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Impact of Curcumin and Flaxseed Oil on Bone Turnover in Glucocorticoid-Injected Female Rats

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Abstract: **The present study was conducted to evaluate the possible protective effect of flaxseed oil, cur cumin and their combination against GCs-induced osteoporosis in female rats. Healthy female *albino* rats (n=70) weighting (160-200 g) were divided into six groups; group I: (n=10) negative control, Groups (second-sixth) (n=60) rats were injected with glucocorticoids (dexamethasone sodium phosphate) (2mg/kg.b.w/day) for 3 weeks daily. Second group: (n=15) untreated rats (positive control).Third group: (n=10) rats were fed diet containing flaxseed oil (15g/ kg diet). Fourth group: (n=10) rats were orally administered with curcumin (5mg/kg.b.w/day). Five group: (n=10) rats fed on diet containing flaxseed oil (15g/kg diet) and orally administered with cur cumin (5mg/kg.b.w/day). Sixth group: (n=15) rats were treated with estrogen (25mg /kg.b.w/day). Data revealed that GCs exhibited significant decrease in body weight, femoral weight, calcium (Ca^{++}) and phosphorus (P) concentration in serum and femur bone, vitamin D, serum osteocalcin (OC), activity of alkaline phosphatase (ALP) ($p<0.001$) for each, elevated in parathyroid hormone (PTH) ($p<0.001$). Treatment with flaxseed oil, curcumin and mixture showed a significant elevation in Ca^{++}, P^{+++}, Mg^{++} in serum and femur, vitamin D, OC, ALP and decrease in PTH ($p<0.01$) compared with untreated group. Histopathological examination showed features of osteoporosis (cracks - wide Halverson canals - thin trabecular).Treatment with the present substances preserves normal structure of both types of bone, which was more evident in combined treatment. It was concluded that, regular utilization of flaxseed oil and cur cumin as an adjuvant supplement during prolonged Gcs therapy to protect against osteoporosis.**

Keywords: **Curcumin, flaxseed oil, osteoporosis, rats, glucocorticoids.**

1. INTRODUCTION

Bone is a highly specialized form of connective tissue. It is a complex living tissue where the extracellular matrix is mineralized, giving strictness and strength to the skeleton with some degree of elasticity[1].The development of the skeleton is relatively complex. It depends on the simultaneous activity of several different types of cells working together in close consort. Three main cell types are involved in forming and maintaining bone tissue. Osteoblasts are bone-forming cells responsible for synthesizing and depositing bone material. Osteoclasts are accountable for the resorption of bone tissue. Osteocytes cells reside in lacunae and accountable for maintaining bone tissue. Remodeling of bone takes place at the

cellular level as osteoclasts remove bone tissue and osteoblasts construct bone tissue. These conflicting processes of bone formation and resorption allow bones to maintain or change their shape and size during development.Another cell type was described in literatures and was known as lining cells which have no contribution to bone formation, they are found to contribute to the bone remodeling, and to affect the concentration of minerals in blood and bone tissues [2].

Osteoporosis (OP) is a skeletal disorder characterized by low bone mass and microarchitecture deterioration of bone tissue, leading to improved bone weakness and consequent risk of fracture [3]. The definition proposed by a World Health Organization "A disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to improved bone weakness and a consequent increase in fracture risk" .The OP fractures accounted for 0.83 % of the worldwide disability caused by non-infectious diseases [4], internationally, about 200 million women are affected via OP, and in the developed countries one in three women and one in five men over the age of 50 years will sustain an OP-related fracture . The OP is the main health problem in postmenopausal women, who knowledge a sharp low level in estrogen concentration that leads to an increased rate of bone turnover (Nielsen *et al*. 2004). Bone turnover increased is associated with both decreased bone mineral density (BMD) and increased risk of fracture .Twenty-five percent of Saudi Arabian women over 50 years are reported to have osteopenia and the estimated prevalence of OP is 23-34% [5].

Hormone Replacement Therapy (HRT) is an effective treatment for menopausal symptoms that also offers protection against fractures at both hip and spine during menopause [6]. The doses of HRT in the prevention of bone loss are higher than those required in treating menopausal symptoms . For these reasons, the new trend to treat OP without side effects many researchers are interested in medicinal properties of natural herbs with fewer side effects [7].

Diet plays an important role in the skeletal growth and maintenance of bone health throughout life. Poor bone health will result in increased risk of osteoporotic fracture. The OP is a major public health problem through its association with the fragility fracture [8]. Mounting evidence indicates that the amount and type of fat in the diet can have important effects on bone health.Omega-3(ω-3) is considered as essential fatty acid, cannot synthesize inside the body. For that reason, ω-3 fatty acids must be consumed from a dietary source [9]. The ω-3 polyunsaturated fatty acids (PUFA) inhibit the activity of osteoclasts and enhance the activity of osteoblasts in animals [10].Also, the relation of omega-6 (ω-6) to ω-3 fatty acids in the diet may be important .Lowering the dietary relation of ω-6 / ω-3 PUFA increased bone marrow cellularity and bone strength in animals [11].

Flaxseed is a popular traditional food and remedy, as flaxseed and flaxseed oil (FO), or linseed oil, contains α-linolenic acid, ω-3 fatty acid, short chain PUFA, soluble and insoluble fibers, phytoestrogenic lignans, proteins and an array of antioxidants [12]. The FO is readily available in the diet as flaxseed is incorporated into many commonly consumed foods such as bread, muffins and cereals. Previous research has suggested that dietary supplementation of FO, rich in α-linolenic acid, the essential ω-3 PUFA, appears to be more beneficial toward improvements in bone mineral content and BMD . Flaxseed is a rich source of mammalian phytoestrogen lignans [13]. When estrogen level decreased in the body as postmenopausal women, lignans may act like weak estrogen but when natural estrogen is rich, lignans may decrease estrogen's effects by displacing it from the body [14].

Curcumin (CUR) is found in the popular spice turmeric it is a yellow polyphenolic pigment obtained from the rhizome of *Curcuma longa* Linn (Family: *Zingiberaceae*), is a member of the curcuminoid family of compounds [15]. It is well known that curcumin exerts different biologic effects, including anti-inflammatory, antioxidant, antiviral and anti-infection [16]. In addition, some studies investigated the effects of CUR on the regulation of bone turnover .

