MATERIALS AND METHODS

Chapter 3
Materials and Methods

The Materials and Methods chapter is divided into three sections:

3.1 Materials and Methods for Phytochemical Investigations.

3.2 Materials and Methods for *in vitro* cell migration and cell invasion studies.

3.3 Materials and Methods for *in vitro* cell death studies.

3.1 Materials and Methods for Phytochemical Investigations.

3.1.1 Documentation of biological material: Leaves of *Bauhinia variegata* L. were collected from Waghai Botanical Garden, Waghai, Dang. The collected material was submitted for authentication and authentication certificate was obtained from BARO Herbaria of the Department of Botany, The M.S. University of Baroda, Vadodara, Gujarat, India. The voucher specimen is stored for future reference. The authentication number for the plant samples is Auth/30514. The herbaria and authentication certificate for the plant samples are shown in figure 3.1 and figure 3.2 respectively.

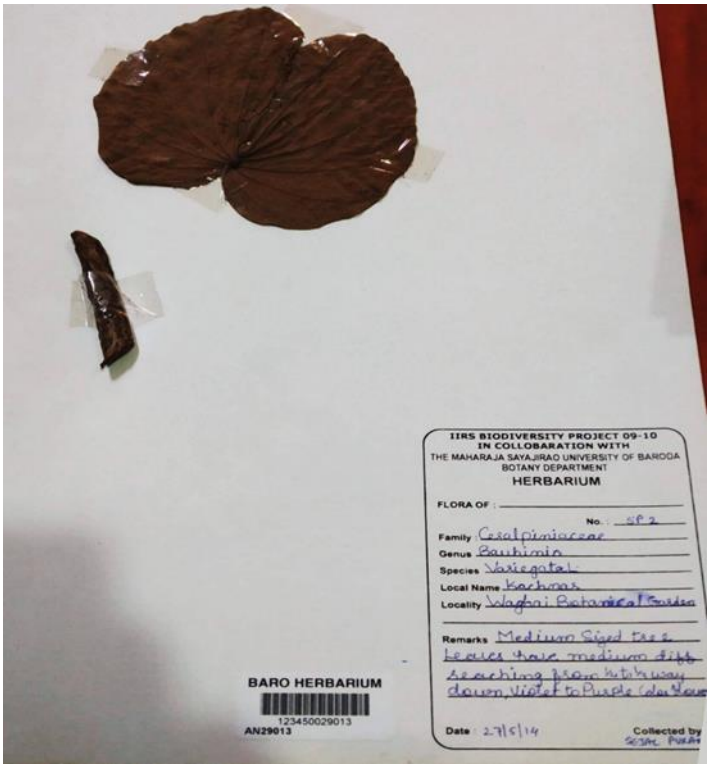
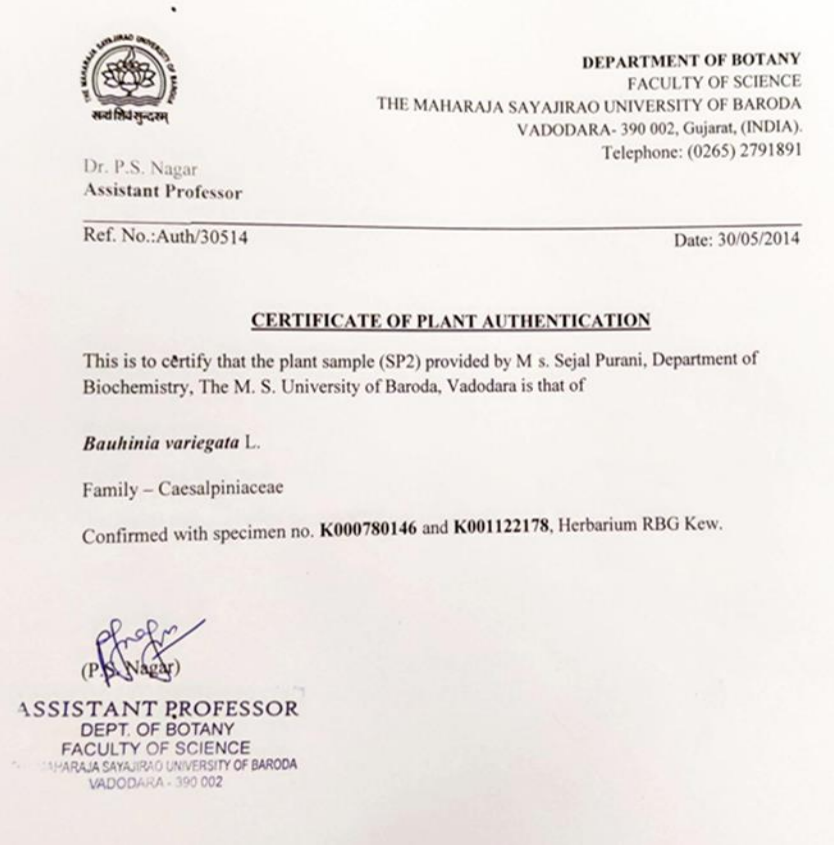


