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Methanol Extract of the Fruits of *Morinda citrifolia* Linn., Restores Bone Loss in Ovariectomized Rats

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Abstract: The objective of this study was to evaluate the effect of methanol extract of the fruits of *Morinda citrifolia* Linn., on osteoporosis induced by ovariectomy in female albino rats at two different dose levels of 500 and 750 mg/kg/day. Healthy female albino rats in the age group of 90 days were selected and randomized into five groups of six animals each. Group 1 was sham operated and served as control while all the remaining groups were ovariectomized. Group 2 was fed with an equivolume of saline and served as ovariectomized control. Group 3 was orally treated with standard Raloxifene (5.4 mg kg^{-1}) whereas the methanol extract of *Morinda citrifolia* (500 and 750 mg kg^{-1}) was administered to the groups 4 and 5. The findings assessed on the basis of biomechanical, biochemical and histopathological parameters, showed that the methanol extract significantly reduced bone loss, as evidenced by a reduction in Tartrate Resistant Acid Phosphatase (TRAP) and urine Hydroxyproline (Hp) levels while simultaneously increasing bone formation [high serum Alkaline Phosphatase (ALP) levels], thereby restoring bone mineralization. The restoration of bone strength was confirmed by biomechanical parameters viz., the three point bending of tibia, load testing of femoral head and compression of IV lumbar vertebra and it was further endorsed by histopathological findings i.e., bone microarchitecture. The extract significantly increased the osteoblastic activity on one hand while on the other it retarded the osteoclastic function thereby contributing to a positive bone balance and hence enhanced mineralization.

Key words: Osteoporosis, *Morinda citrifolia*, bone fragility, bone mineral density, tartrate resistant acid phosphatase (TRAP), alkaline phosphatase (ALP)

INTRODUCTION

Osteoporosis, a disease characterized by high bone fragility and increased risk of fractures with high mortality and morbidity rates, has become an expensive health menace worldwide (Meryl, 1997). Depletion of ovarian hormone following menopause is believed to be a major cause of brittle bones. Hormone Replacement Therapy (HRT), perhaps the most effective treatment, suffers the risk of breast cancer and cardiovascular diseases (Genant *et al*., 1989; Nand *et al*., 1999). A number of therapeutic agents based on various mechanisms of action, other than HRT have been developed for the management of osteoporosis e.g. antiresorptive agents [calcium and vitamin D (Jackson *et al*., 2006), bisphosphonates (Huq, 2007) including Selective Estrogen Receptor Modulators (SERMs) like Raloxifene (Delmas *et al*., 2005)] and bone forming agents [fluorides (Riggs *et al*., 1990, 2002) androgens (Christiansen and Riis, 1990), Parathyroid Hormone (PTH) (Lane *et al*., 1998) and phytoestrogens (Knight and Eden, 1996; Jaffery *et al*., 2006)]. The discovery of estrogenic activity of natural products (El-Halawany *et al*., 2010; Sreeja *et al*., 2010; Kim *et al*., 2010) and its scientific investigation have paved the way to a promising alternative mode of prophylaxis/treatment for osteoporosis. Many phytoconstituents have been designated as phytoestrogens and a number of plants containing these constituents have been identified and investigated in preclinical models for their estrogenicity (Cornwell *et al*., 2004; Hong *et al*., 2009).

Morinda citrifolia L. (Rubiaceae), popularly known as Noni in India, is a reputed medicinal plant used to treat a wide variety of ailments in Polynesia, South east Asia, Australia and the Caribbean (Krishnaiah *et al*., 2009).

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While applications have been reported for all parts of the plant, the leaves have been extensively used, mostly, in the form of juice. Interestingly, in contrast to the traditional use, the fruit juice has currently become popular and is widely used as a general tonic. Traditionally, the roots of *M. citrifolia* have been used as a cathartic and febrifuge internally and externally as a pain killer in gout (Kirtikar and Basu, 1995) the charred leaves with mustard have been used as an antidiarrheal in infants while its juice is used externally to relieve pain in gout. In Guinea the decoction of the roots is used as an emetic and laxative while an infusion of leaves is considered to be emollient, sedative, coolant and stomachic. In Indo-Chinese medicine, the baked fruits are given in dysentery and asthma (Kritikar and Basu, 1991). The root bark is also beneficial in hypertension and lumbago (Prajapati and Kumar, 2003). The fruits are also reported to be stomachic, anti-dysentric, anti-neuralgic etc. The plant has been reported to contain, anthraquinones in its heart wood (Balakrishna *et al*., 1961), sterols (β sitosterol, stigma sterol, etc.) and β carotene in its leaves (Ahmed and Bano, 1980; Aalbersberg *et al*., 1993; Takashima *et al*., 2007) along with flavonoids (rutin, quercetin, kaempferol), iridoids (asperuloside and asperulosidic acid) etc. in its fruits (Farine *et al*., 1996; Deng *et al*., 2007; Kamiya *et al*., 2005; Dalsgaard *et al*., 2006).

