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A Micro-Computed Tomography (micro-CT) Analysis of Postmenopausal Osteoporotic Rat Models Supplemented with *Ficus carica*

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ABSTRACT

Estrogen replacement therapy (ERT) is known to be effective as an anti-osteoporotic treatment for postmenopausal women but exerted many adverse effects. *Ficus carica* (FC) or fig is a fruit rich in antioxidants and anti-inflammatory sources that are believed to contribute to its potential as ERT alternative. This study aimed to evaluate the effects of FC supplementation on the trabecular microarchitecture of postmenopausal osteoporotic rat models. Fifty-six adult female Sprague-Dawley rats were randomly divided into groups of Sham-operated (Sham), ovariectomized control (OVX), ovariectomized with 64.5 µg/kg estrogen Premarin (ERT), ovariectomized with 50 mg/kg aqueous extract of FC (FC50), ovariectomized with 100 mg/kg aqueous extract of FC (FC100), ovariectomized with 50 mg/kg raw extract of FC (RAW50) and ovariectomized with 100 mg/kg raw extract of FC (RAW100). The treatments were given daily via oral gavage for eight weeks. After the treatment, rats were euthanized and femora were dissected out for micro-computed tomography (micro-CT) analysis. Trabecular structure of OVX femur showed significant osteoporotic deterioration in bone volume, trabecular separation, structural model index and connectivity density. ERT significantly reversed the osteoporotic-induced bone changes comparable to Sham level. RAW50 showed the best FC treatment in preserving bone microarchitecture. In conclusion, raw extraction of FC has high potential as an alternative against osteoporosis due to its high mineral contents and bioactive compounds.

INTRODUCTION

Bone mass is regulated by the balance between osteoclast and osteoblast activities. Osteoporosis is a state where bone resorption activity overwhelms the bone formation activity. In women, estrogen deficiency after menopause causes acceleration of bone resorption activity and this lead to osteoporosis which is a common skeletal disorder among the women (Manolagas, 2010; Miyauchi *et al.*, 2013). Therefore, osteoporosis is more prevalent among postmenopausal women than men as estrogen plays a crucial role in bone regulation (Faienza *et al.*, 2013; Tian *et al.*, 2017). It was recorded in Europe in 2010, that 21% of women aged above 50 years old are classified as osteoporosis which was three times greater than men that recorded only 6% (Hernlund *et al.*, 2013).

Since estrogen plays a crucial role in bone regulation, hormone replacement therapy is one of the common conventional treatments used against postmenopausal osteoporosis. Even though it is proposed as an effective treatment for reducing bone osteoporotic changes in postmenopausal osteoporotic women, its long-term use may be accompanied by adverse effects such as breast cancer, stroke, thromboembolism and ovarian cancer (Bernabei *et al.*, 2014; Sood *et al.*, 2014). Other treatments used against osteoporosis include selective estrogen receptor modulators (SERMs) such as raloxifene, and biphosphonates such

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as alendronate and risedronate (Goldhahn *et al.*, 2010; Huang *et al.*, 2010; Inderjeeth *et al.*, 2012). Both SERMs and bisphosphonates are anti-resorptive agents that inhibit the acceleration of bone resorption activity, thereby increasing bone mineral density and preserving the bone structure (Das and Crockett, 2013). However, most of these drugs showed detrimental side effects

