International Research Journal of Biotechnology (ISSN: 2141-5153) Vol. 2(2) pp.047-057 February, 2011
Available online http://www.interesjournals.org/IRJOB

Full Length Research Paper

Ethanolic extract of cinnamon potentiates *in vitro* pparγ reporter activity in presence of pgc1α and src1 and improves glucose tolerance in mice

Rajesh Gupta[a] Sameer Walunj[a] Shyam Awate[b] Roshan Kulkarni[c] Swati Joshi[c], Sushma Sabharwal[a] , A. S. Padalkar[b] and Kalpana Joshi[b]

[a] Department of Chemistry, Division of Biochemistry, University of Pune, Pune, India- 411007
[b] Department of Biotechnology, Sinhagad College of Engg, Pune, India-411041
[c] National Chemical Laboratory , Pune ,India- 411008

Accepted 18 December 2010

Cinnamon is one of the oldest spices in the world and widely used as an anti-diabetic medicine. We have investigated the effect of *Cinnamon zeylanicum* extracts on involvement of important coactivators like PGC1α and SRC1 in potentiation of PPARγ activity. Present study demonstrates that 50:50 ethanol aqueous extract of *Cinnamomum zeylanicum* bark contains specific PPARγ agonists that activate PPARγ in HEK293/T cell based reporter assay and also dose-dependently potentiate the extract mediated PPARγ activity in presence of co-activators like PGC1α and SRC1, overexpressed in the cells. Knockdown of PGC1α gene by specific shRNA against PGC1α, significantly reduced the transactivation potentiation effect of extract induced by coactivator. The ability to activate PPARγ receptor is also supported by its adipogenic potential observed in mouse 3T3-L1 adipogenesis assay. The CZE3 extract improved glucose tolerance in the 12 h fasted C57BL/6 male mice. In summary aqueous ethanolic extract of *Cinnamomum zeylanicum* exhibits part of its glucose lowering effect via activation of PPARγ and extract mediated PPARγ activity is increased upon overexpression of PGC1α and SRC1 in a cell based *in vitro* assay.

Key words: Cinnamon, Peroxisome proliferator activated receptors, Anti-hyperglycemic reporter assay, PGC1α, SRC1

INTRODUCTION

Type 2 Diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia and abnormalities in the carbohydrate fat and protein metabolism. Considerable clinical and experimental evidence supports the anti-diabetic activities of cinnamon (Solomon and Blannin, 2007., Anderson, 2008., Khan et al.,2003., Hlebowicz et al.,2007).

Aqueous cinnamon extract improves glycated hemoglobin HbA (1_c) fasting plasma glucose total cholesterol low-density lipoprotein (LDL) high-density lipoprotein (HDL) and triacylglycerol concentration in patients with T2DM (Hannover et al.,2006). Cinnamon displays insulin-like activity (Roffey et al.,2006) and helps to regulate blood glucose and improves glucose utilization *in vitro* (Anderson et al.,2004., Qin et al.,2003). Despite the widely known biological activity of Cinnamon the details of molecular mechanism by which it brings

*Corresponding Author, Email: joshikalpana@gmail.com

ABBREVIATIONS

PPAR: Peroxisome proliferators activated receptor
CZE: Cinnamon zeylanicum extract
PGC1α: Peroxisome proliferators activated receptor gamma co-activator 1 alpha
oGTT: Oral glucose tolerance test
AUC: Area under curve
T2DM: Type 2 diabetes mellitus
shRNA: short hairpin ribonucleic acid

about glucose lowering is unknown.

The Peroxisome proliferator activated receptors viz PPARα, PPARγ and PPARδ play key roles in the glucose metabolism and lipid homeostasis (Lin et al.,2005., Moller, 2001.,Berger and Moller, 2002., Tjokroprawiro,2006). Synthetic agonists that activate PPARγ such as the thiozolidinediones (TZD's) - Pioglitazone is being effectively used in clinics as "insulin sensitizers" in type 2 diabetics (Moller, 2001).

The regulation of gene expression by PPAR's is largely dependent on the recruitment of accessory proteins called as co-activators or co-repressors to the transcriptional heterodimer complex (PPAR-RXR or PPAR-LXR) (Kersten e*t al*.,2000). PPARγ Co-activator 1alpha (PGC1α) is one such crucial coactivator which is expressed in all the tissues expressing PPARγ like muscle, adipose tissue, intestine etc. PGC1α also plays a role in fatty acid oxidation and lipolysis. PPARγ agonists control blood glucose by increasing the cellular glucose uptake, reducing cellular glucose production and increasing insulin sensitivity in resistant tissues. It has been reported that decreased skeletal muscle PGC1α activity is associated with impaired mitochondrial function and the development of insulin resistance in humans (Hammond *et al*.,2005). Physical activity and exercise causes an increase in muscle PGC1α activity.

In this study, we have evaluated various extracts of *Cinnamomum zeylanicum* for its PPARγ transactivation potency in HEK293/T cell based luciferase reporter assay. Cinnamon bark was extracted successively with two solvents, viz. acetone and methanol: water (7:3), to achieve extraction of less polar, medium polar (fats, carotenoids, fatty acids, steroids, phenyl propanoids, etc.) and polar group of molecules (glycosides, polyphenols, polysaccharides, etc.) respectively. Cinnamon bark was also extracted with aqueous ethanol (1:1) in accordance with Traditional Systems of Medicine. The aqueous ethanol (1:1) extract (CZE-3) was further evaluated for glucose tolerance test (OGTT) in overnight fasted C57BL/6 male mice.

Use of above three solvents for extraction made it possible to extract and evaluate molecules with broad polarity and hence structural diversity.

MATERIALS AND METHODOLOGY

Reagent and Chemicals

Solvents acetone , methanol , ethanol and water were distilled prior to use. DMSO was purchased from Merck. DMEM medium, FBS, Trypsin-EDTA and PBS were procured from Invitrogen. HEPES buffer, sodium chloride, ONPG (β-Galactocidase substrate), sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, β-mercaptoethanol, MTT, rosiglitazone, WY-14643 and Glyburide (G2539) were purchased from Sigma-Aldrich. 5x Reporter lysis buffer, D-Luciferin, Luciferase assay reagent and DNA marker for gel electrophoresis were purchased from Promega. Glucose measuring strips were purchased from Roche.

Plant material

Bark of *Cinnamomum zeylanicum* Blume was purchased from local commercial source. The plant material was authenticated and a voucher specimen was deposited at the Botanical Survey of India, Western Circle, Pune. (No. SPCIV7).

