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# *Withania somnifera* improves bone calcification in calcium-deficient ovariectomized rats

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## Abstract

Osteoporosis, characterized by reduction in bone density, is a significant source of mortality among the elderly, particularly in oestrogen-deficient women. We studied the effect of *Withania somnifera* (WS) root extract (ethanolic), which contains oestrogen-like withanolides for anti-osteoporotic activity. Female Sprague–Dawley rats were either sham operated (n = 12) or ovariectomized (n = 12) and treated with WS/vehicle (65 mg kg$^{-1}$), orally for 16 weeks (n = 12). All rats were allowed free access to a calcium-deficient diet (0.04% Ca) and distilled water. At termination, urinary excretion of calcium (Ca) and phosphorus (P) and serum levels of Ca, P and alkaline phosphatase (ALP) were measured. Femur and tibia bones were processed for histological (histology), morphological (scanning electron microscopy, SEM), biomechanical strength (impact test) and mineral composition (ash) analysis. Ovariectomized (OVX) rats showed a significant increase in serum ALP levels and urinary Ca and P excretion. Histological findings revealed narrowed, and disappearance of, trabeculae with widened medullary spaces in the OVX group. Ash analysis showed a reduction in ash weight, percent ash, ash Ca, ash P and ash magnesium levels in the OVX group. Further, SEM examination revealed metaphyseal bone loss in femurs and impact test showed a reduction in biomechanical strength of tibias in OVX rats. WS treatment markedly prevented the above changes in OVX rats and thus may be a potential agent in the treatment of osteoporosis.

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## Introduction

Osteoporosis, characterized by reduction in bone mineral density, is a significant source of morbidity and mortality among the elderly, particularly in postmenopausal women (Melton et al 1993). The aetiology of human osteoporosis is multifactorial, including heredity, hormonal excesses or deficiency, dietary components and physical activity (Riggs & Melton 1986). Cessation of ovarian function and, more particularly, oestrogen deficiency as a consequence of menopause results in elevated bone turnover, an imbalance between bone formation and bone resorption resulting in net bone loss (Heaney et al 1978). Oestrogen replacement therapy (ERT) is approved for the prevention of bone loss in postmenopausal women and is effective in reducing the incidence of skeletal fracture (Turner et al 1994). However, the American Heart Association, in its recent guidelines for cardiovascular disease prevention in women, recommended a conservative approach to the use of oestrogen hormone therapy alone until further research is available (Mosca et al 2004). Newer oestrogen therapies, such as selective oestrogen receptor modulators or low dose oestrogens, are effective in treating osteoporosis and relatively free from side effects (Mosca et al 2004). Given the positive effects of lower dose oestrogen, it is plausible that the herbal drugs or nutritional supplements that contain substances that have less affinity for the oestrogen receptor than estradiol can reduce menopausal symptoms and, perhaps, benefit bone without the adverse effects associated with oestrogen replacement therapy (ERT) (Prestwood 2003). Further, continual uncertainty and lack of consensus regarding ERT has driven many women to seek alternative sources of oestrogen, particularly the herbal remedies (Oerter Klein et al 2003).

Herbal drugs act holistically to improve symptoms, slow disease progression, correct imbalance and adjust the immune system to restore health and quality of life. They also have a reputation of being both safe and efficacious. The purpose of herbal treatment in many disease states is not to replace the conventional medicines but to supplement the therapy since the combination of the two is believed to be a better choice. The booming sales figures of herbal drugs beyond wildest expectations is therefore not surprising (Grunwald 1995; Brevoort 1996).

