Materials and Methods
Reagents and chemicals

Ketamine hydrochloride was purchased from Neon laboratories Ltd. (Mumbai, India) and xylazine was obtained from Indian Immunologicals Ltd. (Hyderabad, India). Chloroform, isopropanol, Tris, glycine, EDTA, sodium bicarbonate, BSA, TRI reagent were purchased from Sigma-Aldrich (St. Louis, USA). Oligonucleotide primers for BMP-2, 4, 5, 6, 7, 14, IGF-I, IGF-II, IGF-IR, IGF-IIR, IGFBP-3, IGFBP-5, VEGF and β-actin were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). iScript cDNA synthesis kit (Bio-Rad, USA) and quantitative real-time RT-PCR reaction KAPA SYBR® FAST PCR master mix kit (Kapa Biosystems, USA) were purchased. NP-40, sodium deoxycholate, sodium dodecyl sulphate (SDS), Tris base, acrylamide, Bis-acrylamide, TEMED, glycerol, β-mercaptoethanol, glycine, NaCl, bromophenol blue, Tween-20, ammonium per sulphate (APS) and protein marker were purchased from BioRad, UK. Polyvinyl difluoride (PVDF) membrane were purchased from Millipore (Billerica, MA, USA). Primary and secondary antibodies for BMP-2, BMP-4, IGF-IR, IGFBP-3, IGFBP-5, VEGF and α-tubulin (Santa Cruz Biotechnology, California, USA), Super signal Femto enhanced chemiluminescence substrate (ECL reagent) (Pierce Biotechnology Inc., Rockford, USA) were also used in the present study. All other chemicals were of molecular biology grade and purchased from Sisco Research Laboratories (Mumbai, India).

Plant material and extraction

The stem of C. quadrangularis was collected from Vellore District of Tamil Nadu, India, identified and authenticated by a botanist (MUCHS-H104). A voucher specimen was deposited in the Centre for Advanced Studies in Botany, University of Madras, Guindy campus, Chennai 600 025. The fleshy stems (2.5 kg) were washed,
chopped into small pieces, air-dried and crushed into powder. The stem powder was exhaustively extracted with 95% ethanol using a Soxhlet apparatus and the extract yield of 225 g was obtained.

**Animals**

Healthy adult female Sprague-Dawley rat, weighing about 225-250 g were used. Rats were maintained as per national guidelines and protocols, approved by our Institutional Animal Ethical Committee (IAEC No. 02/012/2011). The animals were housed in clean polypropylene cages, maintained in air-conditioned animal house with constant photoperiod of 12 h light/dark cycle. The rats were fed with pellet diet (Gold Mohur Ltd, Mumbai, India) and drinking water *ad libitum.*

**Fracture induction procedure**

Unilateral fractures were produced in the left femur by anesthetizing the female Sprague-Dawley rats with ketamine 80 mg/kg/bw/ip and xylazine 10 mg/kg/bw/im and using a scaled-down version apparatus originally described by (Bonnarens and Einhorn, 1984). A midline anterior knee incision was made and a 23G-gauge needle (1.63 mm in diameter) was inserted into the left femur. Distally, the pin was cut with the cortex of the patella so as not to interfere with knee motion and after the intramedullary pinning procedure, the surgical wound was closed by the surface and the rat were kept in their cages. After the wounds were closed, X-ray radiograph was taken to confirm the proper pin placement.

The mid-diaphysis of the pinned femur was fractured by a force generated by dropping a 500 g weight from a height of 35 cm to produce transverse fracture on the left femur and the fracture induction was confirmed by a dental X-ray machine.
(Dental Trophy 70 CCX, Iri Paris, France: exposure 0.10 s, 70 Kv, 80 mA). When the fracture was not transverse, the rat was removed from the experiment. After awakening from the anesthesia, the rats were permitted for unrestricted full weight-bearing activity. Once the closed femoral fractures were made, the rats were divided into 2 groups, with 18 animals in each group:

**Group-1:** Fracture control (Vehicle)

**Group-2:** Fracture + *C. quadrangularis* (750 mg/kg/bw/day), orally

Oral administration of Vehicle or *C. quadrangularis* was given daily for 21 days from the induction of fracture. Control group received acacia gum in distilled water as vehicle; while other group received *C. quadrangularis* extract in vehicle. Six animals from each group were killed on post fracture (PF) day 7, 14 and 21.

**Fracture callus sample collection**

Bones were removed by disarticulation of the hind limb through the knee joint. After removal of skin, superficial layers of soft tissues were removed. After removal of the intramedullary pin, the callus tissue formed in the fractured femur was harvested at the PF day 7, 14 and 21.

**Callus homogenate preparation**

Callus was snap frozen with liquid nitrogen and powdered by mortar and pestle and homogenized with 1 ml of Trizol (Total RNA isolation reagent) for the RNA isolation and real-time RT-PCR.
Isolation of total RNA

Total RNA was isolated from the callus harvested at PF day 7, 14, and 21 using total RNA isolation reagent (TRI, Sigma) kit as described earlier (Chomczynski and Sacchi, 1987).

Principle

Single step guanidium acid phenol method emphasizes the ability of guanidium isothiocyanate (GITC) to lyse callus tissues, denature protein and inactivate intracellular ribonuclease rapidly. The presence of β-mercaptoethanol in the mixture enhances the solubilization properties of the GITC extraction buffer. Acid phenol extraction (pH < 5.0) selectively retains cellular DNA in the organic phase and aids in the extraction of proteins and lipids. The addition of chloroform further removes lipids and establishes two distinct phases containing the DNA, proteins and lipids and an aqueous phase containing the RNA. The aqueous phase was separated and the RNA was precipitated by adding an equal volume of isopropanol.