Figure 3.1: Authentication record of *Bauhinia variegata* Linn leaves used in the present study.



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CERTIFICATE OF PLANT AUTHENTICATION

This is to certify that the plant sample (SP2) provided by M s. Sejal Purani, Department of Biochemistry, The M. S. University of Baroda, Vadodara is that of

Bauhinia variegata L.

Family – Caesalpiniaceae

Confirmed with specimen no. **K000780146** and **K001122178**, Herbarium RBG Kew.

(P.S. Nagar)
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Figure 3.2: Authentication certificate of *Bauhinia variegata* Linn leaves used in the present study.

3.1.2 Extraction of Phytocomponents: *B. variegata* leaves were washed with distilled water to remove dirt and kept for shade drying. The dried leaves were powdered (300g) and was subjected to extraction using different solvents of increasing polarity. The extraction from the *Bauhinia variegata* L. leaves was carried out by soxhlet extraction method for all solvents (petroleum ether, n-hexane, chloroform, ethyl acetate, methanol) (SRL-chemicals) and cold maceration (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017) for water. Approximately 5 L of each solvent is used during procedure. The extraction was carried out at 40°C -60°C for 8-12 hrs until its colourless. Following eluotropic series, cold maceration using autoclaved distilled water was carried out to obtain aqueous extract. The sugars were precipitated out of aqueous extract with addition of acetone. The filtrate was evaporated to dryness and used for phytochemistry experiments.

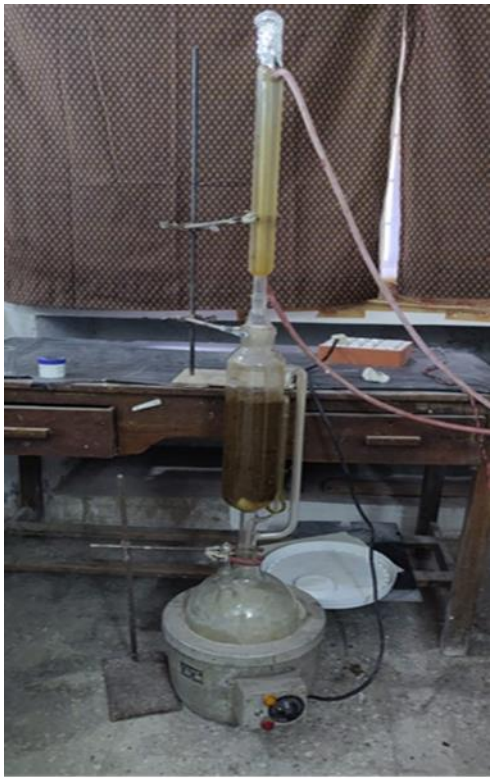

Figure 3.3: Soxhlet extraction setup used for extraction of phytocomponents from *B. variegata* leaves.

The extracts were concentrated at 40°C using hot air oven and aqueous extract was subjected to lyophilization using Lyophilizer (Labconco). The concentrated extracts were kept in refrigerator at 4°C and aqueous extract was kept in -20°C until further use.