Preparation of Extracts

The bark of *C. zeylanicum* was shade dried and then ground to a fine powder. Three extracts; acetone, aqueous methanol (3:7) and aqueous ethanol (1:1) were prepared.

Preparation of Acetone extract

Powdered bark 717 g was extracted for 12 h with acetone under intermittent stirring at room temperature (28 ^{0}C) and the extraction process was repeated three times. The extracts were combined and concentrated in vacuo at 45 ± 2 ^{0}C in a rotavapor (Buchi Model R-210 Germany) and dried to yield extract 66.5 g (9.274%). This was labeled as Acetone extract (CZE-1).

Preparation of Methanol extract

Residual plant material from above extraction process was air-dried in shade to remove residual acetone. It was then extracted for 12 h with aqueous methanol (3:7 v/v) under intermittent stirring at room temperature (28^{0}C) and the extraction process was repeated three times. The extracts were combined and concentrated in vacuo as above and dried to yield extract 57.6 g (8.033%). This was labeled as Methanol extract (CZE-2)

Preparation of Ethanol extract

Powdered bark, 1000 g was extracted for 12 h with ethanol: water (1:1 v/v) under intermittent stirring at room temperature (28^{0}C) and the extraction process was repeated three times. The extracts were combined and concentrated in vacuum as above and dried to yield extract 93.7 g (9.37%).This was labeled as Ethanol extract (CZE-3).

Expression vector and Cell culture transfections

Expression vector for PPARα, γ and PGC1α were constructed using pCI-neo vector (Promega Inc.) The primers for amplification of PPARα (NCBI ref seq: NM_005036), PPARγ1 (NCBI ref seq: BT007281 & L40904.2) and PGC1α (NCBI ref seq: NM_013261.2) gene were designed using online oligonucleotide properties calculator software and synthesized from Sigma. PGC1α, SRC1 and ShRNA for PGC1α were procured from Origene technologies USA. The basic reporter vector was procured from Panomics Inc. USA and then 3x PPRE-TATA reporter vector (Jpenberg et al.,1997) was constructed using random annealing of equimolar ratio of synthetic oligonucleotide primers. RNeasy RNA isolation kit and midi plasmid isolation kit were procured from Qiagen. Sequencing of cloned PPARγ (Matched against NCBI ref seq: BT007281) and alpha (Matched against NCBI ref seq: NM_005036) and reporter vectors were confirmed by automated DNA sequence analyzer (ABI). HEK293/T (ATCC No: CRL11268) and 3T3-L1 cell lines were procured from ATCC. Hep-G2 cell line (used for isolation of total RNA and preparation of cDNA) was procured from NCCS Pune. All cell culture disposable culture flasks and pipettes were purchased either from Nunc or Corning. Cell culture 96 well flat bottom clear plate (cell bind surface) was purchased from corning. Lipofectamine-2000 and DMEM medium was procured from Invitrogen USA. Coactivator involvement assays with PGC1α, SRC1 and gene silencing experiments (for silencing PGC1α) were carried out using lipofectamine-2000 reagent.

Primers used for amplifying genes:

1) For PCR amplification of PPARγ1 flanking with Kpn-I and Xba-I

Forward primer: 5'…GGGGTACCACCATGACCATGGTTGACAC….3'
Reverse primer: 5'…GCTCTAGAGCTCAGTACAAGTCCTTGTAG….3'

2) For PCR amplification of PPARα flanking with Nhe-I and Sal-I

Forward: 5'….CCTAGCTAGCATGGTGGACACGGAAAGCCCA…3'
Reverse: 5'….ACGCGTCGACTCAGTACATGTCCCTGTAG….3'

3) For PCR amplification of PGC1 using HepG2 cDNA as template flanking with Xho-I and Sma-I enzyme
Forward primer: 5'……CCGCTCGAGATGGCGTGGGACATGTGCAA….3' Reverse primer: 5'….TCCCCCGGGTTACCTGCGCAAGCTTCTCTG….3')

Preparation of oligonucleotide primers for synthesis of 3x PPRE DNA.

Sense strand
5'…CTAGCCCAAACTAGGTCAAAGGTCACATCCAAACT AGGTCAAAGGTCAGGGCCCCAAACTAGGTCAAAGGTCAAA….. 3'

Antisense
5'…..GATCTTTGACCTTTGACCTAGTTTGGGGCCCTGACCTTTG ACCTAGTTTGGATG TGACCT TTGACCTAGTTTGGG….3'

Adipogenesis Assay

TG measurement kit was procured from Merck. Dexamethasone, IBMX, porcine insulin, ONPG (β-galactosidase substrate), Wyeth compound (WY-14643) & oligonucleotides were purchased from Sigma. Rosiglitazone was procured from Biocon India.
Method of differentiation of 3T3-L1 preadipocytes: Cells (10,000 c/well) were seeded in 24 well plates prior to induction of adipogenesis and supplemented with DMEM (Low glucose with 10% Bovine Serum). On day-3, (~68 h post seeding) medium was replaced and supplemented with Induction medium (DMEM with 10% FBS containing 0.6 µM IBMX 1.0 µM Dexamethasone and 5.0 µg/ml insulin) for differentiation of preadipocytes. This step also included the addition of test compounds (either reference compound or extract) and plates were incubated in CO_2 incubator for additional 48 h). Medium was aspirated after 48 h and replaced with DMEM containing 10% FBS + respective test compounds/extract in each well for an additional 3 day. On day-8, the plates were observed under inverted microscope for visualizing the adipogenesis effect caused by compounds. Plates were then washed twice with PBS followed by 0.2 ml of lysis buffer/well (0.1% triton in PBS pH 7.4) and kept on shaker with moderate speed (50-60 rpm) for 20 min. Wells were then analyzed for TG content (using TG estimation kit from Ecoline or Merck) after brief centrifugation at 3000 rpm for 10 min at 4^0C.