such as esophageal cancer, hypocalcemia, gastrointestinal intolerance, atrial fibrillation, venous thromboembolism, and stroke (Abrahamsen, 2010; Rizzoli *et al.*, 2011; Mcclung *et al.*, 2013). Hence, the use of natural products with anticipating fewer side effects might provide an alternative approach in managing postmenopausal osteoporosis.
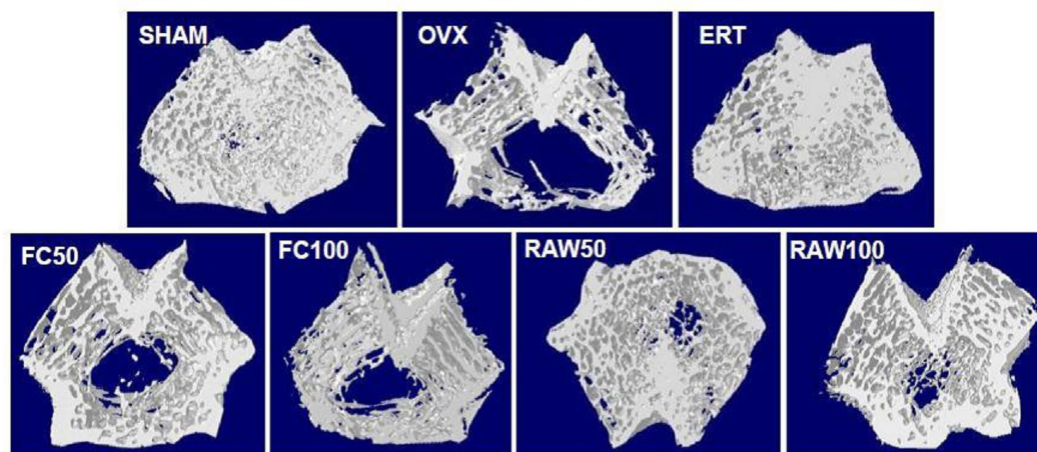

Fig. 1: The 3D image of bone microarchitecture of each group.

Table 1: The minerals and phytochemicals contents of fresh fruit *F. carica.*

Mineral contents	
Calcium	22.44 mg/100 g
Magnesium	9.42 mg/100 g
Zinc	0.53 mg/100 g
Phytochemical contents	
Alkaloids	-
Saponins	+
Flavonoids	+
Tanins	+
Steroids	+
Triterpenes	-

+ Indicate the presence of content, - indicate the absence of content.

Some of the new natural alternatives that were proposed to have anti-osteoporotic properties with minimal side effects are extracts of Labisia pumila, virgin coconut oil, mulberry and blackcurrant (Abujazia *et al.*, 2012; Zheng *et al.*, 2016; Nadia *et al.*, 2014; Jao *et al.*, 2016). These plants contain antioxidative bioactive compounds such as polyphenols, flavonoids, anthocyanins, *ferulic* acid and p-coumaric acid, which were reported to have the capability in improving bone strength as well as preventing or reducing the bone loss of ovariectomized rat models (Levis and Lagari, 2012; Romero and Rivas, 2014). They inhibited the bone resorption activity by scavenging the superoxide radical and suppressed the lipid peroxidation (Abujazia *et al.*, 2012).

Ficus carica or common fig is another fruit which is believed to contain a high level of antioxidant properties after berries (Çalışkan and Aytekin Polat, 2011; Mawa *et al.*, 2013; Harzallah *et al.*, 2016). *F. carica* is also known to contain

an abundance of minerals such as calcium, magnesium, and strontium which are important for bone health (Joseph and Raj, 2011; Pareira *et al.*, 2017). Due to its high antioxidant and nutritional contents, *F. carica* has potentials as an alternative treatment in preserving and preventing postmenopausal osteoporosis. This experimental study is aimed to evaluate the effects of *F. carica* on the bone microarchitecture in osteoporotic rat models. Bone microarchitecture evaluations were accessed using micro-computed tomography (micro-CT) replacing the conventional methodology, histomorphometry which is limited to two-dimensional information, destructive techniques, and time-consuming (Kochi *et al.*, 2010). Micro-CT is known to be effective in determining the efficiency of pharmacological interventions on bone using three-dimensional structures which give the insight of bone microarchitecture. The microarchitectural structure provided is used as an indicator of bone mass, strength, density which aids in diagnosis the risk of osteoporosis (Kim and Henkin, 2013; Fonseca *et al.*, 2014).

