Original Research Article

Bone turnover and oxidative stress markers in estrogen-deficient rats treated with *Marantodes pumilum* leaves and roots: A comparative study

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Abstract

Purpose: To investigate the relative changes in bone turnover and oxidative status markers of estrogen-deficient rats treated with Marantodes pumilum var. alata (MPva) leaf and root extracts.
Methods: Thirty-six female Sprague-Dawley rats were divided into 6 groups (n = 6), namely: baseline (BL); placebo surgery (PS); ovariectomized control (OVXC); estrogen treatment (ERT); leaf extract treatment (MPv); and root extract treatment (MPr). Rats in all groups, except BL and PS, were ovariectomized to induce menopause. Through the oral route, ERT received 64.5 µg/kg/day estrogen (Premarin®); MPv received 20 mg/kg/day leaf extracts of MPva; MPr received 20 mg/kg/day root extract of MPva; while BL, OVXC, and PS served as untreated controls. At the end of 8 weeks treatment, blood and bone samples were collected for assay of bone turnover markers (osteocalcin and pyridinoline) and oxidative status markers (4-hydroxynonenal, superoxide dismutase, and glutathione peroxidase) using enzyme-linked immunosorbent assay (ELISA).
Results: Significantly higher (p < 0.05) bone level of glutathione peroxidase was seen in MPv when compared with BL and OVXC. MPv also showed lower bone level of pyridinoline (p<0.05) compared to OVXC. Bone level of 4-hydroxynonenal in both MPv and MPr groups was significantly reduced (p < 0.05) when compared with OVXC.
Conclusion: MPva leaf showed more remarkable effects on bone turnover and oxidative stress markers of ovariectomized rats than its roots and estrogen treatment. Thus, the use of MPva leaf, as an alternative to estrogen for treating postmenopausal osteoporosis, would be preferred to its roots.

Keywords: Phytoestrogens, Osteoporosis, Marantodes pumilum

INTRODUCTION

Postmenopausal osteoporosis is a systemic disease of the bone associated with micro-architectural deterioration and loss of bone mass with a consequent reduction in bone quality and increase in risk of fracture [1]. With the increasing population of the elderly, osteoporosis now poses a huge economic and social burden. It is estimated to affect over 200 million women

worldwide with 1 out of every 3 above the age of 50 reported to have experienced osteoporosis-related fracture [2,3]. Fall in bone level of estrogen, a female sex hormone known to stimulate osteoblast differentiation and suppress osteoclast resorptive activity through a cascade of biochemical events, is believed to play a primary causative role in the development of postmenopausal osteoporosis [4].

Drug treatment of osteoporosis previously relied on the use of estrogen as a replacement therapy (ERT). Due to the debilitating nature of adverse reactions associated with ERT, alternative antiresorptive agents such as bisphosphonates (e.g. alendronate) are preferred [5]. These alternatives have also been reported to cause important adverse reactions, thus resulting in a surge in demand for safer alternative or complementary medicine for the management of postmenopausal osteoporosis [6].

For decades*, Marantodes pumilum* var. alata (*MPva*) had been used to manage female reproductive health problems in Malaysia [7]. Pharmacological studies have shown that it possesses osteoprotective properties [8]. A recent study has shown that aqueous leaf and root extracts of *MPva,* differentially preserved bone mineral density and micro-architecture of estrogen-deficient rats [9]. However, despite reports of varying nature and distribution of bioactive phytochemicals in the leaves and roots of *MPva,* very little is known about its mechanism of action, and the plant parts that might be responsible for its proven activity. In the current study, the comparative effects of the leaf and root extracts of *MPva* on bone turnover and oxidative stress markers in osteoporotic rat model were investigated in an attempt to evaluate the relative osteoprotective potentials of its leaves and roots. The animal model utilised in this study provided the opportunity to assay these markers in a characteristic estrogen deficiency state that mimics natural postmenopause.

EXPERIMENTAL

Plant materials

The leaves and roots of *Marantodes pumilum* (Blume) Kuntze were collected on 04/06/2016 from Delima Jelita Herbs, a cultivated site in Kedah, Malaysia. Professor Emeritus (Dr.) Abdul Latiff Mohamad of Department of Biological Sciences and Natural Resources, Universiti Kebangsaan Malaysia (UKM) identified the collected plant materials. A voucher specimen was deposited at UKM herbarium (no. UKM-

HF131). The leaf and root specimens were air-dried under shade, size-reduced with a rotary grinder and extracted in distilled water using reflux method at 60 ºC for 2 h. After extraction, the leaf and root extracts were freeze-dried so that they could be converted to dry extracts and stored at -20 ºC.