Cell culture and transient co-transfection reporter assays

HEK 293/T cells (ATCC No: CRL11268) were routinely maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA). Prior to transfection, the cells were seeded in 6 well plates at a density of 1.25 x 10^6 cells/well in DMEM supplemented with 10% charcoal dextran treated FBS (Hyclone, USA). After 18-20 h of growth at 37^oC and 5% CO_2 cells were transfected (total 4 µg DNA) with respective PPAR expression plasmid (5% to total DNA i.e. 200 ng) PPRE reporter vector (50% of total DNA i.e. 2000ng) β-galactosidase plasmid and empty vector DNA (to make up total DNA charge) using Lipofectamine-2000 reagent according to manufacturer's protocol for 5 h. Cells were trypsinized counted and reseeded (100 µl/well) in 96 well plates for evaluation of PPARα and PPARγ transactivation potency of cinnamon extracts as compared to reference PPARγ and α drug used in the experiments viz Rosiglitazone and Wyeth compound (WY-14643). Different fractions of cinnamon extract and reference drug were first prepared as 1000x stock in 100% DMSO (e.g 10 mg/ml for getting 10 µg/ml final concentrations in cell culture plate). Reference drug was also prepared as either 10 mM stock (for rosiglitazone and concentrations used were: 0.001, 0.01, 0.1, 1, 10 & 25 µM) or 75 mM stock (WY-14643 and concentrations used were: 0.1, 1, 10, 25, 50 & 75 µM). The cells were incubated with respective ligands/CZE extracts and reference compounds for 18 h in CO2 incubator with set parameters. Next day the plates were removed from the incubator and lysed using 1x reporter lysis buffer (Promega Inc. USA). The plates were centrifuged to settle the cell debris and cell lysate was analyzed for luciferase assay (using Perkin Elmer Victor Light 96 well plate Luminometer) and β-galactosidase assay (using SpectraMax reader from Molecular Devices). The β-galactosidase expression plasmid was used in the experiment for normalization of transfection efficiency and also as an indicator of toxicity by ligands at higher doses if any. All the experiments were performed at least twice in triplicate wells. The MTT based cytoxicity assay for extracts at various concentrations was carried out separately.

Coactivator reporter assays and gene silencing by specific shRNA

29mer shRNA directed against PGC1α was used in the experiment to knockdown the PGC1α expression in transient transfection assays and check the reduction of transactivation potentiation effect of CZE-3 and relative comparison with vehicle control. All 4 shRNA constructs against PGC1α were tested for its arget specific gene silencing effect in a cell based assay over expressing PGC1α gene. Out of four constructs, only one construct (ID#2TI341034) with sequence(5'…GATAGATGAAGAGAATGAGGCAAACTTGC…3') showed gene specific silencing in HEK 293/T cellular assays over expressing PGC1α and thus used in the experiments. The shRNA constructs were used in a (1:10 molar ratio of PGC1α expression plasmid). Transfection was carried out using Lipofectamine-2000 as per manufacturer's instruction.

Effect of CZE on adipogenesis in mouse 3T3-L1 cell line

The 3T3-L1 cell line was maintained in DMEM medium supplemented with 10% bovine serum and 1x penicillin streptomycin. Cells were seeded at a density of 15000 c/well and incubated in CO_2 incubator set at 37oC 5% CO2 for 2 days. The cells were differentiated using a combination of Dexamethasone (1 µM) IBMX (0.6 µM) & 5 µg/ml insulin in absence of extract (vehicle control) and presence of extract and rosiglitazone for 3 days. The effect of CZE-3 for evaluation of adipogenesis in cells was tested at 5 10 and 25 µg/ml concentration and compared to vehicle control and reference drug rosiglitazone. After complete differentiation cells were lysed and measured for accumulated triglyceride content (TG) using the TG measurement kit (Merck or Ecoline kit). The TG

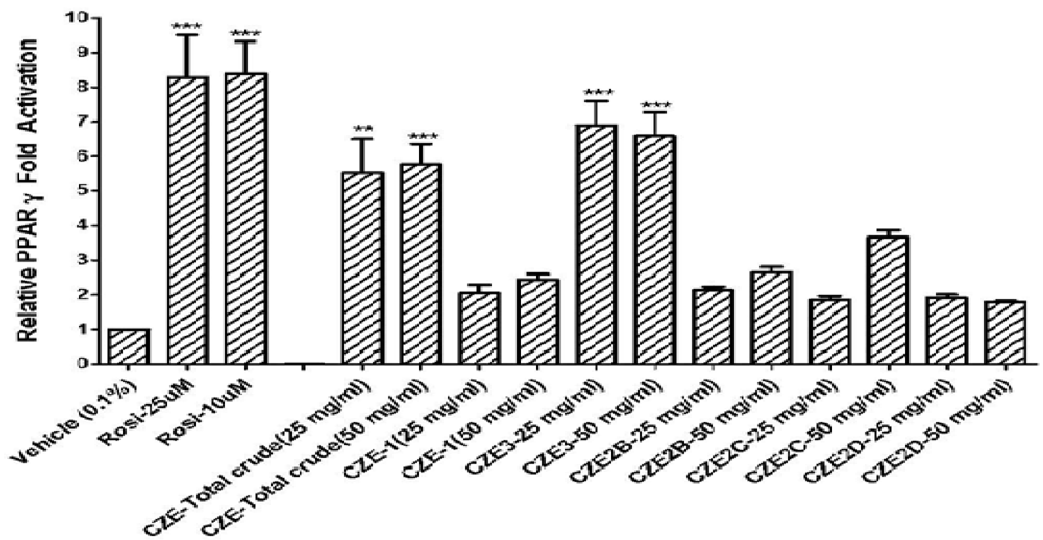

Figure 1. In vitro PPARγ transactivation potency of CZE total crude extract and various fractions in HEK293/T cells. Crude extract of CZE shows more than 5 fold activation compared to vehicle control. CZE-3 shows significant (***p <0.001 and **p <0.01vs vehicle control) PPARγ activity at indicated concentrations.

content was normalized against total protein and finally TG content was expressed as mg /mg protein.

Oral glucose tolerance test in C57BL/6 male mice

Six-week-old C57BL/6 male mice were obtained from commercial suppliers. All in vivo animal model experimental protocols were in adherence with government regulations. The mice were fed normal commercial diet and water ad libitum. For the oral glucose tolerance test (oGTT) overnight (12 h) fasted mice (n=8 per treatment) were orally dosed with either vehicle (0.2% aqueous carboxy methyl cellulose) or test compounds/extract at desired doses via oral gavage. Glucose bolus (3g/kg orally) was then administered to all groups except the baseline control group (n=8) which received water alone. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Bartlett's test for equal variance. The value of **P ≤ 0.01 and *P ≤ 0.05 was used as criterion of statistical significance.

Sampling of Blood

Blood samples (20 µl) were collected from mouse tail veins under ether anesthesia. Food deprivation was continued throughout the measurement of blood glucose (till 120 min.). Blood glucose was measured using strips in ACCUcheck Glucometer instrument (Roche Diagnostics).

Statistical Analysis

Experimental values are expressed as mean ± standard deviation of at least two experiments in triplicate or mean ± SEM of three independent experiments in triplicate for in vitro experiments. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison. The value of P ≤ 0.05 was used as criterion of statistical significance. Graph pad prism software (version 4.03) was used for calculation of EC_{50} values of fractions in various assays.

