



Science

## RELATIVE POTENCY OF ANTIDIABETIC EFFECT OF COSTUS SPECIOSUS (KOEN. EX RETZ.) SMITH PLANT EXTRACT IN MOUSE FIBROBLAST CELL LINE

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### ABSTRACT

*Plant-derived compounds have been used clinically to treat type 2 diabetes for many years as they also exert additional beneficial effects on various other disorders. PI3K pathway is the major pathway activated by insulin receptor (IR). It induces glucose uptake, glycogen synthesis, protein synthesis, cell growth and differentiation. Hence metabolic assay was employed to assess glucose uptake based on the property of 3T3-L1 cells to differentiate into adipocytes which can take up the glucose in medium due to the effect of insulin or insulin like molecules. The results of the current research showed that plants extract probably exerts its anti-diabetic properties by stimulating glucose uptake in adipocytes with significant inhibition of adipogenesis demonstrating reliable relative potency in comparison to the commercial insulin. In this preliminary in-vitro study we report Costus speciosus plant extract to have significant relative potency in reference to commercial Insulin which can be exploited to treat type II diabetes. This plant source could to be a potential, sustainable, natural anti diabetic product after structured preclinical and clinical studies. Current study leads researchers to elucidate the chemical structure, isolate active ingredients in the crude extract for such biological activities.*

### Keywords:

*Costus speciosus, mouse fibroblast, cell differentiation, relative insulin potency.*

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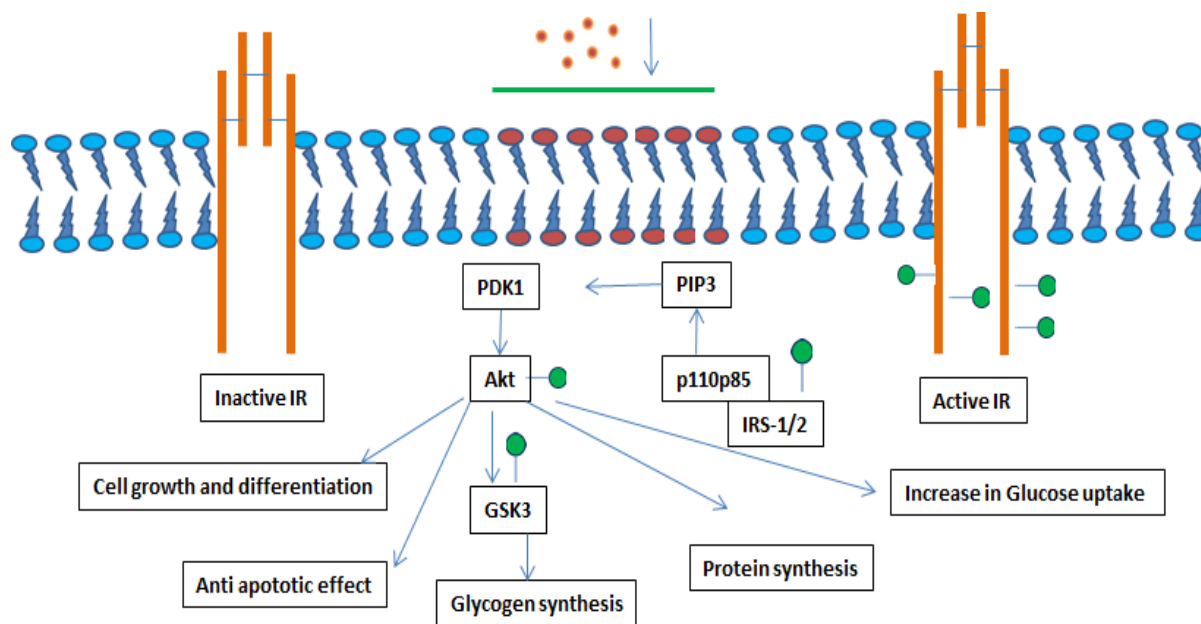
## 1. INTRODUCTION

