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Phytoestrogens of *Pachyrhizus erosus* prevent Bone Loss in an Ovariectomized Rat Model of Osteoporosis

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Abstract

The effects of the etyl acetate extract of root of *Pachyrhizus erosus* (L) Urb (EPE) on bone loss and in ovariectomized (ovx) rats model of osteoporosis were investigated. Forty-two 6-weeks-old female Sprague–Dawley rats were randomly assigned to six groups as followed, sham-operated, OVX, OVX-Estradiol (2 μg/day), OVX-EPE 200 mg/kg BW, OVX-EPE 400 mg/kg BW, OVX-EPE 800 mg/kg BW for 4 weeks. The administration of EPE was given orally using a stomach tube. The results demonstrated that the administration EPE 200, 400, and 800 mg/kg BW significantly prevented bone loss in OVX rats which these effect equivalent to estradiol. These effects were described in increased length of femur and tibiae, bone density, and mineral content of calcium and phosphorous in bone ash. EPE also significantly prevented OVX-induced uterine atrophy and increased in body weight gain. The femur mechanical testing significantly increased the ultimate load and stiffness of femurs of ovariectomized-rats that its effect was greater than OVX or sham-operated rats. Increased bone density may lead to enhanced bone strength, reducing the risk of fracture, which is evident in the administration of EPE due to high content of mineral density and content and increase the ultimate load. This effect seems to be pro-estrogenic compound, which suppress bone resorption by directly acting on estrogen receptor in bone sites. This study suggest that phytoestrogen compound from *Pachyrhizus erosus* may offer a potential alternative therapy for the treatment of health problems such as osteoporosis in post-menopausal women.

Keywords: phytoestrogen, *Pachyrhizus erosus*, ovariectomized-rat, osteoporosis

Introduction

Osteoporosis is one of the major health problem, and expected to increase dramatically in the recent decades. National Osteoporosis Foundation reported that about 44 million Americans are at risk of osteoporosis by having low bone mineral contents and densities. Ten million among these adult patients have osteoporosis and the majority of these patients are women. Recent epidemiological studies have

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suggested that the incidence of osteoporosis is a complex interaction due to many factors such as variety of genetic, geographic, and ethnic factors [1-3]. Estrogen deficiency is generally not one of the major of the main risk factors for osteoporosis, but it is indirect and strongly related with the many recognized osteoporosis risk factors especially in women such as thin, advanced age, postmenopausal, amenorrhea, and more drinking alcohol.

Several line of evidence reported the importance of estrogen in bone remodeling and metabolism. Furthermore, the evident from the clinical used that the administration of hormone replacement therapy (HRT) in a dose dependent manner effectively prevents bone loss in postmenopausal women [4,5] and reduces the incidence of osteoporosis [6-9]. Unfortunately, the use of HRT for long term caused several unwanted side effect associated with these powerful steroids and increased risk for breast and endometrial cancers [10,11]. Therefore, further exploration of alternatives and/or adjunctive approaches that can produce clinically relevant prevent bone loss like in osteoporosis would be interest. Non-hormonal theraphy or natural product therapy may more acceptable for the treatment and prevent osteoporosis.

Recently, much attention has been focused on phytoestrogens, especially isoflavones, as a potential safe alternative for pharmaceutical HRT [12]. Phytoestrogen is one of the natural alternatives that appear to offer the most potential for the prevent bone loss. Phytoestrogen is non-steroid plant-derived compounds which structurally similar to estrogen and possesses both weak estrogenic and antiestrogenic effects [13,14]. Previous study in animals showed that phytoestrogen had a protective effect against bone loss due to estrogen deficiency. The consumption of natural phytoestrogen from soybean instead of a casein-based diet had been demonstrated to prevent bone loss in ovariectomized (OVX) rats [15,16]. Similarly, genistein, a phytoestrogen found predominantly in soybean, prevented bone loss in OVX rats [17-19].

