

# Probing key targets in insulin signaling and adipogenesis using a methanolic extract of *Costus pictus* and its bioactive molecule, methyl tetracosanoate

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**Abstract** A methanolic extract of *Costus pictus* (CPME) showed optimum anti-diabetic activity at 100 ng/ml. Bioactivity-guided purification of CPME led to the isolation of methyl tetracosanoate (MT) which showed an optimum glucose uptake at 1 ng/ml. CPME at 10 µg/ml inhibited adipogenesis whereas fully differentiated adipocytes exhibited a 3-fold increase in lipid accumulation compared to pre-adipocytes. Gene and protein expression of key targets in insulin signaling and adipogenesis pathway revealed that CPME exhibited anti-diabetic activity along with anti-adipogenic activity whereas MT demonstrated only anti-diabetic activity.

**Keywords** Adipogenesis · *Costus pictus* ·  
Glucose uptake · Methyl tetracosanoate ·  
Type 2 diabetes

## Introduction

Type 2 diabetes, characterized by insulin resistance, is caused largely due to obesity, affecting around 80% of the entire diabetic population. Increasing knowledge on the biochemical and cellular alterations occurring in diabetes has led to the development of novel and potentially more effective therapeutic approaches to treat this metabolic disorder. Many pharmaceutical companies also pursue drug discovery using natural products, as traditional knowledge follows a reverse path which helps reduce time and developmental cost (Jachak and Saklani 2007). The reverse path is advantageous in elucidating drug targets and helps in identification of bioactive molecule. Metformin is one such commercially available anti-diabetic drug derived from *Galega officinalis*.

The present study focuses on identifying an anti-diabetic molecule from *Costus pictus* belonging to the family of Zingiberaceae and mostly grown as an ornamental plant in many countries (Meléndez-Camargo et al. 2006). This plant has hypoglycemic activity and can regenerate beta cells of pancreatic islets (Jothivel et al. 2007). The existing challenge is to study the role of *Costus pictus* methanolic extract (CPME) and the isolated bioactive molecule on key regulators involved in the process of glucose uptake and adipogenesis, using an in vitro model.

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## Materials and methods

### Process of extraction

*Costus pictus* leaves were shade dried and extracted using methanol yielding methanolic extract (CPME), which was concentrated under reduced pressure.

### Measurement of 2-deoxy-D-[1-<sup>3</sup>H]glucose

3T3-L1 preadipocytes, grown in 24-well plate were differentiated to adipocytes and were incubated with the plant extracts, column fractions or bioactive molecule and were subjected to glucose uptake assay using radiolabelled 2-deoxy-D-[1-<sup>3</sup>H]glucose (Muthusamy et al. 2008).

### Isolation and purification of bioactive fraction

A column (5 cm × 25 cm) was packed using silica gel (60–120 mesh) in hexane. The ratio of the extract slurry loaded to silica gel was 1:20 (w/w) (using 2 g plant material). The column was eluted by an increasing polarity of the mobile phase solvent mixture hexane/ethyl acetate (100:0, 95:5 to 0:100 v/v), elution was continued in the same column using ethyl acetate/methanol (95:5 to 0:100 v/v) with 25 ml eluate being collected per test-tube. Fractions were concentrated under reduced pressure and pooled based on their TLC fingerprint. The pooled fractions were tested for bioactivity and the bioactive fraction was further purified using second stage column (2 cm × 25 cm) packed with 100–200 mesh silica gel and the obtained fractions were again subjected to bioassay for the determination of the bioactive molecule.

### PTP1B inhibition study

The phosphatase activity was assayed using 20 mM *p*-nitrophenyl phosphatase (pNPP) as substrate and carried out in sodium acetate buffer, in a 96-well format (Burke et al. 1996). The reaction was initiated by the addition of recombinant PTP1B enzyme (Biomol Research Laboratories, Inc., PA, USA) into CPME, MT and sodium orthovanadate pre-treated wells (100 ng/ml, 1 ng/ml and 100 μM, respectively) and incubated for 30 min at 37°C. The

rate of PTP1B-catalyzed hydrolysis of substrate was measured at an absorbance of 405 nm.

### Adipocyte differentiation assay

The ability of 3T3-L1 preadipocyte to differentiate in the presence of plant extract and bioactive molecule were assessed at varying concentrations (1 ng/ml to 10 μg/ml) over untreated cells by measuring the accumulation of triglycerides (Ambati et al. 2007). Triglyceride content was measured using AdipoRed assay kit.

