7 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. (219, 220) 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%. (221, 222)

The major problem with osteoporosis is: it is a "silent disease" because bone loss occurs without symptoms. In many cases, the first "symptom" is a broken bone. Patients with osteoporosis may not know that they have the disease until their bones become so weak that a sudden strain, bump, or fall causes a hip fracture or a vertebra to collapse. Collapsed vertebra may initially be felt or seen in the form of severe back pain, loss of height, or spinal deformities such as kyphosis, or severely stooped posture. (222-224)

As the second most populous country in the world, India is home to a very large population of osteoporosis patients. Although, rather late off the block in terms of economic reforms and development, a steady increase in life expectancy to 66 years is rapidly leading to a very large ageing population, which is at risk of osteoporosis. 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. (225) 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. (226) 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. (227) 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. (228) 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. (229) 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. (230-232) Now a days there is a renewed interest in drugs of natural origin simply because they are considered relatively safer. (233) 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. (9, 226, 234-239) It is also reported that “Plants with anti-inflammatory role can be potent candidates as an osteoprotective agent” (21). Some herbal drugs have been traditionally used in Ayurveda to accelerate the healing of bone fractures and to strengthen the bones. (19) 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.

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. (240, 241) 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. (115) 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 results of the physicochemical determinations are presented in table 6-1. The moisture content (%LOD) of the powdered drugs was found to be AA = 12.50%, TA = 13.5%, CM = 4.8%, BS = 7.6%, which indicates that the drug was properly dried and stored. The determination of moisture content is important for the plant drugs because insufficient drying may lead to possible enzymatic deterioration of active principles. (242) This parameter is therefore essentially used to control the quality of crude drugs and/or herbal drugs/drug products.

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. (170, 242) 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, (71, 94, 115, 135)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 and cold maceration method. For *Terminalia arjuna* and *Acacia arabica*, aqueous and methanolic extract was selected because most of the phytoconstituents will get extracted in water and methanol. (24, 243) The other two drugs, guggul and salai guggul are oleogum-resins and the anti-inflammatory compounds present in them, namely guggulsterones and boswellic acids respectively, are soluble in ethyl acetate and petroleum ether. (244, 245) So, ethyl acetate and petroleum ether were used to prepare extracts of guggul and salai guggul.

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. (30, 246-248)

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. (249) Our study involved bioactivity-guided fractionation which requires the activity of the fractions to be tested at every stage of fractionation. Hence an *in vitro* model, which is cost-effective and rapid, is most suitable for this purpose. The purpose of the model is just to find the relative potency of the fractions and that is fulfilled by this model.
EC50 values are commonly used as a measure of the reasonable expectancy of a drug effect. EC50 indicate the "median effective dose" that produces a quantal effect (all or nothing) in 50% of the population that takes it. From the drug concentration response we found the effective range of Acacia arabica, Terminalia arjuna and Commiphora mukul. Extracts of Acacia arabica and Terminalia arjuna show minimum EC50 value among four drug and and 10-100 µg/ml therapeutic range, Which show higher potency as compare to Commiphora mukul which has EC50 value more as compare to it. Boswellia serrata show very wide EC50 and therapeutic range that indicate it require so much higher dose to produce therapeutic effect, which is not significant important for the further study. 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, (63) 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 (250-253) 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,(254) particularly during the early stages of osteoporosis.(255) 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. (178-184, 256, 257) 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. (258-261)

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. Bone resorption and osteoporosis development is most pronounced in the first 4 to 6 weeks after ovariectomy,(262) it is a reason for selection 40 days as dosing period.

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 (69) and double dorsolateral incisions.(263) 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.

As rodents and rabbits can easily become hypothermic, it is essential that the heating pads, water bottles or isothermic pads be used to provide supplementary heat to recovering animals. If the recovery is delayed, dehydration can be counteracted by subcutaneous or intra peritoneal injection of saline or lactated Ringer’s solution. The recovering animals were turned every ten minutes to stimulate respiration and reduce pulmonary hypostatic congestion. When a crawl reflex had returned, the animal was placed alone in a clean cage with clean dry bedding. Paper towels were used as a bedding material for the first 24 hours as wood shavings or corncob bedding can contaminate the wound. If aseptic conditions are followed, antibiotics are not necessary, and some antibiotics are toxic to rodents. In cases of development of infection, gentamicin was given intra muscularly at a dosage of 5mg/kg s.i.d. (once a day) for five days. In approximately one week circulating androgens or estrogens decrease sufficiently to allow mixing of aggressive animal. Newly introduced animals were observed for up to eight hours to ensure that the others were not attacking it. (264)

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 ovariectionomy 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. (265-269) 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. (270-272) 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. (182, 273) 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. (274, 275) 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. The calcium in the skeleton has the additional role of acting as a reserve supply of calcium to meet the body's metabolic needs in states of calcium deficiency. (276) 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. (277) 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. (278, 279) Increased excretion of phosphorus through urine was due to increased demineralization of bones resulting from Ovariectomy-induced estrogen deficiency. (280, 281) 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.

