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Research Article
Marantodes pumilum Leaves Promote Repair of Osteoporotic fracture In Postmenopausal Sprague-Dawley Rats

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Abstract

Background and Objective: *Marantodes pumilum var alata* (*MPva*), a herb product used in managing female reproductive health problems and postmenopausal symptoms, is known to protect the bone against osteoporosis. Despite convincing evidence of its anti-osteoporotic properties, its role on osteoporotic fracture is yet to be investigated. In this study, the effects of aqueous leaf extract of *MPva* on fracture repair were studied in ovariectomized rats. **Materials and Methods:** Thirty female Sprague-Dawley rats grouped (n = 6) into Sham-operated (Sham), Ovariectomized control (OVXC), Estrogen treatment (ERT) and leaf treatments (MPv20 and MPv100) groups were ovariectomized (except the Sham). Eight weeks post-ovariectomy, the right tibiae of rats were fractured and fixed with titanium plates. The ERT group received 64.5 μg kg^{-1} per day p.o. estrogen (Premarin®) while the MPv20 and MPv100 groups received 20 mg and 100 mg kg^{-1} per day p.o. doses of *MPva* leaf extract, respectively. After 8 weeks treatment, rats were humanely euthanized and their tibiae were excised for investigation of callus bone morphometry, mineralization and biomechanical strength. **Results:** Callus bone volume (BV_{callus}) and volume fraction (BV/TV_{callus}) were found to be higher ($p<0.05$) in MPv20 and MPv100 compared to OVXC. Similar to ERT and Sham, bone mechanical strength parameters, maximum stress and maximum force, were significantly higher in both MPv20 and MPv100 groups compared to OVXC. **Conclusion:** Similar to estrogen treatment and healthy control, treatment with aqueous leaves extract of *Mpva* promotes fracture healing in postmenopausal osteoporotic rats by enhancing the volume of bone callus formed at fracture site and its subsequent remodeling. In view of its safety profile, *Mpva* leaves could be a suitable complementary medicine in the management of osteoporotic fracture in postmenopausal condition.

Key words: Fracture-healing, osteoporosis, phytoestrogens, *Marantodes pumilum*, ovariectomized rats

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Osteoporosis is a systemic bone disease that is characterized by deterioration of bone micro-architecture and loss of bone density with a consequent loss of strength and increase in fracture risk[1]. In women older than 45, it accounts for more hospitalization than diabetes, myocardial infarction and breast cancer[2]. Mortality rates of up to 20-24% in the first year after fracture and greater risk of death, which may persist for at least 5 years have been reported[3]. Higher incidence of osteoporotic fracture has been reported in women than in their male counterparts[4,5]. One out of every 3 women and every 5 men above the age of 50 were reported to experience osteoporotic fractures[6]. In the year 2000, out of an estimated 9 million new osteoporotic fractures, 61% reportedly occurred in women[7]. In view of increasing population of the elderly, these figures are expected to surge. Compared to the rates in 1990, hip fracture incidence has been projected to increase by 310% in women by 2050[8].

Fracture repair processes in postmenopausal condition, through an unclear molecular signaling mechanism, are remarkably delayed or impaired[9]. Estrogen replacement therapy is logically a rational management approach for osteoporotic fracture in postmenopausal women. It has been reported to preserve bone mineral density (BMD) and reduce the risk of fracture[10]. In animal model, treatment with estrogen has resulted in decreased fracture healing time compared to controls possibly due to higher yield of bone calluses with larger chondrocyte areas, greater mineralization, increased trabecular and neocortical thickness[11]. Due to debilitating adverse effects associated with its use[12], estrogen replacement therapy has been replaced by other conventional anti-resorptive drugs such as selective estrogen receptor modulator (e.g., raloxifene) and bisphosphonates (e.g., alendronate). Although conventional antiresorptive agents have shown improvement in BMD and reduction in the risk of related fracture (vertebral by 50%, non-vertebral by 20-25% and hip fractures by 40%), little is known about their clinical role on fracture repair thus casting doubt on their safety[13-15]. In view of this limitation, an increasing number of osteoporotic patients have resorted to the use of supplements and natural medicines thus causing a surge in the demand for alternative or complementary medicines for the management of osteoporosis and its related complications[16].