Several line of evidences reported that estrogen receptors ER_α and ER_β are presence in bone [20,21]. Both *in vitro* and *in vivo* studies have shown that daidzein, genistein, and their glycosides exert a weak estrogenic effect [22]. In addition, raloxifene shown the positive effects of selective estrogen receptor modulators in animals [23] and humans [24]. Because of their similarity to raloxifene in conformational binding to

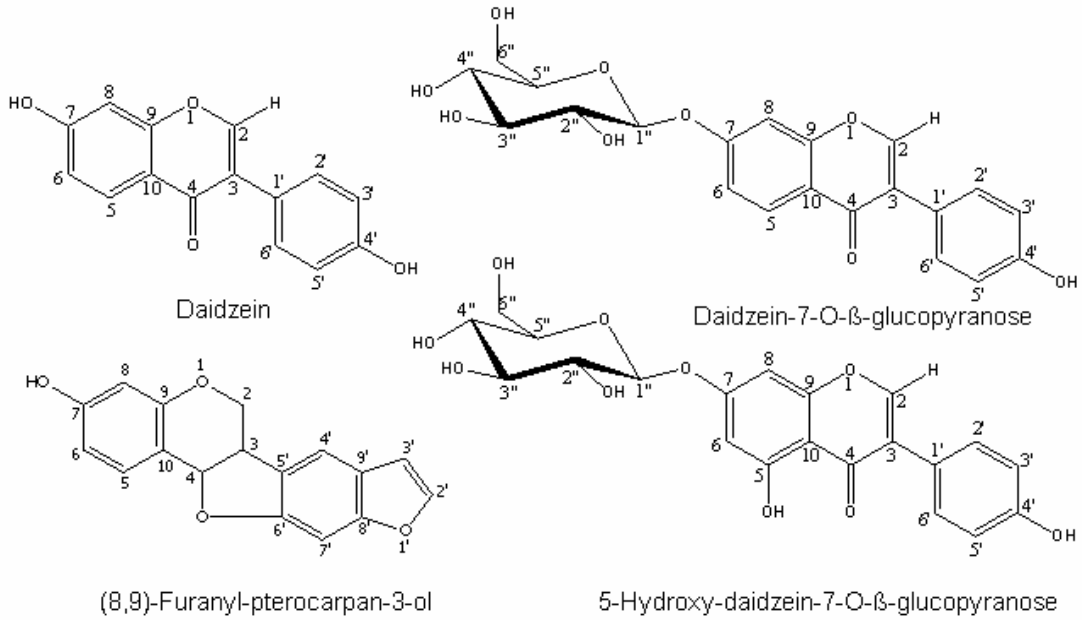

Fig 1. Structure of phytoestrogen compounds from root of *Pachyrhizus erosus* (Lukitaningsih, 2009)

estrogen receptors [25], genistin have selective actions in bone [26,27]. Other studies demonstrated that human dietary studies shown the effects of isoflavone-rich soy protein diets on markers of bone turnover and preventing bone loss as measured from bone mineral density (BMD) and content [28,29]. Recent studies indicate that oral administration of daidzin, genistin, genistein and their succinyl derivatives significantly prevents bone loss in an ovx model of osteoporosis [18,30].

Pachyrhizus erosus (L) Urb or bengkoang is one of the natural plant contain some phytoestrogens. Many years, bengkoang root known and use for traditional cosmetic as sunscreen and whitening. At least four phytoestrogen compounds have been isolated and identified at least 4 phytoestrogen compounds in root of *Pachyrhizus erosus* (L) Urb such as daidzein, daidzein-7-O-β-glucopyranose, (8,9)-furanyl-pterocarpan-3-ol, and 5-hydroxy-daidzein-7-O-β-glucopyranose (Fig.1) [31]. However, the estrogenic effect of phytoestrogen from *Pachyrhizus erosus* (L) Urb have not been investigated especially the novel compound, (8,9)-furanyl-pterocarpan-3-ol. Because of that, there is a great interest to investigate the effect and action of phytoestrogen of root of *Pachyrhizus erosus* (L) Urb on bone and uterine tissue in this osteoporosis model.

Materials and Methods

Plant and Chemical Materials

Pachyrhizus erosus (L) Urb used in the present study were collected from commercial market and authenticated at the Laboratory of Pharmacognosy, Department of Pharmaceutical Biology, Gadjah Mada University, Yogyakarta, Indonesia. Ethanol 96%, methanol p.a, ethyl acetate p.a and petroleum ether p.a. were obtained from Sigma (St. Louis, MO, USA). Estradiol were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

Preparation of Ethyl Acetate Extract of *Pachyrhizus erosus* (EPE)

The dried powders of roots of *Pachyrhizus erosus* were extracted by soxhlet using petroleum ether in order to separate phytosterol. Further, the residue that obtained extracted again using methanol. The methanol extracts evaporated to obtain concentrated extract. This extract further dissolved in water and partition with ethyl acetate. Subsequently, the ethyl acetate fractions obtained were dried at 60°C on steam bath followed by a freeze dried to obtain dried extracts from *Pachyrhizus erosus* (EPE). The extractive value of ethyl acetate from dried powders was calculated as % w/w yield and was found to be 3.71%.