Glucocorticoids (GCs) are classes of steroid hormones. They are generally used in the treatment of different diseases like inflammatory arthritis, for the reason that they possess potent anti-inflammatory actions and suppress immune-competent cell activities [17]. However, long-term uses of GCs are known to cause decrease BMD and OP as results a complex mechanism involving osteoblastic suppression and increased bone resorption, and this is a major problem in clinical practice .This work was conducted to evaluate the protective effect of curcumin alone or combined with flaxseed oil against osteoporosis induced by corticosteroids in female rats.

2. MATERIALS AND METHODS

Chemicals and kits:

Dexamethasone sodium phosphate injection was purchased from Amriya for Pharmaceutical Industries, Alexandria-Egypt. Estrogen tablets (Ethinyloestradiol) were purchased from Kahira Pharmaceutical and Chemical Industries Company, Cario-Egypt. Reagent kits needed for chemistry analysis were purchased from Human Gesellschaft fur

Biochemica and Diagnostica mbH, Germany. These included Ca and P. Mg colorimetric assay kit was purchased from Spinreact, Spain.ALP colorimetric assay kit was purchased from BioVision Incorporated Milpitas Boulevard, Milpitas, USA. OC enzyme linked immunosorbent assay (ELISA) kit and 1,25 $OH)_2$ vitamin D_3 ELISA kit were purchased from Cloud-Clone Corp, assembled by USCN Life Science Inc, USA. 25-OH Vitamin D ADVIA Centaur assay kit was purchased from Siemens Healthcare Diagnostics Inc, USA. PTH ELISA kit was purchased from CUSABIO, China.

Experimental design:

Anesthetic method and animal handling were approved in accordance with the ethical guidelines of Medical Ethics Committee of the King Abdulaziz University and ensure that animals did not suffer throughout the experiment. Healthy female *albino* rats (n=70 rats) weighting about (160-200 g) were obtained from the animal experimental unit at King Fahd a Medical Research Center (KFMRC), King Abdulaziz University. All animals were allowed to one week to acclimatize in animal housing conditions before being used for the study. The rats were housed in standard laboratory conditions at a temperature of (25º± 2°C), relative humidity (50-55%) and a 12 hours light/dark cycle. All animals fed standard nutritionally balanced diet and drinking water *ad libitum*. Standard nutritionally balanced diet was obtained from KFMRC, the diet consists of the following ingredients; protein 20.0%, fat 4.0 %, fiber 5.0 %, vitamin mix 1.0%, mineral mix 3.50%, choline chloride 0.25%, the remained formula up to 100% corn starch, and its energy equals 2850 kcal/kg. The diet manufactured by Grain Silos and Flour Mills Organization, KSA.

After the adaption period, rats were divided into six groups (n=70) according to administrated herbal food or chemical drug as shown in (table.1).

Statistical Analysis:

Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed with one-factor analysis of variance (ANOVA) and paired t-test using MegaStat. The statistical significance difference was considered when (P<0.05, P<0.01 and P<0.001).

3. RESULTS

Results in table 2 showed the body weight, femoral weight in different groups. It was found that there was a significant decrease in body weight and femoral weight in rats induced osteoporosis as compared with negative control group. Treatment with flaxseed oil or curcumin or their combined increased body weight but not returned to normal. A significant increase in femoral weight in treated rats versus untreated. The combined group showed better action than curcumin or flaxseed oil alone or osteoporotic treated with estrogen in body weight and femoral weight.

Data in table 3 showed serum Ca, P and Mg level in different groups. It was found that there was showed a significant decrease in the levels of serum Ca and P in osteoporotic rats compared with negative control (P<0.05) respectively. Rats treated with either flaxseed oil or curcumin or their combined showed elevate the level of serum Ca more than negative control but not return the P to normal value and normalize the Mg level. Administration of curcumin, flaxseed oil or their combined to osteoporotic rats showed elevate the level of serum Ca more than control (+ve), improvement in P and Mg level, there were values showed very highly significant (p <0.001) evaluate in Ca level compared with control (+ve) and showed non- significant differences in P and Mg level.The effect of curcumin or flaxseed oil alone is better than combined (p<0.001) in Ca improvement while curcumin or combined is better than flaxseed oil in P improvement.Also, it was found that no significant changes in serum Mg in osteoporotic rats compared with negative control and treated groups. The action of combined effects is better than estrogen in Ca level.

Data in table 4.showed bone Ca, P and Mg level in different groups. It was found that there was showed a very highly significant (p < 0.001) decrease in Ca accompanied with highly significant (p<0.01) decrease in P level in osteoporotic rats compared with negative control group. Rats treated with either flaxseed oil or curcumin or combined showed elevate the level of bone Ca, P and Mg compared with untreated but not return the Ca level to a normal value. Administration of curcumin, flaxseed oil or their combined to osteoporotic rats showed elevate the level of bone P and Mg level more than control (+ve) and improvement in Ca level, there were values non-significant differences in Ca and showed very highly significant (p < 0.001) evaluate in P and Mg when compared with control (+ve). The effect of curcumin or flaxseed oil alone is better than combined in Ca improvement while curcumin or combined is better than flaxseed oil in P improvement (p<0.001). Also, it was found that no significant changes in bone Mg in osteoporotic rats compared with

negative control groups. The combined treatment with curcumin and flaxseed oil showed a significant elevation of Mg more than control (+ve) and estrogen ($p<0.001$; $p<0.05$) respectively.

Data in table 5. showed 25-OH vitamin D, $1,25(OH)_2$ vitamin D_3 and PTH level in different groups. It was found that, a very highly significant reduction in 25-OH vitamin D and $1,25(OH)_2$ vitamin D_3 accompanied with a very highly significant elevation in PTH level in osteoporotic rats as compared with negative control ($p < 0.001$). Rats treated with flaxseed oil or curcumin or combined tend to elevate the level of parathyroid hormone more than negative control but not return the levels of this vitamin D to a normal value.

Administration of curcumin, flaxseed oil or their combined to osteoporotic rats showed elevate the level of 25-OH vitamin D and $1,25(OH)_2$ vitamin D_3 more than control (+ve) but decrease in PTH level, there were values showed very highly significant ($p < 0.001$) evaluate in 25-OH vitamin D and $1,25(OH)_2$ vitamin D_3 when compared with control (+ve) and showed very highly significant ($p < 0.001$) reduction in PTH level .The effect of curcumin or combined is better than flaxseed oil and estrogen ($p< 0.001$) in 25-OH vitamin D, $1,25(OH)_2$ vitamin D_3 and PTH improvement.

