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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

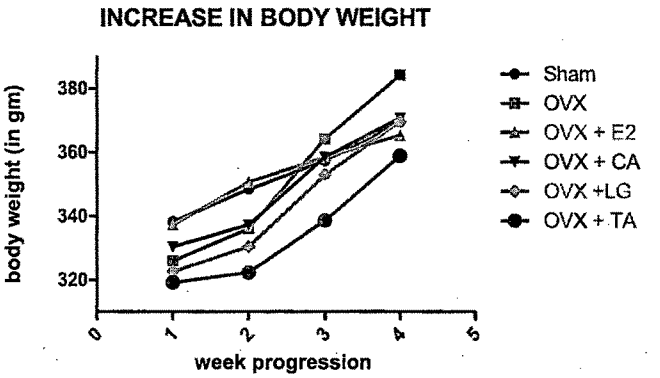

Figure 1 Increase in body weight

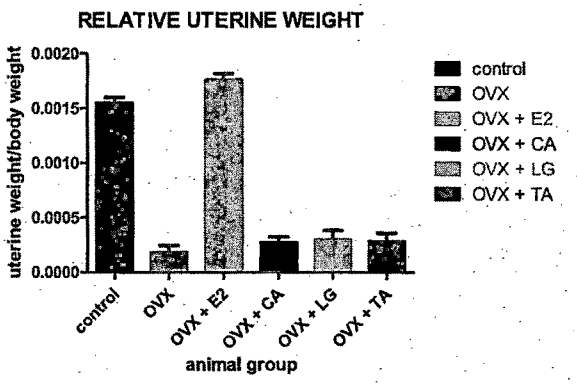

Figure 2: Relative uterine weight

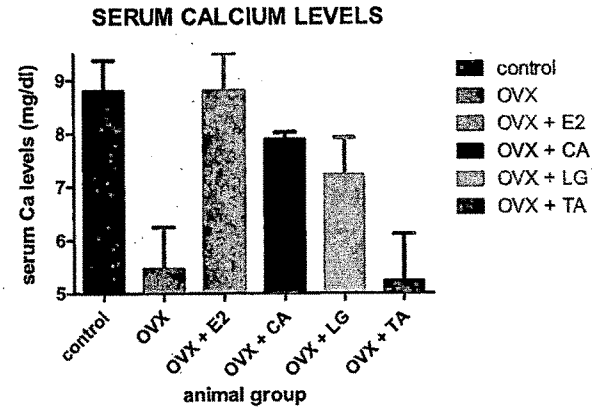

Figure 3: Serum Calcium levels

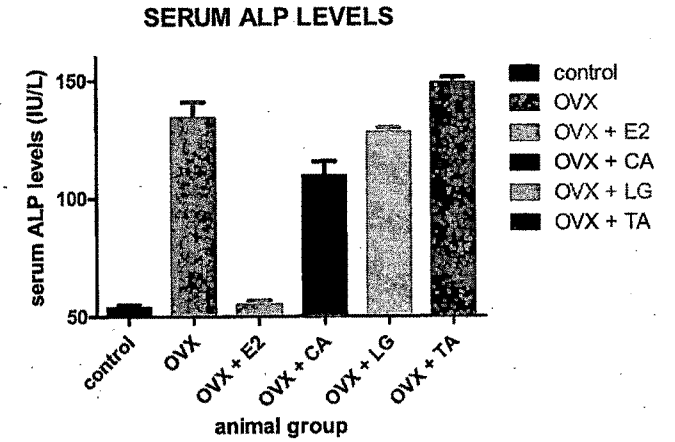

Figure 4: Serum AlP levels

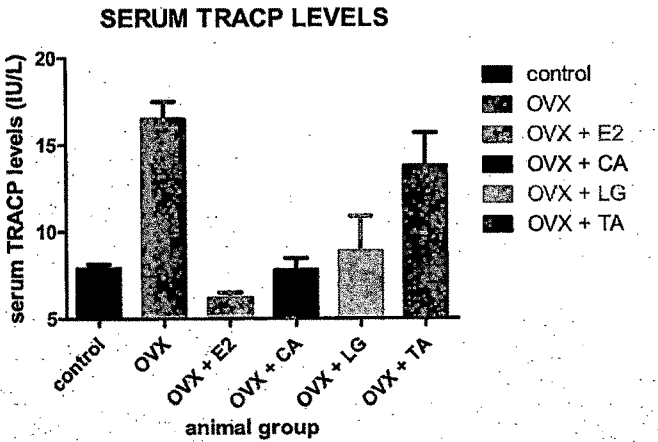

Figure 5: Serum TRACP levels

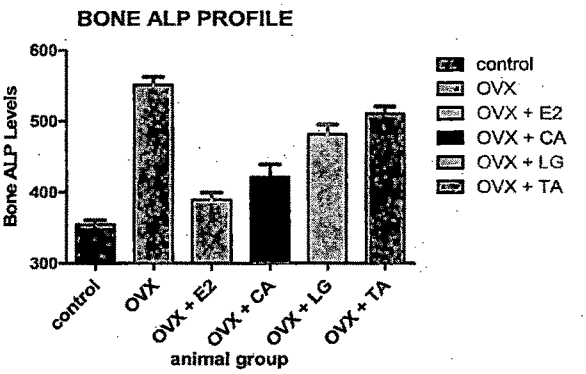

Figure 6: Bone AlP levels

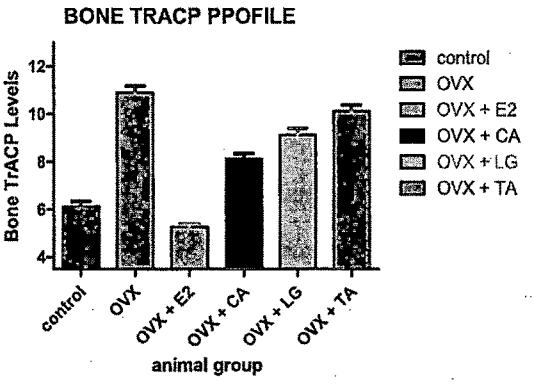

Figure 7: Bone TRACP levels

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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OSTEOPROTECTIVE EFFECT OF *LITSEA GLUTINOSA* IN OVARIECTOMIZED WISTAR RATS

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Abstract: ***Osteoporosis is characterized by a reduction in bone mass with possible alteration in bone architecture and an increased risk of fractures. Various pharmacological interventions are aimed at inhibiting bone turnover and preventing osteoporosis. Litsea glutinosa is a plant commonly used in the traditional Indian medicines for the treatment of osteoporosis. This study is aimed at validating the osteoprotective effect, if any, of this plant in ovariectomized Wistar rats Three months old female Wistar rats were either sham operated or ovariectomized and fed food mixed with L. glutinosa plant bark powder or with normal powdered feed till the end of the study. Our results indicated that L. glutinosa have bone protective effect in a dose dependent manner. It decreased the alkaline phosphatase (ALP) activity in serum as well as bone and also inhibited tartarate resistance phosphatase (TrACP). Other biochemical markers indicated that L. glutinosa have positive effect on bone remodeling. Histology of cancellous bone reaffirmed the protective role of this plant product against osteoporosis. The present data suggest that L. glutinosa bark powder is a promising phytoremedial agent for the treatment of osteoporosis.***

