Osteoprotective Role of Red Clover (*Trifolium Pratense L.*) Isoflavones in Ovariectomized Rats

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Abstract: Osteoporosis (OP) represents most common metabolic bone diseases, bone loss associated with ovarian hormone deficiency considered the most common types of OP. 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 osteoprotective effects of RCI on 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 body weight gain percent (BWG %), feed intake feed efficiency ratio (FER) and fat tissues percent (fat tissues %). Levels of N-terminal propeptide (PINP), osteocalcin and parathyroid hormone (PTH) levels significantly elevation, as well as bone mineral content (BMC) in OVX group compared with sham group. In addition, OVX induced a rise in the number of osteoclasts and noticeable histochemical 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 histochemical 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.

[Hala A. H. Khattab, Mohammed S. Ardawi and Reem A. M. Ateeq. **Osteoprotective Role of Red Clover (*Trifolium Pratense L.*) Isoflavones in Ovariectomized Rats.** *Life Sci J* 2014;11(10s):618-631]. (ISSN:1097-8135). http://www.lifesciencesite.com. 122

Key words: Red clover isoflavones, Ovariectomaized female rats, Bone markers enzyme, Osteocalcin , Parathyroid hormone Bone mineral content, Histochemical changes of bone, Fat tissues.

1. Introduction.

Osteoporosis is a silent painless and weakening of the bones disease, with a harmful impact on morbidity and mortality, that constitutes an enormous socioeconomic crisis **(Reyes and Moreno, 2005** and **Abdulameer et *al.*, 2012).** It defined as "a multi-factorial skeletal disorder characterized by decreased bone mass and deteriorated microarchitecture that lead to increase risk of fracture **(Lau and Guo, 2011)**. Incidence of OP is elevated worldwide as populations age increase. Women are generally affected four times more likely than men, and fracture rates among women are approximately twice as high as men **(Kanis *et al.*, 2008)**. The prevalence of osteoporosis and osteopenia among postmenopausal Saudi Arabian women is common to the extent of over 60% **(Sadat *et al.*, 2004)**. **Greer *et al.* (2008**) estimated the prevalence of OP for Saudi Arabian women aged 50-70 years to be approximately 23%.

Osteoporosis is generally viewed as resulting from a combination of age-related, hormonal, dietary, lifestyle and genetic factors, all of which can lead to reduced bone mass **(Compston, 2004)**. The most common type of OP is the bone loss associated with ovarian hormone deficiency at menopause **(Occhiuto *et al.*, 2007)**, which leads to loss of bone mass **(Riggs *et al.*, 2002)**. Estrogen deficiency has been regarded as a critical cause of OP, which can result from naturally or surgically induced menopause **(Das, 2002)**. Ovariectomized rats (OVX) are widely accepted models for PMO.

Hormone replacement therapy (HRT) has been widely used for relief of menopausal symptoms, prevention and treatment of PMO, as well as it reduce postmenopausal bone loss and reduce fracture incidence **(Ettinger *et al.*, 2004 and Stevenson, 2006).** Although HRT believed to be beneficial, owing to a reduction risk of OP, but the negative side effects, an increased risk of breast cancer, were thought to be outweighed by the advantages. Therefore, HRT has been subject to much discussion and speculation. Treatment with natural herbs is likely to be fraught with lesser side effects compared to the presently used synthetic drugs **(Tenpe and Yeole, 2009)**.

Isoflavones are natural endocrine active phytoestrogens are generally considered to prevent osteoporosis by promoting bone health **(Franke *et al.*, 2009)**. They may be useful as dietary alternative or supplement to postmenopausal HRT, because of their beneficial effects on atherosclerosis **(Foth *et al.*, 2000)**, aging related and hormone-dependent disorders, including cancer risk, menopausal symptoms and

cardiovascular diseases **(Birt *et al.*, 2001 and Yang *et al.*, 2001)**.

Red clover (RC) (*Trifolium pratense*) supplementation have been the subject of much interest for the reduction of menopausal symptoms and conditions related to aging because of their high concentrations of phytoestrogens **(Piersen *et al.*, 2004 and Beck *et al.*, 2005)**. 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).** 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.

Carboxymethyl cellulose sodium salt (CMC), in white and odorless medium viscosity powder and all chemicals used in this study were purchased from Sigma Chemical Co, and all ELISA kits for determination of N- terminal propeptide type I (PINP), osteocalcin (OCN) and parathyroid hormone (PTH) with high grades purchased from different Chemicals 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 standard conditions, temperature of (22±3°C), relative humidity (50-55%) and a 12 h light/dark cycle before being used for the study. Rats were fed standard nutritionally balanced diet according to AIN-93 **(Reeves *et al.*, 1993)** 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.

Experimental osteoporosis (ovariectomy in female rats).

Ovariectomy is considered the procedure that gives reliable model of osteoporosis in female rats. It can be performed in some different ways. After the period of adaptation (one week), first group of female rats (n=40) 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 (n=10) were subjected to sham operation. After the operation, each rat was placed in an individual cage for one week, and then OVX rats were sub-classified to four groups. The operation was done in king Fahd Center for Medical Research.

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 and procedures.

After 1 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 as control negative group. 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). The treatment of the OVX groups with RCI commenced 7 days after the OVX and continued 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 RCI. Percent of body weight gain (BWG%) and feed efficiency ratio (FER) were calculated .

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 enzymes and hormones assay.

