Effect of Phytoestrogens Derived from Red Clover (*Trifolium Pratense L.*) in Ovariectomized Rats

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Abstract: The most common type of osteoporosis is bone loss associated with ovarian hormone deficiency. There is evidence that diets contain high levels of phytoestrogenic isoflavones such as red clover (*Trifolium pratense* L.) isoflavones (RCI) are associated with a low incidence of osteoporosis and reduce menopausal symptoms. The objective of this study was to evaluate the preventive effects of RCI on the progression of bone loss induced by estrogen deficiency (ovariectomy) in rats. Sham operation or bilateral ovariectomy (OVX) was performed on female adult rats (n=50). One week after the operation, OVX rats were treated with an oral dose of 20, 40 or 60 mg of RCI daily for 12 weeks. Results showed that the ovariectomy induced significantly increase on of body weight gain percent (BWG%) and feed intake. Levels of bone specific alkaline phosphatase (B-ALP) significantly elevation, accompanied with significant reduction on estradiol and parathyroid hormone (PTH) levels, as well as bone mineral density (BMD) in OVX group compared with sham group. In addition, OVX showed noticeable histological change in the femur sections compared with sham-operated control. Treatment with RCI significantly ameliorated all tested biological, bone marker enzyme and hormone assay parameters compared with the OVX untreated rats, as well as improved histological alterations induced by OVX. These findings suggest that RCI is effective in reducing bone loss induced by ovariectomy and maintains bone health, probably by reducing bone turnover *via* inhibition of bone resorption.
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Key words: Red clover isoflavones, Female ovariectomaized rats, Bone markers, Bone mineral density, Histopathological changes of bone.

1. Introduction.

Osteoporosis (OP) is a bone metabolic disease characterized by low bone mineral density (BMD) with high risk of fractures. It occurs when there is an imbalance between bone resorption and formation during the bone remodeling process **(Nazrun *et al.*, 2011)**. **Greer *et al.* (2008)** estimated the prevalence of OP for Saudi Arabian women aged 50-70 years to be approximately 23%. The most common type of OP is bone loss associated with ovarian hormone deficiency **(Occhiuto *et al.*, 2007)**, estrogen deficiency influences osteoclast which enhances bone loss by stimulating bone resorption **(NAMS, 2002)**.

There are clear bone-related benefits of hormone replacement therapy (HRT) **(Atkinson *et al.*, 2004)**, however, because a greater incidence of breast and endometrial cancer has been linked to some forms of HRT **(Mahady *et al.*, 2002 and Geller and Studee, 2006)**. Treatment with natural herbs is likely to be fraught with lesser side effects compared to the presently used synthetic drugs **(Tenpe and Yeole, 2009)**. Of all the natural alternatives currently under investigation phytoestrogens, which appear to offer the most potential for the prevention of bone loss and have attracted new attention as a possible agent to prevent and treat postmenopausal osteoporosis (PMO), cancer preventive and relieve menopausal symptoms **(Chen *et al.*, 2003 and Yatkin and Daglioglu, 2011)**.

Red clover (RC) (*Trifolium pratense* L.) is a perennial herb growing in all temperate and subtropical areas around the world **(Booth and Piersen, 2006)**. Red clover botanical dietary supplements have received much attention recently for their potential use in maintenance/ improvement of bone and cardiovascular health. It contains four important estrogenic isoflavones mainly (daidzein, genistein, formononetin and biochanin A) and coumestans **(Sabudak and Guler, 2009)**. Red cloves isoflavones (RCI) are increasingly used in dietary supplements for their purported estrogenic effect in *vivo* and *vitro* assays **(Engelmann *et al.*, 2009)**, hypolipidemic **(Geller and Studee, 2006)**, and is hypothesized to be of potential use as a natural form of HRT (**Occhiuto *et al.*, 2007)**. Therefore the aim of this study to investigate the effectiveness of RCI (*Trifolium pratense* L.) on the progression of bone loss induced by estrogen deficiency in ovariectomized (OVX) female rats.

2. Material and Methods.

Material.

Chemicals and kits.

The chemicals used in this study were purchased from Sigma Chemical Co, and all ELISA kits with high grades purchased from different Chemical Co.

Experiential animals.

Female Wistar rats (n=50 rats) weighing about (200-220g) were obtained from King Fahd

Center for Medical Research. All animals were allowed to one week acclimatize in animal housing conditions before being used for the study and were fed standard nutritionally balanced diet and drinking water *ad libitum*.

Plant materials.

Red clover isoflavone (Promensil) a standardized isoflavone supplement prepared from red clover extract, in tablet form was obtained from Novogen Ltd, United Kingdom. Each tablet contained 40 mg of total isoflavones [genistein (4.0 mg), daidzein (3.5 mg), and their methylated precursors biochanin (24.5 mg) and formononetin (8.0 mg)] **(van de Weijer and Barentsen, 2002)**.

Methods.

Pretreatment with red clover.

Red clover was prepared by dissolving in carboxymethyl cellulose solution (CMC), and an oral dose of 20, 40 or 60 mg/kg were administrated by gavage to rats in 1 ml (of 0.1 % w/v CMC). CMC solution was prepared by dissolving 1g CMC in 1 liter distilled water according to **(Burdette *et al.*, 2002)**.

Experimental design.

After the period of adaptation (one week), first group of female rats were anesthetized with diethyl ether and their ovaries were removed bilaterally according to the method described by **(Waynforth, 1980 and Lasota and Danowska, 2004)**, while the other group of female rats was subjected to sham operator. The operation was done in King Fahd Center for Medical Research. After one week of recovery from surgery, the OVX rats were randomly divided into four groups. The experimental groups were as follows: **Group (1)** (n=10)**:** Control negative (sham operated), rats received daily oral dose of CMC 1 ml (of 0.1 % w/v CMC). **Group (2)** (n=10)**:** Control positive (OVX), rats received the same oral dose of CMC. And **Groups (3, 4 & 5)** (n=30)**:** OVX treated with red clover; rats treated daily with an oral dose of 20, 40 or 60 mg/kg b.wt of RCI, respectively, dissolved in 1 ml (of 0.1 % w/v CMC) for 12 weeks. During the experimental period, food intake (FI) per each group was recorded daily, and all animals were weighed at the beginning and biweekly intervals throughout the 12 weeks to monitor changes and to adjust the dose of RC.

