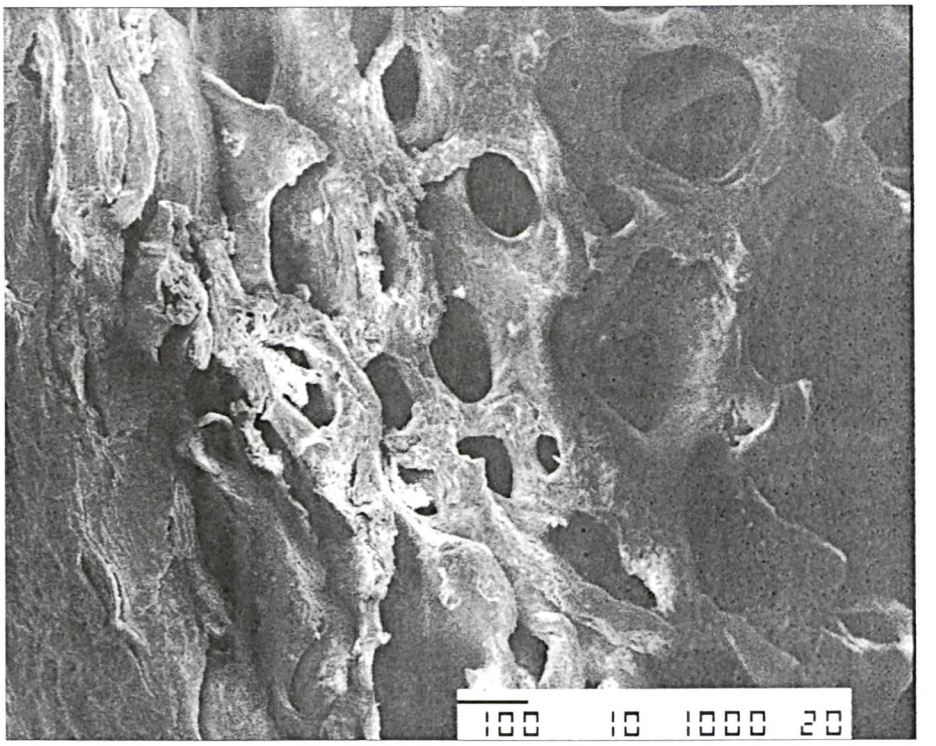

BONE REMODELLING PROPERTY OF CERTAIN BOTANICALS

INTRODUCTION

Bone is mineralized connective tissue in vertebrates that supports body weight, enables locomotion of the organism, protects internal organs from external force, and maintains mineral homeostasis (Giudiceandrea *et al.*, 1998; Lee *et al.*, 2008; Soicher *et al.*, 2010). Bone refers to a family of biological materials, all of which are composed of collagen fibrils, a main component of the three dimensional matrix into which the mineralization occurs (Masse *et al.*, 2010).

Bone is having two structural components; cells and matrix. Bone cells are of three types, osteoblasts which synthesize the bone, osteoclasts which resorb the bone and osteocytes. Osteocytes are the ultimate fate of the osteoblast (Giudiceandrea *et al.*, 1998). A combined activity of these cells maintains the bone homeostasis. The matrix is a lightweight composite material, formed of organic and inorganic constituents. Organic components are approximately 90% type-I collagen and 10% of other organic materials. The organic matrix is combined with inorganic mineral phase consisting of hydroxyapatite [$Ca_5(PO_4)_3(OH)$] (Rho *et al,*. 1998). The size of these plate-shaped crystals is considered the smallest biologically formed crystals known (Wolfie *et al.*, 1985). Collagen gives strength to the matrix in the same way steel is used in reinforced concrete. The bundled collagen is made up of fibrils that are composed of 3 polypeptide chains each about 100 amino acids long. The chains are wound together in a cylindrically shaped triple helix (Lowenstam and Weiner, 1985). These fibrils are bound and impregnated with hydroxyapatite nanocrystals (Rho *et al.*, 1998; Kerschnitzki *et al.*, 2010).

Bone is further organized at the micro-structural scale into repetitive lamellar structures with an alternating fluid phase between them (Weiner and Wagner, 1998). The alternate orientation of the lamellae allows bone to have great torsion strength. Cylindrical motifs are generally oriented along the long axis of bones and are termed secondary osteons, which are tunnels, created by excavation by osteoclasts. These tunnels are then almost completely filled by osteoblastic action, leaving a narrow channel at the center for a blood vessel. In fact, other even smaller capillary-like channels, termed canaliculi, are built into the structure (Kerschnitzki *et al.*, 2010). The canaliculi are numerous and tend to radiate out from the central blood vessel (Martin, 2000).

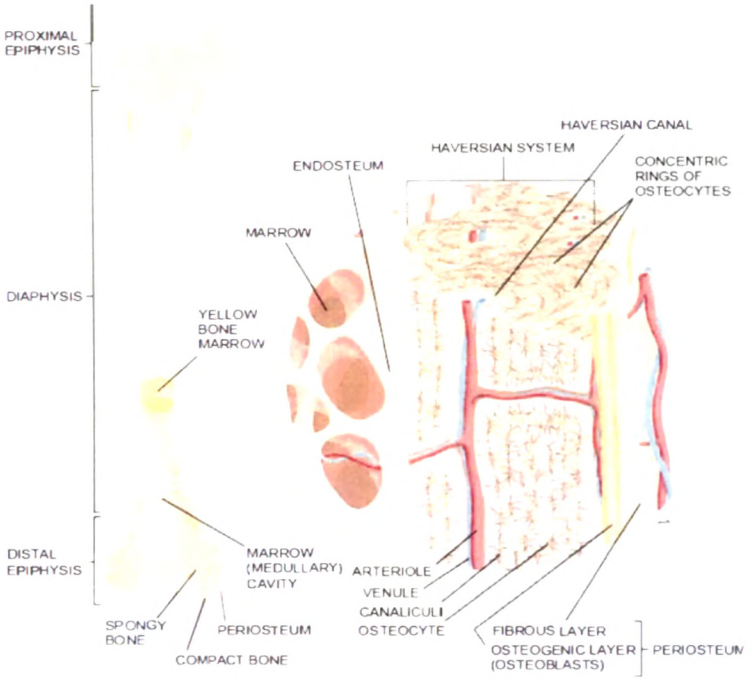

Figure 1 Internal structure of long bones showing cortical and trabecular bone. Cortical bone is formed of Harvesian canal system and separated from trabecular bone by the perosteal membrane. Diagram reproduced from Tabers online Cyclopaedic Medical Dictionary.

Bone is a multifunctional organ, which provides an environment for a heterogeneous population of cells. Bone homeostasis is ensured by the balance between bone formation and bone resorption, which depend on osteoblasts and osteoclasts, respectively (Kerschnitzki *et al.,* 2010). Osteoblasts perform the function of matrix synthesis and mineralization; while in opposition; specialized multinucleated cells called osteoclasts conduct resorption (Miyazaki *et al.,* 2010). Normally there is a dynamic balance between osteoblast and osteoclast activity, resulting in one tenth renewal of bone each year (Sommerfeldt and Rubin, 2001).

