MATERIALS AND METHODS

Protocols for the present experiments were approved by IAEC (Institutional Animal Ethics Committee) of Department of Zoology (827/ac/04/CPCSEA), Faculty of Science, according to CPCSEA, India (Committee for the Purpose of Control and Supervision of Experiments on Animals).

The entire study was carried out in three parts:

1. *In vivo,*
2. phytochemical analysis and
3. *In vitro*.

Part I: *In vivo* studies

Animal Model:

Healthy young adult Wistar rats (*Rattus norvegicus*), nonpregnant females were obtained from the animal breeding facility, SPARC (Sun Pharma Advance Research Center), Vadodara, Gujarat, India. Animals were acclimatized for ten days and then were randomly allocated to control and treatment groups of six by weight. Individual animals were identified with picric acid marking on the body coat and cage card showing experimental group and animal numbers. The animals were housed in clean, sterilized, solid polypropylene cages. Clean rice (paddy) husk was used as the bedding material. The animals were fed with Amrut Brand Laboratory Animal pellet feed (manufactured by Pranav Agrochemicals Ltd., Pune – 411 030) and water was provided in polypropylene bottles. Both feed and water were provided *ad libitum* to all animals. Fresh feed and water was supplied on alternate day.

Environmental Conditions

The animals were maintained in climatically controlled experimental room with a relative humidity range between 50 and 70%. The temperature of the experimental room was maintained between 20° and 24°C. The photoperiod of the experimental room was maintained manually as 12 h artificial light and 12 h darkness.

Route of Administration of Test compound:

The administration of test compound with feed is the most cost efficient method of administration. However, it is difficult to measure feed consumption accurately and concentrations of the feed must be recalculated every week. Moreover, it becomes practically difficult to accurately calculate the herbal intake of individual rats. Hence, the oral route of exposure by gavage (most recommended route of oral dosing) was used in the present study.

Physical Examination

A complete physical examination was performed on each rat. Physical examination was conducted on a daily basis for checking of infection or physiological abnormality. All the rats were examined on daily basis during the entire phase of the experiment.

Body Weight

Individual animals were weighed on the day of commencement of treatment and at weekly intervals thereafter till the end of the experiment.

Feed Consumption

Daily food consumption was taken in to account for entire study periods. After acclimatization, the animals were divided into different groups depending on the experimental protocol and are described in individual chapters.

Ovariectomy (OVX) Procedure/ Experimental Protocol:

Acclimatized rats were then processed for OVX**.** For OVX, rats were kept in aseptic environment and were anesthetized using sodium pentobarbital (40 mg/kg BW) and diazepam (10mg/kg BW). Bilateral ovariectomy was performed on the OVX animals giving dorsal midline incision and ovaries were pulled out. Sham operated rats underwent a sham procedure in which the ovaries were touched only with forceps. In Sham as well as the OVX rats the muscle layer, incision and skin were closed with two stitches and sutured.

After surgery animals were checked for any kind of infection or pathogenesis. Every evening the wounds were cleaned with Dettol and soframycin cream was applied for preventing infection and to facilitate the wound healing. Two weeks after recovery, the OVX rats were separated based on their weights in to different groups of six each. Blood was collected from orbital sinus

puncturing using heparinized capillaries under mild ether anesthesia for biochemical analysis; serum was then prepared by centrifugation of the collected blood (3000 rpm for 20 min) and stored at -80 °C for biochemical analyses.

For collection of tissue, animal was killed by cervical dislocation under ether anesthesia. Once dead, the animal was dissected and bone, uterus and liver tissues were taken out for histology and biochemical analysis. All the tissues were washed thrice in chilled PBS (pH 7.4). Uterus was separated from the surrounding adipose and connective tissues and weighed and sectioned for histological analysis. The femurs were dissected out and cleaned of all soft tissue, then wrapped in saline-soaked tissue blots, sealed in plastic bags and stored at -80 °C for further analysis.

Measurements:

Serum Chemistry

Serum calcium concentrations were measured by standard colorimetric methods as prescribed in the kit using an automatic analyzer, Perkin Elmer and commercial kits (Reckon Diagnostics). Serum Phosphate concentrations were measured by standard colorimetric method as prescribed in the kit using an automatic analyzer, Perkin. Serum creatinine concentrations were measured by standard colorimetric method as prescribed in the kit using an automatic analyzer, Perkin Elmer. Serum Hydroxyproline levels were measured by standard methods of Nueman and Logan and modified by Levin (1972). Breifly, Samples were mixed with $CuSO_4$, NaOH and H_2O_2 and heated at 80°C for 5 min. After heating the tubes were chilled in the ice bath and H_2SO_4 and p-DMAB were added and the tubes were heated for 16 min at 70°C and then cooled. After cooling the pink color formed was read at 540nm using Perkin Elmer auto analyzer and compared with standard hydroxyproline absorbance.

