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Full Length Research Paper

Biomechanical evaluation of fracture healing following administration of *Piper sarmentosum* in ovariectomised rats

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The influence of osteoporosis on fracture healing is a complex phenomenon. Oxidative stress is considered as a pathogenic factor for bone loss and delayed fracture healing. *Piper sarmentosum* is known to possess an antioxidant and anti-inflammatory properties. This study aimed to investigate the fracture healing properties of *P. sarmentosum* aqueous extract in ovariectomised rats by observing changes in the biomechanical properties of femora. Forty female Sprague-Dawley rats (200 to 250 g) assigned into 4 groups: Sham-operated; ovariectomised-control; ovariectomised + conjugated equine oestrogen, 100 µg/kg/day, and ovariectomised + *P. sarmentosum* extract, 125 mg/kg/day. All the rats underwent mid-diaphyseal closed fracture of the right femur with K-wire fixation 6 weeks post-ovariectomy. Following the fracture, all the rats received the aforementioned treatment orally for 6 weeks. Biomechanical analysis revealed that, the flexure load, flexure stress and Young's modulus for the *P. sarmentosum*-treated group increased significantly compared to the control group ($P<0.05$). However, the flexure strain was consistent among all the groups ($P>0.05$). The biomechanical strength parameters in the sham, oestrogen and *P. sarmentosum* groups were identical ($P>0.05$). In conclusion, effective supplementation of *P. sarmentosum* extract restored the biomechanical property of the healed bone. Hence, it may be beneficial for fracture healing in the oestrogen deficient state.

Key words: Antioxidant, biomechanics, fractures healing, osteoporosis, *Piper sarmentosum.*

INTRODUCTION

Osteoporosis is a metabolic disease which occurs as a result of an imbalance of the remodelling process leading to bone fragility and an increase in fracture risk (Raisz, 2005). Asian women have the lowest bone mineral density (BMD) compared to other ethnic groups due to the fact that they have low body weight (Barrett et al., 2005). However, Asian and black women have the lowest relative fracture risk compared with other ethnic groups (Handa et al., 2008).

Reactive oxygen species (ROS) played a role in the remodelling process, whereby overproduction of ROS by osteoclasts accelerated bone resorption (Sontakke and Tare, 2002). Ovariectomy induced oxidative stress and subsequent bone loss by increasing the level of hydrogen peroxide (H_2O_2) (Muthusami et al., 2005). Oxidative stress induced bone loss by increasing the expression of cytokines in ovariectomised mice (Jagger et al., 2005).

The majority of therapeutic agents used to treat osteoporosis acted to inhibit bone resorption rather than

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to induce bone formation (Turner, 2002). Despite the beneficial effects of oestrogen in reduction of fracture risk (Compston, 2005), long-term oestrogen replacement therapy (ERT) increased the risk of endometrial and ovarian cancer (Garrett and Quinn, 2008). Due to the high costs and risk of malignancy following long term use of oestrogen, it may be necessary to discover natural products with less side effects that can be used to replace the current treatment modalities.

Piper sarmentosum (PS) is a creeping plant which belongs to the family of Piperaceae. It is widely distributed in the tropical region of Asia. The plant is widely used in traditional Malay medicine for treating diabetes, hypertension and joint aches (Subramaniam et al., 2003). It has been reported that the extract of the PS plant possesses antimicrobial, anti-inflammatory, anticarcinogenic and hypoglycemic properties (Ariffin et al., 2009; Peungvicha, 1998). An earlier pharmacokinetic study based on active amides compounds isolated from PS, revealed that pellitorine and sarmentine had good bioavailability compared to sarmentosine (Hussain et al., 2009). It was reported that PS extract contains high levels of flavonoid compounds such as naringenin (Subramaniam et al., 2003). It has been found that flavonoids reduce bone loss and increased bone strength in ovariectomised rats (Horcajada et al., 2008). An experimental study revealed that PS extract had fracture healing properties (Estai, 2011b). It was shown that PS prevented cell apoptosis induced by H_2O_2 in human umbilical vein endothelial cells (Hafizah et al., 2010). Furthermore, water extract of PS extract exhibited anti-nociceptive and anti-inflammatory activities *in vivo* (Zakaria et al., 2010).

Keeping in mind the aforementioned facts, the present study was planned to investigate the effects of PS aqueous extract on fracture healing in ovariectomised rats. The results from this study may indicate whether PS is an effective agent to promote fracture healing in the osteoporotic state by restoring the bone strength. This may be beneficial for the effective usage of PS as a supplement to treat fracture healing.