Consequently, researches are being conducted on a wide range of agents with folkloric claims of potency such as virgin coconut oil, soy, blueberry, *Achyranthes bidentata* and *Marantodes pumilum* var. *Alata*[16]. Available evidence has shown that *Marantodes pumilum* var. alata (*Mpva*), a phytoestrogen-rich herb popularly used to manage female reproductive health problems, possess osteoprotective properties[17]. It has been shown to preserve bone density, micro-architecture, mechanical strength and bone turnover markers in estrogen-deficient rats[18-20]. A recent study has revealed that its leaves are more potent than its roots[21]. Phytochemical studies have revealed the presence of useful phenolic compounds with estrogen-like biologic activities such as β-carotene, quercetin, myricetin, kaempferol, catechin, gallic acid, ellagic acid and vanillic acid in *MPva*, which makes it more suitable as research candidate in menopause-related diseases[22-24]. Despite the huge anti-osteoporotic potential of *MPva*, no study has investigated its role on bone fracture repair. In this study, *MPva* leaf extract was investigated for its fracture healing effects in postmenopausal osteoporosis rat model using a combination of Micro-computed tomography (MCT) and mechanical strength test.

MATERIALS AND METHODS

Plant material: The leaves of *Marantodes pumilum* var. *alata* (Synonym: *Labisia pumila*), family myrsinaceae, was used in this study. Plant material were collected from a cultivated site, Delima Jelita Herbs in Kedah, Malaysia and identified by Professor Emeritus (Dr.) Abdul Latiff Mohamad of Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). Voucher specimen was deposited at UKM Herbarium (voucher number: UKM-HF131). Collected leaves and roots were then separately garbled, air-dried under shade, ground using rotary grinder and extracted in warm distilled water using reflux method for 2 h. Resultant extracts were then freeze-dried to obtain dry extracts that were stored at -20°C for subsequent experimental use.

Experimental animals: Healthy female Sprague-Dawley rats (UKM Laboratory Animal Research unit) weighing 250-300 g were used in this study. The US guide for the use and care of laboratory animal as contained in National Institutes of Health publication was strictly adhered to during animal handling[25]. Rats were housed pairwise in wood-shaving furnished plastic cages measuring 45×28×20 cm under room temperature of 25±2°C, natural day-night cycle and humidity. All rats were allowed free access to portable water and standard diet (Gold Coin, Selangor-Malaysia, containing 0.97% calcium, 0.85% phosphorus and 1.05 IU g^{-1} of Vitamin D3). Before commencement of study, all rats were acclimatized to the laboratory environment for 7 days. At the end of the study, all

rats were euthanized in accordance to AVMA guidelines on euthanasia using a combination of ketamine-xylazine mixture overdose and cervical dislocation[26].

Study design: Thirty healthy female Sprague-Dawley rats (4 months old, 250-300 g) were randomly divided into 5 groups (n = 6): Sham-operated (Sham), ovariectomized control (OVXC), estrogen treatment (ERT) and leaf extract treatments (MPv20 and MPv100). All rats, except sham-operated group, were ovariectomized. After ovariectomy, rats were left untreated for 8 weeks, a period recommended for development of osteoporosis[27]. The right tibiae of rats were then fractured using pulse ultrasound and fixed with titanium plates. After osteotomy, via oral route, the ERT group was treated with 64.5 μg kg^{-1} per day estrogen (Premarin®) while MPv20 and MPv100 groups were treated with 20 mg and 100 mg kg^{-1} per day doses of aqueous leaf extract of *MPva*, respectively. After 8 weeks of treatment, all animals were then humanely sacrificed and the fractured tibiae were excised and analyzed for changes in morphometry and mineralization of bone calluses using micro-CT (Skyscan 1076, G015619). After micro-CT, the tibiae were subjected to mechanical strength test using universal strength testing machine (Shimadzu AGS-X 500).