### Lactate dehydrogenase release (LDH) assay for cell death

CPME and MT were assessed for cytotoxicity at 10 μg/ml. The LDH released was measured using the CytoTox 96 (TM) assay (Muthusamy et al. 2008). Triton X was used as positive control.

### Isolation of total RNA and RT-PCR

Cells were co-incubated with optimized concentrations of CPME and MT for 18 h and then homogenized using TRIzol reagent. Isolated RNA was converted to cDNA by reverse transcription (Chomczynski and Sacchi 1987) and cDNA was subjected to PCR with specific primers such as GAPDH, GLUT 4, PPARγ, C/EBPα, SREBP 1c, adiponectin.

### Western blot analysis

Total cell lysates were prepared (Klip et al. 1992) and total protein was estimated using Bradford's method. Equal quantities of protein samples were loaded for carrying out western blot analysis.

### Statistical analysis

All experiments were carried out at least twice in triplicates and data were expressed as mean ± SE of independent experiments. Mean difference between the groups was analyzed by one-way analysis of variance (using GraphPad Prism 5). *P* < 0.05 was considered as statistically significant and are indicated as (\*).

## Results and discussion

3T3-L1 adipocytes were used as a model system since adipocytes mimic fat cells, inducing insulin resistance which is the major contributor of type 2 diabetes (Guilherme et al. 2008). The methanolic extract of *Costus pictus* (CPME) was subjected to primary screening to evaluate its anti-diabetic activity. CPME at 100 ng/ml showed an optimum glucose uptake (Table 1) when compared with rosiglitazone (positive control).

Based on the anti-diabetic activity exhibited by CPME, it was subjected to bioactivity guided purification using conventional column chromatography. The bioactive compound eluted in hexane: ethyl acetate system between ratios of 75:25 (v/v) to 60:40 (v/v) and exhibited an optimum activity at 1 ng/ml (Table 1). The bioactive compound when subjected to glucose uptake assay at lower concentrations such as 100 pg/ml, 10 pg/ml and 1 pg/ml showed a decreased glucose uptake in a dose dependent manner (data not shown). This could be due to saturation limit of the isolated bioactive compound, which was attained at 1 ng/ml and therefore an increase or decrease in concentration did not exert any increment in glucose uptake.

**Table 1** Glucose uptake analysis of *Costus pictus* methanolic extract (CPME) and methyl tetracosanoate (MT) on differentiated 3T3-L1 adipocytes

Sample concentrations	% Glucose uptake upon treatment with CPME	% Glucose uptake upon treatment with MT
1 ng/ml	150 ± 31*	307 ± 15*
10 ng/ml	157 ± 44*	257*
100 ng/ml	295 ± 63*	197 ± 29*
1 µg/ml	178 ± 67*	150 ± 26*
10 µg/ml	108 ± 14*	147 ± 23*

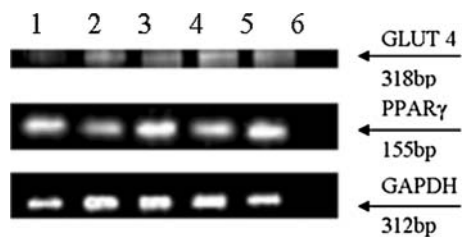
The differentiated 3T3-L1 adipocytes after serum starvation were treated with various concentrations (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml and 10 µg/ml) of extract/compound and incubated for 24 h. The cells were subjected for 15 min stimulation with insulin (10 nM) followed by 2-deoxy-D-[1-3H]glucose. The results were expressed as absolute values of % glucose uptake with respect to the solvent control (methanol). Positive control (rosiglitazone, 50 µM) exhibited 145 ± 8% of glucose uptake. The experiment was performed four times in triplicates. Data are mean ± SE and (\*),  $P < 0.05$  as compared with vehicle control

The purified compound showing optimal activity was subjected to gas chromatography mass spectrometry (GCMS) and proton nuclear magnetic resonance spectroscopy ( $^1\text{H}$  NMR) respectively (Supplementary Fig. 1). The data obtained when compared with SDBS database, revealed that the compound was methyl tetracosanoate (MT) (SDBS No.: 7702; CID: 75546), which is also known as methyl lignocerate, a saturated fatty acid. The molecular formula of the compound was found to be  $\text{CH}_3(\text{CH}_2)_{22}\text{COOCH}_3$ .