RESULTS AND DISCUSSION

PPARγ transactivation reporter assay

PPARα/γ transactivation potency of Cinnamon extracts CZE-1, 2, and 3 were evaluated at two different concentrations viz, 25 and 50 µg/ml. The aqueous ethanol extract, CZE-3 (25 and 50 µg/ml) showed a >7 fold PPARγ activity as compared to reference control (rosiglitazone) which showed >8.2 fold activity at 10 µM & 25 µM. The cytotoxicity analysis of various fractions was assessed by MTT assay (data not shown). The extracts did not show any cytotoxicity till 50 µg/ml. CZE-1 and CZE-2 extract did not show significant PPARγ activity. (Figure **1**). None of the extracts showed PPARα activity.
Effect of various concentrations of CZE-3 was studied in PPARγ transactivation assay in HEK293/T cells and compared with reference control rosiglitazone. CZE-3 exhibited a potent PPARγ activity with an EC_{50} of 12.2 ± 1.5 µg/ml. Fold activation values relative to vehicle control were plotted using graph prism software version 4.03. CZE-3 showed approximately 70% activation (at 25 µg/ml) of PPARγ receptor relative to rosiglitazone set to 100% at 10 µM (saturating concentration) (Figure 2A). Reference compound rosiglitazone (PPARγ activator) activated PPARγ receptor in a dose-dependent manner with an EC_{50} value of 0.078 ± 0.01 µM (Figure 2B). Fold activation values relative to vehicle control were plotted

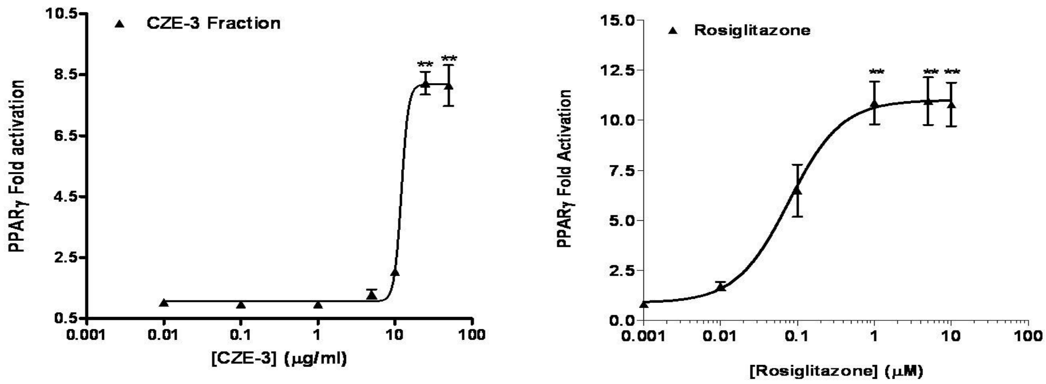

Figure 2A **Figure 2B**

Figure 2. Effect of CZE-3 and rosiglitazone on PPARγ transactivation in HEK293/T cell based reporter assay.
(A) PPARγ activation by different concentrations of CZE-3 and
(B) PPARγ activation by Rosiglitazone. Fold activation values relative to vehicle control were plotted using graph prism software version 4.03. Each concentration is expressed as mean fold increase of luciferase activity ± SD and were treated under similar conditions (n=3 experiments).

using graph prism software. p** value {(<0.01 rosiglitazone saturating dose vs vehicle (0.1% v/v DMSO)}.

Effect of CZE on PPARγ transactivation in cells overexpressing coactivator

CZE-3 showed a potent and specific PPARγ activation comparable to rosiglitazone. This led us to further evaluate CZE-3 for its coactivator recruitment/involvement potential to PPARγ. CZE-3 not only exhibited a potent PPARγ activity but also potentiated the PPARγmediated reporter activity in HEK293/T cell based assay in presence of PGC1α and SRC1 coactivator in a dose-dependent manner(Figure **3**). Similar dose-dependent potentiation of PPARγ activation was observed by rosiglitazone in cells co-expressing PPARγ along with PGC1α and transfected with 3x PPRE reporter vector (p** <0.01 vs vehicle control & p* <0.05 CZE-3 10 µg/ml vs vehicle). PPARγ activity in vehicle control cells transfected with PGC1α was higher as compared to cells transfected with PPARγ alone and reporter vector. This is in agreement with that reported in literature. The PGC1α expression in cells is known to increase the PPARγ activity in a ligand-independent and ligand-dependent manner (at higher ligand concentrations) (Kodera et al.,2000., Burgermeister et al.,2005).
Similar results were observed in experiments with SRC1 transfection reporter assays (Figure 4). HEK 293/T cells over expressing SRC1 and PPARγ demonstrated a significant dose-dependent increase in the potentiation of PPARγ activity at lower concentration of CZE-3 (5 and 10 µg/ml *p <0.05 vs vehicle control cells). Rosiglitazone and troglitazone (withdrawn) both compounds have been shown to recruit co-activators like PGC1α and SRC-1 and regulate expression of genes involved in glucose metabolism. Different PPARγ ligands have differential coactivator recruitment/involvement capability (this is indeed ligand driven due to differential receptor conformation induced by ligand and thus involving a specific set of coactivators in the receptor-ligand complex) and thus this is indeed responsible for differential regulation of set of genes in different tissues in body. The ability of aqueous ethanolic CZE-3 extract to potentiate PPARγ activity in presence of PGC1α and SRC1 demonstrates that *in vivo* the PPARγ activation (a differential confirmation induced by CZE-3) could preferentially involve these coactivators and thus could activate a different and preferential set of genes involved in glucose metabolism in diabetic patients (reported to have reduced levels of PGC1α) and genes involved in oxidative metabolism (Arany et al., 2005). Resveratrol has been shown to improve mitochondrial function and impart protection against metabolic diseases by activating PGC1α and SIRT1 (Lagouge et al.,2006).