The plant has been studied for its antitumor (Furusawa *et al*., 2003), anti-inflammatory (Akihisa *et al*., 2007), analgesic (Younos *et al*., 1990), phytoestrogenic (Chearskul *et al*., 2004), wound healing (Nagori and Solanki, 2011), insulinotrophic (Hamid *et al*., 2008) and antioxidant activities (Chanda *et al*., 2011). The estrogenicity on specific sites, for instance ER (estrogenic receptor) α and ER β has been implicated in the mechanism of action of HRT (Zin *et al*., 2002). However, certain minor mechanisms of action have also been indicated in the treatment of osteoporosis. An interesting association of oxidative stress and osteoporosis has been revealed (Lean *et al*., 2003) wherein antioxidants have been shown to inhibit many inflammatory kinases which are active in osteoporosis. As the selected plant has potential antioxidant and anti-inflammatory activity along with estrogenicity, the present study is an attempt to evaluate the restorative effect of the methanol extract of the fruits of *M. citrifolia*.

MATERIALS AND METHODS

Materials

Plant material: The unripe fruits of the plant *Morinda citrifolia* Linn. were collected from Manipal, Karnataka, during the month of September 2008. The fruits were cut into thin slices and allowed to dry in the shade. Its botanical identity was confirmed by Dr. Gopalakrishna Bhat, Professor of Botany, Poorna Prajna College, Udupi, Karnataka. A voucher specimen (No. 5063) has been deposited at the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal.

Chemicals: Standard β-sitosterol and gallic acid were purchased from Sigma Aldrich, India. All the chemicals and reagents used were obtained from Ranbaxy Fine Chemicals Ltd. Punjab, Fischer Inorganics and Aromatics Ltd. Madras, NICE chemicals Ltd. Cochin and Central Drug House Pvt. Ltd. (CDH), New Delhi (India).

Animals: Swiss albino female rats in the age group of 90 days (170 - 200 g) were acclimatized to the experimental conditions of temperature 23±2°C, controlled humidity and a 12:12 h light and dark cycle. Animals were caged in polypropylene cages with a maximum of two animals per cage. The rats were fed with standard food pellets (Hindustan Lever Ltd., India) and water *ad libitum*. The study was conducted after obtaining animal ethical committee clearance from the Institutional Animal Ethics Committee of KMC, Manipal. No. IAEC/KMC/07/2007-2008.

Preparation of extract and standardization: The shade dried coarse fruit powder (500 g) was extracted with methanol in a soxhlet apparatus for 6 hours. The resultant extract was evaporated under vacuum in a rotary evaporator to a dry mass (yield 94.66 g). The dried solvent free extract was stored in an air-tight labeled container until further use.

Marker based standardization: Estimation of β-sitosterol and gallic acid in methanol extract of fruits of *M. citrifolia* The methanol extract of *M. citirifolia* was standardized using β-sitosterol and gallic acid as standards, by HPTLC.

Preparation of standard solution: The stock solutions of β–sitosterol and gallic acid were prepared by dissolving 10 mg of accurately weighed standards in 5 mL methanol in two separate 10 mL volumetric flasks. The flasks were sonicated for 10 min. and the final volume of the solutions was made up to 10 mL with methanol to get stock solutions containing 1 mg mL^{-1}.

Sample preparation: 100 mg of methanolic extract of *M. citrifolia* Linn., was accurately weighed, dissolved in 5 mL methanol and transferred to a 10 mL volumetric flask.

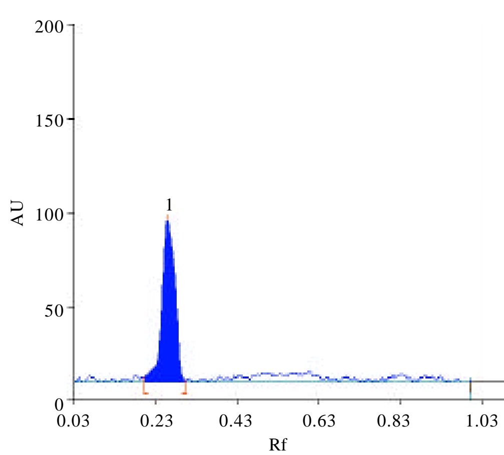

Fig. 1: Chromatogram of standard β-sitosterol

The flask was sonicated for 10 min and the content was filtered through 0.22 μ polyvinylidene fluoride membrane (Millipore, Ireland). The final volume was made up to the mark with m ethanol. This solution (10 mg mL^{-1}) was further used for HPTLC estimation.

HPTLC estimation: HPTLC was performed on 10×10 cm aluminum backed plates coated with silica gel 60 F_{254} (E. Merck, Germany). Standard and sample solutions (2 μL each) were applied on the same chromatographic plate in the form of 6 mm wide bands using Camag Linomat V sample applicator (Muttenz, Switzerland) equipped with a 100 μL Hamilton syringe (USA). Ascending chromatographic development was performed at room temperature (28±2°C), in Camag twin-trough chamber which was previously saturated with mobile phase for 20 min.

Standard β-sitosterol and methanolic extract was eluted in benzene: Ethyl acetate (9.5: 0.5 v/v) for estimation of β-sitosterol. The plate was dried after development and sprayed with 10% methanolic sulphuric acid, followed by heating at 110°C. Scanning was done at 550 nm with Camag TLC Scanner-III (Muttenz, Switzerland) (Fig. 1, 2).

For the estimation of gallic acid, toluene ethyl acetate Formic acid: Methanol (3:3:0.8:0.2 v/v/v/v) was used as a mobile phase. After development, the plate was dried and scanned at 280 nm with Camag TLC Scanner-III (Fig. 3, 4) (Jeganathan and Kannan, 2008).