Serum AlP concentrations were measured by standard enzymatic method using an automatic analyzer, Perkin Elmer and commercial kit purchased from Reckon Diagnostics. Briefly, the tablet from the kits were dissolved in the buffer and allowed to settle at room temperature for 15 min. After 15 min 20 µL of the serum was added to 1 mL of the solution and read at 405 nm for 2 min at 30 second intervals. The mean change in the absorbance per minute were calculated and multiplied with 2713 to obtain the AlP activity in IU/L. Serum TRAcP concentrations were

measured by standard enzymatic method using an automatic analyzer, Perkin Elmer and commercial kit purchased from Reckon Diagnostics. Briefly, the tablet from the kits were dissolved in the buffer and allowed to settle at room temperature for 15 min. After 15 min 100 µL of the serum was added to 1 mL of the solution and read at 405 nm for 10 min at 60 seconds interval. The mean change in the absorbance per minute was calculated and multiplied with 860 to obtain the TRAcP activity in IU/L.

Tissue estimations/Estimation of Enzymes:

At the end of the experiment bone tissue (femur) was removed, freed of adherent tissues. Bones were then homogenized in PBS at 4°C using mechanical homogenizer. The homogenate was then used for assessment of Bone AlP and TRAcP using enzymatic methods on Perkin Elmer autoanalyzer.

Estimation of Calcium and Hydroxyproline

Bone was dissolved in 6N HCl for 48 hours. After complete dissolution, the solution was evaporated on sand bath to yield white crystalline powder. This powder was dissolved in Tris buffer pH 8.8 and analyzed using commercially available test for calcium. Hydroxyproline was estimated following the method of Neuman and Logan (1950) and modified by Levine (1972) which we have already discussed.

Tissue profile:

Preparation of Tissues

The whole process of histopathology was carried out as per the steps described by Brar *et al*. (2000). Uterus and bone were immersed in a solution of 4% neutral buffered paraformaldehyde for fixation and then further processed. The tissues were washed in 70% alcohol, to remove excess fixative. The tissues were then transferred into perforated capsules for further processing. For bones, the tissue was decalcified in 14 % EDTA. Processed tissues were embedded in paraffin using routine method. Representative transverse sections 5 – 7 µm thick sections were cut and stained with haematoxylin and eosin. Histopathological examination of slides was performed using light microscope.

Part II Phytochemical analysis

Plant Material

Preparation of Methanolic extract

Fruits, leaves and flowers of MO was obtained and standardized as per our previous studies (Rangrez *et al.,* 2011). Dried powder was prepared by drying MO in oven at 50º C. 100 gm dried powder of each component was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hours . Methanolic extract was dried on water bath at 55º C. The percentage yield of the plant was found to be 9.8%, 6.3% and 7.7% for fruits, leaves and flowers respectively. The plant extract was freeze dried and stored at -70º C. For *in vivo* assay the working solution was prepared in 0.9% saline, for *in vitro* working solution was prepared by dissolving the extract in DMEM and filtering using 0.23µ filter.

Preliminary screening of phytochemicals:

Preliminary phytochemical analysis was carried according to the methods described by Daniel (1992) and individual bioactive phytochemicals molecules of *L.glutinosa* were preformed as follows:

Test for Flavonoids

Test for flavonoids were carried out as described by (Shastry *et al.,* 2010; Devmurari *et al.,* 2010)

1. Shinoda's test: 1 gm of powder + 10 ml alcohol + a piece of magnesium + 5 ml HCl → red color →indicate presence of flavonoids.

2. Zn-HCl reduction test: 1 ml aqueous extract + zinc dust 100 mg + few drops of concentrated HCl → red color indicates presence of flavonoids.

3. H_2SO_4 test: 5 ml aqueous extract + 5 ml of dilute ammonia solution + 2 ml concentrated H_2SO_4 →A yellow coloration observed in extract indicated presence of flavonoids.

4. Lead Acetate Test: 2 ml of ethanolic extract + 1 ml lead acetate solution
→ Formation of yellow precipitate showed the presence of flavonoids.

Terpenoids:

Test for terpenoids were carried out as described previously (Daneil, 1991).

10 ml chloroform + 20 mg of crude extract → filter + 2 ml acetic anhydride + concentrated H_2SO_4 → a blue green ring should appear on top of the mixture.

Phenol:

Test for phenols were carried out as described previously (Zorica *et al.,* 2009; Reza *et al.,* 2009; Sahu *et al.,* 2010).

$FeCl_3$ Solution Test: 2 ml ethanolic extract + 2 ml of 5% $FeCl_3$ → deep blue black color showed the presence of phenols.