Reduction of CZE mediated PPARγ transactivation potentiation effect in cells silenced with specific shRNA against PGC1α

Transactivation experiments with PGC1α shRNA constructs were conducted to confirm the observed CZE-

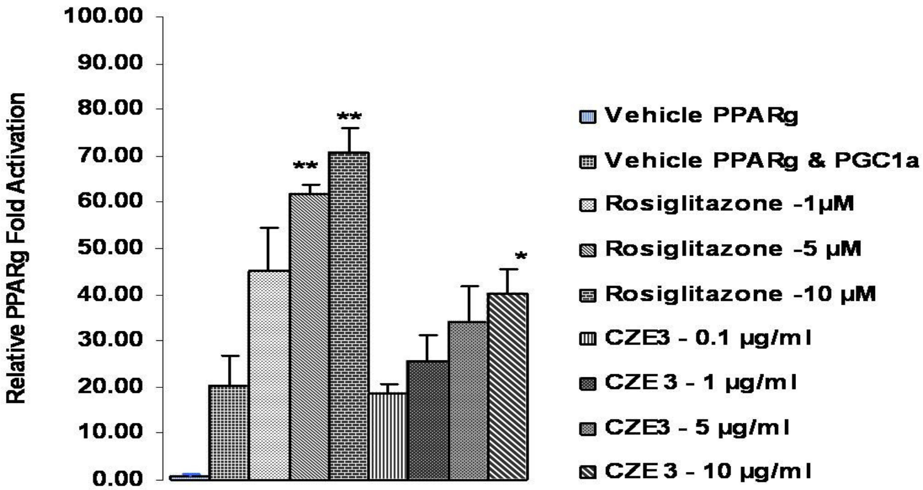

Figure 3. Effect of PGC1α on PPAR gamma mediated transactivation in transfected HEK293/T cell based reporter assay.
Dose dependent transactivation potentiation of PPARγby rosiglitazone and CZE-3 extract in cells co-transfected with full length PPARγPGC1α and 3x PPRE reporter vector. Figure shows potentiation effect by rosiglitazone and CZE-3 vs basal vehicle control. The PPARγactivity in vehicle control cells transfected with PGC1α is significantly higher as compared to cells transfected with PPARγ alone and reporter vector. (**p <0.01 vs. Vehicle control) & (*p <0.05 CZE-3 10 µg/ml vs vehicle). Values are expressed as average fold increase of luciferase activity ± SD compared with vehicle control cells treated under similar conditions (n=2 experiments).

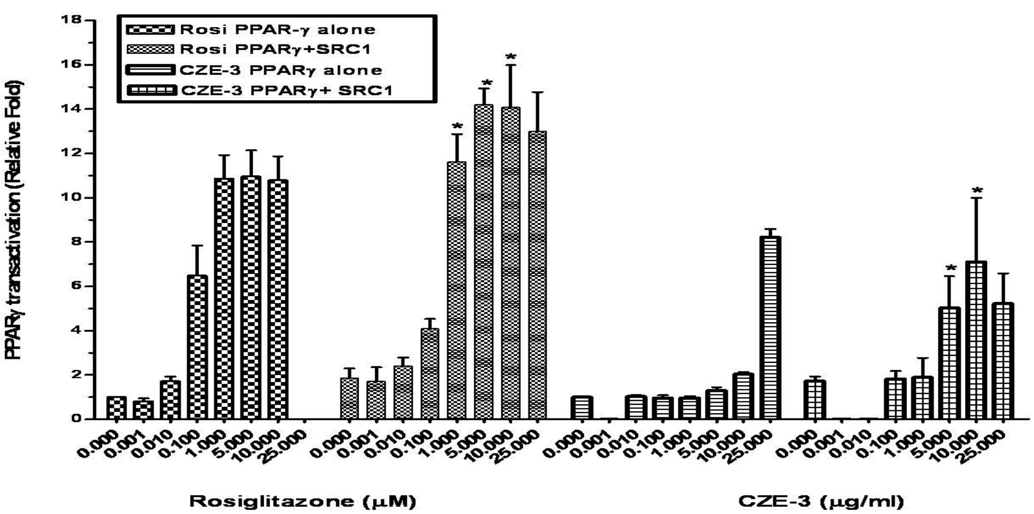

Figure 4. Effect of CZE-3 on PPARγ transactivation assay in HEK 293/Tcells expressing SRC1 and PPARγ.
Rosiglitazone and CZE-3 shows a significant and dose-dependent potentiation of PPARγ transactivation in presence of SRC-1 coactivator vs P PPARγalone (*P<0.05).

3 induced PPARγ potentiation effect by PGC1α. Cells transfected with PPARγ, PGC1α and a shRNA construct against PGC1α showed a marked dose-dependent reduction in the transactivation potentiation effect mediated by both viz, rosiglitazone and CZE-3 confirming crucial role of PGC1α in this process. This effect is due to

Figure 5A & B

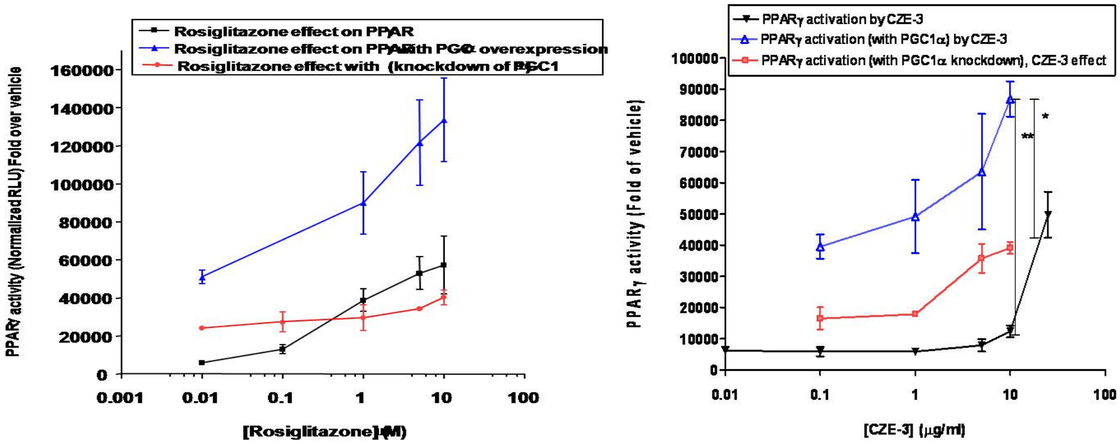

Figure 5A

Figure 5B

Figure 5. PPARγ transactivation by CZE3 in cells transfected with PPARγ, cells expressing PPARγ + PGC1α and in cells silenced for PGC1α.gene

(A) Reduction of rosiglitazone mediated PPARγ transactivation potentiation effect in cells silenced for PGC1α gene. (B) Reduction of CZE-3 mediated PPARγ transactivation potentiation effect in cells silenced for PGC1α gene.

the knockdown (~75%) of PGC1α mRNA in HEK293/T cells. Knockdown or silencing of PGC1α gene by specific shRNA's directed against PGC1α significantly (**p< 0.01 reduced the transactivation potentiation effect mediated by the CZE-3 in presence of coactivator (Figure 5A, Figure 5B). Thus based on the experimental results it is clearly evident that CZE-3 contains powerful PPARγ full agonist (s).