The amount of phytoconstituents were calculated by the formula:

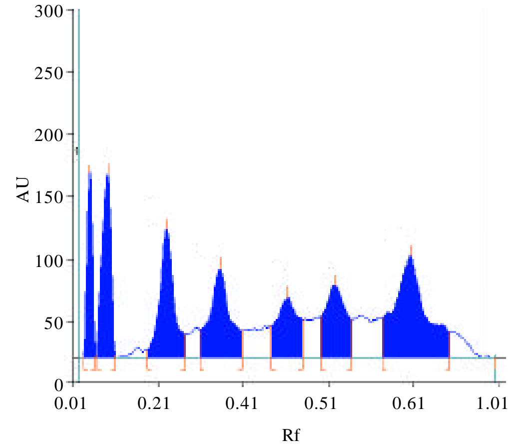

Fig. 2: Chromatogram of β-sitosterol in total methanolic extract

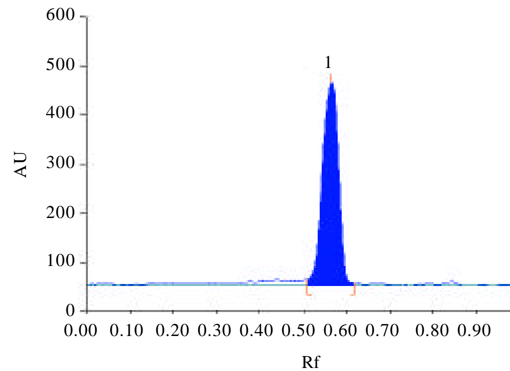

Fig. 3: Chromatogram of standard gallic acid

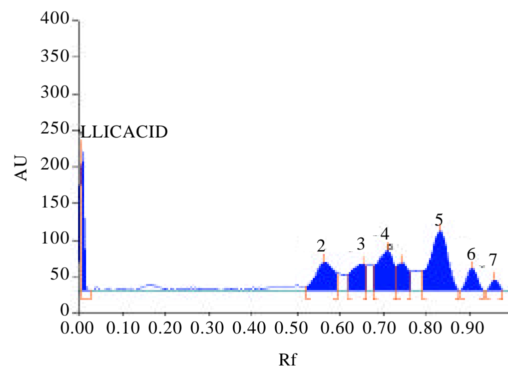

Fig. 4: Chromatogram of gallic acid in total methanolic extract

$$(\text{AUC of standard} = \frac{[(\text{AUC of sample} \times \text{Conc. of std.}}{\text{Conc. of sample})]} \times \%\text{purity})$$

Acute toxicity (Adoption 425): Ten healthy female albino rats were divided into two groups of equal size. Animals

of both the groups were fasted overnight before the test. The first group was administered 5000 mg kg^{-1} body weight of freshly prepared methanol extract of *Morinda citrifolia* suspended in 2% acacia while the other group was given an equivolume of 2% acacia solution. The animals were observed immediately and then after 30 min, 1, 2, 4, 6 h and thereafter daily for 14 days for behavioral, neurological and autonomical changes. At the end of the fourteenth day the animals were sacrificed with euthanasia and dissected for examination of vital organs.

Antiosteoporotic activity: Experimental animals were divided randomly into five groups of six animals each. Group 1 was sham operated and served as basal control. All the other groups were ovariectomized and received treatment for 3 months beginning from the fifteenth day after ovariectomy. Bilateral ovariectomy was carried out according to Waynforth (Lean *et al*., 2003) and the success of ovariectomy was confirmed by uterine atrophy. Group 2 received vehicle and served as ovariectomized control. Group 3 was orally administered Raloxifene, 5.4 mg kg^{-1} (Dr. Reddy's Laboratory, Hyderabad, India). Groups 4 and 5 were orally fed with suspension (2% acacia) of methanol extract of *M. citrifolia* at two different doses levels of 500 and 750 mg kg^{-1} body weight, respectively. At the end of the treatment blood and urine samples from all the groups were withdrawn by tail vein method to assess biochemical parameters. The animals were then sacrificed using sodium pentathionate and bones were isolated for biomechanical and histopathological studies.

Evaluation parameters: Osteoporosis was assessed with the help of various specific and non specific markers including biochemical and biomechanical parameters. Histopathological changes in the bone architecture were also studied.

Biochemical markers: Serum Alkaline Phosphatase (ALP), Calcium (Ca) and Inorganic Phosphorus (iP) were estimated with the help Cobas C111 autoanalyzer using diagnostic kits (Roche, Dignostics, Germany). Tartarate Resistant Acid Phophatase (TRAP) was estimated by King's (Waynforth, 1988) method using diagnostic kit from Span diagnostics (Surat, India). Hydroxylproline (Hp) in urine was estimated by modified Neumann Logan (King and Jegatheesan, 1959) method, in which hydroxyproline was treated with $CuSO_4$ and H_2O_2 in an alkaline solution, resulting in the formation of Δ pyrroline-4-carboxylic acid which upon acidification was converted to pyrrole-2-carboxylic acid. The latter condensed with p-dimethylaminobenzaldehyde (Ehrlich's reagent) to give a coloured complex which was measured at 540 nm.