MATERIALS AND METHODS

Preparation of PS aqueous extract

Fresh leaves of PS were purchased and the plant was then identified by a botanist from the Medicinal Plant Division, Forest Research Institute Malaysia (FRIM). The freeze drying process of the water extract was done following the extraction process of PS. At the end, the freeze dried extract was stored in a dark bottle and kept at 4°C.

Diphenylpicrylhydrazyl (DPPH) free radical scavenging test

In the current study, PS extract was screened for its possible antioxidant and radical scavenging activity by DPPH technique as in previous protocol (Lee et al., 2004). Scavenging of DPPH free radical is considered the main antioxidant assay (Sharma and Bhat, 2009). DPPH has been widely used to investigate the free radical-scavenging activity of natural antioxidants (Wettasinghe and Shahidi, 2000). DPPH (Sigma, USA) is a radical itself with a purple colour, converts into a stable compound with a yellow colour by reacting with hydrogen ions of an antioxidant (Bondet et al., 1997). In this study, butylated hydroxytoluene (BHT) represented DPPH standard whereas vitamin C acted as positive control. DPPH-MeOH solution of 25 ml was prepared by dissolving 20 µg of DPPH (equivalent to 50 µM of DPPH) in 1 ml of methanol (MeOH) (Univar, Australia), mixed well and stored in a dark room (Karioti et al., 2004). Stock solutions of PS extract and vitamin C (1000 µg/ml) were prepared in a dark room by dissolving 1 mg of each in 1 ml of ddH_2O at ratio (1:1). Stock solution of BHT (1000 µg/ml) was prepared in a dark room by dissolving 1 mg of BHT in 1 ml of MeOH at ratio (1:1). The stock solution of PS extract and standards were then diluted to obtain concentration ranges of 500, 100, 50 and 10 µg/ml. One millliter of DPPH-MeOH stock solution was then added to 1 ml of concentration ranges of PS extract, vitamin C and BHT mentioned previously at ratio of (1: 1). Following 30 min of incubation in a dark room, absorbance was measured at 517 nm using microplate reader (Versamax, Sunnyvale, USA). All samples were run in duplicate and averaged. The percentage of DPPH radical-scavenging capacity was then calculated as the following (Kouame et al., 2009):

Scavenging activity (%) = [1- Absorbance of sample (DPPH + sample) / Absorbance of control (DPPH)] × 100

The IC_{50} values were calculated, which is the concentration sufficient to obtain 50% of a maximum scavenging capacity.

Animals and experimental protocol

This study was approved by the Institutional Animal Ethics Committee, Universiti Kebangsaan Malaysia (UKM). Forty female Sprague-Dawley rats weighing 200 to 250 g were obtained and housed individually in cages. All animals had free access to water and rat chow *ad libitium* and were acclimatized for 2 weeks. Rats were randomly allocated into Sham-operated (S) (n = 10) and ovariectomised groups (n = 30). The S group underwent sham operation while the ovariectomised group received bilateral ovariectomy at the beginning of the study. The rats were left untreated for 6 weeks after ovariectomy for osteoporosis to develop (Estai et al., 2011d). The right femora of all the rats underwent closed fracture. All the rats were anaesthetized with a mixture of xylazil and ketamine (1:1) at a dose of 0.1 ml/100 g (Troy Laboratories, Australia) which was given intramuscularly. An incision was made on the right knee to appreciate the anterior intercondylar notch. A Kirschner wire (K-wire) (Jorgensen laboratories, USA) 1.0 mm in diameter was then inserted into the right femoral medullary canal toward the greater trochanter of the femur. Following the insertion of K-wire, a 1.10 lb steel blade (guillotine fracture device) was fallen from a height on the mid-diaphysis of the rat femur to produce closed fracture as per previous protocol (Estai et al., 2011c). Radiograph images were immediately taken post-fracture to confirm the fracture. Twenty four hours after fracture, the ovariectomised rats (n = 30) were randomly subdivided into 3 groups: (i) Ovariectomised-control (C) group was given vehicle normal saline; (ii) Ovariectomised + PS water extract (P) group was treated orally with PS water extract at a dose of 125 mg/kg/day; (iii) Ovariectomised + oestrogen (E) group was treated orally with conjugated equine oestrogen at a dose of 100 µg/kg /day. The sham (S) group (n = 10) was given vehicle normal saline

Table 1. Description of biomechanical measurements of bone.

