5 EXPERIMENTAL

5.1 Collection and characterization of plant materials

5.1.1 Collection of plant materials

The stem bark of *Acacia arabica* and *Terminalia arjuna* were purchased from Lallu Vrajlal Gandhi (LVG) herbal store, Ahmedabad, Gujarat, India. The voucher specimen (Number: KBIPER/2012/V/01 and KBIPER/2012/V/02 respectively) of the stem barks were deposited in the Pharmacognosy department of K. B. Institute of Pharmaceutical Education and Research, Gandhinagar. The dried barks were ground to coarse powder using pulverizer and then stored in air-tight container in cool, dark and dry place till further use.

The oleogum resin of *Commiphora mukul* and *Boswellia serrata* were purchased from LVG, Ahmedabad, Gujarat, India. The voucher specimen (Number: KBIPER/2012/V/03 and KBIPER/2012/V/04 respectively) of the materials were deposited in the Pharmacognosy department of K. B. Institute of Pharmaceutical Education and Research, Gandhinagar.

5.1.2 Authentication of plant materials

5.1.2.1 Macroscopic and Microscopic evaluation

The stem bark of *Terminalia arjuna* and *Acacia arabica*, in whole form and powdered form were studied for macroscopical and microscopical characters. Free hand transverse sections of stem barks were taken and studied.

Oleogum resin of *Commiphora mukul* and *Boswellia serrata* were subjected to macroscopic studies which comprised of study of organoleptic characters of the drugs viz., color, odour, appearance, taste, smell, texture and fractures.
5.1.3 Characterization of the plant materials

The quality parameters were established for the plant materials before commencing the study. This was done with the objective of characterizing the plant materials which can help to maintain uniformity in the quality of different batches of materials collected during the course of study. Following quality parameters were established for each drug:

• Loss on drying
• Total ash value
• Acid-insoluble ash value
• Extractive values (water and alcohol soluble)
• TLC fingerprint

5.1.3.1 Determination of Loss on Drying

Ten g of dried drug was placed in evaporating dish. The drug was dried at 105°C for 5 h and weighed. Drying and weighing was continued at one hour interval until difference between two successive weighing corresponded to not more than 0.25%. Constant weight was considered to reach when two successive weighings after drying for 30 min in a desiccators, show not more than 0.01 g difference. (163)

5.1.3.2 Determination of Ash Values

Ash values of powdered drugs were determined by the following methods. (164)
5.1.3.2.1 Determination of Total Ash

Two g of accurately weighed powder was incinerated in a crucible at a temperature 450°C in a muffle furnace until it was white indicating the absence of carbon. If carbon free ash could not be obtained in this manner, cool the crucible was cooled and the residue moistened with about two ml of water or a saturated solution of ammonium nitrate. It was dried on a water bath and then ignited to constant weight. It was then cooled, weighed and percentage of ash was calculated with reference to the air-dried powdered drug.

5.1.3.2.2 Determination of Acid Insoluble Ash

The ash obtained above was boiled for 5 min with 25 ml of 70 g/l hydrochloric acid and filtered using an ashless filter paper to collect insoluble matter. The ash obtained was washed with hot water and filter paper was burnt to a constant weight in a muffle furnace at 450°C. The percentage of acid-insoluble ash was calculated with reference to the air-dried powdered drug.

5.1.3.3 Determination of Extractive Values

Extractive values of powders of drugs were determined by the following methods: (165)

5.1.3.3.1 Determination of Water Soluble Extractive (Hot Extraction)

Four g of the powdered material was soaked in 100 ml of water in a closed flask for 1 h and was shaken frequently. It was then boiled gently for 1 h on water bath, cooled and weighed and the weight was readjusted. Twenty five ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105°C to a
constant weight. The percentage of water-soluble extractive was calculated with reference to the air-dried powered drug.

5.1.3.3.2 Determination of Alcohol Soluble Extractive (Cold Extraction)

Four g of the air-dried powdered materials was macerated with 100 ml of alcohol in a closed flask for 6 h, shaking frequently at an interval of 1 h. It was then allowed to stand for 18 h and filtered rapidly to prevent any loss during evaporation. Twenty five ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105 °C to a constant weight. The percentage of alcohol soluble extractive was calculated with reference to the air-dried powdered drug.