Biomechanical markers: The freshly isolated bones (femur, tibia and fourth lumbar vertebra) were assessed for the loading test of the femoral neck, three-point bending of tibia (Neuman and Logan, 1950) and the compression test of fourth lumbar vertebra (Peng *et al*., 1994). A suitably modified tablet hardness tester (Cadmach, Ahmedabad, India) was employed to estimate the said parameters.

Histopathological evaluation: Microtome (Leica RM 2245, Germany) sections of decalcified femurs, fixed in 10% neutral buffered formalin and embedded in paraffin wax were stained with haematoxylin and eosin and mounted and observed for microarchitectural changes.

Statistical analysis: The data was analyzed using One Way Analysis ff Variance (ANOVA) followed by Post Hoc Tukey's test using SPSS computer software version 11.5. Level of significance was fixed at 0.05.

RESULTS

HPTLC analysis: The percentage content of β-sitosterol and gallic acid in the total methanolic extract of *Morinda citrifolia* was found to be 2.87 and 1.006%, respectively (Table 1, 2).

Acute toxicity study: Oral administration of the methanol extract of *M. citrifolia* at a dose level of 5000 mg kg^{-1} showed neither mortality nor any signs of clinical abnormality (behavioral, neurological or autonomical) even up to 14 days. At necropsy no gross pathological observations were made in target organs and hence the extract was considered as safe and its LD_{50} was determined to be >5000 mg kg^{-1} body weight.

Table 1: Estimation of β-sitosterol in total methanolic extract of *M. citrifolia*

Fig. No.	Sample	R_f	AUC	% content
1	β-sitosterol (standard)	0.23	9229.6	98
2	β-sitosterol in total methanolic extract	0.23	2706.3	2.87

Table 2: Estimation of gallic acid in total methanolic extract of *M. citrifolia*

Fig. No.	Sample	R_f	AUC	% content
3	Gallic acid (standard)	0.56	12576.4	98
4	Gallic acid in total methanolic extract	0.56	1291.5	1.006

Table 3: Effect of methanol extract of *M. citrifolia* on alkaline phosphatase, tartarate resistant acid phosphatase, serum calcium and inorganic phosphorous and urine hydroxyproline

Group No.	Groups (n = 6)	Dose (mg kg^{-1})	ALP (U L^{-1})	TRAP (KA°)	Calcium (mg dL^{-1})	Phosphorous (mmol L^{-1})	Hydroxyproline (µg mL^{-1})
1	Sham	Vehicle	1.22±0.16	6.69±0.22	9.08±0.17	1.54±0.07	42.13±3.09
2	OVX (control)	Vehicle	2.67±0.34^{a}	12.95±1.03^{b}	9.98±0.08	1.32±0.17	99.40±3.07^{b}
3	Raloxifene (Std.)	5.4	2.22±0.08	8.22±0.39^{d}	9.47±0.19	1.99±0.13^{c}	47.67±5.89^{d}
4	Methanol ext.	500	2.64±0.32^{a}	9.92±0.13^{c}	9.53±0.06	1.59±0.04	52.45±7.40^{d}
5	Methanol ext.	750	3.28±0.37^{a}	8.80±0.70^{c}	9.16±0.17	1.33± 0.11	46.87±4.05^{d}

Results expressed as Mean±SE (n = 6), ^{a}p<0.05 vs. sham, ^{b}p<0.001 vs. sham, ^{c}p<0.05 vs. OVX. ,^{d}p<0.001 vs. OVX

Table 4: Effect of methanol extract of *M citrifolia* on the biomechanical markers

Group.No.	Groups (n = 6)	Dose (mg kg^{-1})	Three point bending of tibia (kg)	Femoral neck load testing (kg)	Compression of 4th lumber vertebra (kg)
1	Sham	Vehicle	8.27±0.17	3.65±0.39	16.85±0.62
2	OVX (Control)	Vehicle	2.42±0.31^{b}	1.25±0.17^{b}	7.62±0.17^{b}
3	Raloxifene (Std)	5.4	7.42±0.29^{d}	2.52±0.20^{c}	15.85±0.71^{c}
4	Methanol ext.	500	4.95±0.69^{c}	2.47±0.21^{c}	13.72±0.88^{c}
5	Methanol ext.	750	6.00±0.78^{d}	2.52±0.25^{c}	15.70±0.90^{c}

Results expressed as Mean±SE (n = 6), ^{a}p<0.05 vs. sham, ^{b}p<0.001 vs. sham, ^{c}p<0.05 vs. OVX, ^{d}p<0.001 vs. OVX

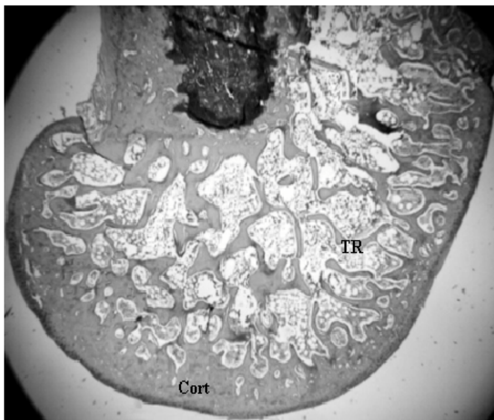

Fig. 5: Photomicrograph of femur section of group 1 (sham group) showing normal, dense and uniform trabaculae (H and E, 50x) (TR: Trabecular, Cort: Cortical Bone)