Key words: *Litsea glutinosa*, Osteoporosis

INTRODUCTION

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 calcium loss associated with estrogen deficiency during menopause. Osteoporosis is a progressive debilitating process that reduces cancellous bone and consequently leads to the weakening of the overall integrity and stability of bone that enhances the bone fragility and consequent increase the fracture risk [1]. The pathogenesis of postmenopausal osteoporosis is manifested by dropping in ovarian estrogen levels and increased bone turn over with an increase in bone resorption by osteoclasts resulting in decreased bone mass which is associated with an increase in the production of pro-inflammatory cytokines such as Interleukin (IL) 1 and 6, which contribute to bone resorption by increasing osteoclastogenesis [2-5].

Until recently, calcium supplementation with vitamin D and hormone replacement therapy (HRT) with estrogen, selective estrogen receptor modulators, calcitonin, raloxifene, amino-bisphosphonates, teriparatide, parathyroid hormone, strontium ranelate, growth hormone, and insulin like growth factor - 1(IGF -1) were the mainstays in the treatment of menopause associated osteoporosis resulting in the prevention or slowing of bone loss [5]. Recent evidence suggest that estrogen replacement therapy (ERT) is associated with increased risk of breast, ovarian and endometrial cancer in postmenopausal women, it is now generally recognized that alternative approaches to the prevention and treatment of osteoporosis might be worth exploring [2]. Hence, recently the attention has been focused on phytoestrogens and phytotherapy for the treatment of osteoporosis.