5.2 Preparation of extracts of herbal drugs selected for study

5.2.1 Aqueous extracts of stem barks - *Acacia arabica* and *Terminalia arjuna* (AAA and TAA)

Barks of *Acacia arabica* and *Terminalia arjuna* were powdered and aqueous extract was prepared using hot maceration technique. Hundred gm of powder was mixed with 1000 ml of distilled water and then it was heated on boiling waterbath for six hours and allowed to stand overnight. The mixture was then filtered and the marc was extracted twice again in the same manner. The filtrates from each extraction step were pooled and concentrated to dryness.

5.2.2 Methanolic extracts of stem barks- *Acacia arabica* and *Terminalia arjuna* (AAM and TAM)

Powdered bark of *Acacia arabica* and *Terminalia arjuna*. 100 g of each, were extracted separately with 1000 ml of methanol by heating under reflux on waterbath for 6 hours at 55°C. The mixture was then filtered and the marc was extracted twice again in the same manner. The filtrates from each extraction step were pooled and
concentrated under vaccum using a rotary vaccum evaporator. The concentrate was evaporated to dryness at temperature not exceeding 60° C.

5.2.3 Petroleum ether extracts of oleogum resins of Commiphora mukul and Boswellia serrata (CMP and BSP)

Oleo gum resins of Commiphora mukul and Boswellia serrata, 100 g each were washed under tap water followed by washing with distilled water. They were further air-dried on filter paper at room temperature and then powdered with the help of pestle and mortar. Further air-dried powder (10 g) of the resins was thoroughly mixed with 500 ml Petroleum ether, and allowed to stand for 24 hrs. Solution was filtered through muslin cloth and then re-filtered by passing through Whatman’s Filter No.1. Then filtrate was concentrated to oily residue at room temperature (25°C) to yield the pure extracts. Then extracts were stored in refrigerator (4°C) until further use.

5.2.4 Ethyl acetate extracts of oleogum resins of Commiphora mukul and Boswellia serrata (CMEt and BSEt)

The ethyl acetate extracts were prepared by following the same procedure described in section 5.2.3, but ethyl acetate was used as a solvent for extraction instead of petroleum ether.

5.3 Preliminary Phytochemical Screening of plant extracts

Extracts were subjected to various qualitative tests to detect the presence of phytoconstituents like alkaloids, flavonoids, saponins, carbohydrates, sterols and terpenoids, anthraquinone glycosides, coumarins and tannins
5.3.1 Preparation of test solution

5 g extracts of entire herb was extracted with 50 ml of 50% v/v methanol for 15 min on a boiling water bath and filtered. The filtrate was used for following tests:

5.3.1.1 Test for Alkaloids

1 g of powder was extracted with 20 ml alcohol by refluxing for 15 min and filtered and the filtrate was evaporated to dryness. The residue was dissolved in 15 ml of 2N H₂SO₄ and filtered. After making alkaline, the filtrate was extracted with chloroform. The residue left after evaporation was tested for the presence of alkaloids with Dragendorff’s reagent. Formation of orange coloured precipitates indicates the presence of alkaloids. (166)

5.3.1.2 Tests for Flavonoids

5.3.1.2.1 Shinoda Test

To the extract of powder, a small piece of Magnesium ribbon and 3 to 4 drops of concentrated Sulphuric acid was added. Development of red colour indicates the presence of flavanones. (167)

5.3.1.2.2 Fluorescence Test

1 g powder was extracted with 15 ml methanol for 2 min on a boiling water bath, filtered while hot and evaporated to dryness. To the residue, 0.3 ml 3% w/v boric acid solution and 1 ml 10% w/v oxalic acid solution were added. The mixture was evaporated to dryness and the residue was dissolved in 10 ml ether. Observe fluorescence in the ethereal layer under U.V. light. Development of greenish fluorescence in ethereal layer under U. V. indicates the presence of flavonoids. (168)
5.3.1.3 Test for Saponins

5.3.1.3.1 Froth Test

0.1 g of powder was vigorously shaken with 5 ml of distilled water in a test tube for 30 sec and was left undisturbed for 20 min. Persistent froth indicates the presence of saponins. (169)

5.3.1.4 Tests for Carbohydrates

5.3.1.4.1 Molisch’s Test

To the extract, α-naphthol and concentrated H\textsubscript{2}SO\textsubscript{4} were added. Development of purple coloured ring indicates the presence of carbohydrates. (170)