Blood collection and biochemical analysis.

One day after the end of treatment, rats from each group were fasted overnight. Blood samples were withdrawn by heparinized capillary tube from the retro orbital plexu of each rat under anesthesia with diethyl ether according to the method of **Cocchetto and Bjornsson (1983)**. Blood samples were allowed to clot, and then centrifuged at 3000 rpm for 15 min to separate serum, which kept at -20 ºC till biochemical analysis.

Bone marker enzyme and hormones.

Serum samples were used for determination of bone marker enzyme including bone specific alkaline phosphatase (B-ALP) according to **(Horn, 1972)**. As well as determination of estradiol and parathyroid hormones according to **Melkko *et al.* (1996**) and **Rizzoli *et al.* (1990)** respectively.

Determination of bone mass.

Bone densitometry were estimated for all experimental groups under anesthetized with intraperitoneal (i.p.) injection of 4 ml of mixture 3:1 (Ketamin 3 mg/kg and Seton 1 mg/kg) according to **(Moshref, 2007),** by Dual-Energy X-Ray Absorption (DEXA) used (LUNAR Prodigy Model, SA1002XR01, General electric., Madison, WI, USA), in the Center of Excellence for Osteoporosis Research (CEOR), KAU.

Histological examination.

The cleaned left femur was fixed in 10% neutral buffered formalin for histological examination. After fixation, the femurs were decalcified in 5% nitric acid for 3 days **(Verdenius and Alma, 1958)**, embedded in paraffin and cut into longitudinal section of 5 µm thickness and stained with hematoxylin–eosin (H&E) then examined by light microscopy **(Bancroft and Cook, 1998)**.

3. Results.

Biological evaluation.

Table (1) showed the effect of different doses of RCI on biological evaluation parameter in OVX female rats. The results indicated that, there were no significant differences in initial body weight (b.wt) between all experimental groups. In OVX untreated group recorded very highly significant elevation at ($p < 0.001$) in all biological evaluation parameters as compared with control (sham) group, with percentage (24.33%, 101.6%, 10.84% and 82.86 in final b.wt, BWG%, FI and FER, respectively) as percent change from control group. While when compared control (sham) group with three doses (20, 40 or 60 mg) of RCI treated OVX groups, there were very high significant differences in biological evaluation parameters at ($p< 0.001$) except the effect of high dose of RCI on food intake (FI) that reported a high significant differences at ($p< 0.01$).

Concerning OVX untreated group compared with OVX treated groups the data showed that, there were very highly significant difference in all treated groups on all biological evaluation parameters at ($p< 0.001$), except the effect of low dose RCI on feed efficiency ratio that showed a highly significant difference at ($p< 0.01$) and significant difference at ($p< 0.05$) with the high dose (60 mg of RCI) on FI, while there was no significant differences between OVX group and low dose of RCI treated group on FI. Regarding the effect of different doses of RCI used in OVX

treated groups, it showed that, there was a very highly significant difference when compare between low (20 mg) to both medium (40 mg) and high dose (60 mg) at (p< 0.001) on final b.wt and BWG%. The comparison between medium dose of RCI and high dose in treated groups, there was significant difference with high dose (60 mg) on final b.wt and BWG% at (p< 0.01 and p< 0.05, respectively).

Table (1): Effect of red clover isoflavones on biological evaluation in ovariectomized rats.

Experimental groups	Initial b.wt (g)	Final b.wt (g)	BWG %	FI (g/day/rat)	FER(g)
Control (sham)	218.0 ± 2.12	287.7 ± 2.28	31.97± 1.95	21.86 ± 0.78	0.035 ± 0.0019
Control (OVX)	217.6 ± 1.90	357.7 ± 3.08 ^{a***}	64.45± 2.49 ^{a***}	24.23± 1.30 ^{a***}	0.064 ± 0.0046 ^{a***}
OVX+ 20 mg RCI	218.4 ± 2.10	350.3 ± 3.25 ^{a***b***d***}	60.39 ± 2.26 ^{a***b***d***}	24.13 ± 0.69 ^{a***}	0.060 ± 0.0019 ^{a***b**}
OVX+ 40 mg RCI	217.5 ± 2.15	341.1 ± 1.46 ^{a***b***c***}	56.87 ± 1.31 ^{a***b***c***}	23.58 ± 0.54 ^{a***}	0.058 ± 0.0015 ^{a***b***}
OVX+ 60 mg RCI	218.2 ± 1.50	338.0 ± 2.02 ^{a***b***c***d**}	54.88 ± 1.45 ^{a***b***c***d*}	23.16 ± 1.17 ^{a**b*c*}	0.058 ± 0.0034 ^{a***b***c*}

RCI: Red clover isoflavone; **OVX:** Ovariectomized; **b.wt:** body weight; **BWG:** Body weight gain **FI:** Food intake and **FER:** Feed efficiency ratio.
Results are presented as the mean ± SD (n= 10).
^{a}**:** Significant differences *vs.* control (sham operated). ^{b}**:** Significant differences *vs.* OVX.
^{c}**:** Significant differences *vs.* OVX+20 mg RCI. ^{d}**:** Significant differences *vs.* OVX+40 mg RCI.
(*: p<0.05; **: p<0.01 and ***: p<0.001).

Bone marker enzyme and hormones.

Table (2) showed the effect of different doses of RCI on serum levels of bone-specific alkaline phosphatase (B-ALP) in ovariectomized (OVX) female rats. It is noticed that, control (-ve) female rats recorded very highly significant differences (p< 0.001) compared with OVX group in B-ALP with the mean values (9.12 ± 0.88 *vs* 11.97 ± 1.18 (U/L) in control (-ve) and OVX, respectively). While OVX female rats that received RCI at low dose (20 mg) showed a significant difference (p < 0.05) as compared with control (sham) in B-ALP. There were non-significant changes in female OVX groups administered medium and high doses in B-ALP compared with control (sham), and their serum levels tended to match with control group (sham).