The ovariectomized (OVX) rat model is a scientifically approved model of osteoporosis. Moreover, various

pathological processes found in this model are similar to those found in humans. In both the species bone loss is quite rapid after the onset of estrogen deficiency. This is characterized by a period of increased bone turnover during which resorption exceeds formation. Also in both the species, bone loss from trabacular bone is greater than cortical bone [3]. These similarities are strong evidence that the OVX rat bone loss model is suitable for studying the prevention and treatment of postmenopausal bone loss [4].

Indian herbal medicine has been widely used for the treatment of many diseases from thousands of years [6]. There are numerous plants which have been shown to have positive effect on bone remodeling. A few of them are used in the prevention of osteoporosis. *Litsea glutinosa* commonly known as "Maida Lakri" is said to be one of the most potent plants for treatment of osteoporosis, and also described in *Ayurveda* for the treatment of osteoporosis as well as bone fracture healing [6]. *L. glutinosa* belongs to the family Lauraceae and many of its members are believed to have osteoprotective effect. In the past, the development of herbal anti-osteoporosis formulations was pursued mainly by scientists in Asian countries, including China, Japan and Korea [2]. Dry bark powder of the *L. glutinosa* 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 [6]. However, no scientific data is available about the osteoprotective effect of this plant. In the present study an attempt was made to explore the osteoprotective property of *L. glutinosa* plant.

MATERIALS AND METHODS

Experimental protocol: Thirty, 3-month-old virgin female Wistar rats brought from Sun Pharma Advance Research Center (300 ± 20gm) were used for this study. The animals were kept for 8 days before the onset of the experiment to acclimatize to laboratory conditions (the room temperature was 22 ± 4 °C with a 12h/12h light/dark cycle). Rats were ovariectomized and sham operated as described previously [4]. Rats were given a lag phase of 10 days to recover from the stress of operation. Thereafter, the rats were divided in into six groups as shown below (table 1). Normal control and OVX control were given simple powdered feed, using water as binder. While experimental groups were given 2 % (w/w) and 5 % (w/w) of the *L. glutinosa* bark powder, mixed with the feed. Food was given every evening at 6 p.m. and the left food was discarded next day and fresh food was given. Every week the feed was checked for any bacterial or fungal infection. Food and water was given *ad libitum.*

Every week blood was collected by orbital sinus puncturing under mild ether anesthesia. 0.5 ml blood was collected; serum was separated and stored at -80° C until assayed. For 8 weeks the treatment was continued and after that the rats were sacrificed by giving overdose of anesthesia. Rats were dissected, bone, uterus and liver were collected, washed in PBS (pH 7.4), and stored in -80° C until assayed. All the assays were carried out using commercial kits purchased from Recon Diagnostics.

