CHAPTER- TWO
MATERIALS AND METHODS
2.1 General Reagents and Chemicals

Solvents like, acetone, methanol, ethanol and water were distilled prior to use. DMSO was purchased from Merck Co. U.K. HEPES buffer, sodium chloride, ONPG (β-Galactosidase substrate), sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, β-mercaptoethanol (β-ME), MTT, Dexamethasone, IBMX, porcine insulin, WY-14643 and Glyburide (G2539) were purchased from Sigma-Aldrich. Rosiglitazone was purchased from Biocon Ltd, India. Microbiological reagents like, Luria-bertani broth (LB broth), sterile water, general antibiotics were purchased from Hi-media. 5x Reporter lysis buffer, D-Luciferin, Luciferase assay reagent and DNA marker for gel electrophoresis were purchased from Promega. G418 was purchased from Calbiochem (Merck, U.K), GW9508; a GPR40 agonist was procured from Tocris (Japan). Ham’s F12 medium, TRI reagent and HI-FBS were purchased from Sigma (UK), 96 well black clear bottom plates and V-bottom dilution plates were procured from Corning (Sigma, U.K). 96 well RT-PCR clear plates and 2x RT-PCR reagent containing Eva green dye and Sso Taq was procured from Bio-Rad laboratories, U.S.A. FLUO-4AM Direct kit was purchased from Invitrogen. USA. Glucose measuring strips used for OGTT studies were purchased from Roche, Germany.

Table 2.1. List of reference compounds used in study.

<table>
<thead>
<tr>
<th>Reference Compound</th>
<th>Chemical name &amp; Synonym</th>
<th>M.Wt</th>
<th>Molecular Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW9508, GPR40 &amp; 120 agonist</td>
<td>Synonym: 4-[[3-phenoxyphenyl]methyl]amino]-benzene propanoic acid</td>
<td>347.41</td>
<td><img src="image1" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>WY-14643, PPARα agonist</td>
<td>Pirinixic acid. Synonym: 4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid</td>
<td>323.8</td>
<td><img src="image2" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Rosiglitazone Maleate, PPARγ agonist</td>
<td>Synonym: 5-{{p-2-(Methyl-2-pyridylamino) ethoxy}benzyl}-2,4-thiazolidinedione maleate</td>
<td>473.12</td>
<td><img src="image3" alt="Molecular Structure" /></td>
</tr>
</tbody>
</table>
2.2 Collection and Standardization of Extracts

2.2.1 Plant material

The plant bark from *Cinnamomum zeylanicum* was obtained from local source, Manakarnika Aushadhalaya, Chinchawad, Pune, Maharashtra. The plant material was authenticated and a voucher specimen was submitted at the Botanical Survey of India, Western Circle, Pune (No. SPCIV7).

2.2.2 Methodology of Extraction

*Preparation of extracts*

The barks of *Cinnamomum zeylanicum blume* (Cinnamon) were shade dried and then ground to a coarse powder. Three extracts; acetone, aqueous methanol (30:70%, v/v), and aqueous ethanol (50:50%, v/v; hydro alcoholic extract) were prepared. Rationale for using different solvents for extraction was to get fractions containing ligands/compounds with varied polarity (high, moderate and least polar compounds) and to assess their activity *in vitro*. Extraction process could also help to isolate the major ingredients like cinnamaldehyde, cinnamic acid, coumarins, palmetic acid etc. from extracts.

*Preparation of acetone extract*

Powdered bark 717 g was extracted for 12 h with acetone under intermittent stirring at room temperature (27±2 °C) and the extraction process was repeated three times. The
extracts were combined and concentrated in vacuum at 45 ± 2 °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) and used for in vitro activity analysis.

**Preparation of aqueous methanol extract (30:70, v/v)**
Residual plant material from above extraction process was air-dried in shade to remove residual acetone. This was then further extracted for 12 h with aqueous methanol (30:70, v/v) under intermittent stirring at room temperature (27±2 °C) and the extraction process was repeated three times. The extracts were combined and concentrated in vacuum at 45 ± 10°C in a rotavapour (Buchi, Model R-210 Germany) and dried to yield final extract 57.6 g (8.033%). This was labeled as aqueous methanol extract (CZE-2) and used for in vitro activity analysis.

**Preparation of aqueous ethanol extract (50:50, v/v)**
Powdered bark, 1000 g was extracted for 12 h with ethanol: water (50:50, v/v) under intermittent stirring at room temperature (27±2 °C) and the extraction process was repeated three times. The extracts were combined and concentrated in vacuum as above and dried to yield final extract 93.7 g (9.37%). This was labeled as aqueous ethanol extract (CZE-3) and used for in vitro activity analysis.

### 2.3 Material for Extraction

Thin Layer Chromatography (TLC) as well as preparative thin layer chromatography (prep-TLC) was carried out on Merck precoated plates. Column chromatography was carried using silicic acid as stationary phase from, Spectrochem. All Solvents were procured from Rankem (Ranbaxy laboratories) and distilled prior to use. Synthesis grade Chloroform was procured from Thomas Baker and used for extraction.

