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Research Article

Hypoglycemic Effect and Finger Printing Analysis of *Costus igneus* Partially Purified Fraction in L6 Cells

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ABSTRACT

Plant secondary metabolites with proved hypoglycemic properties have been used in traditional medicine worldwide. *Costus igneus* is used in traditional medicine as hypoglycemic medicine in India. *Costus igneus* is able to reduce the blood glucose level and possess good antidiabetic activity in *in-vitro* and *in-vivo* models. In the current study the extract from the plant is subjected column chromatography and purified fractions were tested in HPTLC to identify the number of active components. The purified fraction was assessed for antidiabetic activity in L6 myoblasts. The crude extract proved the presence of bioactive various secondary components and HPTLC analysis showed the presence of 5 components. Glucose uptake was significantly increased with the column fraction of *Costus igneus* in L6 myotubes. A maximum 59% of glucose was obtained with the column fraction at 30µg/ml concentration which was compared with insulin and standard rosiglitazone. The current study concluded that column fraction possess hypoglycemic effect on the L6 cells.

Keywords: *Costus igneus* ; hypoglycemic properties; andidiabetic activity; column chromatography.

INTRODUCTION

Costus igneus is a commonly known insulin plant in India and belongs to Costaceae family. According to the previous studies there is a strong belief that by consuming these plant leaves can control the blood glucose levels and diabetics. Those who consume the leaves of this plant reported a drop in the blood glucose levels[1]. Identification of the plant components in therapeutic application of diabetes is of growing interest as they contain many active phytochemical constituents against the hyperglycemic condition. These phytochemical constituents are of organic in nature which is synthesized through the activity of individual activity of cells. The biosynthetic process in plants helps in the conversion of the simple chemical components into complex bioactive secondary metabolites. The phytochemical components isolated from the various plants have applications in medicines and therapeutics[2]. Hence phytochemistry and pharmacognosy has steadily grown in to a dynamic and large multidimensional study.

Plant derived products have been applied in the traditional and folk medicine healing system and are being assessed for their antidiabetic activity[1]. Hence the current study is focused to evaluate the phytochemical composition and antidiabetic activity of *Costus igneus* leaves extract which is partially purified using column chromatography.

MATERIALS AND METHODS

Preparation of crude extract

The air dried, powdered leaves of *Costus igneus* (100.0g) was defatted with petroleum ether by soaking for 24hrs. Then crude extract was prepared by soaking with ethanol for 5 days. On the fifth day, the ethanol filtrate was subjected to soxhlet to get concentrated extract. This crude extract was subjected to solvent-solvent partition extraction using methanol and ethyl acetate (1:1). Methanol layer and ethyl acetate layers was collected and separated then concentrated and subjected to TLC and sprayed with the picric acid reagent to identify the presence of flavonoids. The fraction which showed positive results with picric acid was subjected to column chromatography for separation of the compounds and subjected to HPTLC.

Priliminary phytochemical analysis

The crude extract was subjected to phytochemical analysis was done according to standard procedures.

Purification and identification of flavonoids

Purification of the active compound will be further carried out using column (2.5X50cm) packed with silica gel (60-120 mesh)/neutral alumina of column chromatography grade SRL, Mumbai). After drying, a small piece of absorbent cotton will be placed at the bottom of the column. Silica gel will be mixed with methanol and then packed in the column as stationary phase. Crude extract slurry was prepared by mixing with the methanol and small amount of silica gel powder and allowed to dry in hot air oven. Then the slurry was loaded at the top of the column and eluted using suitable solvent system. Fractions 5 and 6 were collected and subjected to HPTLC.

In vitro α-glucosidase inhibitory Activity)

In vitro α-glucosidase inhibitory activity was evaluated according to standard procedure[3]. α-glucosidase inhibitory assay is based on the breakdown of maltose to glucose. Briefly, procedure is as follows, 200µl of α -glucosidase

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Table 1: Maximum Rf, Maximum Height, Area %

Peak no.	Maximum Rf	Maximum Height	Area %
1.	0.24	182.8	55.70
2.	0.29	18.6	6.40
3.	0.37	15.6	5.09
4.	0.58	57.6	28.40
5.	0.83	10.4	4.41

Table 2: α – glucosidase inhibitory activity

Concentration	% inhibition of glucosidase activity	
(µg/ml)	Plant extract	Acarbose
0.3	7.65±0.23	18.87±0.57
1	14.88±0.11	35.29±0.61
3	23.42±1.02	38.86±0.08
10	44.20±0.19	45.41±0.34
30	58.69±1.13	48.70±0.79
100	71.38±0.57	54.87±0.06
300	84.21±2.32	70.67±0.06
1000	94.15±0.01	97.29±0.12
IC_{50}	24.57µg/ml	43.15µg/ml