Reagents

- Trizol (phenol, guanidiumisothiocyanate, urea, detergents, buffering agents and stabilizers).
- Chloroform (molecular biology grade).
- Isopropanol (molecular biology grade).
- 75% ethanol - 7.5 ml of absolute ethanol was mixed with 2.5 ml of autoclaved deionized water.
Procedure

The callus tissue homogenized in 1ml of Trizol was kept at 4°C for 5 min to permit complete dissociation of nucleoprotein complexes. To this, 0.2 ml of chloroform was added, shaken vigorously for 15 sec and placed on ice at 4°C for 5 min. The lysate was then centrifuged at 12,000 rpm for 15 min at 4°C, which yielded lower organic phase containing DNA and proteins and upper aqueous phase containing RNA. The volume of the aqueous phase was about 40-50% of the total volume of the lysate.

The aqueous phase was carefully transferred to a fresh microcentrifuge tube without disturbing the interphase. Equal volumes of isopropanol were added, mixed and kept at 4°C for 10 min. It was again centrifuged at 10,000 rpm for 10 min at 4°C to precipitate the RNA.

The supernatant was removed and the pellet was washed twice with 75% ethanol and air dried. The RNA pellet was then dissolved in 50 µl of sterile water and placed in a water bath at 60°C for 10 min to ensure maximum solubility of RNA. The RNA sample was subsequently mixed gently and stored at -85°C.

Quantification of RNA

The diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260 nm. An absorbance of 1 OD is equivalent to an RNA concentration of 40 µg/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260 nm with dilution factor and 40 µg. The purity of RNA preparations
was assessed by determining the ratio of absorbance of sample at 260 nm and 280 nm.
The purity of RNA obtained was 1.7-1.8.

**Reverse transcription (cDNA conversion)**

Extracted total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio Rad, USA).

**Kit components**

5 X iScript reaction kit contains 25 μM MgCl₂, 2.5 μM dNTP mix, oligo-dT, RNase inhibitor and RNAse free water.

**Procedure**

(i) iScript cDNA synthesis kit components and extracted total RNA were thawed and placed on ice.

(ii) 1 μg of total RNA was used for 20 μl reaction.

**Preparation of RT reaction mixture**

The following components were added into an autoclaved 1.5 ml vial.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X iScript reaction mix</td>
<td>4.00 μl</td>
</tr>
<tr>
<td>iScript RT enzyme</td>
<td>1.00 μl</td>
</tr>
<tr>
<td>Template</td>
<td>1.0 μg in 15 μl of RNAse free water</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.00 μl</td>
</tr>
</tbody>
</table>
(iii) The reaction mixture was placed in a thermal cycler, which was programmed as follows.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>5 min</td>
</tr>
<tr>
<td>42°C</td>
<td>30 min</td>
</tr>
<tr>
<td>85°C</td>
<td>5 min</td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

(iv) Finally, the cDNA was stored at -20°C until used for real-time PCR.

**Real-time PCR reaction**

mRNA expressions of BMP-2, 4, 5, 6, 7, 14, IGF-I, IGF-II, IGF-IR, IGF-IIR, IGFBP-3, IGFBP-5 and VEGF normalized with β-actin were studied by the method of comparative real-time PCR.

**Principle**

Real time PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA (Rappolee *et al*., 1988) and permits the analysis of different samples from as little as one cell in the same experiment. It is the most sensitive and the most flexible of the quantification methods (Wang and Brown, 1999) and can be used to compare the levels of mRNAs in different sample populations, to characterize patterns of mRNA expression (Bustin, 2000). RT-PCR principle is based on the properties of the reaction kinetics. Real-Time PCR read out is given as the number of PCR cycles (cycle threshold - Ct) necessary to achieve the level of fluorescence. For
this study, the Ct was fixed in the exponential phase of the PCR. During the initial PCR cycles, the fluorescence signal emitted by SYBR Green 1 bound to PCR product is usually too weak to register above the background signal. Indeed, a difference could not be defined until after about 15 PCR cycles. During the exponential phase of the PCR, the fluorescence doubles at each cycle. After 35 cycles, the intensity of the fluorescent signal usually begins to plateau, indicating that the PCR has reached a saturation status. Specificity and RT-PCR product verification can be achieved by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon (Ririe et al. 1997). This is done by slowly increasing the temperature above the Tm of the amplicon and measuring the fluorescence. As the Tm of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product. The ct value is inversely proportional to the initial concentration of the template. The relative concentration of one target with respect to another is reflected in the difference in cycle number necessary to achieve the same level of fluorescence.

As RNA cannot serve as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. Separation of the RT-PCR steps has the advantage of generating a stable cDNA pool that can be stored virtually indefinitely.