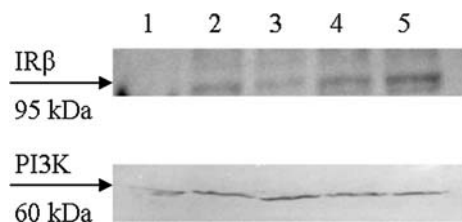
CPME and MT were non-cytotoxic on 3T3-L1 adipocytes (data not shown). Optimum concentrations of CPME (100 ng/ml) and MT (1 ng/ml) were used for further studies to decipher their mechanistic role in activating key diabetic markers like protein tyrosine phosphatase 1B (PTP1B), insulin receptor  $\beta$  (IR $\beta$ ), phosphatidylinositol 3 kinase (PI3K), peroxisome proliferator-activated receptor (PPAR $\gamma$ ) and glucose transporter 4 (GLUT4).

The translocation of GLUT4 is hindered due to increased PTP1B levels resulting in dephosphorylation of IR and IRS-1 thereby retaining PI3K in an inactive state (non-phosphorylated) under insulin resistant conditions (Wu et al. 2005; Laukkanen et al. 2004). CPME and MT showed 76 ± 4% and 76 ± 5% inhibition of PTP1B enzyme respectively and the positive control, sodium orthovanadate (100 µM) showed 96 ± 5% of PTP1B inhibition.

CPME and MT treated cells were found to elevate the GLUT4 mRNA expression compared to the positive controls (Fig. 1). Cells upon treatment with CPME and MT were found to show IR $\beta$  and PI3K protein expressions equivalent to that of positive controls (Fig. 2). The effect of CPME and MT on PPAR $\gamma$  mRNA expression was analyzed, since PPAR $\gamma$  agonists act as insulin sensitizers in resistant tissues (Cho et al. 2008). Activation of PPAR $\gamma$  has been found to influence insulin signaling at various points including the p85 subunit of PI3K (Chuang et al. 2007) which has been reported for enhancement of GLUT4 activity (Sandouk et al. 1993). Cells treated with MT at 18 h exhibited PPAR $\gamma$  expression on par with rosiglitazone (50 µM) whereas CPME exhibited basal level expression (Fig. 1) (Yang et al. 2001). Although increased levels of PPAR $\gamma$  exhibits insulin sensitization, this target is over-expressed in obese conditions and its suppression acts as a potential treatment for type 2 diabetes caused due to obesity (Spiegelman and Flier 1996).



**Fig. 1** Effect of *Costus pictus* methanolic extract (CPME) and methyl tetracosanoate (MT) on GLUT4 and PPAR $\gamma$  mRNA transcripts. 3T3-L1 adipocytes were incubated with insulin (100 nM) for 15 min and rosiglitazone (50  $\mu$ M), CPME (100 ng/ml) and MT (1 ng/ml) for 18 h. Lane 1–6 indicates untreated control, insulin, rosiglitazone, CPME, MT treated cells and PCR negative control, respectively. The expression levels of GLUT4 transcripts (318 bp) were compared with control cells. CPME and MT showed expression similar to the positive controls, Insulin and Rosiglitazone. The levels of PPAR $\gamma$  (155 bp) in CPME treated cells showed basal level expression whereas MT treated cells showed increased expression as compared to rosiglitazone, the positive control. The mRNA of GAPDH (316 bp) in the same sample was used as a loading reference



**Fig. 2** Effect of *Costus pictus* methanolic extract (CPME) (100 ng/ml) and methyl tetracosanoate (MT) (1 ng/ml) on IR $\beta$  and PI3K expression. Equal quantities of protein samples were loaded and resolved in 10% SDS–PAGE. Proteins were transferred onto nitrocellulose membrane. The membrane was blocked with (5% w/v) skimmed milk powder as the blocking agent for overnight at 4°C followed by 1 h incubation with the primary antibody (1:2000 of anti-phospho IR $\beta$ /p85 PI3K antibody (BD Pharmingen)). After PBS washing and secondary antibody (ALP conjugate) incubation, the blot was visualized using NBT/BCIP chromogenic agent and photographed. Lane 1–5 indicates untreated control, insulin, rosiglitazone (50  $\mu$ M), CPME (100 ng/ml) and MT (1 ng/ml) treated cells respectively. 3T3-L1 adipocytes pretreated with CPME and MT for 24 h showed phosphorylation of IR $\beta$  and PI3K on par with positive control insulin (100 nM)

The process of adipogenesis is a major sign of obesity and accumulation of lipid occurs as an end result. CPME (10  $\mu$ g/ml) showed an inhibition of lipid accumulation compared to fully differentiated adipocytes (positive control) (Table 2). Preadipocytes treated with CPME and MT for the entire duration of assay showed no cytotoxicity (data not shown).