Osteoblasts develop from the mesenchymal cell lineage, initially as preosteoblasts, which develop into functional osteoblasts (Minguell *et al.,* 2001). Osteoblasts have three functions; to produce the bone matrix proteins (Ellies and Krumlauf, 2006), express necessary genes for mineralization (Franz *et al.,* 2006), and regulation of osteoclasts (Spencer *et al.,* 2006). Osteoclasts are derived from the monocyte/macrophage lineage (Hwang *et al.,* 2006; Messalli and Scaffa, 2010). The main physiological function of osteoclasts is to degrade mineralized matrix (Hwang *et al.,* 2003). Recruitment, proliferation and differentiation of osteoclasts are

controlled by osteoblastic cells and thus they play an essential role in the maintenance of bone mass (Lindsay, 2010). Osteoblasts secrete various paracrine hormones in response to osteotropic factors (Tanaka *et al.,* 2005). Hence, bone resorption is indirectly under the control of many different hormones such as 1, 25 $(OH)_2$ vitamin D_3, parathyroid hormone (PTH), interleukin-1 (IL - 1), interlukin - 6 (IL - 6) and estrogens which stimulate osteoblasts (Franchimont *et al.,* 1997). Disturbances in any of these processes result in systemic or local bone diseases such as osteoporosis, osteomalacia and periodontal diseases, and impair the healing of fractures (Rodan *et al.,* 2002; Kwan *et al.,* 2004, Ryoo *et al.,* 2006; Lee *et al.,* 2008).

Increased average life span and popularization of western lifestyle among the population has led to increased incidence of chronic diseases. Such diseases include circulatory system disorders, type 2 diabetes, dementia and osteoporosis (Navarro *et al.,* 2010). World Health Organization (WHO) defines osteoporosis as a systemic skeletal disease, characterized by low bone mass and disorders in bone microarchitecture which lead to increased risk of fractures (Stachura and Olchowik, 2008). Bone mass changes with age, reaching maximum during the 30s in both males and females and begins to decrease after the age of forty-five (Fintini *et al.,* 2010). By the age of eighty, bone mass declines to 50% of its maximum value. Xue (1998) opined that bone loss begins between the ages of thirty and forty and continues throughout the aging process. Nattiv *et al.,* (1994) established that normally bone mass peaks in women at age thirty-five and then tends to be lost at the rate of about 1% of bone mass every year (or 10% over 10 years). The peak bone mass of women is less than that of men because of the decrease in bone mass owing to menopause (Hidaka, 2006). After menopause bone loss is typically 5% per year. Major risk factors for osteoporosis are age, genetics, lifestyle (especially nutrition) and menopausal status (North American Menopause Society, 2002).

Peak bone density appears to be achieved during early adult life soon after linear skeletal growth has stopped and it is largely genetically determined (Ellerington and Stevenson, 1993, Stevenson and Marsh, 1992). Other factors include ovarian failure due to menopause, age-related factors, hyperparathyroidism, hypercortiolism, hyperthyroidism, hypogonadism, reticuloendothelial disorder, connective tissue disorder, drugs (eg. corticosteroids, heparin, and alcohol), cigarette smoking, and immobilization (Qin *et al.,* 2003). The prevalence of osteoporosis is increasing dramatically corresponding to an increase in the geriatric population. Menstrual status is an important determinant of peak bone mass as well as the development of bone loss prior to onset of menopause. It has been reported that

postmenopausal women have significantly lower bone mass than pre and perimenopausal women (Khan and Syed, 2004). In their studies on menopause-related changes in bone mineral density (BMD), Sprague *et al.,* (2010) have shown accelerated bone loss in the early stage, associated with decreased level of Estrogen in post menopausal women. Osteoblasts are more sensitive to age related estrogen loss then osteoclast, thereby shifting bone remodelling, which leads to osteoporosis (Luigi and Loredana, 2005). Moreover, Estrogen deficiency promotes bone loss at all ages, increasing the life span of osteoclast and decreasing the osteoblastic activity (Macdonald *et al.,* 2004). When the menstrual cycles get irregular in perimenopause, the serum calcium level rises rapidly and reaches maximum in 2–5 years at postmenopause, and then slightly decreases afterwards suggesting that the balance between blood calcium levels and bone mass is the most important issue in osteoporosis (Sawka *et al.,* 2010; Sprague *et al.,* 2010). The three main factors on which the progression of osteoporosis depends are: an inadequate peak bone mass, excessive bone resorption and inadequate formation of new bone during remodelling (Moe, 2010).

Mackay (1992) indicated that in perimenopausal woman the ovarian follicles become progressively fewer in numbers and more refractory to hormonal stimulation. Hacker and Moore (1998) reasoned that eventually the production of estrogen, progesterone and the other ovarian hormones is reduced leading to a clinical condition defined as menopause. Receptors for estrogen are widely distributed in vagina, cervix, uterus, ovary, pelvic fascia, bladder, skin, bone, heart, arteries, liver, brain, muscle, breasts and other endocrine glands (Huang *et al.,* 2010). When estrogen levels are low or absent, cells within these tissues become relatively inactive. The net result of the inactivity in target cells is an increase in general body dysfunction (Hwang and Putney, 2010).

The underlying mechanism in all cases of osteoporosis is an imbalance between bone resorption and bone formation. In normal bone, there is constant matrix remodeling of bone; up to 10% of all bone mass may be undergoing remodeling at any point in time. The process takes place in bone multicellular units (BMUs) as first described by Frost in 1963. Bone is resorbed by osteoclast cells, after which new bone is deposited by osteoblast cells. Hormonal factors strongly determine the rate of bone resorption; lack of estrogen increases bone resorption as well as decreasing the deposition of new bone that normally takes place in weight-bearing bones (Soicher *et al.,* 2010; Masse *et al.,* 2010).

Activation of osteoclasts during osteoporosis is regulated by various molecular signals, of which RANKL (receptor activator for nuclear factor κB ligand) is one of best studied (Zhang

et al., 2008; Thomas and Martin, 2008; Leibbrandt and Penninger, 2009). This molecule is produced by osteoblasts and other cells (e.g. lymphocytes), and stimulates RANK (receptor activator of nuclear factor κB) (Guidiceandrea *et al.,* 1998; Grzegorzewska and Mlot, 2006; Kapoor *et al.,* 2008; Zhang *et al.,* 2008). Osteoprotegerin (OPG) or Osteoclastogenesis inhibitory factor (OCIS), as name suggests, is a protein that prevents osteoclastogenesis. This protein binds RANKL before it has an opportunity to bind to RANK, and hence suppresses osteoclastogenesis. Hence, the maturation and activation of osteoclasts is controlled by the osteoblastic cells. Under the influence of Estrogen, osteoblasts secrete the Osteoprotegrin (Nannuru *et al.,* 2009) which binds with RANKL, preventing its binding with RANK on osteoclastic precursor cells. This checks the maturation of osteoclasts (Bharti *et al.,* 2004; Chen and Wang, 2006). During postmenopause due to absence of estrogen, the osteoblasts do not secrete osteoprotegrin, causing continuous stimulation of osteoclastic precursor cells into osteoclasts, resulting in unchecked resorption leading to osteoporosis (Kapoor et al., 2008).

According to WHO, osteoporosis is second only to cardiovascular disease as a global healthcare problem. Medical studies show a 50-year-old woman has a similar lifetime risk of dying from hip fracture as from breast cancer (Navarro *et al.,* 2010). 93% of women acknowledge seriousness of osteoporosis, but 8 out of 10 do not believe they are personally at risk; 80% of women with osteoporosis are not aware of their risk before diagnosis (International Osteoporosis Foundation survey, 2000). Since osteoporosis affects the growing elderly population, it will put a bigger burden on the healthcare system as management is expensive. Unless swift action is taken, it can escalate into an economic threat. International Osteoporosis Foundation (IOF) estimates that the annual direct cost of treating osteoporosis fractures of people in the workplace in the USA, Canada and Europe alone is approximately USD 48 billion (Randell *et al.,* 1995, IOF, 2002) The worldwide cost burden of osteoporosis (for all ages) is forecast to increase to USD131.5 billion by 2050. Osteoporosis also results in huge indirect costs that are rarely calculated and which are probably at least 20% of the direct costs. Once a woman suffers a first vertebral fracture, there is a five-fold increase in the risk of developing a new fracture within one year (Lindsay *et al.,* 2001; Navarro *et al.,* 2010).