Effect of CZE on PPARα transactivation

The CZE-3 did not showed any PPARα transactivation activity in HEK293/T cell based reporter assay. (Data not shown). Figure 6. shows the effect of reference compound WY-14643 (concentrations used: 0.1 1 10 25 50 & 75 µM). It shows a dose-dependent activation of PPARα with an EC_{50} value of 22.0 ± 2.3 µM (n=3 independent experiments). Thus we showed that CZE-3 is PPARγspecific and could contribute as one of the mechanism for *in vivo* hypoglycemic effect of cinnamon extract.

Effect of CZE on adipogenesis in mouse 3T3-L1 cells

Adipocytes are the major target cells of PPARγ agonists in vitro and in vivo. Synthetic TZD class of compounds with PPARγ activation potency are known to accumulate triglyceride (TG) in adipose tissues and that is the reason to see weight gain in animal models and is causal of side effect in these group of drugs (weight gain). Thus we carried out experiments to evaluate this possibility (Figure 7A, Figure 7B). CZE-3 and reference compound rosiglitazone were evaluated for their adipogenic potential (TG accumulation) in 3T3-L1 *in vitro* cell line model.

We observed a significantly higher TG loading (~2.01 fold over vehicle) in cells treated with rosiglitazone but, CZE-3 showed only a mild increase (non-significant) in TG accumulation at 25 µg/ml (<1.5 fold over vehicle) and thus less adipogenic compared to rosiglitazone. Enhanced TG accumulation is a characteristic of potent PPARγ agonists (rosiglitazone) but reduced TG accumulation is beneficial in terms of candidate drug potential (desired drug would be weight neutral or weight reduction in animal models and patients). CZE-3 also exhibited a weak DPP-IV inhibitory activity in purified enzyme assay (Data not shown). Partial PPARγ agonists and PPARα agonists do not show adipogenesis in these cells (Burgermeister et al.,2005., Guan et al.,2005). Moderately potent dual PPAR α/γ, weak PPARγ agonist and partial PPARγ agonists have weight neutral property, cause lesser hepatotoxicity and cardiotoxicity related side effects as exhibited by potent PPARγ agonists like rosiglitazone, that has been recently withdrawn. Since PPARγ plays major role for glucose disposal in liver,

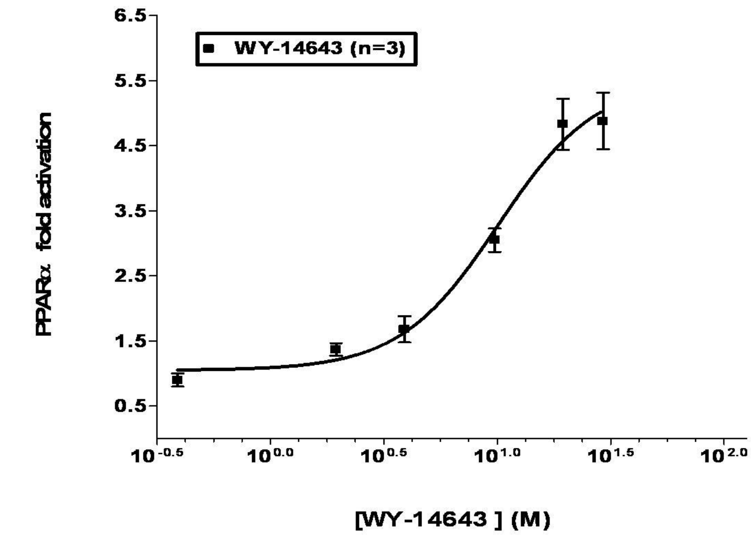

Figure 6. PPARα transactivation by Wyeth compound.
Dose response curve of WY-14643 for PPARα transactivation in HEK 293/T cells. The reference compound shows a dose-dependent activation of PPARα

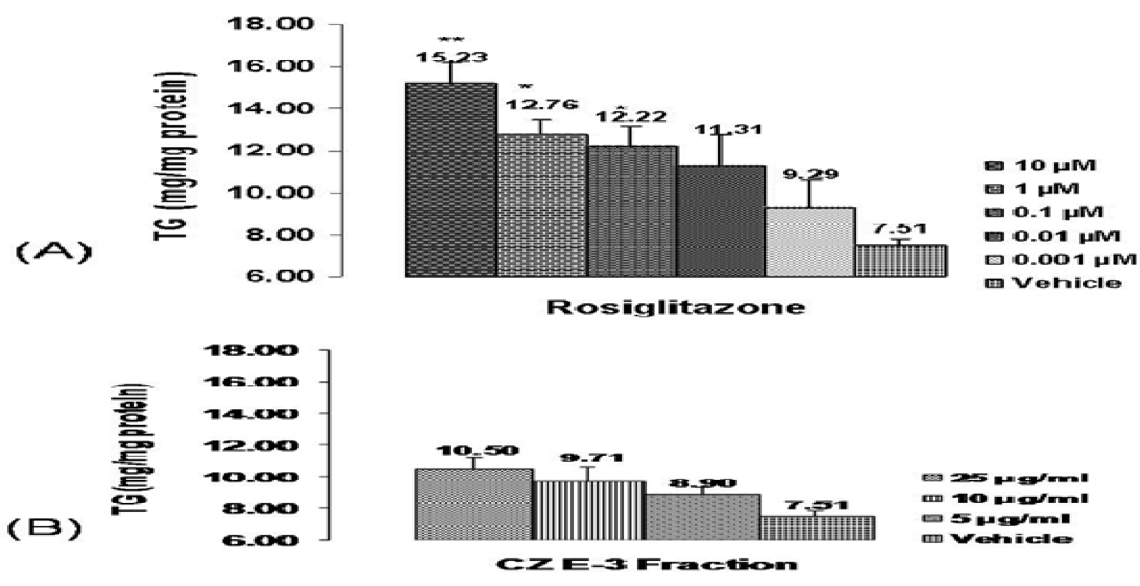

Figure 7. Effect of CZE-3 and rosiglitazone on adipogenesis in 3T3-L1 cells.
Confluent 3T3-L1 cells were incubated for 3 days with induction mixture and PPARγ ligand rosiglitazone (A) or CZE-3 (B) or vehicle as indicated in experimental procedure. Experiments are done in duplicate wells of 24 well plates (in 3 independent experiments). **p (<0.01 vs. Vehicle control; *p (<0.05 vs. Vehicle control). Values are expressed as TG (mg/mg protein) with SD.