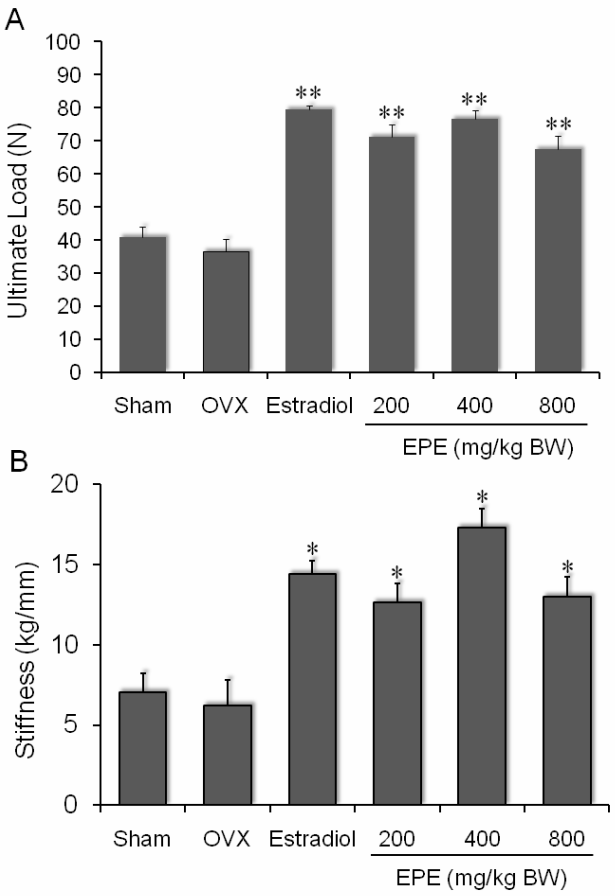

Figure 2. Effect of phytoestrogen of EPE on femoral mechanical strenght [ultimate load (A), Stiffness (B)] in ovariectomized (OVX)-rat model of osteoporosis. Each column represents mean ± S.E.M. of 5 rats. *p<0.05, **p<0.01 significantly different with OVX-rats group.

Animals

Female Sprague–Dawley rats, aged 42 days, were purchased from Animal Laboratory Center Unit (The Laboratory of Research and Assessment, Gadjah Mada University, Yogyakarta, Indonesia). The animals were grouped and housed in polyacrylic cages with not more than five animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C) with dark and light cycle (12/12 h) and allowed free access to commercial pellet diet (PT. Multipala Agrinusa, Indonesia) and water ad libitum.

Administration Procedure

Rats were acclimatized to laboratory condition for 1 week before commencement of experiment. All procedures described conducted in accordance with Guideline for Care and Use of Animals Laboratory of Faculty of Pharmacy, Gadjah Mada University. At 50 days of age, bilaterial ovariectomy was performed *via* a dorsal midline incision under ether anesthesia. Upon recovery from anesthesia, animals were assigned to experimental groups, normal (sham-operated), OVX, OVX-estradiol, OVX-EPE 200, OVX-EPE 400, and OVX-EPE 800, with six to eight animals per group, per experiment. Twenty days after ovariectomy, all the rats were allowed controlled access to a commercial standard pellet and free access to deionized water for 20 days. Normal (sham-operated) and OVX rats were sacrified under light anesthesia to determine the baseline at 70 days. From 70 days, estradiol (2 µg/day), EPE (200, 400 and 800 mg/kg BW/day) was given orally using a stomach tube for 4 weeks. The food intake of all rats was measured every 3 d. At day 28 after first dosing, the urine of each rat was collected over 24 h, using a metabolic cage.

On the day after the last dose, the rats were blood collected from orbital plexus after and sacrified under light anesthesia. The uterus was removed and the wet weight, was determined. The femurs and tibiae were also removed immediately for bone analyses.

Serum and Urine Analysis

Blood samples were centrifuged at 2000 × *g* for 15 min to obtain serum, after standing for 30 min at room temperature. Serum Calcium and Phosphorus: Serum calcium and inorganic phosphate were measured spectrophotometrically, using commercial kits from DiaSys International (Holzheim, Germany). Urinary excretion of creatinine, calcium, and phosphorus were measured with a commercial kit from DiaSys International (Holzheim, Germany).