MATERIAL AND METHODS

Animal and treatment

This study was performed according to the experimental protocol approved by Universiti Sains Islam Malaysia Animal Ethics Committee (USIM/AEC/AUP/2016(4)). A total of 56 female Sprague-Dawley rats aged five to six months weighing between 200 g to 250 g were used in this study. The rats were housed in a ventilated cage at a temperature of $29^{\circ}C \pm 3^{\circ}C$ under natural day/night cycle. They were supplied with normal food pellets (Gold Coin, Malaysia) and tap water *ad libitum*. They were acclimatized to the new environment for a week before the study was started. The rats were randomly assigned into groups of Sham-

operated (Sham), ovariectomized control (OVX), ovariectomized with supplementation of 64.5 µg/kg of estrogen Premarin (ERT), ovariectomized with supplementation of 50 mg/kg *F. carica* aqueous extract (FC50), ovariectomized with supplementation of 100 mg/kg *F. carica* aqueous extract (FC100), ovariectomized with supplementation of 50 mg/kg *F. carica* raw extract (RAW50) and ovariectomized with supplementation of 100 mg/kg *F. carica* raw extract (RAW100). Rats were ovariectomized to induce an osteoporotic state. The treatments started two weeks following the ovariectomy to ensure healing of the rats as well as successful osteoporosis induction. The treatments were given daily, for eight weeks via oral gavages.
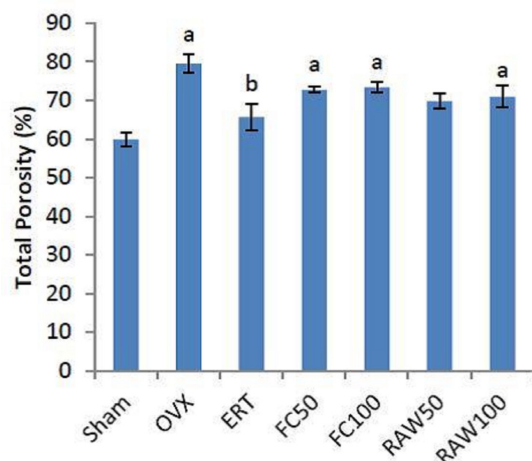

Fig. 2: Mean percentage of total porosity of all groups. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values. 'a' indicates there was significant difference with Sham group and 'b' indicated there was significant difference with OVX group.
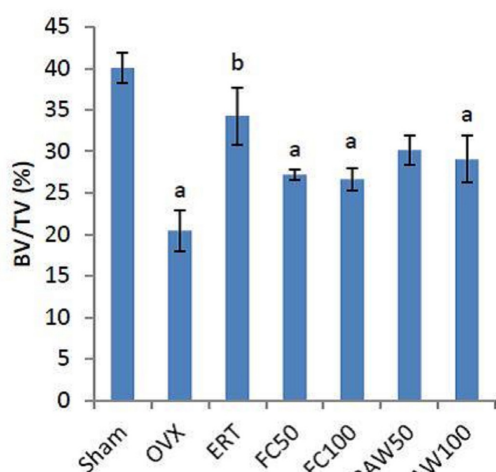

Fig. 3: Mean bone volume fraction percentage of all groups. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values. 'a' indicates there was significant difference with Sham group and 'b' indicated there was significant difference with OVX group.

Ficus carica (FC) and Estrogen (ERT) preparation

Fresh *F. carica* (FC) were obtained from fig Fertigation (Sungai Ramal Dalam Kajang, Selangor). The fresh *Ficus carica* fruits were sent to Malaysian Agricultural Research and