**Instruments:** UV data was taken on Varian 50 Bio UV/VIS spectrophotometer. Ultraviolet visualization of TLCs was done in UV scanner from Biotech Instruments Pvt. Ltd. IR data was collected on Perkin Elmer instrument. Mass spectrum analysis of samples using API-QSTAR-PULSAR at 3Kv ionization potential. HPLC grade methanol from Merck Co. was used to record the mass spectrum.
2.3.1 Phytochemical analysis of Cinnamomum zeylanicum extracts using HPLC-PDA

We referred a previously reported HPLC-PDA method, modified it and optimized as per our laboratory conditions (Kentaro Tsuji-Naito 2008). All experiments were performed with Schimadzu LC-20 AD Prominence HPLC system (Schimadzu, Japan) which included a binary pump an auto sampler, a column oven and a polarized diode array detector along with on-line degasser. The separation of aqueous methanol (CZE-2) and aqueous ethanolic (CZE-3) extract of C. zeylanicum was carried on a Syncronis C-18 analytical column (4.6 mm * 150 mm I.D., 5 um particle diameters, supplied by Thermo scientific (USA). A gradient elution with mobile phase consisting of solvent (A) Water: 0.1% v/v acetic acid and solvent (B) Acetonitrile: 0.1% v/v acetic acid was used for separation of compounds with clear peaks. A non-linear gradient elution program was utilized using varied percentages of solvent B from 1% (in the initial time), 1-10% (0-15 min), 10-100% (15-55 min), and 100-1% (55-65 min). The temperature of oven was set to 25°C and flow rate was set at 1.0 ml/min. 10 mg of CZE-2 and CZE-3 were accurately weighed, dissolved in 10 ml of methanol (to make 1 mg/ml) and filtered with 0.45 micron membrane filter for analysis. A 20 µl injection volume was used in all analysis (20 µg/20 µl).

2.4. Cell and Molecular Biology Reagents

Cell cultivation medium like, DMEM; Ham’s F12; Ham’s F12K, RPMI-1640 were procured from Invitrogen, U.S.A. Trypsin-EDTA, FBS, Lipofectamine 2000 and PBS were also from Invitrogen, U.S.A. Restriction enzymes, 1Kb DNA ladder, cDNA synthesis kits, were purchased from MBI fermentsas. U.S.A. Agarose, TEMED, Sodium-dodicyl sulphate (SDS) and beta-acrylamide from procured from Lonza. β-mercaptoethanol, Taq polymerase (recombinant) and other cell culture plastic-wares from Sigma-Corning. Ampicillin, kanamycin, tetracycline and chloramphenicol were purchased from Hi-Media Labs. TG measurement kit was procured from Merck Co. Lipofectamine-2000 and DMEM medium was procured from Invitrogen U.S.A.

2.4.1 Expression vector and development of 3x PPRE reporter vector construct

Expression vector for PPARα, γ and PGC1α were constructed using pCI-neo vector (Promega Inc.). Full-length DPP-IV gene was procured from Origene Inc. U.S.A and
used as template to PCR amplify (using primers flanking EcoRI and Sal-I enzyme as forward and reverse primer) and directionally clone in pFAST HTa vector from Bacto Bac insect kit from Invitrogen, U.S.A. The primers for PCR amplification and complete sequencing of PPARα (NCBI ref seq: NM_005036), PPARγ1 (NCBI ref seq: BT007281 & L40904.2) and PGClα (NM_013261.2) were designed using online oligonucleotide properties calculator software and synthesized from Sigma. PGClα, SRC1 and ShRNA for PGClα (Cat #TR310260; with tube IDs: T1341033, T1341034, T1341035 and T1341036) were procured from Origene Inc. USA. The basic reporter vector (pTranslucent) was procured from Panomics Inc. USA and then 3x PPRE-pGL3 and 3x PPRE-pTRANS 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), PPAR alpha (Matched against NCBI ref seq: NM_005036), DPP-IV gene (NCBI ref: NM_001935) and 3x PPRE sequence in reporter vectors was confirmed by automated DNA sequence analyzer (ABI) from Genome biotech Pvt.Ltd or Chromous biotech Pvt Ltd, India (CRO companies).

2.4.2 Maintenance of cell lines and generation of stable cell lines

**Maintenance of mammalian and rodant cell lines:** CHO cells (ATCC number: CCL-61) were procured from ATCC, U.S.A and cultivated in Hams F12 medium supplemented with 1x penstrep and containing 10% heat-inactivated FBS (Invitrogen, U.S.A). The cells were routinely maintained in T-25 cm$^2$ flask incubated at 37°C in humidified Co2 incubator with 5% CO$_2$. HEK293 and HEK293/T cells (ATCC No: CRL11268) were also procured from ATCC, U.S.A and routinely maintained in Dulbecco’s modified eagle medium (DMEM) containing 1x penstrep and supplemented with 10% heat-inactivated FBS (Invitrogen). Hep-G2 cell line (for isolation of total RNA and preparation of cDNA) was procured from NCCS, Pune, India. All general cell culture disposable culture flasks and pipettes were purchased from Nunc, U.S.A or Corning, U.S.A. 96-well flat bottom clear plate (cell bind surface) was purchased from Corning, U.S.A. 3T3-L1 (mouse pre-adipocyte cell line) cells were procured from ATCC, U.S.A and maintained in DMEM medium containing low glucose and 1.5 g/lit sodium bicarbonate, containing 10% NBCS or
10% FCS supplemented with 1x penstrep at above mentioned CO₂ incubator conditions.