All values are expressed as mean ±SEM

solution was pre-incubated with the test (Concentration – 1 – 1000µg/ml) and control sample for 5 min. The reaction was started by adding 200µl of sucrose and it was terminated after 30 min incubation at 37^0C by heating at 90–100^0C. The liberated glucose was determined. The enzyme activity is directly proportional to the liberated glucose and the liberated glucose is measured by GOD-POD method at 546nm using semi auto analyzer. The inhibitory activity of the extract was calculated as follows.
% Inhibition= [(control-test)/control)]*100

In – vitro α- amylase inhibitory assay

α-amylase inhibition studies were done according to the method of [4]. 100µl of column fraction 10 was taken (1-1000µg/ml) and allowed to react with 200µlof α-amylase enzyme (Hi media RM 638) and pH was adjusted to 6.9 by adding 100 µL of 2mM phosphate buffer. Then incubated for 20mintes and 100µl of 1% starch solution was added. The same procedure was repeated for control by replacing enzyme with buffer. After 5 minutes incubation, 500µl of DNS reagent was added to control and test samples. Then boiled in water bath for 5min and absorbance was recorded at 540 nm using UV-Vis spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula.
% inhibition = [(Control – Test)/Control]*100
Suitable reagent blank and inhibitor controls were simultaneously carried out.

Cell Culture Studies

Preparation of cell culture

L6, a monolayer myoblast culture ATCC (obtained from NCCS, Pune – Passage no- 8) was cultured in DMEM with 10% fetal bovine serum (FBS) and supplemented with penicillin (120 units/ml), streptomycin (75µg/ml), gentamycin (160µg /ml) and amphotericin B (3µg/ml) in a 5% CO_2 environment. For differentiation, the L6 cells were transferred to DMEM with 2% FBS for 4 days, post-confluence to allow for differentiation. The extent of differentiation was established by observing the multinucleate of cells. In the current study 90% of the myoblasts were fused to form myotubes. After differentiation, the cells were incubated in a high glucose medium for 24h (DMEM medium with a supplement of 25 mM/L glucose) to acquire an insulin resistance stage[5].

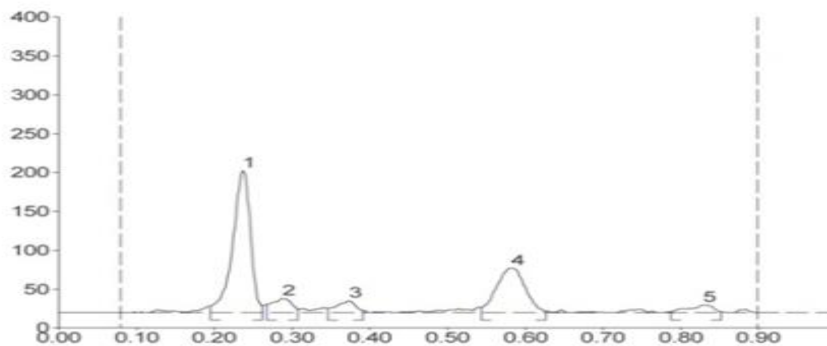
Cytotoxicity study

Cytotoxicity of the column fraction was assessed by MTT assay. MTT assay was performed according to the Gohel[6]. Cells were plated in 96 - well plate at a concentration of 5x 10^4 cells/ well. After 24h of incubation, it was washed with 200µl of 1x Phosphate buffered saline (PBS; pH 7.4) and starved by incubation in serum – free medium for an hour at 37ºC in CO_2 incubator. After starvation, cells were treated with different concentrations (1–1000µg/ml) of the Plant extract for24h. At the end of the treatment, media from Control and column fraction treated cells were discarded and 50µl of MTT containing PBS (5mg/ml) was added to each well. Cells were then incubated for 4h at 37ºC in CO_2 incubator. The purple formazan crystals formed were then dissolved by adding 150µl of DMSO and mixed effectively by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured using Multimode reader (Perkin elmer) at 570nm. Optical density of each sample was compared with control optical density and graphs were plotted.