Bone Length, Density and mineral content

The femurs were also removed immediately after sacrified for bone analyses. The right and left femurs were freed of soft tissue. The removed right femurs were freed of soft tissue using small scissors, tweezers and cotton gauze. The length of each femur was measured with a Vernier caliper. Following the same method as in the previous report [30] bone volume and density were measured by applying Archimedes' principle [32] Then the bones were dehydrated and defatted in acetone and anhydrous ether, dried for 12 h at 110°C and reweighed to obtain the dry bone weights. Bone calcium and phosporus content in ash bone were determined by atomic absorbtion spectrophotometry (AAS).

Femoral Mechanical Testing

Bone Strength (Breaking Force) was measured according to Yao et al. (2005) [33] by means of a three-point bending test on an universal test instrument of the Instron type (Tokyo Testing Machine Mfg Co. Ltd., Tokyo, Japan), as reported previously. The three-point bending test was performed at a displacement rate of 0.05 $mm.min^{-1}$. The load-displacement curve was recorded simultaneously during the test. The ultimate load and the stiffness of the femoral were measured from the load-displacement curve.

Statistical Analysis

Data from the animal experiments were expressed as the mean ± S.E.M. The statistical significance of differences between the groups were assessed with a one-way ANOVA, followed by Bonferroni or LSD post-hoc test analysis using rel 13.0 software SPSS (Chicago, IL, USA). *p* values of less than 0.05 were considered to indicate significant differences.

Results

Body and Uterine Weights

The effect of EPE on average body weight gain and uterus are presented in table 1. As decribed in table 1, ovariectomi caused atrophy of uterus ($p<0.001$). This effect was prevented by the administration of EPE 200 and 400 mg/kg BW, but not 800 mg/kg BW. In other hand, ovariectomy increased average daily body weight gain ($p<0.001$). This effect also was prevented by the administration of EPE 200, 400 and 800 mg/Kg BW. The effect of EPE 800 mg/kg BW was greater than estradiol.

Femoral Length, Density, and Calcium and Phosphorus Content

The activities of EPE on bone were demonstrated in table 2. The result demonstrated that OVX caused bone loss which determined by decreased bone density ($p<0.01$), content of calcium bone ($p< 0.05$) and phosphorous ($p<0.05$) (Table. 2). The results shown that the administration of EPE 200, 400 and 800 mg/kg BW for 28 days capable to increase the length of femur, tibiae, bone density and calcium content in bone (Table 2). These effect of all dose of EPE were greater than estradiol for length of femoral and tibiae. The effect of EPE 400 mg/kg BW on bone density was equivalent with estradiol. Furthermore, the administration of all doses of EPE and estradiol could restore the calcium content loss to the sham-group, but only 400 mg/kg could restore to the sham-group for phosphorous content loss and this effect was equivalent with estradiol.

Table 1. Effect of phytoestrogen of EPE on body weight and uterus in ovariectomized (OVX)-rat model of osteoporosis

Group	Measure			
	Initial body weight (g)	Final body weight (g)	Average Daily Body Weight Gain (g/day)	Uterus weight (g)
Sham	88,70 ± 7,82	141,16 ± 6,05	2,62 ± 0,30	0,199 ± 0,041
OVX	87,90 ± 5,76	160,08 ± 4,66	3,61 ± 0,23###	0,045 ± 0,007###
Estradiol	105,28 ± 6,64	174,74 ± 15,04	3,47 ± 0,75*	0,057 ± 0,015**
OVX+EPE 200 mg/kg	120,20 ± 11,35	187,26 ± 14,38	3,35 ± 0,72**	0,049 ± 0,013*
OVX+EPE 400 mg/kg	118,56 ± 10,16	189,76 ± 12,40	3,56 ± 0,62*	0,063 ± 0,013**
OVX+EPE 800 mg/kg	114,84 ± 11,49	171,42 ± 11,12	2,83 ± 0,56***	0,041 ± 0,015

Values are means±S.E.M., *n*=6–8 rats. Within a column, values with a superscript are significantly different: ## *p*,0.01, ### *p*,0.001 compared with sham rats; *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with OVX rats.