The osteoblastic bone formation is thought to be mediated by two different processes: one is the formation of new osteoblasts, and the other is the activity of osteoblasts to produce bone matrix. Osteoblasts produce collagen, alkaline phosphatase, osteocalcin and other matrix proteins. 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. (282) The total ALP serum pool consists of several dimeric isoforms, which originate from various tissues: liver, bone, intestine, spleen, kidney, and placenta. In adults with normal liver function, approximately 50% of the total ALP activity in serum is derived from the liver, whereas 50% arises from bone. (283) 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. (284) Hence, in the absence of any liver disease, serum levels of total ALP provide a good impression of the extent of new bone formation and osteoblast activity. (285) Bone turnover has been found to be significantly increased in postmenopausal women (286) and can be diagnosed by the increased serum ALP levels.
(285) 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.

(287) 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. (288) It is a membrane bound enzyme, which dephosphorylates bone matrix phosphoproteins and thus, allows migration of osteoclasts. (289) As a bone marker, TRAP is unique in that it reflects the number of osteoclasts. Given the fact that osteoclasts resorb bones, (284) 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. (290) 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. (291) 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. (292) 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 it’s 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. (293-296) 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. (297-302) IL-6 and IL-1 may influence osteoclastogenesis by stimulating self-renewal and inhibiting the apoptosis of osteoclasts progenitors. (302, 303) They promote osteoclast differentiation, which is an important stimulator of bone resorption that has been linked to accelerated bone loss seen in postmenopausal women. (296) Estrogen deficiency leads to upregulation of cytokines, Interleukins, RANK -L, TNF alpha, which are responsible for enhancing osteoclastic activity (304) 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. (305) *Terminalia arjuna* is reported to have anti-inflammatory property. (23) Therefore, it might be causing suppression of these potent inflammatory mediators and hence, preventing further consequences responsible for the bone loss.

Estrogen can be considered as an antioxidant as it was found to exhibit antioxidant protection of lipoproteins in the aqueous system (306) and was also shown to increase the expression of glutathione peroxidase in osteoclasts. (307) That is why decline in estrogen leads to increase in osteoclasts activity resulting in bone loss. Reactive oxygen species (ROS) alter mitochondrial and nuclear DNA integrity and increase the risk of mutations. When DNA repair mechanisms are overwhelmed, cells undergo apoptosis, which leads to tissue damage. (308) This can be applicable in postmenopausal osteoporosis mechanism. When body is subjected to high oxidative stress following estrogen deficiency, lipid accumulation occurs. Lipid peroxidation promotes osteoblast apoptosis and simultaneously up-regulates ROS production. (309, 310) ROS has been shown to promote osteoclast resorption activity either directly or mimicking RANK signaling and stimulating osteoclast differentiation, or indirectly, by stimulating osteoblast/osteoclast coupling and subsequent osteoclast differentiation. (311-313) Oxidative stress may also increase bone resorption through activation of NF-κB, which plays an important role in osteoclastogenesis. (314, 315) 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. (316) 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.(317) 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. (317) 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. (318) 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. (319, 320) Based on these reports, it was postulated that anti-osteoporotic activity of TAM-Nbut could be because of saponins present in it. Hence, saponin fraction (TAM-Nbut-S) was isolated from TAM-Nbut.

Saponins contain sugar portion i.e. the glycone part and the non-sugar portion known as the sapogenin. Generally the pharmacological effects of saponins are due to the aglycone part and the sugar part only serves to make them water soluble. Hence, the saponin fraction (TAM-Nbut-S) was hydrolysed to obtain the sapogenin fraction (TAM-Nbut- A). The sapogenin fraction was subjected to in vitro bone culture assay to assess its pharmacological activity. The sapogenin fraction showed more significant effect on bone calcium deposition in comparison to TAM-Nbut- S. Thus, it was confirmed that the anti-osteoporotic activity of saponin fraction was because of the sapogenin portion.

**Terminalia arjuna** majorly contain four triterpenoid sapogenins - arjunic acid, arjunolic acid, arjunetin and arjungenin (321, 322). Arjunetin is one of the major triterpenoid sapogenins present in *Terminalia arjuna*. Hence, arjunetin was investigated for its effect on bone calcium metabolism using in vitro bone culture experiment. Arjunetin showed significant calcium deposition in bones in in vitro bone culture experiment even at a concentration of 10 μg/ml. This suggests that arjunetin is one of the compounds responsible for anti-osteoporotic activity of *T. arjuna* bark. Further experiments are needed to investigate if compounds other than arjunetin also contribute to the anti-osteoporotic activity of the drug. Further studies on arjunetin using specific target-based *in vitro* biological assays would help to elucidate the mechanism of action of arjunetin.