*Withania somnifera* (WS) Dunal, popularly known as Ashwagandha (Fam. Solanaceace) in Ayurveda, the Indian system of traditional medicine, is widely used in the treatment of variety of ailments (Anon 2004). Anti-inflammatory, immunomodulatory, anti-tumour, antioxidant, cardioprotective, adaptogenic, anticoagulant, anti-rheumatic and anti-osteoarthritic activity are some of the important pharmacological properties of WS (Mishra et al 2000). It also appears to exert positive influence on the endocrine and central nervous systems (CNS) (Mishra et al 2000). WS, like oestrogens, possesses beneficial effects on the cardiovascular, CNS and endocrine systems (Mishra et al 2000; Tohda et al 2000). Further, presence of phytosterols and the low toxicity profile of WS (Mishra et al 2000) may be advantageous for its use in chronic illnesses such as osteoporosis. The active principles of WS are heterogeneous alkaloids that include cuscohygrine, anahygrine, tropine, pesudotropine and anaferine. The plant also contains steroidal lactones, withanolides and withaferin, which are oestrogenic compounds. It is possible that the oestrogenic nature of withanolides, similar to phytosterols, may result in anti-osteoporotic activity.

This study was designed to investigate the potential salutary effects of WS using a calcium-deficient ovariectomized rat model (Hodgkinson et al 1978; Kalu et al 1989). The rationale for combining ovariectomy with calcium deficiency was to accelerate bone loss and to create a condition of severe osteoporosis. The dose of WS used in this study was based on earlier studies (Mitra et al 2000; Prabhakara Reddy & Lakshmana 2003) and toxicity profiles (Mishra et al 2000).

## Material and Methods

### Preparation of *Withania somnifera* extract

The authenticated *Withania somnifera* roots (Dr R. Kannan, Botanist, R & D Centre, The Himalaya Drug Company) were procured from Himalaya Drug Company, ground and extracted with 50% ethanol in a soxhlet apparatus, dried and stored at room temperature in a desiccator until use. Further details regarding the chemical constituents of WS extract and its fingerprint analysis can be found on the Himalaya Drug Company's website (http://www. himalayahealthcare.com/products/ashvagandha).

### Animals, diets and study protocol

Thirty-six, three-month old virgin female Sprague–Dawley rats were purchased from the animal facility at National Institute of Mental Health and Neurosciences (NIMHANS, Bangalore) and maintained under constant laboratory conditions ($24 \pm 2^{\circ}C$, 12-h light–dark cycle) with free access to food and water. After three weeks of adaptation, all rats were sham operated or ovariectomized (OVX) under pentobarbital anaesthesia ($30\,mg\,kg^{-1}$). After surgery, all rats were allowed a week to recover. The animal experimentation ethics committee of the institute approved all the procedures.

The rats were divided into 3 groups as follows: sham-operated control rats ($n = 12$), ovariectomized rats receiving vehicle only (0.5% sodium carboxymethylcellulose, $n = 12$), OVX rats receiving WS extract suspension ($65\,mg\,kg^{-1}$, twice a day p.o., for 16 weeks, $n = 12$). All rats were provided with free access to a low calcium diet (0.04%) and distilled water. The diet consisted of 20% protein, 5% fat, 0.04% calcium and 0.4% phosphorus (w/w) (Reddy et al 2004). Body weights were measured weekly at the same time of the day throughout the treatment period. At the end of 16 weeks, urine was collected from overnight-fasted rats in individual metabolic cages. On the following day, all rats were killed under pentobarbital anaesthesia, their blood was collected and the separated serum was stored at $-80^{\circ}C$ until use. Left legs were separated from the right legs during the collection of bone samples. Femurs and tibias were carefully removed and cleared from the adhering muscle and fat tissues. One half of the right femurs were immediately fixed in 10% neutral buffered formalin (NBF) for histological examination and the remaining half were frozen until scanning electron microscopy (SEM) studies were performed. All the right tibias were preserved in 50% normal saline ethanol for impact test. Left femurs were autoclaved for 15 min at $110^{\circ}C$, cleaned of the remaining adhering tissues, dried and stored for ash analysis.

### Serum and urine analysis

Total calcium and inorganic phosphorus in serum and urine were determined by colorimetry using a commercially available kit (Sigma Aldrich) and serum alkaline phosphatase (ALP) activity was measured using Dialab diagnostic kit in an automatic analyser (Hitachi BM 704).