Osteoporosis is an important public health problem in older adults. Not only does it gives rise to morbidity but also markedly diminishes the quality of life of women after menopause and of both women and men over 65 years of age (Cooper *et al.,* 1992). In the twentieth century the proportion of older persons started to rise and is expected to continue throughout this century. The number of individuals aged 60 and above is projected to grow to almost 2 billion

by 2050, of which fifty-four percent live in Asia and the vast majority of who will be in the developing world. Such accelerated global population aging will increase economic and social demands on all countries (Kanis *et al.,* 2002).

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%. In men risk is 15-30% (Randell *et al.,* 1995). 1 in 3 women over 50 will suffer a fracture due to osteoporosis; over 60, this rate doubles (Navaro *et al.,* 2010). 1 in 5 men over 50 will suffer a fracture due to osteoporosis; this rate triples over 60. The annual incidence rate of osteoporotic fractures in women is greater than the combined incidence rates of heart attack, stroke and breast cancer (Riggs *et al.,* 1995).

With socio-economic development in many Asian countries and rapid ageing of the Asian population, osteoporosis has become one of the most prevalent and costly health problems in the region. Unsurprisingly, Asia is the region expecting the most dramatic increase in hip fractures during coming decades; by 2050 one out of every two hip fractures worldwide will occur in Asia (Riggs *et al.,* 1995). 1 out of 8 males and 1 out of 3 females in India suffers from osteoporosis, making India one of the largest affected countries in the world (Gupta *et al.,* 1967). Expert groups peg the number of osteoporosis patients at approximately 26 million with the numbers projected to increase to 36 million by 2013 (Malhotra and Mittal, 2008). In India higher incidence of osteoporosis in males and lower peak incidence age in females is observed in comparison with Western countries. The incidence of hip fracture is 1 woman to 1 man in India. 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 (Damodaran *et al.,* 2000).

With increasing longevity of the Indian population, it is now being realized that, as in the West, osteoporotic fractures are a major cause of morbidity and mortality in the aged. Based on 2001 census, approximately 163 million Indians are above the age of 50; this number is expected to increase to 230 million by 2015. Even conservative estimates suggest that of these, 20 per cent of women and about 10-15 per cent of men would be osteoporotic. The total affected population would, therefore, be around 25 million. If the lower bone density is shown to confer a greater risk of fracture, as is expected, the figure can increase to 50 million (Gupta *et al.,* 1967; Malhotra and Mithal, 2008).

Life expectancy of female in India is 72.61 years, indicating that an Indian female lives around 26 years after menopause. Development of osteoporotic fracture during this time can

be fatal to the quality of life of female (Malhotra and Mithal, 2008). With the growing aged population, we urgently need to develop new and effective treatments for chronic and protracted diseases, e.g. Chronic pain, diabetes, arthritis, osteoporosis, cancer as well as cardiovascular and brain ailments (Hidaka, 2006). Prevention of disease is preferred to treatment especially in the absence of adequate treatment (Avioli, 1993). This is particularly relevant to osteoporosis as there is no satisfactory treatment, for such a common and debilitating disease in our society. It is therefore much better to use preventive modalities in the early stages of menopause and in the postmenopausal years. Ellerington and Stevenson (1993), claim that rate of bone loss begins to slow down approximately eight to ten years after menopause. So any preventive measures should be taken earlier since they would be of reduced benefit after this time. "Given the health implications of osteoporotic fractures, the primary goal of osteoporosis therapy is to prevent fractures by slowing or preventing bone loss, maintaining bone strength, and minimizing or eliminating factors that may contribute to falls" (North American Menopause Society, 2002). Pharmacological treatments for osteoporosis are classified as anti-resorptive agents that primarily decrease bone resorption and anabolic agents that primarily stimulate bone formation thus restoring bone mass previously lost. Therapeutic agents such as estrogen (with or without progesterone), calcitonin, bisphosphonates (alendronate), and selective estrogen receptor modulators (raloxifene) have been approved as antiresorptive agents for the prevention of osteoporosis (Ke, 1998). Hormone replacement therapy (HRT) has shown a strong correlation with increasing bone mineral density and lowering fracture incidence. However, definitive measures for the prevention of bone loss are still required (Genazzani and Gambacciani, 1999) as these agents have both advantages and disadvantages. The major issue is the relationship between HRT and breast cancer (Forsblad and Carlsten, 2010). Long-term and current HRT use is followed by a slight though significant increase in the risk of breast cancer. HRT is based on the inhibition of bone resorption to prevent further bone loss; however, it is of no significance to many patients which have already lost substantial amount of bone (Yin *et al.*, 2004). Progestogens can modify the cellular response of normal as well as cancerous breasts (Genazzani and Gambacciani, 1999). Combined HRT is associated with a higher risk of breast cancer as compared to estrogens alone. Panidis, *et al.*, (2001) stated that although HRT has beneficial clinical effects and a positive benefit to risk ratio, awareness of the side effects such as weight gain and fear of cancer, limit compliance. Recent study data from the Heart and Estrogen/progestin Replacement Study (Grodstein *et al.*, 2001) has revealed that the use of

HRT in women with established risk factors for coronary heart disease increases in the short term. Kaplan *et al.,* (2002) recommended that until definitive guidelines became available, an individualized approach should be applied with careful consideration of both the benefits and risks of treatment. Although this situation could be improved to some extent with HRT the treatment cannot be used in all situations. Western medicine advises women with breast cancer to avoid HRT (Forsblad-d'Elia *et al.,* 2010). The application of HRT is of limited value for postmenopausal women and patients who already suffer from osteoporosis (Chen, 2003a). Bone loss with different regimes: lifestyle modification, separately or in combination, have been suggested to prevent, delay, or attenuate bone loss via osteoporosis. However, till date there is no established medication for the treatment of osteoporosis, and the existing medication are still facing the problem of side effect and compliance of the patient.

Plants have been a source of medicine and a major resource for health care since ancient times, with some traditional herbal medicines having been in use for more than 2,000 years. Currently, the modern pharmaceutical industry is paying more and more attention to plants as scientists re-discover that plant life is an almost infinite resource for medicine development (Sharma *et al.,* 2003). One fourth of the modern medicines that are available on prescription today owe their origins to higher plants of tropical forests; out of which, 74% are derived from plants that have some related use in traditional herbal medicine (Sukh Dev, 1997). There is a great deal of interest developing in natural estrogens, particularly plant derived phyto-estrogens (Chen and wong, 2006; Zhang *et al.,* 2008). These compounds have weak estrogen like effects and some animal studies are promising as they have both osteoclast inhibiting and osteoblast stimulating effect (Hidaka *et al.,* 2006). Apart from phyto-estrogens, there have been several phytochemicals isolated from plant and are found to have beneficial effect in bone healing and osteoporosis (Domínguez *et al.,* 2011). These plants have been used in traditional systems for the treatment of variety of diseases and described very well with their properties in Chinese and Indian traditional medicinal system. *Ayurveda*, the Indian traditional system of medicine, is based on empirical knowledge of the observations and the experience over millennia. More than 1200 diseases are mentioned in different classical Ayurvedic texts. Management of various diseases is done with more than 1000 medicinal plants (89.93%); 58 minerals, metals, or ores (5.24%) and 54 animal and marine products (4.86%) (Sharma *et al.,* 2003).