Results in table 6. showed serum OC level and activity of ALP in different groups. It revealed that, a very highly significant ($p<0.001$) reduction in serum OC level accompanied with significant ($p<0.05$) decrease in activity of ALP in osteoporotic rats as compared with negative control group. Rats treated with either flaxseed oil or curcumin or their combined showed elevate the level of activity of ALP more than negative control but not return OC level to a normal value. Administration of curcumin, flaxseed oil or their combined to osteoporotic rats showed elevate the level of OC and activity of ALP more than control (+ve), there were values showed non- significant differences except curcumin in OC level compared with control (+ve) and showed highly significant differences ($p<0.01$) in activity of ALP. The effect of curcumin or combined is better than flaxseed oil and estrogen ($p<0.01$) in OC and ALP improvement.

Histological examination of femoral bone:

In the present study, cortical bone of female rat from negative control group showed the normal histological structure of compact bone described in previous literature. The Haversian system is the structural characteristic. It consists of the central Haversian canal around which lacunae of osteocytes are arranged in a concentric pattern.Volkman canals looked elongated. Bone matrix is homogeneously stained pink (acidophilic). Both the surface and inner lining of cortical bone is smooth. Osteocytes lacunae near those regions are arranged parallel to both surfaces [Figure 1 (A and B)].Trabecular bone consists of a bony branching trabeculae of normal thickness separated by bone marrow spaces. Osteocyte lacunae are randomly scattered within the acidophilic bone matrix [Figure 15 C].

Administration of dexamethsone sodium phosphate to adult female rat for 3 weeks result in histological changes much similar to those described for OP in animal models. There was widening of Haversian canals. Disorganization of the concentric lacunae lamellae. Bone matrix showed unstained rarefied regions. There is roughness and increase fibrous tissue in both the inner and outer surfaces [Figure 2 (A and B)].Trabecular bone showed marked thinning of the individual trabeculae and widening of marrow spaces [C].

Administration of flaxseed oil in diet to animals receiving dexamethsone sodium phosphate result in protection against OP induced histological changes seen in non-treated groups. Both cortical and trabecular bones showed a normal structure which is nearly similar to those seen in negative control animals. Notice that cortical bone matrix still showed unstained rarefied regions [Figure 3 (A and B)].

Curcumin in the present study was found to prevent osteoporotic changes induced by dexamethsone sodium phosphate. The outer surface is regular and smooth; Haversian canals looked of normal size. Bone matrix is homogeneously stained pink (acidophilic). The cortical bone of femur showed signs of the new bone formation around cracked bones and as dark lines near the inner surface of the bone [Figure 4 A].Trabecular bone showed branching trabeculae with normal thickness and separated by marrow spaces of normal width which are similar to those seen in negative control group [Figure B].

The combination of curcumin and flaxseed oil also provided protections against osteoporotic changes induced by dexamethsone sodium phosphate. Haversian canals, osteocytes lamellae looked similar to negative control. In this group features of new bone formation in the form of layers marked by basophilic calcified lines were observed [figure 5 (A)]. Bone matrix was homogeneous, but few regions still showed unstained rarefied appearance. Trabecular bones also showed normal trabeculae thickness and marrow spaces [Figure 5 B].

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Examination of bone sections of rat femur of dexamethsone sodium phosphate and estrogen treated animals showed protection against induced OP in both cortical and trabecular bone, but it was observed that bone matrix still showed numerous unstained rarefied regions and bone lamellae looked less organized compared to other treated groups. The inner surface showed irregular outlines with aggregation of bone marrow cells [Figure 6.(A)].Trabecular bone showed normal thickness but also bluish regions indicating a lack of complete ossification [Figure B].

Summary: notice that the effect of dexamethsone sodium phosphate was more observed in trabecular bone (thin trabecular and wide marrow spaces) while in cortical bone random disturbance of Haversian system, widening of Haversian canals and occasionally bone fissuring and cracking were observed.Protection from those changes were observed in all groups but was more evident in the group treated with curcumin followed by those treated by curcumin and flaxseed oil and then flaxseed oil alone. The effect of used substances (curcumin and combined treatment) seemed to be similar or even better than those observed in animals treated with estrogen .

Table.1: The examined rats were divided into six group (n=70) according to administrator food or drug.

Animal Group	Number	Administrated (Food or Drug)
Group I (Normal)	10 rats	Rats were fed standard diet served as a negative control group.
Group (II- VI)	60 rats	Rats were injected with (2mg/kg.b.wt/day) dexamethsone sodium phosphate, intraperitonial for 5 weeks.[*]
Group II(Osteoporotic untreated)	15 rats	Rats were fed on a standard diet and served as a positive control group.
Group III(Osteoporotic treated with flaxseed oil)	10 rats	Rats were fed on diet containing flaxseed oil (15g/ kg.diet).[**,1]
Group IV(Osteoporotic treated with curcumin)	10 rats	Rats were orally administered with curcumin (5mg/kg.b.wt/day) in 2ml phosphate-based saline.[2]
Group V (Osteoporotic treated with curcumin and flaxseed oil).	10 rats	Rats fed on diet containing flaxseed oil (15g/kg.diet) and orally administered with curcumin (5mg/kg.b.wt/day) in 2ml phosphate based saline.
Group VI (Osteoporotic treated with estrogen).	15 rats	Rats were injected with estrogen (25mg/kg.b.wt/day) in10 ml tween 80.[3]

Table.2: Initial and final body weight (g), bone weight (g) and relation between bone weight / body weight (final) in all studied groups (Mean ± SD).