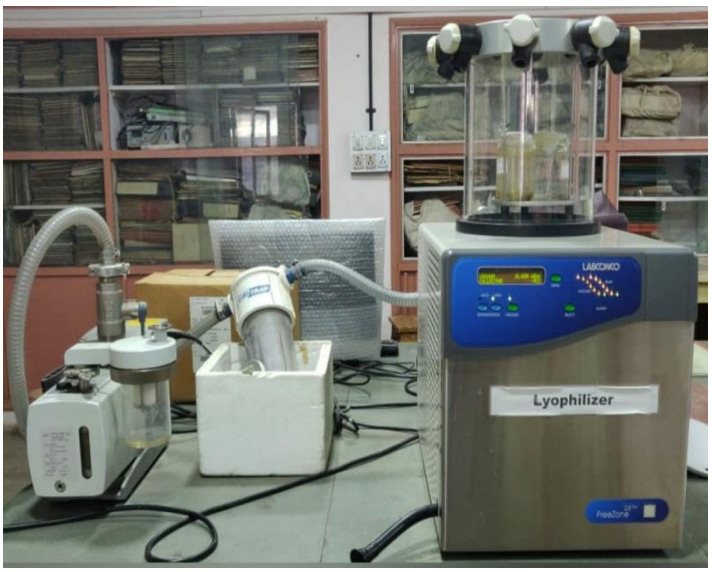

Figure 3.4: Lyophilizer used for the lyophilisation of the aqueous extract

Solvent was recovered from the crude extract and %yield was calculated as below:

$$\% \text{ Yield} = \frac{\text{Weight of precipitate remaining after recovery of solvent} \times 100}{\text{Weight of dried powder used for extraction}}$$

Polar and mid-polar fractions were dissolved in DMSO (< 0.5%) and aqueous extract was added to sterile DMEM, filtered, and used for further experiments.

3.1.3 Phytochemical studies: The class of phytochemical present were qualitatively analysed to understand their nature and chemical composition.

3.1.3.1 Qualitative Analysis

The qualitative chemical tests were performed to establish phytochemical profile of given extracts. Phytochemical screening of various extracts from *B. variegata* leaves were carried for the presence of metabolites such as alkaloids, flavonoids, quinones, glycosides, phenols, carbohydrates, sterols, terpenoids, and saponins.

Table 3.1 Preliminary qualitative tests for phytocompounds

Phytocomponents	Test	Positive result
Alkaloids	Wagner's test: 1mg of extract + 1ml alcohol+ few drops of Wagner's reagent	Red brown precipitate
Carbohydrate	Molish test: 2ml filtrate+ 2 drops of alcoholic solution of α-naphthol (Shake well) + 1ml of concentrated H2SO4 (allow to stand)	Violet ring
Protein	Biuret: 2ml filtrate+2% CuSO4+add 1ml 90% ethanol+ excess of KOH pellets	Pink colour in ethanol
Phenol	Ferric Chloride test: 500mg extract+ 5ml d/w+ few drops of neutral 5% FeCl3	Dark green colour
Flavonoid	Alkaline reagent test: 10% (NH4)3OH+ aqueous solution of extract	Yellow Fluorescence
Steroids	Lieberman-Buchard test: extract in chloroform+1ml of acetic acid & 1ml of acetic anhydride(heated in water bath)+ few drops of H2SO4	Blue green colour
Saponins	Few mg of extract + Distilled water (Shaken)	Stable froth formation
Terpenoids	0.5ml of chloroform extract + 0.5ml conc. H2SO4.	Cherry red colour
Quinones	1ml crude extract + Dilute NaOH	Blue green or red colour
Cardiac Glycosides	Keller Killani test: 5ml of extract+ 2ml of glacial acetic acid containing drop of FeCl3 solution+ 1ml of concentrated H2SO4	Brown ring, violet may appear bellow brown ring

3.1.3.2 Anti-oxidant activity: The anti-oxidant potential of crude extracts was evaluated using 2, 2-Diphenyl- 1-picrylhydrazyl (DPPH) free radical assay (Neha Sharma, Bhardwaj, Kumar, & Kaur, 2011). DPPH (0.3 mM) was added to each tube of extracts, prepared in methanol with final volume 1ml and was incubated at 37°C for 30mins in dark. Ascorbic acid was used as standard. The solution therefore looses colour from purple to yellow which is measured spectrometrically at 517 nm The activity was monitored using a spectrometer at 517nm. Experiment was performed in triplicates. The percentage scavenging of the DPPH free radical was calculated.