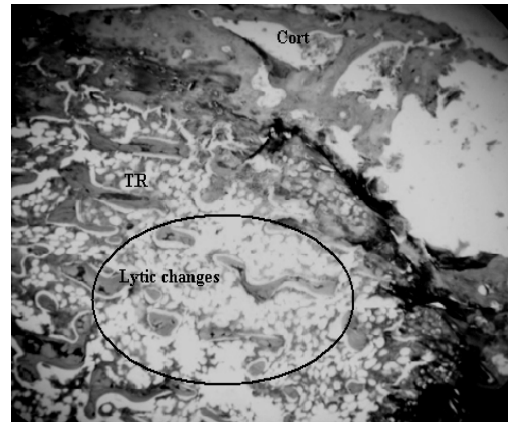

Fig. 6: Photomicrograph of femur section of group 2 (ovariectomized control group) showing disruptive and lytic changes (H and E, 100x) (TR: Trabecular, Cort: Cortical bone)

Biochemical parameters: The study showed no significant variation in the Ca levels of any of the groups (Table 3). The iP which fell marginally in the ovariectomized group (2), showed a significant rise in the standard treated group (Table 3). The recovery recorded in the extract treated groups 4 and 5 however were statistically insignificant. In group 2, a significant elevation was observed in the serum levels of ALP, TRAP and in the Hp (Table 3) as compared to the normal control (p< 0.05). The ALP levels in the extract treated groups (4 and 5) remained significantly elevated (on par with group 2). Though a marginal reduction was observed in the group 3, it was statistically insignificant. A significant reduction in the elevated levels of TRAP and Hp was observed in all the treated groups (p<0.001 and 0.05, respectively).

Biomechanical parameters: The biomechanical tests (Table 4) viz. load bearing on femoral neck, three point bending of tibia and compression of IV lumbar vertebra showed a significant loss in strength in the ovariectomized group as compared to the sham (p<0.001). The treated groups showed a clear and significant recovery in the bone strength (p<0.001 and 0.05) in all the parameters studied.

Histopathology: Histopathological studies of the femora of group 2 showed a marked disruption of bone microarchitecture in the trabecular as well as a visible loss in the cortical bone (Fig. 6). All the treated groups showed a significant reversal and make over of the loss both in trabecular as well as cortical bone (Fig. 7-9) as compared to sham operated group (Fig. 5).

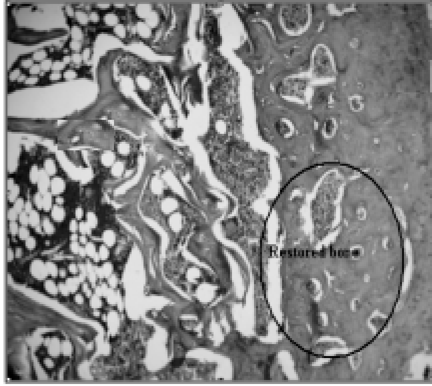

Fig. 7: Photomicrograph of femur section of group 3 showing dense cortical portion (H and E, 100x). (standard group) (TR: Trabecular, Cort: Cortical Bone)

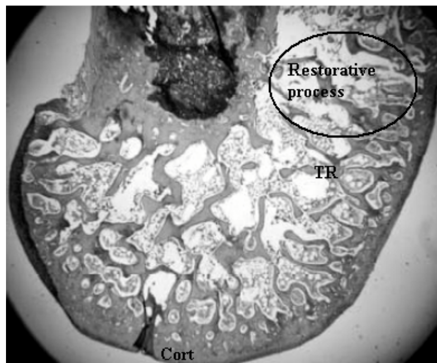

Fig. 8: Photomicrograph of femur section of group 4 (MC 500 mg kg^{-1}) showing progressive restoration (H and E, 50x) (TR: Trabecular, Cort: Cortical Bone)

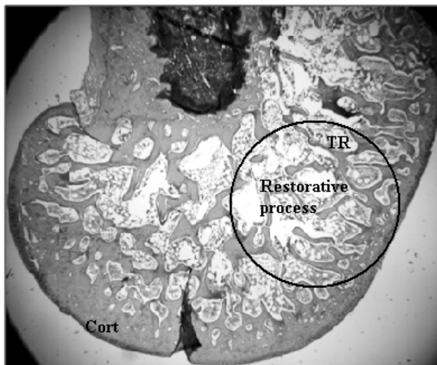

Fig. 9: Photomicrograph of femur section of group 4 (MC 750 mg kg^{-1}) showing restoration of bone (H and E, 50x) (TR: Trabecular, Cort: Cortical Bone)

DISCUSSION

The methanol extract of the fruits of *M. citrifolia* was selected for the study as it is rich in antioxidant flavonoids like kaempferol, quercetin, rutin and in iridoids with anti-inflammatory and anti-nociceptive potential (Farine *et al*., 1996; Deng *et al*., 2007; Kamiya *et al*., 2005; Dalsgaard *et al*., 2006). The fruits have been reported to have weak estrogenic (Chearskul *et al*., 2004) activity similar to selective estrogenic receptor modulators. β-sitosterol has been shown to have estrogenic properties (Ju *et al*., 2004) and hence it was estimated by HPTLC. Antioxidants have shown to inhibit certain inflammatory kinases involved in osteoclastogenesis and hence gallic acid (Li *et al*., 2005), the established antioxidant was estimated as standard for the extract. It is also reported to contain rutin (Yang *et al*., 2008) which also has a good antioxidant potential. The extract when tested for its toxicity was found to be safe even at a dose of 5000 mg kg^{-1} body weight. Hence one-tenth of the safe dose was selected as the lower therapeutic dose in this study. Healthy female albino rats of wistar strain were selected for the study due to ease in handling and availability. The ovariectomized rat model for osteoporosis, a most commonly used preclinical model to simulate postmenopausal osteoporosis (Hartke, 1999) in women, was adopted to evaluate antiosteoporotic activity.