5.3.1.4.2 Fehling’s Test

To 1 ml extract of the powder, 1 ml of the Fehling solution (Fehling A + Fehling B) was added. The mixture was heated on boiling water bath for 5-10 min. Development of yellow precipitates, changing to brick red precipitates indicates the presence of reducing sugars. (170)

5.3.1.5 Test for Sterols and Triterpenoids

5.3.1.5.1 Libermann Burchardt’s Test

1 g powdered drug was moistened with 1.0 ml of acetic anhydride and 2 drops of sulphuric acid on a clean tile. The powder was mixed well. Development of red color in the powder indicates the presence of sterols and triterpenoids. (171)

5.3.1.5.2 Salkowski Reaction

To the 2 ml of extract, 2 ml Chloroform and 2 ml concentrated H\textsubscript{2}SO\textsubscript{4} were added and shaken well. Appearance of red colour in Chloroform layer
and greenish yellow fluorescence in acid layer indicates the presence of sterols and triterpenoids. (171)

5.3.1.6 Tests for Tannins

5.3.1.6.1 Test with Gelatin

To 2-3 ml of extract, 1% w/w gelatin solution containing NaCl was added. Formation of heavy white precipitates indicates the presence of tannins. (172, 173)

5.3.1.6.2 Test with Lead acetate

To the extract, 2 ml of 10 %w/w solution of lead acetate was added. Formation of heavy dull yellowish precipitates indicates the presence of tannins. (171)

5.3.1.7 Tests for Coumarins

5.3.1.7.1 With Ammonia

A drop of ammonia was taken on a filter paper. To this, a drop of extract of powder was added. Appearance of fluorescence indicates the presence of coumarins. (174)

5.3.1.7.2 With Hydroxylamine hydrochloride

To the extract add a drop of saturated alcoholic hydroxylamine hydrochloride solution and a drop of alcoholic Potassium hydroxide. The mixture was heated, cooled and acidified with 0.5N Hydrochloric acid and a drop of 1% w/v FeCl₃ solution was added. Development of violet color indicates the presence of coumarins. (174)
5.4 Evaluation of extracts for anti-osteoporotic activity using *in vitro* model

5.4.1 *In vitro* model

The study protocol was approved by IAEC - Protocol Number: PIPH01/10

5.4.1.1 Male Rats for Bone Tissue Cultures

Male Wistar rats weighing 90–100 g (4 weeks old) were used. The animals were fed with a commercial laboratory chow containing 1.1% calcium and 1.1% phosphorus at a room temperature of 25°C, with free access to distilled water.

5.4.1.2 Bone Culture Experiments

The rats were sacrificed under ether anesthesia by cervical dislocation, and the femurs were removed aseptically after bleeding and soaked in ice-cold 0.25M sucrose solution. The soft tissue and marrow were cleaned-off from the femur, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated by a morphological tool. The femoral diaphyseal and femoral-metaphyseal tissues were cut into small pieces (the size of about 2 x 2 mm) by a pair of scissors. Diaphyseal and metaphyseal fragments (3 or 4 pieces) were cultured for 48 h in a 35 mm petridish in 2.0 ml of medium consisting of Minimum essential medium (MEM) (high glucose, 4.5 g/dl) supplemented with antibiotics (penicillin 100 units and streptomycin 100 mg/ml). In order to determine the effects of drugs on bone calcium content, bone tissues were cultured in a medium containing drug.

Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ and 95% air. After incubation period, the diaphyseal or metaphyseal tissues were removed, washed with ice-cold 0.25 M sucrose solution and dried for 16 h at 110°C. The calcium content was determined by atomic absorption
spectrophotometry. (175-177) The calcium content in the bone tissue was expressed as mg/g of dry bone. (177)

5.4.2 Experimental Protocol

Effect of extracts on the calcium contents in the femoral-diaphyseal and femoral-metaphyseal tissues were measured using in-vitro bone culture method. Diaphyseal or metaphyseal bone tissues were cultured in the presence of 10 μg/ml, 100 μg/ml, 500 μg/ml and 1000 μg/ml concentration of test extracts. After incubation period calcium content was measured in bone tissues.

