidase inhibition activity of extracts
Modified method of Kim et al. was used to test the inhibition of alpha glucosidase [11]. To all the labeled test tubes 200 microlitre of each extract must be added separately. To these tubes 200 microlitre of alpha glucosidase should be added. To the above solution 200 micro litres of tris buffer is to be added. Incubate the solution for 1hr at room temperature followed by incubation at 65^0C for 5 min. The solution must be centrifuged followed by testing the OD at 540nm.

Control: A control was prepared using the above same procedure replacing the extract with distilled water.

Results and Discussion

Based on the OD values obtained in spectrophotometer the percentage% of inhibition of both the enzymes was calculated as shown below:

α - amylase inhibition
% inhibition = control Abs − Test Abs ÷ contol Abs × 100
(Control abs = standard OD, Test abs = obtained OD)

α - Glucosidase Inhibition
% inhibition = control Abs − Test Abs ÷ contol Abs × 100

(Control abs = standard OD, Test abs = obtained OD)

Table 1, 2: Shows the % inhibition values calculated for all the extracts of leaf and flower samples against both alpha amylase and alpha glucosidase

Inhibition of Alpha amaylase

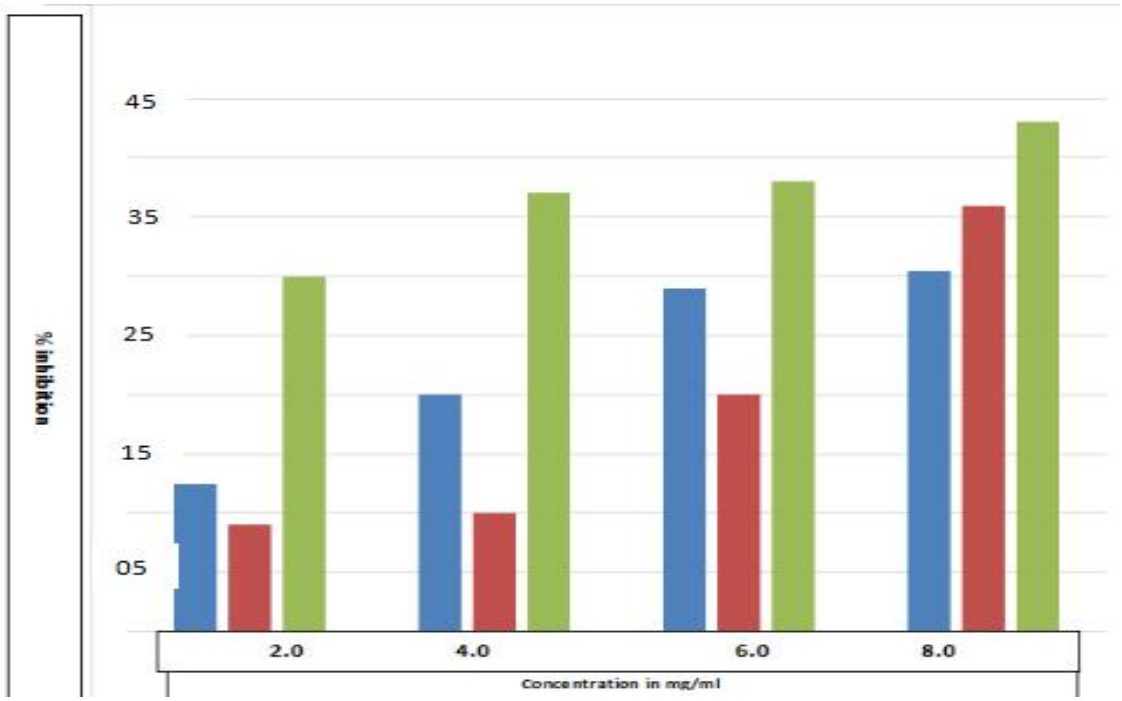
	Chloroform		Ethanol		Aqueous	
	Leaf	**Flower**	**Leaf**	**Flower**	**Leaf**	**Flower**
2mg/ml	30%	26%	13%	11%	9%	8%
4mg/ml	36%	34%	20%	17%	10%	9%
6mg/ml	37%	33%	29%	26%	20%	18%
8mg/ml	43%	38%	30%	28%	36%	30%