In the present study following ovariectomy, an elevation was observed in the levels of ALP, TRAP and Hp thereby suggesting an increase in bone turnover in accordance with the earlier study by Shirwaikar *et al*. (2003). The iP and Ca levels however failed to show any statistically significant changes. In the extract treated groups the levels of iP and Ca showed no statistically significant changes implying normal calcium and mineral homeostasis (Shoback *et al*., 1993) and a well regulated hormonal (PTH and calcitonin) secretion. Marcus (1994) discussed the importance of bone and serum TRAP levels in osteoclastic function and suggested that increased ALP levels can be viewed as a marker of higher osteoblastic activity, in agreement with that study, the present study showed a dose dependant reduction in both TRAP and Hp levels suggesting a significant reduction in osteoclastogenesis. Tauchert *et al*. (2009) further emphasized the effect of elevated serum TRAP levels in higher resorption. Higher osteoclastic activity can also be evidenced by increased serum and urine Hp according to Calvo *et al*. (1996). Though ALP is a non-specific marker of bone formation, elevated levels of the enzyme in extract-fed groups (4 and 5) under the present experimental

conditions signify the formation of new bone. Phytoestrogens are generally considered as bone forming agents however, in the present study the extract also showed a dose dependant anti-resorptive potential by suppressing the elevated TRAP and urine Hp levels. In comparison to raloxifene treated group (group 3), treatment with the higher dose of *M. citrifolia* extract (group 5) demonstrated significant osteoblastic and a similar antiosteoclastic activity. This signifies restoration of mineralization and hence antiosteoporotic activity.

High bone turnover in the absence of estrogen creates a negative bone balance, consequently leading to a loss in bone strength (Ezzat *et al*., 2007). A good antiosteoporotic drug must change the negative bone balance to a positive bone balance i.e., an eventual gain in bone strength (Hartke, 1999). The bone strength, a direct measure of osteoporosis (Katsumata *et al*., 1995) when estimated by three point bending, load testing of femoral head and compression of IV lumbar vertebra (Neuman and Logan, 1950; Peng *et al*., 1994), showed a sharp decrease in the bone strength of the ovariectomized group. Following treatment with our extract and raloxifene, higher bone strength almost equal to sham was observed in all the groups thereby suggesting mineralization or otherwise positive bone balance which support the claims made in earlier studies.

Histopathological findings of the ovariectomized group also illustrated a clear bone loss, both in trabecular and cortical areas. The treated groups showed a significant reversal of the bone loss and almost a complete restoration of bone architecture. The histopathological findings therefore endorse the biomechanical and biochemical results.

CONCLUSION

The methanol extract of the fruits of *M. citrifolia* was studied for its possible antiosteoporotic activity for the first time. It was observed that the extract significantly accelerates the bone formation on one hand while on the other it potentially reduces the bone resorption. Further studies are warranted to predict the mechanisms of action involved in the antiosteoporotic activity of this potent drug.

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REFERENCES

Aalbersberg, W.G.L., S. Hussein, S. Sotheeswaran and S. Parkinson, 1993. Carotenoids in the leaves of *Morinda citrifolia*. J. Herbs Spices Med. Plants, 2: 51-54.

Ahmed, V.U. and M. Bano, 1980. Isolation of β-sitosterol and ursolic acid from *Morinda citrifolia* Linn. J. Chem. Soc. Pak., 2: 71-72.

Akihisa, T., K. Matsumoto, H. Tokuda, K. Yasukawa and K.I. Seino *et al*., 2007. Anti-inflammatory and potential cancer chemopreventive constituents of the fruits of *Morinda citrifolia* (Noni). J. Nat. Prod., 70: 754-757.

Balakrishna, S., T.R. Seshadri and B. Venkataramani, 1961. Special chemical components of commercial woods and related plant materials. Part X. Heartwood of *Morinda citrifolia* Linn. J. Sci. Ind. Res., 20: 331-333.

Calvo, M.S., D.R. Eyre and C.M. Gundberg, 1996. Molecular basis and clinical application of biological markers of bone turnover. Endocr Rev., 17: 333-368.

Chanda, S., R. Dave and M. Kaneria, 2011. *In vitro* antioxidant property of some Indian medicinal plants. Res. J. Med. Plant, 5: 169-179.

Chearskul, S., S. Kooptiwut, S. Chatchawalvanit, S. Onreabroi, M. Churintrapun, P. Saralamp and N. Soonthornchareonnon, 2004. *Morinda citrifolia* has very weak estrogenic activity *in vivo*. Thai. J. Physiol. Sci., 17: 22-29.