5.4.3 Statistical analysis of data

All the test extracts were studied in triplicate at each concentration level. Data were expressed as mean ± standard error of mean. Statistical evaluation was done by Graphpad Prism 5 software. The nonlinear regression analysis of log drug concentration vs. response-variable slope (four parameter) and EC$_{50}$ value was determined. Statistical differences between the mean EC$_{50}$ values of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey test. Data were considered statistically significant at P value ≤ 0.05.

5.5 Evaluation of AAA, AAM, TAA and TAM extracts for anti-osteoporotic activity in rats

5.5.1 Selection of dose of the extracts

The doses of the extracts to be evaluated in the rats were selected based on the human dose range given in Ayurvedic texts and effective dose range mentioned in published research paper. (78, 107, 109, 126)
5.5.2 Selection of Animal Model

The anti-osteoporotic activity of extracts were evaluated using ovariectomized Wistar rats, following industrial guidelines of FDA, USA, for pre-clinical evaluation of anti-osteoporosis drugs.

The striking resemblance of the ovariectomized rats to humans with respect to estrogen deficiency related pathophysiology—i.e., increase in bone turnover, bone loss—osteopenia (178-181) and prevention of the same by estrogen replacement (182-184) make the ovariectomy rat a gold standard model of human osteoporosis. Since the ovariectomy model has effectively demonstrated the skeletal response of known agents such as conjugated equine estrogen (183) and parathyroid hormone (185, 186) in a fashion similar to that in postmenopausal women, this has been an animal model of choice for evaluation of new therapies for both prevention and treatment of osteoporosis. (187-200) In fact, the Food and Drug Administration has also recommended the utilization of the ovariectomy model for pre-clinical evaluation of anti-osteoporotic drugs. (201)

5.5.3 Animals

Three months old female Wistar albino rats, weighing between 250 to 300 g, were used. The animals were allowed to acclimatize for ten days prior to the study. Then, the rats were randomly dividing into groups, six rats in each group.

5.5.4 Ovariectomy Procedure

Ovariectomy was done under anesthesia, using a ketamine 50 mg/kg intraperitonially. Two dorso-lateral incisions, approximately 1 cm long were made above the ovaries. With the use of a sharp dissecting scissors, the skin was cut
together with the dorsal muscles and the peritoneal cavity was accessed (Figure 5-2) and the ovary was located. The connection between the fallopian tube and the uterine horn was cut and the ovary was removed. Because of muscle bleeding, the incision requires suturing. (69)

![Figure 5-1 Incision site for ovariectomy in rats and anatomy of the urogenital system of the female rat.](image)

**5.5.5 Treatment Protocol**

The rats from all groups were ovariectomized except one group, which served as sham operated control and was treated with vehicle. One ovariectomized group received vehicle (2 ml/kg) and served as ovariectomized control. Second ovariectomized group, which served as positive control, treated with estrogen (2 mg/kg p.o.). The other groups were treated with the test extracts given orally. The treatment was started on the day of ovariectomy and was continued for 40 days.

The body weight was recorded on the first day of treatment and at weekly intervals throughout the experiment. (202) Twenty–four hour urine samples were
collected after 40 days by placing each rat in a metabolic cage. Urine samples were acidified with 2 ml of 1 M HCl and centrifuged at 100 g for 10 min at 4°C to remove contaminating sediments. Samples aliquots were stored at -20°C until further analysis. (203) After 40 days of treatment, blood samples from all the groups were withdrawn by retroorbital bleeding, allowed to clot at room temperature and centrifuged at 1000 g for 20 min to separate serum. Serum samples were stored at -20°C until analysis. (203) The left and right femur along with tibia were dissected out from each rat. The left femur was thawed, autoclaved for 15 min at 110°C and divested of soft tissue for the measurement of weight, length, volume and density. The right femur was immediately fixed in 10% neutral buffered formalin for histopathological examination. (202) The fourth and fifth lumbar vertebrae were isolated from each rat for measuring the mechanical strength. (15) The uterus of each rat was also removed out and weighed.

5.5.6 Parameters to be assessed for Antiosteoporotic study

5.5.6.1 Serum Analysis

All the serum parameters were determined using diagnostic kits.