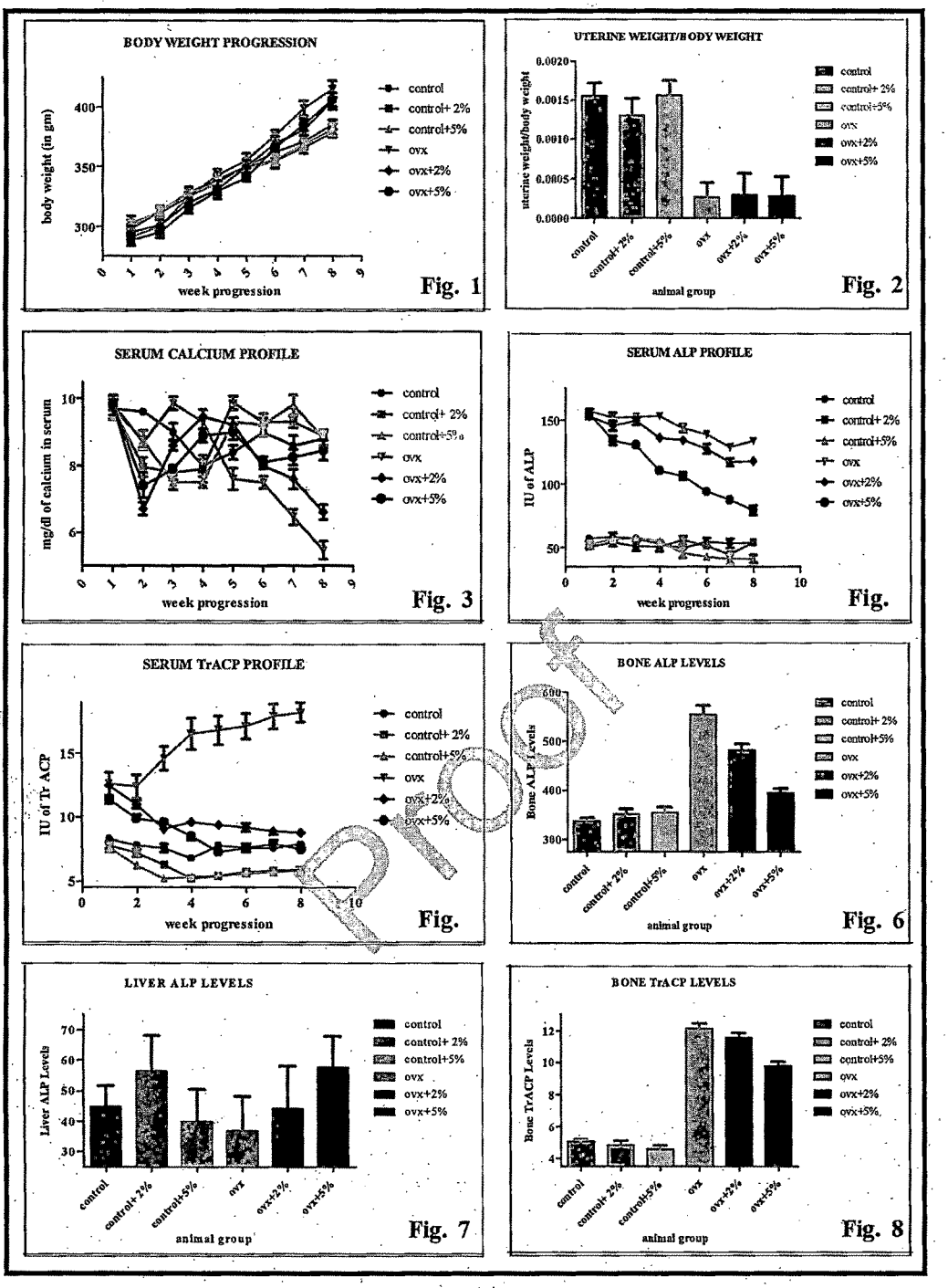
The calcium (Ca) and phosphorus (P) concentrations of serum samples were estimated using standard colorimetric methods and analyzed using Perkin Elmer spectrophotometer [7-11]. Serum ALP and TrACP were estimated using pNPP method [12,13]. For histology of the bone, tissues were fixed in 4% para-formaldehyde, washed in tap water, decalcified in EDTA – G as described [14]. Paraffinized sections were cut at 7 µm and stained using standard Haematoxilin eosin staining. For uterine histology, tissues were fixed in 10% neutral buffered formalin, and paraffin sections of 5 µm thickness were cut and stained using standard Haematoxilin eosin staining.

Statically analyses: All the data were analyzed by GraphPad Prism 5 software, using two way ANOVA followed by Bonferroni post hoc test to compare the results ($p<0.05$).

RESULTS

Body and uterine weight ratio: As compared to the body weight of the Sham group, the body weights in the OVX group were increased significantly after 8 weeks (Fig. 1). Though a decrease in body weight was observed in plant treated OVX group it was statistically not significant. Uterine weight did not show any alteration between treated and control groups. However, uterine weight to body weight ratio showed a slight increase in the plant treated animals, suggesting a possible protective effect of plant (Fig 2). Food consumption by the OVX animals was higher compared to Sham animals however, treated did not show any alteration in the food consumption.

Serum calcium and phosphate levels: The effect



Figures 9 to 20 are histological preparations of uterus and bone of control and experimental rats. X 100

Fig. 9: Histology of the uterus of Sham operated animals showing normal uterine epithelium (arrow) and uterine glands (ug).

Fig. 10: Histology of the uterus of Sham operated 2% plant treated animals showing normal uterine epithelium (arrow) and uterine glands (ug).

Fig. 11: Histology of the uterus of Sham operated 5% plant treated animals showing normal uterine epithelium (arrow) and uterine glands (ug).

Fig. 12: Histology of the uterus of OVX animals showing loosen stromal tissue (st).