$$\%\text{Anti-oxidant activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

3.1.4: Identification of phytocomponents:

3.1.4.1 Thin Layer Chromatography: The mobile phase optimization was carried out using TLC silica gel 60 F_{254} plates (Merck). Various combinations of the solvents chloroform, ethyl acetate and methanol were tested to obtaining a band separation. The optimized mobile phase was found to be chloroform, ethyl acetate and methanol (4:6:5) as a solvent system. The chamber was pre-saturated with mobile phase for 30 minutes. After completion of the elution, the dried plate was subjected to visualization under UV chamber (254nm and 366 nm) and sprayed using spray reagent (5% Anisaldehyde Sulphuric acid solution). Rf values were determined by using following formula (Rf = Distance travelled by the solute / Distance travelled by the solvent). The standards Berbamine dihydrochloride, Rhapontin and Papaverine hydrochloride were used as a positive control.

3.1.4.2 Flash Chromatography: Flash chromatographic system (Isolera-I, Biotage) was used for the separation of compounds from the aqueous extract. It was loaded in OROCHEM EZYFLASH plastic cartridge containing 25 g silica as stationary phase. Elution in flash chromatography was started with mobile phase of chloroform and ethyl acetate (90:10–10:90) with varying ratios and throughout ten-fold increase of methanol, with flow rate of 5 ml/min and pressure 1.5 to 2 bars. Elutes were scanned continuously at 254 nm and 280 nm throughout the experiment. The fractions (each of 20 ml) were collected using fraction collector in test tubes unless an absorbance drift was observed at the set wavelengths. Fractions were concentrated and subjected to TLC.

3.1.4.3 HRLC-MS analysis: High Resolution Liquid chromatography- Mass Spectroscopy (HRLC-MS) analysis of the aqueous leaves extract of *Bauhinia variegata* was carried out using 6550 iFunnel Q-TOFs system equipped with a HiP Sampler, binary pump, and column component. The column outlet was coupled to a Dual AJS ESI for ion source. The mobile phases constituted of water with 0.1% formic acid (A) and 90% acetonitrile in water with 0.1% formic acid (B) at a flow rate of 300 µL /min and the injection volume was 5 µl. The following parameters remained the same throughout the MS experiment: for electro spray ionization (Dual AJS ESI) with positive and negative ion polarity, the nozzle voltage was set to 1000 V, the gas temperature to 250°C, the nebulizer pressure to 35 psi, and the drying gas flow rate to 11 L/ min. The column was held at 95% Solvent A (0.1% formic acid in water) and 5% Solvent B (0.1% formic acid in acetonitrile) for 1 min, followed by a 20 min step gradient from 5% B to 100% B, then 5 min with 100% B. Finally, elution was achieved with a linear gradient from 100% B to 5% B for 5 min. The details of acquisition and evaluation of mass spectrometric data were provided by IIT (SAIF), Mumbai.

3.1.4.4 FTIR analysis: Fourier-transform infrared spectroscopy was performed to know the possible functional groups present in the aqueous extract of *B. variegata* leaves. The lyophilized aqueous extract (10 mg) was encapsulated in of KBr (100 mg) pellet to organize translucent sample discs. The prepared sample was loaded in FTIR spectroscope (FTIR spectrophotometer, SHIMADZU) with a scan range from 600 to 4000 cm^{6}.

3.*2* Materials and Methods for *in vitro* cell migration and cell invasion studies.

3.2.1 Cell culture conditions: Two metabolically different human breast cancer cell-lines (MCF-7 and MDA-MB-231) were procured from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Himedia) supplemented with 10% fetal bovine serum (Gibco - Invitrogen) and 1% antibiotic (PSN- 5mg of Penicillin, 5mg of Streptomycin and 10 mg of Neomycin per ml (Gibco - Invitrogen). Cell lines were maintained at 37^{o}C in a 5% (v/v) CO2 atmosphere with 95% (v/v) humidity in a humidified incubator (Thermo

Scientific, India). Cultures were maintained in 25 cm2 tissue culture flasks and passaged thrice weekly using trypsin–EDTA (Himedia) to detach the cells.