Development Institute (MARDI) and Forest Research Institute Malaysia (FRIM) for the mineral content and phytochemical test (Table 1). The *F. carica* raw extraction was prepared by using 1:1 ratio of raw figs and deionized water (100 mg: 100 ml) for 100 mg/ kg solution and 1:2 ratio of raw figs and deionized water (100 mg: 200 ml) for 50 mg/kg solution. Aqueous extraction of *F. carica* was performed by Phytes Biotek Sdn Bhd (Shah Alam, Malaysia). The sample was extracted using an extractor with ratio 1 kg (raw FC): 10 L (reversed osmosis water) at 95°C for 3 hours. Then, the extracted sample was freeze-dried and evaporated at 70°C-80°C in vacuum condition until the minimum volume of concentrate was achieved. The concentrated liquid was further concentrated using rotary evaporator and freeze-dried into powder at a temperature between −20°C to 30°C for three days. The extracted powder was dissolved in deionized water for 50 mg/kg and 100 mg/kg dosage of rat weight. Estrogen Premarin (Wyeth-Ayerst, Canada) tablets containing conjugated estrogen were crushed, dissolved in deionized water and were given via oral gavage at the dose of 64.5 µg/kg rat weight. The treatments were given daily for 8 weeks via oral gavage.
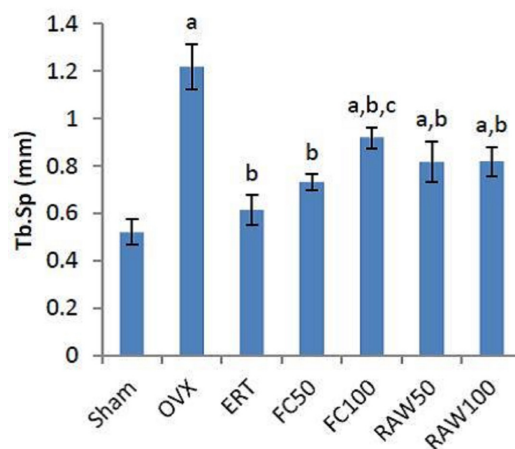

Fig. 4: Trabecular separation values of each group. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values. 'a' indicates there was significant difference with Sham group; 'b' indicated there was significant difference with OVX group and 'c' indicates there was significant difference with ERT group.

Bone sampling

Rats were put into euthanasia by an overdose of diethyl ether via inhalation. After the rats were sacrificed, the muscles and tissues of the limb were cut to expose the femora. Then, the hip and knee joints were cut to remove the femora. The muscle tissues attached around the femora were cleaned and removed. The clean femora were kept in 10% formalin solution at room temperature for preservation prior to bone microarchitecture tests.

Bone microarchitecture analysis

Bone microarchitecture analysis was done using micro-CT scanner (Bruker SkyScan 1176, Belgium) at the Department of Medical Imaging, UiTM Puncak Alam, Selangor. The femur was dried superficially and wrapped with parafilm to maintain normal hydration of bone during the scanning. The femur was put horizontally into a 6.0 cm polystyrene foam tube

to hold the sample and mounted on the sample bed in the vertical axis of the scanner. The scanner was set at medium Al 0.5 mm filter, 18 m pixel resolution and 0.4 rotation degree. The current, voltage, and exposure were selected depending on the bone where the transmission profile value was read within the range of 27%-32%. The projected images after scanning were reconstructed using NRecon V1.6.4.0 (SkyScan) software and the datasets of reconstructed images were then analyzed using CT Analyser (CTAn V1.11.10; SkyScan). The region of interest (ROI) was set at 1.347 mm from the growth plate extended to a further 2.200 mm with total of 200 slices. This ROI is the trabecular region which is rich in blood supply and has high bone turnover activities. The 3D morphometric parameters were measured which include total porosity, bone volume fraction (BV/TV), bone surface fraction (BS/BV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), connectivity density (Conn.Dn), structural model index (SMI) and degree of anisotropy (DA).
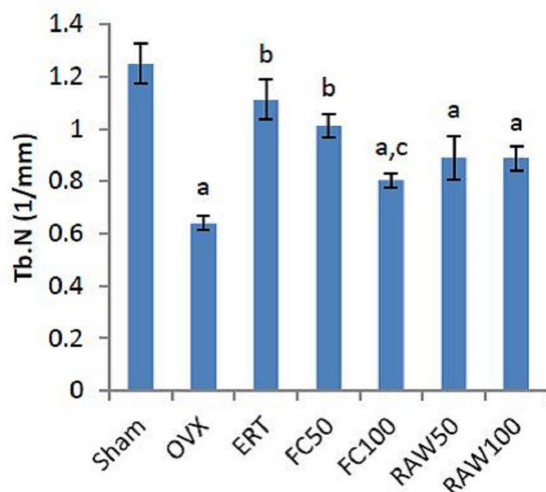

