Comparative anti-osteoporotic properties of the leaves and roots of *Marantodes pumilum var. alata* in postmenopausal rat model

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ABSTRACT

Background: Marantodes pumilum var. *alata* (*MPva*), popularly known as Kacip Fatimah, is widely used to maintain female reproductive health, facilitate post-partum recovery and manage symptoms of menopause and osteoporosis in South-East Asia. This study aims to further evaluate the osteoprotective potential of *MPva* in view of reports of its bone-protective properties in postmenopausal condition.
Methods: Thirty female Sprague-Dawley rats were sorted into 5 groups (n = 6) namely: MPv (leaf treatment); MPr (root treatment); ERT (estrogen treatment); OVXC (untreated ovariectomized control) and Sham (untreated sham-operated control). All rats (except the Sham) were ovariectomized to induce a state of estrogen deficiency that simulates menopause. Two weeks after ovariectomy, the rats were treated for 8 weeks with oral gavages of estrogen and plant extracts. The ERT group received 64.5 μg/kg/day dose of estrogen while *MPv* and *MPr* groups received 20 mg/kg/day dose of leaf and root extracts, respectively. At the end of treatment, left femora were excised from euthanized rats and investigated for changes in bone micro-architecture, mineral density, and biomechanical properties.
Results: Bone volume fraction, degree of anisotropy and structure-model-index of bone were significantly improved ($p < 0.05$) in the MPv group compared to OVXC. Breaking force and maximum stress of bone were also significantly higher ($p < 0.05$) in the MPv group compared to the OVXC.
Conclusion: Treatment with *MPva* leaf protected bone microarchitecture and density against osteoporosis-related changes in postmenopausal rats. Similar to estrogen, the protective effects of *MPva* leaf translated into better-enhanced bone mechanical properties compared to the root treatment.
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1. Introduction

Osteoporosis, a bone condition that is characterized by decreased bone strength and increased risk of fracture, is said to be clinically present when bone mineral density (BMD) falls more than 2.5 standard deviation (SD), below the standard reference for the maximum bone mineral density of young adult female.[1] It is caused by deterioration of bone micro-architecture and loss of bone mass due to bone remodeling deficit.[2] Due to increasing life expectancy, osteoporosis now poses a huge social and economic burden.[3] Higher prevalence of osteoporosis has been recorded in women than in their male counterparts.[4] With over 200 million women affected by this condition worldwide, 1 out of every 3 above 50 years was reported to experience osteoporotic fracture.[5,6] In 2006, 61% of 8.9 million new cases of bone fracture occurred in women.[7] Estrogen deficiency due to menopause or surgery (bilateral ovariectomy) has shown to be the underlying cause of osteoporosis in women. It has been reported to cause an upsurge in bone remodeling with a consequent higher bone resorption by osteoclast cells than bone formation by osteoblast cells.[8]

Estrogen replacement therapy (ERT) has been a logical and valuable treatment option in the management of postmenopausal osteoporosis. Studies have shown that ERT was able to reduce the risk of fracture in postmenopausal women by preserving BMD and

decreasing rate of bone loss.[9] Due to high risk of cancer (breast, colorectal and endometrial) and other serious side effects associated with chronic use of estrogen, ERT has been reserved for management of symptoms of menopause such as hot flashes and vaginal dryness.[10] A number of agents that showed anti-resorptive activities and decreased risk of bone fracture such as bisphosphonates e.g. alendronate are now recommended as alternatives to ERT.[11] Although these alternatives have yielded positive clinical outcomes such as delayed progression of the disease and reduced risk of fracture, they are however known to be associated with debilitating side effects that often affect compliance to drug regimen.[12] Due to the questionable safety of available anti-resorptive treatments, there is an increasing demand for safer alternative and complementary medicine by osteoporotic patients.

Research is being conducted on a wide range of agents with folkloric claims of bone-protective activities such as virgin coconut oil, soy, *Marantodes pumilum* var. *alata* (*MPva*), blueberry, *Achyranthes bidentata* and many more. *MPva*, a herb belonging to family "Primulaceae", is widely used to maintain female reproductive health and as a post-partum medicine in Malaysia.[13] Its root extract was reported to preserve bone micro-architecture when compared with estrogen-deficient and healthy controls while its whole plant extract was reported to enhance the bone mechanical strength of estrogen-deficient rats.[14,15] As phytoestrogens have been shown to improve bone density,[16,17] the presence of phenolic compounds with estrogenic activity (β-carotene, quercetin, gallic acid, myricetin, kaempferol, ellagic acid, catechin, syringic acid, and vanillic acid) in *MPva* makes it desirable for management of osteoporosis.[18–20] A previous study has reported a differential pattern and dose dependent decline of bone-protective effect of *MPva* according to its plant parts.[21] However, despite reports of varying pharmacological activity of the leaves and roots of *MPva*, attributable to the varying nature of phytoestrogens in its leaves and roots, previous studies have not investigated the relative osteoprotective effects of its leaves and roots. This study is designed to evaluate the relative osteoprotective potential of the leaves and roots of *MPva* in postmenopausal osteoporosis rat model. Using a combination of micro-computed tomography (micro-CT) and biomechanical strength tests, effects of *MPva* on bone micro-architecture and mineral densities were further investigated for biomechanical strength.

2. Materials and methods

2.1. Extract preparation

The leaves and roots of *MPva* (family: Primulaceae, synonyms: *Labisia pumila, Labisia pothoina*) were obtained on the 16th of February 2016 from Delima Jelita Herbs Simpang Empat, Kedah, Malaysia and authenticated by Professor Emeritus (Dr.) Abdul Latiff Mohamad at the Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). Voucher specimens (No. UKM-HF131) were deposited at the UKM Herbarium. The plant specimens were individually garbled, air-dried under shade and ground using a rotary grinder. Reflux extraction in distilled water was done based on the modified method[22] at 60 °C for 2 h. Resultant extracts were then freeze-dried to obtain dry extracts that were stored at −20 °C for experimental use.