5.5.6.1.1 (a) Calcium determination (204-207)

Assay Principle:

In alkaline solution, calcium binds with metal complexing dye O- Cresolphthalein Complexone (OCPC) to form a bluish-purple complex, which was measured at 578 nm. The intensity of colour formed is proportional to calcium concentration in the sample. Hydroxyquinoline acts as a masking agent and eliminates the interference of magnesium.
Reagents:

(1) Reagent no. 1 (OCPC reagent)

(2) Reagent no. 2 (AMP (2-amino-2-methyl-1-propanol) Buffer)

(3) Reagent no. 3 (Calcium standard)

Procedure:

Working reagent preparation:

Reagents 1 was diluted with reagent 2 in equal proportion i.e. 1 ml of reagent 1+ 1 ml of reagent 2. Mixed properly by gentle swirling. It was a stable for 15 days at 2-8º C. Reagent 3 was ready to use.

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>Pipette In To Tube</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td>--</td>
<td>--</td>
<td>20 µl</td>
</tr>
<tr>
<td>Reagent 3</td>
<td></td>
<td>--</td>
<td>20 µl</td>
<td>--</td>
</tr>
<tr>
<td>Working calcium reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td></td>
</tr>
</tbody>
</table>

Reagents were mixed well and incubated at 37 ºC for 5 minutes. The absorbance of standard was measured at 578 nm, the results were calculated as per given formula.

Calculation:

Serum / plasma calcium (mg/dl) = Absorbance of test / Absorbance of standard * 10

5.5.6.1.2 (b) Inorganic Phosphorous (208, 209)

Assay principle:
In acidic medium, inorganic phosphorous reacts with ammonium molybdate to form phosphomolybdate complex is measured at 340 nm and is directly proportional to the concentration of inorganic phosphorous in the sample.

Inorganic phosphorous + Ammonium molybdate $\rightarrow$ Phosphomolybdate complex

Reagents:

1. Reagent no. 1 (Molybdate Reagent)
2. Reagent no. 2 (Sample blank reagent)
3. Reagent no. 3 (Phosphorous standard)

Procedure:

<table>
<thead>
<tr>
<th>PROCEDURE</th>
<th>Pipette In To Tube</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>--</td>
<td>--</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Reagent 3</td>
<td>--</td>
<td>10 µl</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Reagent 1</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td></td>
</tr>
</tbody>
</table>

The reagents were mixed as above and incubated at 37° C for 5 minutes. The absorbance was measured at 340 nm of standard followed by the test. The results were calculated as per given formula.

Calculation:

Serum Inorganic Phosphorous (mg/dl) = Absorbance of test / Absorbance of standard * 5
5.5.6.1.3 (c) Alkaline Phosphatase (ALP) (210, 211)

**Principle:**

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in presence of the oxidizing agent potassium ferricyanide and forms an orange red coloured complex, which can be measured colourimetrically. The colour intensity is proportional to the enzyme activity. The reaction can be represented as:

\[
\text{Phenyl Phosphate} \xrightarrow{\text{alkaline phosphatase}} \text{Phenol + Phosphate}
\]

\[
\text{Phenol + 4- Aminoantipyrine} \xrightarrow{\text{Pot- Ferricyanide}} \text{Orange- Red complex}
\]

**Reagents:**

1. **Reagent no. 1** (Buffered substrate pH 10)
2. **Reagent no. 2** (Chromogen Reagent)
3. **Reagent no. 3** (Phenol standard 10 mg %)

**Preparation of working reagent:**

One vial of reagent 1, buffered substrate was reconstituted with 2.2 ml of purified water. Reagents 2 and 3 were ready to use.

**Procedure:**

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working buffer substrate</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Purified water</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>
Mix well and incubate at 37 °C for 3 minutes

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Reagent 3</th>
<th></th>
<th></th>
<th>0.05 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>--</td>
<td>0.05 ml</td>
<td>--</td>
<td>--</td>
<td>---------</td>
</tr>
</tbody>
</table>

Mix well and incubate at 37 °C for 15 minutes

<table>
<thead>
<tr>
<th></th>
<th>Reagent 2</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>--</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

Mix well after addition of each reagent and measure the O.D. of blank, standard, control and test against purified water at 510 nm.