Fig. 5: Mean trabecular numbers of each group. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values. 'a' indicates there was significant difference with Sham group; 'b' indicates there was significant difference with OVX group and 'c' indicates there was significant difference with ERT group.
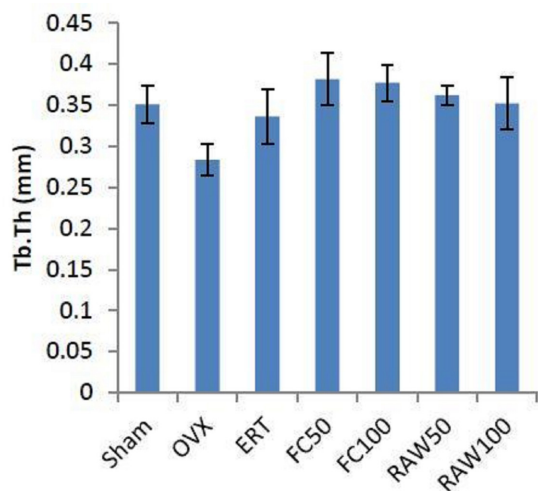

Fig. 6: Mean trabecular thickness values of all groups. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values.

Statistical analysis

The data was analyzed using Statistical Package for Social Science software version 24.0 (SPSS 24.0, Chicago, USA). The normally distributed data were tested using analysis of variance (ANOVA) followed by Tukey's HSD test. For not normally distributed data were tested using Kruskal-Wallis and Mann-Whitney tests. All the results were expressed as mean standard error of the mean (SEM).

RESULT AND DISCUSSION

Osteoporosis is a global health issue due to its long-term outcome which may affect the quality of life of a person as well as country's economic development. It is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration resulting in bone fragility and susceptibility to fracture (Peck, 1993). Once a fracture occurred, an individual will experience pain, limited physical function, immobility, and social and emotional problem. A study reported that after one year of hip fracture, 25% of the patients have died, 40% can walk poorly or wheelchair bound, and 60% can walk more or less the same as before the fracture (Klein-Nulend *et al.*, 2015).

Higher incidence of osteoporosis among the women is highly associated with the decreased estrogen level when they hit menopause (Khosla *et al.*, 2010; Svejme *et al.*, 2012). Estrogen replacement therapy (ERT) is one of the conventional treatments used by the postmenopausal women in order to preserve bone from deteriorating and to avoid fracture from occurring. However, this therapy is compromised by long-term side effects such as gastrointestinal intolerance, ovarian and breast cancer (Narod, 2011; Ritte *et al.*, 2012; Chung *et al.*, 2016). Hence, previous studies were proposed alternatives from the natural plants with fewer side effects in managing osteoporosis. *Ficus carica* was proposed in this study as an alternative treatment against osteoporosis due to its mineral content and antioxidant properties (Table 1) which believes to improve bone health as demonstrated by other past studies.

The morphometric change in bone structure is a key indicator for determining osteoporosis status and the risk of fracture to occur. In the present study, the trabecular bone was assessed using micro-CT. Micro-CT is a tool that produces high measurement accuracy of trabecular bone calculation with three-dimensional images. Micro-CT provides both metric and non-metric bone parameters. Metric parameters include bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Non-metric parameters include connectivity density, structural model index (SMI) and degree of anisotropy (DA) (Nishiyama *et al.*, 2010; Ito, 2011).

Ovariectomy has been proved and used by many studies as estrogen-deficiency osteoporosis model. As expected, OVX group showed a massive loss of trabecular connections (Figure 1) and recorded the most porous bone structure (Figure 2). This is supported by previous studies which also reported on the deterioration of ovariectomized rat's trabecular bone structures (Mohamed *et al.*, 2012; Bouxsein *et al.*, 2010). Estrogen supplementation group (ERT) showed reversing bone osteoporotic changes with denser trabecular microarchitecture as shown by the 3D images (Figure 1) and significantly better trabecular

connection with low porosity (Figure 2). Both aqueous and raw extracts of FC treatments were found to have a better 3D image of bone microarchitecture than OVX group. RAW50 showed the best microarchitecture among the treatment groups with fewer bone deteriorations (Figure 1).