Serum samples were used for determination of bone marker enzymes including N- terminal propeptide of procollagen type I (PINP) according to the method described by **(Atkinson *et al.*, 2004)**. As well as determination of osteocalcin and parathyroid hormones according to **Miles *et al.* (1974)** 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. Bone mineral content (BMC) was determined by DEXA in different positions Body fat tissues percentage was estimated by DEXA for each experimental group using the following equation:

$$\textbf{Fat tissues percent (Fat \%)} = \frac{\text{Total tissue (g)}}{\text{Total fat (g)}} \times 100$$

Histochemical examination.

All histochemical stains were performed on the sections of left femur bone rats in all groups. Histochemical staining procedures for toluidine blue; stain utilized for optimal demonstration of mineralized bone, osteoid seams, osteoblasts and osteoclasts **(Bancroft *et al.*, 1994)**. Masson trichrome method stains differently a mineralized bone (blue) and an osteoid (red) **Asonova and Migalkin (1996)**. Osteoclasts were identified as multinucleated cells **(Drury, 1980)**.

Statistical analysis.

Results were expressed as (mean ± SD). Data were analyzed statistically by analysis of variance, one way ANOVA followed by post hoc multiple comparisons using L.S.D. test, according to **Snedecor and Cochron (1989)**. An IBM computer with a software system SPSS version 20 was used for these calculations.

3. Results.

Biological evaluation.

Table (1) showed the effect of different doses of RCI on biological evaluation parameter (BWG%, FI and FER) and fat tissues percent in OVX female rats. The results indicated that, 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 (101.6%, 10.84% and 82.86 in BWG%, FI and FER, respectively) and 55.35 % in fat tissues % as percent change from control (sham) group. While when compared between control (sham) group with three doses (20, 40 or 60 mg) of RCI treated OVX groups, there was very high significant differences in biological evaluation parameters at ($p< 0.001$) except the effect of high dose of RCI on feed 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 with all treated groups on all biological evaluation parameters at ($p< 0.001$), except the effect of low dose RCI on feed efficiency ratio and fat tissues% 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. In addition, the low dose (20 mg) revealed a a significant difference as compared with the high dose (60 mg) RCI on FI and FER at ($p< 0.05$), and high significant difference compared with high dose (60 mg) of RCI on fat tissues% at ($p< 0.01$).

Table (1): Effect of RCI on biological evaluation and fat tissues % in ovariectomized female rats.

Experimental groups	BWG %	FI (g/day/rat)	FER (g)	Fat tissues %
Control (sham)	31.97± 1.95	21.86 ± 0.78	0.035 ± 0.0019	23.36 ± 2.34
Control (OVX)	a^{***} 64.45± 2.49	a^{***} 24.23± 1.30	a^{***} 0.064 ± 0.0046	a^{***} 36.29 ± 3.61
OVX+ 20 mg RCI	$a^{***}b^{***}d^{***}$ 60.39 ± 2.26	a^{***} 24.13 ± 0.69	$a^{***}b^{**}$ 0.060 ± 0.0019	$a^{***}b^{**}$ 32.00 ± 2.01
OVX+ 40 mg RCI	$a^{***}b^{***}c^{***}$ 56.87 ± 1.31	a^{***} 23.58 ± 0.54	$a^{***}b^{***}$ 0.058 ± 0.0015	$a^{***}b^{***}$ 30.10 ± 2.14
OVX+ 60 mg RCI	$a^{***}b^{***}c^{***}d^{*}$ 54.88 ± 1.45	$a^{**}b^{*}c^{*}$ 23.16 ± 1.17	$a^{***}b^{***}c^{*}$ 0.058 ± 0.0034	$a^{***}b^{***}c^{**}$ 28.29 ± 2.10

RCI: Red clover isoflavone. **OVX:** Ovariectomized. **FI:** Feed intake. **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+20mg RCI. ^{d}**:** Significant differences *vs.* OVX+40mg RCI.
(*: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$).

Bone marker enzymes and hormones assay parameters.

Table (2) showed the effect of different doses of RCI on serum levels of N-terminal propeptide (PINP) in ovariectomized (OVX) female rats. It is noticed that, the control (sham) female rats recorded very highly significant differences ($p< 0.001$) compared with OVX group in PINP, with the mean value (7.01 ± 0.65) *vs.*

(10.00 ± 0.98) (ng/ml) in control (sham) and OVX, respectively. Concerning OVX untreated female rats it showed that, PINP recorded very highly significant difference ($p< 0.001$) when compared with treated OVX groups at all administered doses of RCI (20, 40 and 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 female rats, showed significant improvement in bone marker enzymes, a dose response trend was observed with various levels of RCI. The serum levels of PINP in RCI treated OVX female rats at three used doses recorded a very high significant difference ($p< 0.001$) between low and high dose, and non-significant change where found between low and medium doses.

The effect of different doses of RCI on serum levels of bone homeostasis hormones; osteocalcin (OCN) and parathyroid hormone (PTH) in OVX female rats shown in Table (2). The results revealed that, ovariectomy resulted in a very high significant decrease in serum OCN and PTH levels as compared to control (sham operated) group at ($p< 0.001$), with percentage (33.58% and 69.26 % in OCN and PTH, respectively) as percent change from the control group. After treatment, there were highly significant changes in OVX treated with RCI at low and medium doses and sham control group in all hormonal assay parameters. While in OVX group received high dose of RCI, there was a significant difference in both OCN and PTH at ($p< 0.01$ and $p< 0.001$, respectively) as compared with sham control group.

Administration of RCI induced an improvement in hormonal assay compared with the OVX untreated group. In OCN levels, there was non-significant changes at low doses comparing with untreated OVX group. A mild improvement at ($p< 0.05$) was found when compared between OVX untreated and treated with RCI at medium doses, while at high dose of RCI it reached very high significant difference at ($p< 0.001$). Comparing the effect of the different used doses of RCI it was found that, there was a significant change ($p< 0.05$) between low and medium as well as between 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 them.