\( (Ct \; - \; \text{means threshold cycle which reflects the cycle number at which the fluorescence generated within a reaction, crosses the threshold. It is inversely correlated to the logarithm of the initial copy number).} \)
**Primers for real-time PCR**

The following primers (forward and reverse) were selected based on available literature given in parenthesis.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Gene name &amp; accession No.</th>
<th>Primers (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BMP-2 NM_017178.1</td>
<td>Sense: ACACCGTGCTCAGCTTCCATCA Antisense: AGCTGGCTGTGGGAGGCTTTAT</td>
<td>228</td>
</tr>
<tr>
<td>2</td>
<td>BMP-4 NM_012827.2</td>
<td>Sense: TGCCCGTGCCATTCCGCTCTA Antisense: AGGTGGTGCCCGGAGTGGAAA</td>
<td>125</td>
</tr>
<tr>
<td>3</td>
<td>BMP-5 NM_0011081 68.1</td>
<td>Sense: TTTGGGCTTTTACAGCTCTGCCA Antisense: TTCCGGTTGCGGCTGCTCTCA</td>
<td>168</td>
</tr>
<tr>
<td>4</td>
<td>BMP-6 NM_013107.1</td>
<td>Sense: TCGCACCCCAAAGGCTACGCT Antisense: GGTTGGTGCGCAGCAGGT</td>
<td>152</td>
</tr>
<tr>
<td>5</td>
<td>BMP-7 NM_0011918 56.2</td>
<td>Sense: ATGGAGAGGGCCCTTCTTTCCGTTT Antisense: AACACCCCTAAGGCCCACATCCCT</td>
<td>154</td>
</tr>
<tr>
<td>6</td>
<td>BMP-14 XM_0010663 44.5</td>
<td>Sense: AAGAACCTCAAGGCTCGCTGCA Antisense: ACTGCATGGTGTGCGGCTTCA</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>Gene</td>
<td>Accession Number</td>
<td>Sense Sequence</td>
</tr>
<tr>
<td>----</td>
<td>----------</td>
<td>------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>7</td>
<td>IGF-I</td>
<td>NM_178866.4</td>
<td>GACCGGGACGTACAAAT</td>
</tr>
<tr>
<td>8</td>
<td>IGF-II</td>
<td>NM_001190162.1</td>
<td>CTGTGAGAACCTTCCAGCTT</td>
</tr>
<tr>
<td>9</td>
<td>IGF-IR</td>
<td>NM_052807.2</td>
<td>ACCCATCAGAAAGTACGCCG</td>
</tr>
<tr>
<td>10</td>
<td>IGFBP-3</td>
<td>NM_012588.2</td>
<td>AAACAGTGTCGCCCTCCA</td>
</tr>
<tr>
<td>11</td>
<td>IGFBP-5</td>
<td>NM_012817.1</td>
<td>GTGTACCTGCCAACTGTGA</td>
</tr>
<tr>
<td>12</td>
<td>VEGF</td>
<td>NM_001287107.1</td>
<td>ACGCAGCGACAAGGCAGACTAT</td>
</tr>
<tr>
<td>13</td>
<td>β-actin</td>
<td>NM_031144.3</td>
<td>TCCACCCGCGAGTAACAACCT</td>
</tr>
</tbody>
</table>

**Real-time PCR reaction**

RT-PCR was carried out in a CFX 96 Touch Real Time PCR (Bio Rad, USA). The reaction was performed using Kapa SYBR green fast PCR master mix kit (It contains all the PCR components along with SYBR green dye).
Procedure

Template, primers, SYBR Green dye and RNAse free water were thawed and placed on ice and the reaction mixture was prepared by mixing SYBR green (10 µl) and template (5 µl).

Reaction protocol

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAPA SYBR FAST qPCR Master Mix (2 X)</td>
<td>10.0 µl</td>
<td>1 X</td>
</tr>
<tr>
<td>Forward Primer (10 µM)</td>
<td>2.0 µl</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Reverse Primer (10 µM)</td>
<td>2.0 µl</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2.0 µl</td>
<td>1 in 10 dilution</td>
</tr>
<tr>
<td>Water</td>
<td>4.0 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20.0 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

(i). The reaction mixture was mixed by pipetting up and down for few times.

(ii). All reactions were performed in triplicate along with no template control (NTC). Reaction mixture was placed in the thermal cycler, which was programmed as mentioned in the table below.

(iii). Melt curve analysis was performed to confirm that only one product is amplified during PCR.

(iv). The data were analyzed by the CFX 96 software. The results were expressed as fold change.
### Segments and Thermal Parameters

<table>
<thead>
<tr>
<th>Segments</th>
<th>Thermal parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Segment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Denaturation - 1 cycle</td>
<td>95°C for 3 min</td>
</tr>
<tr>
<td><strong>Segment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C for 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58°C for 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C for 45 sec</td>
</tr>
<tr>
<td><strong>Melting curve</strong></td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C for 30 sec</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C for 30 sec</td>
</tr>
</tbody>
</table>

### Amplification plot

This is a plot of amplification cycles on the X-axis versus fluorescence units on the Y-axis, for each ramp or plateau on which data are gathered. At every cycle, the fluorescence emitted is calculated and the PCR cycle at which fluorescence measured by the instrument is determined to be at a statistically significant level above the background signal. The threshold cycle (Ct) is inversely proportional to the log of the initial copy number.

### Melting curve analysis

All products generated during the PCR amplification reaction were melted at 95°C, then annealed at 55°C and subjected to gradual increases in temperature changes. Fluorescence data were collected until the reaction reached 95°C. The result is a plot of raw fluorescence data unit’s versus temperature. There is a rapid linear
increase in fluorescence between 82°C and 84°C, where the major product melts usually.

Two steps are required to interpret results from a SYBR green I melt curve analysis. The first step is to review the PCR products i.e. gene of interest in the reaction. The presence of a single homogenous melt peak for all sample reactions confirms specific amplification. The second step is to evaluate primer-dimer formation. It is acceptable to observe a small but if there is a corresponding peak in the sample amplification plots the Ct’s from these wells cannot be trusted as accurate.

