OSTEOPROTECTIVE EFFECT THREE ANTI INFLAMMATORY PLANTS IN OVARIECTOMIZED WISTAR RATS

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Summary

Osteoporosis is a metabolic bone disease, characterized by increased porosity of the skeleton resulting from reduced bone mass. The drug discovery process in this direction is very attractive, because of non-availability of suitable, safe and effective means for the management of this condition. The objective of the study was to evaluate antiosteoporotic activity of three medicinal plants described in Ayurveda, namely, *Litsea glutinosa*, *Curcuma aromatica* and *Terminalia arjuna*. Ovariectomized rats model was used in this study for evaluation. Effects were evaluated by using serum and tissue biochemical parameters. The effect on health status during the treatment was evaluated by regularly checking the overall body weight of the ovariectomized rats.
Litsea and *Curcuma* showed significant effects on ameliorating the changes induced by Ovariectomy, while *Terminalia* had no effect. Both these plants had potent inhibitory activity, similar to that was observed by Estrogen supplementation. Bone remodeling markers were upregulated in OVX animals and their ameolioration was achieved by plant treatment. Thus, our study is first to provide scientific evidence that *Litsea* and *curcuma* are having osteoprotective effect as they ameliorate changes induced by Ovariectomy

Key words: Osteoprotective, Ovariectomy, Osteoblasts, Osteoclast.

Introduction

Osteoporosis is a complex, multi-factorial disease 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 (1). 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, as well as increase in urinary Ca loss associated with estrogen deficiency (2). In osteoporosis, the formation and function of osteoblasts decreases whilst osteoclast formation and recruitment increases. 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; while bone resorption is associated with osteoclast formation and differentiation, and tartrate-resistant acid phosphatase activity (TRAP) (1).

Bone is a tissue maintaining itself through continuous osteogenesis and osteolysis by osteoblast and osteoclast (3) 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 (3, 4 and 5).

The ovariectomized rat model is a scientifically accepted model of 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 trabecular bone is greater that 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 (6, 7).

Hormone replacement therapy (HRT) has been an established regime for prevention of postmenopausal bone loss, (8, 9) 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 (10, 11). 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, including osteoporosis. 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* (which means the science of long life) 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. Curcumin is one such medicine. Its history goes back over 5000 years, to the heyday of Ayurveda. 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 (12). 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. (13). 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 (14). Curcumin has also proved to prevent osteoclastogenesis (15).

L. glutinosa is described in *Ayurveda* for its bone protecting effect and used in traditional medicine in healing the fractures. It is commonly known as "Maida Lakri" and said to be one of the most potent plants for treatment of osteoporosis, (16). *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 (17). 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 (16). 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 (16). 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 (18). Casuarinin, a tannin identified from this plant has antiviral activity against Herpes virus, while ellagic acid is known to have antihaemorrhagic effect (19). 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 (20). Plants with anti inflammatory role can be potent candidates as an osteoprotective agent (21). 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 – I).

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 (2 mg/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 and blotted weighed. Uterus index was measured immediately. At sacrifice, blood was taken from 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 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 Graph Pad PRISM (Version 5.0). A p value of less than 0.05 was considered statistically significant (17).

Results

Body weight and uterine weight
Percentage yield of the plants is shown in Table I. *T. arjuna* was the highest yielding followed by *C. aromatica* and the least yield was of *L.glutinosa*. As shown in Fig 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. In addition, OVX caused significant atrophy of the uterus in rats as anticipated (Fig 2). E_2 significantly increased uterine weight in OVX rats ($p < 0.001$ vs sham) but the weight remained substantially lower than that of the sham rats ($p < 0.01$). 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 ($p < 0.01$) (Fig 1)

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 ($p <0.05$ vs sham). E_2 significantly reversed the OVX-induced changes in serum calcium levels; Extracts treatment also suppresses serum calcium levels. AlP, an osteoblastic function marker increased significantly in OVX ($p < 0.001$ vs sham). E_2 replacement reduced these changes to normal. Of the three treated botanicals *C. aromatica* showed a significant decrease in serum AlP levels while L. glutinosa showed decrease in AlP but, it was statistically non significant (Fig 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. (Fig 5)

Bone chemistry

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 treatment as well as E_2 treatment significantly suppressed the OVX-induced increase in bone AlP and TRACP levels. However, the *C.aromitica* 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 (Fig 6 and 7)

Table 1 Percentage yield of aquous extract of plants.