Table (2): Effect of RCI on serum levels of bone marker enzymes and hormones in ovariectomized female rats.

Experimental groups	PINP (ng/ml)	OCN (ng/ml)	PTH (pg/ml)
Control (sham)	7.01 ± 0.65	1.34 ± 0.06	10.02 ± 1.00
Control (OVX)	^{a***} 10.00 ± 0.98	^{a***} 1.79 ± 0.16	^{a***} 16.96 ± 1.20
OVX + 20 mg RCI	^{a***b***} 9.04 ± 0.82	^{a***d*} 1.68 ± 0.15	^{a***b***} 13.24 ± 1.06
OVX + 40 mg RCI	^{a***b***} 9.03 ± 0.85	^{a***b*c*} 1.63 ± 0.15	^{a***b***} 12.63 ± 1.12
OVX + 60 mg RCI	^{a***b***c***d***} 8.76 ± 0.88	^{a**b***c*} 1.54 ± 0.12	^{a***b***} 12.84 ± 1.07

RCI: Red clover isoflavone. **OVX:** Ovariectomized. **PINP**: N- terminal propeptide of procollagen type I.
OC: Osteocalcin. **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 ***: $p<0.001$).

Bone mass results.

Effect of RCI on bone mineral content (BMC) in 3 positions: head, legs and spine of OVX female rats in Table (3) and Figure (1). Results of DEXA showed that, BMC in control (sham operator) indicated very high significant differences compared with OVX untreated group at ($p< 0.001$) in the head, legs and spine. The values of BMC in both medium and high doses of RCI recorded non-significant changes as compared with control (-ve) in the three positions. Low dose recorded a significant decrease ($p< 0.05$ and $p< 0.01$ in BMC of head and legs, respectively) compared with control (-ve) group, while BMC in spine recorded non-significant difference as compared with control (-ve) group.

Administration of RCI to OVX groups induced an elevation in BMC as compared with untreated OVX group. In BMC of head, legs and spine there were high significant differences at ($p< 0.01$) between the medium and high doses of RCI. BMC of head and legs in low dose of RCI (20 mg/ day) recorded significant difference at ($p< 0.05$ and $p< 0.01$, respectively) compared with untreated OVX group. The same

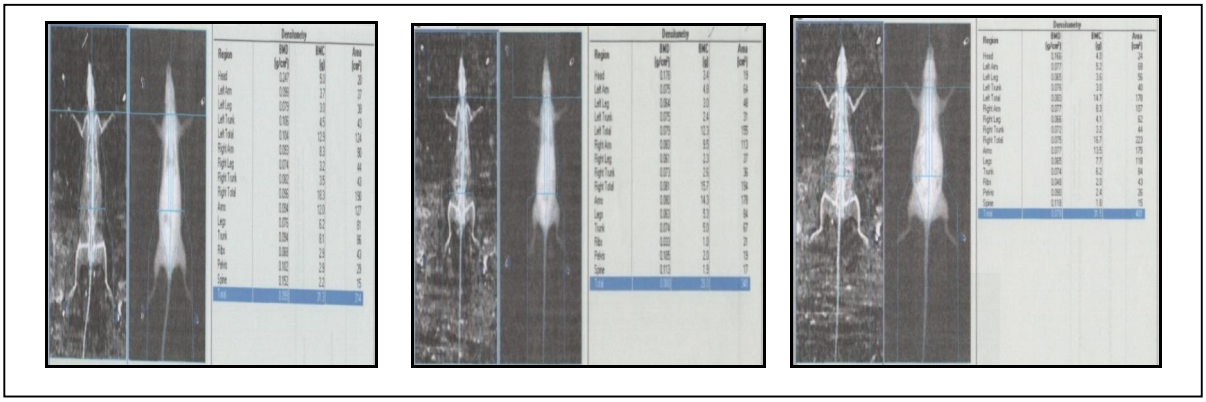
significant difference was recorded in both medium and high doses of OVX treated groups at ($p< 0.01$ and $p< 0.001$, respectively) as compared with the corresponding untreated OVX group.

These results indicated an improvement in BMC 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. Regarding the different doses of RCI used to treat OVX it was showed that, there was a non-significant difference between those doses, expect when compare low with high dose of RCI on BMC of the legs at ($p< 0.05$).

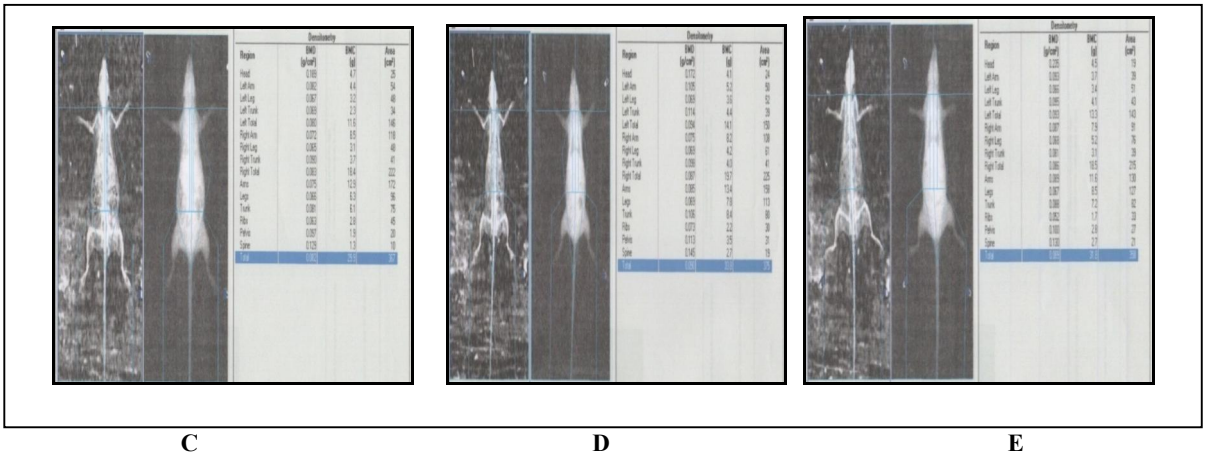
Table (3) Effect of RCI on bone mass in ovariectomized female rats.