2.2. Quantification of phenolics

Simultaneous quantification of the aqueous extracts was performed using an LC/MS/MS assay against seven phenolic standards, namely, gallic acid, caffeic acid, apigenin, kaempferol, quercetin, ellagic acid and myricetin on an AB Sciex 5500 QTrap liquid chromatography tandem mass spectrometer coupled to Agilent 1290 Infinity UHPLC system. The standards and extracts were separated on a Phenomenex Synergi Fusion C_{18} column (100 × 2.1 mm, 5 μm) by gradient elution with a different ratio of a mixture of (A) water with 0.1% formic acid and 5 mM ammonium formate, and (B) acetonitrile with 0.1% formic acid and 5 mM ammonium formate as the mobile phase over a 15-min period at a flow rate of 250−400 μL/min. A turbo spray source was applied and operated in negative ion mode. Multiple reaction monitoring (MRM) scan was used for quantification by monitoring the precursor-product ion transitions of the respective m/z of the standards; gallic acid (m/z 169.011), caffeic acid (m/z 179.000), apigenin (m/z 269.000), kaempferol (m/z 284.916), quercetin (m/z 300.923), ellagic acid (m/z 300.923) and myricetin (m/z 317.000). All standard calibration curves achieved good regression at > 0.99.

2.3. Drug and chemicals

Conjugated estrogen (Wyeth-Ayerst, Canada) was obtained from Caring Pharmacy, No. 12 Jalan Raja Haroun, Kajang, Selangor, Malaysia.

2.4. Dose selection

The choice of doses: 64.5 μg/kg/day estrogen and 20 mg/kg/day of *MPva* used in this study was based on outcome of previous study.[14] Another study revealed that 20 mg/kg/d dose of *MPva* confers more protection on the bone of rats than higher doses of 50 and 100 mg/kg/day.[21]

2.5. Experimental animals

Healthy female Sprague-Dawley rats (4 months old) weighing 200−250 g were obtained from the laboratory animal unit, UKM. Animal handling was conducted in accordance with the US guide for the use and care of laboratory animal as contained in National Institutes of Health (NIH) publication.[23]

Rats were housed pairwise in plastic cages (45 cm × 28 cm x 20 cm) furnished with wood shavings as bedding and under room temperature of 25 ± 3 °C, natural day-night cycle and humidity. The cages were cleaned every 48 h. Rats were allowed free access to filtered portable water and standard diet containing 0.97% calcium, 0.85% phosphorus and 1.05 IU/g of Vitamin D3 (Gold Coin, Selangor-Malaysia). Prior to commencement of experimentation, all rats were allowed to acclimatize to laboratory environment for 7 days. After experimentation, rats were humanely euthanized using an overdose of ketamine-xylazine mixture intraperitoneally (IP) and cervical dislocation.[24]

2.6. Study design

All procedures involving animals in current study were approved by Universiti Kebangsaan Malaysia Animal Ethics Committee, UKMAEC (FP/FAR/2016/NORAZLINA/28-JAN./720-JAN.-2016-DEC.-2017).

Except for the Sham, rats were ovariectomized. Drugs were administered daily orally for 8 weeks. (n = 6).

Thirty Sprague-Dawley rats were divided into five groups. Before commencement of treatment, all rats (except the SHAM) were ovariectomized under anaesthesia to induce a state of estrogen deficiency that simulate menopause. After 2 weeks healing period, the rats were given daily oral gavages of estrogen and plant extracts (Table 1), for a period of 8 weeks.[15] After treatment, left femora were harvested from humanely sacrificed animals for investigations. Changes in bone microarchitecture (quantitative

Table 1
Study design.

Group	Treatment
Sham-operated (SHAM)	Untreated
Ovariectomized control (OVXC)	Untreated
Estrogen treatment (ERT)	64.5 µg/kg/d estrogen
Leaf extract treatment (MPv)	20 mg/kg/d aqueous leaf extract
Root extract treatment (MPr)	20 mg/kg/d aqueous root extract

morphometry) and mineral densities (densitometry) were investigated using Micro-CT. After Micro-CT, the femora were further subjected to mechanical strength test using universal mechanical strength testing machine. All report sections of this study are in compliance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for reporting animal research.[25]

2.7. Surgical protocol

The method previously described by Kajuria et al. was adopted.[26] Under anaesthesia (IP ketamine: xylazine cocktail 8:1), the abdominal fur was shaved with an electronic clipper (Domotec MS-4612, Germany) and sterilized with 70% alcohol. Using surgical scalpel blade (No. 11), a small peritoneal incision measuring 0.4–0.6 cm was made vertically on the outer skin of the middle part of the abdomen that lies between the 2nd and 3rd nipples. Through the opening, the underlining muscle tissue was incised vertically to create 0.3 cm opening through which surrounding adipose tissue was pulled out to expose underlying uterine tube from which exteriorized right and left ovaries were gently retracted and ablated. The uterine horns were sutured and returned to the peritoneal cavity. Open wound was then sutured in two layers (muscle and skin) and cleaned with Povidone iodine solution. Postoperatively, rats were injected subcutaneously 0.1 mg/kg buprenorphine daily for 3 days to control pain[27] and intramuscularly 5 mg/kg enrofloxacin (Baytril®) 12 hourly for 7 days to prevent infection.[28]

2.8. Bone sample collection

Left femora were harvested from humanely sacrificed animals using a surgical blade (No. 11) and scissors. Dissected bone samples were cleansed of all surrounding soft tissues, wrapped with sterile gauze soaked in phosphate buffer solution and immediately stored at −70 °C.