Biomechanical measurement	Description
Maximum stress (σ)	Intrinsic - strength
Maximum strain (ε)	Intrinsic - reciprocal of brittleness
Young's modulus (E)	Intrinsic - stiffness
Maximum load	Extrinsic - strength

Biomechanical description of bone fragility includes strength, brittleness, work to failure and stiffness. Bone tissue properties are called intrinsic biomechanical properties which include maximum flexure stress, maximum flexure strain and Young's modulus. The whole bone properties are called extrinsic biomechanical properties which include maximum flexure load or ultimate force (Adopted from Turner, 2002).

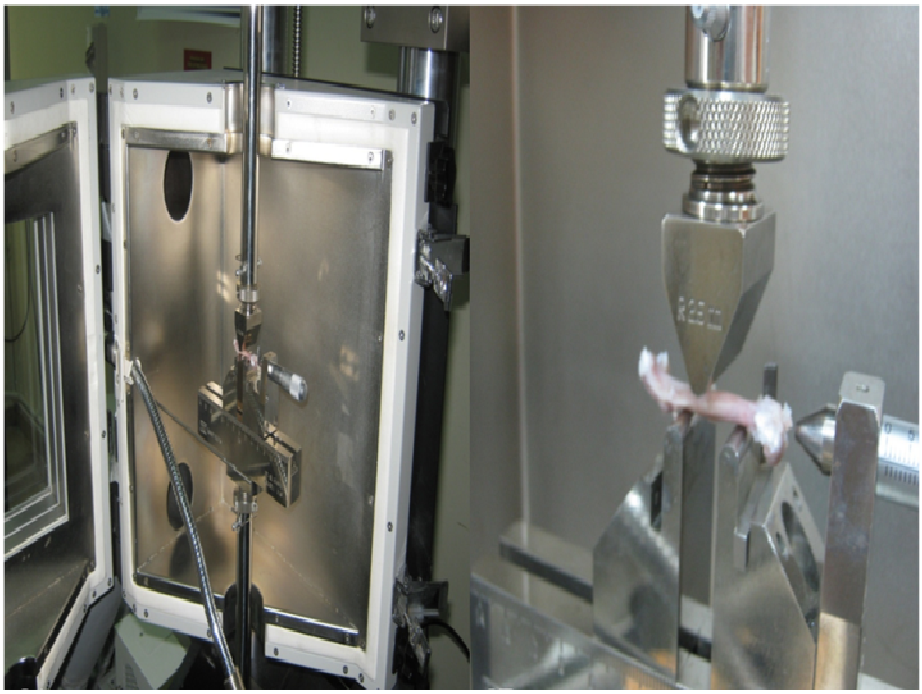

Figure 1. Photograph of three point bending test (Instron Microtester), showing preparation for biomechanical testing of fractured femur sample.

only. All treatments were given orally immediately after fracture of right femur for 6 weeks. At the end of the study, all the rats were sacrificed with an overdose of diethyl ether. The right femora were dissected and each bone sample was then wrapped with sterile gauze soaked in phosphate buffered saline solution and stored at -70°C (Shuid et al., 2010a).

Treatment

The dose of PS extract used in this study was adopted from previous studies which were given at a dose of 125 mg/kg/day (Ima-Nirwana et al., 2009). The extract was dissolved in normal saline before being administered orally to rats. Oestrogen (Conjugated equine estrogen, Premarin-Wyeth, Canada) was given orally at a dose of 100 µg/kg /day; the oestrogen dose was adopted from an earlier study (Estai et al., 2011a). Premarin tablets were crushed and dissolved in normal saline to get the desired concentration.

Measurement of biomechanical bone parameters

Three point bending test of the right fractured femora of all rats was done to determine the maximum flexure load, maximum flexure stress, maximum flexure strain, and elastic modulus (Table 1). As per previous protocol (Shuid et al., 2010a), the frozen right femora were thawed slowly at room temperature. The bone samples were then tested using Instron Microtester 5848 machine (Instron Corp, USA) (Figure 1). The femur was mounted horizontally in the material testing machine on two supports and a load was applied to the middle of the diaphysis corresponding to the site of fracture with the span length of 10 mm and loading speed of 5 mm/min until the bone was fractured. The data were transferred to the computer