Quantification of phenolics

Aqueous leaf and root extracts of *MPva* were screened for phytochemicals using liquid chromatography-tandem mass spectrometry (LC/MS/MS) assay [10]. Seven standards, namely: gallic acid, quercetin, caffeic acid, kaempferol, ellagic acid, apigenin and myricetin were used. The array of LC/MS/MS used consists of AB Sciex 5500 QTrap liquid chromatography tandem mass spectrometer coupled to Agilent 1290 Infinity Ultra-high Performance Liquid Chromatography (UHPLC) system. On a Phenomenex Synergi Fusion C_{18} column (100 × 2.1 mm, 5 μm), the plant extracts as well as the standards were separated by gradient elution using different ratio of mixtures as mobile phase: (A) acetonitrile with 0.1 % formic acid and 5 mM ammonium formate, and (B) water with 0.1 % formic acid and 5 mM ammonium formate.

The mobile phase was allowed to run over 15 min at 250 - 400 µL/min flow rate. Quantification was done by monitoring the precursor-product ion transitions of the respective m/z of the standards using multiple reaction monitoring (MRM) scan; caffeic acid (m/z 179.000), gallic acid (m/z 169.011), kaempferol (m/z 284.916), apigenin (m/z 269.000), ellagic acid (m/z 300.923), quercetin (m/z 300.923) and myricetin (m/z 317.000).

Experimental animals

Healthy female Sprague-Dawley rats weighing 200 - 250 g were involved in this study. The animals were obtained from the Laboratory Animal Unit of Universiti Kebangsaan Malaysia (UKM). All rats were housed in plastic cages with wood shavings bedding at room temperature of 25 ± 3 °C, under natural day-night cycle and humidity. The cages were cleaned every 48 h. Before commencement of the experiment, the rats were acclimatized to the laboratory environment for 7 days. In the course of animal treatment, the rats were allowed free access to standard feed, Gold Coin® (0.97 % calcium, 0.85 % phosphorus and 1.05 IU/g vitamin D3) and filtered portable water. All experimental procedures were conducted in accordance with

the provision of US Guide for the Use and Care of Laboratory Animals [11].

Study design

Thirty-six rats were sorted into six groups of 6 rats each (n = 6): Baseline (BL); placebo surgery (PS); untreated ovariectomized (OVXC); estrogen treatment (ERT); leaf extract treatments (MPv) and root extract treatments (MPr). With exception of the placebo surgery and BL groups, all rats were ovariectomized to induce a state of menopause. The ERT group was treated with 64.5 µg/kg/day dose of estrogen (Premarin®), while MPv and MPr groups were treated with 20 mg/kg/day dose of leaf and root extracts, respectively. Treatments were administered to rats as oral gavages (0.1 ml/100 g) for 8 weeks [12]. At the commencement of treatment and at the end of treatment, blood samples were collected from the tail vein of rats for assay of bone turnover markers (BTMs), pyridinoline (PYD) and osteocalcin (OC). Using a combination of ketamine-xylazine overdose and cervical dislocation, rats in the BL (at the commencement of treatment) and other groups (after treatment) were humanely sacrificed according to AVMA guidelines on euthanasia [13]. The right femora were harvested for assay of oxidative stress markers, 4-hydroxynonenal (4-HNE), superoxide dismutase (SOD) and glutathione peroxidase (GPx). During the treatment period, changes in animals' body weight were also monitored with an electronic balance (Fisher Scientific, model no. 51100213). This study was approved by the Animal Ethics Committee of UKM (UKMAEC, no. FP/FAR/2016/NORAZLINA/28-JAN./720-JAN.-2016-DEC.-2017).

Ovariectomy

The method previously described by Khajuria was adopted [14]. After anesthetizing the animals with ketamine-xylazine mixture (80:10 mg/kg), the abdominal area was shaved and cleaned with 70% alcohol solution. Using a surgical blade (no. 11), a vertical incision creating 0.4 - 0.6 cm wide opening into the peritoneum was made. Through this opening, the underlying uterine tubes were pulled out by gentle retraction to expose the right and left ovaries. The ovaries were cut-off, and the uterine tubes were sutured and returned back to the peritoneum. The opening was then closed using suture and treated with Povidone iodine solution. Postoperatively, the animals were injected 0.1 mg/kg buprenorphine (0.342 /mL) subcutaneously once daily for 3 days to control pain and 5 mg/kg enrofloxacin (Baytril®) intramuscularly every 12 hours for 7 days to prevent infection.