Herbal medicine is still the mainstay of about 75 - 80% of the world population, mainly in the developing countries, for primary health care (Kamboj, 2000).Many of the drugs thus

discovered are still used in the modern system, and many more carry the structural imprint of the parent molecular prototype which had led to their discovery. Dramatic discoveries in instrumentation and computation have led to widening of the horizons of drug discovery and development from natural sources thus benefiting society.

According to the WHO, the use of herbal remedies throughout the world exceeds that of the conventional drugs by two to three times (Evans, 1994). It has been estimated that 75-80% of world population depends on crude plant drug preparations to tackle their health problems, though this may be due economic considerations (Sukh Dev, 2006). However, in countries like India, China, and other states with well founded traditional systems of medicine, plant based therapeutic agents occupy an important niche in health management. The last three decades are a witness to a new development. The economically developed countries, for whatever reasons, are seeing an ever growing interest in natural remedies, which have come to be known as herbal drugs or phytomedicines. Their preparations are invariably single plant extracts, or fractions thereof, as distinct from pure chemical entities which may be called molecular drugs (Kamboj, 2000). This new breed of plant derived products are carefully standardized, and their efficacy and safety, for a specific application, fairly demonstrated. It has been estimated that the present global market for these products may be of the order of 20 billion U. S. dollars, and is growing at the rate of 10 – 15% annually.

Looking at the situation in India a large segment of the population still depends on Ayurvedic drugs for their health care needs, and the situation will continue to be so for many more years to come. The first and foremost consideration, therefore, is to put the materia medica of Ayurveda on a modern scientific footing to provide drugs of proper standard quality (Sukh Dev, 2006). Standardization of herbal drugs, which are multi-component, is a daunting task. The use of plants for healing purposes predates human history and forms the origin of much modern medicine. Many conventional drugs originated from plant sources: a century ago, most of the few effective drugs were plant based. Examples include aspirin (willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy) (Vickers and Zollman, 1999).This is the first major task that needs to be undertaken in research and development on Ayurvedic drugs, so that patients get drugs of standard quality.

Traditional Ayurvedic therapeutic formulations draw on an impressive array of plants, many of which have been scrutinized by modern scientific methods. The first Ayurvedic herb which attracted international attention was *Rauvolfia serpentine*, when it was found that its constituent alkaloid, reserpine, had the twin effect of lowering high blood pressure and acting

as a tranquillizer. In its traditional usage, it has been employed to treat insanity. This was in the nineteen fifties. Currently, *Curcuma longa* (turmeric), another Ayurvedic crude drug, is being evaluated for several therapeutic applications. In the classic Ayurveda literature several plants with therapeutic claims as immunomodulators, memory enhancers, neuroprotectives, antiobesity, and antiageing agents, etc have been described, and which have now received some modern scientific attention (Sukh Dev, 2006).

Structural novelty and new modes of action are common features of plant drugs. This has been shown by anticancer agents like vinblastine, vincrisitne and paclitaxel; cardiovascular agents like forshkolin; anti-HIV agent like calanolid and latest to add to this list is gugulsterone, the active constituent of Guglip, a hypolipidemic drug, which has been shown to act through inhibition of farnesoyl nuclear receptors, causing an increase of bile acid excretion and thus, increasing the metabolism and mobilization of cholesterol in the liver (Pal and Shukla, 2003). One of the important current emphases in new drug discovery research is to get products with new modes of action, and plant drugs most often fulfill this requirement admirably (Sukh Dev, 1997).

Ayurvedic drugs are also attracting much attention for diseases with no or inadequate drugs for treatment in modern medicine, such as for metabolic and degenerative disorders. Most of these diseases have multifactorial causation, and there is a growing realization that in such conditions combination of drugs, acting on a number of targets simultaneously is likely to be more effective than drugs acting at one target; one drug paradigm is not likely to be satisfactory in such cases (Pal and Shukla, 2003). Ayurveda drugs which are most often multi component have a special relevance for such conditions and are attracting much attention. No doubt this would need much detailed study to get proof of concept but these are opportunities offered (Sukh Dev, 1997).

Since ipriflavone was approved for the treatment of osteoporosis in the 1980s, natural plants have been researched extensively for their osteoprotective effect (Yin *et al.,* 2004). There are many natural agents that are found to have osteoprotective effect which includes plant and animal products. It has been shown that the fruits of *Cnidium monnieri* are having positive effect on induced osteoporosis both *in vivo* and *in vitro* by inhibiting the formation and differentiation of osteoclastic cells (Qin *et al.,* 2003). Water extract of *Dioscorea spongiosa* was also found to have positive effect on bone metabolism (Yin *et al.,* 2004). *Curcuma longa* is one of the proven miracle plants and had been used in treatment of variety of diseases (Bharti *et al.,* 2004). Curcumin, the active ingredient of this plant is found to have

osteoprotective effect by directly suppressing the NF κB ligand activated pathway in osteoclastogenesis (Anto *et al.,* 2002). A study by Hidaka *et al.,* (2006) revealed that Royal jelly is also found to have osteoprotective effect by favoring the intestinal absorption of dietary calcium. It has also been reported that extracts from the medicinal plant *Emblica officinalis* induces apoptosis in human primary osteoclastic cells (Penolazzi *et al.,* 2008). Yin *et al.,* (2006) in their studies confirmed that steroidal glycosides from *Dioscorea septemloba* have inhibitory activity on bone resorption. It has also been proved that *Eucommiae* cortex has dual effect on osteoporosis, i.e. it promotes osteoblasts and inhibits osteoclasts (Hyekyung *et al.,* 2003). Drugs that promote proliferation and differentiation of osteoblastic cells are having osteoprotective effect (Lee *et al.,* 2008).

The review of the literature has thus shown that herbals do have osteoprotective effects. Hence, the first aim of the present study was to do the preliminary screening of the botanicals for their osteoprotective efficiency (Chapter 1) and shortlist them for detailed analysis. The four plants selected for studying their osteoprotective effect are as follows.

Listea glutinosa (LG)

Family *Lauraceae* consists of 50 genera and 2000 species of trees, shrubs, and herbs, of which 70 are of medicinal value in the Asia–Pacific region. Lauraceae are well-known for elaborating isoquinoline alkaloids and sesquiterpenes. *Litsea odorifera* is used to treat biliousness, to promote lactation, and to heal boils and furuncles. In China, the seeds are eaten to promote digestion and treat cough and bronchitis. In Vietnam, Cambodia, and Laos, a decoction of the plant is used to treat mental disorders such as hysteria and forgetfulness. In Taiwan, the plant is used to treat athlete's foot and other skin diseases. A methanolic extract of bark of *Litsea cubeba* and its fractions (0.01 mg/mL) from bark inhibited nitric oxide and prostaglandin E_2 production in LPS-activated RAW 264.7 macrophages without significant cytotoxicity at less than 0.01 mg/mL concentration. The methanol extract decreased the enzymatic activity of myeloperoxidase (0.05 mg/mL). These findings suggest that *L. cubeba* is beneficial for inflammatory conditions and may contain compound(s) with anti-inflammatory properties (63). *Litsea glutinosa* has been described as promoter of longevity of semen generation and emollient. Sap of fresh bark or its decoction is prescribed as a remedy for diarrhea, dysentery, rheumatism, and as an aid to longevity. In addition, in current usage, a paste prepared by grinding bark with water is used as a plaster in cases of sprain, bruises, wounds, inflammation, back pain, rheumatic and gouty joints, bone fractures etc. It has analgesic, antiseptic and emollient effects. Methanolic extract, aqeuous extract as well as the

ethanolic extracts of various part of the *L. glutinosa* have proved to have multipotential role. The pharmacological potential of this plant as an osteoprotective agent is unexplored, and it would be interesting to know whether the plant possesses such activity. Hence, along with the screening of the other botanicals, *L.glutinosa* was selected as the plant to study its osteoprotective efficacy *In vivo* (Chapter 1, 2 and 3) and *In vitro* (Chapter 5 and 6). Further, the phytochemical analysis of the plant was also done (Chapter 4)