**Real-time PCR – Data analysis**

Efficiency of amplifications was determined by running a standard curve with serial dilutions of cDNA. For each measurement, Ct was determined. This was defined as the number of cycles necessary to reach a point in which the fluorescent signal is first recorded as statistically significant above background. In this study, the threshold value was determined with a baseline set manually at 100 relative fluorescence units (RFU). Results were analyzed using the comparative critical threshold (ΔΔCt) method in which the amount of target RNA is adjusted to a reference (internal target RNA). The fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001).

**Analysis of protein expression of BMP-2, BMP-4, IGF-IR, IGFBP-3, IGFBP-5 and VEGF by western blot analysis**

**Preparation of callus tissue homogenate**

Protein expression in the callus was assessed by western blot analysis as described by Muthusami et al. (2011a).
Fracture callus tissue from each group (Control and *C. quadrangularis*) were excised and rinsed with saline. One ml of ice-cold RIPA buffer with protease inhibitor was added. Then they were homogenized with mortar and pestle and the homogenate was centrifuged for 15 min at 12,000 X g at 4°C. The supernatant was aspirated into fresh tubes and protein concentration was determined.

**Estimation of protein**

The protein concentration was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin (BSA) as the standard.

**Principle:**

In alkaline solution, protein molecules form a complex with copper ions and the amino acids containing phenolic hydroxyl group *viz.*, tyrosine and tryptophan present in the copper-protein complex reacts with Folin-Ciocalteau reagent to give a blue colour due to the reduction of phosphomolybdate. The intensity of the colour is proportional to the concentration of protein.

**Reagents:**

1. **Sodium hydroxide (0.1 N):** 4 g of sodium hydroxide was dissolved in 1000 ml of distilled water (w/v).
2. **Sodium carbonate, 2% (Reagent A):** 2 g of sodium carbonate was dissolved in 100 ml of 0.1 N NaOH (w/v).
3. **Sodium-potassium tartrate, 1.35%:** 1.35 g of sodium-potassium tartrate was dissolved in 100 ml of distilled water (w/v).
4. **Copper sulphate, 0.5% (Reagent B):** 5 mg of copper sulphate was dissolved in 1 ml of 1.35% sodium potassium tartrate (w/v) just prior to use.
5. **Alkaline copper reagent (Reagent C):** This was prepared fresh at the time of protein assay by mixing 50 ml of reagent A with 1 ml of reagent B (v/v).

6. **Folin-ciocalteau phenol reagent (1 N):** Commercially available 2 N Folin reagent was diluted to 1 N solution by adding an equal volume of distilled water (v/v).

7. **Bovine serum albumin (BSA) standard:** A standard solution of BSA containing 250 µg/ml was prepared in 0.1 N NaOH. 12.5 mg of bovine serum albumin was dissolved in 50 ml of 0.1 N NaOH in a 50 ml standard flask.

**Procedure**

Callus homogenate sample (5 µl) was taken in a clean glass tube. The volume in each tube was made up to 0.1 ml with water. Then 0.5 ml of alkaline copper reagent was added, mixed and allowed to stand for 10 min at room temperature. 0.5 ml of 1 N Folin-ciocalteau phenol reagent was added to each tube, shaken well and incubated for 20 min at room temperature. The intensity of blue colour developed was read at 720 nm against reagent blank containing all the reagents except the sample. A set of standards were run on each batch of assay. The amount of protein present in the samples was calculated by referring the standard graph obtained or by plotting the standard graph. A set of standards containing 25, 50, 75, 100, 125 and 150 µg of bovine serum albumin was taken in a series of test tubes and treated similarly to that of the sample tubes. The standard graph was drawn by plotting the concentration of the standard protein solution on the X-axis and the optical density on the Y-axis.

**Calculation**

The protein content in the callus homogenate was calculated using the formula:
Protein concentration = \frac{\text{O.D of unknown}}{\text{O.D of known}} \times \text{Standard concentration}

Western blot analysis

Reagents

1. **Radio immuno precipitation buffer** (RIPA): (150 mM NaCl, 50 Tris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% DS, pH 7.4).

   For 500 ml,

   - Tris base: 3.029 g
   - NaCl: 4.383 g
   - EDTA: 0.186 g
   - Na-deoxycholate: 2.5 mg
   - SDS: 500 mg
   - NP-40: 5.0 g

   All these chemicals were dissolved in 400 ml double distilled water, pH was adjusted to 7.4 with HCl and then the volume was made up to 500 ml. The buffer was stored in a refrigerator.

2. **Protease inhibitor cocktail**: One tablet of protease inhibitor (Roche Pharmaceuticals, USA) was dissolved in 2 ml of double distilled water (DDW) at stored in -20°C.
3. **Running gel buffer: (1.5M Tris, pH 8.8):** For 250 ml, 45.375 g Tris was dissolved in 200 ml DDW, pH was adjusted with HCl and the volume was made up to 250 ml and stored at room temperature.

4. **Stacking gel buffer (0.5 M Tris, pH 6.8):** For 250 ml solution, 15 g Tris was dissolved in DDW and the pH was adjusted with HCl before adjusting the volume up to 250 ml.

5. **SDS 10%:** For 100 ml solution, 10 g SDS was dissolved in DDW and stored at room temperature.

6. **Acrylamide 30%:** 29 g acrylamide (29%) and 1 g bis-acrylamide (1%) were dissolved in 100 ml DDW.

7. **Ammonium per sulfate 10%:** 100 mg ammonium per sulfate was dissolved in 1 ml DDW.