The biological evaluation of plant products on the basis of their use in the traditional herbal system of medicine develops a basic platform for the recent and newer drug discovery methods, development of new drugs from different plant sources. From the innumerable plants being researched since time immemorial, *Costus speciosus* is important one. This plant of Costaceae (Zingiberaceae) family, is commonly known as keukand (hindi), Variegated Crepe Ginger (English). It is an erect, succulent, perennial herb, upto 2.7 meters in height, arising from a horizontal rhizome, found in tropical region of India and also cultivated for ornament. Various medicinal properties are attributed to it, particularly in the treatment of asthma, fungal diseases, rheumatism, Diabetes (Bhat, 2010).

Insulin is a ~ 6 kDa polypeptide hormone secreted by  $\beta$ -cells of the 'islets of Langerhans' in the pancreas. Together with glucagon, a hormone secreted by  $\alpha$ -cells of pancreas, insulin maintains glucose homeostasis in the blood. Insulin has been demonstrated to have multiple biological effects in virtually all tissues. Its actions can be broadly categorised as metabolic actions and mitogenic actions. Metabolic actions of insulin comprise increased uptake of glucose, amino acids and fatty acids by target cells, increased expression and activity of enzymes involved in synthesis of glycogen (e.g., phosphofructokinase-2 and glycogen synthase), proteins and lipids, and decreased activity of enzymes involved in degradation of carbohydrates, proteins and lipids (Baldea, 2010). These varied metabolic effects make insulin the most potent anabolic hormone known. The main target tissues for metabolic actions of insulin are skeletal muscles, cardiac muscles, adipose tissue and liver. In muscles and adipose tissue, insulin induces translocation of the GLUT4, insulin-dependent glucose transporter, to the plasma membrane (James & Piper, 1994).

### **PI3K signaling pathway:**

The PI3K signalling pathway is the major pathway activated by insulin. This pathway elicits both metabolic and mitogenic responses in the cells (Klein, 2007). PI3K is the most upstream signalling molecule of this pathway. It consists of two subunits – p85 (regulatory subunit) and p110 (catalytic subunit). The regulatory subunit, p85, has been reported to have at least eight isoforms. The exact role of these different isoforms in the regulation of insulin action is not clear. Binding of the p85 subunit to IRS results in the recruitment of p110 subunit to p85 and thus in the activation of the catalytic activity of PI3K (Fig.1). The p110 subunit catalyzes phosphorylation of phosphatidylinositol (Adams,2000)-bisphosphate (PIP2) and generates phosphatidylinositol-trisphosphate (PIP3). Inhibitors against PI3K or transfection with dominant negative constructs of the gene block insulin-mediated glucose transport, glycogen and lipid synthesis thus highlighting the importance of PI3K in the metabolic actions of insulin . PIP3 binds to the pleckstrin homology (PH) domain of a variety of signalling molecules and thus influences glucose uptake, glycogen synthesis, and cell growth. The most important binding partner of PIP3 is phosphoinositide-dependent kinase 1 and 2 (PDK1/2), for which PIP3 is an allosteric regulator.



**Figure 1:** PI3K pathway activation by active insulin receptor.

PI3K pathway is the major pathway activated by insulin receptor (IR). It induces glucose uptake, glycogen synthesis, protein synthesis, cell growth and differentiation. In addition, it has anti-apoptotic effect.

#### **Choice of cell line for invitro assay:**

Several cloned lines of mouse 3T3 fibroblasts which are capable of differentiating into adipocyte-like cells invitro was previously established (Green et al.,1975). Cell lines have been used as model systems to understand various mechanisms of plants in animal and human health as they provide a continuous source of large numbers of cells necessary for proliferation and differentiation. The 3T3-L1 cell line was selected for this study because it displays relevant features including lipid storage and glucose homeostasis. During differentiation, 3T3-L1 pre-adipocytes become adipocytes with a 20-fold increase in the number of insulin receptors and acquire the ability to utilize glucose in response to insulin [Tom, 2011].

