Materials and Methods

Animal maintenance and ethics statement

Albino morph of *Poecilia latipinna* of both the sexes, of the same age, with a mean ± S.D. standard length (LS) of 4.721 ± 0.340cm and weight of 2.890 ± 0.696g were purchased from a commercial supplier (Oskar fish, Vadodara, Gujarat, India). All animals were screened for parasitic infections and the healthy ones were used for the experiments. Only the healthy animals were further maintained in glass aquaria (12"×7"×9") containing dechlorinated water with constant aeration. Tank water was monitored continuously and maintained for their physiochemical parameters as described by Rajaram *et al*. (2016). In detail, the animals were maintained in the temperature range of 26 ± 2°C, pH 7.0-7.4, salinity 0.25ppt, dissolved oxygen 7.8mg/L, phosphate 0.36-2.38mg/L, total nitrate 1.0-2.2mg/L and 12:12 hour light to dark cycles. About 10% of the aquarium water was changed every day and replaced with fresh charcoal-UV purified water from a water purification system (Aquaguard, Eureka Forbes, Mumbai, India). The behaviour of the fish was observed on daily basis. Handling and processing of fish were carried out according to the ethical principles (Drugs and Cosmetics Rule 1945) approved by the Institutional Animal Ethics Committee (IAEC) (Project no. MSU-Z/IAEC/14) constituted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (Reg. No. 827/GO/Re/S/04/CPCSEA).

Drug administration and experimental procedure

A 5mg/ml stock solution of LDN193189 (Sigma-Aldrich, USA) was prepared in warm distilled water and stored at -20ºC. Freeze-thaw cycles were minimized by preparing aliquots of the stock solution. The dose of 2.5mg/kg of body weight was selected based on dose range study performed earlier in the lab and also published by Rajaram *et al*. (2016). After acclimatization of fish for a period one week, fish were separated in two groups named as control and LDN193189 treated. For each methodology, ten animals were included in each group. Fish of the treated group were transferred to another aquarium and the tank water was replaced gradually with ice-cold water until the fish became listless. Further, the fish were placed on an ice block and the distal tip (approximately one third region) of the caudal fin was amputated using a sterile chilled surgical blade. The use of chemical anaesthetics was avoided in order to eliminate their influence on the expression of various signalling molecules to be studied. Nonetheless, enough care was taken not to cause

any distress to the animals during the surgical procedure. The fishes were continuously monitored during the surgery and thereafter for another 30min for signs of abnormal behaviour, whereas the ones that showed symptoms of distress, were immediately sacrificed by an overdose of MS-222 (Murawala *et al*., 2018). Control animals received an injection of 25µL of physiological saline (0.6N) while the treatment group received single injection of the BMP receptor inhibitor, LDN193189 (water-soluble) at 2.5mg/kg body weight of the subject (25µL) (Patel *et al*., 2019). Dosing pattern and tissue collection for each study has been detailed in the materials and methods section of individual chapter.

Morphological observations and morphometry

Morphological observations were made using light microscope DM2500 coupled to the in-built camera (Leica, Germany). The lengths of fin regenerates were measured using digital calliper (Mitutoyo, Japan).

In vivo BrdU labelling

Materials

BrdU (Sigma-Aldrich, USA)

2N HCl

4% PFA

0.1M Borate buffer

10% BSA (prepared in PBS)

Citrate buffer (10mM Citric acid, 0.05% Tween 20, pH 6)

Mouse anti-BrdU primary antibody (0.15µg/ml)

Goat anti-Mouse secondary antibody (0.5µg/ml)

Anti-fade solution

20%(w/v) n-propyl gallate in dimethyl sulfoxide.

1:10 dilution of 10XPBS with 1 part of 10X PBS and 9 parts of glycerol.