2.9. Microcomputed tomography

With slight modification, the method described previously was adopted.[29] The distal femora, 1.5 mm below the growth plate and extending 2.0 mm towards the bone mid-diaphysis, was scanned with an X-ray scanner system (Skyscan 1076, G015619) at a scanning mode of 9 µm voxel size, 82 kVp voltage, 112 µA current and 0.5 mm AL filter. Scanned x-ray images were reconstructed using NRecon software and processed with 3D analyzer software (CTAN). The region of interest (ROI) was set at 200 slices off an offset slice that was 100 slices away from a reference slice at the growth plate. Bone morphometric parameters: bone volume fraction (BV/TV); trabecular thickness (Tb.Th); trabecular separation (Tb.Sp); trabecular number (Tb.N); structure model index (SMI); degree of anisotropy (DA); medullary area (Ma.A); average cortical bone area fraction (Ct.Ar/Tt.Ar); average cortical thickness (Ct.Th) and connective density (Conn. D) were measured. Bone mineral density (BMD) of trabecular and tissue mineral density (TMD) of cortical bone were also measured by comparing attenuation values of bone samples to that of a phantom rod containing a known density of calcium hydroxyapatite.

2.10. Mechanical strength test

Employing 3-point bending test, universal mechanical strength testing machine (AGS-X 500N, Shimadzu) was used to evaluate biomechanical properties of the left femora.[14] The machine was first calibrated while bone parameters (diameter, length, and mid-point) were measured using a digital caliper. Bone samples were then placed on 2 perpendicular lower support of the machine (10 mm apart) such that the mid-point coincided with the center of the two supports. Incremental load was gradually applied downward (10 mm/s) at the mid-point of the femora until it fractures. Using data analysis software, Trapezium X, bone strength parameters: maximum load, displacement, stiffness, stress, strain and Young's modulus were measured.

2.11. Statistical analysis

Results obtained from all rats were expressed as mean ± SEM. Using SPSS (Version 20), data were first tested for normality of distribution using Shapiro-Wilk test before analysis with one-way analysis of variance (ANOVA) and Tukey's post hoc test. At $p < 0.05$, results were considered significant.

3. Results

3.1. Quantification of phenolics in the aqueous extracts of Marantodes pumilum var. alata

Indicating that the extracts differ in their phytochemical compositions, different profiles of LC/MS/MS chromatograms of aqueous extracts of MPv and MPr were obtained (Fig. 1). Quantitative analysis revealed that the plant extracts contained mainly gallic acid and ellagic acid (Table 2). The leaves contained more gallic acid than the roots and vice versa for ellagic acid. However, the content of caffeic acid, myricetin, apigenin, kaempferol, and quercetin was found to be < 0.00.

3.2. Micro-CT imaging

The OVXC group possessed the lowest density of trabeculae compared to the Sham and ERT. Both MPv and MPr also appeared to possess higher densities of trabeculae when compared to OVXC (Fig. 2a and b). No visible difference was seen in the Micro-CT images of the cortical bone part (Fig. 3).

3.3. Quantitative morphometry

MPv group showed significantly improved ($p < 0.05$) morphometric parameters of trabecular bone, BV/TV, SMI and DA compared to OVXC group (Table 3). When compared to MPr group, DA was significantly higher ($p < 0.05$) in the *MPv* group. On the cortical bone, both *MPv* and *MPr* groups showed significantly ($p < 0.05$) higher values of Ct. Th and Conn. D when compared to OVXC group (Table 4).