Concerning OVX untreated female rats it showed that, the B-ALP recorded very highly significant difference (p < 0.001) when compared with treated OVX groups at all administered doses of RCI (20, 40 or 60 mg/d). Thus, indicated the noticeable improvement effect of RCI at all used doses, but the most noticeable improvement was showed when used RCI at a dose level of 60 mg/d. Administration of RCI to OVX rats, showed significant improvement in bone marker enzyme. A dose response trend was observed with various levels of RCI, where it showed significant difference (p< 0.05) in B-ALP between low (20 mg RCI) and both medium and high doses (40 and 60 mg RCI) in OVX treated group, while the medium and high doses recorded non-significant difference.

Table (2): Effect of red clover isoflavones on serum levels of bone marker enzyme and hormones in ovariectomized female rats.

Experimental groups	B-ALP (U/L)	Estradiol (pg/ml)	PTH (pg/ml)
Control (sham)	9.12 ± 0.88	48.68 ± 1.52	10.02 ± 1.00
Control (OVX)	11.97 ± 1.18 ^{a***}	35.16 ± 1.49 ^{a***}	16.96 ± 1.20 ^{a***}
OVX + 20 mg RCI	10.08 ± 1.09 ^{a*b***d*}	38.08 ± 2.39 ^{a***b**d***}	13.24 ± 1.06 ^{a***b***}
OVX + 40 mg RCI	9.11 ± 0.91 ^{b***c*}	45.56 ± 2.92 ^{a**b***c***}	12.63 ± 1.12 ^{a***b***}
OVX + 60 mg RCI	8.92 ± 0.75 ^{b***c*}	46.85 ± 2.36 ^{b***c***}	12.84 ± 1.07 ^{a***b***}

RCI: Red clover isoflavone; **OVX:** Ovariectomized; **B-ALP:** Bone specific alkaline phosphatase and **PTH:** Parathyroid hormone.
Results are presented as the mean ± SD (n= 10).
^{a}**:** Significant differences *vs.* control (sham operated). ^{b}**:** Significant differences *vs.* OVX.
^{c}**:** Significant differences *vs.* OVX+20 mg RCI. ^{d}**:** Significant differences *vs.* OVX+40 mg RCI.
(*: p<0.05; **: p<0.01 and ***: p<0.001).

The effect of different doses of RCI on serum levels of bone homeostasis hormones; estradiol, and parathyroid hormone (PTH) in OVX female rats is illustrated in Table (2) the results revealed that, ovariectomy resulted in a very high significant decrease in serum estradiol concentration accompanied with a very high significant increase in PTH levels as compared to control (sham operated) group at ($p< 0.001$), with percentage (-27.77% and 69.26 % in estradiol and PTH, respectively) as percent change from the control group. After treatment, there were highly significant changes in OVX treated with low and medium doses of RCI and sham control group in all hormonal assay parameters, at ($p< 0.001$, except in estradiol at $p< 0.01$ in medium dose). While in OVX group received high dose of RCI, there was a non-significant change in estradiol level, but a significant difference in PTH at ($p< 0.001$) as compared with sham control group.

Administration of RCI induced an improvement in hormonal assay compared with the OVX untreated group. There were significant elevation in estradiol levels at ($p< 0.01$ and $p< 0.001$ in low and both medium and high doses of RCI groups, respectively). Regarding the effect of the three doses of RCI used to treat OVX groups on estradiol levels, there was a high significant difference at ($p< 0.001$) between low to medium and high dose of RCI, while no significant difference was shown in medium and high dose of RCI. Meanwhile, in PTH levels, it reported that in all used doses of RCI, showed a very high significant elevation in PTH level ($p< 0.001$) as compared with the OVX untreated group. On the other hand, the values of PTH at the three doses recorded non-significant changes between treated OVX groups.

Bone mass results.

Effect of RCI on bone mineral density (BMD) in 3 positions: head, legs and spine of OVX female rats in Table (3) and Figure (1). Results of DEXA showed that, BMD in control (sham) indicated very high significant differences compared with OVX untreated group at ($p< 0.001$) in the head, legs and spine, expect BMD at the legs that noted significant difference at ($p< 0.05$). While in OVX group treated with low dose of RCI, BMD recorded a significant reduction at ($p< 0.01$) for the head, and in the medium RCI dose, the head BMD recorded a significant ($p< 0.05$) compared with control (sham). The results of the spine BMD recorded non-significant changes in all used RCI doses compared with sham control (-ve) group. At the same time both head and legs BMD recorded a non-significant difference between high dose and control sham (-ve), where their values tended to match with control values.

Table (3): Effect of red clover isoflavones on bone mass in ovariectomized female rats.

Experimental groups	BMD (g/cm^2)		
	Head	Legs	Spine
Control(sham)	0.217 ± 0.021	0.072 ± 0.006	0.151 ± 0.013
Control (OVX)	^{a***} 0.172 ± 0.015	^{a*} 0.067 ± 0.002	^{a***} 0.121 ± 0.010
OVX+ 20 mg RCI	^{a**} 0.187 ± 0.012	0.069 ± 0.002	^{b***} 0.144 ± 0.010
OVX+ 40 mg RCI	$^{a*\,b**}$ 0.195 ± 0.016	^{b*} 0.073 ± 0.007	^{b***} 0.141 ± 0.011
OVX+ 60 mg RCI	^{b**} 0.201 ± 0.019	^{b*} 0.073 ± 0.007	^{b***} 0.147 ± 0.007