Method

BrdU powder (25µg/L) was dissolved in aquarium tank. After incubating animals in BrdU dissolved water for 24hours, caudal fin was amputated. The zone of interest was fixed in 4% PFA for 30sec. Following this, tissue was embedded in OCT medium at -20°C and thin sections of 8-10microns were taken using cryocut E microtome (Reichert Jung, Leica,

Germany). The sections were collected on the slide and subjected to antigen retrieval using citrate buffer method for 20min at 30ºC temperature in oven. Sections were allowed to cool down for 20min at RT, rinsed thrice in PBS for 5min each and permeabilised with 2N HCl for 60min at RT for denaturation of DNA. Again, the sections were rinsed thrice in PBS for 5min each. The acid was neutralised by immersing sections in 0.1M Borate buffer for 15min. Sections were washed again with PBS and were blocked using 5% BSA for 1hr at RT. The sections were then incubated in primary antibody of Mouse Anti-BrdU overnight (approximately for 16hr) in moist chamber at 4°C in complete darkness. Next day, sections were washed with PBS (three times, each for 5min) and incubated with Fluorescein isothiocyanate conjugated secondary antibody (1:50 dilution of Goat Anti-Mouse IgG-FITC in 5%BSA) (GeNei, Merck, USA) for 90min at room temperature. The sections were washed again with PBS. Finally, sections were mounted with anti-fade solution and images were captured using the fluorescent microscope DM 2500 (Leica, Germany).

Whole mount immunolocalization

Materials

Acetone

5X Blocking buffer (GeNei, Merck, USA)

PBS

Primary antibodies

ALP conjugated secondary antibodies

BCIP/NBT substrate (Sigma-Aldrich, USA)

Method

Regenerating caudal fin tissues were collected at specific time points (either 24 or 60hpa), from both control as well as LDN193189 treated groups. Tissues were fixed with cold acetone for 10sec. After fixation, tissues were blocked with 100µL of blocking buffer for 1hr at RT in a moist chamber. Following blocking, tissues were incubated with 20µL of respective primary antibody (as detailed in Appendix I) in a moist chamber at RT for 1hr. Tissues were washed with cold PBS thrice, each for 10min. Further, tissues were incubated with 20µL of appropriate secondary antibody conjugated with ALP (details mentioned in Appendix 2) for 30min at RT. Three washes with PBS were repeated as mentioned earlier.

20µL of BCIP/NBT Substrate was added to the tissues for colour development. Reaction was stopped by adding PBS to the tissues when background colour was seen.

Single cell suspension preparation

Materials

PBS

20mM EDTA

Method

Fin tissue was collected freshly in PBS with 20mM EDTA (14-20mg/ 200µL). It was then minced into fine pieces using sharp scissors. The minced tissue was then centrifuged at 1000rpm for 5min to remove debris and large chunks of tissues. Supernatant was collected into a fresh vial. 10µL of the supernatant containing cells was loaded onto Neubauer's chamber to ensure the preparation of single cells as well as to quantify the cells.

Comet assay

Materials

LMA: Low melting agarose in 0.5% PBS.

NMA: Normal melting agarose in 1% PBS.

Lysing solution:

2.5M NaCl

0.1M EDTA Na_2

10mM TrisHCl

Electrophoresis buffer:

Tris Borate EDTA buffer (pH- 8)

Method

1) Slide preparation:

Slides were dipped in methanol and wiped with cotton and cleaned properly. One third area of the slides was dipped in melted 1% NMA and removed ensuring formation of a thin coating on the slides. Slides were allowed to dry in dust free environment.

2) Sample preparation:

Samples were taken from single cell suspension preparation (about 5×10^4 cells/ml). Cells were then added in pre-melted LMA in 1:10 ratio (where 10 parts of LMA and 1 part of

cells). Tubes containing cells in LMA were incubated in water bath at 40°C.

3) Loading of sample:

Slides coated with NMA were kept over the water bath at 40°C. 50µl of the sample prepared was loaded onto the precoated slides. Slides were titled immediately after applying sample onto it to ensure a uniform spreading of cells on the coated part of the slide. Following which, slides were allowed to air dry.

4) Cell lysis:

Slides were fully immersed in pre-chilled lysing for 30min at 4°C for cell lysis to occur.