8. **10 X SDS Electrophoresis buffer (2.5 M Tris, 1.92 M glycine and 1% SDS):** For 1L solution, 30 g Tris, 144 g glycine and 10 g SDS were dissolved in 800 ml DDW and volume made up to 1L and stored at room temperature.

9. **1 X SDS Electrophoresis buffer:** For 500 ml, 50 ml 10 X SDS electrophoresis buffer was mixed with 450 ml of DDW.

10. **10 X Transfer buffer (250 mM Tris and 1.92 M Glycine):** For 1L, dissolve 30.3 g Tris and 144 g glycine in 800 ml DDW and adjust the volume up to 1L and stored at room temperature.

11. **1 X Transfer buffer with 20% methanol:** For 500 ml, 50 ml 10X transfer buffer, 350 ml DDW and 100 ml methanol were mixed and kept cold until use.
12. **2 X Sample buffer with reducing agent:** (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.004% bromophenol blue). For 10 ml, 2.5 ml 0.5 M Tris-HCl (pH 6.8) and 4.0 ml of 10% SDS, 2.0 ml glycerol, 1.0 ml 2-mercaptoethanol, 0.4 mg bromophenol blue and made up the volume to 10 ml with DDW. 1 ml aliquots were made and stored at -20°C.

13. **10 X Phosphate buffered saline (0.1 M Sodium phosphate):** For 1L solution; 13 g NaH$_2$PO$_4$.4H$_2$O dissolved in DDW, pH adjusted to 7.2 with NaOH, the volume made up to 1L and stored at room temperature.

14. **Blocking buffer:** For 500 ml: To 500 ml of 1X PBS, 900 mg I-Block™ (Tropix, Bedford, MA, USA) was added, dissolved by heating/stirring (heat should not exceed 100°C). Then add 0.5 ml Tween-20 and stored in refrigerator.

15. **Tris buffered saline (TBS): (20 mM Tris, 500 mM NaCl, pH 7.5):** For 500 ml, 1.21 g Tris and 14.62 NaCl were dissolved in 400 ml double distilled water and adjusted pH to 7.5 with HCl and made up to 500 ml.

16. **T-TBS (0.2% Tween-20 in TBS):** For 100 ml, add 200 µl Tween-20 to 100 ml of TBS. The solution was kept cold.

17. **Prestained SDS-PAGE standard:** Commercially available prestained protein standard was obtained and this molecular weight standard was stored at -20°C.

**Low range molecular weight standards**

1. β-galactosidase 126,000
2. BSA 92,000
3. Ovalbumin 52,900
4. Carbonic anhydrase  35,400
5. β-lactoglobulin  26,900
6. Lysozyme  21,500

**Preparation of gel**

**Running gel (12%)**
- Acrylamide (30%)  3.96 ml
- Running gel buffer  3.0 ml
- DDW  4.8 ml
- SDS (10%)  0.12 ml
- APS (10%)  0.12 ml
- TEMED  0.012 ml

**Stacking gel (5%)**
- Acrylamide (30%)  0.5 ml
- Stacking gel buffer  0.38 ml
- DDW  2.1 ml
- SDS (10%)  0.03 ml
- APS (10%)  0.03 ml
- TEMED  0.003 ml

**Procedure**

1. 50 µg of total protein was mixed with 2 X sample buffer and boiled for 5 min.
2. The sample mixture was run on 12% SDS-PAGE gel in 1 X running gel buffer at 100 V for 2.5 h and electrotransferred on to a PVDF membrane at 30 V for 1 h.
3. The membrane was blocked with blocking buffer for overnight.
4. After overnight blocking, the membrane was incubated with a primary antibody (1:1000 dilutions) for overnight incubation.

5. After incubation with the primary antibody, the membrane was washed three times with blocking buffer for 10 min each.

6. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000 dilutions) for 45 min.

7. The membrane was then washed in TBS and T-TBS thrice.

8. Then the signals were detected and visualized using the enhanced chemiluminescence system.

9. The band was visualized and subjected to densitometry scanning using a densitometer (Bio-Rad Chemidoc-Gel Documentation).

10. The band intensity for BMP-2, BMP-4, IGF-IR, IGFBP-3, IGFBP-5, and VEGF was normalized with the internal control α-tubulin.

**Estimation of alkaline phosphatase (Orthophosphoric monoester phosphohydrolase E.C.No.3.1.3.1)**

Alkaline phosphatase (ALP) activity was determined by the method of Andersch and Szecypinski (1947) using p-nitrophenyl phosphate as the substrate.

**Principle**

p-nitrophenyl phosphate is colourless in solution, but upon hydrolysis, the phosphate group liberates p-nitrophenol which is highly coloured in an alkaline solution. The rate of hydrolysis of p-nitrophenyl phosphate is proportional to the concentration of the enzyme present in the sample.
Phosphatase

\[ \rho\text{-nitrophenyl phosphate} + \text{NaOH} \rightarrow \rho\text{-nitrophenol} + \text{phosphate} \]

Reagents

1. **Glycine buffer (pH 10.5, 0.1 M):** 7.5 g of glycine and 0.095 g of magnesium chloride was weighed and transferred to 1L volumetric flask containing about 750 ml of distilled water. 85 ml of 1 N NaOH was added, mixed thoroughly and the solution was made up to 1L. pH was checked and the solution was stored at 4°C.

2. **ρ-nitrophenyl phosphate (0.4%):** This was freshly prepared on the day of use at a concentration of 4 mg/ml in distilled water.

3. **1 N (NaOH):** 40 g of NaOH in 1000 ml of distilled water (w/v).