In vitro glucose uptake activity

6-(N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose (6 – NBDG) assay

In vitro glucose uptake was studied by Kuhn et al.,



Graph:1 HPTLC graph

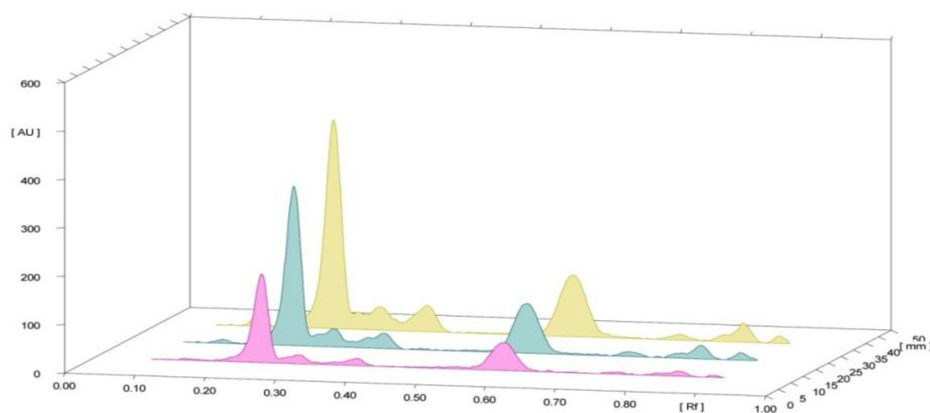


Figure 1: HPTLC chromatogram of flavonoids isolated from *costus igneus*

Table 3: α - amylase inhibitory activity

Concentration (μg/ml)	%Inhibition of Amylase activity	
	Plant extract	Standard Acarbose
1	8.24±0.20	13.73±0.63
3	16.53±0.05	23.84±0.09
10	23.76±0.13	39.03±0.19
30	34.35±0.07	50.26±0.09
100	52.18±0.21	69.25±0.06
300	63.51±0.11	76.51±1.51
1000	83.26±0.27	93.32±1.08
IC_{50}	78.06 μg/ml	30.83 μg/ml

All values are expressed as Mean±SEM

Table 4: Cytotoxicity assay of the extract against L6 myotubes

Concentration (μg/ml)	% inhibition of cell growth	
	Plant extract	Standard rosiglitazone
Control	0.07±0.30	0.07±0.30
0.1% DMSO	11.27±0.93	11.27±0.93
1	0.20±0.08	1.46±0.49
3	8.58±0.04	2.05±1.25
10	9.13±0.03	1.50±0.69
30	11.00±0.11	3.71±0.67
100	18.93±0.72	10.37±0.54
300	24.13±0.07	15.26±0.85
1000	27.79±0.79	18.08±0.68
IC_{50}	>1000μg/ml	>1000μg/ml

All values are expressed as Mean±SEM

2011[7]. L6 myoblasts was grown in 96 - well plate and was subjected to glucose uptake was assessed using fluorescent tagged 6-NBDG - 6-(N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose as reported. When semi confluent monolayer was formed, the culture was renewed with the respective differentiation media. Different concentrations (3, 10, 30μg/ml) of the column fractions were added to the wells along with the standard rosiglitazone. At the end of treatment, 10nM of Insulin was added to stimulate glucose uptake and incubated for 15 minutes. 20μg/200mL of 6-NBDG was added and incubated for 10min at dark. Glucose Uptake (in %) was measured using fluorometer with an excitation/emission filter 466/540 nm.

Statistical analysis

Analysis of statistical significance of differences in measurements between samples was done by one-way ANOVA with Tukey's post hoc test (Graph Pad Prism version 4) and $P<0.05$ was considered statistically significant.

RESULTS

Phytochemical analysis

Costus igneus extract phytochemical analysis was Proved for the presence of the various secondary metabolites like saponins, flavonoids, alkaloids, betacyanin, phytosteroids

HPTLC analysis

In the present studies HPTLC is applied to obtain Finger print patterns of the phytochemical constituents, estimation of the active secondary metabolites and also identification. PTLC chromatogram was developed using a solvent system (ethylacetate: acetic acid: water (15:3:5)). The HPTLC chromatogram and table were shown in (Table1, fig1) and the graph was shown in graph 1 and the HPTLC analysis revealed the presence of 5 flavonoids.