**HIT-T15 (Pancreatic β- cell line):** HIT-T15 cell line (Passage 54, CRL-1777) was procured from ATCC and cultured at 37°C with 5% CO₂ using Ham’s F12 medium supplemented with 2.5% HI-FBS (Sigma), 10% horse-serum (Newzealand source, Lonza. USA), 100 IU/ml penicillin and 0.1 mg/ml streptomycin. The cells were passaged every 3-4 days with the split ratio of 1:3 and maintained in T-25 cm² TC² flask from Sarstedt, Germany.

**Maintenance of SF-9 cells (Insect):** SF-9 cell line was procured from ATCC, U.S.A and routinely maintained in Graces insect medium containing 0.5x penstrep and supplemented with 10% certified FBS (Invitrogen, U.S.A), 1x lactalbumin hydrolysate (Invitrogen, U.S.A) and 1x yeastolate (Invitrogen, U.S.A). These cells do not require trypsization for passaging and are very loosely adhered to flask (15-20% cells in suspension). Cells were passaged using regular 10 ml serological pipette from Nunc, U.S.A.

**hGPR40-CHO stable cell line:** hGPR40-CHO cell line, stably expressing hGPR40 was developed using wild-type CHO cells. Transfection was done using lipofectamine 2000 reagent (Invitrogen, U.S.A). The stable cell clones that were resistant to 600 μg/ml G418 were initially verified by semi-quantitative RT-PCR. The stable cell clones and wild-type CHO cells were routinely cultured in T-25 cm² tissue culture flasks (Sarstedt, Germany) using Ham’s F12 medium (Sigma, UK) supplemented with 10% heat-inactivated FBS (HI-FBS), 1% penstrep. Cultivation medium for stable cells was additionally supplemented with 300 μg/ml of G418.

### 2.4.3 Method for total RNA isolation and cDNA preparation

Total RNA isolation from human liver cell line (Hep-G2) was done using RNeasy mini kit from Qiagen. Hep-G2 cells were cultivated in T-25 cm² flask in complete DMEM containing 10% HI-FBS and 1x penstrep. Cells were trypsinized (Recovery: ~5 x10⁶ cells) from T-25 cm² flask using 0.25% trypsin-EDTA (0.05%) solution and centrifuged in 15 ml tube for 5 min at 180g. Further steps were as mentioned below.

- Cell pellet was resuspended in 5 ml of phosphate-buffer saline (PBS, pH-7.4) and centrifuged again as above.
Supernatant was aspirated off gently and 600 µl of freshly prepared RLT buffer (take 1 ml of RLT and add 10 µl of β-mercaptoethanol) and was added to the pellet. Tube was vortexed for 2 min and then pipetted up and down to mix. To this, 1 volume of freshly prepared 70% ethanol was added and mixed.

The solution was transferred to spin column (700 µl) and centrifuged for 30 sec at 10,000 rpm. The step was repeated 3 times to spin down all the solution.

The spin column was then washed twice with RLW buffer and centrifuged.

RPE solution was then added to give final wash and removal of salts from column membrane and then spun at 10000 rpm for 2 min. The bottom tube (reservoir) was changed and spun again and left for 5-7 min at RT.

RNA was eluted by adding DEPC treated water (60 µl) to the centre of column and kept for 2-3 min, followed by centrifugation at 10000 rpm. The step was repeated again with 25 µl of water to enhance the RNA recovery. The obtained RNA yield was 929 µg/ml.

4.5 µg of total RNA was then used to prepare the cDNA using 2-step cDNA kit from MBI fermentas using oligo dT primers as follows.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (4.5 µg)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>6.0 µl</td>
</tr>
<tr>
<td>Oligo dT&lt;sub&gt;18&lt;/sub&gt; primer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>12.0 µl</td>
</tr>
<tr>
<td>5 x reaction buffer</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>Ribo RNase inhibitor</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>-</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>2.0 µl</td>
</tr>
</tbody>
</table>

The mixture of 19 µl was then incubated at 37°C for 5 min and then 1ul of Revert aid HMMul LV reverse transcriptase enzyme from kit was added to make up the reaction volume to 20.0 µl. This mixture was incubated at RT for 60 min, followed by incubation at 46°C for 15 min. Finally the reaction was terminated by incubation at 65°C for 5 min, chilled on ice and dispensed in aliquots in 0.5 ml tube and stored at -80°C freezer. 2.0 µl was used for quantification in Nanodrop.