Table.2 Effect of phytoestrogen of EPE on femoral and tibiae lenght, bone density and bone mineral content in ovariectomized (OVX)-rat model of osteoporosis

Group	Measure				
	Femoral lenght (mm)	Tibiae lenght (mm)	Bone density (g/cm^3)	Bone ash content of calcium (%)	Bone ash content of phosphorus (%)
Sham	27,92 ± 0,64	32,57 ± 0,48	1,231 ± 0,071	47,03 ± 8,95	33,09 ± 0,64
OVX	29,22 ± 0,60	34,14 ± 0,52	1,178 ± 0,067##	40,42 ± 2,48#	31,67 ± 1,02#
Estradiol	31,58 ± 1,01*	35,66 ± 0,88*	1,447 ± 0,012***	49,76 ± 2,27**	32,99 ± 1,73*
OVX+EPE 200 mg/kg	33,42 ± 0,75**	37,87 ± 1,19*	1,410 ± 0,036***	47,01 ± 4,62**	30,40 ± 1,86
OVX+EPE 400 mg/kg	32,51 ± 0,92**	37,25 ± 0,57**	1,441 ± 0,064***	50,02 ± 6,55**	32,73 ± 1,09*
OVX+EPE 800 mg/kg	32,83 ± 0,99**	37,60 ± 0,75*	1,377 ± 0,0,84***	48,93 ± 1,87**	31,55 ± 3,25

Values are means±S.E.M., *n*=6–8 rats. Within a column, values with a superscript are significantly different: ## $p<0.01$, ### $p<0.001$ compared with sham rats; *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with OVX rats.

Table.3 Effect of phytoestrogen of EPE on serum calcium, phosphorus and alkaline phosphatase (ALP) in ovariectomized (OVX)-rat model of osteoporosis

Group	Measure		
	Serum calcium (mg/dl)	Serum phosphorus (mg/dl)	Alkaline phosphatase ALP (U/l)
Sham	20,91 ± 1,79	11,04 ± 1,79	605,84±190,83
OVX	21,70 ± 2,63	11,60 ± 1,98	788,03±151,11
Estradiol	14,78 ± 2,62*	19,36 ± 7,27*	364,68±102,21*
OVX+EPE 200 mg/kg	13,13 ± 1,94**	24,72 ± 9,72**	346,70±81,61*
OVX+EPE 400 mg/kg	14,57 ± 2,77*	15,42 ± 2,41*	404,22±73,99*
OVX+EPE 800 mg/kg	14,10 ± 2,88*	15,48 ± 2,59*	439,80±100,53*

Values are means±S.E.M., *n*=6–8 rats. Within a column, values with a superscript are significantly different:

*p<0.05; **p<0.01; ***p<0.001 compared with OVX rats.

Table 4. Effect of phytoestrogen of EPE on urinary calcium and phosphorus in ovariectomized (OVX)-rat model of osteoporosis

Group	Measure				
	Creatinine (mg/dl)	Urinary calcium (mg/dl)	Calcium/ creatinine	Urinary phoshorus (mg/dl)	Phosphorus/ Creatinine
Sham	100,00±64,23	15,87 ± 5,86	0,19 ± 0,09	233,64±61,11	3,08 ± 1,91
OVX	106,00±45,19	14,89 ± 5,85	0,15 ± 0,04#	163,83±64,40#	1,65 ± 0,47#
Estradiol	118,00±25,64*	8,47 ± 2,99*	0,07 ± 0,01***	195,14±86,70	1,65 ± 0,90
OVX+EPE 200 mg/kg	119,00±64,56*	12,38 ± 11,09	0,09 ± 0,06**	136,20±122,00*	0,90 ± 0,62*
OVX+EPE 400 mg/kg	106,25±71,11	9,23 ± 6,26*	0,11 ± 0,06*	144,98±55,02*	1,95 ± 0,83
OVX+EPE 800 mg/kg	117,00±26,36*	10,54 ± 3,79	0,09 ± 0,02**	194,48±44,95*	1,67 ± 0,22

Values are means±S.E.M., *n*=6–8 rats. Within a column, values with a superscript are significantly different: ## p<0.01 compared with sham rats. *p<0.05; **p<0.01; ***p<0.001 compared with OVX rats

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Femoral mechanical testing

In OVX rats, ovariectomy slightly reduced ultimate load, an indicator of the material properties of bone, compared with sham rats (Fig. 1). The results demonstrated that the administration of EPE 200, 400, and 800 mg/kg BW significantly increased the ultimate load and stiffness of femurs of ovariectomized-rats that its effect was greater than OVX or Sham (Fig 1 and 2). EPE 400 mg/kg BW is the optimum dose to achieved greatest ultimate load and stiffness of femoral and its effect was equivalent to estradiol.