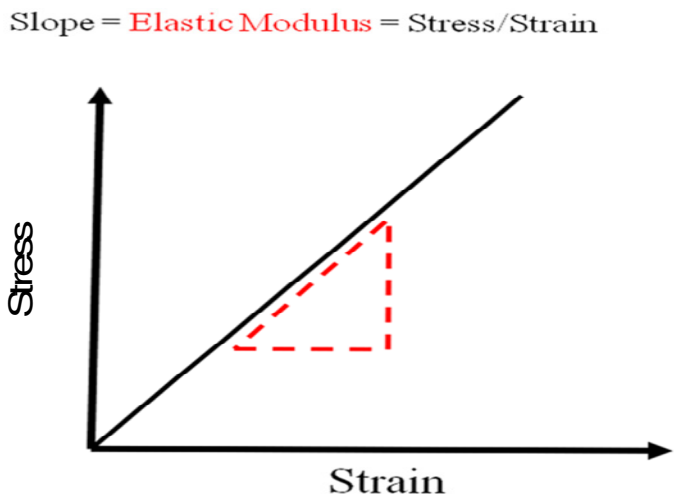

Figure 2. Stress–strain curve. The load-deformation curve was converted to stress-strain curve. Young's modulus is the slope of the linear part of the stress–strain curve.

Table 2. Biomechanical strength measurements of bone.

Biomechanical measurement	Formula	Unit
Maximum stress (σ)	$\sigma = F/A$	MPa (megapascals)
Maximum strain (ε)	$\varepsilon = \Delta L / L0$	mm/mm
Young's modulus (E)	$E = \sigma / \varepsilon$	MPa (megapascals)
Maximum load		N (Newton)

σ, Maximum flexure stress; F, failure load (N); A, cortical area of the specimen (m^2) ; ε, maximum flexure strain, ΔL, change in the length (mm); L0, original length (mm); E, elastic modulus (Adopted from Comelekoglu et al., (2007)

which translated the numerical signals. The Bluhill software package was used to generate a load-deformation curve thus identifying the elastic and plastic zones, which were separated by the yielding point. Bone strength (maximum flexure load) is defined as "the height of the curve" (Turner, 2002), and it represents the maximum compressive force applied until a fracture occurs. The slope of the linear portion of the load–displacement curve defines the stiffness, and the area under the load–displacement curve defines the energy absorption capacity. Load–displacement recordings were then converted to a stress–strain curve (Comelekoglu et al., 2007) (Figure 2). Stress–strain curve was generated, and the following values were calculated: Maximum stress, maximum strain, and elastic (Young's) modulus (Chesnut and Rosen, 2001) (Table 2).

Statistical analysis

Statistical analysis was carried out using the SPSS statistical package version 17. Normal distribution of all variables was examined by using Shapiro-Wilk test. Normally distributed (Parametric) data were analyzed by using one way ANOVA test followed by Tukey's post-hoc test. For all analysis, differences considered were significant at $P<0.05$. The results were presented as mean ± SEM.

RESULTS

DPPH free radical scavenging assay

The PS extract showed dose-dependant increase in activity in accordance with the other tests. Aqueous extract of PS exhibited stronger scavenging activity compared to the BHT, that is, the scavenging activity of PS extract (IC_{50} 54.21 µg/ml) was found to be higher than that of BHT (IC_{50} 93.76 µg/ml). However, it was lower compared to that of vitamin C (IC_{50} 8.03 µg/ml) (Figure 3).

Biomechanical measurements

The mean maximum flexure load (N) for the P group was significantly higher compared to the C group (Figure 4), but it did not differ from the S and E groups. The mean maximum flexure stress (MPa) of the P group increased significantly compared to the C group (Figure 5), but it was identical to the S and E groups. A very high positive