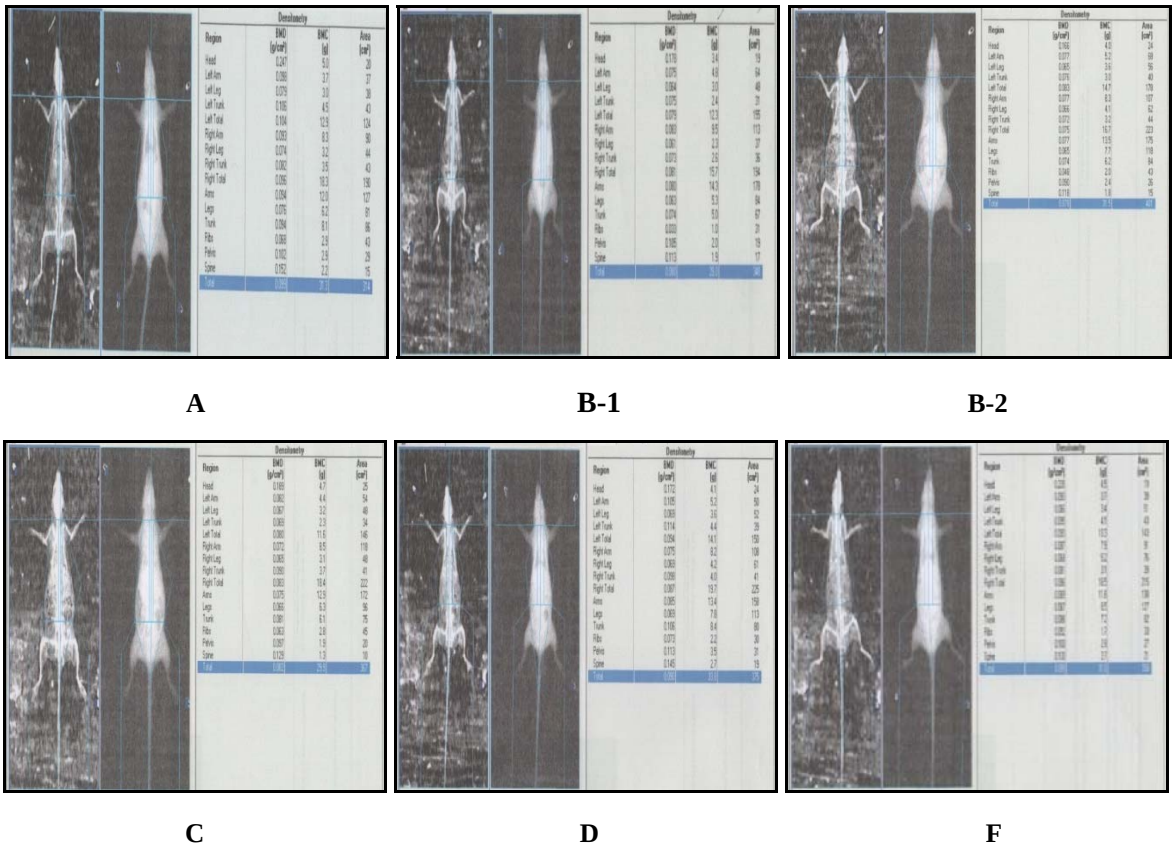
RCI: Red clover isoflavone; **OVX:** Ovariectomized; **BMD:** Bone mineral density.
Results are presented as the mean ± SD (n= 8).
^{a}**:** Significant differences *vs.* control (sham operated).
^{b}**:** Significant differences *vs.* OVX.
^{c}**:** Significant differences *vs.* OVX+20 mg RCI.
^{d}**:** Significant differences *vs.* OVX+40 mg RCI.
(*:$p<0.05$; **: $p<0.01$ and ***: $p<0.001$).

Administration of RCI to OVX groups induced an elevation in BMD as compared with untreated OVX group. In BMD of head, legs and spine there were high significant differences at ($p< 0.01$) between the medium and high doses of RCI, except for BMD of legs which recorded a significant difference at ($p< 0.05$) as compared with OVX untreated groups. These results indicated an improvement in BMD when used RCI to treat OVX clearly on the spine, where treated OVX groups with RCI at the three used levels induced a very high significant improvement ($p< 0.001$) as compared with untreated OVX group.

Histopathological changes in bone.

Sections of distal femur bone of control rats (sham) untreated group revealed no histopathological changes and normal bone cortex the femur bone was formed of an outer shell of cortical bone to which the periosteum was attached to its external surface and endosteum was attached to its internal surface. The endosteal surface of the cortical bone appeared smooth and was lined with osteoprogenitor cells (Fig. 2). The trabecular and bone marrow have thick and smooth outlines and homogenous acidophilic stained matrix (Fig. 3). Femur cortical bone of OVX untreated rat showed

osteoproteotic regions with bone destruction, loss of normal Haversin system pattern, numerous resorption cavities and distinct changes in femur cortical bone (Fig. 4). The trabecular bone thinned and cracks out showing rarefied regions and numerous splitting with the presence of osteoclast cells (Fig. 5). In addition, dilatation of bone marrow cavity and thin bone trabecular appeared (Fig. 6). The histological structure of femur female rats treated with 20 mg RCI revealed a potential protection from osteoporotic changes induced by ovariectomy as shown in (Fig. 7), except few trabecular with rarified regions and bone marrow spaces containing numerous fat cells (Fig. 8), while no histopathological changes (Fig. 9). Distal femur bone of OVX rats treated with 40 mg RCI showed a marked improvement with no histopathological changes and a thick bone cortex (Fig. 10 and 11). The examination sections of femur OVX female rats treated with 60 mg RCI, showed protection from osteoporotic changes induced by ovariectomy as thick bone cortex (Fig. 12), with no histopathological changes and normal trabecular and bone marrow cavity (Fig. 13 and 14).



A **B-1** **B-2**

C **D** **F**

Fig (1): Examples of bone mass results from DEXA in control different OVX untreated and treated groups. (A): control (sham) group, (B-1 & B-2): control (OVX) group, (C): OVX+ 20 mg RCI group, (D): OVX+ 40 mg RCI group and (E): OVX+ 60 mg RCI group.