Fig. 13: Histology of the uterus of OVX 2% plant treated animals showing loosen stromal tissue (st).

Fig. 14: Histology of the uterus of OVX 5% plant treated animals showing loosen stromal tissue (st).

Fig. 15: Histology of the bone of Sham operated animals showing normal cancellous bone (cb).

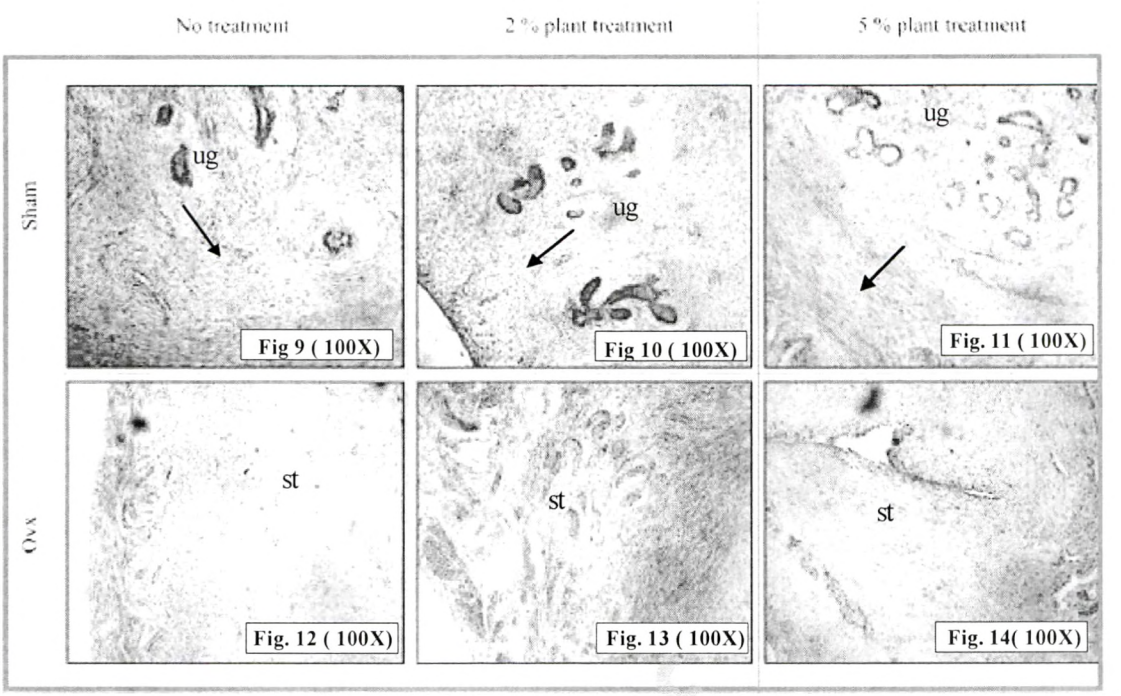


Fig. 16: Histology of the bone of Sham operated 2% plant treated animals showing cancellous bone (cb) similar to normal.
Fig. 17: Histology of the bone of Sham operated 5% plant treated animals showing cancellous bone (cb) similar to normal.
Fig. 18: Histology of the bone of OVX animals showing osteoporotic cancellous bone (ocb) with prominent features of thinning and breakage of trabecules (trb).