Blood and bone sample collection

Before and after treatment, 3 ml blood samples were collected from the tail vein of each rat and allowed to stand for 3 h in a 5 mL plain vacutainer tube. The collected blood samples were then centrifuged at 3000 rpm (Hereus Labofuge 400) for 10 min, and the supernatant was collected with Pasteur pipette and stored at -70 °C. At the end of the treatment, the right femora were excised from the humanely euthanized rats. After removing all surrounding soft tissues, the dissected bone samples were wrapped with sterile gauze soaked in phosphate buffer solution and stored at -70 °C.

Enzyme-linked immunosorbent assay (ELISA)

Biochemical assay of BTMs was carried out on animal bone collected from animals before and after treatment while screening of bone oxidative status markers was carried out on right femora samples homogenized with Omni bead ruptor 24 (Omni International Inc). Enzyme-linked immunosorbent assay (ELISA) was utilized using commercially prepared kits from licensed distributors. Rat PYD (Elabscience biotech, cat. no. E-EL-R0833) and rat OC/BGP (Elabscience biotech, cat. no. E-E-R0243) kits were used to assay PYD and OC, respectively. Rat 4-hydroxynonenal (Cusabio Biotech, cat. no. EQ027232RA), superoxide dismutase (Cayman Chemical, cat. no. 706002), and glutathione peroxidase (Cayman Chemical 703102) kits were used to assay 4-HNE, SOD, and GPx, respectively. The ELISA protocol used to assay each marker was based on manufacturer's specification as stipulated in the 'user guide'. Depending on the wavelengths requirement, ELISA readers by TECAN Sunrise Instrument (model No. 16039400) or Thermo Fisher Scientific Sdn. Bhd (model no. 1510-01170) were used to read the sample absorbance.

Statistical analysis

The results obtained are expressed as mean ± SEM. Using SPSS software (version 20.0), the data were tested for normality of distribution using Kolmogorov-Smirnov test, analyzed with one-way analysis of variance (ANOVA) tool, and Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Levels of phenolics

The chromatograms obtained from liquid chromatography/mass spectroscopy (LC/MS) revealed different profiles of aqueous extracts of leaves and roots. It revealed the presence of 5.540 µg/mL gallic acid and 0.070 µg/mL ellagic acid in the leaf extract. In the root extract, 2.240 µg/mL gallic acid and 0.115 µg/mL ellagic acid were found.

Oxidative stress markers

Bone levels of 4-HNE were significantly lower ($p < 0.05$) in MPv and MPr groups as well as ERT and BL groups when compared with OVXC and PS groups. MPv and MPr groups further showed significantly lower levels ($p < 0.05$) of 4-HNE when compared with ERT and BL groups (Table 1). Bone levels GPx enzyme were significantly higher ($p < 0.05$) in MPv group when compared with OVXC and BL groups (Table 1). However, no significant difference in bone levels of SOD was seen across all experimental groups (Table 1).

Table 1: Bone oxidative stress markers of ovariectomized rats treated with *MPva* leaf and root extracts

Group	HNE (pg/ml)	GPx (nmol/min/ml)	SOD (U/ml)
BL	114.2 ± 8.0[b,d]	21.40 ± 7	0.19 ± 0.03
PS	209 ± 13.8	45.36 ± 10	0.30 ± 0.05
OVXC	132.8 ± 16.0[b]	20.12 ± 9	0.22 ± 0.03
ERT	217.2 ± 11.8	66.33 ± 21	0.32 ± 0.06
MPv	58.3 ± 10.8[b,c,d]	100.99 ± 28[a]	0.28 ± 0.06
MPr	65.8 ± 19.8[b,c,d]	56.49 ± 25	0.24 ± 0.02

Key: HNE: 4-hydroxynonenal; GPx: Glutathione peroxidase; SOD: Superoxide Dismutase; a' indicates [a] significant difference compared to OVXC and BL groups; [b] significant difference compared to PS; [c] significant difference compared to ERT. Data presented as mean ± SEM ($p < 0.05$, ANOVA).

Bone remodeling markers

Significant reduction in bone levels of OC was seen in animals in the OVXC group when pre- and post-treatments levels were compared ($p < 0.05$). But, no significant difference ($p < 0.05$) was seen in the pre and post-treatment bone levels of OC in both MPv and MPr groups, as well as the ERT and PS groups (Figure 1). However, post-treatment bone levels of pyridinoline were significantly lower ($p < 0.05$) in MPv, ERT and PS groups when compared to OVXC and MPr groups.
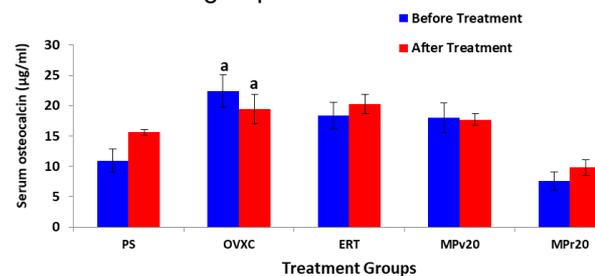