Terminalia Arjuna

Terminalia arujuna is described in Ayurveda for variety of diseases including heart diseases and obesity (Sukh Dev, 2006). Medicinally valuable part of the plant is bark, also known as Arjunsal. Clinically it has been proved as a cardio tonic agent and also used in congestive cardiac failure. Many workers explored it for variety of diseases including antioxidant, hypolipidemic, free radical scavenging, wound healing and antibacterial activity (Kumar and Prabhakar, 1987). *Terminalia arjuna* is extensively used for the treatment of osteoporosis and other bone related disorders as it improves the synthesis and secretion of female hormones (Nadkarni, 1996a). It helps in relieving the pains associated with osteoporosis frequently encountered in old age and is also useful in cases of general debility, nervous exhaustion and muscle pains (Nadkarni, 1996b). When 50% ethanolic extract of the bark was fed to diet induced hyperlipidaemic rabbits for 60 days, significant hypolipidaemic effect was noticed (Ram *et al.,* 1997). Bark extract was found to have wound healing effect when tested in incision and excision wound models in rats (Rane *et al.,* 2003). This plant is also proved to improve heart conditions; liver problems; digestive disorders; edema; bone fractures; diarrhoea and venereal diseases. Besides its wound healing, antiviral and antiinflammatory property preliminary scientific study to determine the efficacy of the plant as an osteoprotective agent was thought pertinent so as to have the comparative efficacy along with other botanicals (Chapter 1).

CURCUMA AROMATICA

Curcuma aromatica, commonly known as 'Jangli Haldi' belonging to genus curcuma, consist about 70 species of rhizomatous herbs. It is widely used as a flavoring agent, condiment and a source of yellow dye (Ambastha, 1995). The essential oils of *Curcuma* revealed the presence of various mono and sesquiterpenes. Early studies also showed the presence of curcumol in oil extracted from rhizome. The plant has been extensively studied for its pharmacological properties and has been proved to be anti antigenic, anthelminthic, anti microbial, wound healing, antitumor, antioxidant and cytoprotective (Ahmed *et al.,* 2010). However, its role as

an osteoprotective agent is not well explored and hence, the plant was selected to see the efficacy *In vivo* (chapter 1) and *in vitro* (chapter 5) analysis was carried out to see the osteoprotective effect of this plant.

MORINGA OLIFERA

Moringa oleifera is a plant described in *Ayurveda* (Indian system of medicine) and belonging to the family Moringaceae (Khare, 2007; Aney *et al.,* 2009). Moringa is a small or medium sized tree cultivated for its fruits. *M. oleifera* is a highly valued plant, distributed in many countries of the tropics and subtropics. *Moringa* is nature's medicine cabinet. It is best known as an excellent source of nutrition and is a natural energy booster. Different parts of this plant are being employed for the treatment of various ailments in the indigenous system of medicine, and its fruits are used as food in entire India (Aney *et al.,* 2009). Moreover, *M. oleifera* is reported to regulate a myriad of cellular activities (Ezeamuzie *et al.,* 1996; Hukkeri *et al.,* 2006; Sukh Dev, 2006; Sutar *et al.,* 2008) and anti inflammatory activity (Hukkeri *et al.,* 2006; Sukh Dev, 2006; Khare, 2007). Further, it has been documented that plants with strong anti inflammatory properties also exhibit osteoclast inhibiting property and can be used for the treatment of osteoporosis and rheumatoid arthritis (Penolazzi *et al.,* 2008). Checking the osteoclasts from activation therefore, could be a possible avenue to probe for better management of osteoporosis and rheumatoid arthritis. Recently, anti cancer compounds have been isolated from this plant and it is known to be a potent inhibitor of tumour promoter teleocidin B-4-induced Epstein Barr Virus (EBV) activation in Raji cells (Murakami *et al.,* 1998). Methanolic extract of this plant has been found to have radioprotection to bone marrow chromosomes in mice. In current studies, this plant has been shown to have osteoprotective effect in Ovariectomy induced osteoporosis (Burali *et al.,* 2010). Thus, an *in vitro* study (chapter 5) was carried out to see the osteoprotective role of this plant.

CHAPTER 1

PRELIMINARY SCREENING OF BOTANICALS FOR THEIR OSTEOPROTECTIVE EFFECT IN OVARIECTOMIZED WISTAR RATS

It has been a well known fact that anti inflammatory agents are potent candidates for the treatment of osteoporosis. The objective of the study presented in this chapter was to explore the osteoprotective effect of three botanicals, *Litsea glutinosa*, *Curcuma aromatica* and *Terminalia arjuna*, which were previously reported as anti inflammatory agents. Their effect was assessed *in vivo* on OVX wistar rats.

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INTRODUCTION

Osteoporosis is a complex, multi-factorial condition characterized by reduced bone mass and impaired micro-architectural structure, leading to an increased susceptibility to fractures. Although most of the bone strength (including bone mass and quality) is genetically determined, many other factors (nutritional, environmental and life-style) also influence bone (Jasminka *et al.,* 2000). Postmenopausal osteoporosis is a major age-related health problem for women who often have negative calcium balance due to decrease in intestinal calcium absorption, insufficient dietary calcium intake and increase in urinary Ca loss associated with estrogen deficiency during menopause (Kalpan and Hirsch, 2004). In osteoporosis, the formation and function of osteoblasts decreases whilst osteoclast formation and recruitment increases, and this causes a relative increase of osteoclastic bone resorption over osteoblastic bone formation. The bone formation is related to osteoblastic proliferation, alkaline phosphatase (AlP) activity, osteocalcin and collage synthesis; and the bone resorption is associated with osteoclast formation and differentiation, and tartrate-resistant acid phosphatase activity (TRAP) (Jasminka *et al.,* 2000). Bone is a tissue maintaining itself through continuous osteogenesis and osteolysis by osteoblast and osteoclast (Dempster and Lindsay, 1993) respectively. The unbalance between osteoblast and osteoclast activities is caused by the reduction of estrogen in a woman at the menopause, aging, administration of corticoid preparations, smoking, drinking and the like. It increases osteolysis rather than osteogenesis and consequently induces osteoporosis (Spencer *et al.,* 1986; Ryan *et al.,* 1992; Dempster and Lindsay, 1993).

The ovariectomized rat model is a scientifically accepted model for osteoporosis. The various pathological processes found in this model are similar to those found in humans. In both species bone loss is most rapid after the onset of estrogen deficiency. This is characterized by a period of increased bone turn over during which resorption exceeds formation. Also in both species, bone loss from trabacular bone is greater than cortical bone. These similarities are strong evidence that the ovariectomized (OVX) rat bone loss model is suitable for studying the prevention and treatment of postmenopausal bone loss (Xiao *et al.,* 2002; Hidaka *et al.,* 2006)).

Hormone replacement therapy (HRT) has been an established regime for prevention of postmenopausal bone loss, (Stevenson, 2005; Prelevic *et al.,* 2005) but recent evidence indicates that its long-term use is accompanied by side effects, such as the increased risk of breast, ovarian and endometrial cancer (Wiseman 2004; Shi 2004). Thus, alternative means of

proven efficacy and safety should be developed for prevention and treatment of postmenopausal osteoporosis. Herbal medicine is one of the potent candidates for the treatment of variety of diseases. Although these herbal medicines are seen as cost-effective alternatives by their traditional users, their international acceptance as a major regimen for prevention and treatment of osteoporosis would require extensive research using modern science.