Tannin:

Test for tannins were carried out as described previously (Zorica *et al.,* 2009; Reza *et al.,* 2009; Sahu *et al.,* 2010).

Lead Acetate Test: 2 ml of ethanolic extract + 2 ml lead acetate → white precipitates indicate the presence of tannins.

Gelatin test: 2 ml of ethanolic extract + 2 ml of 1% gelatin + 4 ml 10% sodium chloride → white precipitate.

$K_2Cr_2O_7$ Test: 5 ml plant extracts + 1ml 10% $K_2Cr_2O_7$ → Yellowish brown precipitate.

Saponin:

Test for saponins were carried out as described previously (Daniel, 1991)

1. Foam Test: 1 ml of alcoholic extract + 20ml distill water → a thick layer of foam indicated presence of saponin.

2. Lead Acetate Test: 1ml extract + 1 ml of 1% lead acetate → Formation of white precipitate indicated the presence of saponins.

Glycosides

Test for glycosides were carried out as described previously (Kodangala *et al.,* 2010; Sahu *et al.,* 2010)

1. Molisch's test: 2 mg of ethanolic extract + 10ml of water + 2-3 drops of Molisch's reagent + 2ml concentrated sulphuric acid → Reddish violet ring appear, indicating the presence of glycosides.

2. H_2SO_4 Test: 2 ml of extract + 3 ml of 3% ferric chloride + 3 ml of acetic acid → green precipitate + 1 ml sulphuric acid → Formation of a brownish red layer after effervescence at the interface was due to aglycone and the development of greenish blue color in the upper layer (acetic acid) layer was due to sugar.

Test for Cardiac Glycosides

Test for cardiac glycosides were carried out as described previously (Zorica *et al.,* 2009; Reza *et al.,* 2009; Sahu *et al.,* 2010).

1. Keller killiani test [test for Deoxy sugars]: 2 ml of the extract + glacial acetic acid + one drop 5% $FeCl_3$ + conc. H_2SO_4 → Reddish brown color appeared at junction of two liquid layers and upper layer turned bluish green indicating the presence of glycosides.

2. Kedde's test: chloroform extract + add one drop of 90% alcohol and 2 drops of 2% 3, 5-dinitro benzoic acid (3, 5-dinitro benzene carboxylic acid-Kedde's reagent) in 90% alcohol + alkaline 20% sodium hydroxide solution → a purple color is produced.

Alkaloids:

Tests for alkaloids were carried out as described previously (Kam *et al.,* and Khaleque *et al.,* 2001). 20 mg of plant crude extract was added to 10 ml methanol and placed in a sonic bath to dissolve. The extract was then filtered using a Whatman filter paper No.1; 2 ml of filtrate was taken and mixed with 1% HCl. Three different tests were performed for alkaloids.

1. Mayers' test: 1 ml of mixture + 6 drops of Mayer's reagent → yellowish creamish precipitate indicating the presence of alkaloids.
2. Wagner's Test: 1 ml of mixture + 6 drops of Wagner's reagent → brownish red precipitate

indicating the presence of alkaloids.

3. Dragendroff's test: 1 ml of mixture + 6 drops of Dragendroff's reagent → orange precipitate indicating the presence of alkaloids.

GC MS analysis:

Gas chromatographic analysis

GC/MS analysis was carried out using Perkin Elmer autosystem XL with turbo mass system equipped with PE 5 MS 30m X 250 micron silica capillary. Injector and detector temperatures were 250º and 300ºC, respectively. The temperature started from 70º C for 5 min and then rose to 290º C at the rate of 10º C per minute. Helium was used as carrier gas. The MS was taken at 70 eV. Scanning speed was 0.84 scans s^{-1} and the scanning period was from 40 to 550 s. Sample volume was kept 3 µL.

PART III *IN VITRO*

Preparation of Bovine Cortical Bone Slices:

Bovine cortical bones slices were prepared as described by Richard and coworkers (1997). The bones were collected fresh from a local slaughterhouse in anti microbial anti fungal liquid, obtained from Himedia Chemicals, Mumbai. Bone slices were cut into 8 x 8 x 3 mm^3 (l x b x w). The slices were then defatted by sonicating for two 30 min periods in 5% solution of Triton X 100 in double distilled water for 30 minutes and rinsed in distilled water, then sterilized with 75% ethanol, dried, and left under ultraviolet light for 15 minutes before use.