muscle and other tissues (Moller, 2001., Berger and Moller, 2002., Tjokroprawiro, 2006.,Miller and Etgen, 2003., Gerhold et al.,2002), Its activation/upregulation by CZE-3 in combination with involvement of beneficial coactivators like PGC1α and SRC1 could probably be one of the factors for observed plasma glucose reduction (Ammon, 2008) and triglyceride reduction in animal models, enhanced insulin sensitivity, improved

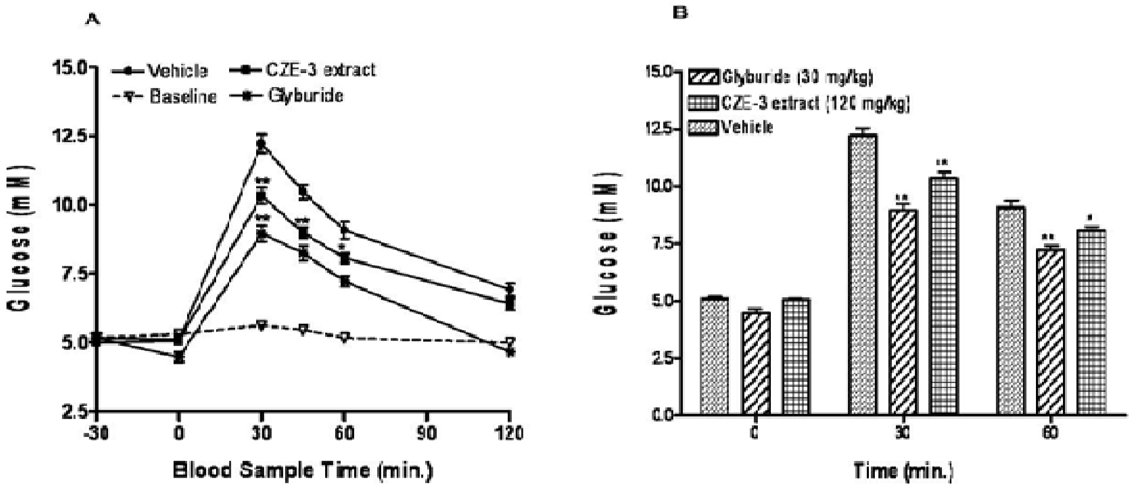

Figure 8. OGTT study in male C57BL/6J mice with CZE3 and Glyburide.
CZE-3 improves glucose tolerance in C57BL/6 male mice. A Oral glucose tolerance test (3g/kg glucose) in C57BL/6 mice that were treated with vehicle (●) CZE extract (120mg/kg ■) or Glyburide (30mg/kg *). Animals were first dosed with compounds/CZE-3 extract or vehicle 30 min (time -30 min) before oral dosing of glucose bolus (3g/kg orally). Blood glucose levels (measured as mg/dl & plotted in mM) were measured at each specified time point till 120 min after the administration of CZE-3 or reference compound or vehicle and then averaged. Values represent the means ± SEM (n = 8). *P < 0.05 **P<0.01 significantly different from values at 30 min 45 min and 60 min between vehicle and test groups by one-way repeated measure ANOVA. B Reduction in blood glucose levels by CZE-3 (120 mg/kg) and glyburide (30 mg/kg) at 30 and 60 min (post dose) as compared to vehicle.

hyperglycemia, reduced serum/hepatic lipid (Kim SH et al., 2010) and diabetic patients. Cinnamon water extract was reported to increase the expression of PPAR gamma/alpha and their target genes such as LPL, CD36, GLUT4, and ACO in 3T3-L1 adipocyte (Sheng X et al.,2008). In our study we observed that CZE-3 had no significant PPARα activity (data not shown) possibly demonstrating the differences in activity of ligands of varying polarity extracted by different solvents. Water extract from *C. cassiae* has been most extensively studied by various labs however; *C. zeylanicum* ethanolic extract is less explored.

Oral glucose tolerance test in C57BL/6 male mice

C57BL/6 male mice which received CZE-3 (120 mg/kg) and glyburide (30 mg/kg) showed improved glucose tolerance (Figure 8). Oral glucose tolerance test (oGTT) in overnight (12 h) fasted mice (n=8 per treatment) demonstrated approximatly 28% glycemic suppression (AUC) and antihyperglycemic property as compared to vehicle (0.2% aqueous carboxy methyl cellulose). Data were analyzed by using one-way repeated measure ANOVA followed by Bartlett's test for equal variance. The **P value was 0.0057 and statistically significant. By bartlett's test variance were significantly different (*P ≤ 0.05) between vehicle glyburide and CZE-3 group.

CZE-3 contains PPARγ agonists which caused ligand induced potentiation of PPARγ activity upon SRC1 and PGC1α expression in cell based reporter assay and thus have potential coactivator involvement ability.

In a 12 week study carried out in C57BIKsj db/db (Kim SH et al., 2010) cinnamon extract treated group showed a significantly lower fasting glucose and postprandial 2 h blood glucose levels as compared to control group. However the studies and data were limited to the mRNA expression and measurement of biochemical parameters affected by PPAR gene activation. PPAR's regulate the genes involved in glucose and lipid metabolism, however certain coactivators and transcriptional repressors are crucial factors with respect to regulation of different genes in various tissues and genes are triggered differentially (depending on the nature of ligand) by the ligands for these receptors. Binding of ligand to a PPAR receptor causes an involvement/recruitment of coactivators and repressors to receptor that brings about a confirmation change of receptor and then triggering different genes of glucose or lipid metabolism. Our studies show that PGC1□ and SRC1 are the major coactivators that could probably be involved *in vivo* in the PPAR mediated gene regulation and overall glycemic control.

Pioglitazone, an approved TZD class of drug is being used to treat T2DM patients is a moderately potent PPARγ agonist and known to involve PGC1α and SRC coactivator for PPARγ mediated gene regulation and glycemic control. It does not show significant wt gain in animal models and has much lesser side effects and much lower risk on heart rate. Other drugs (weak or partial PPARγ and dual activators, SPARMS) that are in different clinical phases have been demonstrated to recruit/involve PGC1α and SRC1 for gene regulation and also show a TG lowering effect along with glucose reduction and HDL elevation effect. These candidate drugs have much lower side effects as compared to rosiglitazone and muraglitazar (dual agonist with more of PPARγ activity). CZE-3 being moderately potent PPARγ agonist is expected to have lower side effects, however, further work on its cardiac safety is warranted. The oral glucose tolerance test (oGTT) in C57BL/6 male mice showed marked improvement of glucose tolerance at different time points in mice post glucose (3g/kg) administration after CZE-3 treatment. CZE-3 was efficacious in the animal model tested and caused approximately 27- 28% glycemic suppression (AUC at 30, 45, 60 min. of blood sample post oral glucose bolus administration) as compared to vehicle control group. This result is very well correlated with extract's PPARγ activity along with coactivator involvement/recruitment capability. PPARγ agonists are known to have anti-hyperglycemic effect and are reported to be insulin sensitizers. It is therefore likely that CZE-3 can ameliorate diabetic hyperglycemia. Glyburide or Glybenclamide (30 mg/kg) improved the glucose tolerance more efficaciously in C57BL/6 male mice under similar time points (post oral glucose dose), but also showed glucose reduction below basal levels. Rosiglitazone (withdrawn) was not included in this study for complete doses as glyburide (more potent, sulphonylurea class of drug) shows robust glucose reduction.