Surgical protocol: With an exception of the sham-operated group, ovariectomy was performed on all rats following previously described method[28]. Eight weeks post-ovariectomy, under anaesthesia (Ketamine: Xylazil, 8:1), the left tibiae of all rats were fractured as previously demonstrated[29]. Before surgery, the left hind limbs of rat were shaved of fur and sterilized with 70% alcohol. The skin was incised with a surgical scalpel (No. 11) in distal direction from the tuberositas tibia to expose the flexor and extensor muscles, which were carefully elevated from the tibia to expose two-thirds of the proximal tibial bone. A T-shaped titanium fixation plate XS (57-05140 Stryker Trauma, Selzach, Switzerland) was fixed to the anterior-medial surface of the tibia with two proximal and two distal screws (1.2 mm) leaving the mid central plate hole without screw (Fig. 1). Osteotomy was performed at the position of the mid central plate hole using pulsed ultrasound (Piezosurgery®, Mectron Medical Technology, Carasco, Italy) after temporarily removing the plate. The plate was then returned to its previous position to stabilize the fracture thus producing a fracture gap of 0.5 mm. After surgery, the animals were injected with 5 mg kg^{-1} enrofloxacin (Baytril®) intramuscularly every 12 h for 7 days to prevent infection and 0.1 mg kg^{-1} buprenorphine (0.324 mg mL^{-1}) subcutaneously daily for 3 days to control pain.

Fig. 1: Fractured tibia of rats showing fracture gap before and after fixation

Micro-CT assessment: Before commencement of MCT investigation, the fixation plates were carefully unscrewed and removed from bone samples without much distortion of the bone calluses. With slight modification, method previously demonstrated was adopted[30]. During Micro-CT assessment, the entire tibia was scanned with MCT X-ray scanner system (Skyscan 1076, G015619) at a scanning mode of 9 μm voxel size, 82 kVp voltage and 112 μA present. Following reconstruction of X-ray images using Skyscan NRecon software, the fracture line was identified and analyzed with 3D analysis software (CTAN). The region of interest (ROI) was set at 100 axial slices above and below the fracture line. Total callus volume (TV_{callus}), mineralized callus volume (BV_{callus}), fraction of the mineralized tissue within the total callus volume (BV_{callus}/TV_{callus}), connective density of callus ($Conn.D_{callus}$) and the volumetric BMD of the mineralized tissue comprising the callus (mBMD) were measured.

Mechanical testing: Three-point bending test using universal mechanical strength testing machine (Shimadzu AGS-X 500N) was conducted to evaluate the biomechanical strength of the fractured tibia after micro-CT scanning[30]. After the universal strength-testing machine was calibrated, bone samples of measured diameter and length (using digital caliper) were then placed on the lower perpendicular support of the machine (6 mm apart). The samples were adjusted to ensure the osteotomy line aligns with the center of the two supports. Then an incremental load was gradually applied downward (10 mm sec^{-1}) at the fractured tibia until it fractured. Bone biomechanical strength parameters such as maximum breaking force, maximum stress and strain,

displacement, stiffness, elasticity and Young's modulus were measured using Trapezium X data analysis software.

All experimental procedures of this study were approved by UKM Animal Ethics Committee, UKMAEC (FP/FAR/2016/NORAZLINA/28-JAN./720-JAN.-2016-DEC.-2017) and executed from February to November, 2017. All report sections are in compliance with animal research reporting of *in vivo* experiments (ARRIVE) guidelines for reporting animal research[31].

Statistical analysis: All results were expressed as Mean±SEM. Using statistical package for social science software (SPSS, version 20), data were first tested for normality of distribution using Shapiro-Wilk test before analyzing with one-way analysis of variance (ANOVA) and followed by Tukey's *post hoc* test. At $p<0.05$, results were considered significantly different.

RESULTS

Micro-CT Imaging: Radiological images revealed disrupted distribution pattern of bone callus in the OVXC group compared to the other experimental groups that showed more uniform distribution pattern of bone callus along the fracture line. Both MPv20 and MPv100 groups were visually seen to show better closure of fracture gap (Fig. 2). Additionally, enlargement of the fibula bone in both Sham and OVXC groups were seen.

The 3D Micro-CT images further revealed a non-union of fracture healing ends in the ovariectomized control (OVXC) group (Fig. 3). Although the Sham and ERT groups possessed better periosteal repair compared to OVXC, the MPv20 and MPv100 groups showed more complete periosteal healing with almost complete disappearance of osteotomy line (Fig. 3).