5) Unwinding and electrophoresis:

Following cell lysis, slides were immersed in electrophoresis buffer for 15min at 4°C. Slides were placed in the electophoretic unit taking care that slides do not touch each other or the wall of the unit. Electrophoresis was performed at 21V for 40min.

6) Staining of slides:

Upon completion of electrophoresis, slides were taken out form the unit and immersed in distilled water for 5min followed by immersion in 70% ethanol for 5 min. Slides were stained with 25mM EtBr for 2-3min. Later, slides were observed under fluorescence microscope and images were captured at 20X and 40X magnification.

Each slide was scored for minimum score of 30. The entire experiment was performed thrice with samples from both the groups (control and LDN193189 treated for 60hpa only) as well as from positive control (cells treated with 30% H_2O_2). The protocol for comet assay was adapted from Dhawan *et al*. (2013). The comet assay parameters were evaluated by CaspLab software version 1.0.0. as instructed by Olive and Banáth (2006).

DNA fragmentation

Materials

SNET lysis buffer:

TrisCl	20mM
EDTA	5mM
NaCl	400mM
SDS	1% w/v
Proteinase K	400µg/ml of SNET buffer

Method

DNA isolation from fin tissue (n=10 animals per group) was performed using SNET lysis buffer (25 mg of tissue/ml of SNET buffer) as mentioned by Sambrook and Russell (2006). 25mg of tissue was collected in 1ml of SNET buffer and was incubated at 37°C for 48hours or until the solution turned milky white. Following this, 25:24:1 phenol:chloroform:isoamylalcohol was added to the vial in 1:1 ratio and was incubated on a rocking platform at room temperature for 30min. The mixture was then centrifuged at 8000 rpm for 15min at 4°C. The aqueous phase was then transferred to a new vial and isopropanol was added in equal ratio. DNA was precipitated by centrifuging the vial at 8000rpm for 15min at 4°C. The pellet was washed with 70% ethanol. Pellet was air dried and dissolved in 50µL of distilled water. DNA was quantified using UV-VIS spectrophotometer at 260nm. 50ng of DNA from each group was loaded in 1% agarose gel. Agarose gel electrophoresis was carried out at 100 V for 30 min. The gel image was taken by GeNei imaging system (Merck, USA).

Protein estimation (Bradford, 1976)

Materials

Bradford reagent

100mg of Coomassie Brilliant Blue G-250 was dissolved in 50ml of 95% ethanol. To the above, 100ml of 85% (w/v) ortho-phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre (Final concentration: 0.01 % w/v Coomassie Brilliant Blue G-250, 4.7 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid).

Lysis buffer

Tris (pH 7.5)	50mM
NaCl	200mM
$CaCl_2$	10mM
Triton X-100	1%

To prepare 100ml of lysis buffer, 0.60g of Tris, 1.16g of NaCl and 0.1g of $CaCl_2$ was weighed and transferred into a 250mL flask. The added contents were dissolved in 70ml of distilled water. pH was adjusted to 7.5 using digital pH meter (Analytical Instruments, USA). Finally, 1mL of Triton X-100 was added to the lysis buffer and volume was made up to 100mL.

Method

Fin regenerates from each group (n=10) of each stage of epimorphic regeneration were collected and 10% homogenate was prepared using lysis buffer (25mg/ml of lysis buffer). The homogenate was kept at 8-10°C for 1hr. After incubation, the homogenate was centrifuged at 4°C for 15min at 8000rpm. The supernatant was transferred to a new vial and protein was estimated by Bradford method (Bradford, 1976). 10µL of supernatant was taken in a 96well plate in triplicate wherein 200µL of Bradford reagent was added and mixed well. Blank contained 10µL of distilled water instead of protein sample. After 5min, the absorbance was read at 595nm in iMark microplate reader (BioRad, USA). The standard curve was used to determine the protein in unknown samples. Bovine Serum Albumin (BSA) was used as standard.