C. zeylanicum bark was extracted sequentially with two solvents acetone for extraction of less polar and medium polar molecules and methanol for extraction of polar molecules. Both the extracts were found to exhibit comparable activities but aqueous ethanolic (1:1) extract which effectively extracted polar molecules was found to be most active. These results indicated that less polar and medium polar molecules such as coumarin, cinnamaldehyde, cinnamic acid, sterols and terpenoids might not be contributing towards the activity and major role is probably played by polar molecules such as glycosides and polysaccharides. This is being confirmed by isolating active principle(s) by bio-assay directed fractionation technique.

CONCLUSION

It is concluded that aqueous ethanolic extract of *Cinnamomum zeylanicum* exhibits part of its glucose lowering effect through of PPARγ activation and this ligand induced effect is potentiated upon overexpression of coactivators (PGC1α and SRC1) *in vitro* in 293/T cells. CZE-3 (120 mg/kg) improve glucose tolerance in C57BL/6 male mice but with lower efficacy (27% glycemic suppression (AUC at 30 min. post oral glucose dose of 3g/kg) as compared to glyburide (30 mg/kg 46% glycemic suppression AUC at 30 min post oral glucose dose) in normal mice. Capability to involve/recruit important coactivator like PGC1α in the cells is beneficial because it is reported to be down-regulated in diabetic patients (T2DM). CZE-3 demonstrates potential for further fractionation and isolation of compounds.

ACKNOWLEDGEMENTS

We thank the President, STES, Pune, India, Head, Department of Chemistry, University of Pune, India and Director, NCL, Pune for providing us necessary support to carry out the study.

REFERENCES

Ammon HP. (2008). Cinnamon in type 2 diabetics Med. Monatsschr. Pharma. 31: 179-183.
Anderson RA (2008). Chromium and polyphenols from cinnamon improve insulin sensitivity Proceedings. Nutri. Society. 67: 48-53.
Anderson RA, et al. (2004). Graves D J Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. Jour. Agri. Food. Chem. 52: 65-70.
Arany Z, et al. (2005). Transcriptional coactivator PGC-1 alpha controls the energy state and contractile function of cardiac muscle. Cell. Metab. 1: 259-271.
Berger J, Moller D E. (2002) The mechanisms of action of PPARs. Ann. Rev. Medicine. 53: 409-435.
Burgermeister E, et al. (2005). A novel partial agonist of PPAR-γ recruits PGC1α prevents Triglyceride accumulation and potentiates insulin signaling in vitro. Mol. Endocrinology. 20: 809-830.
Guan H P et al. (2005). Corepressors selectively control the transcriptional activity of PPARγ in adipocytes. Genes. Dev.19: 453-461.
Gerhold D L, et al. (2002). Gene expression profile of adipocyte differentiation and its regulation by peroxisome proliferator-activated receptor-gamma agonists. Endocrinology. 143: 2106-2118.
Hammond LE, et al. (2005). Mitochondrial glycerol-3-phosphate acyltransferase-1 is essential in liver for the metabolism of excess acyl-CoAs. J. Biol. Chem. 280: 25629-25636.
Hannover BM, et al. (2006). Effects of a cinnamon extract on plasma glucose HbA1c and serum lipids in diabetes mellitus type 2. Eur. Jour. Clin. Invest. 36: 340-344.
Hlebowicz J, et al. (2007). Effect of cinnamon on postprandial blood glucose gastric emptying and satiety in healthy subjects. American. J. Clin. Nutri. 85: 1552-1556.
Jpenberg AI, et al. (1997). Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor heterodimer binding to DNA A functional analysis of the malic enzyme gene PPAR response element. J. Biol. Chem. 272: 20108-20117.
Khan A, et al. (2003). Cinnamon improves glucose and lipids of people with type 2 diabetes. Diabetes Care; 26: 3215-3218.
Kersten S, Béatrice D, Wahli W. (2000). Roles of PPARs in health and disease. Nature. 405: 421-424.
Kim SH, Choung SY (2010). Antihyperglycemic and antihyperlipidemic action of Cinnamomi Cassiae (Cinnamon bark) extract in C57BL/Ks db/db mice. Arch Pharm Res 33(2):325-333.

Kodera Y, et al. (2000). Ligand type-specific interactions of peroxisomes proliferator-activated receptor-γ with transcriptional coactivators. J. Biol Chem. 275: 33201-33204.
Lin J, Handschin C, Spiegelman B M (2005). Metabolic control through the PGC1 family of transcription coactivators. Cell Metab.1: 361-370.
Lagouge M, et al. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha Cell. 127: 1109-1122.
Miller A R, Etgen GJ. (2003). Novel PPAR ligands for type 2 diabetes and metabolic syndrome. Expert. Opin. Invest. drugs.12: 1489-1500.
Moller D E. (2001). New drug targets for type 2 diabetes and the metabolic syndrome. Nature. 414: 821-827.
Qin B, et al. (2003). Cinnamon extract (traditional herb) potentiates in vivo insulin-regulated glucose utilization via enhancing insulin signaling in rats. Diabetes. Res. Clin. Practice. 62: 139-148.
Roffey B, Atwal A, Kubow S. (2006). Cinnamon water extracts increase glucose uptake but inhibit adiponectin secretion in 3T3-L1 adipose cells. Mol. Nutrition. Food. Res. 50: 739-745.
Sheng X, Zhang Y, Gong Z, Huang C, Zang YQ. (2008). Improved Insulin Resistance and Lipid Metabolism by Cinnamon Extract through Activation of Peroxisome Proliferator-Activated Receptors. PPAR Res. 581,348.
Solomon TP, Blannin AK (2007). Effects of short-term cinnamon ingestion on in vivo glucose tolerance. Diabetes. Obes. Metab. 9: 895-901.
Tjokroprawiro A. (2006). New approach in the treatment of T2DM and metabolic syndrome (focus on a novel insulin sensitizer) Acta. Med. Indones. 38:160-166.