Experimental groups	BMC (g)		
	Head	Legs	Spine
Control(sham)	4.81± 0.41	7.84 ± 0.79	2.36 ± 0.24
Control (OVX)	^{a***} 3.90 ± 0.38	^{a***} 5.59 ± 0.41	^{a***} 1.51 ± 0.11
OVX+ 20 mg RCI	^{a*b*} 4.34 ± 0.40	^{a**b**} 6.65 ± 0.53	^{b***} 2.31± 0.15
OVX+ 40 mg RCI	^{b**} 4.59 ± 0.34	^{b***} 7.33 ± 0.74	^{b***} 2.29 ± 0.11
OVX+ 60 mg RCI	^{b**} 4.56 ± 0.42	^{b***c*} 7.48 ± 0.76	^{b***} 2.28 ± 0.20

RCI: Red clover isoflavone. **OVX:** Ovariectomized. **BMC:** Bone mineral content.
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+20mg RCI. ^{d}**:** Significant differences *vs.* OVX+40mg RCI.
(*:p<0.05; **: p<0.01 and ***: p<0.001).



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



C **D** **E**

Fig (1): Examples of bone mass results from DEXA in control (shame), OVX untreated and OVX 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.

Histochemical examination in bone.

Histochemical structure of femur bone in control (sham) female rats stained with Masson's trichrome showed no histological changes and a normal structure (Fig 2 and 3), while the examination of distilled femur bone stained with toulidine blue showed negative histochemical reactions and absence of osteoclast cells (Fig.12). Femur cortical bone of OVX untreated rats showed positive, severe and darkly blue reactions stained, decalcified osteoid bone as showed in the figures stained with Masson's trichrome (Fig 4 and 5). Also, showing multiple osteoclast cells along the cortical bone in figures stained with toulidine blue (Fig 13& 14).

While histochemical structure of femur female rat treated with 20 mg RCI, potential protection from osteoporotic changes induced by ovariectomy was observed as shown in the figures stained with Masson's trichrome, there were moderate positive reaction of decalcified bone (osteoid tissue) (faint blue positive trichrome reaction) figures (6 and 7). In (Fig 15) stained with toulidine blue showed multiple osteoclast cells along the cortical bone, but was less compared with OVX untreated rats. In OVX treated with 40 mg RCI, bone of rats from group OVX treated with 40 mg RCI showed between mild to moderate trichrome positive reaction (blue stain of osteoid tissue) shown in figures stained with Masson's trichrome (Fig 8 and 9). Also, in figures stained with toulidine blue it were noticed that single positive stained osteoclast cells in thick cortical bone (Fig 16) which is an evidence of the extent of improvement compared with ovariectomized group.

Meanwhile, the histochemical structure of femur female rat treated with 60 mg RCI the potential protection from osteoporotic changes induced by ovariectomy was observed as faint or mild positive trichrome reaction as showed in figures stained with Masson's trichrome (Fig 10 and 11). Furthermore, ovariectomized rats were treated with 60 mg RCI were characterized by negative histochemical reaction (absence of osteoclast) as in figures (17) along with a thick cortex, which is a marker of significant improvement compared with OVX untreated rats.

4. Discussion.

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 bone formation during the bone remodeling process **(Nazrun *et al.*, 2011)**. 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 hormone replacement therapy (HRT) **(Atkinson *et al.*, 2004)**, although HRT helps to prevent the development of pathologies in postmenopaus women, however, because a greater incidence of breast and endometrial cancer has been linked to some forms of HRT, increased attention has been placed on finding viable and safe alternatives. Because of their selective estrogenic like activity, soy and red clover are hypothesized to have a positive effect on BMD as women age. Phytoestrogens, such as those derived from RC, have more evidence-based studies than most herbal medicinal products, although the literature is beset by difference in methodologies making direct comparison between them difficult **(Wronski and Yen, 1991)**.

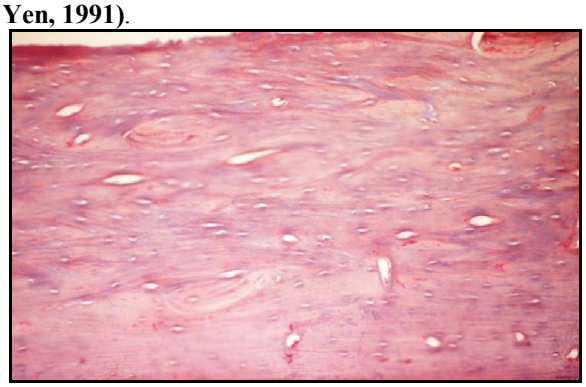


Fig (2): Distilled femur bone of rats from group control (sham) untreated showing no histochemical reaction. (M.T.S x 200)