2.4.4 Primers used for PCR amplification, cloning and qPCR studies

Human PPARγ1 gene was PCR amplified using primers flanking with Kpn-I and Xba-I. Human PPARα gene was PCR amplified using primers flanking with Nhe-I and Sal-I. Human PGC1α gene was PCR amplified using HepG2 cDNA and primers flanking with Xho-I and Sma-I enzyme. These genes were used for transient
transfection based co-expression studies in HEK293/T cells. Full-length human DPP-
IV gene sequence was PCR amplified using DPP-IV cDNA expression clone
(Origene, U.S.A) and primers flanking with EcoRI and Sal-I. The PCR amplified full-
length gene of DPP-IV was cloned in pFAST HTa vector, characterized and
sequenced in ABI machine; from a CRO company (Genome Biotech, Pvt. Ltd,
Pashan, India). Two clones (# 1 & 2) were sequenced and found to be completely
matching with the NCBI reference sequence (NM_001935) except for a single change
in base that leads to change in 4\textsuperscript{th} amino acid, alanine (Ala-4 to Pro-4). But, since
amino acid 1 to 26 is involved in the membrane anchoring, this change would not
affect the enzyme activity. The clones (DPP-IV /HTa clone # 1 & 2) were then
transformed in DH10 Bac competent cells to generate bacmid DNA (harbouring DPP-
IV gene). Bacmid DNA clones were characterized by restriction digestion analysis
and used for transfection in Sf-9 cells using cellfectin (Invitrogen Inc. U.S.A). DPP-
IV expression conditions were standardized from initial P1 and then P2 virus lot,
using an MOI of 4 and purified using Ni-NTA affinity columns from Qiagen and GE
Healthcare. Real-time qPCR primers for expression studies were designed and
synthesized from Sigma Inc. U.S.A

Table 2.2. Primer sequence used for cloning of various genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'..3')</th>
<th>Reverse Primer (5'..3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPPAR\text{y1}</td>
<td>GGGGTACCATCACATGACCATGGTTGACAC</td>
<td>GCTCTAGAGGTCCTCTACAGTGCCAGC</td>
</tr>
<tr>
<td>hPPAR\text{a}</td>
<td>CCTAGCTAGCATGGTGGACACGGAAAGCCCA</td>
<td>AGCCGACTGATCATGCGTCTCGTAG</td>
</tr>
<tr>
<td>hPGC\text{1a}</td>
<td>CCGCTCGAGATGGCCCGAATGGTGGCAAAAGCA</td>
<td>TCCCCGGGTACCTGCGCAAGCTCTCG TAG</td>
</tr>
<tr>
<td>hDPP-IV</td>
<td>CCGGAATTCCATGAAGAGCGCTGGAAGGTTTC</td>
<td>GGGCCGTCGACCTAAGGGAAGAACATTG</td>
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</tbody>
</table>

Table 2.3. HuSH 29 mer shRNA sequences in construct against PGC\text{1a}

<table>
<thead>
<tr>
<th>Tube ID</th>
<th>Sequence (5'..3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1341033 (#1)</td>
<td>TGCTCCACCAGACAGATGACTCTC</td>
</tr>
<tr>
<td>T1341034 (#2)</td>
<td>GATAGATGAAAGGAATGAGGCGAAACCTTC</td>
</tr>
<tr>
<td>T1341035 (#3)</td>
<td>CTCTTACTGCAAGCAGGACATGCCTAGCT</td>
</tr>
<tr>
<td>T1341036 (#4)</td>
<td>CAGGAGGAGAAGAGCCGCTCTCTGAA</td>
</tr>
</tbody>
</table>

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

\textit{Sense strand}

5'…CTAGCCCAACTCAGGTCAAAGGGTCATCCAACAGGTAAGGTCGAGGTCGGCCCCAA
ACTAGGTCAAAGGTCAAAA…3'
2.5 **In vitro Assays**

2.5.1 **Stock Solutions**

**Phosphate Buffer Saline (PBS) 10X**: 80.0g NaCl, 2.0 gm KCl, 11.5g Na2HPO4 and 2.0g KH2PO4. All the components were weighed accordingly and added serially in 1000 ml distilled water (D/W). pH of the solution was adjusted to 7.4 before autoclaving. The 10X buffer solution was diluted with autoclaved Milli-Q water to make the 1x working concentration. This buffer was used for general assays.

**1x Phosphate Buffer Saline (PBS) for cell culture**: Calcium and magnesium free PBS was procured from Invitrogen, Inc. USA. This was used for washing cell monolayer and during maintenance and trypsinization of mammalian/insect cells.

**Krebs Ringer’s Bicarbonate-HEPES buffer, pH 7.4 (KRBH)**: 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.1 mM MgCl2, 10 mM HEPES and 2.5 mM NaHCO3 and 0.2% bovine serum albumin (BSA). All the components were weighed accordingly and added serially in appropriate volume of Milli-Q water except CaCl2. After adjusting pH to 7.4, CaCl2 solution was added drop by drop with constant stirring. If precipitation occurred, the buffer solution was bubbled with CO2. Solution (100 ml) was sterilized with 0.22 micron syringe filter and stored at 4°C. The buffer was pre-warmed at 37°C prior to initiation of insulin secretion assays. The method of same is described in below section, 2.6.4.

**3 (4,5 dimethylethiazolyl-2)-2, 5-diphenyle tetrazolium (MTT) Stock Solution**: 5 mg MTT dissolved in 1 ml PBS. Solution was filter sterilized using 0.22 μm filter (Milipore). Solution was wrapped in aluminium foil and stored at 4°C.

**RNaseA Stock Solution**: 20 mg of RnaseA (Sigma-Aldrich) was dissolved in 1 ml of Nuclease Free water and stored at -20°C.