SDS-PAGE

Materials

30% Gel stock

29% Acrylamide	29g
1% Bis-acrylamide	1g
Distilled water	100mL

4% Stacking gel (3mL)

30% Gel stock	0.5mL
1.0M Tris-HCl (pH 6.8)	0.380mL
10% SDS	0.02mL
10% APS	0.02mL
TEMED	0.003mL
H_2O	2.077mL

Resolving gel (5mL)

Component	Volume (mL) for 10%	Volume (mL) for 12%	Volume (mL) for 15%
30% gel stock	1.7	2.0	2.5
1.0M Tris-HCl (pH 8.8)	1.3	1.3	1.3
10% SDS	0.05	0.05	0.05
10% APS	0.05	0.05	0.05
TEMED	0.002	0.002	0.002
H_2O	1.898	1.598	1.098

5X Tank buffer (1L)

0.125M Tris base	15.1g
1.25M Glycine	94g
10% SDS	50mL

5X Sample buffer

0.25M Tris-Cl (pH 6.6)

β- Mercaptoethanol

10% SDS

50% Glycerol

0.5% Bromophenol blue

Method

The glass plates and spacers were cleaned with the methanol before assembling them. They were clamped and sealed from the bottom with 1% agar. It was allowed to solidify for 15min. Then the resolving gel mixture was poured into the assembly and allowed to polymerize for 45min at RT followed by stacking gel casting. Comb was inserted immediately after pouring stacking gel mixture onto the polymerized resolving gel. Upon complete polymerization of the gel, the assembly was kept into electrophoresis unit containing tank buffer. Samples and molecular weight markers were loaded in the sample wells. The gel was allowed to run at a constant voltage of 100V until the dye front reached at the bottom of the gel. Following completion of the run, the gel was removed from the unit. The glass plates were separated carefully and the gel was transferred into a staining box for further procedure.

Silver staining method

Materials

Methanol: Water: Glacial acetic acid (50:40:10) (Fixative)

0.02% $AgNO_3$

1.4% EDTA (Stop solution)

Developing solution

0.02% $Na_2S_2O_3$

2.19% Na_2CO_3

125μL of Formaldehyde

Method

The gel was incubated in fixative for 30min at RT following which the gel was washed with 5% methanol for 5min. Further the gel was washed twice during each wash for 5min with distilled water. The gel was incubated with 0.02% $Na_2S_2O_3$ for 3-5min and after that the gel was washed with distilled water for 10min. The gel was further incubated with 0.02% $AgNO_3$ for 20-30min. 30mL of developing solution was added to the gel following the previous incubation. Upon development of bands in the gel, the stop solution was added. The staining of the gel was done to assess the quality of the protein isolated from the tissues. Protein preparations with sufficient yield and unaffected integrity were used in the western blot experiments.

Western blot

Materials

Methanol

Whatmann filter papers

Nitrocellulose membrane/ Polyvinylidene difluoride (PVDF) membrane

Distilled water

5X Semidry transfer buffer (pH 9.2)

240mM Tris

195mM Glycine

0.1% Ponceau S staining solution in 5% acetic acid (100mL)

0.1g Ponceau S

5mL acetic acid

Volume made up to 100mL with double distilled water

Blocking buffer

TBS

Method

A stack of four whatmann filter paper was socked in 1X transfer buffer. Nitrocellulose membrane (5cm×8cm) was also kept in transfer buffer for 5-10min. In case of PVDF, the membrane was incubated with methanol to activate the chemical group of the membrane, so that it can interact with the proteins being transferred onto it. Following this, PVDF membrane was washed with transfer buffer to remove excess of methanol. The gel obtained