Experimental groups Variables …...	Control (-ve)	Osteoporotic (+ve)	Osteoporotic+ Flaxseed oil	Osteoporotic+ Curcumin	Osteoporotic+ (Flaxseed oil &Curcumin)	Osteoporotic+ Estrogen
Body weight (initial) (g)	162.14±11.39	188±8.06	186.71±12.78	188.29±9.34	189.43±8.30	173.2±10.59
Body weight (final) (g)	196.43±11.72	126.8±20.05	141.86±16.72	150.57±11.70	159.86±18.26	143±20.25
Bone weight (g)	0.560± 0.033	0.496±0.034	0.724±0.086	0.722±0.080	0.761±0.145	0.573±.079
Relation between Bone weight / Body weight (final)	0.002850	0.003911	0.005103	0.004795	0.004760	0.004006

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Table.3:Levels of calcium, phosphorus and magnesium in serum of different groups (Mean ± SD)

Experimental groups Parameters	Control (-ve)	Osteoporotic (+ve)	Osteoporotic+ Flaxseed oil	Osteoporotic+ Curcumin	Osteoporotic+ (Flaxseed oil & Curcumin)	Osteoporotic+ Estrogen
Ca (mg/dl)	4.211± 1.26	7275 ± 1.42.	6.739±0.66	6.691 ± 0.89	6.361 ±0.67	5.506 ±1.49
p-value	---- ----	* a143.0 ----	a***0.00 .000 b***	a***0.00 .000 b***	a**3.001 .000 b***	a *425.0 .000 b***
P (mg/dl)	10.000± 2.65	7.652± 0.50	7.954± 1.20	8.011± 0.61	8.051± 0.87	9.886± 1.96
p-value	---- ----	.0142 a** ----	.0188 a* .6681 b	.0220 a* .6103 b	.0247 a* .5703 b	.8912 a .0033b**
Mg (mg/dl)	3.737± 1.06	3.005± 0.37	3.347± 0.41	3.151± 0.49	3.326± 0.70	3.774± 0.40
p-value	---- ----	.101a ----	.848 a .652 b	.503 a .935 b	.837 a .667 b	1.000 a .070 b

Ca: Calcium**, P:** Phosphorus**, Mg:** Magnesium.[a:] Comparison between p-value of negative control and p-value of different groups.
[b:] Comparison between p-value of osteoporotic (+ve) and p-value of osteoporotic treated groups.[c:] Comparison between p-value of osteoporotic treated with (Flaxseed oil &Curcumin) and p-value of osteoporotic treated (Estrogen).(* P< 0.05, ** P < 0.01 and *** P< 0.001).

Table.4:Levels of calcium, phosphorus and magnesium in bone of different groups (Mean ± SD).

Experimental groups Parameters	Control (-ve)	Osteoporotic (+ve)	Osteoporotic+ Flaxseed oil	Osteoporotic +Curcumin	Osteoporotic +(Flaxseed oil &Curcumin)	Osteoporotic+ Estrogen
Ca (mg.Ca/g. bone)	1076.095±138.26	764.274±72.24	805.764±50.19	831.291±87.64	775.751±41.87	1032.801±95.47
p-value	---- ---- ----	.000 a*** ---- ----	.000 a*** .3369 b ----	.000 a*** .1258 b ----	.000 a*** .7889 b .000 c***	.3626 a .000 b*** ----
P P/g (mg. .bone)	572.694±112.96	364.827±73.84	610.719±81.02	632.341±77.69	635.203±40.51	547.027±185.15
p-value	---- ---- ----	.0022 a** ---- ----	.5108 a .000 b*** ----	.3045 a .000 b*** ----	.2823 a .000 b*** .2644 c	.6565 a .01 b** ----
Mg (mg. Mg/g .bone)	30.100±3.44	24.337±2.58	36.554±4.39	34.560±6.99	41.391±2.24	30.540±10.34
p-value	---- ---- ----	.1038 a ---- ----	.0481 a* .001 b*** ----	.1656 a .001 b*** ----	.0010 a*** .000 b*** .0350 c*	.8897 a .1033 b ----

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Ca: Calcium**, P:** Phosphorus**, Mg:** Magnesium.[a:] Comparison between p-value of negative control and p-value of different groups.
[b:] Comparison between p-value of osteoporotic (+ve) and p-value of osteoporotic treated groups.[c:] Comparison between p-value of osteoporotic treated with (Flaxseed oil &Curcumin) and p-value of osteoporotic treated (Estrogen).([*] P< 0.05, [**] P < 0.01 and [***] P< 0.001).

Table.5: Levels of 25-OH vitamin D, 1,25(OH)$_2$ vitamin D$_3$ and parathyroid hormone in different groups (Mean ± SD).

Experimental groups Parameters ……..	Control (-ve)	Osteoporotic (+ve)	Osteoporotic + Flaxseed oil	Osteoporotic + Curcumin	Osteoporotic + (Flaxseed oil &Curcumin)	Osteoporotic+ Estrogen
25-OH Vitamin D (pg/ml)	24,570.947± 2,669.94	10,930.539 ± 823.30	13,825.158 ± 1,096.21	15,675.609 ± 1,580.29	14,019.823 ± 1,094.08	12,642.011 ± 1,556.05
p-value	---- ---- ----	.000 [a***] ---- ----	.000 [a***] .000 [b***] ----	.000 [a***] .000 [b***] ----	.000 [a***] .000 [b***] .0843 [c]	.000 [a***] .0314 [b*] ----
1.25(OH)$_2$ Vitamin D$_3$ (pg/ml)	14,364.286± 2,886.28	6,302.380 ± 1,360.33	12,148.170 ± 3,797.30	13,671.429 ± 2,275.75	13,207.143 ± 2,318.48	7,301.784 ± 2,459.89
p-value	---- ---- ----	.000 [a***] ---- ----	.1306 [a] .000 [b***] ----	.6313 [a] .000 [b***] ----	.4242 [a] .000 [b***] .000 [c***]	.000 [a***] .5215 [b] ----
PTH (pg/ml)	±959.27775.321	957.139 ± 214.02	887±41310.652	316.920 ± 211.19	360.368 ± 222.72	574.401 ± 230.75
p-value	---- ---- ----	.000 [a***] ---- ----	[a]2486. [*b**]0.00 ----	[a]7386. .000 [b***] ----	[a]4815. .000 [b***] .1051 [c]	[a*]151.0 [*b*]084.0 ----

PTH: Parathyroid hormone. [a:]Comparison between p-value of negative control and p-value of different groups.
[b:] Comparison between p-value of osteoporotic (+ve) and p-value of osteoporotic treated groups. [c:] Comparison between p-value of osteoporotic treated with (Flaxseed oil &Curcumin) and p-value of osteoporotic treated (Estrogen). ([*] P< 0.05, [**] P < 0.01 and [***] P< 0.001).

