INVESTIGATION OF SOME AYURVEDIC HERBAL DRUGS FOR EFFICACY IN POST-MENOPAUSAL OSTEOPOROSIS USING OVARIENTOMIZED RAT MODEL

A SYNOPSIS SUBMITTED TO
KADI SARVA VISHWAVIDHYALAYA
GANDHINAGAR, GUJARAT.

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(PHARMACOGNOSY)

BY
NIMISHA PARSHOTTAMBHAI KAKADIA

UNDER THE GUIDANCE OF
DR. NIRANJAN S. KANAKI, PhD
DEPT. OF PHARMACOGNOSY,
K. B. INSTITUTE OF PHARMACEUTICAL EDUCATION AND RESEARCH,
SECTOR – 23, GANDHINAGAR

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“INVESTIGATION OF SOME AYURVEDIC HERBAL DRUGS FOR EFFICACY IN POST-MENOPAUSAL OSTEOPOROSIS USING OVARIETOMIZED RAT MODEL”

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FACULTY : PHARMACEUTICAL SCIENCES

SUBJECT : PHARMACOGNOSY

RESEARCH STUDENT : NIMISHA P KAKADIA

RESEARCH GUIDE : DR. NIRANJAN S. KANAKI

REGISTRATION NO. : 10G0211

DATE OF REGISTRATION : 02/02/2010

SIGN OF GUIDE

DR. NIRANJAN S. KANAKI
RESEARCH SUPERVISOR

SIGN OF STUDENT

NIMISHA P. KAKADIA
RESEARCH STUDENT
This is to certify that the thesis entitled “INVESTIGATION OF SOME AYURVEDIC HERBAL DRUGS FOR EFFICACY IN POST-MENOPAUSAL OSTEOPOROSIS USING OVARIETOMIZED RAT MODEL” represents the bonafide work of Ms. Nimisha P. Kakadia. The work mentioned in this synopsis is original to best of my knowledge. I have supervised the work at laboratory of institute during entire research period and it has not been plagiarized in any context. This work is up to my satisfaction and submitted from Department of Pharmacognosy, K. B. Institute of Pharmaceutical Education and Research, (Constituent college of Kadi Sarva Vishwavidyalaya), Gandhinagar.

SUPERVISOR

DR. NIRANJAN S. KANAKI

Forwarded through,

DR. GAURANG B. SHAH
Principal and Dean,
K. B. Inst. of Pharmaceutical education and Research
Gh-6, Sector-23 Gandhinagar
I hereby declare that the work entitled “INVESTIGATION OF SOME AYURVEDIC HERBAL DRUGS FOR EFFICACY IN POST-MENOPAUSAL OSTEOPOROSIS USING OVARIETOMIZED RAT MODEL” is my own original experimental work conducted at the laboratory of K. B. Institute of Pharmaceutical education and research, Gandhinagar and Parul institute of Pharmacy, Vadodara. The work has not been reported anywhere to the best of my knowledge. The write up in the thesis is my own with original and proper references are quoted wherever experimental details are taken or referred from anywhere. The work presented here has not been submitted previously to this university or any other university/institute for the award of any degree or diploma.

NIMISHA PARSHOTTAMBHAI KAKADIA
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<th>ABBREVIATIONS</th>
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<tbody>
<tr>
<td>AAA</td>
<td>Aqueous extract of <em>Acacia arabica</em></td>
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<tr>
<td>AAA 250</td>
<td>Aqueous extract of <em>Acacia arabica</em> (250 mg/kg/Body weight)</td>
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<tr>
<td>AAA 500</td>
<td>Aqueous extract of <em>Acacia arabica</em> (500 mg/kg/Body weight)</td>
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<td>AAM</td>
<td>Methanolic extract of <em>Acacia arabica</em></td>
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<tr>
<td>AAM 250</td>
<td>Methanolic extract of <em>Acacia arabica</em> (250 mg/kg)</td>
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<tr>
<td>AAM 500</td>
<td>Methanolic extract of <em>Acacia arabica</em> (500 mg/kg)</td>
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<tr>
<td>BSEt</td>
<td>Ethyl acetate extract of <em>Boswellia serrata</em></td>
</tr>
<tr>
<td>BSP</td>
<td>Petroleum ether extract of <em>Boswellia serrata</em></td>
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<tr>
<td>CMP</td>
<td>Petroleum ether extract of <em>Commiphora mukul</em></td>
</tr>
<tr>
<td>CPCSEA</td>
<td>Committee for Purpose of Control and Supervision on Experiments on Animals</td>
</tr>
<tr>
<td>DP</td>
<td>Diaphyseal</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
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<tr>
<td>IAEC</td>
<td>Institutional Animal Ethics Committee</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit</td>
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<tr>
<td>KBIPER</td>
<td>K. B. Institute of Pharmaceutical Education and Research</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>MP</td>
<td>Metaphyseal</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>p.o.</td>
<td>Per Oral</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SERMs</td>
<td>Selective estradiol receptor modulators</td>
</tr>
<tr>
<td>TAA</td>
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<tr>
<td>TAA 250</td>
<td>Aqueous extract of <em>Terminalia arjuna</em> (250 mg/kg/Body weight)</td>
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<td>TAM 500</td>
<td>Methanolic extract of <em>Terminalia arjuna</em> (500 mg/kg/Body weight)</td>
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<tr>
<td>TAM-Et</td>
<td>Ethyl acetate soluble fraction from TAM</td>
</tr>
<tr>
<td>TAM-Nbut</td>
<td>n- butanol soluble fraction from TAM</td>
</tr>
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<td>TAM-Nbut-A</td>
<td>Aglycone fraction from TAM-Nbut</td>
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<td>TAM-Nbut-S</td>
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<td>TAM-P</td>
<td>Petroleum ether soluble fraction from TAM</td>
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<td>Residue after fractionation of TAM</td>
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<tr>
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<td>Toluene soluble fraction from TAM</td>
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1 ABSTRACT

1.1 Background

Osteoporosis which means "porous bones," is a skeletal disease in which bones become brittle and prone to fracture. In other words, the bone loses density. Bone density is the amount of bone tissue (such as calcium and minerals) in a certain volume of bone. Osteoporosis is diagnosed when bone density has decreased to the point where the risk of fractures is high even without severe stress or injury to the bones. Many herbal drugs in India, has been traditionally used in Ayurveda to accelerate the healing of bone fractures and to strengthen the bones. However, no scientific study has been done to validate their usefulness in the alleviation of osteoporosis.

1.2 Aim

Investigation of some Ayurvedic herbal drugs for efficacy in post-menopausal osteoporosis using ovariectomized rat model

1.3 Materials and method

In vitro studies were performed on metaphyseal and diaphyseal femur bone to study effect of different extracts of Acacia arabica, Terminalia arjuna, Commiphora mukul and Boswellia serrata. The extracts (AAA, AAM, TAA and TAM) which showed significant effect in in vitro study were selected for in vivo studies using bilateral ovariectomized rat model. The animals were divided in to groups: Group I [SHAM Control (SHAM)], II [Disease control (OVX)], III [Standard estrogen (2 mg/kg/day/p.o.) (STD)], IV [Aqueous extract of Acacia arabica (250 mg/kg/Body weight) (AAA250)], V [Aqueous extract of Acacia arabica (500 mg/kg/Body weight) (AAA500)], VI [Methanolic extract of Acacia arabica (250 mg/kg) (AAM250)], VII [Methanolic extract of Acacia arabica (500 mg/kg) (AAM500)], VIII [Aqueous extract of Terminalia arjuna (250 mg/kg/Body weight) (TAA250)], IX [Aqueous extract of Terminalia arjuna (500 mg/kg/Body weight) (TAA500)], X [Methanolic extract of Terminalia arjuna (250 mg/kg/ Body weight) (TAM250)], XI [Methanolic extract of Terminalia arjuna (500 mg/kg/ Body weight) (TAM500)]. TAM showed significant activity in in vivo study so, it was further fractionated to isolate active fraction. Bioactivity guided fractions of TAM (TAM-P, TAM-T, TAM-Et, TAM-
Nbut, TAM-R) obtained by successive extraction with different solvents were evaluated using bone culture method. TAM-Nbut fraction was found to be most effective. Saponin (TAM-Nbut-S) fraction and aglycone fraction (TAM-Nbut-A) were isolated from TAM-Nbut. And both the fractions showed significant activity in in vitro efficacy study. The aglycone fraction (TAM-Nbut-A) was found to contain arjunetin. Hence, arjunetin was evaluated for its efficacy on bone turnover. The content of arjunetin in aglycone fraction was estimated by HPLC.

1.4 Results

Macroscopic, microscopical and Phytochemical analysis of *Terminalia arjuna*, *Acacia arabica*, *Commiphora mukul* and *Boswellia serrata* confirmed their authenticity. The calcium content in the diaphyseal or metaphyseal tissues was significantly increased when the bone tissues were cultured in the presence of different extracts of *Terminalia arjuna* and *Acacia arabica*. Similar results were found with *Commiphora mukul*. However, extracts of *Boswellia serrata* did not show significant activity in in vitro bone-culture experiments. The methanolic extracts of *Terminalia arjuna* showed maximum anti-osteoporotic activity in in vivo study using ovariectomized rat model, among different extracts of *Terminalia arjuna* and *Acacia arabica*. TAM-Nbut fraction was found to be the most effective amongs all other fractions of TAM. Phytochemical screening showed the presence of saponins and tannins in n-butanol fraction of *Terminalia Arjuna*. The saponin and sapogenin fractions from TAM-Nbut, significantly increased the calcium content in the metaphyseal tissues when the bone tissues were cultured in their presence. Arjunetin, which was one of the components of sapogenin fraction, showed significant effect on bone turnover. The content of arjunetin in TAM-Nbut-A was found to be 0.5790 %w/w.

1.5 Conclusion

The methanolic extract of Terminalia arjuna possesses significant anti-osteoporotic activity and arjunetin is one of the compound responsible for the activity. The possible mechanism of antiosteoporotic activity of *Terminalia arjuna* appears to be due to anti-inflammatory activity, anti-oxidant activity and phytoestrogen compound present in it.