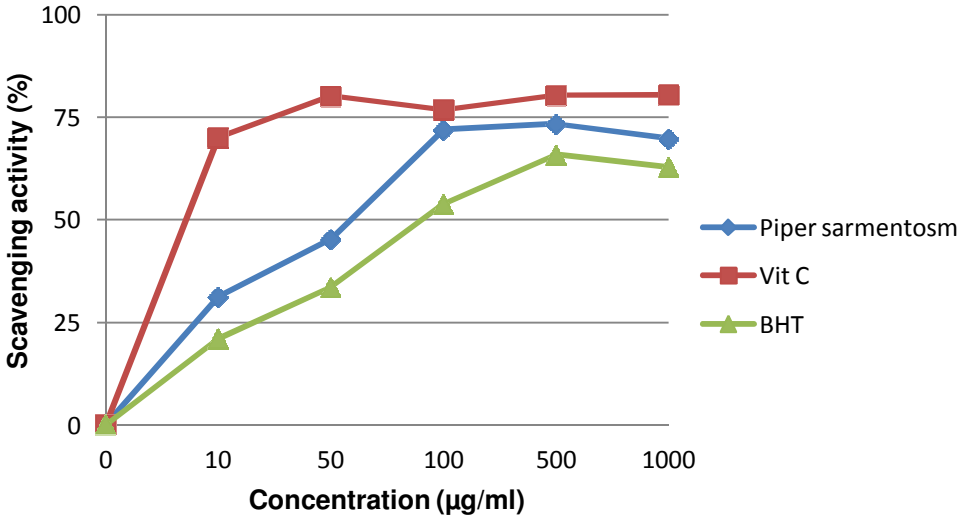

Figure 3. DPPH radical-scavenging assay of Piper sarmentosum aqueous extract. Vitamin C, Vitamin C (positive control); BHT, Butylated hydroxytoluene (DPPH standard). The IC_{50} of antioxidant activity of PS aqueous extract was 54.21 µg/ml.

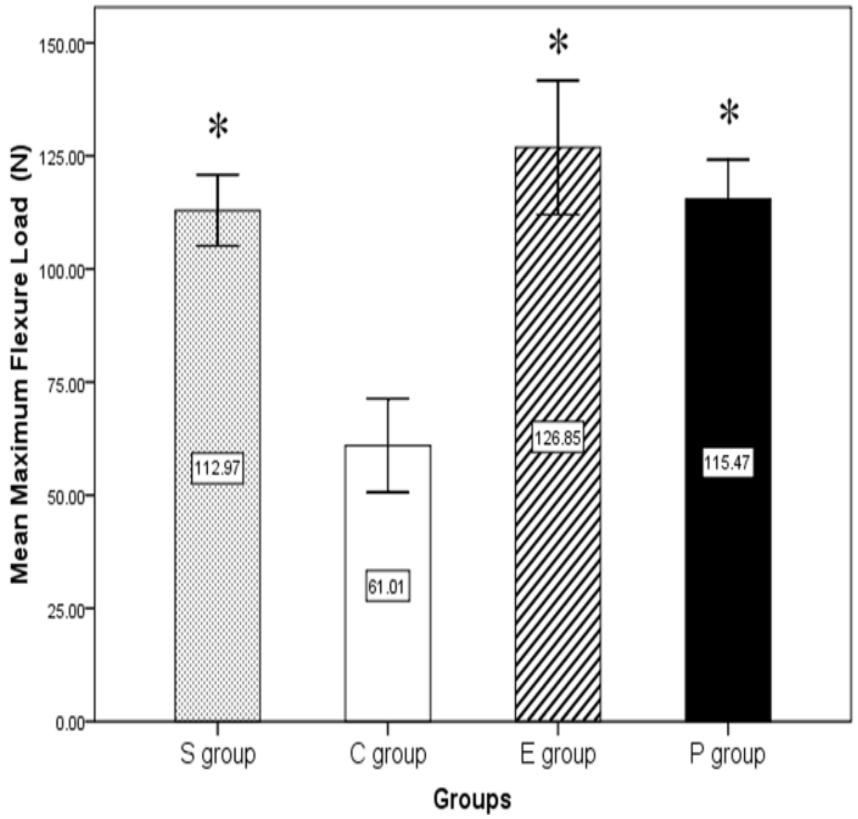

Figure 4. Mean maximum flexure load, after six weeks of treatment following fracture of the right femora. S, sham-operated group, treated with normal saline; C, ovariectomised group, treated with normal saline; E, ovariectomised group treated with conjugated equine oestrogen 100 µg/kg/day; P, ovariectomised group, treated with PS extract 125 mg/kg/day for another 6 weeks; n = 10. P< 0.05 is considered significant.*P< 0.05 as compared to the C group. All values were expressed as Mean ± SEM.