**Calculation:**

Serum alkaline phosphatase = [\((\text{O.D. test} - \text{O.D. control})/ (\text{O.D. standard} - \text{O.D. Blank})\)]

* 10

5.5.6.1.4  (d) Serum Tartarate-Resistant Acid Phosphatase (TRAP) (211, 212)

The test was carried out using diagnostic reagent kit (Span diagnostic Ltd., Surat) for the *in vitro* determination of TRAP by King’s method. Acid phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 4.8, Phenol so formed reacts in alkaline medium with aminoantipyrine in presence of oxidizing agent potassium-ferricyanide and forms an orange red colored complex.
5.5.6.2 Urine Analysis

5.5.6.2.1 (a) Calcium

Analysis of calcium was done as per method described in section 5.5.6.1.1..

Urine calcium content was determined by given formula

\[
\text{Urine Calcium (mg/dl) = Absorbance of test / Absorbance of standard} \times \text{dilution factor}
\]

5.5.6.2.2 (b) Phosphorous

Analysis of calcium was done as per method described in section 5.5.6.1.1..

Urine calcium content was determined by given formula

\[
\text{Urine Inorganic Phosphorous (mg/day) = Absorbance of test / Absorbance of standard} \times \text{dilution factor}
\]

5.5.6.2.3 (c) Creatinine (213, 214)

**Principle:**

Creatinine in a protein free solution, reacts with alkaline picrate and produces a red colored complex, which is measured colorimetrically.

**Reagents:**

1. Reagent no. 1 (Picric acid)
2. Reagent no. 2 (Sodium Hydroxide, 0.75 N)
3. Reagent no. 3 (Stock Creatinine Standard, 150 mg %)

**Preparation of working reagent:**

0.1 ml of reagent 3 was diluted to 10 ml with purified water and mixed well.

All other reagents were ready for use.
Procedure:

Step: A Deproteinization of test sample

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>0.5 ml</td>
<td></td>
</tr>
<tr>
<td>Purified water</td>
<td>0.5 ml</td>
<td></td>
</tr>
<tr>
<td>Reagent 1: Picric acid</td>
<td>3 ml</td>
<td></td>
</tr>
</tbody>
</table>

The reagents were mixed with urine and kept in a boiling water bath exactly for one minute, cooled immediately under running tap water and centrifuged.

Step: B. Color Development

<table>
<thead>
<tr>
<th></th>
<th>BLANK</th>
<th>STD</th>
<th>TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate /supernatant (from step A)</td>
<td>--</td>
<td>--</td>
<td>2 ml</td>
</tr>
<tr>
<td>Working reagent</td>
<td>--</td>
<td>0.5 ml</td>
<td>--</td>
</tr>
<tr>
<td>Purified water</td>
<td>0.5 ml</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Reagent 1 : picric acid</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>--</td>
</tr>
<tr>
<td>Reagent 2 : sodium hydroxide</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

The supernatant was mixed with reagents, allowed standing at room temperature exactly for 20 minutes and the optical density was measured at 520 nm of blank, standard and test against purified water.

Calculation:

Urine creatinine (g/litre) = O.D. Test - O.D. Blank/ O.D. Std - O.D. Blank * 0.75
5.5.6.3 Physical parameters

5.5.6.3.1 (a) Body weight

The animals were weighted every week during the treatment and the percentage changes in body weight were calculated by following formula:

\[
\% \text{ change in body weight} = \frac{(\text{body weight on day } X - \text{body weight on day } 0)}{\text{body weight on day } X} \times 100
\]

5.5.6.3.2 (b) Uterine index

On last day of treatment all the animals were sacrificed using overdose of ether. Uterus of each animal was isolated and washed with saline and weighed and ratio of Uterus weight to Body weight was measured & The uterine Index was calculated by following formula:

\[
\text{Uterine Index} = \frac{\text{Weight of uterus}}{\text{total body weight}}
\]

5.5.6.4 Biomechanical parameters

5.5.6.4.1 (a) Femur length

The femur length, defined as the distance between the greater trochanter and the medial condyle, was measured for the left femurs length using vernier calipers. (202)

5.5.6.4.2 (b) Bone volume and densities

Bone volume and densities were measured by Archimedes’s principle. Each bone was placed in unstoppered vial filled with deionized water, and the vial was put in a desiccators connected to a vacuum for 90 min. The desiccators was agitated
periodically to ensure that all trapped air diffused out of the bone, at which time the bone was removed from the vial, blotted with tissue paper, weighed, and returned to the vial containing deionized water. The bone was reweighed in a boat suspended, but completely immersed, in water previously equilibrated to room temperature, and the density was calculated (grams/volume). (215)