1.6 Keywords

Post menopausal osteoporosis, Ovariectomy, Arjunetin, Saponin
2 INTRODUCTION

Osteoporosis is a disease characterized by low bone mass, micro-architectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk. It is a major cause of morbidity and mortality and medical expense worldwide. In India, it has been found that 29.9% of women and 24.3% of men aged between 20 and 79 years, have low bone mass. Furthermore, about 50% women and 36% of men over 50 years of age are noted to have low bone mass. Osteoporosis affects an estimated 75 million people in the United States, Europe, and Japan combined, including one in three postmenopausal women and majority of the elderly. Osteoporosis causes more than 1,300,000 fractures annually in the United States alone. The disease will be a greater problem in the future, because the world population is aging and the incidence of osteoporotic fractures is increasing in many geographic area. (1, 2)

The goals of management of osteoporosis are to prevent fractures, to decrease the pain when present and to restore function. Pharmaceutical agents are used to minimize further bone loss (3). In premenopausal conditions, calcium and vitamin D supplementation and estrogen are given for prophylaxis (4). Hormone replacement therapy (4, 5), bafilomycin A1 (6), Tibolone (7), Phytoestrogens (8), Strontium (9), HMG-CoA reductase inhibitors (9), and selective estrogen receptor modulators (4) are used in the treatment of osteoporosis. However, most commonly calcium and Vitamin D supplementation, estrogen treatment, bisphosphonates and calcitonin administration are widely used for management of osteoporosis (4).

The efficacy of currently used anti-osteoporotic drugs is compromised in several ways. Vitamin D supplementation at higher dose causes hypocalcaemia, hypercalciuria and kidney stone formation. Most commonly estrogen is used for a long term in hormone replacement therapy, but this causes severe adverse effects like breast cancer, endometrial cancer, vaginal bleeding, blood pressure, thromboembolism and blood clot. Therapy with bisphosphonates is associated with irritation
of esophagus, abdominal or musculoskeletal pain, nausea and heart burn. Parenteral calcitonin can lead to potential adverse effects including, nausea, vomiting, diarrhoea, anorexia, facial flashing, tingling, skin rash, edema of the feet and pain at the site of injection. The adverse effects of current therapeutic options of osteoporosis, along with their inability to restore lost bone mass and bone remodeling on the other, has raised interest in the study of alternative and complementary medicines for management of osteoporosis. (4)

India has an ancient heritage of traditional system of medicine. Materia medica of India provide lots of information on the folklore practices and traditional aspects of therapeutically important natural products. With the emerging interest in the world to adopt and study the traditional systems of medicine and to exploit their potentials, the evaluation of the rich heritage of the traditional medicine is essential. Compared to synthetic drugs, herbal preparations are generally considered to be less toxic with fewer side effects (10). Therefore the search for more effective and safer therapeutic agents has become an area of current research.

Ayurvedic system of medicines is one of the oldest system of medicine having a history of more than 2000 years. A wide array of herbal and mineral drugs have been reported in ayurvedic texts for osteoporosis, but only few of them have been scientifically evaluated, importantly, Cissus quadrangularis Linn (11) Withania somnifera (12), Epimedium brevicornum Maxim and Asparagus racemosus (13)

However, the crude drugs like Acacia arabica (Babul), Terminalia Arjuna (Arjun), Commiphora mukul (Guggul), Commiphora myrrha (Hirabol), Boswelia serrata (Salai guggul), Symlocos racemosa (Lodhra), Polygonatum cirrhifolium (Medalakdi), lac (resin from the lac bug, Laccifer lacca), Uraria picta (Prisnaparni), Curcuma amada (Mango ginger), Grewia hirsuta (Nagbala), Aloe vera (Aloe) (14), which are claimed to be efficacious in bone remodelling, have still not been evaluated scientifically for their efficacy against osteoporosis. Hence, it is proposed to undertake a project to evaluate some of these drugs for their efficacy against osteoporosis
3 AIM AND OBJECTIVE

3.1 Aim

- Investigation of some ayurvedic herbal drugs for efficacy in post-menopausal osteoporosis using ovariectomized rat model

3.2 Rationale for selection of drug

- The stem bark of *Acacia arabica*, known as Babool and *Terminalia arjuna*, known as Arjuna, Oleo gum resin of *Boswellia serrata* known as Salai guggul and *Commiphora mukul* known as guggul in India, have been traditionally used in Ayurveda to accelerate the healing of bone fractures and to strengthen the bones. (15)

- It is reported that “Plants with anti-inflammatory role can be potent candidates as an osteoprotective agent” (16). The selected herbal drugs for are reported as anti-inflammatory action so, they may act as a osteoprotective agent. (17-21)

- *Terminalia arjuna* holds a reputed position in both Ayurvedic and Unani Systems of medicine. Vagbhata places it in the vellantaradi & nyagrodhadi groups indicated in urinary disorders and broken bones, respectively. According to the Sarangadhara Samhita, *Terminalia arjuna* belongs to the nyagrodhadi group specific for fractures, ulcers, uterine & urological complaints, skin diseases etc. (22)

- Literature reports suggest that the selected drugs used to heal fracture and osteoarthritis condition. (20, 23-27)
4 METHODOLOGY

4.1 Collection and characterization of plant materials

4.1.1 Collection of plant materials

The stem bark of *Acacia arabica* and *Terminalia arjuna*; oleogum resin of *Commiphora mukul* and *Boswellia serrata* were purchased from Lallu Vrajlal Gandhi (LVG) herbal store, Ahmedabad, Gujarat, India. The voucher specimen (Number: PIP/2011/PCG-V/01, PIP/2011/PCG-V/02, PIP/2011/PCG-V/03 and PIP/2011/PCG-V/04 respectively) of the stem barks and oleogum resins 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.

4.1.2 Authentication of plant materials

4.1.2.1 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.

4.1.3 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. Quality parameters such as Loss on drying, Total ash value, Acid-insoluble ash value and Extractive values (water and alcohol soluble) were established according to standard guideline (28-30) for each drug.

4.2 Preparation of extracts of herbal drugs selected for study

4.2.1 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.

4.2.2 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.
4.2.3 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 500ml 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.

4.2.4 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 4.2.3, but ethyl acetate was used as a solvent for extraction instead of petroleum ether.

4.3 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 (31).

4.4 Evaluation of test extracts for anti-osteoporotic activity using *in vitro* model

4.4.1 *In vitro* model

The study protocol was approved by IAEC - Protocol Number: PIPH01/10
4.4.1.1 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.

4.4.1.2 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. (32-34) The calcium content in the bone tissue was expressed as mg/g of dry bone. (34)
4.4.2 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.

4.4.3 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.

4.5 Evaluation of test extracts for anti-osteoporotic activity in ovariectomized rats

4.5.1 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. (35-38)

4.5.2 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.
4.5.3 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 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. (39)

4.5.4 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. (40) 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. (41) After 40 days of treatment, blood samples from all the groups were withdrawn by retrorbital 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. (41) 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. (40) The fourth and fifth lumbar vertebrae were isolated from each rat for measuring the mechanical strength. (11) The uterus of each rat was also removed out and weighed.

4.5.5 Parameters to be assessed for Antiosteoporotic study

4.5.5.1 Serum and Urine Analysis

Serum (calcium, phosphorous, alkaline phosphatase and TRAP) and urine (calcium, phosphorous and creatinine) parameters were determined using diagnostic kits. (42-48)

4.5.5.2 Physical parameters

4.5.5.2.1 (a) Body weight

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

4.5.5.2.2 (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

4.5.5.3 Biomechanical parameters

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. (40)T The same femurs were then dried in an evacuated oven at 110°C for 48 h and weighed using a digital weighing balance. The femur bone density and bone volume was determined. The fourth lumbar vertebra and tibia were isolated. The fresh vertebra and rtibia were placed in digital pfizer hardness compressor and pressed, until it fractured. The force required to fracture it was recorded in Newtons. Femoral neck load testing also done in same manner. (11, 49, 50).

4.5.5.4 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 (51)

4.5.6 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.

4.6 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 24° 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.

4.7 Pharmacognostical and pharmacological evaluation of fractions of TAM

Fractions of TAM were analyzed separately for the presence of various phytoconstituents. The effect of fractions on bone calcium metabolism was evaluated as per the, in vitro bone culture experiment

4.8 Isolation of saponin fraction (TAM-Nbut-S) and aglycone fraction (TAM-Nbut-A) from TAM-Nbut fraction

4.8.1 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 and dilute H₂SO₄ was added to precipitate excess lead as lead sulphate. The precipitates were removed by filtration and filtrate was neutralizes 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 vaccum 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 4-1)
4.8.2 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, evaporate to dryness and labeled as TAM-Nbut-A.

4.9 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

4.10 Study of arjunetin in TAM-Nbut-A fraction

4.10.1 TLC analysis of arjunetin and 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 °C for 5 min, and the colours of the bands resolved and compared.

4.10.2 In vitro Pharmacological evaluation of arjunetin

The effect of arjunetin on bone calcium metabolism was evaluated as per the, in vitro bone culture experiment

4.11 Determination of content of arjunetin in TAM-Nbut-A by HPLC

4.11.1 Preparation of standard solutions

5 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.

4.11.2 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.
4.11.3 Chromatographic condition

Analysis was performed 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. 4-1)

4.11.4 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.
Table 4-1 Chromatographic conditions for HPLC analysis

<table>
<thead>
<tr>
<th>Specification</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>C 18 , 250 * 4.6 mm, 5 μm pore size</td>
</tr>
<tr>
<td>Mobile phase preparation</td>
<td>A: Mix 30 volume of ACN and 70 volume of water, filter and degas</td>
</tr>
<tr>
<td></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</td>
</tr>
<tr>
<td></td>
<td>0.0 min</td>
</tr>
<tr>
<td></td>
<td>10.0 min</td>
</tr>
<tr>
<td></td>
<td>30.0 min</td>
</tr>
<tr>
<td></td>
<td>35.0 min</td>
</tr>
<tr>
<td></td>
<td>40.0 min</td>
</tr>
<tr>
<td>Flow rate</td>
<td>Gradient</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10 µl</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>220 nm</td>
</tr>
</tbody>
</table>

4.11.5 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.
5 RESULTS

5.1 Authentication and Characterization of plant material

Plant material were authenticated by comparing their morphological and microscopical characters with those reported in the literature. (28, 52) The materials were characterized by measuring their physicochemical properties like ash values, extractive values and moisture content.