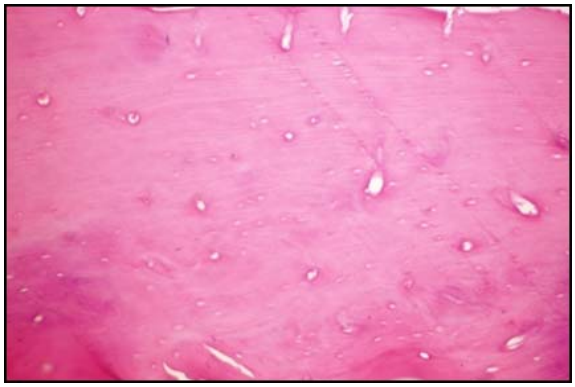


Fig (2): Distilled femur bone of control (sham) untreated group showing normal bone cortex. (H&E x200)

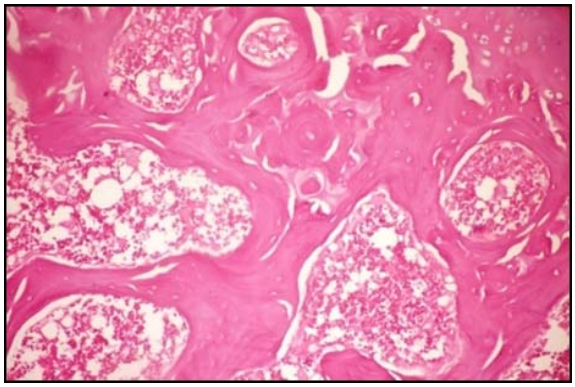


Fig (3): Distilled femur bone of control (sham) untreated group showing normal bone marrow and normal bone trabecular. (H&E x200)

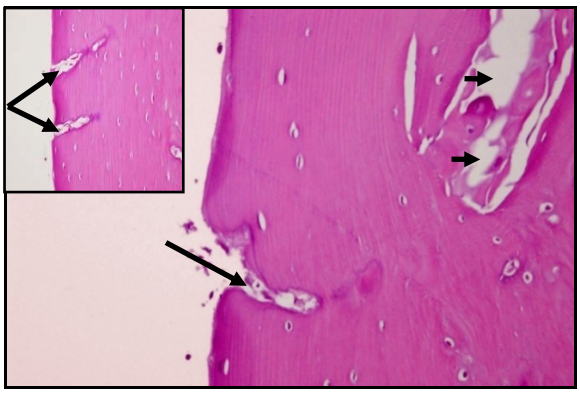


Fig (4): Distilled femur cortical bone of (OVX) untreated group showing osteoproteotic regions with bone destruction (small arrows), loss of normal Haversin system pattern and numerous resorption cavities (large arrows). (H&E x200 & 400)

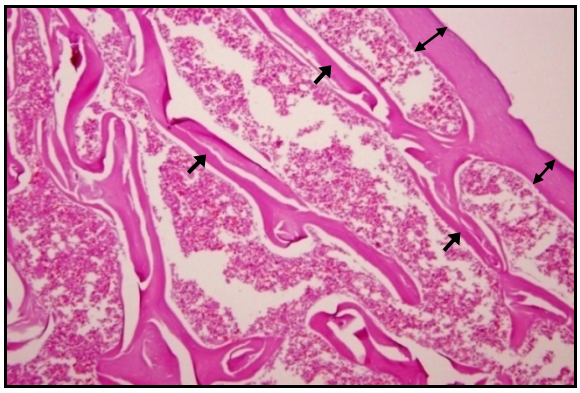


Fig (5): Distilled femur bone of (OVX) untreated group showing thin cortical bone (double head arrows) and the trabecular bone are thinned out showing rarefied regions and numerous splitting (small arrows). (H&E x200)

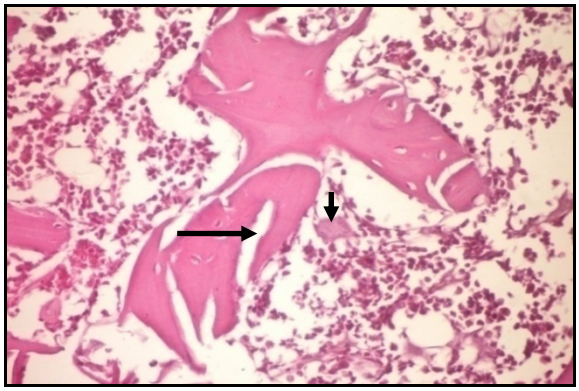


Fig (6): Distilled femur bone of (OVX) untreated group showing cracks in bone trabecular (large arrows) with presence of osteoclast cell (small arrows). (H&E x200)

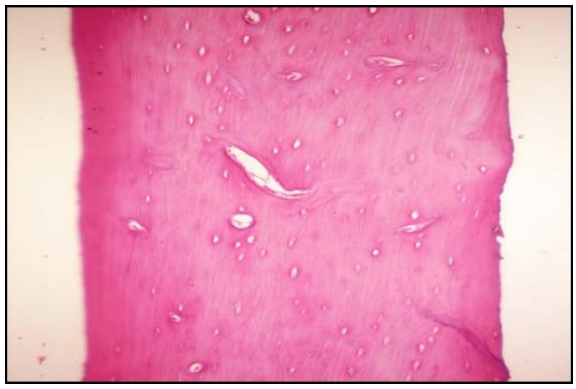


Fig (7): Distilled femur bone of OVX+ 20 mg RCI group showing no histopathological changes. (H&E x 200)

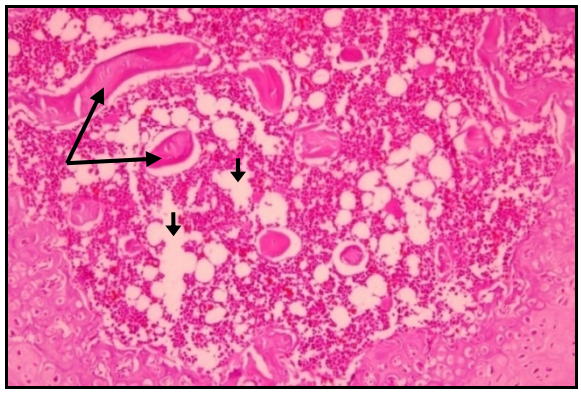


Fig (8): Distilled femur bone of OVX+ 20 mg RCI group showing few trabecular with rarified regions (large arrows) also bone marrow spaces contains numerous fat cells (small arrows). (H&E x 200)

