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Bone Indices and Body Weight Changes of Ovariectomized Rats Treated with *Marantodes pumilum* var. *Alata*

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Marantodes pumilum var. *alata* (MPva) is a popular female aphrodisiac and known source of phytoestrogens. It is traditionally used to manage postmenopausal crisis and preserve bone health in women. The study aims to investigate the effects of MPva on bone indices and bodyweight of postmenopausal rats. Thirty female Sprague-Dawley rats were divided into groups (n=6): Healthy control (Sham); ovariectomized control (OVXC); Estrogen treatment (ERT); plant leaf extract treatment (MPv) and plant root extract treatment (MPr) groups. All rats (except the Sham-operated group) were ovariectomized. After 2-weeks healing period, the ERT group was treated with 64.5 µg/kg estrogen while MPv and MPr groups received pre-determined optimal daily dose of MPva leaf and root extracts, respectively. Changes in bodyweight of rats were monitored through an eight-weeks treatment period after which rats were euthanized for testing of bone parameters. Both leaves and roots of MPva (20 mg/kg) were found to show optimal preservation of bone mineral density and mechanical strength. Untreated estrogen-deficient rats (OVXC) showed significant weight-gain when compared with the healthy control (Sham) ($p < 0.05$). Treatment with MPva leaf and root extracts, similar to treatment with estrogen, significantly suppressed weight-gain observed in the estrogen-deficient (OVXC) group ($p < 0.05$). In conclusion, treatment with MPva leaf and root extracts preserved bone strength. In addition, it suppressed weight gain in postmenopausal rats, an outcome that makes MPva more beneficial to postmenopausal women that may experience weight gain.

Keywords: Kacip Fathimah, osteoporosis, menopause, bodyweight.

1. Introduction

The bone, in order to accommodate mechanical loading imposed by our body weight or external load, undergoes constant modelling that consequently results in increased bone mass [1]. High body weight or body mass index (BMI) has been shown to correlate with higher bone mass. Conversely, low body weight is directly related to bone loss [2, 3, 4]. Although the molecular mechanism through which weight impacts on bone is not fully understood, increased adipose tissue associated with increased BMI was reported to induce a corresponding increase in estrogen production, osteoclast suppression, and a resultant increase in bone mass due to bone modelling [3]. Furthermore, obesity has been shown to be associated with insulin resistance that is characterized by high plasma levels of insulin that may cause androgen and estrogen overproduction in the ovary, and reduced

production of sex hormone-binding globulin by the liver. These changes may result in an increase in bone mass due to reduced osteoclast activity and possibly increased osteoblast activity induced by the elevated sex hormone levels [5]. Thus, weight gain commonly seen in postmenopausal women can be said to be a blessing in disguise. Although epidemiological data show positive correlation between high BMI and bone mass, obesity or excessive body fat is well known to cause or exacerbate several public health problems associated with menopause [6]. In view of these challenge, the effects of medications on the weight of postmenopausal women is equally as important as its primary efficacy. Thus, in order to avoid other complications, it's important to ensure that medications administered to women after

menopause do not enhance their weight-gain tendency.

Marantodes pumilum var. *alata* (MPva, family: Primulaceae) is a popular Asian herb with folkloric claim of efficacy against a myriad of medical conditions including osteoporosis in postmenopausal women. In our previous investigations, we have reported its ability to preserve bone micro-architecture and enhance bone strength [7]. However, in view of the fact that, increase in bodyweight secondary to hormonal dysregulation is common in postmenopausal women, it's important to ensure that medications administered to women after menopause do not enhance their weight-gain tendency in order to avoid other complications. This study aims to investigate the effects of MPva treatment on bodyweight and bone indices of postmenopausal rats in an attempt to evaluate its suitability as an alternative medicine for the maintenance of bone health in postmenopausal women.

2. Materials and Methods

2.1 Preparation of plant extracts

Marantodes pumilum var. *alata* leaves and roots were obtained from a cultivated site at Delima Jelita Herbs in Kedah, Malaysia. As described by [8], the leaf and root specimens were garbled, dried and ground using a milling machine. The dried powder of the leaves and roots were then individually extracted by reflux method at 60 °C for 2 hrs with distilled water (plant to water ratio used was 1:10 and 1:15 for roots and leaves, respectively) and then freeze-dried to obtain dry water extract.