5.1.1 Macroscopic and Microscopic studies

5.1.1.1 Stem bark of Acacia arabica

5.1.1.1.1 MACROSCOPY

Bark was hard, dark brown or black, deeply fissured transversely and longitudinal; inner surface was reddish brown, longitudinally striated and fibrous; broke with difficulty and exhibited a fibrous fracture; taste was astringent. These macroscopic characters were comparable with those mentioned in literature. (28) (Figure 5-1)

5.1.1.1.2 MICROSCOPY

Transverse section of mature bark shows, 15-25 layers of thin-walled, slightly flattened mostly rectangular, brown coloured cork cells, a few lenticels formed by rupturing of cork cells, secondary cortical cells ovate to elongated, many tanniferous stone cells, variable in shape and size present in large groups, secondary phloem consists of sieve tubes, companion cells, fibres, crystal fibres and phloem parenchyma phloem fibres in many groups and thick-walled, phloem tissues

![Figure 5-1 Stem bark of Acacia arabica (a- Outer surface ; b- Inner surface)](image)
Results

K.B.I.P.E.R

filled with reddish or brown contents present, crystal fibres thick-walled, elongated, divided by transverse septa into segments, each contains a prismatic crystal of calcium oxalate, medullary rays uni to-multi-seriate run almost straight, ray cells radially elongated, 20-24 cells high and 2-5 cells wide, crystals of calcium oxalate found scattered amongst the stone cells of secondary cortex and phloem parenchyma. (Figure 5-2) The microscopical characters were comparable with those mentioned in the literature (28) for stem bark of *Acacia arabica*.

![Figure 5-2 Transverse section of the *Acacia arabica* stem bark](image-url)
5.1.1.2 Stem bark of *Terminalia arjuna*

5.1.1.2.1 MACROSCOPY:

Bark available in pieces, flat, curved, recurred, channelled to half quilled, 0.2-1.5 cm thick, 10 cm in length and upto 7 cm in width, outer surface somewhat smooth and grey, inner surface somewhat fibrous and pinkish, transversely cut smoothened bark shows pinkish surface, fracture, short in inner and laminated in outer part; taste, bitter and astringent. These macroscopic characters were comparable with those mentioned for stem bark of *Terminalia arjuna* in literature. (28, 52) (Figure 5-3)

![Stem bark of *Terminalia arjuna*](image)

**Figure 5-3 Stem bark of *Terminalia arjuna* (a. Outer surface, b. Inner surface)**

5.1.1.2.2 MICROSCOPY:

Mature stem bark shows cork consisting of 9-10 layers of tangentially elongated cells, a few outer layers filled with brown colouring matter; cork cambium and secondary cortex not distinct and medullary rays observed traversing almost upto outer bark; secondary phloem occupies a wide zone, consisting of sieve tubes, companion cells, phloem parenchyma and phloem fibres, traversed by phloem rays, usually uniseriate but biseriate rays also occasionally seen; in the middle and outer phloem region, sieve tubes were collapsed to ceratenchyma; phloem fibres distributed in rows and present in groups of 2-10; rosette crystals of calcium oxalate were present in most of the phloem parenchyma, alternating with fibres; idioblasts consisting of large cells having aggregates of prismatic and rhomboidal crystals of...
Results

calcium oxalate in row throughout the zone, measuring 260-600 μ in dia., starch grains, mostly simple, compound of 2-3 components, sometimes upto 5 components, round to oval, elliptical, measuring 5-13 μ in dia., distributed throughout the tissue  (Figure 5-4) The microscopical characters were comparable with those mentioned in the literature (28, 52) for stem bark of Terminalia arjuna.

Figure 5-4 Transverse section of the Terminalia arjuna stem bark (Left- Unstained, Right- stained)
5.1.1.3 Oleogum resin of *Commiphora mukul*

5.1.1.3.1 MACROSCOPY:

light to dark brown vermicular or stalactitic pieces, tears in varying sizes; Slightly sticky to touch; Resinous lump which turned darker in color on long storage; fracture brittle; burns with smoking flame in fire. Odour aromatic and balsamic, taste acrid, characteristic and slightly bitter. These macroscopic characters were comprable with those mentioned for oleogum resin of *Commiphora mukul* in literature. (52)

5.1.1.4 Oleogum resin of *Boswellia serrata*

5.1.1.4.1 MACROSCOPY:

Globular or club shaped agglutinated tears of greenish white and yellow color covered with brown or black coarse powder; fracture brittle, fractured surface waxy and semitransulent; odour characteristic, balsamiferous; Taste bitter and pungent; Burns readily with balsamic fragrance. These macroscopic characters were comprable with those mentioned for oleogum resin of *Boswellia serrata* in literature. (52)
5.1.2 Physico-chemical analysis of selected herbal drugs plant material

The selected herbal drugs were powdered and analysed for moisture content (loss on drying), ash values (Acid insoluble ash, Water soluble ash, Total ash) and extractive value (Alcohol soluble and water soluble extractive value), as per guidelines of WHO. The results are given in Table 5-1

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>HERBAL DRUGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>LOSS ON DRYING</td>
<td>12.50%</td>
</tr>
<tr>
<td>ASH VALUES</td>
<td></td>
</tr>
<tr>
<td>Acid insoluble ash</td>
<td>1.33% (Not more than 2%)</td>
</tr>
<tr>
<td>Total ash</td>
<td>10.66% (Not more than 15%)</td>
</tr>
<tr>
<td>EXTRACTIVE VALUES</td>
<td></td>
</tr>
<tr>
<td>Alcohol soluble extractive value</td>
<td>25.6% (Not less than 6%)</td>
</tr>
<tr>
<td>Water soluble extractive value</td>
<td>13.6% (Not less than 4%)</td>
</tr>
</tbody>
</table>

All values are in terms of % w/w of herbal drug.
The values in parenthesis are reference value as per literature (28, 52)

AA = Acacia arabica, TA = Terminalia arjuna, CM = Commiphora mukul, BS = Boswellia serrata
5.2 Preliminary phytochemical screening of Plant extracts

The powdered herbal drugs were extracted with different solvents using hot maceration method as per procedure described in 4.2. All the extracts were subjected to various chemical tests to detect the presence of compounds of different chemical groups. The % yield of each extract obtained is given in Table 5-2.

Table 5-2 Phytochemical Screening of herbal extracts

<table>
<thead>
<tr>
<th>Chemical groups</th>
<th>AA (15.40%)</th>
<th>AAM (22.62%)</th>
<th>TA (17.60%)</th>
<th>TAM (21.84%)</th>
<th>CM (40.84%)</th>
<th>CMEt (42.36%)</th>
<th>BS (38.78%)</th>
<th>BSEt (34.00%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>- Absent</td>
<td>+ Present</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>+ Present</td>
</tr>
<tr>
<td>Tannins</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+ Present</td>
<td>+ Present</td>
<td>- Absent</td>
<td>+ Present</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>+ Present</td>
</tr>
<tr>
<td>Saponins</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
</tr>
<tr>
<td>Gums and Mucilage</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>- Absent</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
</tr>
<tr>
<td>Steroid</td>
<td>- Absent</td>
<td>+ Present</td>
<td>- Absent</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
<td>+ Present</td>
</tr>
</tbody>
</table>

(-) Absent  (+) Present (%). Percentage

The values in parenthesis are percentage yield value.

AA = Acacia arabica, TA = Terminalia arjuna, CM = Commiphora mukul, BS = Boswellia serrata, AAA = Aqueous extract of Acacia arabica , AAM = Methanolic extract of Acacia arabica , TAA = Aqueous extract of Terminalia arjuna , TAM = Methanolic extract of Terminalia arjuna , CMP = Petroleum ether extract of Commiphora mukul, CMEt = Ethyl acetate extract of Commiphora mukul, BSP = Petroleum ether extract of Boswellia serrata, BSEt = Ethyl acetate extract of Boswellia serrata
5.3 Evaluation of test extracts for anti-osteoporotic activity using *in vitro* model

Effects of different drug extracts on the calcium contents in the femoral-diaphyseal and femoral metaphyseal tissues were evaluated using *in-vitro* bone culture method. The calcium content in the diaphyseal and metaphyseal tissues significantly increased when the bone tissues were cultured in the presence of aqueous and methanolic extracts of *Acacia Arabica* and *Terminalia arjuna* at concentrations 10 and 100 μg/ml. The calcium content in the diaphyseal or metaphyseal tissues significantly increased when the bone tissues were cultured in the presence of 100 μg/ml petroleum ether and ethyl acetate extracts of *Commiphora mukul* and *Boswellia serrata* did not cause any significant change in the calcium content. At 500 - 1000 μg/ml concentration of extracts of *Boswellia serrata*, the calcium content was decreased in the diaphyseal or metaphyseal tissues. (Figure 5-5)
Results

Effects of herbal extracts on the calcium contents in the diaphyseal and metaphyseal tissues of femur bone in \textit{in vitro} experiment.

Each bar represents Mean ± S.E.M. Number of samples in each group = 3.