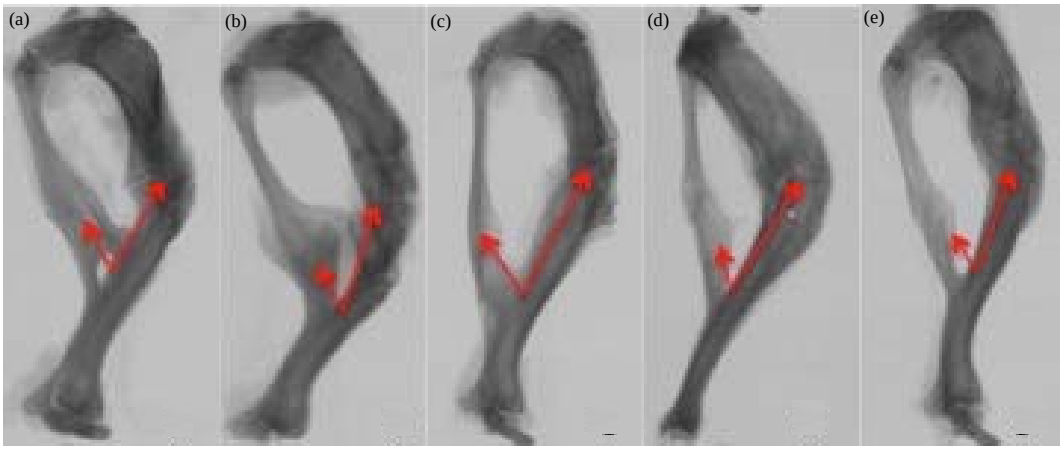

Fig. 2(a-e): X-ray images of fractured tibiae after 8 weeks of treatment. Arrow indicates stabilization of fibula and healed fracture line of the tibiae, respectively

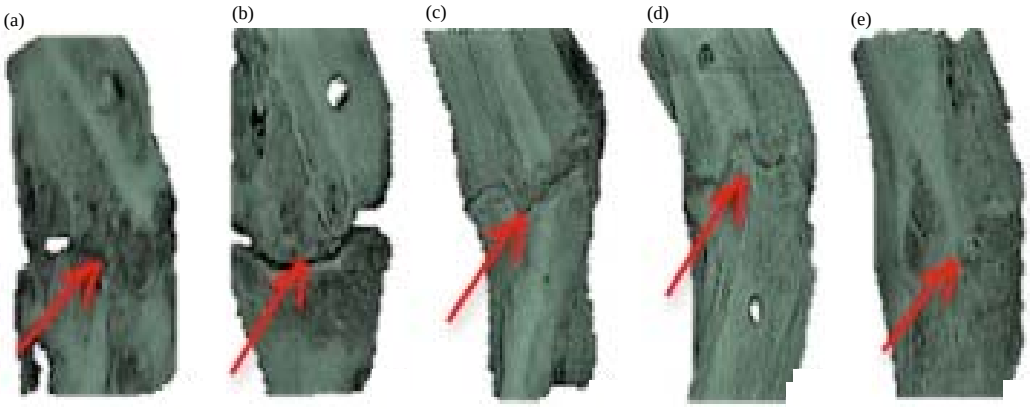

Fig. 3(a-e): Micro-CT images of fractured tibiae showing healing pattern along fracture line, (a): Sham (Sham-operated), (b): OVXC (ovariectomized control), (c): ERT (estrogen treatment), (d): MPV_{20} (20 mg kg^{-1} *Mpva* leaf treatment) and (e): MPv_{100} (100 mg kg^{-1} *Mpva* leaf treatment)

Table 1: Quantitative morphometry of fracture callus of ovariectomized rats' tibiae

Group	BV (mm^3)	BV/TV (%)	Obj.S/TV (1/mm)	Conn.Dn ($1/mm^3$)	Po (tot) (%)
Sham	12.33±0.50*	11.01±0.90*	4.27±0.50	163.20±12.50#	87.42±0.80
OVXC	4.42±1.10	4.72±1.00	2.32±0.60	68.49±17.12	92.06±2.20
ERT	7.81±2.20	11.72±3.04*	3.70±0.80	80.20±17.41	88.28±3.00
MPv20	11.30±2.20*	14.28±2.90*	4.19±0.60	106.63±19.60#	85.73±2.80
MPv100	9.35±1.60	13.45±1.50*	3.22±0.40	57.45±12.70	86.79±1.50

*Signifies significant difference compared to OVXC group. #Signifies significant difference compared to OVXC and MPv100 group ($p<0.05$, ANOVA). TV: Total callus volume, BV: Mineralized callus volume, BV/TV: Mineralized tissue fraction within the total callus volume, Obj.S/TV: Bone surface density, Conn.D.: Connective density of callus, Po (tot): Total pore volume fraction