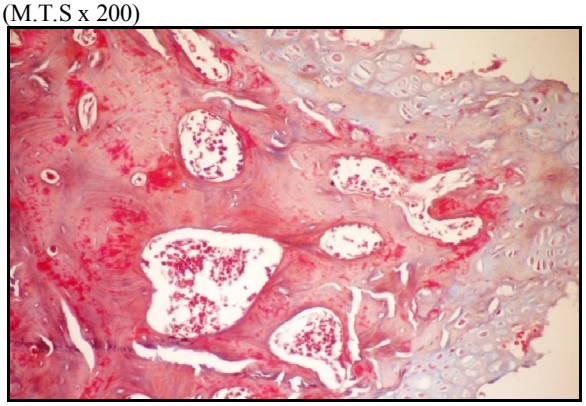


Fig (3): Distilled femur bone of rats from group control (sham) showing no histochemical reaction. (M.T.S x 200)

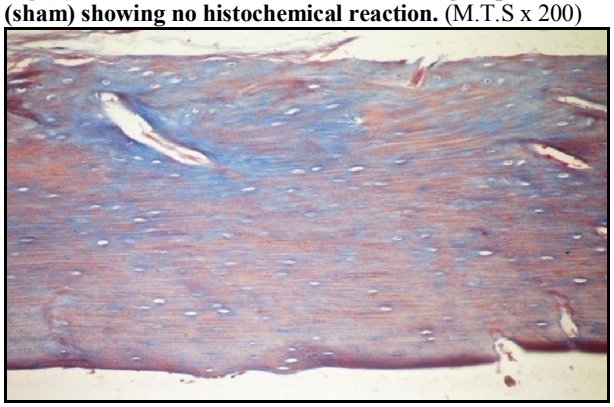


Fig (4): Distilled femur bone of rats from group control (OVX) untreated showing positive trichrome reaction. (M.T.S x 200)

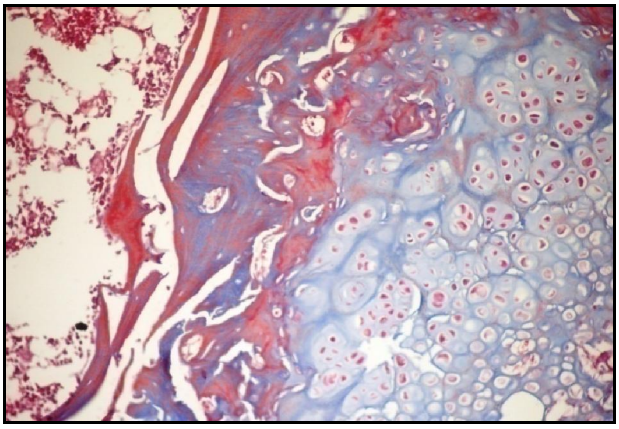


Fig (5): Distilled femur bone of rats from group control (OVX) untreated showing severe, darkly and positive blue stained decalcified bone. (M.T.S x 200)

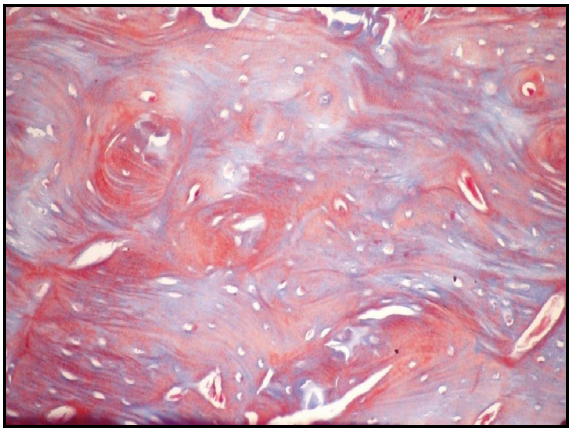


Fig (8): Distilled femur bone of rats from group OVX+40 mg RCI showing moderate trichrome positive reaction. (M.T.S x 200)

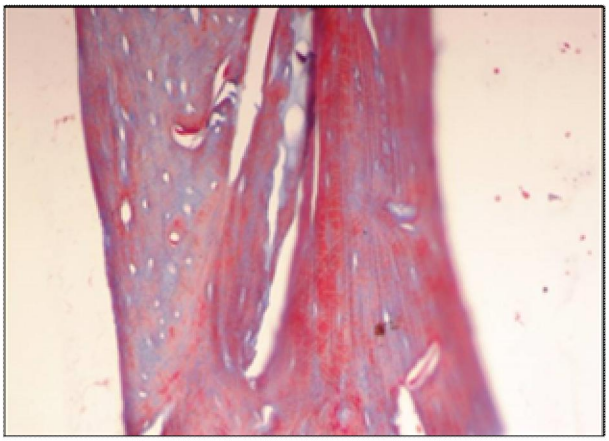


Fig (6): Distilled femur bone of rats from group OVX+20 mg RCI showing moderate positive reaction of decalcified bone (osteoid tissue) (faint blue positive trichrome reaction). (M.T.S x 200)

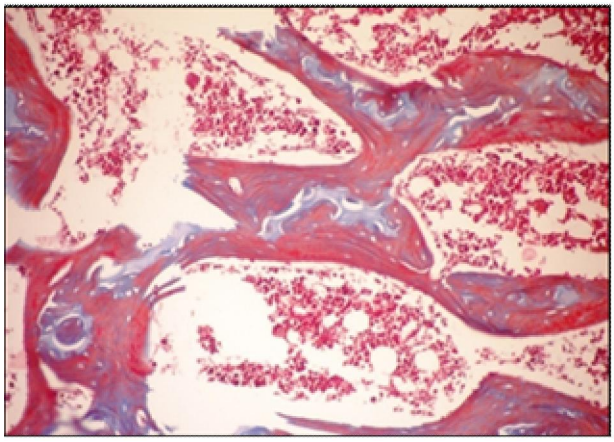


Fig (9): Distilled femur bone of rats from group OVX+40 mg RCI showing mild positive blue stain of osteoid tissue. (M.T.S x 200)