\textbf{SHAM} = Normal control, \textbf{AAA 10} = Aqueous extract of \textit{Acacia arabica} (10 µg/ ml), \textbf{AAA 100} = Aqueous extract of \textit{Acacia arabica} (100 µg/ ml), \textbf{AAM 10} = Methanolic extract of \textit{Acacia arabica} (10 µg/ ml), \textbf{AAM 100} = Methanolic extract of \textit{Acacia arabica} (100 µg/ ml), \textbf{TAA 10} = Aqueous extract of \textit{Terminalia arjuna} (10 µg/ ml), \textbf{TAA 100} = Aqueous extract of \textit{Terminalia arjuna} (100 µg/ ml), \textbf{TAM 10} = Methanolic extract of \textit{Terminalia arjuna} (10 µg/ ml), \textbf{TAM 100} = Methanolic extract of \textit{Terminalia arjuna} (100 µg/ ml), \textbf{CMP 10} = Pet ether extract of \textit{Commiphora mukul} (10 µg/ ml), \textbf{CMP 100} = Pet ether extract of \textit{Commiphora mukul} (100 µg/ ml), \textbf{CMEt 10} = Ethyl acetate extract of \textit{Commiphora mukul} (10 µg/ ml), \textbf{CMEt 100} = Ethyl acetate extract of \textit{Commiphora mukul} (100 µg/ ml), \textbf{BSP 10} = Pet ether extract of \textit{Boswellia serrata} (10 µg/ ml), \textbf{BSP 100} = Pet ether extract of \textit{Boswellia serrata} (100 µg/ ml), \textbf{BSEt 10} = Ethyl acetate extract of \textit{Boswellia serrata} (10 µg/ ml), \textbf{BSEt 100} = Ethyl acetate extract of \textit{Boswellia serrata} (100 µg/ ml)

* Significantly different from SHAM (p< 0.05)
5.3.1  **EC$_{50}$ value determination**

The data of above in vitro experiment was subjected to four parameter non-linear regression analysis using graph pad Prism 5.0 software and EC$_{50}$ value of each extract was determined. (Table-5-3)

<table>
<thead>
<tr>
<th>DRUG</th>
<th>MP</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acacia arabica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA</td>
<td>22.14</td>
<td>24.53</td>
</tr>
<tr>
<td>AAM</td>
<td>11.97</td>
<td>12.46</td>
</tr>
<tr>
<td><strong>Terminalia arjuna</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAA</td>
<td>16.03</td>
<td>12.47</td>
</tr>
<tr>
<td>TAM</td>
<td>12.43</td>
<td>10.93</td>
</tr>
<tr>
<td><strong>Commiphora mukul</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMP</td>
<td>43.29</td>
<td>48.62</td>
</tr>
<tr>
<td>CMEt</td>
<td>41.02</td>
<td>40.20</td>
</tr>
<tr>
<td><strong>Boswellia serrata</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSP</td>
<td>Very wide</td>
<td>Very wide</td>
</tr>
<tr>
<td>BSEt</td>
<td>Very wide</td>
<td>Very wide</td>
</tr>
</tbody>
</table>

DP = Diaphyseal, MP = Metaphyseal, AAA = Aqueous extract of *Acacia arabica*, AAM = Methanolic extract of *Acacia arabica*, TAA = Aqueous extract of *Terminalia arjuna*, TAM = Methanolic extract of *Terminalia arjuna*, CMP = Petroleum ether extract of *Commiphora mukul*, CMEt = Ethyl acetate extract of *Commiphora mukul*, BSP = Petroleum ether extract of *Boswellia serrata*, BSEt = Ethyl acetate extract of *Boswellia serrata*
5.4 Evaluation of test extracts for anti-osteoporotic activity in ovariectomized rats

5.4.1 Effect of test extracts on Body weights of Ovariectomized Rats

Ovariectomy in female rats produced cardinal signs of postmenopausal osteoporosis i.e. increase in body weight throughout the period of study. The changes in the body weights are expressed as % of the initial body weight. Although food intake of rats in all the groups was similar, the ovariectomized rats showed significant increase in the body weight as compared to the sham control (p<0.05). Estrogen treatment inhibited the ovariectomy-induced gain in the body weight (p<0.05). Treatment with AAM at 250 and 500 mg/kg b.w. and AAA at 250 mg/kg b.w. also significantly inhibited the ovariectomy induced weight gain (p<0.05). (Figure 6-13). Treatment with TAM at 250 and 500 mg/kg b.w. and TAA at 250 mg/kg b.w. also significantly inhibited the ovariectomy induced weight gain (p<0.05). (Figure 5-6)

5.4.2 Effect of test extracts on uterine index in Ovariectomized Rats

As expected, ovariectomy significantly caused atrophy of the uterus compared to the sham rats. From the uterus weight uterine index calculated.

The decrease in the uterine index (the weight of the uterus in proportion to the body weight) caused by ovariectomy was significantly reversed by treatment with estrogen and AAM at both the doses. The uterine index in ovariectomized rats given TAA 500, TAM250, TAM500 and estrogen was also significantly increased than OVX group but the estrogen has a more prominent effect than all other treatments on uterine index. (Figure 5-6)
Results

Figure 5-6 Effect of *Acacia arabica* and *Terminalia arjuna* on body weight in ovariectomy induced osteoporosis in rats.

Each bar represents Mean ± S.E.M. Number of animals in each group = 6.

**SHAM** = Normal control, **OVX** = Disease control, **STD** = Osteoporosis treated with estrogen (2 mg/kg b.w./day), **TAA 250** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (250 mg/kg), **TAA 500** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (500 mg/kg), **TAM 250** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (250 mg/kg), **TAM 500** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (500 mg/kg)

* Significantly different from SHAM (p<0.05), ** significantly different from OVX (p< 0.05)
5.4.3 Effect of test extracts on biochemical serum markers of osteoporosis in ovariectomized rats

5.4.3.1 Serum calcium

No significant change in serum calcium levels was found in ovariectomized rats as compared to SHAM rats. Neither AAA250, AAA500, AAM250, AAM500, TAA250, TAA500 and STD extracts had any significant effects on serum total calcium. However, TAM250 and TAM500 slightly decreased serum calcium level when compared to ovariectomized rats. (Figure 5-7)

5.4.3.2 Serum phosphorous

Ovariectomy decreased serum inorganic phosphate levels compared to SHAM group. Both estrogen and TAM (at both doses) treatments, significantly inhibited the ovariectomy induced decrease in serum inorganic phosphate. However, the effect on serum phosphate levels was not significant in other treatment groups. (Figure 5-7)

5.4.3.3 Serum ALP

Ovariectomy increased the serum alkaline phosphatase when compared to that in Sham rats. Treatment with STD and TAM500 significantly lowered the serum ALP levels when compared to that in the OVX group. (Figure 5-7)

5.4.3.4 Serum Tartarate resistant acid phosphatase (TRAP)

The TRAP levels in serum were significantly increased after ovariectomy indicating increased bone turnover. However, treatment with AAM and TAM caused significant decrease in the levels of this enzyme as compared to the OVX group. (Figure 5-7)
Figure 5-7 Effect of *Acacia arabica* and *Terminalia arjuna* on Serum calcium in ovariectomy induced osteoporosis in rats.

Each bar represents Mean ± S.E.M. Number of animals in each group = 6.

**SHAM** = Normal control, **OVX** = Disease control, **STD** = Osteoporosis treated with estrogen (2 mg/kg b.w./day), **AAA 250** = Osteoporosis treated with Aqueous extract of *Acacia arabica* (250 mg/kg), **AAA 500** = Osteoporosis treated with Aqueous extract of *Acacia arabica* (500 mg/kg), **AAM 250** = Osteoporosis treated with Methanolic extract of *Acacia arabica* (250 mg/kg), **AAM 500** = Osteoporosis treated with Methanolic extract of *Acacia arabica* (500 mg/kg), **TAA 250** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (250 mg/kg), **TAA 500** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (500 mg/kg), **TAM 250** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (250 mg/kg), **TAM 500** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (500 mg/kg).

* Significantly different from SHAM (p< 0.05), ** significantly different from OVX (p< 0.05)
5.4.4 Effect of test extracts on biochemical urine markers of osteoporosis in ovariectomized rats

5.4.4.1 Urine calcium

There was no significant change observed in the serum calcium levels after ovariectomy. However, the urinary excretion of calcium was found to be increased in ovariectomized rats and it was significantly reduced by treatment with STD, AAM 500, TAA500, TAM250 and TAM500. (Figure 5-8)

5.4.4.2 Urine phosphorous

The serum phosphorus levels were significantly reduced after ovariectomy and this coincided with the increase in urinary excretion of phosphorus. The ovariectomy-induced changes in the phosphorus levels in serum and urine were reversed by treatment with STD. Similar effect was observed on urinary excretion of phosphorus after treatment with AAM500, TAA250, TAA500 and TAM250 (Figure 5-8)

5.4.4.3 Urine creatinine

Ovariectomy resulted in a significant reduction in urinary excretion of creatinine in rats. None of the treatments including the STD, could significantly reverse the OVX-induced reduction in urinary creatinine excretion. (Figure 5-8)
Results

Figure 5-8 Effect of *Acacia arabica* and *Terminalia arjuna* treatment on Urine parameters in ovariectomy induced osteoporosis in rats.

Each bar represents Mean ± S.E.M. Number of animals in each group = 6.

**SHAM** = Normal control, **OVX** = Disease control, **STD** = Osteoporosis treated with estrogen (2 mg/kg b.w./day), **AAA 250** = Osteoporosis treated with Aqueous extract of *Acacia arabica* (250 mg/kg), **AAA 500** = Osteoporosis treated with Aqueous extract of *Acacia arabica* (500 mg/kg), **AAM 250** = Osteoporosis treated with Methanolic extract of *Acacia arabica* (250 mg/kg), **AAM 500** = Osteoporosis treated with Methanolic extract of *Acacia arabica* (500 mg/kg), **TAA 250** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (250 mg/kg), **TAA 500** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (500 mg/kg), **TAM 250** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (250 mg/kg), **TAM 500** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (500 mg/kg)

* Significantly different from SHAM (p<0.05), ** significantly different from OVX (p<0.05)
5.4.5 Effect of test extracts on biomechanical markers of osteoporosis in ovariectomized rat

5.4.5.1 Three point bending of tibia

The mechanical parameters of the tibiae measured by three point bending tests were significantly different among various groups. It was found to be significantly lower in OVX group as compared to that of SHAM group. The mechanical strength of bones in ovariectomized rats was restored to normal after treatment with STD and all the extracts except AAA250. (Figure 5-9)

5.4.5.2 Compression of IV lumbar vertebra

The mechanical strength of the bones, measured by, compression of lumbar vertebra was found to be significantly lower in OVX group as compared to that of SHAM group. The mechanical strength of bones in ovariectomized rats was restored to normal after treatment with STD and all the extracts excepts AAM250. (Figure 5-9)

5.4.5.3 Loading test of femoral neck

Ovariectomy caused a significant decrease of the maximal load of the femoral neck compared to the sham rats. Treatment of estrogen remained the maximal load of the femoral neck to the levels near of the sham rat. AAA500, AAM250, AAM500, TAA250, TAA500, TAM250 and TAM500 also increase femoral neck strenght to the significant level. (Figure 5-9)The maximal load required to break femoral neck was significantly lower in OVX rats as compared to SHAM rats.