Table.6:Levels of osteocalcin and activity of alkaline phosphatase in different groups (Mean ± SD).

Experimental groups Parameters……..	Control (-ve)	Osteoporotic (+ve)	Osteoporotic+ Flaxseed oil	Osteoporotic+ Curcumin	Osteoporotic+ (Flaxseed oil &Curcumin)	Osteoporotic+ Estrogen
OC (pg/ml)	1,789.379 ± 429.99	510.838 ± 65.20	741.136 ± 263.56	989.2571 ± 532.81	754.575 ± 258.07	602.243 ± 102.06
p-value	---- ---- ----	.000 [a***] ---- ----	.000 [a***] .2121 [b] ----	.000 [a***] .0123 [b*] ----	.000 [a***] .1872 [b] .1897 [c]	.000 [a***] .6171 [b] ----
ALP (U/ml)	111.6 ± 46.6	49.20± 11.78	149.9± 52.65	176.1 ± 63.16	154.7± 30.76	142.7± 65.99
p-value	---- ---- ----	.0415 [a*] ---- ----	.1635 [a] .0022 [b**] ----	.0219 [a*] .000 [b***] ----	.1177 [a] .0015 [b**] .6743 [c]	.2547 [a] .0041 [b**] ----

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OC: Osteocalcin**, ALP:** Alkaline phosphatase.[a:] Comparison between p-value of negative control and p-value of different groups. [b:] Comparison between p-value of osteoporotic (+ve) and p-value of osteoporotic treated groups.[c:] Comparison between p-value of osteoporotic treated with (Flaxseed oil &Curcumin) and p-value of osteoporotic treated (Estrogen).([*] P< 0.05, [**] P < 0.01 and [***] P< 0.00)

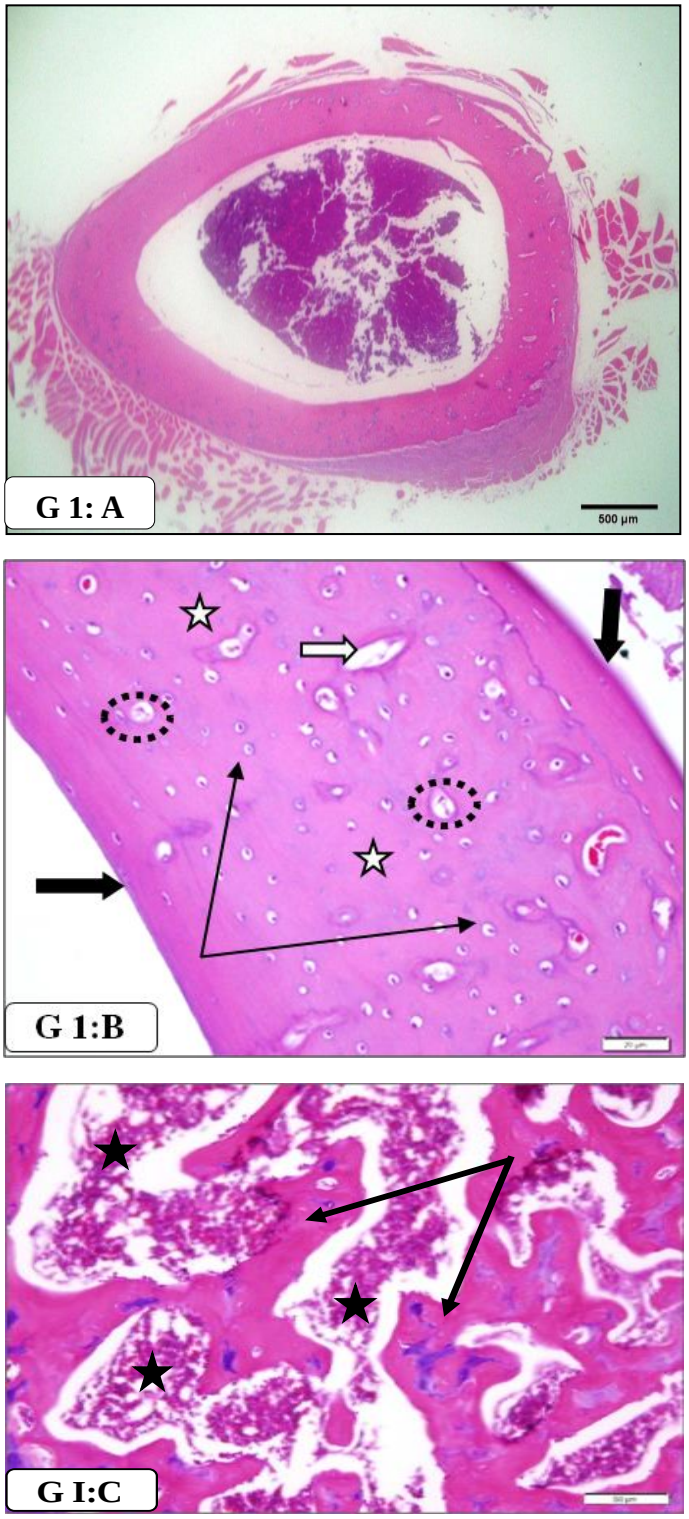

Figure 1 Sections from the middle part of rat femur of the negative control group showing: **A.** low power of a cross section of the femur. **B.** Magnified power to show normal regular Haversian canals (rounded dotted circles) and Volkman canals (white arrow). Bony lacunae showing nuclei of osteocytes as small dark dots (thin black arrows). Bone matrix is homogeneously stained pink or acidophilic (white stars).Notice the smooth outer and inner surfaces of bone (thick black

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arrows). **C.** Bony trabeculae are of normal thickness (black arrow). Notice the marrow spaces with a normal width (black stars).