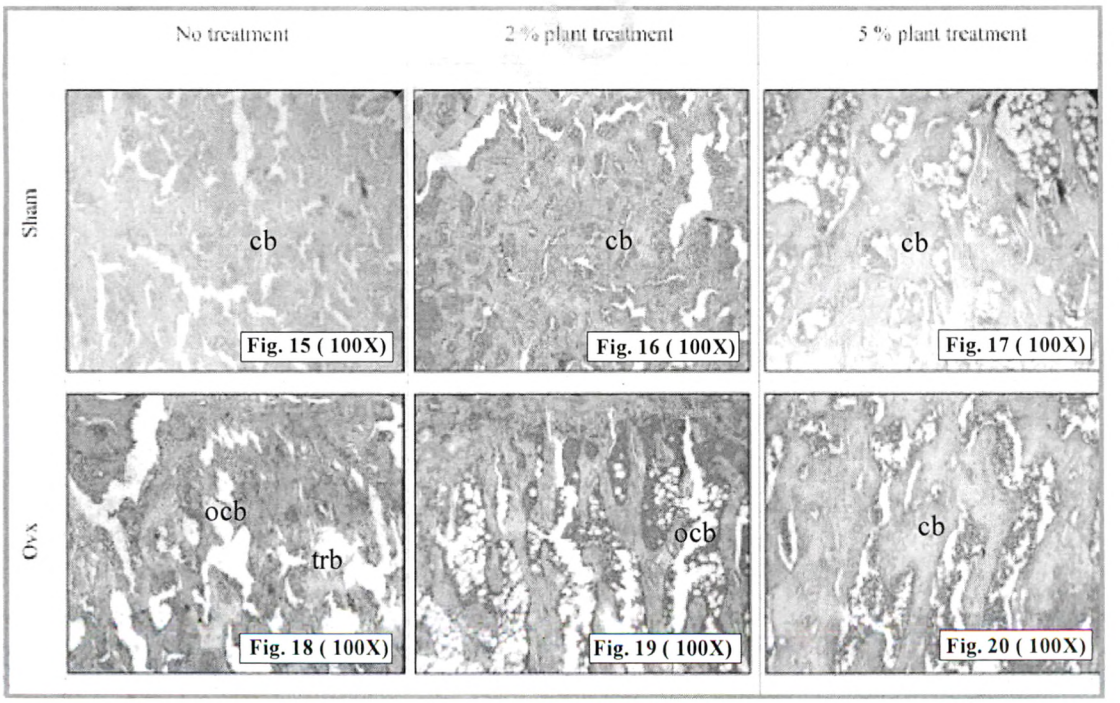


Fig. 19: Histology of the bone of OVX 2% plant treated animals showing osteoporotic cancellous bone (ocb) with lesser thinning and breakage of trabecules (trb) compared to figure 18.
Fig. 20: Histology of the bone of OVX 5% plant treated animals showing cancellous bone (cb) similar to figure 15.

Group	Name of the group	No. of animals	Treatment	Abbreviation
A	Normal control	5	Sham Operated	Sham
B	Normal treatment	5	Sham Operated + 2% herbal treatment	Sham + 2%
C	Normal treatment	5	Sham Operated + 5% herbal treatment	Sham + 5%
D	Ovariectomized control	5	Ovariectomized	Ovx
E	Ovariectomized treatment	5	Ovariectomized + 2% herbal treatment	Ovx + 2%
F	Ovariectomized treatment	5	Ovariectomized + 5% herbal treatment	Ovx + 5%

Table 1: Group of animals with name number and mode of treatment

of *L. glutinosa* bark powder on serum calcium level is presented in Fig. 3. In first two weeks the calcium level was fluctuating, but later on it stabilized. OVX appeared to reduce serum calcium level and the plant bark powder treatment ameliorated the same. The control group did not show any significant change. Further, OVX also led to the gradual decrease in phosphate levels.

Serum ALP levels: OVX caused a significant increase in the serum ALP levels, which remained high till the end of the experiment (8 weeks). Herbal treatment lowered the serum ALP levels in a dose dependent manner. Control treatment did not show any significant alteration (Fig. 4).

TrACP levels: TrACP levels increased with duration in OVX animals and showed the increasing trend till the end of the experiment. At 2% dose the TrACP activity reduced significantly and at 5% dose it is reduced to the control values. In control treatment also the plant lowers the TrACP activity (Fig. 5).

Bone and liver ALP levels: Bone ALP levels were significantly increased in the OVX group as compared to that of controls. Herbal treatment showed decrease in ALP levels in a dose dependent manner (Fig. 6). The alteration in liver ALP levels was found insignificant. Therapeutic treatment did not show any significant variation (Fig. 7).

Bone TrACP levels: Bone TrACP level was higher in OVX animals as compared to that of control. In therapeutic animals, TrACP level was reduced in a dose dependent manner (Fig. 8).

Histology of the uterus: The appearance of uterine epithelium and stroma was normal in control animals. The covering epithelial cells were a mixture of ciliated and secretory simple columnar cells and the connective tissue of the lamina propria which was rich in fibroblasts and contained abundant amorphous ground substance. Connective tissue fibers were mostly reticular (Fig. 9). No significant differences were observed in the control 2% or 5% treatment groups (Figs 10 and 11). Study reveals that in OVX groups the length of the epithelium and the number of the uterine glands were decreased. Along with this, the appearance of stromal tissue was looser in OVX groups (Fig.12). Herbal treatment (2 or 5 %) to OVX group did not show any improvement (Figs. 13,14).