4. **0.1 N NaOH:** 4 g of NaOH in 1000 ml of distilled water (w/v).

5. **0.02 N NaOH:** 0.8 g of NaOH in 1000 ml of distilled water (w/v).

6. **Standard solution (stock):** 0.1391 g (10 mmol) of ρ-nitrophenol was dissolved in 6 ml of 0.1 N NaOH (w/v) solutions and diluted to 90 ml with distilled water in a 100 ml volumetric flask and made up to 100 ml. 1 N HCl was added drop by drop until the solution turned pale yellow. The stock is stable for about one year when refrigerated.

7. **Working standard solution:** 10 ml of stock standard was diluted to 100 ml with 0.02 N NaOH. The working standard solution was prepared on the day of use.

Procedure

Into clean glass tubes marked ‘blank’ and ‘test’, 0.5 ml of ρ-nitrophenyl phosphate substrate and 0.5 ml glycine buffer (pH 10.5) were added. The tubes were placed in a water bath at 37°C for 5 min. The reaction was initiated by the addition of
callus homogenate to the ‘test’ and water in the ‘blank’ marked tubes and the time was noted. Exactly after 30 min of incubation at 37°C, the reaction was arrested by the addition of 10 ml of 0.02 N NaOH. The tubes were mixed well and the colour developed was read at 410 nm against the reagent blank. The ALP activity was expressed as μmoles of ρ-nitrophenol formed per hour per mg of protein.

**Calculation**

The ALP activity was calculated using the formula:

\[
\frac{\text{O. D of unknown}}{\text{O. D of known}} \times \frac{\text{Standard Time}}{X} \times \frac{\text{Concentration}}{X} \times \frac{\text{Time correction factor}}{X} \times \frac{1}{\text{mg protein}}
\]

\[
= \mu \text{moles of ρ-nitrophenol formed/h/mg protein}
\]

**Estimation of tartrate resistant acid phosphatase (Orthophosphoric 2 Phosphohydrolase (EC 3.1.2.2))**

Tartrate resistant acid phosphatase activity was determined by the method of Tenniswood *et al.* (1976) using ρ-nitrophenyl phosphate as the substrate.

**Principle**

ρ-nitrophenyl phosphate is colourless in solution but upon hydrolysis, the phosphate group liberate ρ-nitrophenol which is highly coloured in an alkaline solution. The rate of hydrolysis of ρ-nitrophenyl phosphate is proportional to the enzyme activity present in the sample.
Reagents

1. Citrate buffer (0.1 M; pH 4.85): 21.04 g of citric acid was weighed and transferred to 1L volumetric flask containing 600 ml of distilled water. To this, 180 ml of 1 N NaOH and 100 ml of 0.1 N HCl (9.909 ml of Con.HCl was made up to 1L with water, the pH was checked and stored at 4°C.

2. ρ-nitrophenyl phosphate (0.4%): This was freshly prepared on the day of use at a concentration of 4 mg/ml in distilled water.

3. Sodium hydroxide (0.1 N): 0.4 g NaOH in 100 ml distilled water.

4. Tartrate (0.2 M): 30.018 g tartaric acid was dissolved in 1000 ml of distilled water.

Procedure

Into clean glass tubes marked ‘blank’ and ‘test’, 0.5 ml of 0.4% ρ-nitrophenyl phosphate substrate, 0.5 ml of 0.1 M citrate buffer (pH 4.85) and 0.2 ml of DL–tartrate were added. The tubes were pre-incubated at 37°C in a water bath for 5 min. The reaction was initiated by the addition of 0.1 ml callus homogenate and water to the ‘test’ and ‘blank’ tubes, respectively and the time was noted.

After 30 min of incubation at 37°C, the reaction was arrested by the addition of 3.8 ml of 0.1 N NaOH. The reaction product, ρ-nitrophenol was measured spectrophotometrically at 415 nm against reagent blank. The enzyme activity was calculated from the standard curve and expressed as μmoles of ρ-nitrophenol formed per hour per milligram of protein.
Calculation:

The TRAP activity was calculated with the following formula.

\[
\frac{\text{O. D of unknown}}{\text{O. D of known}} \times \frac{\text{Standard Concentration}}{\text{Factor}} \times \frac{\text{Time}}{\text{mg protein}} = \mu\text{moles of } \rho\text{-nitrophenol liberated/h/mg protein.}
\]

Estimation of antioxidant enzyme activities

Superoxide dismutase (EC 1.15.1.1, SOD)

The enzyme was assayed according to the method of Marklund and Marklund (1974).

Principle

The degree of inhibition of auto-oxidation of pyrogallol, at an alkaline pH, by superoxide dismutase was used as a measure of the enzyme activity.

Reagents

1. Tris-HCl buffer (0.1 M; pH 8.2) containing 2 mM EDTA (w/v): 1.121 g of Tris was dissolved in 100 ml of distilled water and the pH was adjusted to 8.2 with HCl, then 74.4 mg of EDTA was added.
2. Tris-HCl buffer (pH 7.5; 0.05 M): 605.7 mg of Tris was dissolved in 100 ml of distilled water pH was adjusted to 7.5 with HCl.
3. Absolute ethanol
4. Chloroform
5. Pyrogallol stock solution: 25.2 mg pyrogallol was dissolved in 1 ml of 0.05 M Tris-HCl buffer, (pH 7.5) wrapped in an aluminium foil and stoppered test tube.

6. Pyrogallol working solution: At the time of assay, 0.5 ml of the above stock solution was diluted to 5 ml with 0.05 M Tris-HCl buffer to give a 2 mM working solution and use immediately.