Table 2: Mechanical strength parameters of fractured tibiae

Group	Maximum force (N)	Maximum stress (N/mm^2)	Maximum strain (%)	Displacement (mm)	Elasticity (N/m^2)	Young's modulus (N/mm^2)
Sham	69.5±6.0*	4.3±0.5*	6.5±0.4	5.7±0.4	296.4±58.0	48.5±8.0
OVXC	22.5±3.0	1.6±0.4	4.9±0.4	4.9±0.4	297.4±51.0	51.6±8.0
ERT	49.7±9.0*	3.0±0.6*	6.0±0.8	5.6±0.6	416.4±71.0	63.2±8.0
MPv20	65.0±11.0*	4.2±0.7*	5.6±0.7	5.0±0.5	257.2±58.0	56.2±14.0
MPv100	61.1±16.0*	3.0±1.0*	8.2±1.3*	5.8±0.7	434.8±73.0	78.0±12.0

*Signifies significant difference compared to OVXC group ($p<0.05$, ANOVA), Sham: Sham-operated group, ERT: Estrogen treatment group, OVXC: Ovariectomized control group, MPv20: 20 mg kg^{-1} day *MPva* leaf extract treatment group, MPv100: 100 mg kg^{-1} day *MPva* leaf extract treatment group

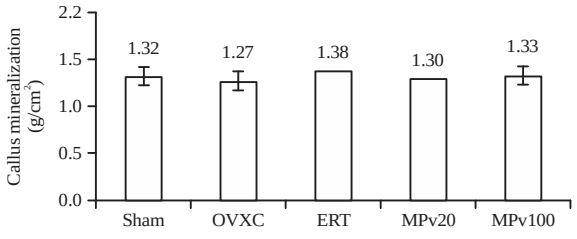

Fig. 4: Mineralization of fracture callus of ovariectomized rats' tibiae. Values are expressed as Mean±SEM

Bone morphometry: Quantitative morphometry revealed significantly lower ($p<0.05$) BV_{callus}, BV_{callus}/TV_{callus} and Conn. D. in the OVXC group when compared to the Sham group (Table 1). MPv20 group possessed significantly increased BV_{callus}, BV_{callus}/TV_{callus} and Conn. D. compared to the OVXC group ($p<0.05$) while the ERT and MPv100 groups were found to have significantly higher ($p<0.05$) BV_{callus}/TV_{callus} when compared to the OVXC group (Table 1).

Bone densitometry: Bone densitometry revealed lower mBMD in OVXC group compared to other groups. This outcome was, however, not significantly ($p<0.05$) different across all experimental groups (Fig. 4).

Bone mechanical strength: Significantly lower ($p<0.05$) bone breaking force and maximum stress was recorded in the OVXC group compared to the Sham group. Similar to the Sham group, significantly higher ($p<0.05$) breaking force and maximum stress was seen in both MPv20 and MPv100 groups, as well as the ERT group, when compared with the OVXC group (Table 2). Additionally, the MPv100 group showed significantly higher ($p<0.05$) bone maximum strain when compared to OVXC and no significant difference ($p<0.05$) in bone displacement, elasticity and Young's modulus was recorded across all treatment groups (Table 2).

DISCUSSION

Radiological examination of X-ray images revealed that treatment with both 20 and 100 mg kg^{-1} per day doses of *MPva* leaves caused a complete closure of fracture gap compared to the untreated ovariectomized rats that had disrupted callus formation around the fracture line (Fig. 2). Additional evidence from 3D micro-CT imaging revealed a non-union of fracture ends in the untreated ovariectomized rats while rats treated with 20 and 100 mg kg^{-1} per day doses of *MPva* leaves, similar to estrogen treatment and the healthy control, showed union of fracture ends with almost complete disappearance of fracture line (Fig. 3).

Micro-CT analysis, quantitative morphometry, further revealed that treatment with 20 mg kg^{-1} per day dose of *MPva* leaves caused significantly increase in BV_{callus}, BV_{callus}/TV_{callus} and Conn. D. compared to the OVXC group ($p<0.05$) while treatment with estrogen and 100 mg kg^{-1} per day dose of *MPva* leaves only caused significantly increase ($p<0.05$) in BV_{callus}/TV_{callus} when compared to the OVXC group (Table 1). BV_{callus}/TV_{callus} represents the amount of bone callus (BV) within the total callus volume (TV) while connective density (Conn. D) denotes the density of trabecular connections that is needed to be broken to sever a bone tissue into two parts[32,33]. These parameters, owing to the high resolution, excellent reproducibility and accuracy of micro-CT, have been used to assess bone callus in monitoring of fracture healing in small animals like rats and mice[34].