#### **Metabolic assay principle:**

Metabolic assay is based on the property of 3T3-L 1 cells to differentiate into adipocytes which can take up the glucose in medium and the effect of insulin or insulin like molecules. Differentiated cells , adipocytes have a very high expression of insulin receptors and show the presence of lipid droplets in their cytoplasm indicating synthesis of lipids [Alonso-Castro, 2008].Supplementing the medium with increasing doses of insulin and/ crude extract will cause a direct propotional increase in Glut 4 tranported at the plasma membrane of the cell, enabling the cells to take up glucose for fat metabolism. The uptake of glucose by adeposites, results in decreas of glucose content in the medium, the remaining glucose concentration is directly proportional to the dose of insulin given which can be measured using glucose oxidase / peroxidase (GOPOD) reagent.

GOPOD forms colored product in proportion to the glucose concentration present in the medium. The intensity of the colour is measured by absorbance at 550nm using a spectrophotometer. Relative potency analysis is performed using obtained OD values in parallel line assay analysis software (PLA).

The aim of the present study was to investigate the possible mechanism of anti-diabetic activity of *Costus speciosus* plant extracts in comparison to commercial insulin( Actrapid) as standard by dose dependent glucose uptake measurement in 3T3 L1 cells and to assign relative potency of the extract to be potential anti diabetic compound.

## 2. MATERIALS AND METHODS

### **MATERIALS**

#### ***Costus speciosus* plant extracts sample preparation:**

Fresh aerial parts of *Costus speciosus* for the proposed work were collected from western ghats of Karnataka and authenticated by KVG college of Ayurveda, Sullia, Dakshina kannada, Karnataka India. The whole plant material was dried under shade, segregated for leaves, rhizome and shoot and mechanically reduced to moderate coarse powder and stored in air tight containers for further use in extraction process (Chan, 2012).

#### **Preparation of crude extract:**

The coarse powder (100g) was mixed with 500ml of distilled water and allowed to extract overnight at room temperature. The resultant dark green-brown mixture was filtered through filter paper (Whatman No. 1) and the filtrate was evaporated to dryness at 40°C under reduced pressure in a Rotavapor-R rotary evaporator. The resultant brown powder weighed 12 g. The residue was redissolved in distilled water at a concentration of 0.2 g/ml and kept frozen as one ml aliquots at -20°C until required. This was termed the crude extract.

#### **Preparation of the aqueous, ethanol extract of *Costus speciosus*:**

The dried crude extract (14g), produced as above, was mixed with absolute ethanol ( 80 ml) and allowed to stand overnight in a dark place. The resultant light green-brown mixture was then filtered and the filtrate was evaporated to dryness at 40°C. Quantity of 2g dry material was obtained and redissolved in absolute ethanol (0.2g/ml). This was termed the "ethanol extract". The residue from the extraction was redissolved in distilled water (0.2g/ml ).

#### **Preparation acidified stock of *Costus speciosus* extract:**

A quantity of 10 mg was dissolved completely in 0.1 N HCl and stored at -20 deg C freezer until use.

#### **Reagents for metabolic assay:**

**3T3L1 cell line:** ATCC ( Catalogue number : CL173).

Actrapid® (commercial insulin): Novo Nordisk, Bagsvard, Denmark.

**Complete medium:** DMEM High glucose medium( Gibco,11995-065) + 10 % H1-FBS + Penicillin Streptomycin (100 IU/100ug)+10 mM HEPES (Invitrogen, 15630).

**Differentiation medium:** DMEM High glucose medium + 10 % H1 –FBS + Penicillin Streptomycin (100 IU/100ug)+10 mM HEPES + 0.25uM Dexamethasone +0.5 mM Isobutyl methyl xanthine+ defined ug/mL sample .

**Metabolic assay medium:** DMEM Low glucose medium (Sigma Aldrich,D5921 without phenol red) + penicillin streptomycin+0.5% H1- FBS +10mM HEPES +2 mM- Glutamine.