3.4. Bone density

The outcome of bone densitometry revealed a significantly lower ($p < 0.05$) bone mineral density in OVXC group compared to Sham and ERT groups (Fig. 4a). Similar to SHAM and ERT, significantly higher ($p < 0.05$) bone mineral density was seen in *MPv* group when compared with OVXC group (Fig. 4a). On the cortical bone, tissue mineral density values in both MPv and MPr, as well as
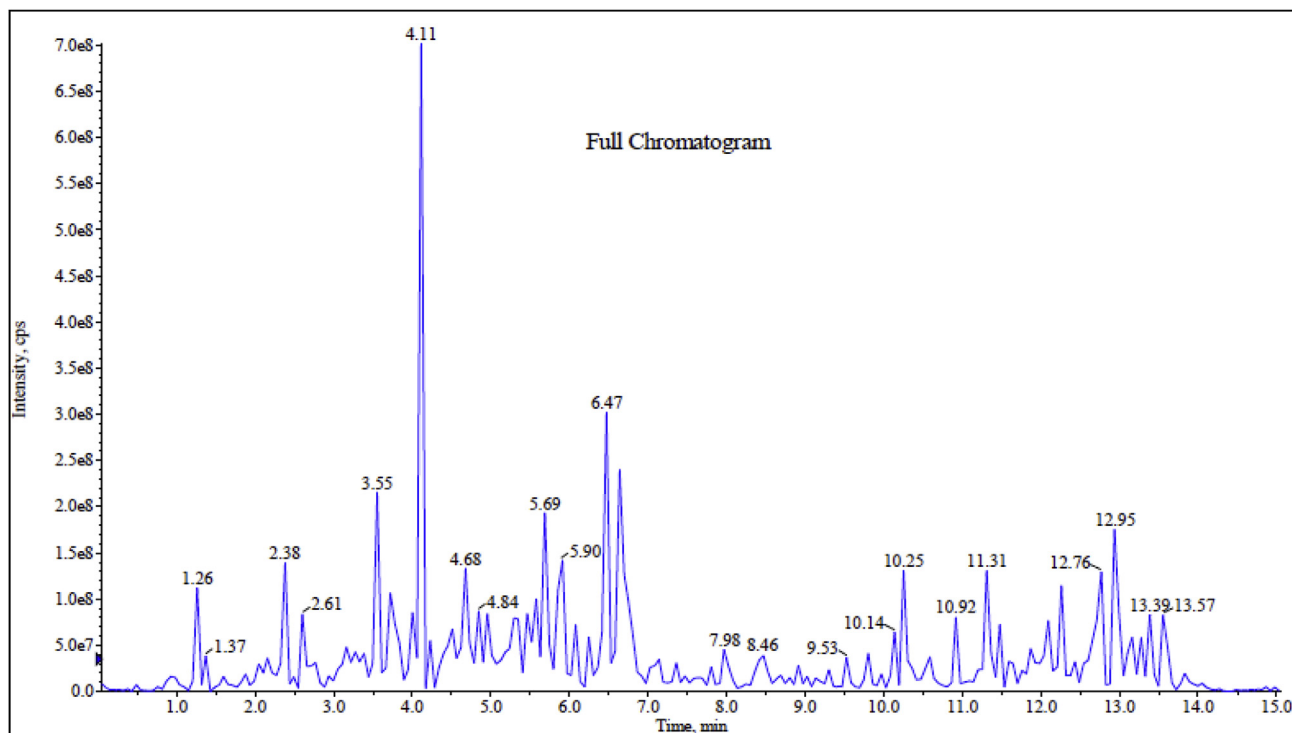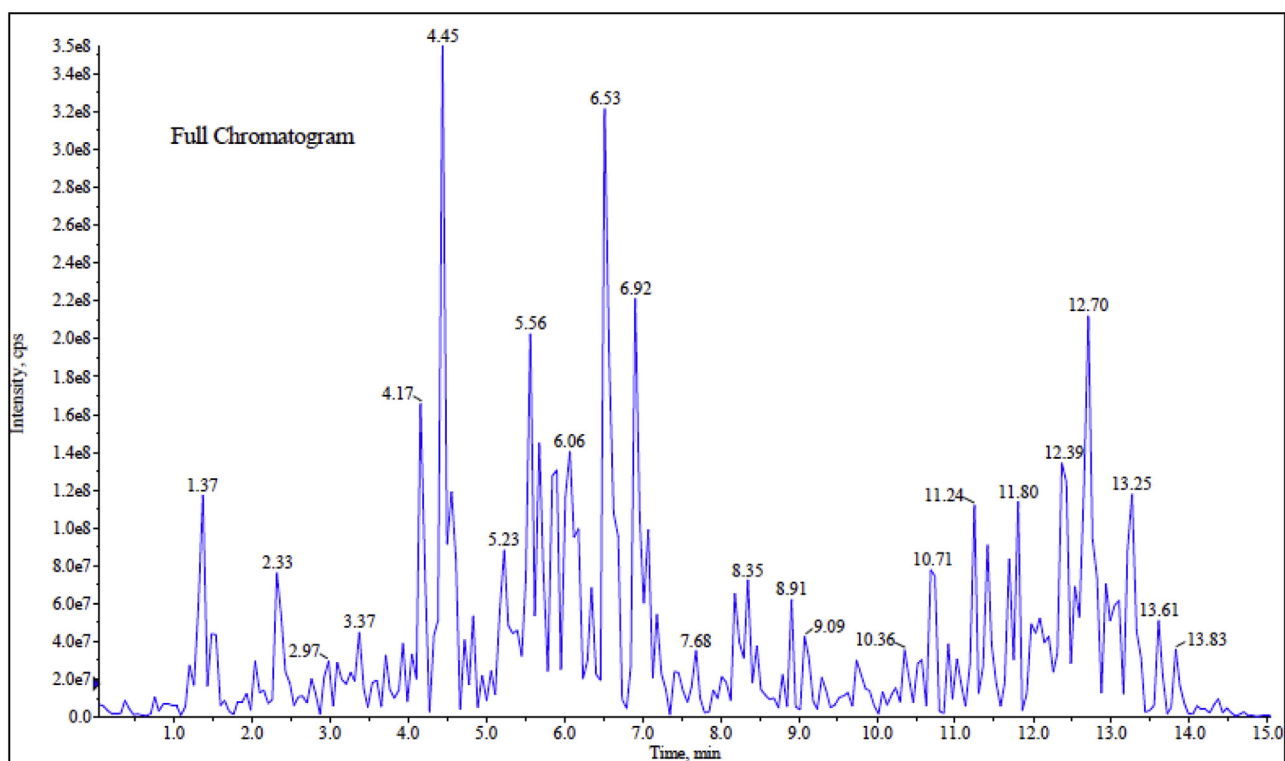
Fig. 1. LC/MS/MS chromatograms of *Marantodes pumilum* var. *alata* extracts. a: Leaves, b: roots.

Table 2
Content of targeted phenolic compounds in water extract of leaves and roots of *Marantodes pumilum* var. *alata* by using LCMS/MS.

Phenolic compounds	Content in extract (µg/mL)	
	MPv	MPr
Gallic acid	5.540	2.240
Caffeic acid	<0	<0
Apigenin	<0	<0
Kaempferol	<0	<0
Quercetin	<0	<0
Ellagic acid	0.070	0.115
Myricetin	<0	<0

MPv: leaves, MPr: roots.

ERT and Sham groups, were significantly higher ($p < 0.05$) compared to OVXC group (Fig. 4b).

3.5. Bone biomechanical strength

Significantly lower ($p < 0.05$) maximum force and maximum stress of bone were recorded in OVXC group when compared to the SHAM and ERT groups (Fig. 5a and b). Similar to SHAM and ERT, maximum force and stress of bone were significantly higher ($p < 0.05$) in the *MPv* group when compared with OVXC group (Fig. 5a and b). No significant changes were observed in maximum strain values (Fig. 5c). For Young's modulus, SHAM group had a

significantly higher value ($p < 0.05$) compared to OVXC and MPr (Fig. 5d).

4. Discussion

As we are aware of the influence of several plant collection factors on the phytochemical composition of plants,[30] we conducted LC/MS/MS assay of plant material in order to know the specific phytochemical constituents in our plants. Contrary to previous phytochemical studies outcomes, probably due to differences in plant collection area, time and season, LC/MS/MS of plant material revealed the presence of only gallic and ellagic acid in the plant leaves and roots.