### Histological examination

The right femurs fixed in 10% NBF for 12 h at $4^{\circ}C$ were decalcified in 5% ethylenediaminetetra-acetic acid (EDTA, pH 7.4) for 7 days, embedded in paraffin and cut into longitudinal sections of $5\,\mu m$ thickness. The sections were stained with haematoxylin and eosin (H&E) (Bancroft 1980) for observation.

### Scanning electron microscopy (SEM) studies

The frozen right femurs were trimmed on the frontal plane to expose the growth plate using a rotating

diamond saw and placed in 5% sodium hypochlorite (commercial bleach) for 4 h. The bones were then dehydrated in ethanol and dried, mounted on stubs and coated with gold using a sputter coater (Miller & Bowman 1998). The samples were then examined using a JEOL JSM-840A SEM to determine the cancellous bone loss at the metaphysis.

### Ash weights and mineral content of bone

The left femurs were placed in tared silica crucibles, weighed, dried to constant weight at 110°C and ashed for 24 h at 650°C. The ash weights were determined and the samples were suitably diluted with de-ionized water to estimate calcium, phosphorus and magnesium by colorimetry using an automatic analyser (BM Hitachi).

### Biomechanical strength of tibia by impact test

The biomechanical strength of tibia was determined by using an impact test apparatus (Tinius Olsen, PA, USA) as described previously (Reddy Nagareddy & Lakshmana 2005). Briefly, each sample was placed in a specially designed sample holder and held firmly by means of two screws, one on each end of the sample. The pendulum of the impact test apparatus was dropped from a fixed angle, which had been determined in preliminary studies. Since tibias are irregular in shape, care was taken to ensure that the site of impact was at the midpoint. The impact sensor (detector) was placed in an appropriate position so as to pick up the data upon collision. The results of this test were analysed using specific software (Instron Dynatup, 930-I). Using the software, various parameters such as impact velocity, impact energy, maximum load, energy to maximum load, time to maximum load and total fracture energy were calculated.

### Statistical analysis

Results are expressed as mean with their standard error (s.e.m.). All data were analysed using GraphPad Prism software (Microsoft, San Diego, CA, USA). One-way analysis of variance was first performed to test for any significant difference among the groups. When significant, the Dunnet multiple comparison test was used to determine the specific differences between means. The level of significance was $P < 0.05$ for all statistical tests.

## Results

### Serum and urine analysis

The effect of WS on serum calcium, phosphorus and ALP and urine calcium and phosphorus is presented in Table 1. Serum calcium and phosphorus levels were similar in all groups. WS treatment did not affect these variables. OVX rats showed a significant increase in serum ALP levels compared with sham-operated controls. Treatment with WS prevented the elevation of serum ALP. Urinary excretion of calcium and phosphorus were also elevated in OVX rats; these increases were significantly reduced by WS treatment.

**Table 1** Effect of *Withania somnifera* (WS) extract on serum and urinary biochemical markers in calcium-deficient ovariectomized rats

| | Sham | OVX | OVX-WS |
|---|---|---|---|
| Serum | | | |
| Calcium ($mmol\,L^{-1}$) | $2.56 \pm 0.08$ | $2.36 \pm 0.06$ | $2.57 \pm 0.07$ |
| Phosphorus ($mmol\,L^{-1}$) | $1.61 \pm 0.06$ | $1.69 \pm 0.03$ | $1.60 \pm 0.07$ |
| ALP (IU) | $106.1 \pm 2.68$ | $151.4 \pm 3.51^{a}$ | $106.4 \pm 2.11^{b}$ |
| Urine | | | |
| Calcium (mg/24 h) | $0.45 \pm 0.01$ | $0.75 \pm 0.03^{a}$ | $0.57 \pm 0.013^{b}$ |
| Phosphorus (mg/24 h) | $4.31 \pm 0.19$ | $7.52 \pm 0.12^{a}$ | $5.18 \pm 0.31^{b}$ |

All value are expressed as mean ± s.e.m., n = 10. $^{a}P < 0.05$ compared with sham-operated (Sham) group; $^{b}P < 0.05$ compared with ovariectomized (OVX) group.