Figure 1: Bone levels of osteocalcin in ovariectomized rats treated with *MPva* leaf and root extracts. Data presented as mean ± SEM ($p < 0.05$, ANOVA). Bars with the same alphabet indicate significant difference between pre- and post-treatment levels
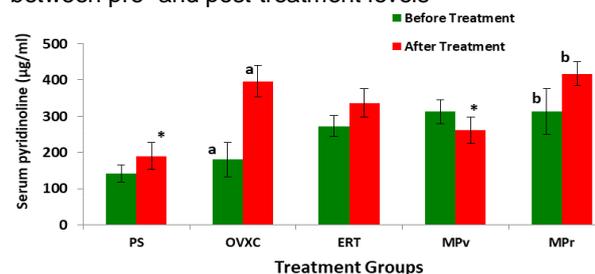

Figure 2: Bone levels of pyridinoline in ovariectomized rats treated with *MPva* leaf and root extracts. *significant difference compared to OVXC and MPr groups. Data presented as mean ± SEM ($p < 0.05$, ANOVA). Bars with the same alphabets indicate significant difference between pre- and post-treatment bone levels ($p < 0.05$, ANOVA)

Table 2: Weight changes in ovariectomized rats treated with *MPva* leaf and root extracts

Group	Weekly animal weight (g)								
	0	1	2	3	4	5	6	7	8
PS	252±3	256±2	256±2	255±3	258±4	261±5	258±5	260±6	264±5
OVXC	265±11	270±12	281±13*	283±14*	288±15*	290±15*	290±16*	288±16*	291±17*
ERT	263±8	266±8	264±9	264±8	267±9	266±8	273±10	273±10	273±10
MPV	260±10	259±10	264±11	268±12	269±11	269±12	272±13	274±13	277±13
MPr	259±17	262±16	261±17	261±16	264±17	268±16	269±16	270±17	272±18

Data presented as mean ± SEM ($p < 0.05$, ANOVA). '*' indicates significant difference compared to other treatment groups ($p < 0.05$, ANOVA). **PS**: placebo surgery group, **OVXC**: ovariectomized control group, **ERT**: estrogen treatment group, **MPv**: 20 mg/kg *MPva* leaf treatment group, **MPr**: 20 mg/kg *MPva* root treatment group

Body weight

Significantly lower animal weight ($p < 0.05$) was seen in MPv, MPr, ERT, and PS groups when compared with OVXC group as early as week 2 (Table 2).

DISCUSSION

Phytochemical investigation revealed the presence of differential quantities of phytoestrogenic constituents, gallic and ellagic acid in the leaves and roots of *MPva*. Phytoestrogens, a diverse group of plant-derived phenolic compounds with estrogenic biological properties, are widely self-prescribed as safe dietary supplement to postmenopausal hormone replacement therapy (HRT) in the management of menopause and postmenopausal osteoporosis. Isoflavones of soy, genistein, has been shown to conserve bone and prevent loss of BMD in ovariectomized rat model [15]. Like estrogens, phytoestrogens are known to act as female sex hormone by displacing oestradiol binding to antibodies raised against oestradiol. They are also known to possess antioxidant effects, a property that makes them more desirable in the management of osteoporosis [16].

Oxidative stress, a prime risk factor in the development of osteoporosis, has been reported to cause an accelerated bone loss due to induced osteoclast resorptive activities from the accumulated harmful free radicals [17]. Ligands generated from lipid oxidation during oxidative stress are also thought to interact with Wnt signaling pathway to cause decreased osteoblastogenesis and age-dependent decreased in bone formation [18]. As the bone remodels, bone-specific biochemical markers known as bone turnover markers (BTMs) are released into the bloodstream and eventually excreted in the urine. Bone or urine levels of BTMs have shown correlation with bone turnover activities, thus providing useful information on bone metabolism that complements bone density and morphology in the assessment of skeletal status, and monitoring of anti-resorptive drug therapy [19]. Clinicians and researchers have assayed BTMs and bone oxidative stress markers in both blood and urine samples to assess bone quality, predict the risk of fracture and monitor responses to drug treatment less invasively [6,20].