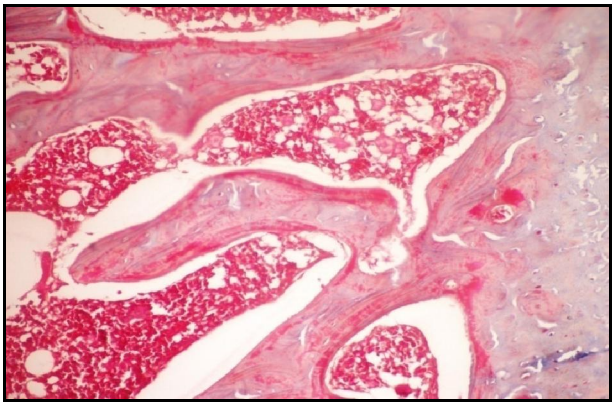


Fig (7): Distilled femur bone of rats from group OVX+20 mg RCI showing faintly stained osteoid tissue. (M.T.S x 200)

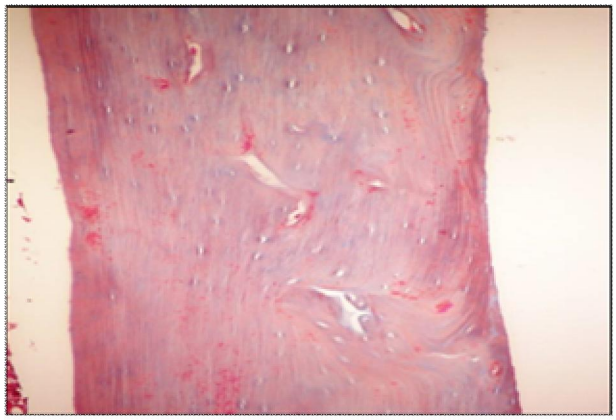


Fig (10): Distilled femur bone of rats from group OVX+60mg RCI showing mild histological reaction (faint blue positive trichrome reaction). (M.T.S x 200)

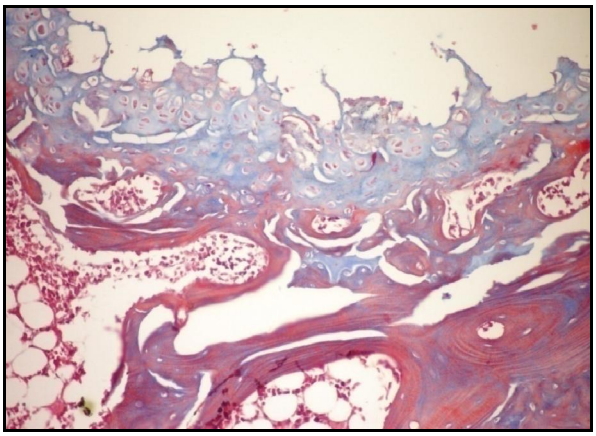


Fig (11): Distilled femur bone of rats from group OVX+60mgRCI showing faint or mild positive trichrome. (M.T.S x 200)

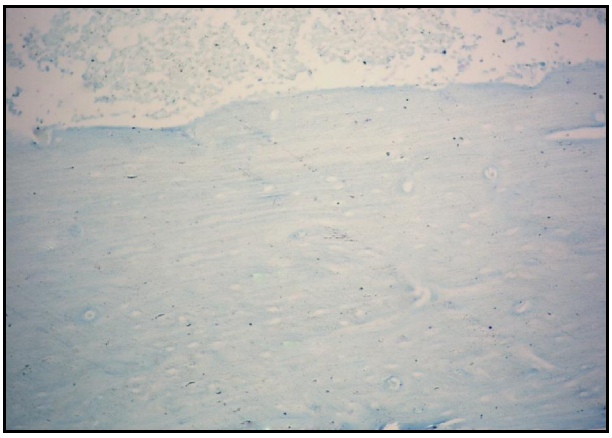


Fig (12): Distilled femur bone of rats from group control (sham) untreated showing no histochemical reaction (absent of osteoclast cells). (T.B. x 200)

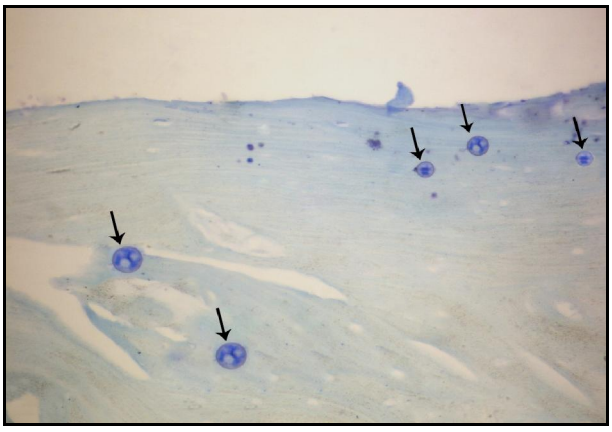


Fig (13): Distilled femur bone of rats from group control (OVX) untreated showing multiple osteoclast cells along the cortical bone. (T.B. x 400)