5.4.5.4 Femur weight

Ovariectomy-induced osteopenia in the rat produces skeletal responses like increase bone turnover which leads to bone loss and subsequently decrease bone weight. In the ovariectomized (ovx) rats, femur weight was significantly decrease and this condition was reversed by treatment with STD as well as with AAA500, AAM250, TAM250 and TAM500 (Figure 5-9)
Figure 5-9 Effect of *Acacia arabica* and *Terminalia arjuna* on mechanical parameters in ovariectomy induced osteoporosis in rats.

Each bar represents Mean ± S.E.M. Number of animals in each group = 6.

**SHAM** = Normal control, **OVX** = Disease control, **STD** = Osteoporosis treated with estrogen (2 mg/kg b.w./day), **AAA 250** = Osteoporosis treated with Aqueous extract of *Acacia arabica* (250 mg/kg), **AAA 500** = Osteoporosis treated with Aqueous extract of *Acacia arabica* (500 mg/kg), **AAM 250** = Osteoporosis treated with Methanolic extract of *Acacia arabica* (250 mg/kg), **AAM 500** = Osteoporosis treated with Methanolic extract of *Acacia arabica* (500 mg/kg), **TAA 250** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (250 mg/kg), **TAA 500** = Osteoporosis treated with Aqueous extract of *Terminalia arjuna* (500 mg/kg), **TAM 250** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (250 mg/kg), **TAM 500** = Osteoporosis treated with Methanolic extract of *Terminalia arjuna* (500 mg/kg)

* Significantly different from SHAM (p< 0.05), ** significantly different from OVX (p< 0.05)
5.4.6 Effect of test extracts on bone mineral content in ovariectomized rats

5.4.6.1 Percentage Ash

There were differences in the right femur ash% among the groups by the end of the treatment period. However, ovariectomy significantly decreased the right femur ash% compared with the sham group. The AAM500, TAM250, TAM500 and STD treatments significantly increased the right femur ash% compared with the OVX group. (Figure 5-10)

5.4.6.2 Bone calcium

Bone calcium of Sham and OVX model had significant difference, This indicated that ovariecomy decreased ash calcium. The administration of estrogen to the OVX rats significantly recovered calcium level. AAA500, AAM250, AAM500, AAM250, TAA500, TAM250 and TAM500 also restored BMD almost completely to the level of Sham group. (Figure 5-10)

5.4.6.3 Bone phosphorous

The Phosphorous of the bones, measured by prepared ash of it. There was not any difference in OVX group and other treated groups as compared to that of SHAM group. (Figure 5-10)

5.4.6.4 Bone magnesium

At 6 weeks after operation, ovariectomy caused significant decrease in the ash magnesium level compared to the sham rats. Both STD and other treatment groups cause not any change in the ash magnesium level as compare to OVX rats. (Figure 5-10)
Figure 5-10 Effect of Acacia arabica and Terminalia arjuna on bone mineral contents in ovariectomy induced osteoporosis in rats.

Each bar represents Mean ± S.E.M. Number of animals in each group = 6.

**SHAM** = Normal control, **OVX** = Disease control, **STD** = Osteoporosis treated with estrogen (2 mg/kg b.w./day), **AAA 250** = Osteoporosis treated with Aqueous extract of Acacia arabica (250 mg/kg), **AAA 500** = Osteoporosis treated with Aqueous extract of Acacia arabica (500 mg/kg), **AAM 250** = Osteoporosis treated with Methanolic extract of Acacia arabica (250 mg/kg), **AAM 500** = Osteoporosis treated with Methanolic extract of Acacia arabica (500 mg/kg), **TAA 250** = Osteoporosis treated with Aqueous extract of Terminalia arjuna (250 mg/kg), **TAA 500** = Osteoporosis treated with Aqueous extract of Terminalia arjuna (500 mg/kg), **TAM 250** = Osteoporosis treated with Methanolic extract of Terminalia arjuna (250 mg/kg), **TAM 500** = Osteoporosis treated with Methanolic extract of Terminalia arjuna (500 mg/kg)

* Significantly different from SHAM (p< 0.05), ** significantly different from OVX (p< 0.05)
5.5 Bioactivity guided fractionation of TAM

Methanolic extract of *Terminalia arjuna* (TAM) was found to be most effective in *in vivo* study, amongst all the extracts. Hence, it was further fractionated by successive extraction with organic solvents of increasing polarity viz. Petroleum ether (60-80°C), toluene, ethyl acetate and n-butanol. The marc left after extraction with n-butanol was denoted as residual extract. (Figure 5-11)

![Figure 5-11 Fractionation of TAM by successive extraction with solvents of increasing polarity](image)

**TAM** = Methanolic extract of *Terminalia arjuna*, **TAM-P**= Petroleum ether soluble fraction of TAM, **TAM-T**= Toluene soluble fraction of TAM, **TAM-Et**= Ethyl acetate soluble fraction of TAM, **TAM-Nbut**= n-butanol soluble fraction of TAM, **TAM-R**= Residual fraction of TAM
5.6 Pharmacognostical and pharmacological activity of fractions of TAM

5.6.1 Phytochemical screening of fractions of TAM

Preliminary phytochemical analysis showed that TAM-Nbut mainly contain saponin and tannin wherever residual fraction contain mainly tannins.

5.6.2 Effect of fractions of TAM on bone resorption

Effect of different fractions of TAM on the calcium contents in the femoral-diaphyseal and femoral-metaphyseal tissues, was evaluated using \textit{in-vitro} bone culture method. The results are shown in Figure 5-12. The calcium content in the diaphyseal and metaphyseal tissues significantly increased when the bone tissues were cultured alongwith TAM-Nbut fraction. Other fractions did not cause significant increase in bone calcium content.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure512}
\caption{Effect of fractions of TAM on the calcium contents in \textit{in vitro} experiments using diaphyseal and metaphyseal tissues obtained from femur bone.}
\end{figure}

Each bar represents Mean ± S.E.M. Number of samples in each group = 3.

SHAM = Normal control, TAM-P= Petroleum ether soluble fraction of TAM, TAM-T= Toluene soluble fraction of TAM, TAM-Et= Ethyl acetate soluble fraction of TAM, TAM-Nbut= n-butanol soluble fraction of TAM, TAM-R= Residual fraction of TAM

* Significantly different from SHAM (p< 0.05)

5.7 Isolation of TAM-Nbut-S and TAM-Nbut-A

Saponin fraction was isolated from TAM-Nbut fraction and its yield was 50% of the mother fraction. Moreover, saponin fraction was hydrolysed to obtain the aglycone fraction (Yield: 33% of TAM-Nbut)
5.8 Pharmacological activity of TAM-Nbut-S and TAM-Nbut-A

Effect of saponin and aglycone fractions of TAM-Nbut on the calcium contents in the femoral-metaphyseal tissues, was investigated using *in-vitro* bone culture method. The calcium content in the metaphyseal tissues significantly increased when the bone tissues were cultured in the presence of different concentrations of TAM-Nbut-S and TAM-Nbut-A. (Figure 5-13)

![Figure 5-13](image_url)

**Figure 5-13** Effect of TAM-Nbut-S and TAM-Nbut-A on the calcium contents in *in vitro* experiments using metaphyseal tissues obtained from femur bone.

Each bar represents Mean ± S.E.M. Number of samples in each group = 3.

SHAM = Normal control, **TAM-Nbut-S (10)**= Saponin fraction of TAM-Nbut (10 µg/ml), **TAM-Nbut-S (25)**= Saponin fraction of TAM-Nbut (25 µg/ml), **TAM-Nbut-S (50)**= Saponin fraction of TAM-Nbut (50 µg/ml), **TAM-Nbut-A (10)**= Aglycone fraction of TAM-Nbut (10 µg/ml), **TAM-Nbut-A (25)**= Aglycone fraction of TAM-Nbut (25 µg/ml), **TAM-Nbut-A (50)**= Aglycone fraction of TAM-Nbut (50 µg/ml).

* Significantly different from SHAM (p < 0.05)

5.9 Study of arjunetin in TAM-Nbut-A fraction

5.9.1 TLC analysis of arjunetin and TAM-Nbut-A

After trying various combination of mobile phase, we finally selected Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.8:0.2) mixture as mobile phase to achieve adequate resolution and effective separation of compounds in TLC plate. (Figure 5-14)
5.9.2 *In vitro* Pharmacological evaluation of arjunetin

Since arjunetin is major aglycone found in *Terminalia arjuna* and TLC analysis showed its presence in TAM-Nbut-A, arjunetin was investigated for its effect on bone calcium content in *in vitro* using bone culture method. The calcium content in the metaphyseal tissues significantly increased when the bone tissues were cultured in the presence of different concentrations of arjunetin. (Figure 5-15)

![Figure 5-15](image)

**Figure 5-15** Effect of Arjunetin on the calcium contents in *in vitro* experiments using metaphyseal tissues obtained from femur bone.

Each bar represents Mean ± S.E.M. Number of samples in each group = 3.