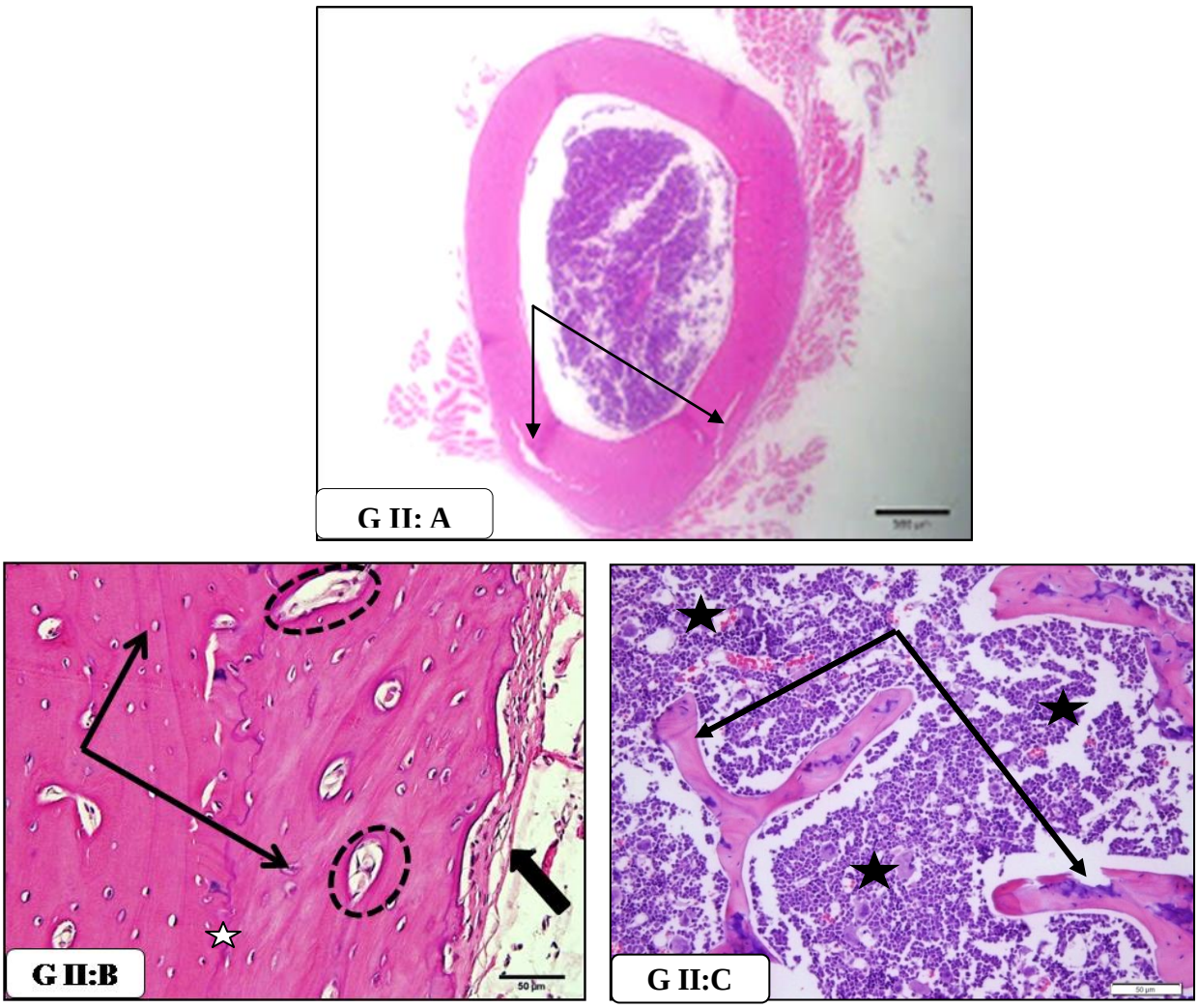

Figure 2 Sections from the middle shaft of rat femur of osteoporotic (positive control) group showing: **A.** low power with fissures or cracks in cortical bones (thin black arrows). **B.** Magnified power to show wide Haversian canals (dotted circles).Disorganization of concentric osteocytes lamellae (thin black arrows). Bone matrix (white star) showed regions of weak staining (rarefactions). There are irregularity and fibrosis of the inner bone lining (thick black arrow).**C.** Marked thinning of bone trabeculae (black arrows) and wide marrow spaces (black stars).

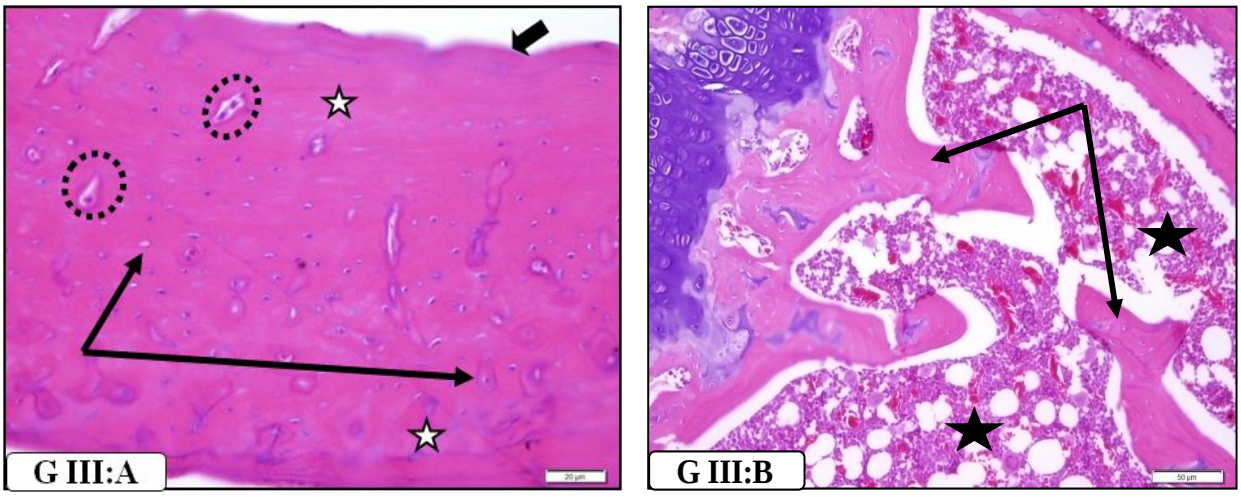

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Figure 3 Sections from the middle shaft of rat femur of osteoporotic + flaxseed oil group showing: **A.** cortical bone with normal Haversian canals width (dotted circles).Osteocyte lamellae are of normal appearance (thin black arrows). Bone matrix still showed unstained rarefied regions (white stars). The bone surface is smooth with no features of irregularity or fibrosis (thick black arrow). **B.** Trabecular bone with nearly normal thickness trabeculae (black arrow). Bone marrow spaces return to the normal width (black stars).