There are many plants described in *Ayurveda* for the treatment of myriad of diseases. *Ayurveda* mentions a number of plants with anti inflammatory and osteoprotective effect. However, the scientific base behind their osteoprotective effect is still not clear. The questions of whether medicines discovered today are safer, more efficacious, and more affordable than generic medicines (whose patents have expired) or medicines that are centuries old could be answered "no" for most of the modern medicines. If so, then it is logical to revisit and revive these age-old medicines for the welfare of mankind. Curcumin is one such medicine. Its history goes back over 5000 years, to the heyday of Ayurveda (which means the science of long life). One of such plant is *Curcuma aromatica*, commonly known as 'Jangli Haldi' belonging to genus curcuma, consisting about 70 species of rhizomatous herbs. It is widely used as a flavoring agent, condiment and a source of yellow dye (Ambasta, 1995). The essential oils of *Curcuma* revealed the presence of various mono and sesquiterpenes. Early studies also showed the presence of curcumol in oil. The plant has also widely studied various pharmacological activities like anti angiogenic, cholerctic and cholagougic, anthelmintic, anti microbial, wound healing, antitumour, antioxidant, cytoprotective etc. (Ahmed *et al.,* 2010). Numerous lines of evidence suggest that curcumin is a potent anti-inflammatory agent, Its pharmacological safety combined with its anti-inflammatory action, makes it an ideal agent to explore for preventive and therapeutic situations (Aggrawal *et al.,* 2007). Curcumin has also proved to prevent osteoclastogenesis (Bharti *et al.,* 2004).

L. glutinosa is described in *ayurveda* for its bone protecting effect and used in tradition medicine in healing the fractures. It is commonly known as "Maida Lakri" is said to be one of the most potent plants for treatment of osteoporosis, (Sukh Dev., 2006). *L. glutinosa* belongs to the family Lauraceae and many of its members are believed to have osteoprotective effect. Bark of the *L. glutinosa* is used for the preparation of the dried bark powder (Parikh *et al.,* 2009). This bark powder is prescribed directly or used in the formulation for the treatment of osteoporosis. Many herbal formulations which are used for the prevention of osteoporosis are

having Maida Lakri as their main herb (Sukh Dev, 2006). However, very less scientific data is available about the osteoprotective effect of this plant.

Terminalia arjuna is a plant described in ayurveda for variety of diseases including heart diseases and obesity (Sukh Dev, 2006). Medicinally valuable part of the plant is bark, also known as Arjunsal. It has been used as a cardiotonic agent in clinical trials far back in 1951. From then it has been explored for variety of diseases including anti oxidant, hypolipidemic, free radical scavenging, wound healing and antibacterial activity (Kumar and Prabhakar, 1987). Casuarinin, tannin identified from this plant has antiviral activity against Herpes virus, while ellagic acid is known to have anti-haemorrhagic effect (Cheng *et al.,* 2002). Terminoside A, a constituent of the bark extract potently inhibited nitric oxide production, suggesting the probable mechanism behind the anti inflammatory activity of this plant (Ali *et al.,* 2003). Plants with anti inflammatory role can be potent candidates as an osteoprotective agent (Penolazzi *et al.,* 2008).

Thus in the present study an attempt is being made to look in the osteoprotective efficacy of the crude extracts of three botanicals *viz, C. aromatica, L. glutinosa* and *T. arjuna.*

MATERIALS AND METHODS

Crude plant drugs were obtained from local drug market and aqueous extracts were prepared by boiling 100 gm of plant in 5 liters of water for 24 hours and then filtered. The filtrates were evaporated on water bath at 60° C to yield semi solid paste. These semi solid pastes were then freeze dried to yield powdered extract (Table – 1.1).

Animals and Treatments:

Thirty six 3-month-old virgin female Wistar rats, weighing about 225 g, were obtained from Sun Pharma Advance Research Center. Rats were housed in a room with alternating 12 h periods of light and dark, ambient temperature of 23 ± 3 °C and humidity of 55 ± 5%. All animals were allowed free access to distilled water and fed on a commercial diet (Pranav Agro food). The acclimatized rats underwent either Sham operated (n= 6) or bilaterally OVX (n= 6). Two weeks after recovering from surgery, the OVX rats were randomly divided into three groups: vehicle-treated (1 ml DW/100 g bw/d); estrogen (E_2)-treated (2m g/kg/d); Plant extracts treated (200 mg/kg/d). Powdered extract of plants were dissolved in distilled water and was orally administered to rats at the dosage of 200mg/kgbw. Body weights were measured once a week during the experimental period. The time for measuring daily food consumption and body weight was the same during the entire period. After euthanizing the rat with cervical dislocation under ether anesthesia the femur was dissected from each animal and

cleaned of all soft tissue, then wrapped in saline-soaked tissue blots, sealed in plastic bags and stored at -80 °C for further analysis. The uterus of each rat was also dissected, separated from the surrounding adipose and connective tissues, blotted and weighed. Uterus index was measured by calculating relative uterine weight. At sacrifice, blood was taken by orbital sinus puncturing under ether anesthesia; serum was then prepared by centrifugation of the collected blood (3000 rpm for 20 min) and stored at -80°C for biochemical analyses. The study was approved by Institutional animal ethical committee.

Serum Chemistry:

Serum calcium concentrations were measured by standard colorimetric methods using an automatic analyzer, Perkin Elmer and commercial kits (Reckon Diagnostics).

The femur bone was dissolved in 6 N HCl and HCl was dried at120 °C for 6 h on a sand bath. The resultant powder was then buffered in tris buffer and analyzed for calcium content using automatic analyzer.

Statistical Analysis:

Data are expressed as mean values and S.E.M. One-way ANOVA was used to compare data from all groups and student's T test was used as post test after ANOVA was performed to compare pairs of groups by the statistical software of GraphPad Prism (Version 5.0). A p value of less than 0.05 was considered statistically significant.

RESULTS

Body weight and uterine weight

Percentage yield of the plants is shown in Table 1.1. *T. arjuna* was the highest yielding followed by *C. aromatica* and the least yield was of *L. glutinosa.* As shown in Table 1.2 and Figure 1.1, rats in all experimental groups had almost similar initial body weights. Four weeks after operation, there was a significant increase in the body weight of the OVX rats ($p< 0.01$, *vs* sham). Treatment of OVX rats with E_2 significantly suppressed the increase in body weight associated with E_2 deficiency and returned body weight to the level maintained by sham group four weeks after treatment (Table 1.2).In addition, OVX caused significant atrophy of the uterus in rats as anticipated (Table 1.3). E_2 significantly increased uterine weight in OVX rats (Table 1.3, Figure 1.2). In contrast, treatment of OVX rats with extracts did not affect the uterine weight. *Litsea* and *Curcuma* had no significant effect on the body weight, whereas the *T. arjuna* showed significant decrease in the body weight (Table 1.3, Figure 1.2)

Serum Chemistry

At the end of the experiment, the serum levels of several bone markers were measured as indicators of the protective effects of the botanicals.OVX significantly decreased serum calcium level (Table 1.4, $p < 0.05$ vs sham). E_2 significantly reversed the OVX-induced changes in serum calcium levels; treatment of extracts also suppresses serum calcium levels (Figure 1.3). AlP an osteoblastic function marker increased significantly in OVX ($p < 0.001$ *vs* sham) (Figure 1.4). E_2 replacement reduced these changes to normal. Of the three treated botanicals *C. aromatica* showed a significant decrease and non significant decrease in response to *L. glutinosa* decrease in the serum AlP levels (Figure 1.4). TRAcP levels increased in OVX rats compared to sham operated rats indicating excess resorption. ($p < 0.001$) E_2 supplementation potently inhibited the TRAcP levels and reduced them significantly lower than even normal animals. All the three botanical treated groups showed a significant decrease ($p < 0.001$) as compared to the OVX group of rats (Table 1.4, Figure 1.5)

Bone chemistry

The effect of the botanicals on bone AlP and TRAcP are summarized in Table 1.5. OVX rats showed a significant increase in both bone AlP as well as the TRAcP levels as compared to sham ($p < 0.001$). Both botanical treated as well as E_2 treated significantly suppresses the OVX-induced increase in bone AlP and TRAcP levels. However, the *C. aromatica* and *L. glutinosa* showed a significant change whereas, *T. arjuna* showed insignificant increase as compared to the OVX rats. This result indicated that the botanical extracts prevented the induction of high bone turnover associated with the E_2 deficiency in OVX rats (Figure 1.6 and 1.7).