Christiansen, C. and B.J. Riis, 1990. 17β-estradiol and continuous norethisterone: A unique treatment for established osteoporosis in elderly women. J. Clin. Endocrinol. Metab., 71: 836-841.

Cornwell, T., W. Cohick and I. Raskin, 2004. Dietary phytoestrogens and health. Phytochemistry, 65: 995-1016.

Dalsgaard, P.W., O. Potterat, F. Dieterle, T. Paululat, T. Kuhn and M. Hamburger, 2006. Noniosides E-H, New trisaccharide fatty acid esters from the fruit of *Morinda citrifolia* (Noni). Planta Med., 72: 1322-1327.

Delmas, P.D., R. Rizzoli, C. Cooper and J.Y. Reginster, 2005. Treatment of patients with postmenopausal osteoporosis is worthwhile. The position of the International Osteoporosis Foundation. Osteoporosis Int., 16: 1-5.

Deng, S., A.K. Palu, B.J. West, C.X. Su, B.N. Zhou and J.C. Jensen, 2007. Lipoxygenase inhibitory constituents of the fruits of Noni (*Morinda citrifolia*) collected in Tahiti. J. Nat. Prod., 70: 859-862.

El-Halawany, A.M., M.H. Chung, H.M. Abdallah, T. Nishihara, M. Hattori, 2010. Estrogenic activity of a naringinase-treated extract of *Sophora japonica* cultivated in Egypt. Pharm. Biol., 48: 177-181.

Ezzat, W.M., H.M. Raslan, A. Elsherbiny, M. Mokhles, M.M. Ahmed, N. Abd El-Ghaffar and E.A. Rasheed, 2007. Bone mineral density and bone turnover markers in chronic liver disease. J. Med. Sci., 7: 748-754.

Farine, J.P., L. Legal, B. Moreteau and J.L.L. Quere, 1996. Volatile compounds of ripe fruits of *Morinda citrifolia* and their effects on *Drosophila*. Phytochemistry, 41: 433-438.

Furusawa, E., A. Hirazumi, S. Story and J. Jensen, 2003. Antitumor potential of a polysaccharide-rich substance from the ripe fruit juice of *Morinda citrifolia* (Noni) on sarcoma 180 ascites tumor in mice. Phytother. Res., 17: 1158-1164.

Genant, H.K., D.J. Baylink and J.C. Gallagher, 1989. Estrogens in the prevention of osteoporosis in postmenopausal women. Am. J. Obset. Gynecol., 161: 1842-1846.

Hamid, M., S.P.M. Bohari, M.S. Bastami, A.M. Ali, N.M. Mustapha and K. Shari, 2008. Evaluation of the insulinotrophic activity of Malaysian traditional plants extract. J. Boil. Sci., 8: 201-204.

Hartke, J.R., 1999. Preclinical development of agents for treatment of osteoporosis. Toxicol Pathol., 27: 143-147.

Hong, G.E., P.K. Mandal, C.W. Pyun, K. Choi and S.K. Kim *et al*., 2009. Isoflavone aglycone from fermented soy pulp prevents osteoporosis in ovariectomized rats. Asian J. Anim. Vet. Adv., 4: 288-296.

Huq, F., 2007. A molecular modelling analysis of toxicity of fosamax and risedronate. J. Pharmacol. Toxicol., 2: 489-495.

Jackson, R.D., A.Z. La Croix, M. Gass, R.B. Wallace and J. Robbins *et al*., 2006. Calcium plus vitamin D supplementation and the risk of fractures. N. Engl. J. Med., 354: 669-683.

Jaffery, F., A. Ghannadi and H. Najafzadeh, 2006. Evaluation of the prophylactic effect of fennel essential oil on experimental osteoporosis model in rats. Int. J. Pharmacol., 2: 588-592.

Jeganathan, N.S. and K. Kannan, 2008. HPTLC Method for Estimation of Ellagic Acid and Gallic Acid in *Triphala churanam* Formulations. Res. J. Phytochem., 2: 1-9.

Ju, Y.H., L.M. Clausen, K.F. Allred, A.L. Almada and W.G. Helferich, 2004. β-sitosterol, β-sitosterol glucoside and a mixture of β-sitosterol and β-sitosterol glucoside modulate the growth of estrogen-responsive breast cancer cells *in vitro* and in ovariectomized athymic mice. J. Nutr., 134: 1145-1151.

Kamiya, K., Y. Tanaka, H. Endang, M. Umar and T. Satake, 2005. New anthraquinone and iridoid from the fruits of *Morinda citrifolia*. Chem. Pharm. Bull., 53: 1597-1599.

Katsumata, T., T. Nakamura, H. Ohnishi and T. Sakurama, 1995. Intermittent cyclical etidronate treatment maintains the mass, structure and the mechanical property of bone in ovariectomized rats. J. Bone Miner Res., 10: 921-931.

Kim, H.J., H.J. Suh, J.H. Kim, S.C. Kang, S. Park, C.H. Lee and J.S. Kim, 2010. Estrogenic activity of glyceollins isolated from soybean elicited with *Aspergillus sojae*. J. Med. Food., 13: 382-390.

King, E.J. and K.A. Jegatheesan, 1959. A method for the determination of tartrate labile prostatic acid phosphatase in serum. J. Clin. Path., 12: 85-89.