Changes in bone micro-architecture that causes thinning and loss of trabeculae of the trabecular bone and increased porosity of the cortical bone are typical of estrogen deficiency in postmenopausal osteoporosis. Such changes are often reflected as deterioration of bone micro-architecture.[31] Micro-CT imaging revealed differences in the density of trabeculae within the femur bone. Both the leaf and root extracts treatment groups, like the estrogen treatment and Sham groups, possessed a higher density of trabeculae compared to OVXC group (Fig. 2). Partly in consonance with outcomes of Micro-CT imaging, quantitative morphometry of the femur revealed further details on the effects of *MPva* on bone microarchitecture. Results revealed significantly higher ($p < 0.05$) BV/TV and DA and significantly lower (p<0.05) SMI in the *MPva* leaf
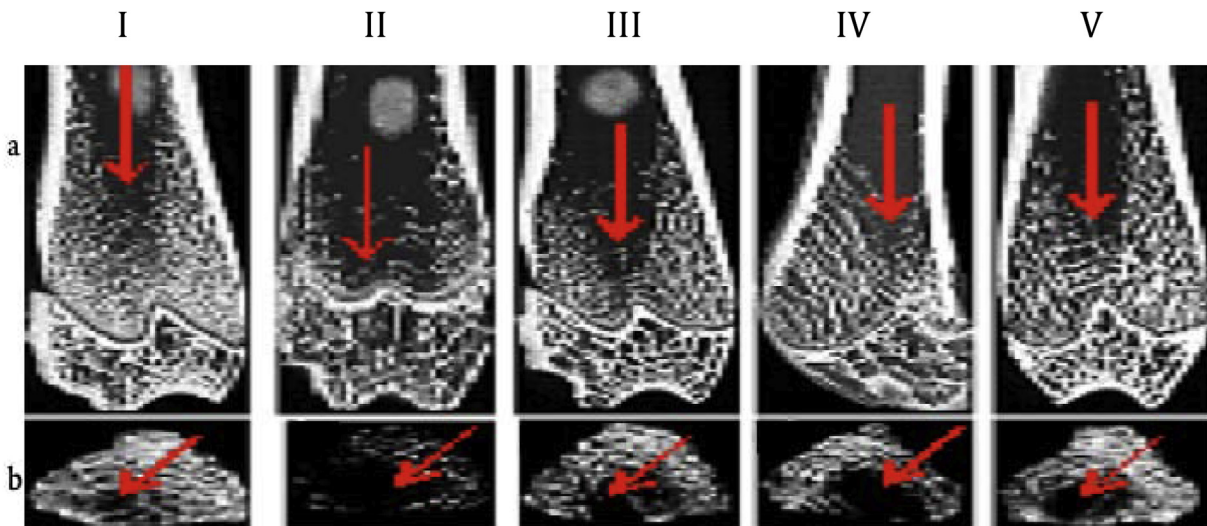
Fig. 2. Micro-CT images showing trabeculae of the distal femur of ovariectomized rats (a: sagittal view, b: transverse view). Red arrows indicate groove showing loss of trabecular structures.
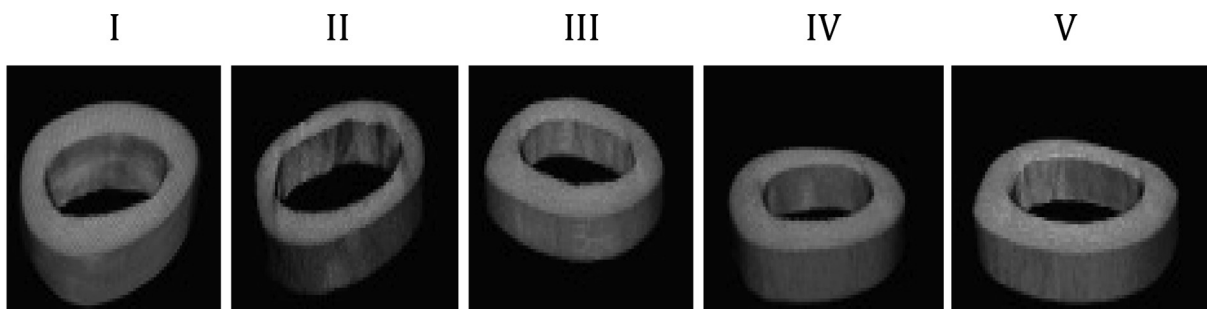

Fig. 3. Micro-CT images showing cortical bone of distal femora of ovariectomized rats. **Legend: I (SHAM)**: Sham-operated group, **II (OVXC)**: Ovariectomized control group, **III (ERT)**: Estrogen treatment group, **IV (MPv)**: *M. pumilum* var. *alata* leaf extract treatment group, **V (MPr)**: *M. pumilum* var. *alata* root treatment group.

Table 3
Trabecular bone morphometry of ovariectomized rats.

Parameters	Treatment Groups				
	SHAM	OVXC	ERT	MPv	MPr
BV/TV	17.33 ± 1.18[a,b]	7.73 ± 0.98	13.31 ± 0.91[a]	13.17 ± 0.93[a]	10.79 ± 1.42
Tb.Th	0.09 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.09 ± 0.01	0.10 ± 0.00
Tb.N	1.95 ± 0.14[d]	0.87 ± 0.11	1.28 ± 0.08[a]	1.36 ± 0.07	1.09 ± 0.12
Tb.Sp	0.63 ± 0.03	0.79 ± 0.08	0.67 ± 0.03	0.68 ± 0.03	0.80 ± 0.05
DA	1.97 ± 0.05[a,b]	1.59 ± 0.05	1.99 ± 0.05[a]	1.87 ± 0.04[a,b]	1.65 ± 0.04
SMI	0.82 ± 0.10[a,b,c]	1.68 ± 0.09	1.27 ± 0.08[a]	1.13 ± 0.07[a]	1.26 ± 0.11[a]

[a] Significantly different from OVXC.
[b] Significantly different from MPr.
[c] Significantly different from ERT.
[d] Significantly different from all other groups. BV/TV: Bone volume fraction; Tb.Th: Trabecular Thickness (mm); Tb.N: Trabecular Number (1/mm); Tb. Sp: Trabecular Separation (mm); DA: Degree of Anisotropy; SMI: Structure model index.

Table 4
Cortical bone morphometry of ovariectomized rats.