DISCUSSION

Natural medicines derived from plants have aroused increasing interest in the prevention and treatment of osteoporosis. This is due to their unique characteristics as these are more suitable for long-term use compared with synthesized chemicals and have apparently fewer adverse effects. In the present study we evaluated 3 plants for their osteoprotective effect against high bone turnover, loss of bone Ca and reduced serum Calcium associated with E_2 deficiency in OVX animals. This study is the first to check the osteoprotective efficacy of the selected botanicals. The present study demonstrated that all the botanicals could prevent high bone turnover and calcium loss caused by E_2 deficiency, without substantial effects on the uterus. OVX of young rats is a model for studying postmenopausal osteoporosis (Kalu, 1991; Thompson *et al.,* 1995). As expected, OVX animals in the present study exhibited all the

characteristics associated with E_2 deficiency, such as weight gain, negative calcium balance, high turnover and uterine atrophy. These conditions were almost recovered with Estrogen replacement, showing the condition almost similar to Sham. These results affirmed the reports of previous studies (Lee *et al.,* 2004) that showed OVX induced changes can be reverted by E_2 supplementation. Our results confirmed with the findings of others that the OVX rat model was characterized by high bone turnover rate (Park *et al.,* 2008; Zhang *et al.,* 2006b). A comparative study of the three botanicals demonstrated that *L. glutinosa* and *C. aromatica* could effectively prevent high bone turnover and calcium loss caused by E_2 deficiency, without substantial effects on the uterus; but *T. arjuna* had no significant effect on OVX induced changes. The increase in bone turnover was the result of increase in both bone formation and bone resorption associated with E_2 deficiency. In the present study it has been proved that serum as well as bone AlP level, which is used as a clinical marker for detecting bone formation *in vivo*, was significantly increased in OVX rats. Treatment of OVX rats with the botanicals for four weeks significantly reduced the serum AlP levels, suggesting that botanicals acts on bone as a potent inhibitor of high bone turnover. Whether the effects of botanicals on bone turnover are primarily mediated by its actions on osteoblastic cells (cell formation) and/or osteoclastic cells (bone resorption) requires further investigation. Regardless of its mechanism of action, the drastic decrease in rate of bone turnover provides a direct explanation for the observed increase in serum and bone calcium content.

It is of interest to note that the botanical treatment demonstrated selective estrogen-like effects on bone without the detrimental stimulatory effects in the uterus. Previous workers (Setchell, 2001; Debbie and Wendy, 2004) have reported that phytoestrogens act as selective estrogen receptor modulator because they exhibit estrogen activity in one tissue (bone), but act as estrogen antagonist in other tissues (breast, uterus). Thus, it is possible that botanicals tested in the present study might be behaving like phytoestrogens that possess selective activity towards bone tissue and uterus. Further, botanicals treated OVX rats had decreased uterine weight indicating uterine atrophy; thus, possibly the botanicals also may reduce the risk of breast and ovarian cancer associated with ERT/HRT. And thus could be a potent alternative therapy for osteoporosis. In the present study *C. aromatica* and *L. glutinosa* were seen as competent osteoprotective agents. Although, *T.arjuna* was not found to have any osteoprotective effect, it showed significant reduction in the body weight of the animals, affirming its hypolipidemic and its anti obesity role. This plant was found to be rich in tannins

and tannins are known for reducing the food consumption, explaining the reason why during our study weight gain was least in *T. arjuna* treated animals (Sukh Dev, 2006). *C. aromatica* is known to be rich in curcumin, which is a potent anti inflammatory agent and a proved osteolysis inhibitor by inhibiting osteoclastogenesis (Bharti *et al.*, 2004). Curcumin is an established osteoprotective agent due to its osteclast inhibiting property which acts through NF κβ ligand signaling pathway (Bharti *et al.*, 2004) and; Folwarczna *et al.*, (2009) have shown that the curcumin is having osteoprotective effects in OVX rats. While *L. glutinosa* has been widely used in India, no data is available to substantiate its beneficial effect on osteoporosis. To our knowledge, the present study is the first demonstration of osteoprotective property of this plant. This plant ameliorated OVX induced changes without affecting the uterus. Though effects observed were not as significant as that of E_2, this plant might be worth exploring. Further in depth analysis is needed to identify the mechanism that mediates the action of *L. glutinosa.*

Table 1.1 Percentage yield of aquous extract of plants.

	Curcuma aromatica	*Litsea glutinosa*	*Terminali arjuna*
% yield	8.19	6.66	12.38

Table 1.2: Mean body weight.

Week	Sham	OVX	OVX + E_2	OVX + CA	OVX +LG	OVX + TA
1	338.3	326.0	337.2	330.4	322.6	319.2
2	348.4	336.0	350.6	337.2	330.4	322.3
3	357.3	364.1	358.5	358.5	352.9	338.6*
4	369.5	384.2	365.3	370.8	369.7	358.9*

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$

Table 1.3: Relative uterine weight

Parameter	Sham	OVX	OVX + E_2	OVX + CA	OVX +LG	OVX + TA
Relative Uterine weight	0.001550 ± 0.000167	0.001300*** ± 0.000220	0.001559*** ± 0.000190	0.000270 ± 0.000183	0.000280 ± 0.000299	0.000273* ± 0.000283

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$

Table 1.4: serum profile

Parameter	Sham	OVX	OVX + E_2	OVX + CA	OVX +LG	OVX + TA
Serum calcium levels (mg/dl)	8.800 ± 0.569	5.458*** ± 0.786	8.800*** ± 0.699	7.890 0.130	7.230 ± 0.689	5.230 ± 0.880
Serum AlP levels (IU/L)	53.790 ± 1.230	133.990*** ± 6.780	54.920*** ± 1.860	109.640*** ± 5.940	127.910 ± 1.990	148.690 2.430
Serum TRAcP levels (IU/L)	7.900 ± 0.234	16.500*** ± 0.990	6.200*** ± 0.322	7.800*** ± 0.670	8.890*** ± 1.990	13.790 ± 1.890

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$

Table 1.5: Bone tissue enzyme profile

Parameter	Sham	OVX	OVX + E_2	OVX + CA	OVX +LG	OVX + TA
Bone AlP levels (IU/L)	354.000 ± 7.000	551.000*** ± 11.667	389.000*** ± 10.666	421.000*** ± 18.333	481.280** ± 14.000	510.000 ± 10.666
Bone TRAcP levels (IU/L)	6.123 ± 0.234	10.89*** ± 0.2896	5.273*** ± 0.123	8.129*** ± 0.2384	9.119*** ± 0.2996	10.123 ± 0.2616