after electrophoretic run was washed with water once before placing it in transfer buffer for an incubation time of 15min. The stack was prepared with four whatmann filter papers, membrane, gel and another four whatmann filter papers in the said sequence only. Air bubbles were removed at each step while making the stack to avoid any interference in the transfer. Once the stack was ready, the lid of the semidry transfer unit (Cat. no. MX-1295-01, Medox Biotech, India) was closed and the electrodes were connected to the power pack (PowerPac Basic, BioRad, USA). The time required to transfer proteins from gel to membrane was standardized based on the size of protein of interest. Proteins below 30kDa, between 30-90kDa and above 90kDa were transferred with 90mA for 8min, 20min and 30min respectively. Following transfer, the membrane was stained with ponceau stain for 20sec (reversible) to check whether the proteins are transferred or not. The membrane was washed with distilled water following staining. The membrane was incubated with blocking buffer for 45min at RT on a rocking platform (MedoxBio 1290-02 Gel rocker, Medox Biotech, India). Blocking buffer was discarded after incubation and the membrane was incubated overnight at 4°C with respective primary antibody (details given in Appendix I). Next day, the membrane was washed with 1X TBS thrice each for 20min. After washes, the membrane was incubated with respective secondary antibody (biotinylated) at RT for 45min. The membrane was again washed with 1XTBS as mentioned earlier. The membrane was then incubated with ALP conjugated streptavidin for 45min at RT. The membrane was washed thrice with 1X TBS following incubation with ALP conjugated streptavidin. Finally, 1mL of BCIP-NBT substrate was added to the membrane and observed for colour development. Once the specific bands were observed on the membrane, the excess of substrate was discarded in order to avoid nonspecific colour development.

Gelatin zymography

Materials

2.5% Gelatin stock solution

Lysis Buffer

10mM Tris (pH 8)

150mM NaCl

1% Triton X-100

10mM $CaCl_2$

5X Sample buffer

1M Tris (pH 6.8)

10% Glycerol

2% SDS

0.002% Bromophenol blue

7.5% resolving gel (5mL)

30% Gel stock	1.25mL
1.5M Tris Cl (pH 8.8)	1.25mL
2.5% Gelatin	1mL
10% SDS	0.05mL
10% APA	0.05mL
TEMED	0.005mL
Distilled water	1.395mL

4% stacking buffer (5mL)

30% Gel stock	2mL
1.5M Tris Cl (pH 6.8)	0.85mL
10% SDS	0.05mL
10% APA	0.62mL
TEMED	0.002mL
Distilled water	1.478mL

Wash buffer (200mL)

20mM Tris Cl (pH 8)	0.484g
0.15M NaCl	1.75g
5mM$CaCl_2$	0.11g
2.5% Triton X-100	5mL

Final volume was made up to 200mL with distilled water.

Incubation buffer (300mL)

50mM Tris Cl (pH 7.5)	1.81g
0.2M NaCl	3.5g
5mM $CaCl_2$	0.165g

0.02% NaN_3	0.06mL
0.02% Brij35	0.06mL

Final volume was made up to 300mL with distilled water.

0.25% Coomassie Brilliant Blue R250 staining solution (100ml)

0.25g Coomassie Brilliant Blue R250

90ml Methanol: H_2O (1:1v/v)

10ml Glacial acetic acid

Gel destaining solution (100ml)

Methanol: Glacial acetic acid: Distilled H_2O (50:10:40)

Method

7.5% gel was made according to standard procedures of SDS-PAGE as explained above. 20µg total protein was loaded on the gel and electrophoresed at 100V till it was resolved up to a certain level. After this, the gel was washed twice with wash buffer (each wash for 1hr) to remove SDS from gel. Further, the gel was rinsed with incubation buffer twice for 15min each and then left in incubation buffer overnight at 37°C. The gel was then stained with Coomassie Brilliant Blue R250 for 2-4hr, followed by destaining with the destaining solution. The gel was destained till clear bands were seen against a dark blue background.