Inhibition of Alpha Glucosidase

	Chloroform		Ethanol		Aqueous	
	Leaf	**Flower**	**Leaf**	**Flower**	**Leaf**	**Flower**
2mg/ml	32%	30%	16%	16%	12%	11%
4mg/ml	38%	37%	25%	19%	16%	13%
6mg/ml	39%	38%	32%	34%	26%	22%
8mg/ml	45%	42%	36%	36%	39%	34%

The above table 1 and 2 depict the inhibition percentage of all extracts on both the enzymes.

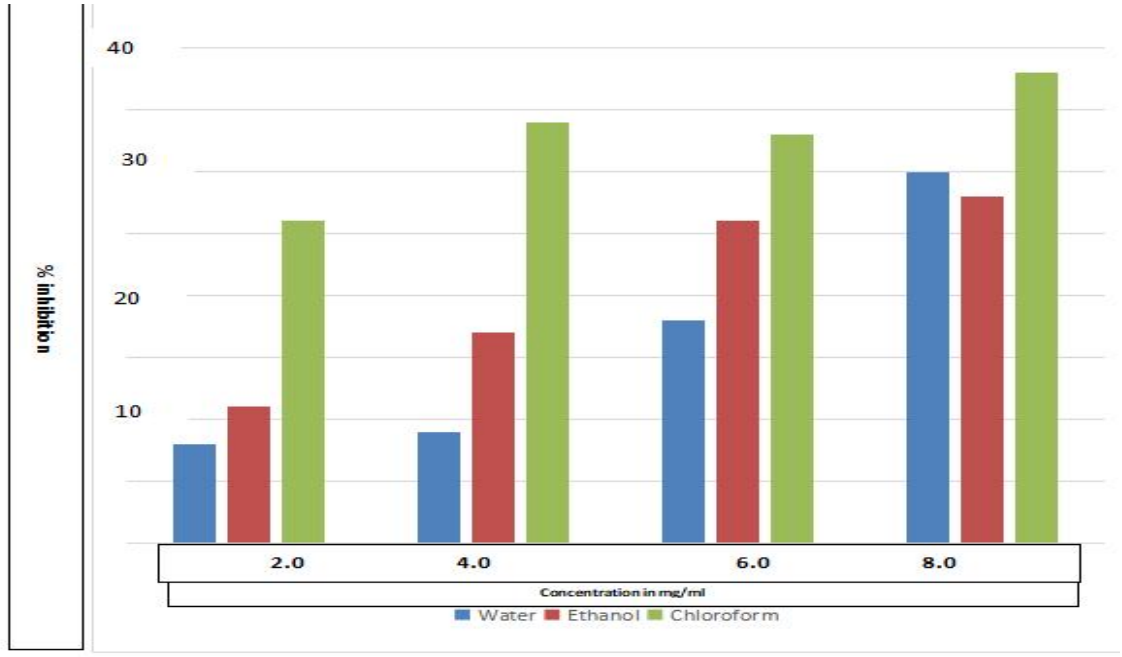
Figure 1 Graph showing the % inhibition of alpha amylase by various concentrations of leaf extracts:



Blue: Ethanol, Brown: Water, Green: chloroform

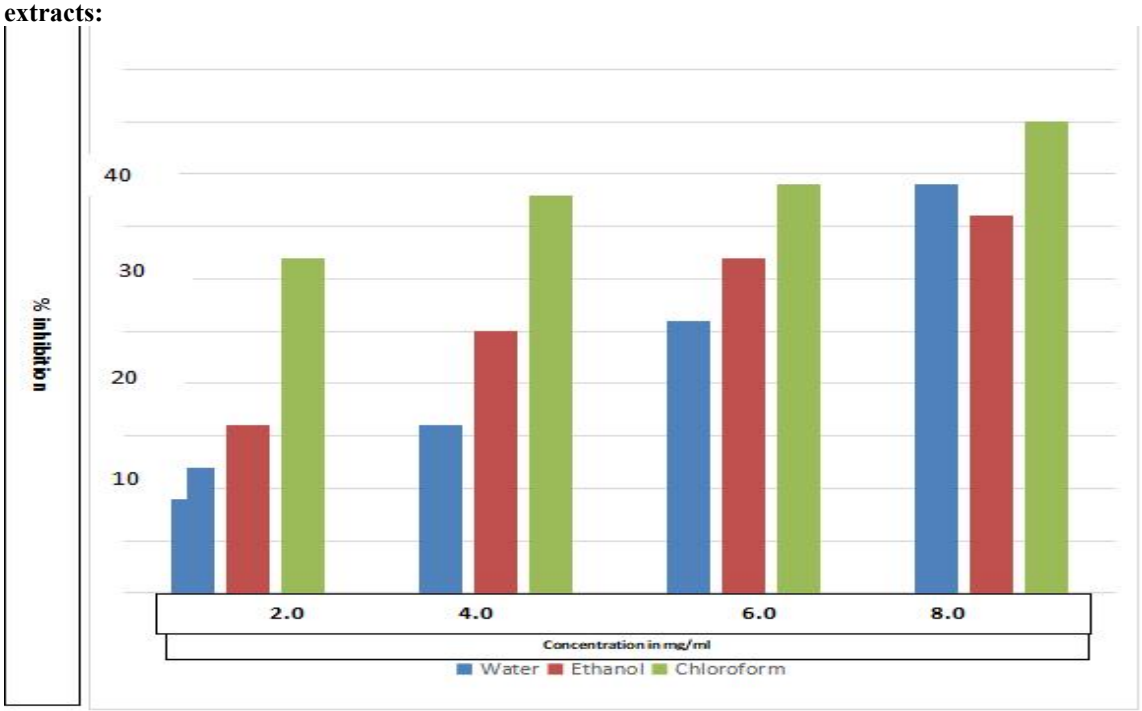
Inference: The graph plotted shows an increasing inhibitory activity of the extracts with concentration. Further the highest inhibition was observed for chloroform extract. Aqueous and ethanolic extracts were showing moderate inhibition.

Figure 2 Graph showing % inhibition of alpha amylase by various concentrations of flower extracts:



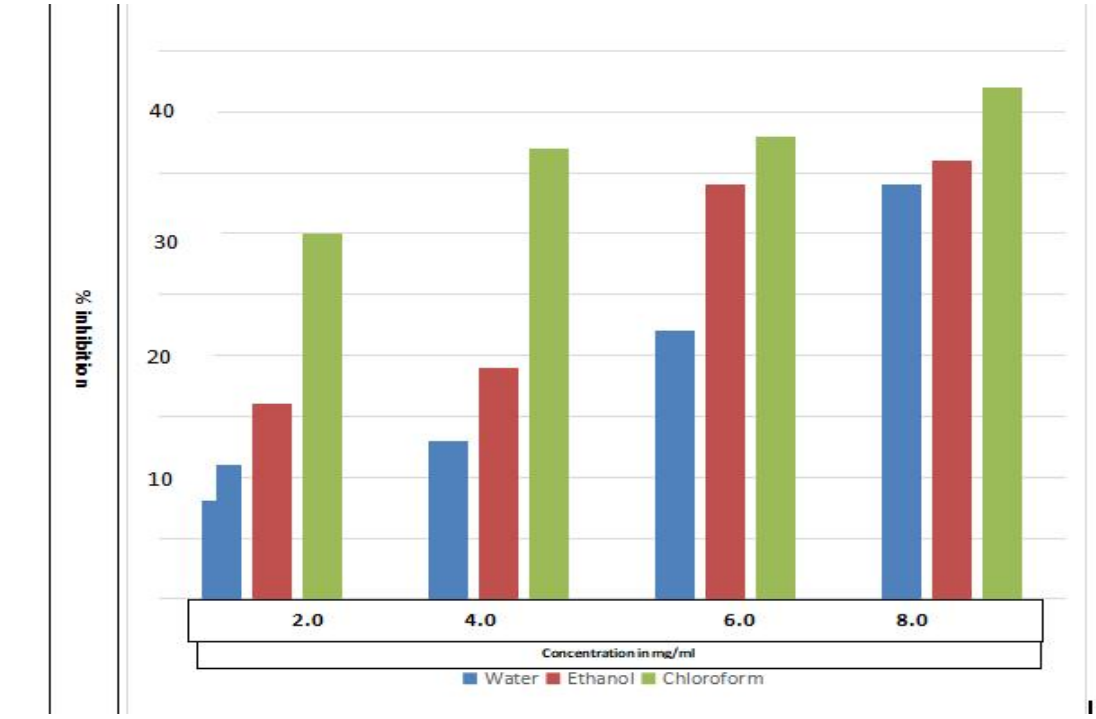
Inference: The extracts at various concentration showed inhibition with chloroform. Ethanolic extracts exhibited maximum activity.