Procedure

To 0.5 ml of tissue extract, 0.25 ml cold absolute ethanol and 0.15 ml chloroform were added. After 15 min of shaking in a mechanical shaker, the suspension was centrifuged for 15 min at 13,000 rpm and 0.5 ml of supernatant was used for the assay.

The reaction mixture for auto-oxidation consisted of 2 ml Tris-HCl buffer (pH 8.2), 0.5 ml 2 mM pyrogallol and 2 ml water. Initially, the rate of auto-oxidation of pyrogallol was noted at an interval of 1 min for 3 min. This was considered as 100% auto-oxidation.

The assay mixture for the enzyme assay contained 2 ml Tris-HCl buffer (pH 8.2), 0.5 ml of 2 mM pyrogallol, 0.5 ml aliquot of the enzyme preparation and water to give a final volume of 4.5 ml. The blank was prepared simultaneously, which contained 2 ml of Tris HCl buffer (pH 8.2) and 2.5 ml of distilled water. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted. The percentage inhibition in the auto-oxidation of pyrogallol in the presence of tissue extract was converted to units of inhibition. The amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation is considered as 1 unit of enzyme activity.
The unit of enzyme activity was then calculated using the formula

\[
\text{Assay volume} \times \frac{\text{Units of inhibition}}{\text{Volumes of tissue extract}} \times \frac{1}{\text{X dilution factor}} \times \frac{\text{X mg protein}}{\text{mg protein}}
\]

The enzyme activity was expressed as Units/mg protein. (One enzyme units correspond to the amount of enzyme required to bring about 50% inhibition of pyrogallol auto-oxidation).

**Glutathione peroxidase (EC 1.11.1.9, GPx)**

The activity of glutathione peroxidase (GPx) was determined by the method of Rotruck *et al.* (1973).

**Principle**

Glutathione peroxidase converts reduced glutathione (GSH) into oxidized form using hydrogen peroxide during its reaction. The amount of GSH utilized is measured in the assay mixture before and after the enzyme activity. GSH reacts with dithio-bis-nitrobenzoic acid (DTNB) to give yellow colour, which was then measured at 412 nm.

**Reagents**

1. **Sodium phosphate buffer (pH 7.0; 0.4 M, w/v)**
   a) 6.24 g of sodium dihydrogen phosphate was dissolved in 100 ml of distilled water.
   b) 7.11 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.

   39 ml of ‘a’ + 61 ml of ‘b’ were mixed and pH was adjusted to 7.0.
2. **Sodium azide (10 mM, w/v):** 65 mg of sodium azide was dissolved in 100 ml of distilled water.

3. **Reduced glutathione (4 mM, w/v):** 12.3 mg of GSH was dissolved in 10 ml of distilled water.

4. **Hydrogen peroxide (2.5 mM, v/v):** 0.085 ml of H$_2$O$_2$ was made up to 100 ml with using distilled water.

5. **10% Trichloro acetic acid (w/v):** 10 g of TCA was dissolved in 100 ml of distilled water.

6. **Disodium hydrogen phosphate solution (0.3 M, w/v):** 5.34 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.

7. **Dithio-bis-nitrobenzoic acid (DTNB) reagent:** 40 mg of DTNB was dissolved in 100 ml of 1% sodium citrate solution.

8. **Standard reduced glutathione:** 20 mg of reduced glutathione was dissolved in 100 ml of distilled water.

**Procedure**

To 0.5 ml buffer, 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml H$_2$O$_2$, and 0.1 ml of 1:10 diluted aliquot of the tissue extract were added and the total volume was made up to 2.0 ml with distilled water. The tubes were incubated at 37°C for 3 min and the reaction was terminated by the addition of 0.5 ml 10% TCA. To determine the residual glutathione content, the supernatant was removed after centrifugation (10,000 rpm for 15 min) and from this 0.1 ml was taken to this, 4.0 ml of disodium hydrogen phosphate (0.3 M) solution and 1 ml of the DTNB reagent were added. The colour developed was read at 412 nm against a reagent blank containing only phosphate solution and DTNB reagent. Suitable aliquots of the standard were also treated similarly.
The enzyme activity was calculated from the formula

\[
\text{OD of unknown} \times \text{Concentration} \times \text{Dilution factor} = \text{OD of the known} \times \text{Volume of tissue extract} \times \text{mg protein}
\]

The enzyme activity was expressed as Units/mg protein (one unit is the amount of enzyme that converts 1 µmole GSH to GSSG in the presence of hydrogen peroxide/min).

**Glutathione- S-transferase (EC 2.5.1.18, GST)**

The enzyme activity was assayed by the method of Habig *et al.* (1973).

**Principle**

GST catalyses the transfer of sulfhydryl group from GSH to CDNB (1-Chloro-2, 4 dinitrobenzene) from forming GSH-CDNB conjugate, which has maximum absorbance at 340 nm. The absorbance is directly proportional to enzymatic activity.

**Reagents**

1. **Potassium phosphate buffer (pH 6.5; 0.5 M, w/v)**
   
   (a) 3.405 g of KH$_2$PO$_4$ was dissolved in 50 ml of distilled water.
   
   (b) 4.360 g of K$_2$HPO$_4$ was dissolved in 50 ml of distilled water.

   Equal volume of ‘a’ and ‘b’ were mixed and pH was adjusted to 6.5

2. **1-Chloro-2, 4 dinitrobenzene (CDNB: 30 mM, w/v in ethanol):** 6 mg of CDNB was dissolved in 1 ml of ethanol.
3. Reduced glutathione (GSH; 30 mM, w/v): 22.35 mg of GSH was dissolved in 3 ml of distilled water.