Parameters	Treatment Groups				
	SHAM	OVXC	ERT	MPv	MPr
Ma.Ar	12.93 ± 1.4	15.72 ± 1.9	14.70 ± 1.6	12.02 ± 1.1	10.85 ± 1.6
Ct.Ar/Tt. Ar	34.85 ± 1.2[a]	27.04 ± 1.9	31.12 ± 1.0	30.66 ± 1.6	31.30 ± 1.8
Ct.Th	0.38 ± 0.01[a]	0.29 ± 0.02	0.36 ± 0.01[a]	0.36 ± 0.02[a]	0.35 ± 0.00[a]
BS/TV	3.19 ± 0.14[a,b]	2.75 ± 0.11	2.88 ± 0.05	2.77 ± 0.08	2.90 ± 0.11
Conn. D.	64.34 ± 9.7[c]	15.5 ± 2.60	39.51 ± 4.8[a]	39.06 ± 3.2[a]	36.69 ± 1.5[a]

[a] Significantly different from OVXC.
[b] Significantly different from MPv.
[c] Significantly different from all other groups. Ma. Ar: Medullary Volume (mm^{2}); Ct. Ar/Tt.Ar: Average cortical bone area fraction (%); Ct. Th: Average cortical thickness (mm); BS/TV: Bone surface density; Conn. D: Connective Density.
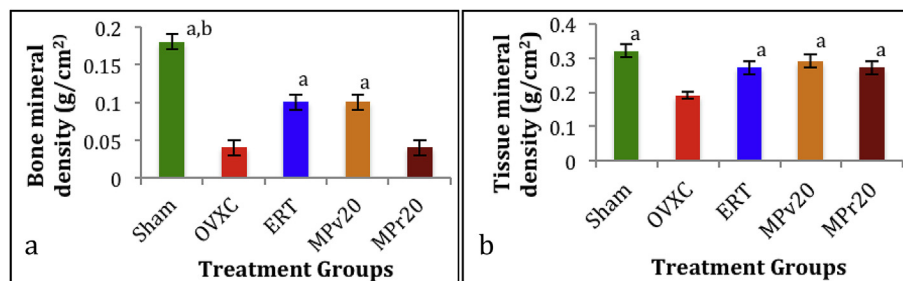
Fig. 4. Mineral densities of distal femora of ovariectomized rats (a: Bone mineral density, b: Tissue mineral density). a significantly different compared to OVXC; b significantly different compared to other groups (p < 0.05).

treatment group compared to the OVXC group. However, when compared to the MPr group that only showed significantly lower SMI compared to OVXC, the MPv group further showed significantly higher ($p < 0.05$) DA (Table 3). BV/TV represents the amount of trabeculae in the total trabecular bone volume of interest while SMI reflects plate-to-rod-to-sphere changes in bone structures. Higher values (normal: 0–4) of SMI reflect a loss of bone strength as a result of changes in bone structures from plates to rods to spheres. Conversely, DA, which indicates the extent of anisotropy of bone structures, increases with increasing bone strength. Qualitative morphometry of the trabecular bone revealed that the leaf extract, similar to estrogen, preserved micro-architectural structures and prevented bone loss induced by estrogen deficiency better than the root extract. Contrarily, on the cortical bone, both MPv and MPr groups, as well as ERT group, showed similar effects by possessing significantly higher ($p < 0.05$) Ct. Th and Conn. D when compared to OVXC group (Table 4). Ct. Th represents the average thickness of the cortical bone within the total bone area of interest while Conn. D is an index of bone connections density that needs to be broken to sever a bone into two parts.[32,33]

Bone mineral density, a direct indicator of bone density, has been used as an assessment tool for bone strength and fracture risk. For every 10% fall in bone density of vertebra and hipbones, 2 and 2.5 fold increase in the risk of fracture, respectively, has been reported.[34] In addition to quantitative morphometry, micro-CT has been used to estimate bone tissue mineralization by comparing X-ray attenuation in bone samples with hydroxyapatite phantom of known density.[35] Results of densitometry revealed a significantly higher ($p < 0.05$) bone mineral density in MPv group when compared with OVXC group (Fig. 4a), which was similar to ERT and Sham groups. Although similar effects on TMD were seen between MPv and MPr as well as ERT and SHAM groups compared to OVXC group (Fig. 4b), densitometry outcome seen in this study also revealed that the leaf extract, similar to estrogen, prevented loss of BMD induced by estrogen deficiency better than the root extract.

Because bone outcomes of morphometry and densitometry do not always reflect bone strength, it is advisable to follow up outcomes of quantitative morphometry and densitometry with further tests. Mechanical bone strength test has been shown to be one of the most appropriate tools for assessing bone strength and
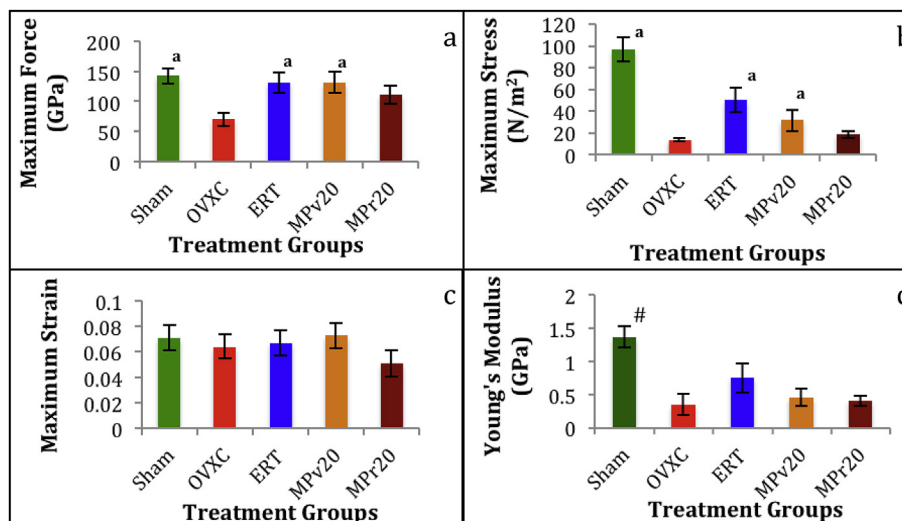
Fig. 5. Mechanical strength parameters of femora of ovariectomized rats. a significantly different when compared with OVXC. # Significantly different when compared with OVXC and MPr (p < 0.05).
Legend: SHAM: Sham-operated group, **ERT**: Estrogen treatment group, **OVXC**: Ovariectomized control group, **MPv**: *M. pumilum* var. *alata* leaf extract treatment group, **MPr**: *M. pumilum* var. *alata* root treatment group.