Gene expression study

Materials

0.1% DEPC-treated water

Chloroform

Isopropanol

70% Ethanol prepared in DEPC water

1% Agarose prepared in DEPC water

25mM Ethidium bromide

10X TBE buffer (1L)

Tris base	108g
Boric acid	55g

0.5M EDTA	40mL
DEPC water	Adjusted to 1L

5X RNA loading dye (1mL)

500mM EDTA	8µL
40% Formaldehyde	72µL
Glycerol	200µL
Formamide	30µL
2.5% Bromophenol blue	30µL
10X TBE	400µL
DEPC water	260µL

6X DNA loading dye (1mL)

30% Glycerol	300µL
0.25% Bromophenol blue	2.5mg
Distilled water	700µL

Method

Total RNA isolation

Tissue (n=10 from each group) was collected and homogenised in TRIzol (1ml TRIzol per 14-18mg tissue) and left for 5min at room temperature. The homogenate was centrifuged at 12000g for 20min at 4^{o}C. Supernatant was collected and incubated with 200µL of chloroform (per 1mL of TRIzol) for 20-30min in ice with intermittent invert-mixing. Following incubation, the tubes were centrifuged at 12000g for 20min at 4^{o}C. The upper aqueous layer was carefully transferred to a new vial containing 500µL of isopropanol. The mixture was allowed to incubate for 45min at 4°C. Following incubation, the vials were centrifuged at 12000g for 20min at 4°C. The supernatant was discarded and pellet was washed with 0.5mL of 70% Ethanol by centrifuging the tubes at 7000g for 5min at 4°C. This step was repeated for one more time to get rid of any residual impurities left. The pellet was air-dried for 5-7 min. Finally, the pellet was dissolved in 25-30µL of DEPC-treated water depending on the size of pellet. The pellet was allowed to dissolve completely by storing it at 8-10°C overnight.

RNA quantification and quality check

After completely dissolving of RNA into DEPC treated water, it was estimated at 260nm and 280nm by UV spectrophotometer. $O.D._{260}/O.D._{280}$ was taken as a measure of purity check. A ratio of 1.8-2.0 was considered to be of good quality of RNA. Additionally, the quantification of RNA was also done by using Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA). For this, first of all Qubit 3.0 fluorometer was calibrated with Quantiflour RNA estimation kit (cat # E3310, Promega, USA). The details are given below for the calibration:

Step1: 1X TE buffer was prepared from 20X TE buffer.

(For 1 ml of 1X TE buffer: 50µl of 20X TE buffer + 950µl of nuclease free water)

Step2: Quantiflour RNA dye working solution was prepared as per the requirement.

(1µl of RNA dye + 400µl of 1X TE buffer)

Step3 (A): Blank was prepared (was placed in Qubit as Standard#1 during calibration)

(In a 0.5ml tube: 100µl of RNA dye working solution (prepared in step2) + 100µl of 1X TE buffer; Total volume of the system should be 200µl)

Step3 (B): Preparing standard#2

(In a 0.5 ml tube: 2µl of RNA standard (given in kit: 100µg/ml) + 98µl of 1X TE buffer + 100µl of RNA dye working solution; total volume of the system should be 200µl)

Step3 (C): Unknown sample preparation

(In a 0.5 ml tube: 1µl of RNA (1:10 diluted) from fin tissues + 99µl of 1X TE buffer + 100µl of RNA dye working solution; total volume of the system should be 200µl)

The standards and unknown samples were incubated at room temperature for 5 min in dark. After incubation, the tubes were inserted in the fluorometer and readings were recorded in µg/µL. Finally, the quality of RNA was checked by running the samples on 1% agarose gel at 50V for 50min using 1X TBE buffer.

cDNA synthesis

After estimating RNA of each sample, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Firstly, 2X reverse transcription master mix was prepared as mentioned below. RNA was added to the 1X

reverse transcription mater mix prepared. Following this, the tubes were kept in thermal cycler for the reaction to occur.

2X Master mix preparation (per 20µL reaction):

The kit components were thawed on ice. The volume of the components needed to prepare the required number of reactions was calculated.