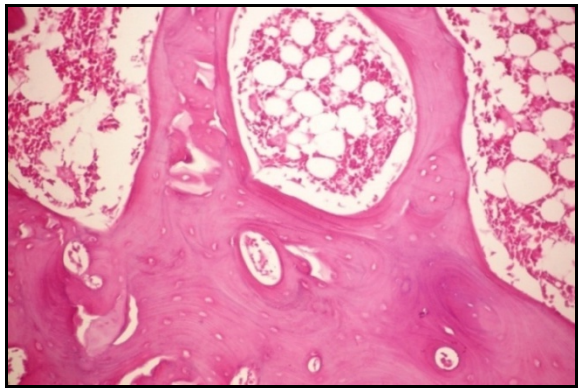


Fig (9): Distilled femur bone of OVX+ 20 mg RCI group showing no histopathological changes. (H&E x 200)

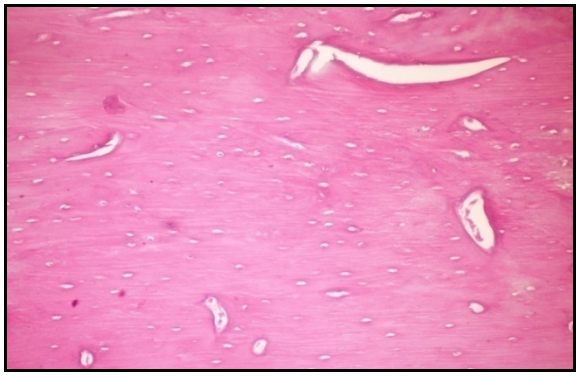


Fig (10): Distilled femur bone of OVX+ 40 mg RCI group showing thick bone cortex. (H&E x 200)

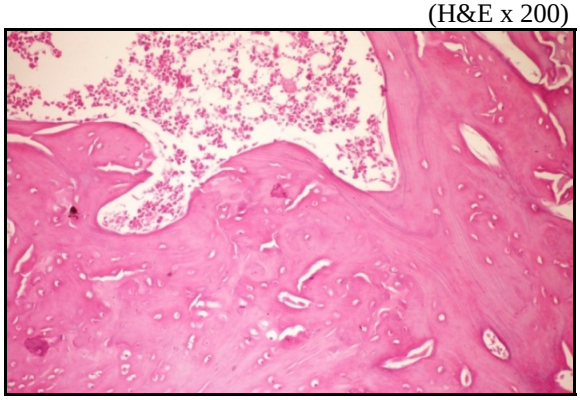


Fig (11): Distilled femur bone of OVX+ 40 mg RCI group showing no histopathological changes. (H&E x 200)

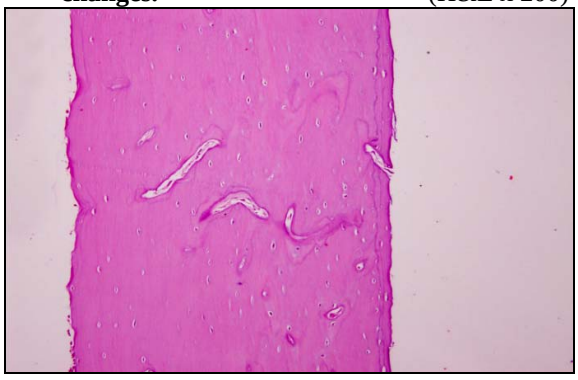


Fig (12): Distilled femur bone of OVX+ 60 mg RCI group showing thick bone cortex. (H&E x 200)

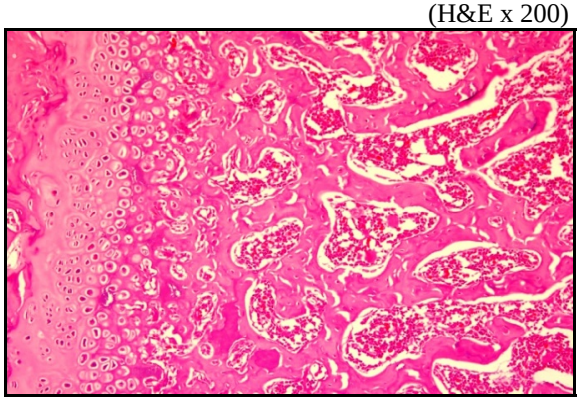


Fig (13): Distilled femur bone of OVX+ 60 mg RCI group showing no histopathological changes. (H&E x 200)

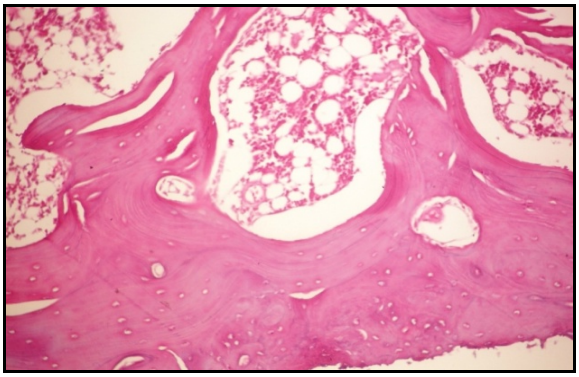


Fig (14): Distilled femur bone of OVX+ 60 mg RCI group showing normal bone marrow cavity and normal bone trabecular. (H&E x 200)

4. Discussion.

Osteoporosis is the most common metabolic bone diseases associated with disharmonic bone remodeling due to decrease in bone formation or accelerated bone resorption **(Stanworth and Jones, 2008)**. Estrogens play an important role in skeletal homeostasis, and ovarian hormone deficiency is one of the most important risk factors for OP. There are clear bone-related benefits of HRT, but given the uncertain popularity of this approach, there is a clinical need for alternative well tolerated nutritional treatments that can safely be used after the menopause and which effectively prevent bone loss and the development of OP **(Wronski and Yen, 1991)**.

In the present study, the results indicated that, OVX untreated group recorded very high significant elevation at ($p< 0.001$) in all biological evaluation parameters as compared with control (sham) group. Consistent with the previous results, the study by **Zaid *et al.* (2010)** who reported that, ovariectomized induced significant higher in overall biological evaluation parameters. **Zhang *et al.* (2012)** found that OVX rats induced significant increase in the b.wt compared to the sham rats. Furthermore, **Ferreri *et al.* (2011)** found an association between b.wt and increased food consumption in OVX and sham rats. Therefore, FI appears to be the primary means through which accelerated weight gain is achieved post-ovariectomy, which is consistent with the present findings. This effect may be explained by **(Liang *et al.*, 2002)**, who mentioned that estrogen has been implicated in feeding behavior and adiposity, therefore ovariectomy-induced hyperphagia results in BWG and adiposity. Moreover, the increase in b.wt of OVX postulated to the regulatory effect of estrogen on the adipose tissues **(Stanworth and Jones, 2008)**.