**Table 2** Effect of *Costus pictus* methanolic extract (CPME) and methyl tetracosanoate (MT) on lipid accumulation in 3T3-L1 adipocytes after differentiation/induction

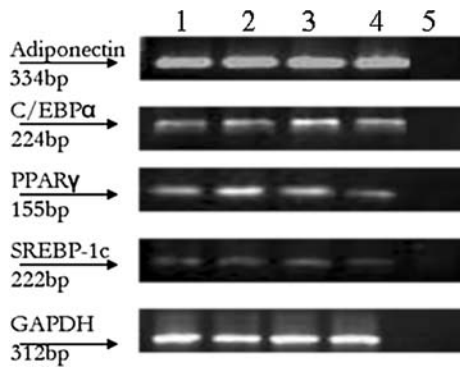
Sample concentrations	% lipid accumulation upon treatment with CPME	% lipid accumulation upon treatment with MT	% lipid accumulation upon differentiation (no treatment)
1 ng/ml	626 $\pm$ 15*	363 $\pm$ 53*	262 $\pm$ 23*
10 ng/ml	686 $\pm$ 85*	453 $\pm$ 72*	
100 ng/ml	562 $\pm$ 36*	645 $\pm$ 3*	
1 $\mu$ g/ml	287 $\pm$ 64*	729 $\pm$ 26*	
10 $\mu$ g/ml	66 $\pm$ 14	580 $\pm$ 41*	

Two-day post-confluent 3T3-L1 cells were differentiated according to the protocol followed by treatment with described concentrations of *Costus pictus* methanolic extract (CPME) and methyl tetracosanoate (MT) for 2 days. Eight days after induction of differentiation, cells were assayed for total triglyceride content using AdipoRed reagent. Pre-adipocytes were separately maintained. The results were expressed as absolute values of % lipid accumulation with respect to the solvent control (methanol). This experiment was performed thrice in triplicates. Data shown reflect the mean  $\pm$  SE and (\*),  $P < 0.05$  as compared with pre-adipocytes group

Based on the ability of CPME (10  $\mu$ g/ml) to inhibit adipogenesis, effect of CPME (10  $\mu$ g/ml) on mRNA transcription levels of various markers affecting adipogenesis (PPAR $\gamma$ , CCAAT/enhancer-binding proteins (C/EBPs), and adipocyte determination and differentiation factor-1c/sterol regulatory element-binding protein 1c (ADD1/SREBP1c)) was analyzed. In general increased C/EBP $\alpha$ , PPAR $\gamma$  and SREBP1c levels are observed during the process of adipocyte differentiation (Wu et al. 1998). Adiponectin, a cytokine secreted from adipocytes acts as an insulin sensitizer. Its expression in obese condition is found to be decreased and under normal condition its levels are found to be high (Nadler and Attie 2001). CPME (10  $\mu$ g/ml) showed a down-regulation of adipogenic markers such as PPAR $\gamma$ , C/EBP $\alpha$ , SREBP 1c and increased adiponectin expression levels compared to fully differentiated adipocytes (Fig. 3) confirming the fact that CPME has the ability to inhibit adipogenesis without affecting its anti-diabetic activity.

## Conclusion

The above results show that the anti-diabetic activity exhibited by CPME and MT is aided together by PTP



**Fig. 3** Effect of *Costus pictus* methanolic extract (CPME) (10  $\mu\text{g/ml}$ ) on genes involved in adipogenesis. CPME inhibited adipocyte differentiation by altering the expression of key genes involved in adipogenesis. Lane 1–5 indicates untreated control, insulin (100 nM), rosiglitazone (50  $\mu\text{M}$ ), CPME (10  $\mu\text{g/ml}$ ) treated cells and PCR negative control, respectively. Maximal expression of C/EBP $\alpha$  (224 bp), PPAR $\gamma$  (155 bp) and SREBP1c (222 bp) were observed with rosiglitazone and CPME showed less expression on comparison with untreated adipocytes

1B inhibition and IR $\beta$ –PI3K activation. During the study it was also found that CPME has the ability to inhibit differentiation of adipocytes, whereas MT exhibited no such activity, which shows the possibility of finding an anti-adipogenic molecule from CPME.

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