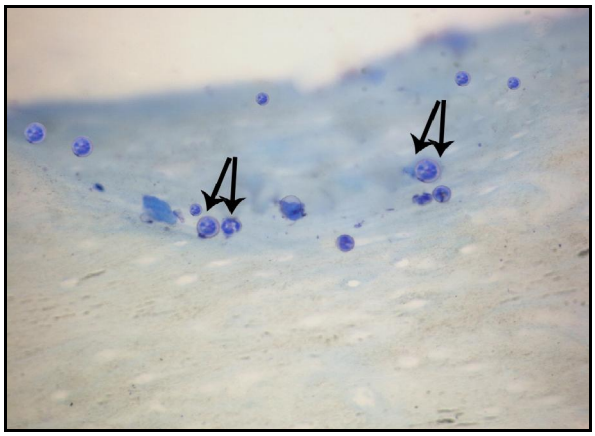


Fig (14): Bone of rats from group control (OVX) showing multiple osteoclast cells along the cortical bone. (T.B. x 400)

Fig (15): Distilled femur bone of rats from group OVX+20 mg RCI showing multiple positive stained osteoclast cell in thick cortical bone. (T.B. x 400)

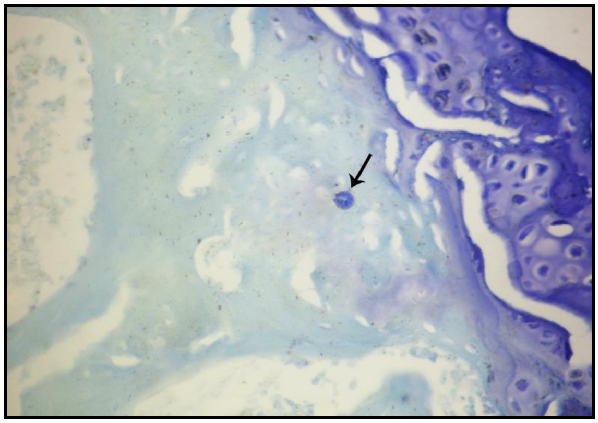


Fig (16): Distilled femur bone of rats from group OVX+ 40 mg RCI showing single positive stained osteoclast cell in thick cortical bone (T.B. x 400)

Fig (17): Distilled femur bone of rats from group OVX+60 mg RCI showing positive single stained osteoclast cell in thick cortex. (T.B. x 400)

In the present experiment, the results indicated that, OVX untreated group recorded very high significant elevation at ($p< 0.001$) in all biological evaluation parameters and fat tissues % as compared with control (sham) group. Consistent with the previous results, the study by **Jiang *et al.* (2008)** and **Zaid *et al.* (2010)** who reported that ovariectomized induced significant higher in overall biological evaluation parameters and in fat mass. Furthermore, 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 adiposity.

Several transcription factors were identified as important regulators of the differentiation pattern of gene expression and the lipid content of fat cells. Hormones including estrogen, growth hormone, thyroid hormone, insulin, and insulin-like growth factor are some of the regulators of a dipogenesis **(Hausman *et al.*, 2001)**. Adipose tissue is highly responsive to estrogen, human and mouse adipose tissues express both estrogen receptors ERα and ERβ **(Anwar *et al.*, 2001 and Naaz *et al.*, 2002)**. Loss of circulating estrogen after ovariectomy leads to increase adipose weights, and this is prevented or reversed by estrogen replacement **(Mohamed and Abdel-Rahman, 2000)**. Estrogen can affect adipose tissue indirectly through modulating appetite or energy expenditure **(Heine, 2000 and Jones *et al.*, 2000)**.

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 ($p<0.001$). 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 **(Asarian and Geary, 2002, Eckel *et al.*, 2002 and Gao *et al.*, 2007)**. Furthermore, estrogen effectively enhances the satiating potency of cholecystokinin (CCK), leading to reduction in meal size and overall FI, CCK is a peptide released from the small intestine during meals and binds to receptors on vagal afferents of pylorus and proximal duodenum to initiate a negative-feedback satiation signal **(Eckel *et al.*, 2002)**. 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 adiposity **(Chen and Heiman, 2001 and Torto *et al.*, 2006)**.

Serum PINP has emerged as a reliable marker of bone turnover in humans and is routinely used to monitor bone formation **(Chen *et al.*, 2005)**. In the present study, it showed significant increase in PINP levels after ovariectomy which is entirely consistent with the studies by **(Stewart *et al.*, 2000, Kneissel *et al.*, 2001 and Rissanen *et al.*, 2008)**. Administrating RCI prevented the rise of serum PINP level compared with OVX untreated rats. These results were supported with **Nikander *et al.* (2004)** and **Rissanen *et al.* (2008)** who reported that treatment of OVX with 17β-estradiol prevented the increase the level of PINP caused by OVX. **Atkinson et *al.* (2004)** who revealed that on postmenopausal women the changes on N–propeptide of collagen type I (PINP) as bone formation marker in treatment group was significant improved ($p< 0.03$) compared with placebo postmenopausal women. Further **Srivastava *et al.* (2013)** evaluated the effect of isoformononetin (isoformo), a naturally occurring methoxydaidzein, for its bone anabolic effect on Sprague-Dawley OVX female rats. They reported that isoformo treatment increased new bone formation, decreased resorptive marker (urinary C-terminal telopeptide of type I collagen) and diminished osteoblast apoptosis in bone, this effect of isoformo may be explained by its pro-survival effect on osteoblasts.

Osteocalcin (OCN) is a sensitive marker of bone formation and unique to bone tissue. It is the most abundant non collagenous bone protein that is synthesized almost exclusively by mature osteoblasts and odontoblasts and deposited in bone matrix. Its activity and synthesis increases when bone mineral density is decreased due to higher bone resorption, so the osteoblastic activity is ascertained by measurement of OCN **(Kumm *et al.*, 2008, Jagtap *et al.*, 2011 and Miao *et al.*, 2012)**.