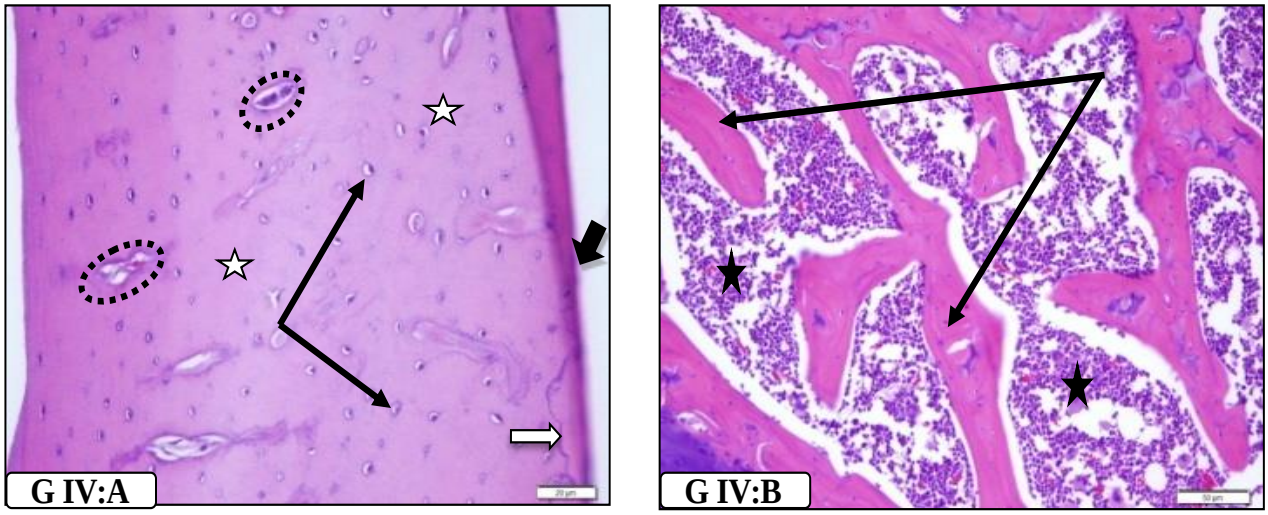

Figure 4 Sections from the middle shaft of rat femur of osteoporotic + curcumin group showing: **A.** cortical bone with normal Haversian canals width (dotted circles).Osteocytes lacunae showed regular arrangement (thin black arrows). The bone matrix is homogeneously pink (acidophilic) indicating good ossification (white stars).The outer surface is smooth (thick black arrow).Signs of new bone formation (blue regions and lines due to deposition of calcium were seen) are observed (white arrow). **B.** Trabecular bone showed nearly normal thickness (black arrows).Bone marrow spaces return to normal width (black stars).

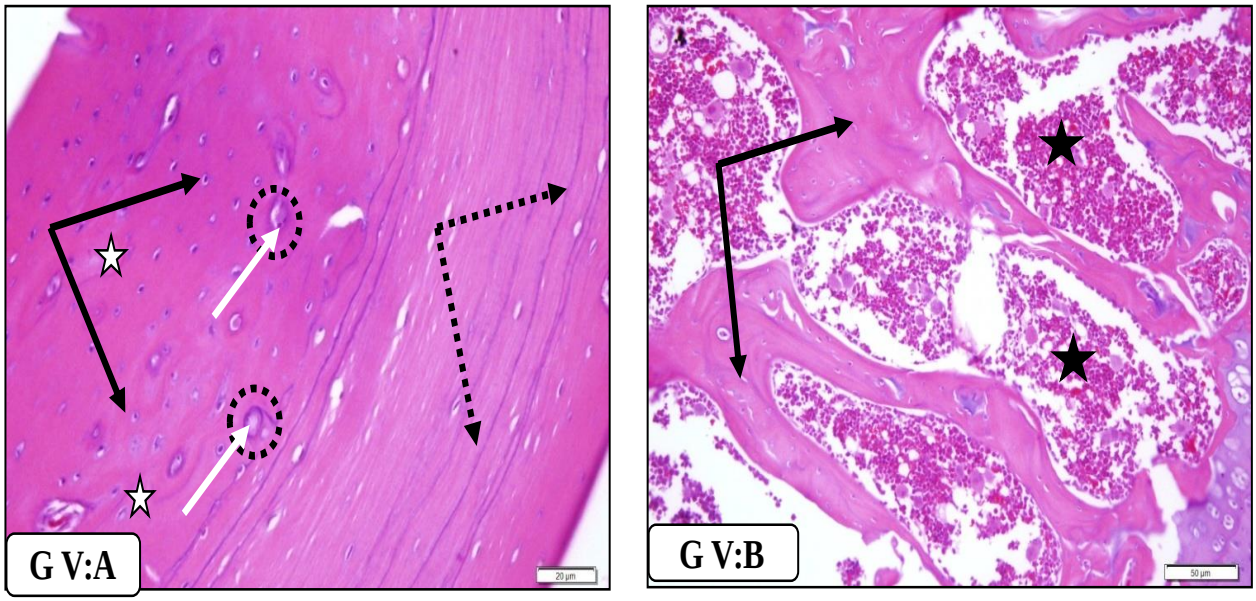

Figure 5 Sections from the middle shaft of rat femur of osteoporotic + (curcumin and flaxseed oil) showing: **A.** cortical bone with narrow Haversian canals (dotted circles) surrounded by bluish ossified regions (white arrows). Also numerous layers of bone deposition marked by bluish calcified lines (dotted arrows). Both indicated new bone formation. Osteocytes lamellae are of normal appearance (black arrows).Bone matrix was homogeneous but few regions still showed unstained rarefied appearance (white stars). **B.** Trabecular bone with normal thickness trabeculae (black arrows) and marrow spaces (black stars).