### Histological examination of decalcified femur bone

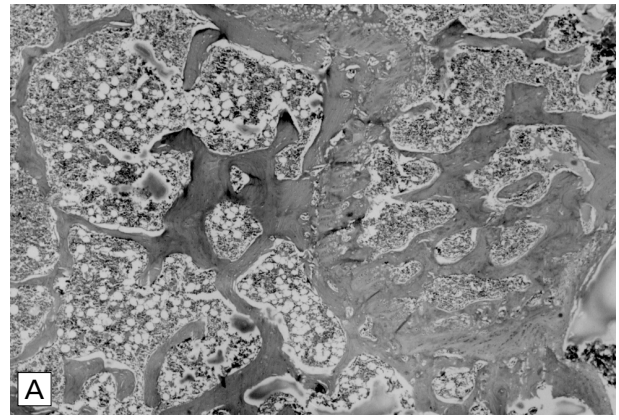
Histological sections of the distal third of femurs in the region proximal to the epiphyseal growth plate were examined for any histological changes. Observations revealed a normal compactness of diaphysis and competent trabeculae (Figure 1A) in all the rats of the sham group. However, the rats in the OVX group showed sparse, uniform thinning of trabeculae and widened inter-trabecular spaces (Figure 1B). Also found were the cartilaginous proliferates in the areas of softened plates of focal to restricted islets in OVX rats. Treatment with WS (Figure 1C) showed a minimum number of thin trabeculae and less frequent cartilaginous proliferation, demonstrating its beneficial effects on bone histology.

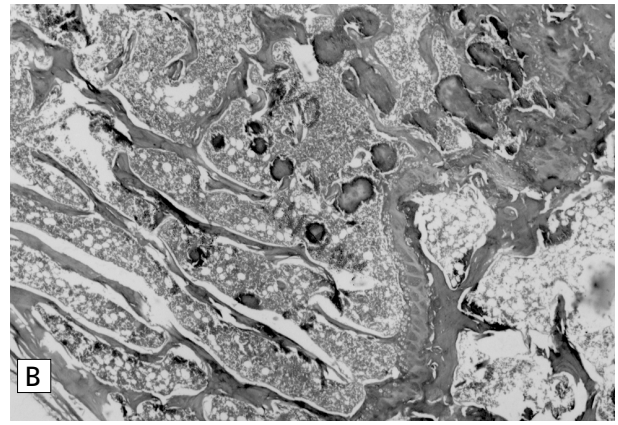
### Scanning electron microscopy studies

The scanning electron photomicrographs of frontal view of the metaphyseal regions of the distal femur from a sham, OVX and WS-treated rat are shown in Figure 2. These photomicrographs revealed that there was a significant reduction in the amount of the metaphyseal cancellous bone in the OVX group compared with the sham and WS-treated group. Photomicrographs of the OVX group femurs also indicated an erosive appearance, greater trabecular separation and significant changes in the intactness and integrity of the bone surface.

### Ash analysis

The results of ash analysis are presented in Table 2. A significant reduction in ash weight, percent ash, ash