**GOPOD substrate:** 4Ap( 3.6 mg/mL) and ESA (4.9 mg/mL) of 0.1 mM sodium phosphate buffer , pH 6.0±0.2 in 1:1 ratio.

**GOPOD reagent:** Prepared by dissolving lyophilized capsule of GOPOD in 39.2 mL of Milli Q filtered water.

**Sodium phosphate buffer, pH 6.0±0.2:** Prepared by dissolving 6g of NaH<sub>2</sub>PO<sub>4</sub> in 50 mL Milli Q purified water to prepare 1M NaH<sub>2</sub>PO<sub>4</sub> buffer. Prepared 0.1M stock by dissolving 5 mL of 1M NaH<sub>2</sub>PO<sub>4</sub> in 50 mL of Milli Q water. Prepared 0.1 M NaH<sub>2</sub>PO<sub>4</sub> by dissolving 0.71g in 50 mL. Adjust 50 mL NaH<sub>2</sub>PO<sub>4</sub> to pH using 0.1 M NaH<sub>2</sub>PO<sub>4</sub>.

#### Cell passaging and seeding:

- 3T3L1 cells were grown to ~ 60-70 % confluence in T flasks in complete medium.
- Prior to trypsinization, cells were washed by adding 1XPBS to respective T flasks.
- Trypsin was added and the cells were incubated for 5-10 in 5% Co2 incubator.
- Trypsin neutralization was done by adding 2 times the existing volume using complete medium.
- Cells were centrifuged at 1500 rpm for 5 minutes at room temperature.
- Supernatant was decanted and cells were resuspended in ~ 15 mL of complete medium .
- Cell count was taken using automated cell counter( Countess, Invitrogen).
- Cells were diluted to final count of 1.25\*10<sup>5</sup> cells/mL.
- A volume of 200ul/well of cell stock was seeded to 96 well plates and incubated at 37 deg C in CO2 incubator for ~ 75 hours.
- Media is removed gently from the wells and 200ul/well of differentiation medium was added and incubated at 37 deg C in 5% CO2 incubator for 96 hours .
- Media is removed gently from the wells and 200ul/well of complete medium was added and incubated at 37 deg C in 5% CO2 incubator for upto 75 hours .
- Preparation of Actrapid standard and *Costus speciosus* sample

100 uL of Actrapid Concentration (µg/mL)	Metabolic assay medium µL	100 µL of crude <i>Costus speciosus</i> plant extract Concentration (µg/mL , W/v)	Metabolic assay medium µL
250	150	250 ethanol extract	150
100	150	100 ethanol extract	150
50	150	250 Aqueous extract	150
25	150	100 Aqueous extract	150

15	150	250 Acidified sample	150
15	150	100 Acidified samples	150
5	150	100 µl of Sterile water	150

### Insulin and plant extract dependent glucose uptake response:

Media was removed carefully without disturbing the cells and washed with 1X DPBS.

A Volume of 100ul/well of each Insulin Actrapid and *Costus speciosus* extract were added to cells and incubated for upto 25 hours at 37 deg C in 5% CO<sub>2</sub> incubator. Volume of 10 ul of the supernatant from each wells were transferred to fresh 96 well plates and 85µl of sterile MQ water is added to all the wells followed by 40 ul of substrate ( sigma Aldrich E8506: A4382) ( ESA: 4-AP) and 65 µl of GOPOD(G3660, Sigma Aldrich ). This mixture was incubated at RT for 10 mins . Colour developed was read at 550nm in a plate spectrophotometer. A graph was plotted using absorbance values (OD) against concentration of Insulin and plant extract sample using PLA software ( soft max pro 5.4.1 V ).

### 3. RESULTS AND DISCUSSIONS

#### Relative Potency of *Costus speciosus* plant extract in 3T3 L1 cells

In order to study the dose-dependence of the proliferative response of 3T3 L1 cells to the regular insulin and *Costus speciosus* plant extract, cells were treated with various concentrations of each standard and sample.