Another important parameter of bone strength, $mBMD_{callus}$, which was measured with micro-CT[35] further revealed that treatment with *MPva* extracts caused an increase, although not statistically significant ($p<0.05$), in $mBMD_{callus}$ when compared to untreated ovariectomized control similar to estrogen treatment, as well as healthy control (Fig. 4).

Outcomes of callus morphometry obtained in this study complement findings of radiology and 3D micro-CT imaging in revealing that treatment with *Mpva* leaves enhanced fracture repair in estrogen-deficient rats to a similar extent as the healthy control. However, although both doses of *MPva* leaves recorded positive outcomes, treatment with 20 mg kg^{-1} per day dose showed better healing properties than higher dose of 100 mg kg^{-1} per day and estrogen.

Results of bone strength test conducted in this study revealed a significant preservation ($p<0.05$) of mechanical strength properties (maximum force/breaking force and maximum stress) of tibia following treatment with 20 and 100 mg kg^{-1} per day doses of *MPva* leaves extract compared to untreated ovariectomized control (Table 2). This outcome was found to be similar to estrogen treatment and healthy control. Bone breaking force is a structural property that reflects general bone integrity while maximum stress-strain and Young's modulus are bone material properties that reflect bone elasticity and intrinsic stiffness, respectively[36]. Because outcomes of bone morphometry and densitometry do not always translate into bone strength, it is advisable to follow up micro-CT analysis with further investigations. Biomechanical strength test has become increasingly useful in assessing bone metabolic disorder and predicting fracture risk in small animals as it provides useful information on structural and material properties of bone material[37]. From a wide range tests applicable, 3-point bending test has been the most commonly used in small animals[38]. Outcomes of bone mechanical strength test in this study revealed that treatment with *MPva* leaves was able to enhance restoration of mechanical strength of the healed tibia similar to healthy control and estrogen treatment. However, contrary to outcomes of Micro-CT, treatment with 100 mg kg^{-1} per day dose of plant extract showed slightly better bone strength parameters as it, additionally, recorded significantly higher ($p<0.05$) bone maximum strain compared to untreated ovariectomized control.

Data gathered in this study reveals that treatment with *MPva* leaves promoted fracture repair in postmenopausal osteoporotic rats. Although lower dose of 20 mg kg^{-1} per day yielded better bone morphometric outcome, higher dose of 100 mg kg^{-1} per day yielded greater restoration of bone mechanical strength, a more critical parameter in fracture repair. The mechanism via which *MPva* may have elicited these effects is unclear. Due to its richness in phytoestrogens, it is believed to mimic estrogen by acting on estrogen receptor to induce bone-remodeling responses. It is believed to stimulate osteoblast differentiation and suppress osteoclastic activities by inducing down-regulation of immuno-modulatory factors such as IL-1, IL-6 and TNF-α[39]. However, a better understanding of the mechanism of action of *MPva* would be achieved if its fracture healing effects were studied through the entire regenerative process of fracture repair.

CONCLUSION

MPva leaves promote healing of osteoporotic fracture in postmenopausal rats by enhancing the volume of bone callus formed at fracture site and its subsequent remodeling. Similar to estrogen treatment and healthy control, treatment with higher dose (100 mg kg^{-1} per day) of *MPva* leaves resulted in better restoration of mechanical strength of fracture bone compared to untreated control. Thus, *Mpva* leaves possess the potential of being used as a complementary medicine in the management of osteoporotic fracture in postmenopausal condition.

SIGNIFICANCE STATEMENT

This study, the first of its kind, investigates the effects of aqueous leaf extract of *Marantodes pumilum* var. alata (*MPva*) on fracture healing in post-menopausal rat model. Study outcome revealed that *MPva* leaves promote healing of osteoporotic fracture in postmenopausal rats by enhancing bone callus volume at fracture site and subsequently restoring bone mechanical strength. This data showed that *Mpva* leaves enhanced fracture-healing activity, in addition to its anti-osteoporotic properties, in postmenopausal condition and may find usefulness as an alternative for management of osteoporotic fracture in postmenopausal condition.

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