BV/TV is the fraction of mineralized bone which occupies a given volume of interest (Perilli *et al.*, 2010). It can be used as predictors of bone stiffness, density and mechanical properties (Maquer *et al.*, 2015). OVX group showed significantly lower BV/TV (Figure 3). This finding is supported by the past studies where ovariectomized rats recorded low BV/TV due to bone perforation following estrogen deficiency (Li *et al.*, 2013; Spilmont *et al.*, 2014; Liu *et al.*, 2015). Other metric parameters are Tb.Th which measures the mean thickness of trabeculae, Tb.N measures the average number of trabeculae per unit length and Tb.Sp measures the mean distance between trabeculae (Bouxsein *et al.*, 2010). An osteoporotic bone is porous due to the thinning of Tb.Th caused by bone resorption which then degenerates and leads to the loss of Tb.N. As Tb.N start to decrease, the distance between the bone (Tb.Sp) is increased. As shown by the current study, the ovariectomized control group had significantly lower Tb.N (Figure 5) and higher Tb.Sp values (Figure 4). ERT group improved the deteriorated bone with significantly higher BV/TV and Tb.N, and lower Tb.Sp than OVX group. RAW50 recorded higher BV/TV (Figure 3) and significantly lower Tb.Sp (Figure 4) compared to OVX group which was comparable to ERT and Sham groups. All treatment groups showed significantly lower Tb.Sp (Figure 4) than OVX group and FC50 group showed significantly higher Tb.N than OVX group (Figure 5), but not significantly different with the other treatment groups. This concludes that all FC extracts showed potential in improving the trabecular structure of osteoporotic bone.
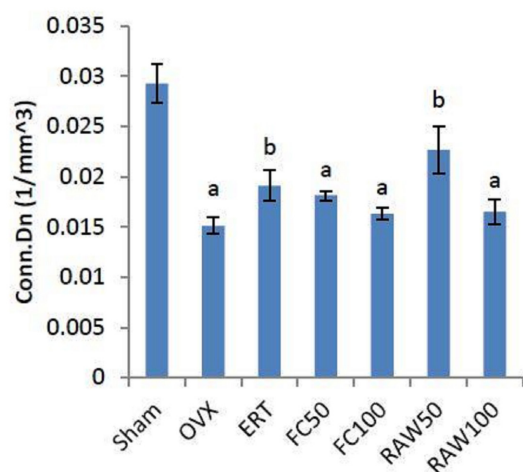

Fig. 7: Connectivity density values of each group. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values. 'a' indicates there was significant difference with Sham group and 'b' indicated there was significant difference with OVX group.

Tb.Th was not significantly different between all the groups even though the treatment groups showed an increment trend higher than OVX group (Figure 6). Studies were done by Francisco *et al.* (2011) reported that ovariectomy caused a reduction in a total number of BV/TV and Tb.N and increased

Tb.Sp while Tb.Th did not change significantly. Other findings also showed that increasing of bone connectivity does not significantly increase trabecular thickness. This is supported by studies done by Ahn *et al.* (2012) and Nadia *et al.* (2017) which reported no significant difference of Tb.Th value between the intervention groups and negative control group. These similar findings may be due to ovariectomy which caused a gradual loss in bone structure where the trabecular structures changed radically from plate-like to rod-like hence, making the Tb.Th measure not significant (Parkinson and Fazzalari, 2013).
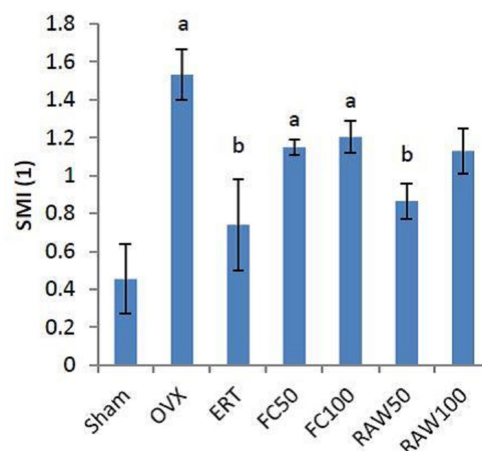

Fig. 8: Structural model index values of each group. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values. 'a' indicates there was significant difference with Sham group and 'b' indicated there was significant difference with OVX group.