5.5.6.4.3 (c) Compression 4th Lumbar Vertebra

The fourth lumbar vertebra was isolated. The fresh vertebra was placed in digital pfizer hardness compressor and pressed, until it fractured. The force required to fracture the vertebra was recorded in Newtons. (15)

5.5.6.4.4 (d) Femoral neck load testing

In the loading test of femoral neck, the head of the femur was loaded with the force parallel to the shaft of the femur until fracture. A metallic clamp with flat surface was used to fix the proximal part of the femur perpendicularly and tightly. A concave compressing head (2.5 mm) was used for loading the head. Force required to break the head was noted. (216)

5.5.6.4.5 (e) Three point bending of tibia

A supporter with two loading points, 13mm apart from each other was used on the stage of the testing machine. Lateral surface of the tibia at tibio-fibular junction was placed upon the first point and proximal tibia upon the other. A rounded press head compressed the middle of the tibial shaft until fracture occurred (216).
5.5.6.5 Measurement of Ash Weight and Mineral Content of Bone

After measuring the bone length, the bone was placed in tared fused silica crucibles, and kept in muffle furnace, incinerated at 1000°C for 24 hour. The ash was weighed and suitably diluted with deionized water for calcium assay. (217)

5.5.7 Statistical analysis of data

Results are presented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey test. Data were considered statistically significant at P value ≤ 0.05.

5.6 Bioactivity guided fractionation of TAM

55 gm of shade-dried bark of *Terminalia arjuna* was powdered and extracted with 5 L of methanol for 8 h by hot maceration under reflux. The methanolic extract was concentrated to dryness under reduced pressure. Methanolic extract was further fractionated by successively extracting with solvents of increasing polarity *viz.*, petroleum ether, toluene, ethyl acetate and *n*-butanol. The residual extract left after fractionation with *n*-butanol was also collected. The extracts were concentrated to dryness under reduced pressure and stored in air-tight vials at 2-4°C. The fractions prepared were labeled as TAM-P (Pet-ether soluble fraction), TAM-T (Toluene soluble fraction), TAM-Et (Ethyl acetate soluble fraction), TAM-Nbut (n-butanol soluble fraction), TAM-R (Residue fraction) respectively. (Figure 5-3)
Methodology

Figure 5-2 Bioactivity guided fractionation scheme of TAM

5.7 Phytochemical and pharmacological evaluation of fractions of TAM

Fractions of TAM were analyzed separately for the presence of various phytocomponents as per the procedures given in section 5.3. The effect of fractions on bone calcium metabolism was evaluated as per the, *in vitro* bone culture experiment described in section 5.4.
5.8 Isolation of saponin fraction (TAM-Nbut-S) and aglycone fraction (TAM-Nbut-A) from TAM-Nbut fraction

5.8.1 Isolation of saponin fraction (TAM-Nbut-S)

TAM-Nbut fraction contained tannins and saponins. TAM-Nbut fraction was treated with lead acetate solution to precipitate tannins. The precipitates were removed by filtration through whatmann filter paper. Filtrate had two layers: aqueous and n-butanol. N-butanol layer, which contained saponins, was separated. Aqueous layer was again washed with n-butanol, to recollect remaining saponins. The n-butanol extracts were pooled and dilute $H_2SO_4$ was added to precipitate excess lead as lead sulphate. The precipitates were removed by filtration and filtrate was neutralized with sodium bicarbonate solution. The n-butanol layer was separated and acetone was added to precipitate saponins. The precipitates were collected by filtration and dried in vacuum oven at 60° C temperature. The saponin fraction thus obtained was stored in air-tight container at less than 4° C until fraction use. (Figure 5-4)
Figure 5-3 Isolation of TAM-Nbut-S

5.8.2 Hydrolysis of TAM-Nbut-S

TAM-Nbut-S fraction was heated with 2 N HCl for 2 hrs to hydrolyze saponin. The mixture was cooled and shaken with chloroform. Chloroform layer was separated, treated with anhydrous sodium sulfate to remove residual water, evaporated to dryness and labeled as TAM-Nbut-A.