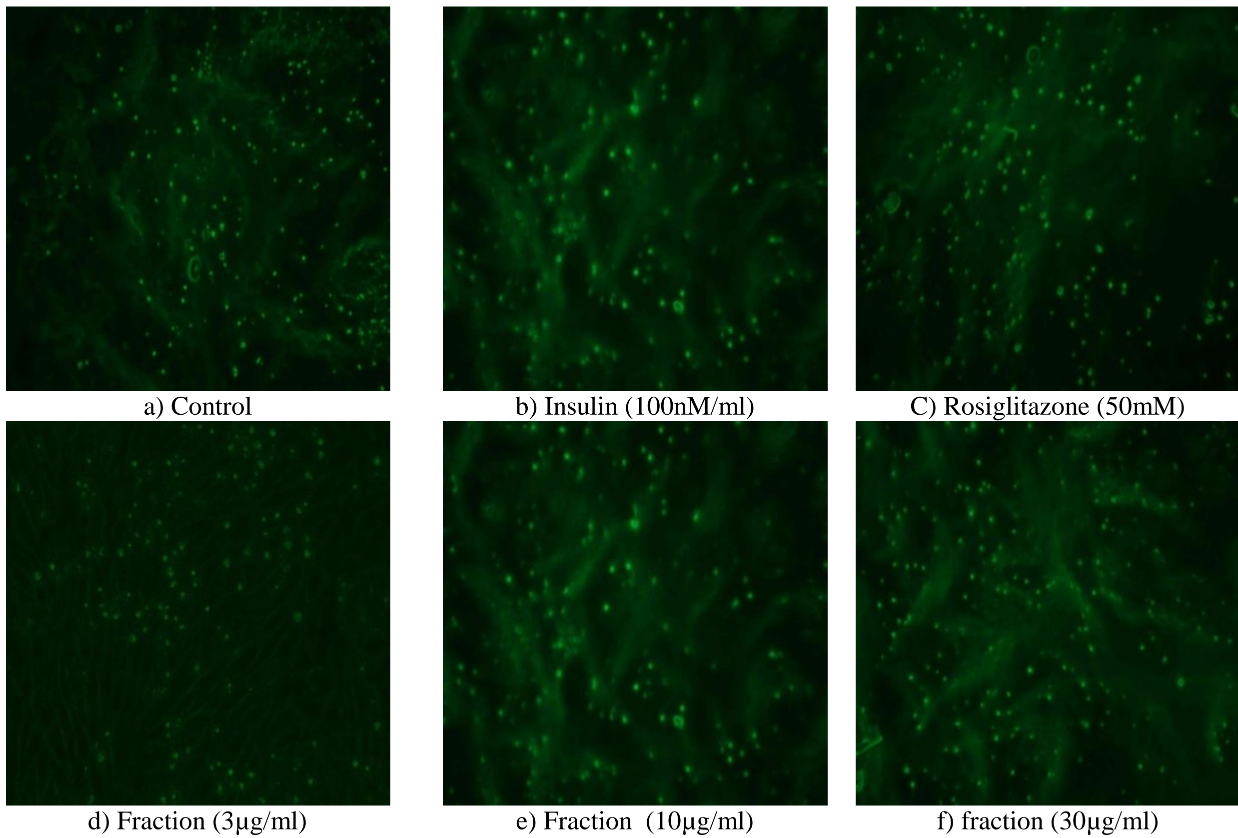
α – glucosidase inhibitory assay

Column fraction showed strong α-glucosidase inhibitory activity against L6 myoblasts when compared with standard acarbose. Column fractions showed 94% of inhibition with 1000μg/ml while standard acarbose showed 97% of inhibitory activity. However, column fraction exhibited strong inhibition towards the α-amylase activity (Table.3). The results of α-glucosidase inhibitory activity were showed in Table.2. Column fractions showed a maximum inhibition of 83% at 1000μg/ml with IC_{50} of 78.06μg/ml.

Cytotoxicity assay

The cytotoxicity of column fraction was determined by MTT assay according to the method mentioned previously. The column fractions concentrations were standardized and up to 1000μg/ml were found to be protective. The column concentrations used as 1μg – 1000μg and compared with rosiglitazone. Effect of column fraction on cytotoxicity was showed in table 4.