**CONTROL** = Normal control, **ARJ 10**= Arjunetin (10 μg/ml), **ARJ 50**= Arjunetin (50 μg/ml), **ARJ 100**= Arjunetin (100 μg/ml)

* Significantly different from SHAM (p< 0.05)
5.10 Determination of content of arjunetin in TAM-Nbut-A by HPLC

5.10.1 Calibration curve

The calibration curve of arjunetin was found to be linear in the concentration range of 5-25 μg/ml with a correlation coefficient ($r^2$) of 0.9951. Average retention time (Rt) of arjunetin in the given mobile phase was 5.7246±0.03 minute. (Figure 5-17 and 18, Table 5-5)

![Figure 5-16 HPLC chromatogram of reference standard - arjunetin (25 μg/ml)](image)

![Figure 5-17 Overlay chromatograms of arjunetin solutions in the concentrations ranging from 5 to 25 μg/ml](image)
Table 5-4 Linearity range of Arjunetin

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Concentration (μg/ml)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>80499</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>162947</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>226963</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>315937</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>369547</td>
</tr>
</tbody>
</table>

Figure 5-18 Calibration curve of arjunetin

5.10.2 Estimation of arjunetin in TAM-Nbut-A fraction

The developed HPLC method was used for determination of arjunetin from TAM-Nbut-A fraction. The sample working solution (10 μL) was injected and the peak area of arjunetin was measured. The peak of arjunetin in the extract was identified by comparing Rt value with that of reference arjunetin. (Figure 5-19)
Figure 5-19 HPLC chromatogram of TAM-Nbut-A fraction

5.10.3 Calculation

From the calibration curve of marker Arjunetin

\[ Y = 14622X + 11853 \] (\( R^2 = 0.995 \)), where \( X \) = Concentration and \( Y \) = Area

From chromatogram of TAM-Nbut-A , \( Y = 230366 \). This value put in above equation so, \( X = 14.94 \) \( \mu \)g/ml. Multiply it with dilution factor 200. Convert the concentration in to gm/ml unit and finally result is converted in to %w/w.

\[
\begin{align*}
    X & = 14.94 \times 200 \, \mu \text{g/ml} \\
    & = 2988 \times 10^{-6} \times 5 \, \text{gm/5ml} \\
    & = 0.014940 \, \text{gm.}
\end{align*}
\]

2.58 gm powder contain 0.014940 gm arjunetin

The TAM-Nbut-A was estimated to contain 0.5790 w/w% arjunetin
6 DISCUSSION

Osteoporosis is a disease in which the density and quality of bone are reduced, leading to weakness of the skeleton and increased risk of fracture, particularly of the spine, wrist and hip. (53, 54) Osteoporosis and associated fractures are an important cause of mortality and morbidity. Osteoporosis is a global problem, which is increasing in significance as the population of the world both grows and ages. Worldwide, lifetime risk for osteoporotic fractures in women is 30-50% and in men it is 15-30%. (55, 56)

As the second most populous country in the world, India is home to a very large population of osteoporosis patients. One out of 8 males and 1 out of 3 females in India suffer from osteoporosis, making India one of the largest affected countries in the world. (57) Expert groups peg the number of osteoporosis patients at approximately 26 million (2003 figures) with the numbers projected to increase to 36 million by 2013. (58) In most Western countries, while the peak incidence of osteoporosis occurs at about 70-80 years of age, in India it may afflict those 10-20 years younger, at age 50-60. Ageing of populations worldwide will be responsible for a major increase in the incidence of osteoporosis in postmenopausal women. (59) To minimize future predicted costs, morbidity, and mortality from increasing numbers of osteoporotic fractures in our rapidly aging population, the American academy of orthopedic surgeons (AAOS) recommends that osteoporosis should become a national public health priority. (60) This was a main concern for selecting osteoporosis as the research area for this project.

Osteoporosis is more common in women than in men. The sudden drop in estrogen levels that characterize the menopause among older women is responsible factor for the postmenopausal osteoporosis. Many genetic and environmental factors influence the fracture risk also. Most of the osteoporosis medications were developed for the treatment of postmenopausal osteoporosis and some are licensed for use only in women. (61) Although hormone replacement therapy and other bone-forming agents have been shown to be effective in prevention and treatment
of post-menopausal bone loss, alternatives are continuously being searched because of actual or possible side effects, or contraindications limiting their use, and poor compliance of patients. (62-64) Now a days there is a renewed interest in drugs of natural origin simply because they are considered relatively safer. (65) So, main center of our attention was to evaluate some Ayurvedic herbal drugs for their efficacy in post-menopausal osteoporosis.

The project was brought into being by an extensive literature review for the selection of herbal drugs for investigation. Certain plant compounds, some of which have been characterized as phytoestrogens, have shown a weak estrogenic effect on bone in human and animal studies. (8, 66-72) It is also reported that “Plants with anti-inflammatory role can be potent candidates as an osteoprotective agent” (16). Some herbal drugs have been traditionally used in Ayurveda to accelerate the healing of bone fractures and to strengthen the bones. (15) On the basis of above literature reports stem bark of Acacia arabica, stem bark of Terminalia arjuna, oleogum resin of Commiphora mukul and Boswellia serrata were selected for our research study.

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The advantages of natural drugs are their easy availability, cost-effectiveness and negligible side effects but the disadvantage is that they do not have consistent quality. A significant factor, which can add to the consistent quality of medicinal plants, is to have satisfactory standardization. Due to the natural heterogeneity such as varied geographical location where these plants grow, problem of diverse vernacular names these plants are known by, the quality of herbal starting materials obtained from wild collections shows great fluctuations. Hence, standardization of herbal products has been extensively required. (73, 74) For standardization and quality assurance of above selected herbal drugs, mainly three attributes were verified: Morphology, microscopy and physicochemical parameters. All morphological and microscopical characteristics were identical to those reported earlier in standard books. (52) Further, the plants were identified and authenticated by Department of Pharmacognosy, KBIPER, Gandhinagar, India. The macroscopic and the microscopic studies of the herbal drugs helped to assume their identity.
Physicochemical studies of the plant drugs are also necessary for standardization, as it helps in understanding the significance of physical and chemical properties of the substance being analyzed in terms of their observed pharmacological activities and especially for the determination of their purity and quality.

The purity of crude drugs could also be evaluated by the determination of ash values, which represent the content of foreign matter such as inorganic salts or silica present as a form of adulterant in the drug sample. Analytical results for total ash were also found similar to standard values noted in compendial literature (AA = 10.66%, TA = 21.33%, CM = 4.66%, BS = 6.00%) The total ash includes both ‘physiological ash’ which is derived from the plant tissue itself, and ‘non-physiological ash’, which is the residue of the extraneous matter adhering to the plant surface. The amount of acid insoluble ash was found to comply with the compendial limit. Acid insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. (31, 75) The ash content gives an idea about the inorganic content of powdered under investigation and thus the quality of the drugs can be assessed.

On the other hand, the water soluble extractive value of the drug was found to be AA = 13.6%, TA = 24.8%, CM= 55.32%, BS= 28.20% (w/w) which indicates the presence of water soluble components such as sugar, acids and inorganic compounds etc.; and the alcohol soluble extractive value was found to be AA = 25.6%, TA = 23.2%, CM= 56.68%, BS= 50.44% (w/w) which indicates the presence of polar and moderately polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids etc. The results of physicochemical analyses lie within the acceptable limits, (52, 76-78)which in turn ascertain the quality as well as purity of selected herbal drugs.

Due to resurgence of interest in herbal drugs demand and hence supply of herbal drugs has increased. In order to maintain trust in herbal medicines, it is also important to ensure that only quality products enter the market. Efforts are being
made by various government agencies and research laboratories to maintain the quality of herbal drugs by proper identification and detailed pharmacognostic, phytochemical investigations and standardization. However, in spite of the continuing efforts, there are no standard methods available for quality control of herbal drugs, which is the main hurdle for India to enter into the multi-million dollar international market. Further, the composition of plant material can vary and it is known to be influenced by the place of origin, soil, climate, season, time of collection, post harvesting conditions, temperature changes, moisture which affect tremendously the quality and therapeutic efficacy of the drug. Therefore, the quality and efficacy of the herbal drugs need to be established through systematic pharmacognostic, phytochemical and pharmacological evaluation and standardization of the drug.

The authenticated powdered plant materials of herbal drugs were extracted with different solvents (Water, methanol, petroleum ether, ethyl acetate) using hot maceration method. Preliminary phytochemical analysis of different extract in our investigation showed the presence of tannins, flavonoids and saponins in AAA extract and Alkaloids, Tannins, flavonoids, saponins and sterols in AAM extract. TAA showed presence of Tannins and saponins and TAM showed presence of tannins, saponins and sterols. CMP and CMEt showed presence of fat and steroid with flavonoids, gums and mucilage, fat and steroid. BSP showed presence of fat and steroid and BSEt showed presence of alkaloid, flavonoids, gums and mucilage, fat and steroid. All this findings are consistent with those reported earlier by other investigators. (24, 79-81)

All above mentioned extracts of selected drugs were screened for their anti-osteoporotic activity using in-vitro bone culture method. In vitro methods reduce the use of animals and some evidence exists that in-vitro studies are capable or potentially capable of providing more rapid, precise, and relevant information than do some animal studies. It is relatively inexpensive. The primary advantage of in vitro work is that it permits an enormous level of simplification of the system under study, so that the investigator can focus on a small number of components. (82)
From the *in vitro* study we conclude that *Acacia arabica* and *Terminalia arjuna* are more effective amongst selected herbal drugs. Although the crude extract of above drugs has been shown to possess anti-osteoporotic activity in *in vitro* model a detailed investigation of the effect of *Acacia arabica* and *Terminalia arjuna* on the metabolic alterations in osteoporosis is required to check which one is more effective among this two, which show more efficacy in *in vitro* model. Thus, the first objective of the present investigation was to perform activity guided phytopharmacological analysis of *Terminalia arjuna* and *Acacia arabica* with special reference to post-menopausal osteoporosis using bilateral ovariectomized rats.

Currently there is no single animal model of postmenopausal osteoporosis that identically represents the stages of osteoporosis in humans, (83) although there are some animals that are relatively close to humans in terms of physiology and can be used for the purpose of comparison. Both small animals and large animals are used depending on which aspects of the osteoporotic condition are being studied. Such animals include rats, rabbits, and sheep. Pathophysiological condition similar to postmenopausal osteoporosis can be produced in these experimental animals by ovariectomy.

Of these animal models, the ovariectomized rat model remains the most popular choice and currently principal laboratory animal, used to investigate this disease, because they are inexpensive to maintain, grow rapidly, have a relatively short lifespan and are widely available (84-87) and as it has been validated to represent the most important clinical features of estrogen deficiency-induced (or postmenopausal) bone loss in the adult human,(88) particularly during the early stages of osteoporosis.(89) These include: increased rate of bone turnover with resorption exceeding formation; an initial rapid phase of bone loss followed by a much slower phase; greater loss of cancellous bone than cortical bone; reduced intestinal calcium absorption; some protection against bone loss by obesity; and similar skeletal response to therapy with estrogen, tamoxifen, bisphosphonates, parathyroid hormone, calcitonin and exercise. (90-98) Moreover, rodents are preffered for ovariectomy induced postmenopausal osteoporosis because the uterus
rapidly regresses in size following ovary removal and incidence of uterine disease is low. The skin of rodents is so loose that the skin incision can be retracted from one side to the other to remove each ovary from the same skin incision. (99-102)

In summary, the striking resemblance of the ovariectomized rats to humans with respect to above criteria (osteopenia) makes the ovariectomized rat, a gold standard model of post-menopausal human osteoporosis. Hence, Ovariectomy-induced osteoporosis in rat was used as the experimental model for in vivo efficacy study of TAA, TAM, AAA and AAM.