Procedure

To 0.4 ml buffer, 0.1 ml homogenate, 1.2 ml water and 0.1 ml CDNB were added and incubated in a water bath at 37°C for 10 min. After incubation, 0.1 ml of reduced glutathione was added. The change in optical density was measured against a reagent blank at 340 nm at 30 sec interval for 2 min.

The enzyme activity was calculated from the formula:

\[
\text{Units/mg protein} = \frac{\text{Volume of assay mixture \times } \Delta \text{OD/min \times } \frac{1}{\text{Dilution factor \times Volumes of the tissue extract}}}{\text{mg protein}}
\]

The activity of GST was expressed as Units/mg protein (one unit is the amount of enzyme that conjugate 1 µmole of CDNB with GSH/ min).

Lipid peroxidation

The lipid peroxidation was measured by the method of Devasagayam and Tarachand (1987).

Principle

Malondialdehyde, an end product of lipid peroxidation reacts with thiobarbituric acid (TBA) to form a pink chromogen ([TBA] 2-malondialdehyde adduct) and is measured by its absorbance at 532 nm.
Reagents

1. **Tris-HCl buffer (0.15 M, pH 7.4):** 1.815 g of Tris was dissolved in distilled water and the pH was adjusted to 7.4 with HCl. Then the volume was made up to 100 ml with distilled water.

2. **Potassium dihydrogen phosphate (KH$_2$PO$_4$) (10 mM, w/v):** 0.136 g of KH$_2$PO$_4$ was dissolved in 100 ml with distilled water.

3. **Trichloro acetic acid (TCA) 10% (w/v):** 10 g of TCA was dissolved in 100 ml of distilled water.

4. **Thiobarbituric acid (TBA) 1% (w/v):** 1 g of TBA was dissolved in 100 ml of distilled water.

5. **Standard malondialdehyde:** A stock solution of malondialdehyde was prepared in distilled water, using 1, 1’, 3, 3’-tetrahydroxypropane. This was stored at 4°C and diluted just before use, to have a working standard containing 50 nmoles/ml.

Procedure

The reaction mixture consisted of 0.5 ml 0.15 M Tris-HCl buffer (pH 7.4), 0.15 ml 10 mM KH$_2$PO$_4$ and 0.1 ml of callus tissue extract and 0.25 ml of distilled water in a total volume of 0.9 ml. The tubes were incubated at 37°C for 20 min with constant shaking. The reaction was stopped by the addition of 1 ml of 10% TCA. The tubes were shaken well and 0.75 ml TBA was added and were heated in boiling water bath for 20 min. Standard tubes containing 10, 20, 30, 40 and 50 nmoles/ml were also run simultaneously. The tubes were centrifuged and the colour developed was measured at 532 nm. The malondialdehyde content of the samples was calculated using the formula:
The malondialdehyde content of the samples was expressed as nmoles of MDA formed/mg protein.

**Hydrogen peroxide**

The hydrogen peroxide generation was assayed by the method of Pick and Keisari (1981).

**Principle**

Horse radish peroxidase converts hydrogen peroxide into water and oxygen. This causes oxidation of phenol red which forms adduct with dextrose which has maximum absorbance at 610 nm.

**Reagents**

1. **Sodium phosphate buffer (50 mM, pH 7.6)**

   - **Solution A**: 890 mg of disodium hydrogen phosphate was weighed accurately and dissolved in 100 ml of DDW.
   - **Solution B**: 692 mg of sodium dihydrogen phosphate was weighed accurately and dissolved in 100 ml of DDW.

   13 ml of solution A was mixed with 87 ml of solution B and volume was made upto 200 ml with distilled water and the pH was adjusted to 7.6 using 1N HCL.

2. **Horse radish peroxidase (HRP) (8 U/ml)**: 1 mg of 80 units of HRP was dissolved in 10 ml of distilled water.

3. **Phenol red solution 28 nM**: 642.9 µl of phenol red solution was made upto 1 ml with DDW.
4. **Sodium hydroxide 10 N:** 40 g of sodium hydroxide was dissolved in 100 ml of DDW.

5. **Dextrose 5.5 nM:**
   - **Stock solution:** 18 mg of dextrose was weighed accurately and dissolved in 100 ml of DDW.
   - **Working solution:** 5.5 ml stock solution was diluted to 100 ml with double distilled water to have the concentration of 5.5 nM.

**Procedure**

The reaction mixture contained 1.64 ml phosphate buffer, 54 µl HRP, 30 µl of 28 nM phenol red, 165 µl of 5.5 nM dextrose and 600 µl of tissue extract. Blank was prepared simultaneously by adding all the reagents except tissue lysate. The reaction mixture was incubated at 35°C for 30 min and the reaction was terminated by adding 60 µl of 10N NaOH solution. Absorbance was read at 610nm against reagent blank on spectrophotometer. For the preparation of standard curve, known amount of hydrogen peroxide and the entire above reagent except enzyme source were incubated for 30 min at 35°C and then 60 µl of 19N NaOH was added and optical density was read at 610 nm.

The hydrogen peroxide content of the sample was expressed as µmol/min/mg protein.
Statistical analysis

The data were subjected to statistical analysis using analysis of variance (ANOVA). When the F-test was found to be significant, the data were subjected to Students’ t test to assess individual variance (Zar, 1974). \( P<0.05 \) was considered statistically significant.