Serum and Urinary Calcium and Phosphorus

The effect of EPE on the mineral serum and urinary was presented in table 3. The administration of EPE 200, 400 and 800 mg/kg BW decreased the concentration of calcium in serum that indicated the bone formation. This effect is greater than estradiol (Table 3). Similarly, the clearance of urinary calcium decreased by the administration of EPE 200, 400, and 800 mg/kg BW and estradiol. The clearance of urinary phosphorus also decreased by administration of EPE 200, 400 mg/kg BW but not 800 mg/kg BW.

Discussion

The main objective of this study was to evaluate whether ethyl acetate extract of *Pachyrhizus erosus* (EPE) is effective in preventing bone loss due to ovariectomy and compare with estradiol. Ovariectomized rats are classically used as an animal model for studying the effect of postmenopausal bone loss [32]. Furthermore, they may provide a useful model for investigating the biological effect of EPE on bone loss in rat-ovariectomized. Ethyl acetate extract from *Pachyrhizus erosus* at least contain isoflavone such as daidzein, daidzein-7-O-β-glucopyranose, 5-hydroxy-daidzein-7-O-β-glucopyranose and (8,9)-furanyl-pterocarpan-3-ol [31]. Isoflavones daidzein in *Pachyrhizus erosus* form in conjugate or glycoside which are degraded by gut microflora, which influences their bioavailability. This enterohepatic cycle leads to a new circulation of daidzein in the blood circulation.

Previous study shown that isoflavone genistin and daidzin prevent bone loss in young ovariectomized rats [30]. Other study reported that daidzein more effective preventing bone loss in ovariectomy- induced bone loss in rats [19]. The present study was investigated the potential preventive effects of ethyl acetate exctract of *Pachyrhizus erosus* (EPE) which contain daizein or its glycoside and the novel compound, (8,9)-furanyl-pterocarpan-3-ol on bone loss in animal model of osteoporosis. The administration of EPE prevented OVX-induced increase average body weight gain in rats. This results also support by previous study that daidzin prevented OVX-induced uterine atrophy and increases in body weight gain, abdominal fat, serum total cholesterol and triglyceride [27]. In addition, other study also reported that soybeans-rich isoflavones dietary interventions effectively reduce cholesterol serum in OVX-induced increased cholesterol serum in rats [15].

According with previous report, rats in the OVX group had lower densities of the right femur and tibiae because of reducing the ovariectomy-induced increase in bone resorption [15,19,32]. The administration of EPE 200, 400 and 800 mg/kg BB effectively prevented OVX-induced lowering bone density and increased lenght of femure and tibiae. These observations are supported by previous study that isoflavones daidzin, genistin and glycitin significantly prevented bone loss in OVX rats, like estrone [27]. In addition, the result also demonstrated that the rats receiving EPE shown greater load strain than sham and OVX. Increased bone density may lead to enhanced bone strength, reducing the risk of fracture, which is evident in the administration of EPE due to high content of mineral density and content and increase the ultimate load. The preventive effect of EPE may be due to enhanced intestinal absorption. Although we did not assess intestinal calcium absorption in this study, the enhanced intestinal absorption of calcium along may be by the modulation of parathyroid hormone and renal function [34]. This finding indicated that the administration of EPE for 28

days increased the ovariectomy-induced rate of bone formation. Previous study proposed that daidzein act as proestrogenic compounds based on bone loss and uterine in OVX rats which that may be tissue-specific 27]. Both *in vitro* and *in vivo* studies have shown that daidzein, genistein, and their glycosides exert a weak estrogenic effect [22]. In fact, estrogen receptors are presence in bone [20,21]. In accord similarity of isoflavone content and structure, we propose that the mechanism of preventing bone loss in ovariectomy rats through the binding of its compounds in *Pachyrhizus erosus* (Fig.1) to estrogen receptor in bone.

In summary, we have demonstrated that the administration of ethyl acetate extract from *Pachyrhizus erosus* prevents bone loss in an ovariectomy rat model of osteoporosis. This effect seems to be proestrogenic compound, which suppress bone resorption by directly acting on estrogen receptor in bone sites. Isolation and use of phytoestrogen compound from *Pachyrhizus erosus* may offer a potential alternative therapy for the treatment of health problems such as osteoporosis in post-menopausal women. The mechanisms of phytoestrogen compounds of *Pachyrhizus erosus* on bone in OVX rats appear to be similar to that of estradiol. Further studies are needed to investigate the efficacy of that phytoestrogen in humans.

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