Components	Volume (µL)
10X RT buffer	2
25X dNTP mix	0.8
10X RT random primers	2
RT enzyme	1
Water	4.2
Total volume	10

2X master mix was placed on ice and mixed gently.

cDNA Reverse Transcription reactions preparation:

- 10µL of 2X master mix was taken into each of an individual tube.
- 10 µL of RNA sample (1µg/µL) was taken into each tube and mixed gently.
- The tubes were briefly centrifuged to spin down the contents.
- The tubes were then placed in the thermal cycler. Following program was run for the reaction.

Thermal cycler Program for Reverse transcription

Conditions	Step1	Step2	Step3	Step4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	α

Semi-quantitative Reverse Transcription Polymerase Chain reaction

After the preparation of cDNA, semi-quantitative PCR was performed in T100 thermal cycler (BioRad, USA). All the components were kept at 4°C while making the reaction mixture. 2X master mix was obtained from Sisco Research Laboratories, Mumbai. All the primer sequences were obtained from NCBI. The primer sequences are listed in appendix II. PCR reaction system was as follows:

Reaction system:

Components	**Volume (µL)**
2X Master mix	5.0
Forward primer (5pmoles/µL)	1.0
Reverse primer (5pmoles/µL)	1.0
cDNA Sample	1.0
Water	2.0
Total volume	10

The tubes were kept in thermal cycler after preparing them as mentioned above. The program used for the study was: 95^0C for 120sec (initial denaturation), 95^0C for 10sec (denaturation), 60^0C for 30sec (annealing), 72^0C for 30sec (extension) followed by 72^0C for 180sec (final extension). The program was run for 40 cycles. At the end, the tubes were incubated at 12^0C in the thermal cycler until further procedure was carried out.

Agarose gel electrophoresis

PCR products were detected by 2% agarose gel electrophoresis. Gel was prepared in 1X TBE buffer. PCR products were mixed with 1X DNA loading dye before loading into gel. The gel was allowed to run at 100V for 30min. After that, the image was taken by GeNei Imaging System (GeNei, Merck, USA) in ultraviolet light. 100bp ladder (Thermo Scientific, USA) was used as marker for confirmation of correct size of PCR products.

Real-time quantitative Reverse Transcription PCR (Real-time qRT-PCR)

After standardizing PCR program in semi-quantitative, quantitative real-time PCR was performed in a LightCycler 96 (Roche Diagnostics, Switzerland) with the following program: 3min at 95°C followed by 45 cycles of 10sec at 95°C, 30sec at 60 °C and 30sec at 72°C. After completion of the last step, melting curve analysis was performed as mentioned below. Melt curve analyses were conducted to assess uniformity of product formation, primer-dimer formation and amplification of non specific products. The PCR product was monitored in real time, using the SYBR Green 1 (BioRad, USA) fluorescence. Threshold for detection of PCR product was set in the log-linear phase of amplification. The comparative cycle threshold (Cq) method was used for relative quantification of gene expression. The amount of mRNA, normalized to the endogenous control (18SrRNA) and relative to a calibrator, was given by $2^{-\Delta\Delta Cq}$, with Cq indicating the cycle number at which

the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR. The fold change in the target gene relative to the 18SrRNA (endogenous control/ reference gene was) calculated using Livak method ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen, 2001). For biological significance, fold change values between 0.5 to 2.0 were not considered as significant up or downregulation.

ΔCq = Cq (target gene) - Cq (18SrRNA)

$\Delta\Delta Cq = \Delta Cq$ (treated) - ΔCq (control)

Fold change = $2^{-\Delta\Delta Cq}$

Thermal cycler program for Melting curve analysis

Temperature (^{0}C)	95	63-68	97
Time (Sec)	10	60	Continuous

Statistical analysis

The data was represented as either Mean ± Standard Deviation (SD) or Mean ± Standard Error of Means (SEM). The control and treated group data was subjected to Unpaired Student's t-test with 95% confidence limit using GraphPad Prism 5 (San Diego, CA, USA). For multiple group comparison, the data was analysed using One Way Analysis of Variance (ANOVA) followed by Bonferroni Post-hoc test. In all the cases, a p value less than or equal to 0.05 was considered statistically significant.