The results presented in this study show that the crude plant extract is known to have relatively significant influence on metabolic potency. Relative potency of *C. Speciosus* was observed to be higher in Acidified sample and lowest in Aqueous extract sample .In contrast, The relatively low in vitro potency of insulin like properties in the crude extract may, however, contribute to explanations of why crude mixture of plant extract is not as effective on a molar basis as human insulin in human

Plant extract sample concentrations demonstrated considerable relative potency when compared to Insulin Actrapid® as shown in the Table 1.

**Table 1:** Relative potency of *Costus speciosus* plant extract in reference to Actrapid.

100 uL of crude <i>Costus speciosus</i> plant extract Concentration (ug/mL , W/v)	Reference standard Insulin	Relative potency
250 ethanol extract	Actrapid®	0.35
100 ethanol extract		0.16
250 aqueous extract		0.24
100 aqueous extract		0.13
250 Acidified sample		0.49
100 Acidified samples		0.24
100 ul of Sterile water		0.06

#### 4. CONCLUSIONS & RECOMMENDATIONS

Insulin's from animal origin was the only source for almost 50 years to treat diabetic conditions. Only in 1980's recombinant DNA technologies and advanced protein chemistry made human insulin preparations available to the world diabetic population. Over the last decade, a number of insulin analogs have been constructed with the aim of providing the diabetic patient with the most efficient, reproducible, and convenient therapy possible. It is imperative that these benefits are not achieved at the cost of the safety profile of the native human hormone. The results presented in this study show that the crude plant extract is known to have relatively significant influence on metabolic or mitogenic potency. The results of the current study showed that plants extract probably exerts its anti-diabetic properties by stimulating glucose uptake in adipocytes with significant inhibition of adipogenesis demonstrating reliable relative potency in comparison to the commercial insulin. The ability of existing therapies to target various aspects of the insulin resistance syndrome induces other metabolic abnormalities, chiefly those involved in lipid metabolism.

In this preliminary in-vitro study *Costus speciosus* plant extract demonstrated to have significant relative potency in comparison to commercial Insulin which can be exploited to treat diabetes using natural herbal extracts. In contrast the relatively low in-vitro potency of insulin like properties in the crude plant extract may, however, contribute to explanations of why crude mixture of plant extract is not as effective on a molar basis as human insulin in human.

Current study leads researchers to elucidate the chemical structures, isolate active ingredients in the crude extract for such biological activities in reference to commercial and recombinant insulins.

There is no doubt that the world is moving towards sustainable, organic and natural therapies for most of the lifestyle diseases where Diabetes ranks number one. Therefore, *Costus speciosus* plant extract is definitely a potential candidate for natural anti diabetic drug for maintenance of diabetes.

#### 5. ACKNOWLEDGEMENTS

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*Conflict of interest: None declared*

*Ethical approval: Not required.*

#### 6. APPENDICES

##### **Quick steps in performing the Glucose uptake assay:**

- a. Cell count : 12500 cells/well/100 ul in DMEM complete medium.
- b. Incubation of cells at 37 deg C CO2 incubator for upto 75 hours.
- c. Addition of Differentiation Medium (DM), 100ul/well.
- d. Incubation of cells at 37 deg C CO2 incubator for upto 96 hours
- e. Media change to Complete Medium (CM), 100ul/well.
- f. Incubation of cells at 37 deg C CO2 incubator for upto 75 hours.

- g. Acidified sample concentrations range used : 250 ng/mL to 5 ng/mL
- h. Dose details: 0.5 times dilution of Acidified samples through the range of samples.
- i. After removing CM, addition of 100ul/Well of each dose in L1 assay buffer.
- j. Incubation of cells with dose concentrations upto 25 hours
- k. Read method: GOPOD assay.
- l. Read wavelength: A550 nm
- m. Instrument: Multimode plate reader (Perkin Elmer, Enspire) .
- n. Result: Calculation of percentage drop in glucose content.
- o. Glucose drop % =  $\{(OD \text{ at highest insulin concentration} / OD \text{ at lowest drug concentration}) * 100\}$

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