2.2 Experimental animals

Before commencement of the study, all procedures were approved by Universiti Kebangsaan Malaysia Animal Ethics Committee, UKMAEC (FP/FAR/2016/ NORAZLINA/28-JAN./720-JAN.-2016-DEC.-2017).

The female Sprague-Dawley rats weighing 300 ± 5 g were obtained from UKM animal laboratory unit. They were housed in plastic cages (at 25 ± 3°C, natural day-night cycle and humidity), and given free access to standard diet (Gold Coin, Selangor-Malaysia; containing 0.97% calcium, 0.85% phosphorus and 1.05 IU/g of Vitamin D3) and filtered tap water *ad libitum*. Before commencement of study, the animals were allowed to acclimatize to the laboratory environment for 7 days. Freshly prepared treatments were administered, according to the

study design, as oral gavages at volume of 0.1ml/100g.

2.3 Animal surgery

Under anaesthesia (Ketamine: Xylazil, 8:1), with an exception of the sham-operated group, all rats were ovariectomized as described previously [9]. To prevent infection and control pain, after surgery, the animals were injected with 5 mg/kg enrofloxacin (Baytril®) intramuscularly every 12 hours for 7 days and 0.1 mg/kg buprenorphine (0.342 /mL) subcutaneously daily for 3 days, respectively.

2.4 Experimental design

A pilot study was first conducted to determine the optimal dose of the extract using twenty-seven (27) rats that were sorted into nine groups (n=3). The first and second groups were untreated sham-operated (Sham) and ovariectomized (OVXC) control groups while the third group was ovariectomized group (ERT) that received 64.5µg/kg dose of estrogen (Premarin®). The fourth, fifth and sixth groups (MPv20, MPv50 and MPv100) were ovariectomized treatment groups that received 20mg, 50mg and 100 mg/kg doses of *Marantodes pumilum* leaf extract, respectively, while the seventh, eighth and ninth groups (MPr20, MPr50, and MPr100) were ovariectomized treatment groups that received 20mg, 50mg and 100 mg/kg doses of *Marantodes pumilum* root extract. All animals received daily drug treatments as oral gavages for a period of 8 weeks. At the end of the study period, animals' weight, bone mineral density and strength were measured.

In the main study (after the pilot study), thirty female Sprague-Dawley rats were sorted (n=6): Sham-operated (Sham); ovariectomized control (OVXC); Estrogen treatment (ERT); plant extract treatment (leaves-MPv, roots-MPr) groups. All rats (except the Sham) were ovariectomized to induce a state of menopause. Treatment was done for a period of 8 wks. The ERT was treated with 64.5µg/kg/day estrogen while the plant extract treatment groups (MPv & MPr) received the optimal dose of aqueous leaf and root extracts of MPva, respectively. The weights of the animals were monitored before commencement, during and after treatment using electronic balance (Fisher Scientific, No. 51100213).

Reports of this study are in compliance with

Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for reporting animal research [10].

2.5 Bone density testing

Bone density was measured employing micro-computed tomography (MCT) techniques. This test was carried out on the femur bone *ex vivo* using Skyscan micro-computed tomography machine (Skyscan 1076, serial no. 09G02065) as previously described [11]. Utilizing scanning mode: 9µm voxel size, voltage: 82 kVp, current: 112 µA, 0.5mm AL filter, 4000 x 2672 resolution, 2050 exposure and 0.8° rotation, the scanning region of interest was set at the distal femur extending 2.0 mm towards the proximal direction [12]. Scanned x-ray images were then reconstructed using NRecon Skyscan software (Skyscan 1076). Reconstructed images were then processed and analyzed to obtain bone mineral density and quantitative morphometric parameters of trabecular bone using 3D Skyscan analyzer software (CTAN). Using attenuation values of a phantom rod containing known density of calcium hydroxyapatite, bone and tissue mineral density (BMD & TMD) were measured.