In the present study, comparison between OVX untreated group and OVX treated groups the data showed that, there were very high significant differences with all treated groups on all biological evaluation parameters at ($p<0.001$), an except the effect of low dose of RCI on FER noticed a high significant difference at ($p< 0.01$) and a significant

difference at ($p< 0.05$) with high dose (60 mg of RCI) on FI. At the same time there were no significant differences between OVX group and both low and medium doses of RCI treated OVX groups on FI.

Loss of circulating estrogen after ovariectomy leads to increase body and adipose weights, and this is prevented or reversed by estrogen replacement **(Mohamed and Abdel-Rahman, 2000)**. Ovariectomy increases LPL activity and lipid deposition in adipocytes, while estrogen also directly decreases the activity of lipoprotein lipase (LPL **(Hamosh and Hamosh, 1975)**. Estrogen can affect adipose tissue indirectly through modulating appetite or energy expenditure **(Jones *et al.*, 2000)**. Limited information on RCI metabolism and bioactivity exists **(Engelmann *et al.*, 2009)**. Consistent with the present results a study by **Burdette *et al.* (2002)** who found that rats fed RC showed a dose-dependent decrease in BWG with increasing concentrations of RC. The RCI effect may be explained by **Uesugi *et al.* (2001)** who found that estrogen down regulate eating behavior.

Meanwhile, in contrast with the present results, findings by **Occhiuto *et al.* (2007)** showed a progressive increase in b.wt during the 14 week experimental period in all groups , there were no significant difference was observed between the sham group, the OVX group, and the ovariectomy treated with oral RCI, this discrepancy with present results is due to the difference in red clover extract that standardized to 11% isoflavone content by weight, thereby exhibiting different biological response.

The improvement showed in biological values in OVX treated groups with RCI, may be attributed to the fact that estrogen regulates FI *via* anorexigenic pathways of the central nervous system and enhances the satiating potency of cholecystokinin (CCK), leading to reduction in meal size and overall FI **((Eckel*et al.*, 2002 and Gao *et al.*, 2007)**. Also, estrogen is thought to exert inhibitory effects on feeding by augmenting glucagon-mediated satiety signaling **(Geary and Asarian, 2001)**. Additionally, the complex interaction between estrogen and leptin in the central nervous system and peripheral tissues also function to control FI and b.wt **(Chen and Heiman, 2001 and Torto *et al.*, 2006)**.

In the present study, ovariectomy induced remarkable changes in bone marker enzyme in female rats, at the 12^{th} week after ovariectomy, OVX untreated female rats showed very high significant increase in levels of both serum B-ALP compared with sham operated group, which supports the occurrence of ovarian deficiency in the OVX model. Furthermore, increased serum levels of B-ALP enzyme were very high significant prevented in the three groups treated with RCI (at a dose level of 20, 40 or 60 mg RCI) compared with OVX untreated groups.

Bone-ALP is a glycoprotein coenzyme linked to the osteoblast membrane; and is a sensitive biochemical marker of bone formation. It has been widely used as a measure of bone remodeling status, B-ALP are released into the circulation during the bone remodeling process **(Eren and Yilmaz, 2005 and Biver *et al.*, 2012)**. Estrogen deficiency increases serum B-ALP, which induces increased bone turn-over. In the present experiment, OVX animals were found to have higher BALP than sham control, indicating increased bone turnover due to OVX-induced estrogen deficiency, which is entirely consistent with studies conducted by **(Szulc and Delmas, 2008 and Ferretti *et al.*, 2010)**. Treatment with RCI prevented the rise of serum B-ALP levels compared with OVX control rats. These results suggest that RCI is effective in reducing bone turnover caused by ovariectomy, which is consistent with the study by **(Occhiuto *et al.*, 2007)**. More evidence was provided by **Kawakita *et al.* (2009)** who showed that RC extracted preparation are effective in reducing ovariectomy-induced bone loss by reducing bone turnover *via* inhibition of bone resorption.

The most important form of estrogen found in the body is estradiol **(Webster, 2006)**. The present results revealed that, ovariectomy resulted in a very highly significant decrease in serum estradiol concentration as compared to control (sham operated) group at ($p< 0.001$). That was consistent with **Kawakita *et al.* (2009)** and **Yoon *et al.* (2012)** who revealed that serum estradiol levels were significantly lower in the OVX group compared with the sham group. The menopause is the consequence of the exhaustion of ovarian follicles which results in decreased production of estradiol and other hormones. There is a close relationship between estrogen deprivation and development of osteoporosis. Low serum levels of 17β-estradiol are associated with lower calcium availability and activation of bone resorption-accelerating cytokines (IL-1, IL-6, IL-11 and TNF-α), leading to the dominance of bone resorption over bone synthesis and subsequent bone decalcification **(Slemenda *et al.*, 1987)**.

Administration of RCI induced improvement in estradiol as compared with OVX untreated group, there were significant elevation at ($p< 0.01$ and $p< 0.001$ in low and both medium and high doses of RCI groups, respectively) as compared with untreated OVX group. The present findings are in accordance with **Kawakita *et al.* (2009)** who found that RCE treatment brought about a comparably and significantly higher level of estradiol in treated OVX rats**.** This effect may be attributed to phytoestrogens that can alter estradiol biosynthesis and metabolism through modulation of steroidogenic enzyme activity and expression, thereby altering serum estradiol levels, increasing the level of estradiol prevent bone loss by depressing bone turnover **(Wronski *et al.*, 1988 and Whitehead and Rice, 2006)**. Another mechanism by which is through stimulation of gastrointestinal deconjugation of estrone, thus leading

to its peripheral reabsorption and conversion to estradiol **(Harrison *et al*., 1999)**.