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$

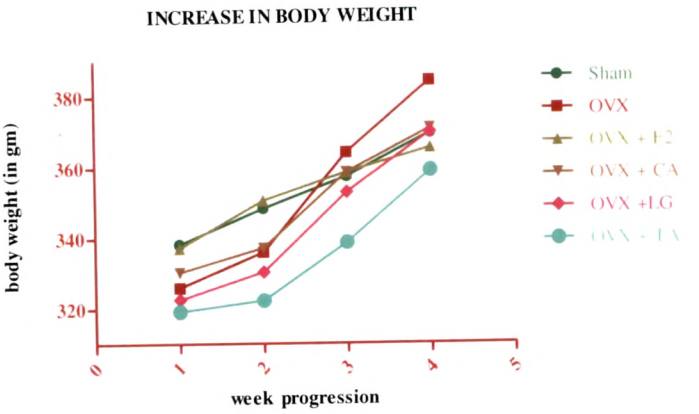

Figure 1.1 Increase in body weight

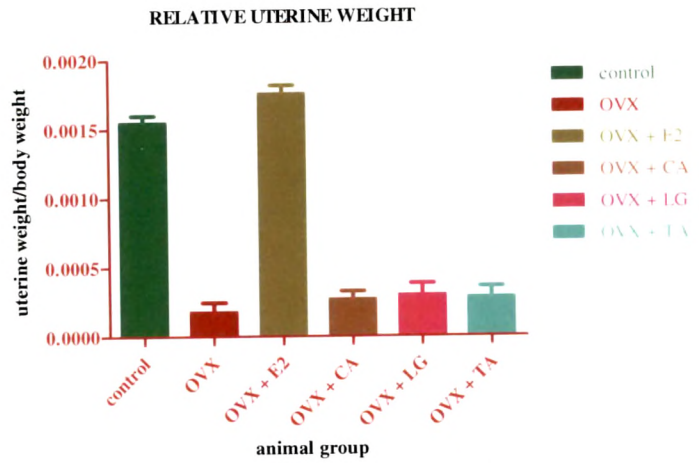

Figure 1.2: Relative uterine weight

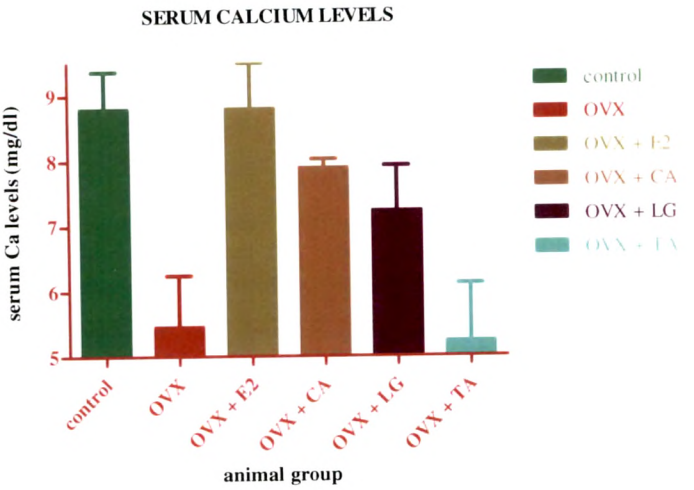

Figure 1.3: Serum Calcium levels

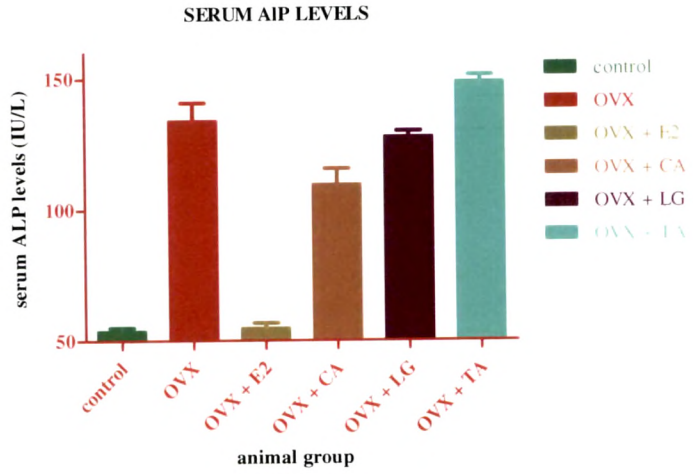

Figure 1.4: Serum ALP levels

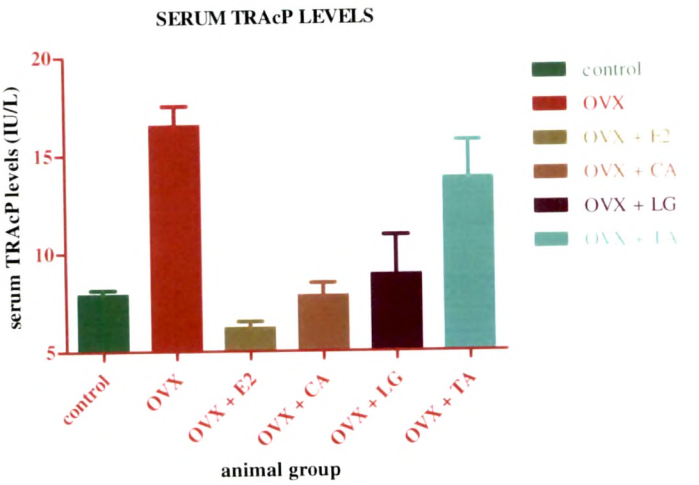

Figure 1.5: Serum TRAcP levels

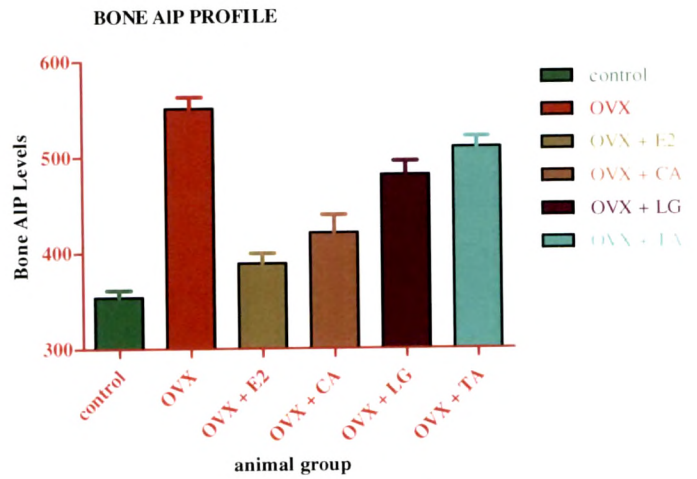

Figure 1.6: Bone ALP levels

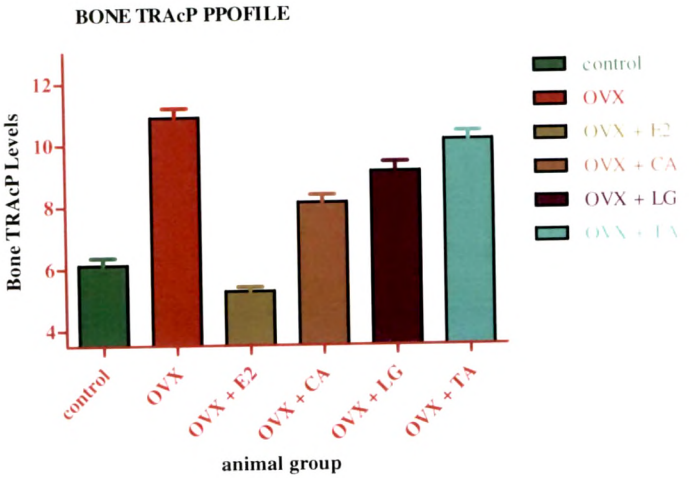

Figure 1.7: Bone TRAcP levels