**Acrylamide/Bis (30%)**: 29.2g acrylamide and 0.8g N'N'-bis-methylene-acrylamide were dissolved in 75 ml Milli-Q water and final volume was made up to 100 ml. The solution was filtered using whatman paper size-2 and stored at 4°C in the dark.
10% (w/v) SDS: 10 g of SDS was dissolved in 90 ml Milli-Q water with gentle stirring and volume was made up to 100 ml in a measuring cylinder, stored at RT.

**Resolving Gel Buffer- 1.5 M Tris-HCl, pH 8.8:** 18.15 g Tris base was dissolved in 80 ml Milli-Q water. The pH was adjusted to pH 8.8 using 6 N HCl and the final volume was made up to 100 ml with Milli-Q water and stored at 4°C.

**Stacking Gel Buffer - 0.5 M Tris-HCl, pH 6.8:** 6 g of Tris base was dissolved in 70 ml Milli-Q water. The pH was adjusted to pH 6.8 with 6N HCl and the final volume was made up to 100 ml with Milli-Q water and stored at 4°C.

10% APS: This solution was prepared fresh every time before use by dissolving 100 mg ammonium persulfate in 1ml of Milli-Q water.

**Sample Buffer (SDS Reducing Buffer):** Mixed 3.55 ml Milli-Q water, 1.25 ml of 0.5 M Tris-HCl, pH 6.8, 2.5 ml of glycerol, 2.0 ml of 10% SDS, 0.2 ml of 0.5%(w/v) bromophenol blue to a total volume of 9.5 ml. Just prior to use, 50μl β-mercaptoethanol was added to 950 μl of sample buffer.

10x Electrode (Running) Buffer, pH 8.3: Added 30.3g of Tris base, 144.0g glycine and 10.0g SDS to 700 ml Milli-Q water and stirred on stirrer. Final volume was made up to 1,000 ml Milli-Q water without adjusting the pH. For routine electrophoresis work, 100 ml of 10x stock was diluted with 900 ml of water.

### 2.5.2 AlamarBlue dye based fluorometric assay for evaluating cytotoxicity:

HEK293 cells were seeded in clear bottom, black 96-well microtitre cell culture plate at a seeding density of 15000 c/well in 100 μl of culture medium (DMEM complete medium supplemented with 10% FBS). Plate was incubated overnight at 37°C in humidified CO2 incubator set to 5% CO2. Next day, plate was removed from incubator and 100 μl of 2x compound solution prepared in DMEM complete medium was added to test wells. Control wells received 100 μl of complete DMEM medium containing 0.5% DMSO (v/v). Thus, the final DMSO in test and control wells was 0.5% (v/v). The plate was again incubated for 18 h in CO2 incubator set to 37°C and 5% CO2. Post incubation, the plate was removed from incubator and medium containing compound was aspirated off using multi-channel pipette and 100 μl/well of AlamarBlue (diluted 10 times in HBSS) was added. The plates were incubated for additional 1 h at 37°C and the fluorescence was measured at 560 nm (excitation wavelength) 590 nm as emission wavelength and in a SpectraMax M5 reader.
(Molecular Devices, U.S.A). AlamarBlue assay is designed to measure quantitatively the proliferation of various human and animal cell lines. The bioassay is routinely used by researchers to establish relative cytotoxicity of agents within various chemical classes. The assay is simple to perform since the dye is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays. The assay incorporates a fluorometric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth.

2.5.3 In vitro reporter assay for assessment of PPAR activation

Transient co-transfection based luciferase reporter assays for evaluation of PPARγ or α activation were developed in HEK293/T cells. Coactivator involvement assays with over-expression of PGC1α or SRC1 and gene silencing experiments were carried out using lipofectamine-2000 in these cells. HuSH 29 mer shRNA construct against PGC1α with tube ID: TI341034 worked well (~80% knockdown) for silencing of PGC1α in transient transfection assays.

Transient co-transfection reporter assay for PPAR

HEK 293/T cells (ATCC No: CRL11268) were routinely maintained in DMEM containing 1x penstrep and 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°C and 5% CO₂, 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. 2000 ng) β-galactosidase plasmid (15% of total DNA, i.e. 600 ng) and empty vector DNA (to make up total DNA) using Lipofectamine-2000 reagent as per kit 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 CZE extracts as compared to reference PPARγ (rosiglitazone) and PPARα (WY-14643) compounds used in the experiments. 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 M5 reader from Molecular Devices). β-gal expression plasmid was used in the experiment for normalization of transfection efficiency. All the experiments were performed at least twice in triplicate wells. Alamar blue dye based fluorometric cytotoxicity assay for extracts at various concentrations were done separately.

**Trypan blue dye exclusion for viable cell count:** Cell viability was assessed using trypan blue dye test using 0.25% (w/v) trypan blue solution containing 0.05% EDTA (Invitrogen, Inc. USA). Blue stained cells were scored as non-viable cells, while unstained cells were counted as viable. Results were expressed as % viability.