Parathyroid hormone (PTH), protein hormone released by the parathyroid gland, is a major regulator of bone metabolism and calcium homeostasis **(Papavasiliou *et al.*, 2003)**. The present results revealed that, ovariectomy resulted in a very highly significant increase in PTH levels compared to the control (sham operated) group at. The obtained results are in accordance with **Taguchi *et al*. (2006)** and **Zhu *et al*. (2012)**.

Parathyroid hormone is a major regulator of ionized calcium and phosphate concentrations in the blood and extracellular fluids and parathyroid hormone receptor 1 (PTHR1) is a specific receptor for PTH and belongs to the G-protein coupled receptor family **(Foord *et al.*, 2005)**. Upon activation in the presence of PTH, PTHR1 triggers calcium and phosphorus mobilization, which leads to osteogenesis and bone turnover. The primary target organs for PTH/PTHR1 are kidney and bone. In bone, PTH/PTHR1 mediate bone resorption by osteoclasts and reduce osteoblast proliferation, resulting in calcium liberation and decreased bone mass **(Potts, 2005)**. Estrogen deficiency increases the rate of bone remodeling which results in high turnover bone loss. **Narayana *et al*. (2012)** reported that estrogen deficiency induces bone resorption by releasing calcium into the extracellular space, which in turn suppresses PTH secretion, calcitriol synthesis, and intestinal absorption of calcium in cancellous bone leading to general bone loss and destruction of local architecture and reduced bone strength resulting in osteoporosis **(Sachdeva *et al*., 2005 and Justesen *et al*., 2006)**.

Administration of RCI induced improvement in PTH, all used doses of RCI showed very high significant elevation in PTH level compared with OVX untreated group. The present data confirmed by the results of **Dong *et al*. (2012)** who found that phytoestrogen treatment significantly decreased the levels of serum PTH in OVX rats ($p< 0.01$) *vs*. OVX untreated rats. The curative role of phytoestrogens could be due to the effect of parathyroid gland and reduced PTH secretion, which is considered as one way in which it is known as a major factor involved in the systemic regulation of bone resorption **(Wong *et al*., 2002)**.

Bone mineral density is one of the most important factors to measure bone quality. Present results of DEXA showed that, BMD in control (sham) group reported very high significant differences as compare with OVX untreated group at ($p< 0.001$) in both head and spine, but in legs it recorded significant difference at ($p< 0.05$). The results are in accordance with the findings of **Xie *et al*. (2006)** who found a decrease in bone mass in the 4th week after OVX in rats, and a typical osteoporosis profile identified in the 8th week after OVX rats. **Occhiuto *et al*. (2007)** reported that after 14 weeks, the ovariectomy reduced bone mineral content and femoral density. In the study by **Lei *et al.* (2009)** they reported that a progressive loss of BMD in OVX rats, and certain morphological features in the bone marrow sections. The obtained results may be explained through OVX induced decrease in estrogen level, which is accompanied by bone mass loss, confirmed by significant deterioration in the levels of bone remodeling tested parameters.

The obtained DEXA data reported amelioration in BMD in OVX rats treated with RCI clearly in the spine, where treated OVX groups with RCI at the three levels used induced very highly significant improvement ($p< 0.001$) as compared with untreated OVX group. In agreement with this result **Occhiuto *et al*.** (**2007)** and **Kawakita *et al*. (2009)** who measured the effect of red clover on total bone mass and found that significant increase in BMD in treated OVX compared with untreated group. The administration of isoflavones or their derivatives prevented bone loss in OVX rats due to its similar structurally to estradiol and their estrogenic-like activity which induced positive effect on BMD **(Kawakita *et al.*, 2009)**. Treatment with isoflavones in OVX group show greater BMD and mechanical bone strength, this effect might be due to the enhancement of intestinal calcium absorption **(Arjmandi, 2001)**. The beneficial effects results from stimulation of bone formation rather than suppression of bone resorption ***(*Fanti *et al.*, 1998 and Harrison *et al.*, 1998)**.

In the present study, the bone histological examination of control rats (sham) revealed no histopathological changes and normal bone cortex, while femur cortical bone of OVX untreated rat showing osteoproteotic regions with bone destruction, loss and distinct changes in femur cortical bone, the trabecular bones are thinned and cracks with presence osteoclast cell. Consistent with this were the results **(Kalleny, 2011 and Saleh and Saleh, 2011)**. The present results may be attributed to an important role of strogens in skeletal homeostasis, and ovarian hormone deficiency is one of most important factors for osteoporosis **(Anastasopoulou and Rude, 2002 and Sharma *et al*., 2006)**.

In the present study, examinations of histological structure of femur female rat treated with 20 mg RCI revealed potential protection from osteoporotic changes induced by OVX and there were marked thick bone cortex, normal bone marrow cavity and bone trabecular. The 40 mg RCI showed marked improvement, no histopathological changes and thick bone cortex, trabecular appeared thick and marrow spaces are narrow compared to OVX untreated rats, while the 60 mg RCI, showed potential protection from osteoporotic changes induced by OVX. Consistent with the previous results **Occhiuto *et al*. (2007).** The protective role of RCI may be attributed to its phytoestrogen effect on bone formation, and a consequence of a genomic and estrogen receptor-mediated effect **(Kuiper *et al.*,**

1997). In addition, a variety of non-genomic mechanisms, including the inhibition of tyrosine kinase, **(Blair *et al.*, 1996)** inhibition of topoisomerase II **(Anderson *et al.*, 1999)**, or activation of a putative membrane-bound receptor for estrogenic molecules **(Watson *et al.*, 1995)**, all have been proposed as mechanisms of action of genistein and other phytoestrogens.

Conclusion:

Although HRT has been a commonly preventive for postmenopausal symptoms, but the negative side effects linked to some forms led to discouraged. In the present study different dosages of red clover isoflavones which are amenable to clinical practice application proved to be effective in increasing the estradiol, bone mineral density together with a decreased bone marker turnover and histological changes of bone. Therefore, dietary supplements of red clover isoflavones have been recommended as an alternative to conventional HRT due to its beneficial effects in the maintenance/ improvement of bone health. More study should be conducted to determine the effect of RCI supplements to alleviate OP for peri and post-menopausal women.

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