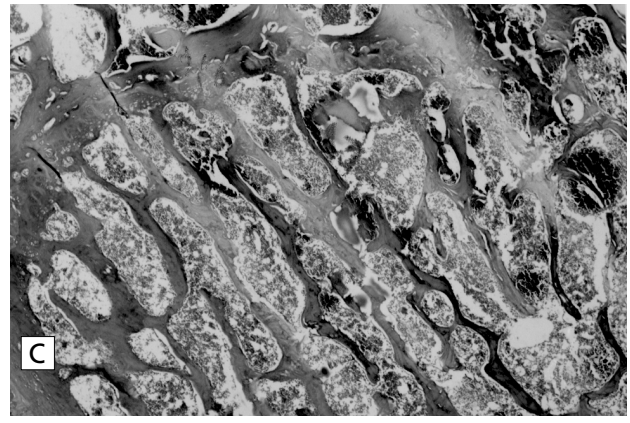
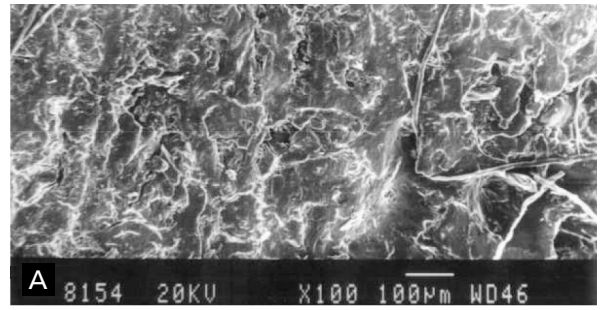
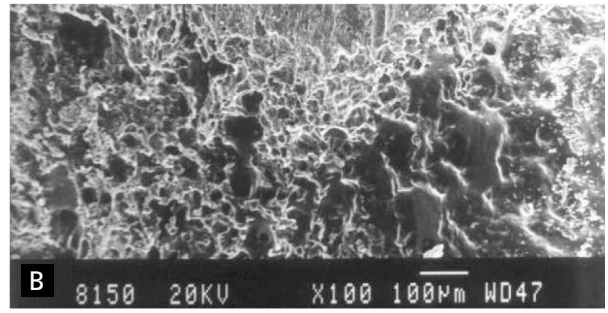




**Figure 1** Histological sections of the distal third of femurs of rats in the region proximal to the epiphyseal growth plate. A. Epiphyseal region showing normal compact trabeculae with inter-trabecular spaces in Sham-operated group (H&E, $\times 100$). B. Epiphyseal region showing sparse, thinning trabeculae with tendency for disappearance, loss of connectivity and widened inter-trabecular spaces in ovariectomized (OVX) group (H & E, $\times 100$). C. Epiphyseal region showing moderately thick elongated trabeculae and narrowed inter-trabecular spaces in ovariectomized group treated with *Withania somnifera* (WS) extract (OVX-WS group) (H & E, $\times$ 100).



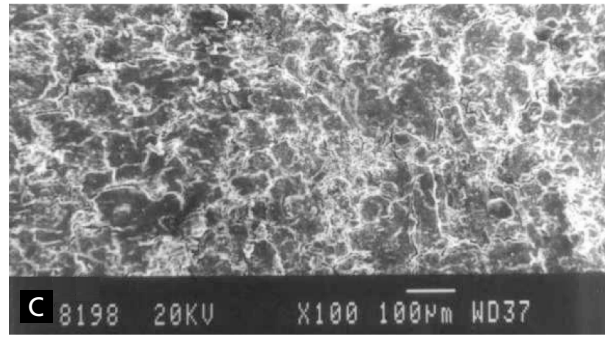




**Figure 2** Scanning electron photomicrographs ($\times 100$) of frontal view of the metaphyseal region of distal femur from a Sham-operated (A), ovariectomized (OVX) (B) and ovariectomized, *Withania somnifera* (WS)-treated (OVX-WS) rat (C).

calcium, ash phosphorus and ash magnesium was observed in OVX rats compared with sham controls. Treatment with WS markedly increased the ash weight and ash calcium and phosphorus levels but did not show any effect on ash magnesium levels.

### Biomechanical strength of tibia by impact test

The results of the impact test (Table 3) demonstrated that less energy was required to break the tibias of the OVX group compared with those of the sham group. Treatment with WS significantly increased the biomechanical strength of tibias as evident from the increased total fracture energy requirements. No significant difference in impact velocity, impact energy, maximum load, time to maximum load or deflection at maximum load were observed between the two groups, indicating that the applied force and collision parameters were constant between the groups.