Figure 3 Graph showing the % inhibition of alpha glucosidase by various concentrations of Leaf extracts:



Inference: In case of alpha glucosidase inhibition all the extracts exhibited good inhibition. .

Figure 4 Graph showing the % of inhibition of alpha glucosidase by various concentrations of flower extracts:



Inference: The inhibition of alpha glucosidase by flower extracts exhibited a good effect by chloroform and ethanolic extracts.

Discussion

Diabetes is an abnormal health condition which causes hyperglycemia characterized by a high elevation in the blood glucose levels. The major metabolic reason is the lack of proper enzyme system to metabolize the sugar produced constantly by food intake. At the cellular level glucose present outside the cell fails to get converted to glucose 6 phosphate and could not enter the cell getting deposited in the blood. These sugar deposits in the blood leads to a condition called diabetes. The glucose deposits are continuously absorbed by the alpha glucosidase present in the intestine [3]. The serous issue of concern in diabetes is its lack of cure. It can only be managed the whole life but not cured. Thus designing suitable mechanisms to manage blood sugar levels is the most efficient method needed in the condition. This can be achieved in several ways. One of the better method is to inhibit the enzymes that are required to absorb the sugar reserves in the body which is done by two vital enzymes alpha amylase and alpha glucosidase [6]. In the current study using the leaves and flower extracts of *Calotropis procera* with 3 different solvents including water, ethanol and chloroform the inhibition of the enzymes is detected. The results revealed the highest inhibitory activity of chloroform extract of both leaves and flowers.

From the results of both alpha amylase and alpha glucosidase inhibition it can be observed that the extract of calotropis is efficient in blocking the activity of these enzymes in spite of the difference in the degree of inhibition. Further the inhibition was comparatively more in chloroform extract proving it to be a best solvent for the extraction when compared to ethanolic and aqueous solvents. The study reveals the special use of calotropis chloroform extract in the treatment of diabetes which is indicated by the inhibition values calculated based on spectrophotometer. Further it was observed from the results that the inhibition of alpha glucosidase is slightly higher than the enzyme alpha amylase.

Conclusion

The current research was undertaken to identify the antidiabetic activity of C*alotropis procera* flower and leaf extracts. The extracts were made in 3 solvents which include water, ethanol and chloroform. The antidiabetic activity was tested invitro by checking for the inhibition of the two major enzymes alpha amylase and alpha glucosidase involved in the processing and absorption of sugars. Results indicated that the chloroform extracts of both flowers and leafs exhibited a considerable inhibition against both alpha amylase and glucosidase indicating its antidiabetic activity. Thus the chloroform extracts can be used as surface applicants in diabetic conditions to regulate the blood sugar levels. Further there was a gradual increase in the inhibitory activity with the increase in the concentration of extract. Thus standardization of highest inhibitory concentration needs to be performed. Glucosidase inhibition was higher when compared to the inhibition of amylase. The study concludes the anti diabetic effect of *Calotropis procera* leaf and flower extracts, it promotes the use of these herbal extracts in the treatment of diabetes.

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