Assay outcome of rats' oxidative status revealed a significantly higher bone level of GPx, an integral component of the body's antioxidant system in rats in MPv group when compared with levels of rats in OVXC group (Table 1). Lower bone levels of Gpx seen in the OVXC group could be attributed to ovariectomy-induced estrogen deficiency in rats as higher level of GPx seen in MPv group may suggest reduced free radical generation and suppression of bone resorptive activities. Furthermore, significantly lower ($p < 0.05$) bone levels of 4-HNE were seen in rats in both MPv and MPr when compared to OVXC and PS groups.

When compared with the ERT group that also showed lower ($p < 0.05$) level of 4-HNE when compared to OVXC group, both MPv and MPr groups showed significantly lower ($p < 0.05$) levels of 4-HNE (Table 1). 4-HNE, a product of lipid oxidation, has been reported to cause changes in proliferation and differentiation of osteoblast cells as well as the activation of osteoblast cells apoptosis. Bone level of 4-HNE has been reported to increase in murine skeleton with age [21]. Significantly lower levels of 4-HNE seen in both MPv and MPr groups revealed that both leaf and root extracts of *MPva* possess better protective effects against oxidative stress than estrogen treatment. However, in view of its effect on bone levels of GPx, the leaf extract of *MPva* possesses better antioxidant property than the root extract.

BTMs assay in this study revealed a significant fall in bone level of OC in the ovariectomized rats when pre- and post-treatment bone levels were compared. Bone level of OC of rats in the MPv and MPr, as well as ERT and PS groups, was preserved throughout the course of treatment (Figure 1). OC, a 49-amino acid bone protein, is a sensitive and specific marker of osteoblast activity that is widely used to assess bone formation activities. It has been used as a surrogate marker in clinical evaluation of bone turnover in a variety of bone metabolic diseases and in describing the effects of anti-osteoporotic drugs. In early post-menopause, a temporary increase in bone levels of OC, which is usually followed by a gradual fall has been reported [22]. Changes in bone levels of OC seen indicates that both the leaf and root extracts of *MPva* were able to preserve bone formation activities against estrogen deficiency to a similar extent as estrogen treatment and healthy control. However, the bone level of PYD, a well-established marker of bone resorption, was significantly increased ($p < 0.05$) in rats in OVXC as well as MPr group after treatment, while rats in MPv groups showed no change in bone PYD levels throughout the treatment period.

Bone PYD level seen in MPv groups was similar to that of PS and ERT groups. Furthermore, the

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post-treatment levels of PYD were significantly lower ($p < 0.05$) in the MPv group when compared to levels of both OVXC and MPr groups (Figure 2). PYD is a non-reducible crosslink proteolytic fragment of matured collagen matrix and a direct indicator of bone resorptive activities. Assay of PYD levels in bone and urine has also been used as a tool for clinical assessment of fracture risk in a variety of bone metabolic conditions such as Paget's disease and primary hyperparathyroidism, and to monitor anti-osteoporotic drug treatment [23]. Assay outcome of PYD shows that the leaf extract exerted better preservation of bone resorptive activities, which implies better anti-resorptive activities than the root extract.

High body mass index (BMI) has been shown to be strongly related to high bone mass. Osteoclast suppression due to the increased estrogen production caused by high BMI is believed to be responsible for the increase in bone mass [24]. Although epidemiological data have shown a correlation between BMI and bone mass, obesity is also known to exacerbate several health problems associated with menopause.

In view of these challenges, the effect of *MPva* extracts on animal weight was monitored in this study. In the second week of treatment, significant weight gain was seen in rats in the OVXC group compared to the healthy control. In both the leaf and root extracts treatment groups, as well as estrogen treatment group, weight of rats was not significantly different ($p < 0.05$) from the OVXC group (Table 2). This implies that both the leaf and root extracts of *MPva* were able to suppress weight gain associated with estrogen loss, an outcome that informs the suitability of *MPva* use in postmenopausal condition.

CONCLUSION

The leaf extract of *MPva* reduces oxidative stress, increases bone antioxidant enzyme and reduces bone-resorbing activity in ovariectomized rats. Its effects are superior to both those of the root extract and estrogen treatment. In view of the safety profile of *MPva*, its aqueous leaf extract has the potential to become an alternative medicine to estrogen in treating postmenopausal osteoporosis.

DECLARATIONS

Conflict of interest

No financial or commercial conflict of interest exists with regard to this work.

Authors' contribution

We declare that the authors named in this article did this work and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved this manuscript for publication. The following is the respective contributions of the authors. NM designed the study, acquired and managed the research grant, wrote and approved the article; TRG conducted the experiments (collected and analysed the findings), and wrote the article; ANS, INS and NM helped in designing the study, editing and approving the article; NA carried out plant extraction, standardization and quantification of plant extracts.

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