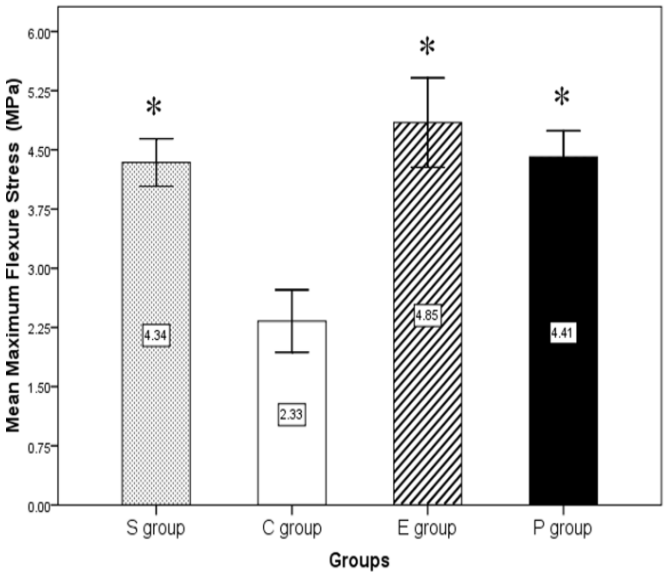

Figure 5. Mean maximum flexure stress, after six weeks of treatment following fracture of the right femora. S, sham-operated group, treated with normal saline; C, ovariectomised group, treated with normal saline; E, ovariectomised group treated with conjugated equine oestrogen 100 µg/kg/day; P, ovariectomised group, treated with PS extract 125 mg/kg/day for another 6 weeks; *n* = 10. P< 0.05 is considered significant.*P< 0.05 as compared to the C group. All values were expressed as mean ± SEM.

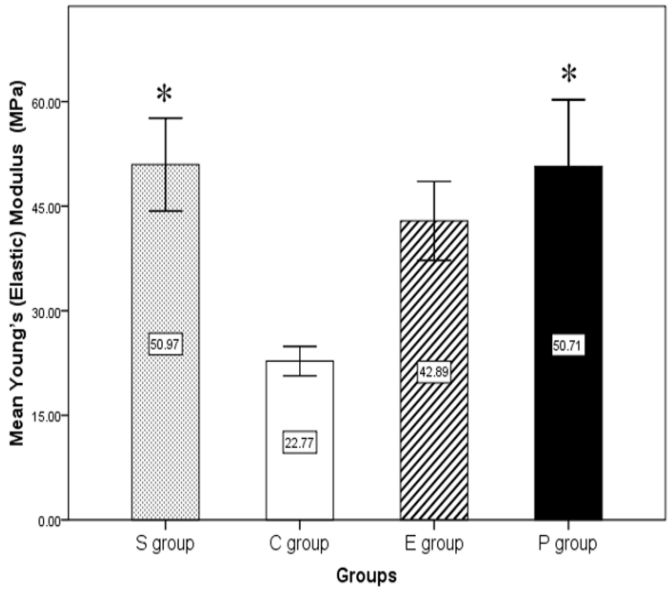

Figure 6. Mean Young's modulus, after six weeks of treatment following fracture of the right femora. S, Sham-operated group, treated with normal saline; C, ovariectomised group, treated with normal saline; E, ovariectomised group treated with conjugated equine oestrogen 100 µg/kg/day; P, ovariectomised group, treated with PS extract 125 mg/kg/day for another 6 weeks; *n* = 10. P< 0.05 is considered significant.*P<0.05 as compared to the C group. All values were expressed as Mean ± SEM.

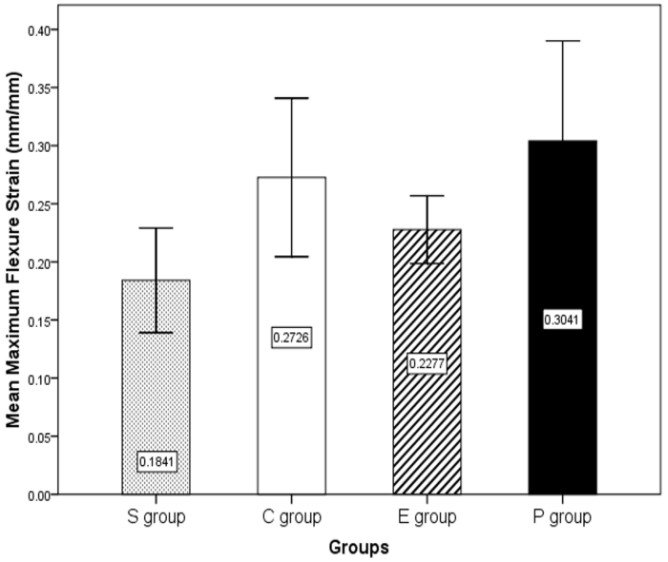

Figure 7. Mean maximum flexure strain, after six weeks of treatment following fracture of the right femora. S, Sham-operated group, treated with normal saline; C, ovariectomised group, treated with normal saline; E, ovariectomised group treated with conjugated equine oestrogen 100µg/kg/day; P, ovariectomised group, treated with PS extract 125 mg/kg/day for another 6 weeks; *n* = 10. No significant difference was observed among all the groups (P>0.05). All values were expressed as Mean ± SEM.

correlation was seen between flexure load and flexure stress which was statistically significant at P<0.01 (r = 1.0). Furthermore, the mean Young's modulus (MPa) of the P group was significantly increased compared to the C group, while it was consistent with the S and E groups (Figure 6). However, no significant difference was observed in the mean maximum flexure strain (mm/mm) among the four groups (Figure 7).