**Table 2.4. Optimization of transfection conditions for PPARγ Co-transfection assays**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Concentration of DNA (ng): Test</th>
<th>Concentration of DNA (ng): Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>3x-PPRE Reporter vector</td>
<td>2000</td>
<td>2000</td>
</tr>
<tr>
<td>β-Gal vector</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>Empty pCI-neo vector</td>
<td>1200</td>
<td>1400</td>
</tr>
</tbody>
</table>

**Table 2.5. Optimization of transfection conditions for PPARα Co-transfection assays**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Concentration of DNA (ng): Test</th>
<th>Concentration of DNA (ng): Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>3x-PPRE Reporter vector</td>
<td>2000</td>
<td>2000</td>
</tr>
<tr>
<td>β-Gal vector</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>Empty pCI-neo vector</td>
<td>1200</td>
<td>1400</td>
</tr>
</tbody>
</table>
2.5.4 **Development of coactivator reporter assay in HEK293/T cells**

HEK293/T cells transfected with PPARγ expression plasmid along with either PGC1α or SRC1 coactivator plasmid and reporter vector to evaluate the effect of CZE on PPARγ mediated transactivation cells over-expressing these key coactivators. A 29 mer shRNA against PGC1α was used to knockdown PGC1α expression in transient transfection based reporter assays to check the effect of CZE-3 mediated transactivation potentiation. All four PGC1α shRNA constructs were tested for its gene silencing effect in cells over expressing PGC1α. Out of four constructs, only one construct (ID#2, TI341034) with sequence as described in Table 2.3, showed specific PGC1α knockdown in HEK 293/T cells transfected with PGC1α along with its shRNA construct and further used in the experiments. The shRNA constructs were used in 10 time’s higher molar ratio. All transfections were carried out using Lipofectamine-2000 as per manufacturer’s instruction.

2.5.5 **Adipogenesis Assay**

**Method for 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 or extract) and plates were incubated in CO₂ 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 additional 3 days. On day-8, the plates were observed under inverted microscope for visualizing the adipogenesis effect. Plates were 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 analyzed for TG content (using TG estimation kit- Ecoline or Merck) after brief centrifugation at 3000 rpm for 10 min at 4°C.

2.5.6 **Measurement of glucose-stimulated insulin secretion in HIT-T15 cells**

HIT-T15 cells were propagated in T-25 flask using Hams’F12 K medium containing 10% horse-serum (HS) and 2.5% heat-inactivated fetal bovine serum (HI-FBS). Cells
(20000 to 25000 cells/well) were seeded in 96-well clear bottom cell-bind surface plate (Corning) using Hams F12 K medium containing 10% horse-serum and 2.5% HI-FBS and incubated in an incubator set at 37°C and 5% CO₂ atmosphere for 18-20 h. Twenty four hours prior to assay, cells were serum starved for 18 h followed by 3 washes with Krebs ringer-HEPES buffer (KRBH, pH 7.4) containing 0.2% BSA and incubated for 1 h without glucose. After 1 h, buffer was aspirated, followed by addition of reference/extract in respective wells prepared in KRBH buffer (with 0.2% BSA) with 0, 3.3 or 15 mmol/l glucose for 1 h at 37 °C. Supernatant was used for insulin estimation by HTRF kit (Cisbio, Germany) as per manufacturer’s instruction.

2.5.7 Fluorometric imaging plate reader based intracellular calcium assay in hGPR40-CHO cells

The functional cell-based FLIPR assay using hGPR40-CHO stable cells was performed to assess the cinnamon extract’s potential for GPR40 receptor activation. hGPR40-CHO cells stably-expressing the hGPR40R were routinely maintained in T-75 cm² flask supplemented with 400 µg/ml of G418. Two days prior to assay, 10,000 cells/well were seeded in black-wall clear-bottom 96-well plates pre-coated with Poly-D-Lysine and washed before use. The cells were serum-starved for 18-20 h, prior to performing assay. The cells were then incubated with 200 µl/well of fluorometric imaging plate reader (FLIPR) assay buffer solution- A containing 0.05% BSA, 2.5 mmol/l probenecid, and 8 µmol/l Fluo-4-AM at 37°C for 1 h and additional 1 h at RT. The entire content of Fluo-4AM bottle was reconstituted in 20 ml assay buffer solution A as per manufacturer’s instruction in manual. Fluo-4 direct calcium assay kit (Invitrogen. USA). Probenecid vial was reconstituted as 250 mM stock using DMSO and 200 µl of this is mixed in the buffer solution A before assay. Reference compound or extracts were dissolved in DMSO as 1000x stock and diluted to desired concentrations in assay buffer solution A and added to cells using FLIPR as 5x solution (50 µl/well) making final desired concentration in well as 1x. Fluorescence was measured for 330 sec in FLIPR tetra machine (Molecular Devices Inc., USA).