**Table 2** Effect of *Withania somnifera* (WS) extract on ash parameters in femur bone of calcium-deficient ovariectomized rats

| Ash parameters | Sham | OVX | OVX-WS |
|---|---|---|---|
| Ash weight (g) | $0.29 \pm 0.034$ | $0.21 \pm 0.010^{a}$ | $0.28 \pm 0.051^{b}$ |
| Percent ash | $73.2 \pm 0.16$ | $65.4 \pm 0.33^{a}$ | $69.7 \pm 0.38^{b}$ |
| Calcium (mg) | $98.9 \pm 1.5$ | $75.2 \pm 0.6^{a}$ | $88.0 \pm 0.5^{b}$ |
| Phosphorus (mg) | $45.7 \pm 0.9$ | $35.5 \pm 0.5^{a}$ | $43.5 \pm 0.5^{b}$ |
| Magnesium (mg) | $2.2 \pm 0.05$ | $1.9 \pm 0.03^{a}$ | $1.8 \pm 0.03$ |

All value are expressed as mean $\pm$ s.e.m., n = 12. $^{a}P < 0.05$ compared with sham-operated (Sham) group; $^{b}P < 0.05$ compared with ovariectomized (OVX) group.

**Table 3** Effect of *Withania somnifera* (WS) extract on biomechanical strength of tibia of calcium-deficient ovariectomized rats, as determined by impact test

| | Sham | OVX | OVX-WS |
|---|---|---|---|
| Impact energy (Joule) | $65.2 \pm 0.24$ | $65.4 \pm 0.36$ | $67.08 \pm 0.64$ |
| Impact velocity ($m\,s^{-1}$) | $2.35 \pm 0.02$ | $2.39 \pm 0.07$ | $2.40 \pm 0.02$ |
| Maximum load (kN) | $0.048 \pm 0.005$ | $0.046 \pm 0.002$ | $0.045 \pm 0.002$ |
| Energy to max. load (Joule) | $0.092 \pm 0.007$ | $0.089 \pm 0.01$ | $0.094 \pm 0.007$ |
| Time to max. load (ms) | $1.28 \pm 0.013$ | $1.23 \pm 0.009$ | $1.31 \pm 0.017$ |
| Defl. at max. load (mm) | $2.54 \pm 0.18$ | $2.39 \pm 0.05$ | $2.60 \pm 0.22$ |
| Total energy (Joule) | $0.118 \pm 0.002$ | $0.071 \pm 0.0006^{a}$ | $0.114 \pm 0.004^{b}$ |

All value are expressed as mean $\pm$ s.e.m., n = 8. $^{a}P < 0.05$ compared with sham-operated (Sham) group; $^{b}P < 0.05$ compared with ovariectomized (OVX) group.

## Discussion

The purpose of this study was to evaluate the potential beneficial effects of an alcoholic extract of *Withania somnifera* (WS) root in osteoporosis caused by ovariectomy and concurrent calcium deficiency. Our findings suggest that OVX rats developed bone changes similar to those seen in oestrogen-deficient osteoporotic women. These changes are probably partly due to inadequate oestrogen levels exacerbated by calcium deficiency, a condition which may also be the cause of bone changes observed in women with postmenopausal osteoporosis (Riggs & Melton 1986). Treatment with WS root extract, which is known to contain oestrogen-like withanolides, particularly withaferin-A, significantly prevented net bone loss in OVX rats.

Biochemical markers of bone turnover reflect the underlying changes in bone histomorphometric parameters or bone mass (Riis 1991). The observation that serum calcium and phosphorus levels were similar in all groups indicates the significance of homoeostatic mechanisms in the maintenance of normal serum levels despite calcium deficiency and ovariectomy. The serum level of calcium depends on the stage and severity of menopause (oestrogen deficiency) and is regulated by many factors, including parathyroid hormone (PTH) and $1,25(OH)_2D$ (Iki et al 2004). Oestrogen deficiency is known to decrease $1,25(OH)_2D$, which in turn causes malabsorption of calcium from the intestine. A reduction in serum calcium level leads to increased secretion of PTH, resulting in rapid mobilization of calcium and phosphorus from bone in an attempt to normalize serum calcium levels. In the process, large amounts of calcium and other minerals are excreted in urine. Since the extent of urinary calcium excretion depends on its serum level, measurement of urinary calcium under fasting conditions may be a useful biochemical marker for determining the net bone loss. The results of our study suggest an increased urinary excretion of calcium and phosphorus in OVX rats. Treatment with WS significantly decreased urinary excretion of both calcium and phosphorus, indicating its anti-resorptive activity. The mechanism by which WS reduces urinary excretion of calcium and phosphorus is not clear and beyond the scope of this study. However, it is possible that the presence of a large number of withanolides, particularly withaferin A, an oestrogen-like compound, may have contributed to anti-resorptive activity (Mishra et al 2000).