The mean values of all biomechanical parameters aforementioned are summarized in Table 3.

DISCUSSION

The aqueous extract of PS revealed stronger inhibitory activity against superoxide anion generation than BHT but it had weaker activity than vitamin C. Subramaniam et al. (2003) found that the Naringenin compounds present in the PS extract had high superoxide scavenging activity. The Naringenin is believed to be responsible for the radical scavenging activities of the PS extract. Furthermore, Hafizah et al. (2010) reported that PS extract had high phenolic contents with potent ferric reducing antioxidant activity. In the oestrogen deficient state, the activity of osteoclasts was increased as a result of the overproduction of ROS, which resulted in acceleration of bone loss (Sheweita and Khoshhal, 2007)

Table 3. The mean values of biomechanical parameters.

Group	Maximum load (N)	Maximum stress (MPa)	Maximum strain (mm/mm)	Young's modulus (MPa)
S	112.9±7.86*	4.33±0.30*	0.18±0.04*	50.9±6.67*
C	61.0±10.35	2.33±0.39	0.27±0.06	22.7±2.11
E	126.8±14.82*	4.84±0.56*	0.22±0.03*	42.8±5.64
P	115.4±8.70*	4.41±0.33*	0.30±0.08*	50.7±9.55*

S, sham-operated (normal saline, 6 weeks); C, ovariectomised-control (normal saline, 6 weeks); E, ovariectomised + oestrogen (100µg/kg/day conjugated equine oestrogen, 6 weeks); P, ovariectomised + PS (125 mg/kg/day PS extract, 6 weeks); n = 10. P< 0.05 is considered significant.*P< 0.05 as compared to the C group. Values were expressed as Mean ± SEM.

2007). The free radical scavenging activity of PS extract most probably reduced the level of ROS by inhibiting the generation of superoxide anion at the fracture site, hence preventing the effect of the oxidative stress. Earlier studies showed that supplementation with palm oil tocotrienol prevented free radical FeNTA-induced bone loss in rats (Ahmad et al., 2005). Bone loss following oestrogen deficiency can be suppressed by the administration of catalase, an antioxidant enzyme (Lean et al., 2005). This was in line with other reports which found that administration of antioxidants may be beneficial for osteoporosis and acceleration of osteoporotic fracture healing (Sheweita and Khoshhal, 2007).

The biomechanical integrity of bone is considered as the main factor associated with the risk of fracture (Reddy et al., 2001). It is important because it has a structural role in the fracture healing process (Liebschner, 2004). Maximum flexure load is the most widely used biomechanical parameter, and it represents the maximum compressive force applied until a fracture occurs (Comelekoglu et al., 2007). The biomechanical description of bone fragility includes strength, brittleness, work to failure and stiffness (Turner, 2002). Osteoporosis affects the biomechanical properties of bone by reducing work to failure (bone absorbs small amount of energy before breaking), and causing brittle bones (Turner, 2002), therefore increasing the risk of fractures. Biomechanically, a drug used to treat bone fragility should increase the strength and reduce the brittleness of bone (Turner, 2002). Many previous studies have investigated the biomechanical properties of healed bone. Biomechanical parameters are used for assessment of the effects of different types of treatment on bone healing (Liebschner, 2004). Biomechanically, bone fragility can be reversed by improving bone strength (Flexure load and stress) and by decreasing brittleness (Flexure strain) of bone (Turner, 2002). Earlier research reports showed that osteoporosis affected the early and late periods of fracture healing in ovariectomised rats (Namkung-Matthai et al., 2001; Kubo et al., 1999). It was found that, in normal rats, closed fractures are completely healed 6 weeks (Tagil et al., 2009). Mechanical restoration of fractured bone is considered as the main target of fracture healing in clinics (Fu et al., 2009). However, restoration of biomechanical properties may not be indicative of ultimate complete healing.