2.6 Bone mechanical strength testing

To measure bone mechanical properties, 3-point bending test was employed as previously described by [11]. Universal mechanical strength testing machine, Shimadzu (AG-X 500N) was used for the tests. The machine was first calibrated while bone parameters (diameter, length and mid-point) were measured using digital caliper. Marked 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. Then incremental of load, at 10 mm/sec, from the top of the machine was gradually applied downward at the mid-point of the femora until it fractures. Bone strength parameters: maximum load, stress and strain were measured while Young's modulus was obtained as the slope of stress-strain curve.

2.7 Animal weight monitoring

In both the pilot and main study, as previously demonstrated, the weight of animals was measured before commencement of treatment, during eight weeks of treatment and at the end of

the treatment period. This task was carefully carried out using electronic balance (Fisher Scientific, No. 51100213). The mean weight and standard errors of each group was then calculated.

2.8 Statistical analysis

All the results obtained were expressed as mean \pm SEM. Analysis was done using SPSS software, version 20. Results were first tested for normality of distribution using Kolmogorov-Smirnov test before analysis with one-way analysis of variance (ANOVA) and tukey's post hoc test. Only results with difference at $p < 0.05$ were considered significant.

3. Results

3.1 Bone mineral density

Rats in the OVXC group were observed to possess significantly lower BMD in both the cortical and trabecular bone parts when compared to the Sham group ($p < 0.05$). Similarly, rats treated with estrogen and both leaf and root extracts of *MPVa* were observed to possess significantly higher ($p < 0.05$) BMD in both the trabecular and cortical bone part when compared with the OVXC (Figure 1a & b). A close look at the differential amount of the mineral densities of the treatment groups, only the 20 mg/kg dose of both leaf and root extract of *MPVa* preserved BMD to a similar extent as recorded in the healthy control ($p < 0.05$).

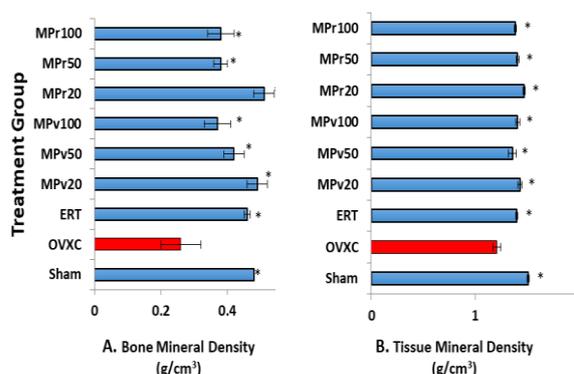


Figure 1: Effects of *Marantodes pumilum* leaves and roots on bone and tissue mineral density. Values expressed as mean \pm SEM. $p < 0.05$ is considered significant (one-way ANOVA) * Indicates significant difference from OVXC group.

3.2 Bone mechanical strength

Maximum load (GPa) and stress (N/m²) parameters of bone were significantly lower ($p < 0.05$) in rats of the OVXC group when compared with rats in the Sham group (Table 1). Similar to the Sham group, these parameters were found to be significantly higher in rats

treated with estrogen ($p < 0.05$). Treatment with both leaf and root extracts of *MPva*, like estrogen treatment, recorded significantly higher maximum load (GPa) of bone when compared with OVXC group ($p < 0.05$).

Table 1: Effects of *Marantodes pumilum* leaves and roots on bone mechanical strength of osteoporotic rats.

Parameters	Treatment Groups								
	Sham	OVXC	ERT	MPr20	PMr50	MPr100	MPv20	MPv50	MPv100
Maximum Force (N)	143.1±	70.3±	131.7±	131.5±	124.0±	129.8±	132.1±	131.7±	127.9±
Maximum Stress (Pa)	12.4*	10.6	16.5*	15.3*	12.9*	11.6*	17.6*	11.2*	10.3*
Maximum Strain	96.37±	13.49±	50.52±	18.42±	22.8±	26.3±	31.31±	27.4±	32.6±
Young's Modulus	11.0 ^a	1.2	7.1*	3.2	4.8	3.9	10.0	6.3	8.2
Maximum Strain	0.071±	0.064±	0.067±	0.051±	0.062±	0.068±	0.073±	0.058±	0.068±
Young's Modulus	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Young's Modulus	1.361±	0.348±	0.754±	0.406±	0.501±	0.463±	0.447±	0.536±	0.612±
Young's Modulus	0.17 ^a	0.16	0.22	0.08	0.13	0.11	0.14	0.11	0.10