predicting fracture risk in small animals.[36] Biomechanical bone strength test results revealed significantly higher ($p < 0.05$) bone breaking force (GPa) and maximum stress (N/m²) in the leaf extract treatment group when compared with OVXC group (Fig. 5a and b). This effect was found to be similar to ERT and SHAM groups. Maximum load (breaking force) is a bone structural property which reflects its integrity while stress-strain curve and Young's Modulus are bone material properties that reflect its elasticity and intrinsic stiffness, respectively.[37] Thus, animals in leaf treatment group, similar to ERT and SHAM groups, possess stronger bones ($p < 0.05$) compared to animals in the root treatment group.

In summary, results of this study revealed that aqueous leaf extract of *MPva* possesses better anti-osteoporotic activities in postmenopausal rats compared to the root extract. Current study provides additional information on the anti-osteoporotic effects of *MPva* that would complement findings of a previous study that reported significant preservation of bone histomorphometric parameters.[15] Similar to estrogen treatment, the protective effects of *MPva* leaf on bone micro-architecture and mineral density further translated into improved bone biomechanical strength. Although the mechanism via which *MPva* elicits these effects is yet to be understood, studies have shown that its phytochemical constituents may be responsible, as they have been shown to possess estrogenic activity.[20] They are believed to act by stimulating estrogen receptor to stimulate osteoblast differentiation during bone formation and suppress osteoclast resorptive activity through down-regulation of immuno-modulatory factors such as interleukin 1 and 6 (IL-1 & IL-6) and tumor necrosis factor alpha (TNF-α). Available evidence has shown that *MPva* conferred anti-oxidant protection on the bone of estrogen-deficient rats and suppressed serum levels of pro-inflammatory factors that may be implicated in the pathogenesis of osteoporosis such as TNF-α.[38] The disparity in pharmacological activities between the leaf and root treatments of *MPva* could be attributed to differences in their content of phytochemicals as the leaves were shown to contain more gallic acid than ellagic acid compared to the roots (Table 2). Gallic acid, a secondary polyphenolic metabolite found in plants, is well known as a natural antioxidant with cardio-protective, anti-cancer, anti-diabetic and anti-inflammatory properties.[39]

5. Conclusion

The leaf extract of *MPva* protected bone micro-architecture and mineral density against osteoporosis-related deterioration in postmenopausal rats. Preserved bone microarchitecture and mineral density outcomes caused by the leaf treatment further resulted in improved bone mechanical strength similar to estrogen treatment but better than the roots. Thus, the leaves of *MPva* carry a great potential to be used as an alternative medicine to estrogen in the management of postmenopausal osteoporosis.

Competing interest

We wish to declare that there was no conflict of interest of any sort amongst all authors and sponsor of this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2019.01.002.

List of abbreviations

BMD   Bone Mineral Density (g/cm²)
BV/TV   Bone Volume Fraction (%)
Conn. D   Connective Density
Ct.Ar/Tt.Ar   Average cortical bone area fraction (%)
Ct.Th   Average cortical thickness (mm)

DA	Degree of Anisotropy
ERT	Estrogen replacement therapy
Ma.Ar	Medullary Volume (mm^2)
SEM	Standard error of the mean
SMI	Structure model index
TMD	Tissue Mineral Density (g/cm^2)
Tb.N	Trabecular Number (1/mm)
Tb.Th	Trabecular Thickness (mm)
Tb.Sp	Trabecular Separation (mm)
TNF-α	Tumor necrosis factor alpha