In this study, the C group showed significant decline in the flexure load, flexure stress and Young's modulus, compared to the S group. Oestrogen loss probably delayed mineralization of the callus and induced osteoporotic changes in the callus. The callus was mainly made up of cartilage, thus leading to a decrease in the biomechanical properties of the callus. This was demonstrated in earlier studies which found that the strength and stiffness of healed bone were decreased in the ovariectomised-control compared to the sham group in the early period of fracture healing (Namkung-Matthai et al., 2001). This was also in agreement with Shuid et al. (2010b) who reported that the sham group had significant improvement in the bone strength and stiffness as compared to the ovariectomised-control and ovariectomised + calcium groups. It was shown that the strength of the healed bone in the ovariectomised-control was inferior compared to the sham group at 12 weeks post-fracture, but was identical at 6 weeks post-fracture (Kubo et al., 1999). The different results may be due to the open fracture method which was used to induce fracture in that study whereas in our study we adopted closed fracture method. Hence, the oestrogen deficient state delayed fracture healing by decreasing bone strength and stiffness of the fractured femur.

There was significant increase in the flexure load, flexure stress and Young's modulus in the P group compared to the group C. Treatment with PS had increased the load and stress as well as increased Young's modulus, but it had no effect on the flexure strain. This may indicate that strength and stiffness are the major determinants in restoring mechanical property rather than strain. Thus, treatment with PS improved the strength and stiffness of bone by restoring its biomechanical properties.

S, sham-operated group, treated with normal saline; C, ovariectomised group, treated with normal saline; E, ovariectomised group treated with conjugated equine oestrogen 100 µg/kg/day; P, ovariectomised group, treated with PS extract 125 mg/kg/day for another

6 weeks

The flexure load, flexure stress and flexure strain in the sham-operated group, treated with normal saline (S), ovariectomised group treated with conjugated equine oestrogen 100 µg/kg/day (E) and ovariectomised group, treated with PS extract 125 mg/kg/day (P) groups were identical. Therefore, treatment with PS and oestrogen improved fracture healing by increasing the bone strength of osteoporotic fractured femora. This was consistent with a very high positive correlation seen between the load and stress of the healed bone. Young's modulus in the P group was slightly higher but was not significantly different compared to the E group. Young's modulus in the E and C groups was identical. This suggested that the P.s extract exhibited better effects by improving the stiffness of healed bone compared to the oestrogen group. Treatment with oestrogen after menopause was shown to prevent bone loss and reduce fracture risk (Compston, 2005). It was found that the flexure stress, flexure stiffness and Young's modulus in the ovariectomised + estradiol and sham-operated groups were consistent at 16 weeks post-fracture (Cao et al., 2002). This may indicate restoration of the biomechanical properties in the ovariectomised state as compared to the control group. Oestrogen decreased bone resorption by inhibiting osteoclast activity and osteoclastogenesis (Chen et al., 2009). Therefore, treatment with oestrogen improved the strength of healed bone most probably by reducing bone resorption. Treatment with PS may have advantage over ERT in terms of improving the stiffness as well as the strength of bone.

There was no significant difference observed in the flexure strain among all the groups. The strain which is a reciprocal to brittleness was consistent in all the groups. This may indicate that fracture healing even in the oestrogen deficient state was able to restore the strain. Kubo et al. (1999) reported that, the strength of healed bone in the ovariectomised-control and sham-operated groups were identical at the early stage of fracture healing, but the bone strength was lower in ovariectomised-control group at the late stage of fracture healing. This may suggest that bone strength was affected mainly in the later period of fracture healing. Consistent strain between the groups may be attributed to the fact that we assessed the fracture healing at 6 weeks post-fracture, the time considered as the beginning of the remodelling process. During this period, the callus is mainly made up of woven bone, which will then be replaced by lamellar bone at the end of the remodeling process. Osteoporotic fracture healing may be able to restore biomechanical pre-fracture properties (Namkung-Matthai et al., 2001), but this restoration may not indicate complete fracture healing. Based on these results, bone fracture may show union even in the oestrogen deficient state, but may end up in the formation of immature callus.

Conclusion

Treatment with PS and oestrogen had beneficial effects on osteoporotic fracture healing by improving the strength (Flexure load, stress). The flexure strain was consistent in all groups which may suggest that osteoporotic fracture healing may occur even in the oestrogen deficient state. The bone strength was probably restored in the late period of fracture healing while the strain was restored in the early period. These results may suggest that the improved fracture healing after administration of PS extract contributed to the bone strength and stiffness rather than to the flexure strain. Treatment with PS extract had some advantage over ERT in that long-term oestrogen treatment has potential to develop tumours. Hence, PS may be used as supplement for patients suffering from osteoporotic fracture. Further studies may be required.

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