Histology of bone: Bone histology showed no significant change in the control groups (Fig. 15). Compact and cancellous bone was found to be completely normal in 2% and 5% treated groups (Figs. 16,17). Bone marrow was also found attached to the cancellous bone. However, in the OVX control group, though the compact bone did not show any significant alteration, the cancellous bone showed altered bone micro architecture (Fig. 18). Intertrabecular bone structure was well maintained in all control groups. In OVX animals however, trabecular bone was seen to be thinning and the quantity of bone was lost. 2 % treatment showed a notable improvement in the quality and micro architecture of bone (Fig. 19). However, signs of osteoporosis were still seen in the bone. 5% plant treatment, the trabecular bone architecture was found to regain the features similar to the control groups (Fig. 20).

DISCUSSION

Ovariectomy of young rat is an approved model for studying post menopausal osteoporosis [3,15]. OVX animals exhibited all the characteristics of post-menopause i.e., weight gain, uterine atrophy, high serum ALP levels etc. Marked reduction in food intake possibly could affect the bone metabolism [3,15]. Food intake and weight gain were higher in all three OVX groups than in control groups, but were similar in OVX groups. The effect of *L. glutinosa* bark powder was much differ-ent on bone and uterus, later is the primary estrogen target organ. It is widely known that estrogen treatment maintains uterine weight. In contrast, feeding with *L. glutinosa* powder did not show any changes in the uterine weight indicating that this plant is not having any phytoestrogens. When the data was taken as uterine weight to body weight ratio, this plant showed ameliorative effect on OVX induced changes. These results were found to be similar to previous report [2].

Serum calcium and phosphate levels decreased in OVX animals. Similar results were reported previously [8]. However, in this study, alteration in the serum ions was statistically not significant. Tartarate resistant ACP is the marker of osteoclastic bone resorption [1]. Main difference seen between TrACP and ALP was that OVX causes a sudden boost in serum ALP levels, while the TrACP levels were observed increasing gradually with time. It is well documented that OVX leads to reduction in circulating levels of estrogen, which in turn play a major role in inhibiting the osteoclastic functioning through RANK RANK L and osteoprotegrin competitive inhibition [15]. Gradually increased TrACP levels are indication of osteoclastic resorption. However, the bone formation event is directly in response to resorption, which is seen in the form elevated ALP levels indicating bone formation. However, this bone formation marker shows a little decrease at the end of two months indicating that the osteoblastic cells had lowered their response to resorption. This could possibly be the cause resulting into osteoporosis that the bone formation is not able to keep up with osteoclastic resorption.

In OVX control, TrACP increased significantly in two months. *L. glutinosa* bark powder prevented this rise in a dose dependent manner indicating the plants osteoclast inhibiting property. OVX causes a significant increase in the serum ALP levels, which remains high for all 8 weeks. As the plant treatment is reducing TrACP, there is parallel decrease observed in the serum ALP levels also in a dose dependent manner. This parallel fall in both the markers clearly suggest that this plant ameliorates the damage caused by estrogen deficiency. To confirm that the elevation in serum ALP levels is because of bone remodeling only, we had estimated liver ALP levels which is the second important source of serum ALP, showed no significant variation between the OVX group and control group.

Observable histological alterations were not marked in uterus indicating that this plant is not having any estrogen potential. Histology of the bone clearly indicated dose dependent osteoprotective effect where 2 % treatment started improving the histological features and 5% treatment almost regained the conditions similar to that of the control. The results were in concomittance with that reported previously [14,16]. The present study established that *L. glutinosa* bark powder prevents bone loss caused by estrogen deficiency, without affecting the uterus. Our results suggest that *L. glutinosa* is having a bone protecting effect without exhibiting substantial effects on the uterus in OVX rats and not having any side effects on the control animals. This plant is having positive effect on bone remodeling and intake of this plant may be useful in preventing bone loss caused by estrogen deficiency. Further, pharmacolocical and phytochemical analysis will help us in understanding the exact mechanistic pathway.

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Abbreviations used: ALP= Alkaline Phosphatase, OVX= Ovariectomy, TrACP= Tartarate Resistant Acid Phosphatase, RANK = Receptor Activated Nuclear factor Kappa, RANK L = RANK Ligand.

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