Serum ALP is an important biochemical marker of bone formation secondary to increased bone turnover (Cosman et al 1996). The levels of this enzyme, particularly the bone-specific ALP, are increased in osteoporosis and other bone metabolic disorders (Victor 1993; Eriksen et al 2000). We observed a similar increase in total serum ALP levels in our OVX rats and treatment with WS restored the elevated levels of ALP, indicating its beneficial effects in reducing bone turnover.

Ovariectomy results in increased bone turnover and net bone loss with a permanent deficit of bone mass at several skeletal sites rich in cancellous bone, such as the proximal femur, vertebral bodies and the metaphyses of long bones (Ammann et al 1988). The microarchitectural alteration in cancellous bone network is very similar to that seen in postmenopausal osteoporosis, including thinning of trabecular elements (Wronski et al 1989). This phenomenon is reflected in our histological findings also as evident from the increased intertrabecular spaces. WS treatment markedly reduced the thinning of trabeculae and also showed a minimum number of such trabeculae. Further, SEM study showed a significant bone loss in the metaphyseal regions of the distal femur in OVX rats. Observation of photomicrographs suggested a decreased bone mass with a number of bone remodelling sites (erosion) in these regions. WS treatment appeared to maintain normal integrity, structure and compactness of the bone.

Calcium deficiency reduces femoral ash weight and ash mineral content, particularly calcium and phosphorus levels (Hodgkinson et al 1978; Donahue et al 1988).

Decreased ash weight and ash mineral content in OVX rats may be due to increased bone resorption, which is attributable to both oestrogen and calcium deficiency. Our results confirmed these findings in that OVX and concurrent calcium deficiency resulted in decreased ash weight, percent ash, ash calcium, phosphorus and magnesium. Treatment with WS prevented bone loss as evident from the preservation of normal levels of ash variables in the WS-treated group. Although we do not have any direct evidence to explain the precise mechanism by which WS prevented bone resorption, it can be speculated that WS, by virtue of its positive effects on the endocrine system, may have increased bone formation and reduced resorption of bone minerals into the systemic circulation and consequent excretion in the urine.

Several different tests have been used for evaluation of bone mechanical properties. Failure strength, which is defined as the maximum stress in a material under a given loading condition (that often coincides with rupture), is an important variable for investigation of any anti-osteoporotic agent (van der Meulen et al 2001). Resistance of bone to fracture or bone strength in turn depends on the amount of bone (density and mineralization) and its architectural spatial organization (Augat et al 1996; Sharp et al 2000). Further, linear correlation between bone density and impact strength has also been reported (Panteliou et al 1999). The results of the impact test indicate that tibias in the untreated OVX group require lower fracture energy than tibias in the sham-operated group. WS treatment significantly improved the tibial bone strength as evident from the increased requirements of fracture energy. The applied force and collision parameters did not differ between the groups, indicating that the samples were subjected to similar simulations, and that the observed differences in fracture energy are attributable to the inherent characteristics of the bone itself.

### Conclusions

In conclusion, WS treatment demonstrated many beneficial effects in ovariectomized rats. However, further investigations are required to elucidate the mechanisms by which WS prevented bone loss. In addition, phytochemical investigations to identify the active constituents and direct mechanistic studies to investigate the pharmacological actions of active constituents may be necessary to further exploit its therapeutic usefulness as an anti-osteoporotic agent.

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