3.2.2 Spheroid generation by Hanging drop method:

Adherent cell cultures were grown to 90% confluence, rinsed twice with PBS, trypsinised and Centrifuged at 200 X G for 5 minutes. Cells were counted using Neubauer chamber. 2×10^6 cells/ml were taken for the spheroid formation. Lid from a 60 mm tissue culture dish was inverted and 2 ml of PBS was added in the bottom of the dish to act as a hydration chamber. The lid of the dish was inverted and 30 µl drops were deposited onto the lid. Cells are suspended in droplets of medium will form coherent 3 D aggregates (spheroids). The lid with droplet was again inverted and placed onto the PBS-filled bottom chamber, incubated at 37°C/5% CO_2/95% humidity, the drops were monitored daily and incubated until either cell sheets or aggregates are formed. A stereo-microscope (Fisher-Scientific) was used to assess aggregate formation. The spheroids were thereafter carefully embedded in 1% agarose without disturbing them. These spheroids embedded in agarose were further monitored for its invasive properties in presence of extract and TNF-α. Bright-field images were captured on Nikon T200 inverted Phase-contrast microscope. The size of the tumor spheroids was measured on Nikon NIS-Elements BR software.

3.2.3 Trypan blue exclusion assay:

Cells were trypsinized using Trypsin EDTA and subjected to centrifugation at 1500 rpm for 3 mins. The supernatant was discarded and pellet was used for further experiments. The pelleted down cancer cells (10 µl) were stained with equal volume of tryphan blue dye (Hi-media). The stained cells were observed under bright field microscope. The live cells were transparent whereas the dead cells were blue in color due to damage of cell membrane. The percentage of live cells were calculated before each experiment.

3.2.4 Cytotoxicity Assay: The cytotoxic potential of plant extracts against breast cancer cell lines (MCF-7 and MDA-MB-231) was measured using MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Cells were seeded in 96-well microplates at a concentration of 10,000 cells/well. Cells were allowed to grow overnight. Untreated cells grown in medium served as control. Fresh media modified with different concentrations of the test extracts was added for different time intervals (24 hrs, 48 hrs and 72 hrs). After incubation period, MTT (5

mg/ml) solution was added followed by 3 hours incubation, medium with MTT was removed and the formazan crystals were solubilized in 100 µl Dimethyl sulfoxide (DMSO). The absorbance of each well was measured at 575 nm by ELISA microplate reader (Thermo Scientific). Paclitaxel (10 nM) was used as a reference drug. IC50 values was calculated and extract which gave best activity was selected for further experiments. MTT was also performed for commercially available standards Berbamine dihydrochloride (sigma-aldrich), Rhapontin (sigma-aldrich) and Papaverine hydrochloride (sigma-aldrich). Cell proliferation was also checked in presence of Tumor Necrosis Factor-alpha (10 nM/ml) (Enzo, Life Sciences) and 17-β-estradiol (10 nM/ml) (sigma-aldrich). The percentage cell viability was calculated and graph was plotted. Experiment was performed in triplicates. Statistical analysis was done using Graph Pad Prism 5.0 software.

3.2.5 Microscopic analysis for morphology studies: Cells were plated on cell culture plates and allowed to grow for 24 hrs in 6 well plate. The cells were monitored using bright field microscope (Nikon eclipse Ti2-E, Japan). For spheroids experiment also, the cells were observed under bright field microscope

3.2.6 Cell Migration Assay

3.2.6.1 Wound Healing Assay:

Cells were allowed to grow till confluency. The Confluent cells were rinsed with PBS and starved in low serum media (2% serum in DMEM) overnight. A scratch in a straight line was made with a 200µl pipette tip to create a wound on the monolayer of cells. The cells were rinsed with PBS and treated with media containing different concentrations of aqueous extracts for 24 hrs. The cells were monitored at different time-point (0, 24,48,72 hr) and stained with crystal violet. The captured images were further analyzed by using ImageJ software.

3.2.6.2 In-house assay developed for cell migration assay:

Boyden's chamber like assemble was arranged to perform cell migration assay with cancer cells. The setup was prepared in lab, holes were made in caps of sterile tarson tubes, which were placed in 6 well culture plate. Low serum media was filled in the caps and non-leaky caps were selected for experiment. A filter (Merck) was placed in

the cap and the cells were loaded on to it. High serum media was added to the surrounding and cells were observed for their migratory characteristics.