Composition of FLIPR assay buffer solution A for 1 Lit:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>126 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>4 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>20 mM</td>
</tr>
</tbody>
</table>

BSA was added to make up 0.05% v/v and adjusted to pH-7.4.
2.6  *In vitro* Enzyme Inhibition Studies

### 2.6.1 Standardization of DPP-IV expression conditions in SF-9 cells

SF-9 cells (2 x 10^6 c/well) were seeded in 6-well plates. Cells were cultured using Grace's insect cell media supplemented with 10% certified FBS, lactalbumin hydrolysate (making final concentration to 1x using 100x bottle from invitrogen) and 1x, Yeastolate, without sodium bicarbonate and adjusted to pH 6.3. Twenty-four hours later the cells were transfected with Bacmid DNA clone #4 (harbouring full-length DPP-IV) using celfectamin transfection reagent (Invitrogen) for 6 h. Similarly another well was transfecteded using mock DNA as negative control. Ninety-six hours post transfection, cells were observed under inverted microscope for visible signs of cell detachment and bulginess or rounding (symptoms of baculovirus infection, post 72 h). P1 virus was then harvested (cell media supernatant) and filtered using 0.22 micron syringe filter. This P1 virus was then used to infect fresh set of SF-9 cells for further baculovirus amplification (harbouring DPP-IV gene) and medium was harvested after 72 h of infection. P2 virus was again infected in SF-9 cells seeded in 6-well plate for quantification using plaque titre assay as per the kit instructions using 1x- agarose overlay. Plaques were counted in different dilutions of virus infection (serial dilutions used from 10^1 till 10^9). An MOI of 4 was found to be optimal for maximal expression of DPP-IV protein. For expression of large amount of DPP-IV protein, T-175 cm2 flasks were used and infected with P2 virus with an MOI of 4. The cells were harvested using pipette (narrow bore 10 ml serological pipette from Nunc) and lysed using lysis buffer (PBS, pH 7.4 with 0.02% triton). Purification was done using Ni-NTA columns from either Qiagen or GE Healthcare. Tetrameric DPP-IV (~84 KDa) was purified under native conditions and assessed for purity using SDS-PAGE. Purified DPP-IV was and validated using DPP-IV reference compounds; MK-403 and vildagliptin (LAF-237) for enzyme inhibition studies.

### 2.6.2 Standardization of DPP-IV enzyme inhibition assay

Purified DPP-IV protein (His-tag-DPP-IV) was evaluated for purity using 10% SDS-PAGE and approximately 84 KDa band was visible on gel. The protein was further characterized using different enzyme concentration to determine the optimal enzyme concentration to be used per well in a 96-well assay format. Later, keeping fixed enzyme concentration we varied the substrate concentration (Gly-pro-amino-methyl...
coumarin) to determine the Km for substrate. The Km was found to be 46 μM for Gly-pro-AMC in our assay and matched close to reported one in literature. The optimum conditions were standardized as mentioned below. The readings were taken in PolarStar Galaxy multiplate reader at an Excitation: 360 nm and Emission: 460 nm. MK403 (Sitagliptin) and LAF-237 were used as reference. 96-well black plates were used and the settings of top read was set in the instrument with fluorescence mode and above Ex and Em settings.

| Buffer: 25 mM Tris-HCl buffer, pH 7.4 with 0.01% BSA, 140 mM NaCl and 10 mM KCl |
| Pre- Incubation with compound/Extract: 20 min at 30 °C |
| Incubation after Substrate addition (50 μM Gly-pro-AMC): 30 min at 30 °C |
| DPP-IV Concentration/well (96-well plate): 10 ng/well/45 μl |
| Final DMSO concentration /well: 0.2% (v/v) |

Flow chart of addition sequence:

```
Enzyme= 45 μl
  ↓
Compound= 45 μl
  ↓
S= 10 μl
  ↓
Measure fluorescence (liberated AMC)
  In Fluorometer
```

2.6.3 Standardization of PTP1B enzyme inhibition assay

Purified PTP1B enzyme (catalytic domain) expressed in E.coli was procured from R&D systems and used for standardization of in-house assay. The enzyme was characterized using different concentrations to determine the optimal enzyme concentration to be used per well in a 96-well assay format. Later, keeping fixed enzyme concentration we varied the substrate concentration (Gly-pro-amino-methyl coumarin) to determine the Km for substrate. The Km was found to be 23 to 26 μM for DiFMUP in different experiments and matched close to reported one in literature. The optimum enzyme inhibition conditions were standardized as mentioned below. The readings were taken in SpectraMax M5 multiplate reader at an Excitation: 358 nm and Emission: 458 nm. Korea 3C was used as reference inhibitor in experiments.
Buffer Used: 50 mM HEPES buffer, pH 7.2 with, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.01% Triton x-100
Pre- Incubation: 15 min at 30 °C
Incubation after Substrate addition (25 μM): 30 min at 30 °C
PTP1B Concentration: 15 ng/well/70 μl
Final DMSO concentration/well: (1%, v/v)
Read Mode: Fluorescence (Top read, Ex:358; Em:458)

Following formulae was used for calculation:

**Calculation:**

\[
\% \text{ PTP1B inhibition} = 100 - \frac{\text{RFU of test} - \text{RFU of blank}}{\text{RFU of control} - \text{RFU of blank}} \times 100
\]

**Flow chart of addition sequence:**

1. Enzyme = 70 μl
2. Compound = 20 μl
3. S = 10 μl
4. Measure the fluorescence of liberated DiFMU in Fluorometer (SpectraMax M5)