Ovariectomy in female rats can be performed in different ways and the selection of the operative method for ovariectomy is very important, especially when the number of animals is very high and the duration of the experiment is short. There are mainly two types of incision used for doing ovariectomy in female rats: single midline dorsal skin incision (39) and double dorsolateral incisions. (103) A short single midline dorsal skin incision method was used for our study because surgery time is significantly less and healing of the wound and recovery is fast as compare to double dorsolateral incision method.

The success of a surgical procedure performed through an abdominal incision depends on careful selection of the incision site and proper closure of the wound. The surgeon needs to consider multiple factors before making an abdominal incision. These factors include the area that needs to be exposed, the disease process, body habits, operative exposure, simplicity, previous scars, cosmesis, the need for quick entry into the abdominal cavity (the elective or emergency nature of the operation) and personal preference.

Preoperative and postoperative care was taken to avoid complications like infections, eviscerations, wound dehiscence, hemorrhage, hypothermia, pulmonary hypostatic congestion, dehydration and anesthetic overdose from these procedures. Only disease free animals were selected as surgical candidates, which can prevent many of these complications. Evisceration and wound dehiscence are often associated with stich removal by the animal by own or by pen mates and it was
avoided by the use of tissue adhesive and auto clips. Infections were avoided by using best sterile techniques and maintained hygienic conditions during and after surgery. Neosporin powder and soframycin cream were carefully selected as a prophylaxis. A best ovariectomy procedure protocol follows to prevent hemorrhage associated with accidental injury to spleen and liver. Ketamine was used in minimum required dose to avoid complication of respiratory distress. All the required postoperative precautions were maintained such as close observation and provision of supplementary heat, fluids and stimulation. All these precautions and care resulted into to zero percent mortality of animals during the study.

Osteoporosis is not only a group of diseases characterized by decrease in bone mineral density resulting from decrease estrogen or other factors but it is also associated with changes in many biochemical markers and biomechanical parameters. Hence, while studying anti-osteoporotic activity of aqueous and methanolic extract of barks of Acacia arabica and Terminalia arjuna, their effects on serum and urine markers of osteoporosis, on biomechanical functions of bone and effect on bone mineral content were investigated in ovariectomized rat.

The ovarian hormones play a significant role in the regulation of food intake and body mass. It has been noted that, during the estrus cycle, food intake tends to be lowest around the time of ovulation when estrogen is the highest, and highest at the diestrus period when estrogen is lowest. Studies have shown that withdrawal of ovarian hormones in rats increases food intake. (104-108) As seen in many studies, ovariectomized rats have significantly higher body weights compared to sham-operated rats due to fat deposition caused by estrogen deficiency. (109-111) The increased body weight provides an additional stimulus for bone neoformation, serving as a partial protection against the osteopenia that occurs in long bones meant for supporting body weight. (90, 112) In this study, the increased body weight in the ovariectomized rat and its reversal in estrogen treated ovariectomized rats indicates that the gain in body weight is due to estrogen deficiency and as a consequence of the partial protection mechanism discussed above. The reversal of
OVX-induced gain in the body weight after treatment with AAM and TAM indicates that these extractsmight have an estrogen-mimetic effect.

Estrogen plays a major role in building the uterine tissue. It accomplishes this by increasing the size and the number of tissues and blood vessels in the uterus. It is responsible for the proliferation of the uterine endometrium and an increased blood, lymphatics and nerve supply to the uterus. (113, 114) The lower uterine index in OVX rats as compared to SHAM control is again due to estrogen deficiency leading to reduced proliferation of endometrium. The reversal of this effect after treatment with AAM and TAM reinforces our presumption that AAM and TAM might have an estrogen-mimetic effect.

Calcium is an essential nutrient that is involved in most metabolic processes and the phosphate salts of which provide mechanical rigidity to the bones and teeth, where 99% of the body's calcium resides. (115) Estrogen is a major female hormone that improves body's ability to absorb calcium from digestive tract. Estrogen also helps maintain calcium levels in skeleton. Menopausal women and women who do not produce sufficient estrogen suffer from decreased bone density because lack of estrogen impairs calcium absorption, resulting in resorption from the skeleton to meet the body's calcium needs. (116) Similar effect was observed in the OVX rats. There was no significant difference in the serum calcium levels of OVX rats as compared to the SHAM control. However, the increased urinary excretion of calcium in OVX rats is suggestive of excessive bone resorption. The increase in bone resorption did not affect the serum calcium levels because many factors other than estrogen play role in homeostasis of calcium in blood. Treatment of ovariectomized rats with STD, AAM 500, TAA500, TAM250 and TAM500 resulted in reduced calcium excretion through urine compared to the untreated rats. This is suggestive of decrease in bone resorption in ovariectomized rats due to these treatments.

Phosphorous is an essential component of bone formation, as calcium phosphate is the primary substance in bone. Phosphorus, along with calcium, constitutes a major portion of the hydroxyapatite crystal in bone. That gives bones and teeth their rigidity. Therefore, serum phosphorous must be part of the initial
evaluation of osteoporotic patients or patients with low bone mass. (117, 118) Increased excretion of phosphorus through urine was due to increased demineralization of bones resulting from Ovariectomy-induced estrogen deficiency. (119, 120) It is thus, concluded that urinary calcium and phosphate can be used as valuable markers of bone loss in postmenopausal women and further studies are necessary to highlight their role in the diagnosis and prognosis of postmenopausal osteoporosis. These biochemical bone markers are inexpensive and valuable predictors of bone loss at all ages especially in the postmenopausal women. Evaluation of bone loss by these biochemical markers also decreases the risk of osteoporotic fractures, which may be due to estrogen deficiency or nutritional deficiencies.

Alkaline phosphatase (ALP) is an important enzyme in the process of bone remodeling. It promotes the mineralization of matrix by decomposing the phosphoric ester into inorganic phosphorous to increase the phosphorous concentration. Thus, ALP plays an important role in osteoid formation and bone mineralization. (121) Serum total ALP is the most widely used marker of bone metabolism due to the wide availability of inexpensive and simple methods. In bones, ALP is specifically secreted by osteoblasts, which promote bone formation. (122) Bone turnover has been found to be significantly increased in postmenopausal women (123) and can be diagnosed by the increased serum ALP levels. (124) The increase of bone-specific ALP levels after menopause in women has been explained by removal of the inhibitory effects of estrogen on bone turnover rate. (125) The increase in serum ALP in untreated OVX rats and its return to normal levels after treatment with estrogen are in agreement with earlier reports discussed above. Though AAM mimicked estrogen in its influence on all other parameters, serum ALP was not restored to normal levels after treatment with AAM. Treatment with TAM (500 mg/kg b.w.) lowered all levels as compared to those in the OVX group. Since ALP is an indicator of osteoblast activity, TAM might be directly promoting the osteoblast activity or the osteogenic differentiation of bone marrow stromal cells.
Osteoclastic bone resorption is mediated by the formation of new osteoclasts and the resorption activity of osteoclasts. TRAP is a histochemical marker of osteoclast, secreted specifically by the osteoclasts in bones. (126) It is a membrane bound enzyme, which dephosphorylates bone matrix phosphoproteins and thus, allows migration of osteoclasts. (127) As a bone marker, TRAP is unique in that it reflects the number of osteoclasts. Given the fact that osteoclasts resorb bones, (122) TRAP is a useful indicator of bone resorption. A significant decline in serum TRAP levels in OVX rats treated with AAM and TAM is suggestive of a reduction in number of osteoclasts and hence reduction in bone resorption.

Post-menopausal estrogen deficiency leads to increased bone resorption, which results in a reduction in the mechanical strength of the bones, which in turn, is related to bone density, micro-architecture, connectivity and mineralization. (128) Similar results were observed in OVX rats, in which the biomechanical strength of the bones was found to be reduced. This was indicated by lesser pressure required to fracture the femoral neck and the tibia and to compress the vertebra, as compared to that for the SHAM group. The excessive de-ossification of the bones in OVX rats was also evident from the lesser bone density and bone calcium content as compared to SHAM rats. Treatment of OVX rats with EST, AAA, AAM, TAA and TAM prevented the de-ossification of bones and there was significant improvement in biomechanical strength, bone density and bone calcium content compared to the untreated OVX rats.

Decreased bone mass is one of the major factors jeopardizing bone integrity, resulting in reduced bone weight, strength and an increased susceptibility to fractures. (129), And the same was observed in the present study. Treatment with AAM and TAM significantly prevented the loss of bone weight in OVX rats. There was no any change in femur length due to ovariectomy or treatment.

Bone mineral density (BMD), bone mineral content (BMC), and bone size have been regarded as important determinants of osteoporotic fractures. BMC is the gold standard for the evaluation of individuals at risk for osteoporosis, as it best predicts the fracture risk in people without previous fractures. (130) In the present
study, ovariectomy was found to significantly decrease the BMC of the total femur as compared to the sham group. AAM and TAM administration prevented OVX-induced calcium loss of the total femur but there was no any significant effect on ash magnesium and phosphorous contents of the bone.

The in vivo efficacy of extracts on OVX rats, assessed on the basis of effects on biochemical, bone mineral density, biomechanical parameters showed that TAM reversed all the pathophysiological changes caused by OVX and its effects resembled those observed with estrogen treatment. Hence, TAM has the potential to be used as an alternative to estrogen replacement therapy in post-menopausal osteoporosis.

Various epidemiologic studies reported an increase in the risk of developing osteoporosis in various inflammatory conditions such as rheumatoid arthritis, haematological diseases, and inflammatory bowel disease. (131-134) Proinflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-6, IL-1, IL-11, IL-15, and IL-17 are elevated in these conditions. (135-140) IL-6 and IL-1 may influence osteoclastogenesis by stimulating self-renewal and inhibiting the apoptosis of osteoclasts progenitors. (140, 141) They promote osteoclast differentiation, which is an important stimulator of bone resorption that has been linked to accelerated bone loss seen in postmenopausal women. (134) Estrogen deficiency leads to upregulation of cytokines, Interleukins, RANK-L, TNF alpha, which are responsible for enhancing osteoclastic activity (142) and down-regulation of osteoprotegrin which is a potent antiosteoclastogenic factor. This results in an increase in inflammatory responses and increase in bone-resorption activity. (143) Terminalia arjuna is reported to have anti-inflammatory property. (18) Therefore, it might be causing suppression of these potent inflammatory mediators and hence, preventing further consequences responsible for the bone loss.