Connectivity density represents the maximum number of trabecular connections that can be broken where the higher the value indicates greater bone connectivity and strength (Chen *et al.*, 2013). The structural model index is a score that reflects the rod versus plate trabecular structure, from 0 to 3 respectively (Roux *et al.*, 2013). During aging and bone remodeling, bone structures change from plate-like to rod-like structure. The plate-like structure is thicker and denser, while the rod-like structure is thinner and less dense due to the loss of bone density (Chen *et al.*, 2013). Sham, ERT, and RAW50 showed significantly higher trabecular connectivity density (Figure 7) and significantly reduced SMI values (Figure 8) compared to OVX group. Ovariectomized rats recorded higher SMI value which indicated that osteoporotic bone had a greater amount of rod-like bone structure than plate-like structure (Isaksson *et al.*, 2011).

The degree of anisotropy (DA) is one another important biomechanical measurement to estimate fracture risk. DA describes how the structural elements are oriented within the bone volume (Francisco *et al.*, 2011). The ideal isotropic structure is valued as 0 and a fully anisotropic structure valued as 1. Osteoporotic bone usually recorded high anisotropy value compared to the normal bone due to loss of the bone structure. OVX group showed higher DA value which indicates that it was prone to fracture when the load is applied. However, the other groups showed no significant difference of DA value compared to OVX (Figure 9). This might be due to the small size of rat's bone where all groups were vulnerable if the load is given.
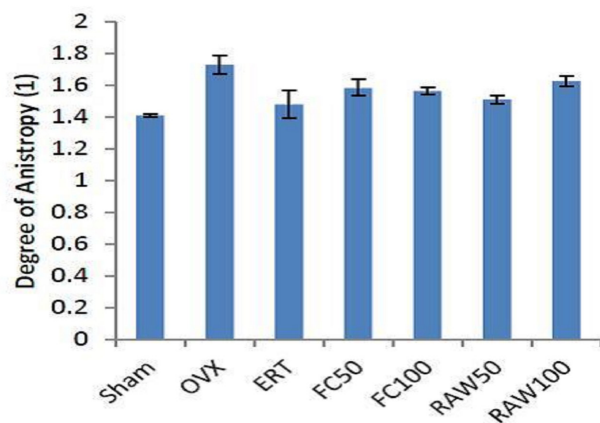
Fig. 9: Degree of anisotropy values of each group. Data presented as mean ± SEM ($p < 0.05$) with standard error bars of the average values.

Osteoporosis associated with estrogen deficiency and highly associated with oxidative stress, a major cause of the imbalance between osteoblast and osteoclast activities. This will consequently result in a reduction of bone density. Antioxidative and anti-inflammatory properties are an important component that may play a role in protecting bone against the damaging effects of free radicals. Polyphenols, phytosterol, anthocyanin, flavonoids, isoflavones, steroids, and alkaloids are examples of antioxidant and anti-inflammatory bioactive compounds found in most plants that have been studied its effectiveness against osteoporosis. FC is one of the fruits that are rich in antioxidant and anti-inflammatory bioactive compounds that may contribute to its potential anti-osteoporotic effect. In this present study, micro-CT analysis was able to provide the insight measurement of the effects of FC on bone quality. From the findings, FC showed promising potential as alternative supplementation against osteoporosis due to its bioactive contents, minerals, antioxidant and anti-inflammatory properties. Further studies are required to identify other aspects of FC as alternative treatment such as by using varieties of extractions and higher doses for better results.

CONCLUSION

Based on the micro-CT analysis, 50 mg/kg raw extract of FC was shown to have the best potential in improving osteoporotic bone changes of ovariectomized rat mode and comparable to ERT. This may due to its phytochemical content of minerals, antioxidant and anti-inflammatory properties. Thus, FC has potential as an alternative treatment for postmenopausal osteoporosis. Further studies are warranted to explore more potentials of FC against osteoporosis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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