References

1. World Health Organization. In: *WHO Scientific Group on the Assessment of Osteoporosis at Primary Health Care Level, Summary Meeting Report*. Brussels, Belgium: WHO Press; 2004.
2. Keating FSJ, Manassiev N, Stevenson JC. Estrogens and osteoporosis. In: Lobo RA, Kelsey J, Marcus R, eds. *Menopause: Biology and Pathology*. California: Academic Press; 2000:509–514.
3. Nancy EL. Epidemiology, etiology and diagnosis of osteoporosis. *Am J Obstet Gynaecol*. 2006;194:3–11.
4. Paves T. Postmenopausal osteoporosis. *JK Pract*. 2004;11(4):281–283.
5. Kanis JA. *WHO Technical Report*. UK: University of Sheffield; 2007:66.
6. Melton III LJ, Chrischilles EA, Cooper C, Lane AW, Riggs BL. Perspective. How many women have osteoporosis? *J Bone Miner Res*. 1992;7:1005.
7. Johnell O, Kanis JA. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporos Int*. 2006;17:1726.
8. Pacific R. Estrogen, cytokines and pathogenesis of postmenopausal osteoporosis. *J Bone Miner Res*. 1996;11:1043–1051.
9. Marx CW, Dailey III GE, Cheney C, Vint II VC, Muchmore DB. Do estrogens improve bone mineral density in osteoporotic women over age 65? *J Bone Miner Res*. 1992;7:1275–1279.
10. Stuenkel CA, Gas MLS, Manson JE, et al. A decade after the Women's Health Initiative - the experts do agree. *Fertil Steril*. 2012;98(2):313–314.
11. Richards JB, Joseph L, Schwartzman K, Kreiger N, Tenenhouse A, Goltzman D. The effect of cyclooxygenase-2 inhibitors on bone mineral density: results from the Canadian Multicentre Osteoporosis Study. *Osteoporos*. 2006;17: 1410–1419.
12. Rao LG, Rao AV. Oxidative stress and antioxidants in the risk of osteoporosis - role of the antioxidants lycopene and polyphenols. In: Valdés-Flores Margarita, ed. *Osteoporosis*. Mexico: IntechOpen; 2013:117–162.
13. Runi SP. Studies on medicinal plant in Sarawak. In: Chang YS, ed. *Seminar on Medicinal and Aromatic Plants*. Kepong: Forest Research Institute Malaysia; 2001:112–119.
14. Nadia ME, Shahrum A, Mohd FMY, Nazrun AS. Time and dose-dependent effects of *Labisia pumila* on the bone strength of postmenopausal osteoporosis rat model. *BMC Complement Altern Med*. 2015;15:58.
15. Fathilah SN, Nazrun AS, Norazlina M, Norliza M, Ima-Nirwana S. *Labisia pumila* protects the bone of estrogen-deficient rat model: a histomorphometric study. *J Ethnopharmacol*. 2012;142:294–299.
16. Gambacciani M, Ciaponi M, Cappagli B, Piaggesi L, Genazzani AR. Effects of combined low dose of the isoflavone derivative ipriflavone and estrogen replacement on bone mineral density and metabolism in postmenopausal women. *Maturitas*. 1997;28:75–81.
17. Albertazzi P, Pansini F, Bonaccorsi G, Zanotti L, Forini E, De Aloysio D. The effect of dietary soy supplementation on hot flushes. *Obstet Gynecol*. 1998;91(1): 6–11.
18. Ali Z, Khan IA. Alkyl phenols and saponins from the roots of *Labisia pumila* (Kacip Fatimah). *Phytochemistry*. 2011;72:2075–2080.
19. Chua LS, Lee SY, Norhanisah A, Sarmidi MR. Review on *Labisia pumila* (Kacip Fatimah): bioactive phytochemicals and skin collagen synthesis promoting herb. *Fitoterapia*. 2012;83:1322–1335.
20. Jamal JA, Houghton JP, Milligan RS, Jantan I. The estrogenic and cytotoxic effects of the extracts of *Labisia pumila* var. *alata* and *Labisia pumila* var. *pumila* in vitro. *J Sains Kesihatan*. 1988;1:53–60.
21. Tijjani RG, Shuid AN, Soelaiman IN, Muhammad N, Mohamed N. Low dose supplementation with *Marantodes pumilum* preserved bone mineral density and micro-architecture in postmenopausal rat model. *Intl J Osteoporos Metab Disorders*. 2017;10(1):1–9.
22. Jamal JA, Sateri AA, Husain K, Jantan I, Mohamad-Arshad MS. Perbandingan pelbagai kaedah pengekstrakan air *Labisia pumila* var. *alata*. *Malaysian Journal of Health Sciences*. 2006;4(1):37–46.
23. *Guide for the Care and Use of Laboratory Animals*. eighth ed. Washington (DC): National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals; 2011. https://doi.org/10.17226/12910. National Academies Press (US).
24. Leary SL. American veterinary medical association. In: *AVMA Guidelines for the Euthanasia of Animals*. edition 2013:26–38.
25. Kilkenny C, Browne W, Cuthill I, Emerson M, Altman D. Improving bioscience research reported: the ARRIVE guidelines for reporting animal research. *PLoS Biol*. 2010;8. e1000412.
26. Khajuria DK, Razdan R, Mahapatra DR. Description of a new method of ovariectomy in female rats. *Rev Bras Reumatol*. 2012;52(3):462–470.
27. Curtin LI, Grakowsky JA, Suarez M, et al. Evaluation of buprenorphine in a postoperative pain model in rats. *Comp Med*. 2009;59(1):60–71.
28. Isaksen M, Mabley D. Drug usage chart for rats. Retrieved March 21, 2015, from http://www.mca.org/Articles/dchart.htm.
29. Steven KB, Peter D, Ralph M, Jurg AG. Monitoring individual morphological changes over time in ovariectomized rats by in vivo micro-computed tomography. *Bone*. 2006;39(4):854–862.
30. Wei L, Dongxue Y, Na L, et al. Influence of environmental factors on the active substance production and antioxidant activity in *Potentilla fruticosa* L. and its quality assessment. *Sci Rep*. 2016;6. Article No: 28591.
31. Cooper DM, Thomas CD, Clement JG, Turinsky AL, Sensen CW, Hallgrímsson B. Age-dependent change in the 3D structure of cortical porosity at the human femoral midshaft. *Bone*. 2007;40(4):957–965.
32. Hildebrand T, Ruegsegger P. Quantification of bone micro-architecture with the structure model index. *Comput Biomech Engin*. 1987;1:15–23.
33. Odgaard A. Three-dimensional methods for quantification of cancellous bone architecture. *Bone*. 1997;20:315–328.
34. Klotzbuecher CM, Ross PD, Landsman PB, Abbot TA, Berger M. Patients with prior fractures have increased risk of future fractures: a summary of the literature and statistical synthesis. *J Bone Miner*. 2000;15:721.
35. Fajardo RJ, Cory E, Patel ND, et al. Specimen size and porosity can introduce error into micro-CT-based tissue mineral density measurements. *Bone*. 2009;44:176–184.
36. Van der Meulen MC, Jepsen KJ, Mikic B. Understanding bone strength: size isn't everything. *Bone*. 2001;29:101–104.
37. Turner CH. Bone strength: current concepts. *Ann N Y Acad Sci*. 2006;1068: 429–446.
38. Nadia ME, Nazrun AS. Time and dose-dependent effects of *Labisia pumila* on bone oxidative status of postmenopausal osteoporosis rat model. *Nutrients*. 2014;6:3288–3302.
39. Zanwar AA, Badole SL, Shende PS, Hegde MV, Bodhankar SL. Role of gallic acid in cardiovascular disorders. *Polyphenols in Human Health and Disease*. 2014;2: 1045–1047.