The present results revealed that ovariectomy resulted in a very highly significant increase in serum OCN levels concentration as compared to the control

(sham) group with percentage (33.58%) as percent change from the control group, indicating increased bone turnover due to OVX-induced estrogen deficiency, which is entirely consistent with the studies by **(Ferretti *et al.*, 2010 and Kim *et al.*, 2011)**. In addition, **Yoon *et al.* (2012)** found that in the 8th week after OVX and sham surgery, OCN level has a 75.4% higher in OVX group than the sham group. In the present study, administration of RCI induced a significant improvement in OCN at (p< 0.05) when compared between OVX untreated and those groups treated with RCI at a medium dose, while at a high dose of RCI it reached a very high significant difference. The present results are supported with the results of **Kawakita *et al*. (2009) and Tyagi *et al.* (2012)** who found formononetin, the highest concentrations in RC, prevented the OVX-induced increase in osteocalcin; bone turnover markers.

Parathyroid hormone (PTH) 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 (p< 0.001), with percentage (69.26%) as percent change from control group. The 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. 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 as compared with OVX untreated group, all used doses of RCI, showed a very high significant elevation in PTH level (p< 0.001) compared with OVX untreated group. On the other hand non-significant change was observed in the values of PTH at the three doses recorded. 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)**.

Long-term administration of isoflavones was found to affect positively bone metabolism **(Arjmandi *et al.*, 1996 and Blair *et al.*, 1996)**. Six-month genistein administration to postmenopausal women led to a significant increase in bone density and concurrent reduction in the concentration of biochemical markers of bone resorption **(Turhan *et al.*, 2008)**. The positive effect of isoflavones on bone metabolism may be mediated by at least two mechanisms, the first is the impact on osteoclasts via activation of apoptosis, and the second is the inhibition of tyrosine-kinase activity via modulation of membrane ER with consecutive changes in the activity of alkaline phosphatase **(Polkowski and Mazurek, 2000)**.

Bone mineral content is one of the most important factors to measure bone quality. The present results of DEXA showed that, BMC in control (sham) group reported very high significant differences in comparison 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 **Jin *et al.* (2003)** and **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. The obtained results reported amelioration in BMC 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 are the studies of **Occhiuto *et al.* (2007)** and **Kawakita *et al.* (2009)** who measured the effect of red clover on total BMC, and reported significant increase in BMC in treated OVX compared with untreated group. Also in animal studies, 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 BMC **(Kawakita *et al.*, 2009)**. 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, histochemical structure of femur bone sections in the control group showed no histological changes with Masson's trichrome, and absence of osteoclast cells with toulidine blue. Femur cortical bone of OVX untreated rat showed positive blue stained, decalcified osteoid bone, severe and darkly blue reaction stained with Masson's trichrome, as well as, showing multiple osteoclast cells along the cortical bone stained with toulidine blue. Consistent with this were the results **(Lane *et al.*, 2003, Weber *et al.*, 2004, Kalleny, 2011 and Saleh and Saleh, 2011)**.

More evidence is provided by **Wang *et al.* (2006)** who showed a decreased number of osteoblasts in the marrow of OVX rats. Differentiation of osteoblast is one of the key events of bone formation **(Deepthi *et al.*, 2012)**.Several factors can cause the appearance of markers of the differentiated osteoblast phenotype, including expression of alkaline phosphatase activity, collagen and osteocalcin **(Behari and Behari, 2009)**. All of these parameters were shown on OVX rats in the present study with decreased level of estrogen led to increase osteoclast formation and enhanced bone resorption, which in turn leads to loss of bone mass and destruction of local architecture resulting in osteoporosis. Moreover, estrogen deficiency is associated with an increased number of osteoclast precursor cells in the murine models' marrow **(Jilka *et al.*, 1992)**. **Lesclous and Saffar (1999)** reported that mast cells accumulated concomitantly with osteoclast generation in estrogen-deficient rats.

The histochemical structure of femur female rat treated with 20 mg RCI, shown by potential protection from osteoporotic changes induced by OVX, There was marked faint blue positive trichrome reaction stained with Masson's trichrome and osteoclast cells along the cortical bone between single and multiple but was less than that in OVX untreated rats stained with toulidine blue. Rats treated with 40 mg RCI, showed a moderate to mild trichrome positive reaction (blue stain of osteoid tissues), as well as in staining Masson's trichrome. In the section stained with tolidin blue single positive stained osteoclast cell were noticed in thick cortical bone. The histochemical structure of femur female rat treated with 60 mg RCI, the potential protection from osteoporotic changes was observed as faint or mild positive trichrome reaction. Furthermore, OVX rats treated with 60 mg RCI characterized by low osteoclast cells or negative histochemical reaction (absent of osteoclast) along thick cortex. Consistent with the previous results, **Occhiuto *et al.* (2007)** in the study to evaluate the preventive effect of red clover isoflavones on the progression of bone loss induced by estrogens deficiency (ovariectomy) in female Wistar rats, histological examination of the femur sections in the region proximal to the epiphyseal growth plate showed that an oral dose of 20 and 40 mg of total isoflavones daily for 14 weeks significantly reduced the number of osteoclasts compared with the OVX untreated rats. The inhibition of osteoclastic bone resorption may result from a direct action of phytoestrogens.

Conclusion. Although, HRT has been a commonly preventive for postmenopausal symptoms, but the negative side effects linked to some forms led to discouraged. 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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10/12/2014