Significant when compared with OVXC. ^a significant when compared with other experimental groups. Values expressed as mean ± SEM. Results considered significant at $p < 0.05$ (one-way ANOVA)

3.3 Weight changes in osteoporotic subject

Two weeks after commencement of treatment, significant increase in body weight of rats in the OVXC group when compared with the Sham group was observed ($p < 0.05$). Like the Sham group, rats treated with estrogen (ERT group) also recorded significantly lower bodyweight compared to the OVXC group ($p < 0.05$). Furthermore, rats in MPr20 group recorded lower bodyweights compared to the OVXC group but no significant increase in body weight compared to both the Sham and ERT groups ($p < 0.05$) (Figure 2). Similar to MPr20, rats in the MPv20 group also recorded significantly lower bodyweight compared to the OVXC group. Though not statistically significant, higher body weight of rats was recorded in the plant extract as well as estrogen treatment groups when compared to the Sham group (Figure 2).

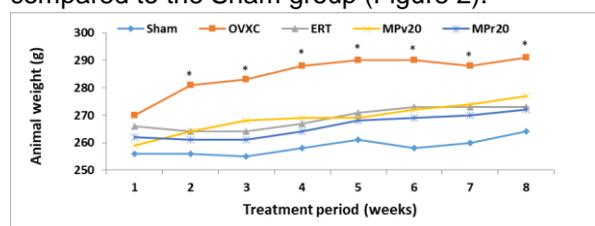


Figure 2: Effects of *Marantodes pumilum* var. alata leaves and roots on bodyweight of osteoporotic rats. Values expressed as mean ± SEM. * Indicates significance at $p < 0.05$ (one-way ANOVA).

4. Discussion

Outcome of the pilot study revealed that rats treated with lower dose of 20 mg/kg of *Marantodes pumilum* var. alata root and leaf extracts possess better bone densities and mechanical strength than those treated with higher doses of 50 and 100 mg/kg when compared to estrogen-deficient rats. This

outcome, which was found to be similar to that of rats treated with the positive control, estrogen, reveals that 20 mg/kg of *Marantodes pumilum* var. alata root and leaf extracts possess better bone protective properties in postmenopausal rats than higher doses of 50 and 100 mg/kg. This outcome also reveals that MPva extract displays declining pharmacology with increasing dose. Thus, 20 mg/kg of *Marantodes pumilum* var. alata root and leaf extracts was adopted for the subsequent investigations.

Compared to the healthy control, rats in the untreated ovariectomized control recorded significant weight gain ($p < 0.05$) from the second week of treatment. Significant increase in rat's body weight observed in the ovariectomized control can be attributed to estrogen deficiency, as a correlation between serum levels of estrogen and weight gain has been previously reported [13]. This point can be further buttressed by study findings that reported a direct link between lower serum estrogen levels and increased food intake and decrease physical activity in laboratory animals [13]. Furthermore, there is evidence suggesting that estrogen hormone therapy increases resting metabolic rate as reduced estrogen level seen during menopause is believed to lower the body's metabolic rate [14]. In current the study, treatment with 20 mg/kg/day dose of both the leaf and root extracts of *MPva*, similar to estrogen treatment, was able to suppress the weight gain seen in the untreated ovariectomized control ($p < 0.05$). Further comparison with the healthy control (sham-operated), the difference in animal body weight amongst the leaf and root extracts of *MPva* as well as estrogen treatment was found to be insignificant ($p < 0.05$). This outcome implies that treatment with 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.

5. Conclusion

Treatment with *MPva* leaf and root extracts preserved bone strength in osteoporotic condition. In addition, it suppressed weight gain in postmenopausal rats. These outcome makes *MPva* extract a potentially beneficial supplementation to postmenopausal women that may experience weight gain.

Conflict of interest

The authors declare no conflict of interest.

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