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

Figure 1 Increase in body weight

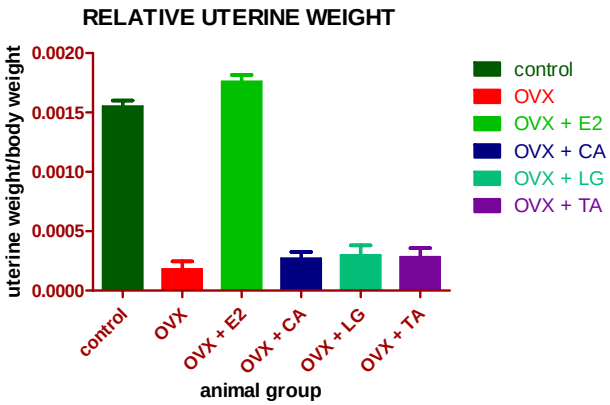

Figure 2: Relative uterine weight

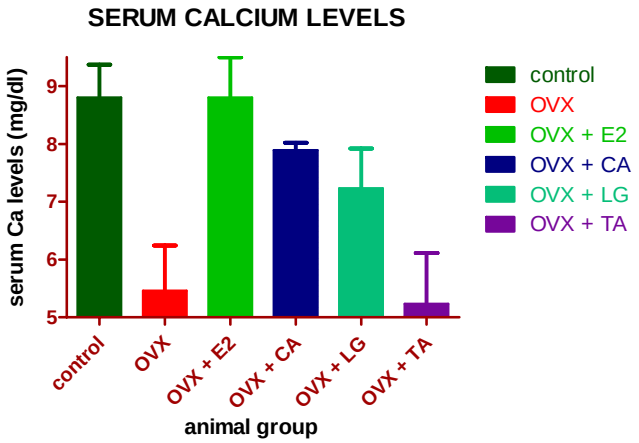

Figure 3: Serum Calcium levels

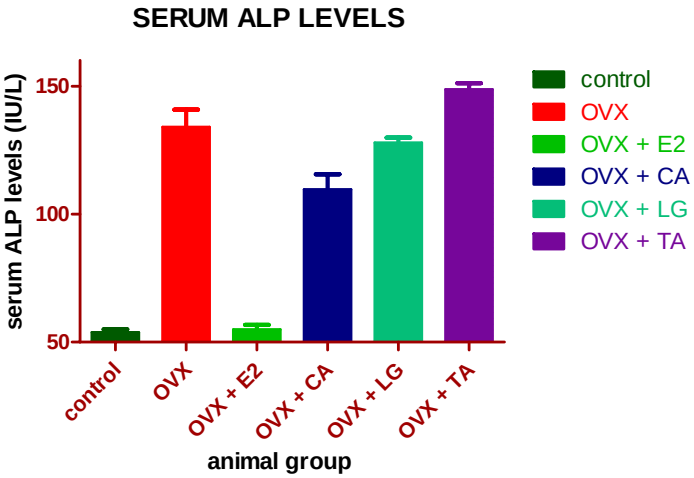

Figure 4: Serum AlP levels

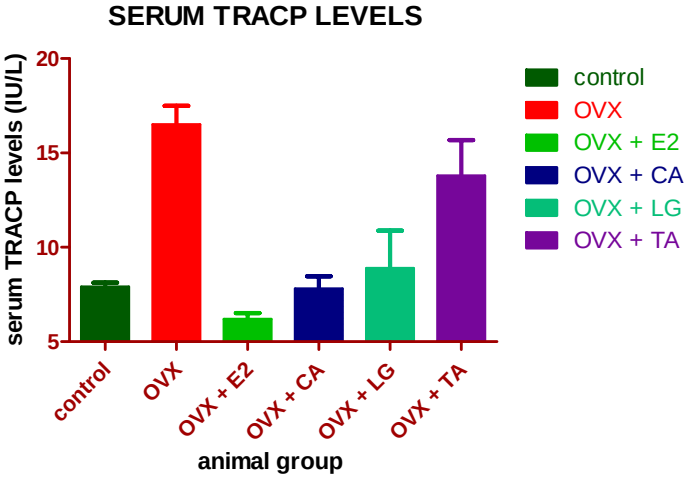

Figure 5: Serum TRACP levels

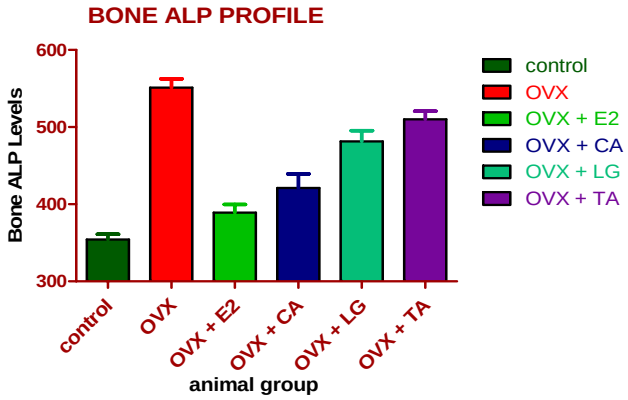

Figure 6: Bone AlP levels

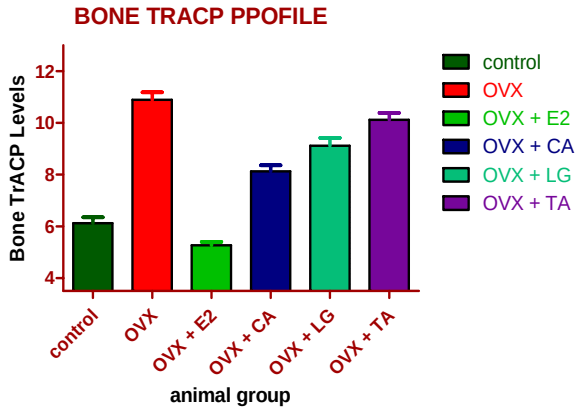

Figure 7: Bone TRACP levels

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 calcium and reduced serum calcium associated with E_2 deficiency in OVX animals. This study is the first to check the osteoprotective efficacy of selected botanicals. The present study demonstrated that all botanicals could prevent high bone turnover and calcium loss caused by E_2 deficiency, without substantial effects on the uterus. Ovariectomy of young rats is a model for studying postmenopausal osteoporosis (22, 23). 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 E_2 replacement, showing the condition almost similar to Sham. These results affirmed the reports of previous studies (24) that showed that OVX induced changes can be reverted with E_2 supplementation.

Our results confirmed with the findings of others that the OVX rat model was characterized by high bone turnover rate (25, 26). 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. However, *T. arujuna* 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 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. Setchell (27) and other co workers (28) have reported that phytoestrogens act as selective estrogen receptor modulator because they exhibit estrogen activity in one tissues (bone), but act as estrogen antagonist in other tissue (breast, uterus). Thus, it is possible that botanicals tested in the present study might be acting 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 these botanicals also may reduce the risk of breast and ovarian cancer associated with ERT/HRT.

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 (16).

C. aromatica is known to be rich in curcumin, a potent anti inflammatory agent and a proved osteolysis inhibitor by inhibiting osteoclastogenesis (15). Curcumin is an established osteoprotective agent due to its osteclast inhibiting property which acts through NF κ β ligand signaling pathway (15) and it 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. 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 for its osteoprotective agent. Further in depth analysis is needed to identify the mechanism that mediates the action of *L. glutinosa.*

In conclusion, our study proved that out of three potent anti inflammatory agents described in *Ayurveda*, *Litsea* and *Curcuma* are having osteoprotective effect, while *Terminalia arjuna* was not found to have any osteoprotective role. Hence, consumption of *Litsea glutinosa* and *Curcuma aromatica* can be helpful in preventing osteoporosis.

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