Isolation and Activation of Osteoclasts:

Osteoclasts were isolated from young rats as per the standard protocol (Richard *et al.,* 1997). The activation of osteoclasts is achieved by using a combination of vitamin D and dexamethasone (Qin *et al.,* 2003). Long bones were removed and freed of adherent muscle and their epiphyses were cut off with a scalpel at the level of the growth plate and discarded. The dissection was performed on a dissecting board using a scalpel and fairly fine forceps to hold the bones. Once clean, the bones are then transferred to 35 mm plastic tissue culture plates containing 3.5 ml chilled Hank's Buffered Salt Solution (HBSS), obtained from Himedia Chemicals, Mumbai,

without serum. Bones are then minced to create a cell suspension, using a scalpel and holding the bones with forceps. The bone debris was then repeatedly sucked in and out of a transfer pipette so as to get maximum number of osteoclasts. The petri dishes were then tilted at a slight angle so as to allow the bulk of the bone matrix to settle for about 10 seconds into the corner of the dish and the cell suspension was then taken up gently from the top of the liquid into a transfer pipette, trying to avoid pieces of bone. These cells were then co cultured with osteoblastic cells isolated from calvarial bones by the method of Liu *et al.,* (1995) in DMEM medium containing 10 % FBS, 1X anti microbial antifungal, 1 gm/l glucose, L glutamine, sodium pyruvate, sodium bi carbonate, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and 1,25-dihydroxyvitamin D3 (10 nmol/l) and dexamethasone (100 nmol/l) at 37ºC in a humidified atmosphere for 48 hrs in 24 well culture dishes (1.5 ml per well). After 24 hrs of activation, culture liquid, dead and non attached cell were removed. Cells were again incubated for 24 hours. After 48 hours attached cells were detached from the wells by washing with 0.1% EDTA Trypsin solution to recover cells. The cells were counted to be 1X 10^9/l. 500 µl of these solution was loaded on each bone slices in 24 well plate containing 1 ml culture liquid to study the resorption. For cell blank only 500 µl of DMEM was added. The formation of osteoclast-like MNC (multinucleated osteoclasts) was confirmed by the staining of TRAP and resorptive pit formed on bone slices. Treatment of *Moringa* plant extract was given in 3 doses *viz.*100 µg/ml, 200µg/ml and 400 µg/ml. After 24 and 48 hrs of culture, solution was collected for analysis of calcium release and enzyme activity. Cold calcium release assay was carried out according to the standard procedure (Sajeda *et al.,* 1997). Alkaline phosphatase (AlP) and tartrate-resistant acid phosphatase (TRAcP) activities were measured spectrophotometrically using the commercial kits purchased from Reckon Diagnostics Pvt. Ltd. Baroda.

SaOS 2 cell line culture:

SaOS 2 cell line was obtained from NCCS, Pune, India and cultured as described by (Thangakumaran *et al.,* 2009). The cells were cultured in a humidified atmosphere (95% air, 5% CO_2) at 37º C. Cell culture medium (DMEM containing 1% anti microbial and antifungal solution) and supplements (10% FBS) were purchased from High Media, Mumbai. Upon reaching confluence, the cells were detached using Accutase (High media) solution and loaded in

96 well plate (Merck Scientific, Bombay).The total duration for culturing was 96 hours. The treatments of the extracts were given in six increasing concentrations i.e.10 µg/mL, 20 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL and 400 µg/mL. After 96 hours, MTT and AlP assays were carried out to understand the effect of the plant extracts on osteoblastic cells. Acridine orange-ethidium bromide staining was carried out to confirm the toxic effects, if any.

MTT test

SaOS-2 were incubated in a DMEM containing 10% FBS under partial pressure of 5% CO_2 at 37 º C. MTT assay was carried out by placing each cell into each well of 96-well plate; adding 0.05% DMSO containing samples into each well and incubating them for 72 h; adding 0.5 mg/mL MTT into each well and further incubating them for 4 h; dissolving produced formazan crystals in DMSO; and measuring their absorbance at 550 nm using ELISA reader as described previously (Ha *et al.,* 2003).

Statistical Analysis

Raw data were processed and analyzed to give group means and standard error with significance, wherever the difference occurs between the controls and treated groups. All the parameters characterized by continuous data were subjected to relevant statistical method (*viz*., Bartlett's test, ANOVA, Dunnett's test or Student's 't' test) using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA (Motulsky, 1999). Microsoft Excel was also used when found necessary. Significance was calculated at 5 % level.

Biochemical estimations

Calcium concentrations were measured by standard colorimetric methods as prescribed in the kit using an automatic analyzer, Perkin Elmer and commercial kits (Reckon Diagnostics). Hydroxyproline levels were measured by standard methods of Nueman and Logan and modified by Levin (1972), as described previously.

AlP concentrations were measured by standard enzymatic method using an automatic analyzer, Perkin Elmer and commercial kit purchased from Reckon Diagnostics. Serum TRAcP

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