3.2.7 Clonogenic cell survival assay: -

Clonogenic activity was assessed by colony forming assay (Jia et al., 2014). 1000 cells per 6-well plate were seeded. After 24 hrs, the cells were treated cells were cultured for 7-15 days (according to cell type) in standard conditions of 5% CO_2 at 37°C. The media was aspirated and supplemented with respective treatments with regular change of respective treatment media every two days. The cells were washed with PBS, fixed with cold methanol, and stained with 0.5% crystal violet. The solution was removed and washed with distilled water. The cells were then left to dry at RT. The images of the plate were captured and number of colonies having more than 50 cells per well counted. The clonogenic capability of each cell line is presented as the percentage of plating efficiency. Plating efficiency is the ratio of the number of colonies to the number of cells seeded and calculated as following:

P.E.= (Number of colonies formed/Number of cells seeded) x 100 %

3.3 Materials and Methods for *in vitro* cell death studies.

3.3.1 Quantification of Reactive oxygen species (ROS):

Cells were plated on a 12-well plate at the density of $1.5x10^5$ cells/well and allowed to grow for 24 hrs. H_2O_2 (50µM) was used as positive control. After treatment with aqueous extract and standards, intracellular ROS production was measured by CM-H_2DCFDA (10 µM) in DMEM (Figueroa, Asaduzzaman, & Young, 2018). The cells were washed thrice using PBS. For intracellular ROS quantification was done at 525 nm using F-7000 Fluorescence Spectrophotometer (Hitachi, Japan).

3.3.2 Determination of Mitochondrial membrane potential: Cells were plated on 12 well plates at the density of $1.5x10^5$ cells/well and allowed to grow for 24 hrs in 24 well plate. After treatment with aqueous extract and standards, and mitochondrial potential was measured using etramethylrhodamine, methyl ester (TMRM) (5µM TMRM). The quantification of fluorescence was done at 510 nm Fluorescence microscopy was done using fluorescent microscope (Nikon eclipse Ti2-E, Japan) and analysed by NIS-elements advanced research imaging software (Version 5.20, Nikon, Japan).

3.3.3 Apoptotic assays: Cells were plated in 24-well plate at density of $1.5x10^5$ cells per well and allowed to grow for 24 hrs in 24 well plate. After treatment with aqueous extract and standards, cells were stained using Annexin V-PI kit (Sigma-aldrich) (Rieger, Nelson, Konowalchuk, & Barreda, 2011). Minimum 100 cells were counted and graph was plotted. The fluorescence microscopy was done using fluorescent microscope (Nikon eclipse Ti2-E, Japan) and analysed by NIS-elements advanced research imaging software (Version 5.20, Nikon, Japan).

3.3.4 Protein expression studies using Western blot

Cells were plated at a density of 4.5×10^5 cells/well in the six well plate and allowed to grow overnight. After 24 hr, the cells were harvested, washed with ice cold PBS (Hi-media) and NP40 lysed in buffer (150 mM NaCl, 50 mM Tris–Cl, 1% NP40, 1mM PMSF) (Roche, Germany). Protein estimation was done by Bradford assay and equal protein was loaded and resolved on 12% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110 V for 1 h at 4 °C. The membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) (Sigma-Aldrich, USA) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody [(Caspase-8, PARP, Bcl2) Cell Signaling Technology, Inc, USA)]. After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween 20) for 10 min and incubated with a secondary antibody [anti-rabbit antibodies (Thermo Scientific, USA)] at room temperature for 1 h. β-Actin (Abcam, USA) was used as housekeeping gene. The membrane was washed three times with TBS-T and signal was visualized by using EZ-ECL chemiluminescence detection kit for HRP (Takara Bio Inc, Japan) by exposing it to X-ray film.

3.4 Statistical Analysis

All experiments have been repeated minimum three times independently and the data is expressed as mean ± SEM. GraphPad Prism was used to perform all the statistical analysis (8.0 version). One-way ANOVA (Dunnett's multiple comparison test) is used to calculate degree of significance wherever there are more than two groups. For Multiple groups comparison two-way ANOVA (Tukey's multiple comparison test) is used. The probability values of $p<0.05$ were considered as statistically significant.