5.9 Pharmacological activity of TAM-Nbut-S and TAM-Nbut-A

The effects of TAM-Nbut-S and TAM-Nbut-A on bone calcium metabolism were evaluated by, *in vitro* bone culture experiment described in section 5.4.
5.10 Investigation of arjunetin

5.10.1 Arjunetin analysis

Arjunetin was purchased on date 20.08.2014 from natural remedies private limited, Banglore, Karnataka. (Product code: A011, Lot No.: T11F163, Storage at 18-25°C, Formula weight: 650.84, molecular formula: C_{36}H_{58}O_{10}, valid up to: November 2015) As per the certificate of analysis provided by the supplier, identity and purity was measured by spectroscopy and chromatography methods. LC and HPTLC gave a single principle spot. No characteristic UV absorption between 230-250 nm. FTIR, NMR and MASS data showed characteristics of arjunetin. Purity was (by area normalization) 98.2 % (by HPLC).

5.10.2 Qualitative analysis of arjunetin in TAM-Nbut-A

Suitably diluted stock solution of TAM-Nbut-A and arjunetin were spotted on pre-coated silica gel 60 F254 TLC plates (E. Merck) using CAMAG Linomat V Automatic Sample Spotter and the plate was developed in the mobile phase - Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.8:0.2). The plates were dried at room temperature and observed in UV light at 254 and 366 nm in a UV chamber. The Rf values of observed bands were recorded. Further, the plates were derivatised by spraying with anisaldehyde sulphuric acid reagent followed by heating at 110 0C for 5 min, and the colours of the bands resolved and theire Rf were recorded.

5.10.3 In vitro Pharmacological evaluation of arjunetin

The effect of arjunetin on bone calcium metabolism was evaluated as per the, in vitro bone culture experiment described in section 5.4
5.10.4 Quantitative analysis of arjunetin in TAM-Nbut-A by HPLC

5.10.4.1 Preparation of standard solutions

1 mg of arjunetin standard was taken in 10 ml volumetric flask, dissolved in acetonitrile and volume was make up to mark with acetonitrile to get a stock solution of 0.1 mg/ml concentration. From this solution, five different aliquots- 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml were diluted separately in 10 ml volumetric flask with acetonitrile and volume was made upto the mark to get working standard solutions of concentrations 5.0 µg/ml, 10.0 µg/ml, 15.0 µg/ml, 20.0 µg/ml, 25.0 µg/ml, respectively.

5.10.4.2 Preparation of Test solutions

250 mg of aglycone fraction was dissolved in acetonitrile in a 10 ml volumetric flask and volume was made upto 10 ml with acetonitrile. One ml of this solution was further diluted upto 10 ml with acetonitrile. All sample solutions were filtered through a 0.45 µm nylon membrane filter, prior to HPLC analysis.

5.10.4.3 Chromatographic condition

Analysis was performed as per reported method (218) on a HPLC solvent delivery system (Varian-9012) with binary gradient system (Varian 9220), a Rheodyne injection valve furnished with 20 µl loop, a UV detector (Varian-9050) and a Chrom-Xuest software. Separation was carried out using a Bischoff column (250 × 4.6 mm i.d., 5 µm pore size). The column was maintained at 27° C throughout the analysis and detection was carried at 220 nm.
<table>
<thead>
<tr>
<th>Specification</th>
<th>Parameter</th>
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</thead>
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<tr>
<td>Column</td>
<td>C 18, 250 * 4.6 mm, 5 μm pore size</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>A: Mix 30 volume of ACN and 70 volume of water, filter and degas</td>
</tr>
<tr>
<td>preparation</td>
<td>B: Mix 70 volume of ACN and 30 volume of water, filter and degas</td>
</tr>
<tr>
<td>Gradient programme</td>
<td>Time (Min)</td>
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<tr>
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<tr>
<td>Detection Wavelength</td>
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</tr>
</tbody>
</table>

5.10.4.4 Calibration curve for Arjunetin

10 μl of each working standard solution of arjunetin was injected in triplicate and peak areas were recorded. Peak identification was achieved by comparison of both the retention time (Rt) and UV absorption spectrum for standards. A calibration curve was obtained by plotting peak area vs concentration of arjunetin injected. The linear regression equation $y = a + bx$ was derived from calibration curve data.
5.10.4.5 Estimation of Arjunetin in TAM-Nbut-A fraction

10 µl of sample solution of TAM-Nbut-A fraction were injected in triplicate and the peak areas were recorded. The content of arjunetin in TAM-Nbut-A fraction was quantified using linear regression equation of arjunetin.