Kirtikar, K.R. and B.D. Basu, 1995. Indian medicinal plants. Int. Book Distribution Dehradun Ind., 4: 758-793.

Knight, D.C. and J.A. Eden, 1996. A review of the clinical effects of phytoestrogens. Obstet. Gynecol., 87: 897-904.

Krishnaiah, D., R. Sarbatly, A.M.M. Hafiz, A.B. Hafeza and S.R.M. Rao, 2009. Study on retention of bioactive components of *Morinda citrifolia* L. Using spray-drying. J. Applied Sci., 9: 3092-3097.

Kritikar, K.R. and B.A. Basu, 1991. Indian Medicinal Plants. 2nd Edn., Periodical Experts Book Agency, New Delhi, India, pp: 856-860.

Lane, N.E., S. Sanchez, G.W. Modin, H.K. Genant, E. Pierini and C.D. Arnaud, 1998. Parathyroid hormone can reverse corticosteroid induced osteoporosis. Results of a randomized controlled clinical trial. J. Clin. Invest., 102: 1627-1633.

Lean, J.M., J.T. Davies, K. Fuller, C.J. Jagger and B. Kirstein *et al*., 2003. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. J. Clin. Invest., 112: 915-923.

Li, L., T.B. Ng, W. Gao, W. Li and M. Fu *et al*., 2005. Antioxidant activity of gallic acid from rose flowers in senescence accelerated mice. Life Sci., 77: 230-240.

Marcus, R., 1994. The Nature of Osteoporosis. In: Osteoporosis, Marcus, R., D. Feldman and J. Kelsey (Eds.). Academic Press, San Diego, California.

Meryl, S.L., 1997. Metabolic Bone Diseases. In: Textbook of Rheumatology, Harris, K. and R. Sludge (Eds.). 5th Edn., W.B. Saunders Co., Philadelphia, pp: 1563-1572.

Nagori, B.P. and R. Solanki, 2011. Role of medicinal plants in wound healing. Res. J. Med. Plant, 5: 392-405.

Nand, S.L., B.G. Wren, B.A. Gross and G.Z. Heller, 1999. Bone density effects of continuous estrone sulfate and varying doses of medroxyprogesterone acetate. Obstet. Gynecol., 93: 1009-1013.

Neuman, R.E. and M.A. Logan, 1950. The determination of hydroxyproline. J. Biol. Chem., 184: 299-306.

Peng, Z., J. Tuukkanen, H. Zhang, T. Jamsa and H.K. Vaananen, 1994. The mechanical strength of bone in different rat models of experimental osteoporosis. Bone, 15: 523-532.

Prajapati, N.D. and U. Kumar, 2003. Agro's Dictionary of Medicinal Plants. Agrobios, India.

Riggs, B.L., S.F. Hodgson, W.M. O'Fallon, E.Y.S. Chao and H.W. Wahner *et al*., 1990. Effect of fluoride treatment on the fracture rate in postmenopausal women with osteoporosis. New Eng. J. Med., 322: 802-809.

Riggs, B.L., S. Khosla and L.J. Melton, 2002. Sex steroids and the construction and conservation of the adult skeleton. Endocr. Rev., 23: 279-302.

Shirwaikar, A., S. Khan and S. Malini, 2003. Antiosteoporotic effect of ethanol extract of *Cissus quadrangularis* Linn. on ovariectomized rat. J. Ethnopharmacol., 89: 245-250.

Shoback, D., R. Marcus, D. Bikle and G. Strewler, 1993. Mineral Metabolism and Bone Disease. In: Basic and Chemical Endocrinology, Greenspan, F.S. and D.G. Gardner (Eds.). 6th Edn., Lang Medical Books/McGraw-Hill Medical Publishing, New York, USA., pp: 273-320.

Sreeja, S., V.S. Anju and S. Sreeja, 2010. *In vitro* estrogenic activities of fenugreek *Trigonella foenum* graecum seeds. Indian J. Med. Res., 131: 814-819.

Takashima, J., Y. Ikeda, K. Komiyama, M. Hayashi, A. Kishida and A. Ohsaki, 2007. New constituents from the leaves of *Morinda citrifolia*. Chem. Pharm. Bull., 55: 343-345.

Tauchert, S., A. di Liberto, T. Cordes, M. Thill, D. Salehin and M. Friedrich, 2009. Tartrate-resistant acid phosphatase (TRAP) as a serum marker for bone resorption in breast cancer patients with bone metastases. Clin. Exp. Obstet. Gynecol., 36: 219-225.

Waynforth, H.B., 1988. Experimental and Surgical Technique in the Rat. Academic Press, London, pp: 160-163.

Yang, J., J. Guo and J. Yuan, 2008. *In vitro* antioxidant properties of rutin. LWT- Food Sci. Technol., 41: 1060-1066.

Younos, C., A. Rolland, J. Fleurentin, M.C. Lanhers, R. Misslin and F. Mortier, 1990. Analgesic and behavioural effects of *Morinda citrifolia*. Planta Med., 56: 430-434.

Zin, Z.M., A. Abdul-Hamid and A. Osman, 2002. Antioxidant activity of extracts from Mengkudu (*Morinda citrifolia* L.) root, fruit and leaf. Food Chem., 78: 227-231.