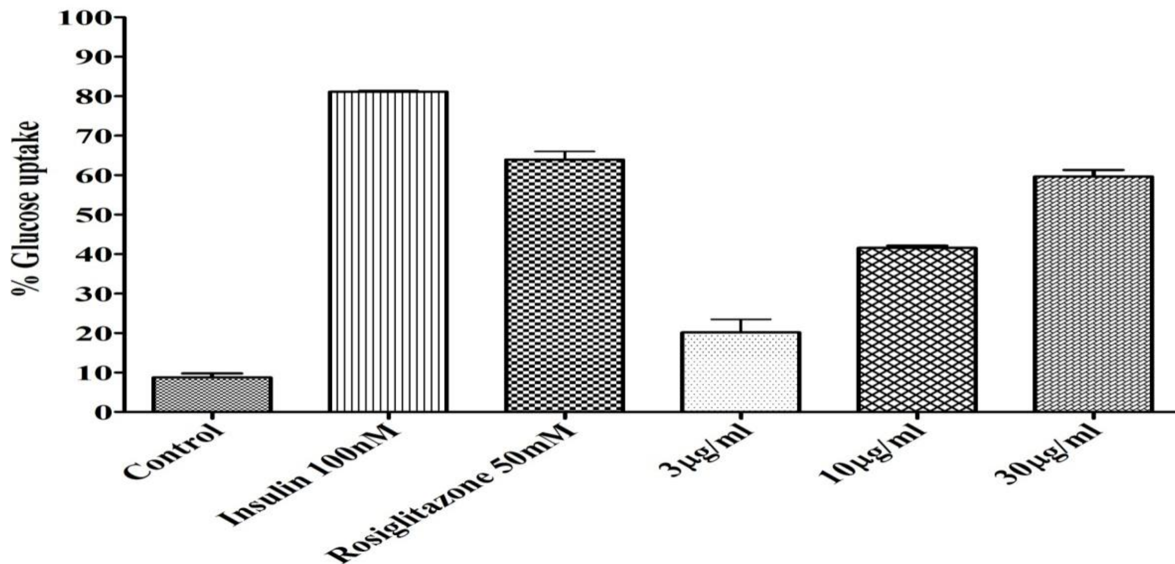
6-NBDG uptake Assay in pretreated L6 myotubes



a) Control b) Insulin (100nM/ml) C) Rosiglitazone (50mM)

d) Fraction (3µg/ml) e) Fraction (10µg/ml) f) fraction (30µg/ml)

Figure 2: Representing 6-NBDG glucose uptake potential of the extract in L6 mytoubes-
Fig2a) control, 2b) insulin, 2c) Rosigitazone, 2d) Fraction (3µg/ml) e) Fraction (10µg/ml) f) fraction (30µg/ml).



Graph2: Effect of the column fraction on 6 – NBDG glucose uptake potential

Antidiabetic potential of column fraction was evaluated by determining 6-NBDG uptake in L6 myotubes. Non metabolizable fluorescent glucose analogue 6-NBDG is used to study increasingly about the cellular transport of glucose. The endocytosis and intracellular accumulation of exogenously applied fluorescent glucose analogue, 6-NBDG by L6 cells and accumulated 6-NBDG fluorescence is shown in Fig.2 and the concurrent gradient-driven uptake of glucose by glucose transporters (GLUTs). Column fraction (3, 10 and 30µg/ml) preincubation significantly increased glucose uptake (P≤0.05). A maximum glucose uptake of 59% was achieved by the column fraction at 30µg/ml concentration which is compared with insulin and Standard rosiglitazone and graph.2 shows the glucose uptake assay by L6 cells.

DISCUSSION

The whole body glucose homeostasis is maintained by the stimulation of glucose uptake by GLUT4 translocation, skeletal muscle is the main key insulin targeted tissues. L6 myotubes are well differentiated skeletal muscle model for understanding the glucose uptake process[8] and hence used for the current study. Natural compounds from plants are well-known platform for developing new drug synthesis with lesser side effects[9].

The current study indicates the anti-diabetic effect of column fraction from *Costus igneus.* Colum fraction was able to stimulated basal glucose uptake in differentiated L6 myotubes. Previous reports on antidiabetic activity of *Costus igneus* leaves showed a hypoglycemic activity. The column faction of *Costus igneus* was able to simulate the differential L6 cells for basal glucose uptake ((fig.2)(2d), (2e) and (2f)). To ensure the behavior of compounds present in the column fraction insulin –mediated glucose uptake was checked (fig.2b). *Costus igneus* column fraction has insulin mimetic compounds but not the insulin-sensitizing compounds. Because glucose uptake is the first vital for maintaining glucose homeostasis, our study could better explain the mechanism behind the hypoglycemic activity of the leaves of *Costus igneus*. In the current study the efficacy of the glucose uptake stimulation of column fraction was compared with rosiglitazone in L6 cell line and the results obtained was significantly equal to the rosiglitazone. So, the current study reveals that *Costus igneus* fractions showed effective antidiabetic activity in L6 differentiated myotubes and is not cytotoxic and can be recommended safe for therapeutic use.

CONCLUSION

The above study concluded that *costus igneus* possess good antidiabetic activity and can be developed as therapeutic drug.

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