Body is subjected to high oxidative stress following estrogen deficiency, lipid accumulation occurs. Lipid peroxidation promotes osteoblast apoptosis and simultaneously up-regulates ROS production. (144, 145) ROS has been shown to promote osteoclast resorption activity either directly or mimicking RANK signaling and stimulating osteoclast differentiation, or indirectly, by stimulating
Oxidative stress may also increase bone resorption through activation of NF-κB, which plays an important role in osteoclastogenesis. (149, 150) Antioxidant compounds such as flavonoids and beta-carotene have been reported to be present in stem bark of *Terminalia arjuna*. Hence, supplementation of *Terminalia arjuna* extracts which contains antioxidant properties can reduce oxidative stress level and thus indirectly prevent bone resorption.

The methanolic extract of *Terminalia arjuna* bark has been reported to contain high concentrations of phytosterols, phenolic compounds, tannins, triterpenoids and saponins. (151) Similar results have been obtained in the phytochemical analysis of TAM. Phytoestrogens are plant-derived substances whose structure results in a chemical nature similar to endogenous estrogens of humans. They have antioxidant property and are adaptogens also. Phytoestrogens act as SERM (Selective estrogen Receptor Modulators), which produce the desired action without side effects. They act as anti-estrogenic in breast and uterine tissue but estrogenic in bone, brain and lipid metabolism and in cardiovascular system.

The positive effects of TAM on the markers of bone metabolism in ovariectomized rats, which are comparable to estrogen and the reported pharmacological and phytochemical properties of *Terminalia arjuna* discussed above, lead to a conclusion that the anti-osteoporotic effect of TAM is due to it’s anti-inflammatory, phytoestrogenic, and antioxidative properties.

The phytochemical investigation of TAM extract showed the presence of mainly saponins and tannins in it. In order to isolate the anti-osteoporotic principle from TAM, a bioactivity-guided fractionation of TAM was done. Bioactivity guided fractionation is an isolation of an active compound or fraction from biomass using a decision tree based solely on bioactivity. In vitro bone culture assay was used to assess the pharmacological activity of fractions at each stage. In the initial stage, TAM was fractionated by successively extracting it with solvents of increasing polarity. The n-butanol fraction of TAM showed most potent effect on bone calcium deposition. The phytochemical investigation of n-butanol fraction of TAM extract
showed the presence of mainly saponin. Including mainly triterpenoid saponins, which were thought to be the reason behind the phytoestrogenic activity of TAM.

Saponin, a widely distributed class of natural product has shown encouraging biological activities. Several discoveries have demonstrated that saponins present in plants are the most important therapeutic agent for the treatment of osteoporosis.(152) Evidence from several human studies also demonstrate that certain dietary phytoestrogen compounds can produce osteogenic effects in postmenopausal women, including oestrogen like effects on vaginal cytology and reductions in hot flushes. So, phytoestrogens increase the overall quality of life. (152) It has been reported that the triterpene-saponins reduced the development of osteoporosis most likely by a reduction of the bone marrow fat load and possibly by reducing the secretion of pro-inflammatory cytokines. (153) Previous studies of saponins fractions from different plant have suggested that it can prevent estrogen deficiency-induced bone loss by dual action: stimulation of new bone formation and inhibition of bone resorption. (154, 155) Based on these reports, it was postuated that anti-osteoporotic activity of TAM-Nbut could be because of saponins present in it. Hence, saponin fraction was isolated from TAM-Nbut.

Arjunetin a triterpenoid sapogenin is one of the major compounds reported in Terminalia arjuna. There are no pharmacological data available to substantiate the therapeutic value of Terminalia arjuna. We made a successful attempt to isolate aglycone fraction and characterize the compound arjunetin in TAM-Nbut-A fraction. We then studied the effect of arjunetin using in vitro model. While studying the anti-osteoporotic activity of various fractions and the aglycone fraction isolated from Terminalia arjuna, it was found that TAM-Nbut-A possess impressive anti-osteoporotic activity. In the end we also made an attempt to correlate the mechanism of action of activity investigated. In addition to estrogen-mimetic effect, TAM might promote the osteogenic differentiation of bone marrow stromal cells. Further studies are needed to substantiate the mechanism of action of TAM and to isolate the compound responsible for its anti-osteoporotic activity.
7 REFERENCES


References


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K.B.I.P.E.R

Kadi Sarva Vishwavidyalaya

References


8 ANNEXURE

8.1 IAEC Certificate

CERTIFICATE

This is to certify that the project entitled “Investigation of some ayurvedic herbal drugs for efficacy in post-menopausal osteoporosis using ovariomized rat model” has been approved by the IAEC.

Protocol No. PIPH 01/10

Name of chairman / member secretary IEAC: Dr. Sajamur Matic

Signature with date

Name of CPCSEA nominee: Yv. Arindakumar Sheth, Link

Chairman / member secretary of IAEC: Dr. Sajamur Matic

CPCSEA nominee: 12-3-10

(Kindly make sure that minutes of the meeting duly signed by all participants are maintained by office)
8.2 List of publications

ABSTRACT

Osteoporosis is a disease characterized by low bone mass, micro-architectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk, it is a major cause of morbidity and mortality and medical expense worldwide. In India, it is found that 29.9% of women and 24.3% of men aged between 20 and 79 year had low bone mass. Furthermore about 50% women and 36% of men over 50 years of age were noted to have low bone mass. The wound healing time of the vertical incision group was slightly shorter than those of the transverse incision group. But didn’t differ significantly. None of the distributions of wound length and healing percentage per day showed significant variation between these two groups. Variation of the wound length is not differing between two groups.

Keywords: Osteoporosis, transverse surgery, vertical surgery

INTRODUCTION

Osteoporosis is a disease characterized by low bone mass, micro-architectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk, it is a major cause of morbidity and mortality and medical expense worldwide. In India, it is found that 29.9% of women and 24.3% of men aged between 20 and 79 year had low bone mass. Furthermore about 50% women and 36% of men over 50 years of age were noted to have low bone mass [1]. Osteoporosis affects an estimated 75 million people in the United States, Europe, and Japan combined, including one in three postmenopausal women and majority of the elderly. Osteoporosis cause more than 1,300,000 fractures annually in the United States alone. The disease will be a greater problem in the future, because the world population is aging and the incidence of osteoporotic fractures is increasing in many geographic area [1-2]. The primary osteoporosis include two entities: one related to menopause, estrogen loss and the other to aging. The primary osteoporosis represents two fundamental different conditions. Type I characterized by loss of trabecular bone owing to estrogen deficiency at menopause and type II osteoporosis characterized by loss of cortical and trabecular bone in men and women owing to long-term remodeling, inefficiency, dietary inadequate, and activation of the parathyroid axis with age [3].
Effect of Acacia Arabica Extracts on Bone Calcium Content

Nimisha Kakadia and Niranjan S. Kanaki

1Department of Pharmacology, K.B. Institute of Pharmaceutical Education and Research, GH-6, Sector-23, Gandhinagar-382023, Gujarat, India.
2Department of Pharmacognosy, K.B. Institute of Pharmaceutical Education and Research, GH-6, Sector-23, Gandhinagar-382023, Gujarat, India.

ABSTRACT
Introduction: Osteoporosis is a disease characterized by low bone mass, micro-architectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk. The available drug treatments have many side effects. So alternative indigenous medicine should be use, which have a lower side effect. Many herbal drugs are reported as anti-osteoporotic agents in ayurveda but they are not scientifically evaluated. Objective: To evaluate the effect of Acacia arabica on bone calcium using in-vitro bone culture experiment. Method: The aqueous and hexanolic extracts of Acacia arabica prepared using hot maceration and soxhlet apparatus method respectively. All extracts evaluate for the effect on bone calcium using in-vitro bone culture experiment. Calcium content was measured and all the data were evaluated using graphpad prism software. Result: The aqueous and methanolic extracts of Acacia arabica caused a significant increase in the calcium content in the diaphyseal and metaphyseal tissues in vitro bone culture experiments. Conclusion: The effect could be due to the steroidal content present in drug extracts. So Acacia Arabica can be used as an anti-osteoporotic agent.

Keywords: Bone calcium, Acacia arabica, osteoporosis.

1. INTRODUCTION
Since ancient times herbal medicines have been used by traditional medical practitioners for the treatment of osteoporosis. Many of these herbal formulations are prepared using a collection of plant materials according to traditional formulas. Although these herbal medicines have been prescribed to patients for so many years, most of them may have not been subjected to scientific investigation to determine whether these herbal drugs truly have the potential to be benefit of the patients. The therapeutic activity of herbal medicinal compounds towards osteoporosis is often attributed depend on their dose concentration. So the present study was carried out to investigate the effective concentration of Indian traditional herbal drugs like Acacia arabica and Terminalia arjuna using in-vitro bone culture.

The term half maximal effective concentration (EC50) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug's potency. The ED50 of a graded dose response curve therefore represents the concentration of a compound where 50% of its maximal effect is observed. The EC50 of a quantal dose response curve represents the concentration of a compound where 50% of the population exhibit a response, after a specified exposure duration. It is also related to IC50 which is a measure of a compound's inhibition (50% inhibition). For competition binding assays and functional antagonist assays IC50 is the most common summary measure of the dose-response curve. For agonist/stimulator assays the most common summary measure is the EC50. Concentration measures typically follow a sigmoidal curve, increasing rapidly over a relatively small change in concentration. The point at which the effectiveness slows with increasing concentration is the IC50. This can be determined mathematically by derivation of the best-fit line. While relying on a graph for estimation is more convenient, it yields less precise and less accurate results. It has some limitation like, The effects of a stressor or drug generally depend on the exposure time. Therefore, the EC50 (and similar statistics) will be a function of exposure time. The exact shape of this time function will depend upon the stressor (e.g., the specific toxicant), its mechanism of action, the organism exposed, and cetera. This time dependency hampers the comparison of potency or toxicity between compounds and between different organisms. According to herbal medicine Acacia arabica is also of great use in dental problems. Vernacularly it is known as a Babool and it possesses anti diabetic properties and often recommended for proper union.