### 2.7 Gene Expression Studies

#### 2.7.1 Quantitative real-time PCR in HIT-T15 cells

HIT-T15 cells (100000 c/well) were seeded in 24 well plates in Hams F12 medium containing 10% HS and 2.5% HI-FBS. Forty-eight hours later the cells were washed with KRBH buffer (pH, 7.4) and then incubated in KRBH buffer for 1 h in presence of reference compound or extract or vehicle (0.1% DMSO). Total RNA was extracted from the cells after 1 h of treatment using the TRI reagent (Sigma-Aldrich Inc. USA) according to the manufacturer’s instruction, and 3 μg of total RNA was reverse transcribed into cDNA using first strand cDNA synthesis kit (MBI Fermentas, USA). Real-time quantitative PCR (qPCR) was carried out using the CFX96 PCR machine (Bio-Rad Laboratories, USA). PCR was performed in a total of 15 μl reaction volume
containing 7.5 µl of 2x Eva green buffer, containing 200 µM deoxynucleotide triphosphate mixture (dNTP), 500 nM primer, 0.05 U/µl SsoTaq polymerase, 2x EVA green. The thermal profile consisted of initial denaturation of 3 min at 94 °C, followed by 40 cycles of 15 sec at 94 °C and 30 sec at 55 °C, for hamster PDX-1 transcription factor, 40 cycles of 15 sec at 94 °C and 30 sec at 52 °C, for hamster insulin and GPR40 gene; 50 cycles of 15 sec at 94 °C and 30 sec at 57 °C, for hamster GLUT2 and Glucokinase gene. The annealing and extension steps were combined in this protocol as per kit instructions. All the results were obtained in at least two independent experiments with PCR in duplicate wells. The mRNA levels of all the genes were normalized using beta-actin as internal house-keeping gene.

Table 2.6. Real-time qPCR primer sequence used for gene expression studies in HIT-T15 cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster Insulin</td>
<td>AGAAGCCATCAGCAAGCAGG</td>
<td>AGAGT6GCTCCACAAGGT6G</td>
<td>173</td>
</tr>
<tr>
<td>Hamster GK</td>
<td>AGGTAGAGGAGATCCTGGCA</td>
<td>ACTTCTGAGCCTTTCTGGGT</td>
<td>192</td>
</tr>
<tr>
<td>Hamster GLUT2</td>
<td>TCTCCTCTCCAGTACATCG</td>
<td>TCTCTCTTCCGGGAATCTCG</td>
<td>183</td>
</tr>
<tr>
<td>Hamster GPR40</td>
<td>TACACTTGCTGCTGCTGGGG</td>
<td>CCTCCTTGAGTCCTTGCTGTA</td>
<td>195</td>
</tr>
<tr>
<td>Hamster PDX-1</td>
<td>CAGCTGCCTTTCCATGGATGA</td>
<td>TCCACTTCATGCGGCCGTTT</td>
<td>227</td>
</tr>
</tbody>
</table>

2.8 In vivo Studies

2.8.1 Procedure for oral glucose tolerance test in male C57BL/6 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 at desired doses via oral gavage. Glucose bolus (3g/kg orally) was administered to all groups except the baseline control (n=8) that received water alone.
2.8.2 Procedure for oral glucose tolerance test in male BKS.Cg-Dock7 db/db mice

Male BKS.Cg-Dock7 <m>+/+Lepr<db> db/db mice (12 week old, 35-45 gm body weight) were used for the study. For studies, db/db mice (11 week old) were obtained from the Research Animal Facility (RAF) at Lupin Ltd. (Lupin Research Park), Pune, and regrouped and acclimatized for at least 1 week at the Experimental animal facility at Lupin Ltd. (Research park), Pune. All experimental procedures were conducted in strict accordance with care and use of laboratory animals as per approved Institutional animal ethics committee (IAEC) protocol no. IAEC/PHR/390. The mice were housed in polypropylene cages with paddy husk as bedding (not more than 8 per cage) in a controlled environment (temperature 23 ± 2 °C; humidity 30-70%; light 300 lux at floor level; noise level 50 decibel) with regular 12 h light and 12 h dark cycle. All mice were given free access to standard pellet laboratory animal diet (Nutrilab, Bangalore) and water before the study. For the oral glucose tolerance test (OGTT) overnight (10 h) fasted db/db mice (n=8) were orally dosed with either vehicle (0.5% aqueous carboxy methyl cellulose containing 0.05% tween-80) or test compounds at desired doses via oral gavage. Glucose bolus (2g/kg, orally) was administered to all groups including vehicle control (n=8).

Experimental Design

12 week old db/db mice fasted for 10 h, having body weight range of 35-45 gm

A) Control group (n=8): Vehicle (0.5% CMC containing 0.05% Tween-80)
B) Test compound (n=8): CZE-3, 150 mg/kg dissolved in above vehicle
C) Reference (n=8): Sitagliptin, 10 mg/kg dissolved in above vehicle

Test compound/reference or vehicle was administered 30 min prior to glucose loading (2g/kg). Blood was collected at 0, 20, 40, 60 and 120 min using glass capillary tubes by occular vein (in eye) in 0.5 ml tubes. The blood was centrifuged at 5000 rpm, 4°C and plasma collected for glucose estimation in automated analyzer (Randox. U.S.A).

2.8.3 Statistical analysis

Data were analyzed by using one-way analysis of variance (ANOVA) followed by Bartlett’s test for equal variance followed by Tukey’s test.

The value of **P ≤ 0.01 and *P ≤ 0.05 was used as criterion of statistical significance.