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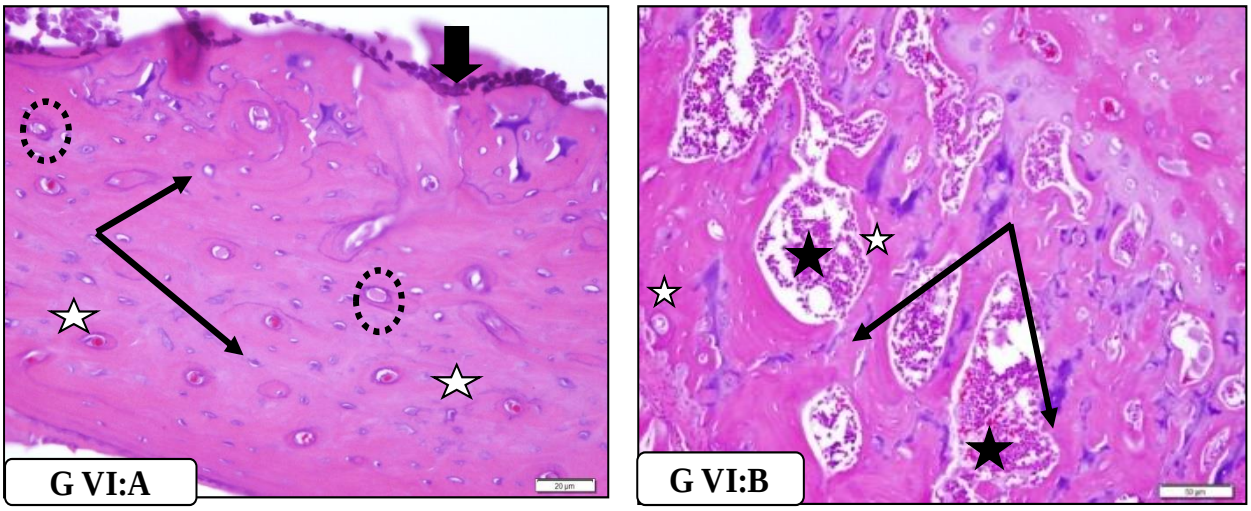

Figure 6 Sections from the middle shaft of rat femur of osteoporotic + estrogen group showing: **A.** cortical bone with narrow Haversian canals (dotted circles), less regular bone lamellae (thin black arrows).Bone matrix is still showed large poorly stained regions (white stars). The inner surface is irregular and showed adherence of cells (thick black arrows). **B.** Trabecular bone is of normal thickness (black arrows) with narrow marrow spaces (black stars).The matrix of trabeculae showed rarified regions (white stars)(H&E stain)

4. DISCUSSION

Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue, predisposing to an increased risk of skeletal fractures (18).It is the most common metabolic disorder of old age in humans (Manolagas and Parfitt 2010). A number of studies have shown a varied prevalence of osteoporosis from 5% to 39% (19)[20].Glucocorticoids (GCs) is widely used to treat a variety of diseases and causes a number of significant side effects[21].They have a beneficial anti-inflammatory and immunosuppressive effect, but their use is associated with decreased bone formation, bone mass, and bone quality, resulting in an elevated fracture risk [21].

The present study was undertaken to evaluate the impact of curcumin and flaxseed oil on calcification in rats injected with GCs to induce experimental osteoporosis. This study was carried out as six groups of rats: negative control group, osteoporotic (+ve control) group, osteoporotic + flaxseed oil group, osteoporotic + curcumin group, osteoporotic + (flaxseed oil and curcumin) group and osteoporotic + estrogen group. The results obtained showed a significant decrease in body weight and bone weight of rats injected with GCs compared with negative control. The previous study reported that rats injected with GCs showed a significant decrease in body weight and bone weight. This is accordance with our result and the significant decrease may be due to the lipolytic effect of GCs as anti insulin action, causes increased catabolic rate and decrease bone mass density [22].Treatment with curcumin and flaxseed oil or combined showed improvement in the body weight and bone weight, the combined group showed better action than curcumin or flaxseed oil alone or osteoporotic treated with estrogen. This may be due to an antagonistic effect of the compounds (curcumin and flaxseed oil) against GCs in improving body weight and bone density.

In the present study, serum Ca, P and Mg of rats injected with GCs showed a significant ($p < 0.05$) decrease in Ca and P level (2.475 and 7.652 mg/dl) as compared with negative control group (4.211 and 10.000 mg/dl) respectively. However showed non-significant differences in Mg level (3.005mg/dl) as compared with negative control group (3.737mg/dl) .The previous study reported that rats injected with GCs showed a significant decrease in P and a similar result was reported that rats injected with GCs showed a significant decrease in Ca. This is in agreement with our result and significant decrease due to decrease Ca absorption from intestinal, decreased renal reabsorption of Ca and P, and may decrease intracellular Ca and P [23].Treatment with curcumin and flaxseed oil or combined showed a significant elevation in the level of serum Ca higher than control (+ve) and improvement in P and Mg level. The effect of curcumin or flaxseed oil alone is better than combined ($p<0.001$) in Ca improvement while curcumin or combined is better than flaxseed oil in P improvement. These results may be due to the action of these compounds that have higher Ca or P content and their

ability in increasing the absorption of these minerals and decreased its loss. In addition, the combined with (curcumin and flaxseed oil) is better than estrogen group may due to their synergistic action.

The results obtained showed also that Ca, P and Mg levels in bone of injected rats with GCs are very highly significant decrease (764.274 mg.Ca/g.bone) ($p < 0.001$) and highly significant decrease (364.827 mg.P/g.bone) ($p<0.01$) as compared with negative control group (1076.095 and 572.694 mg/g.bone) respectively. However there is non-significant differences in Mg level (24.337mg.Mg/g.bone) as compared with negative control group (30.100mg.Mg/g. bone).It reported that rats injected with GCs showed a decrease in Ca and P level. This is in agreement with our result and significant decrease may be due to the GCs that decrease the rate of osteogenesis (or ossification) and calcification via inhibition of PTH [24]. Treatment with curcumin and flaxseed oil or combined showed a significant elevation in the level of bone P and Mg more than control (+ve) and improvement in Ca level. The effect of curcumin or flaxseed oil alone is better than combined in Ca improvement while curcumin or combined is better than flaxseed oil in P improvement ($p<0.001$).Also, the combined treatment with (curcumin and flaxseed oil) showed an increase of Mg more than control (+ve) and estrogen group. These effects may be due to high bioavailability of Ca, P and Mg induced by curcumin and flaxseed oil and deposition of them in bone. In addition high content of omega-3 fatty acids, lignans and polyphenolic may have a positive effect in the rate of calcification and correction Ca and P level.

Previous studies have suggested that vitamin D deficiency or resistance could contribute to the reductions in Ca absorption or, conceivably, also lead more directly to PTH hypersecretion [24].In the present study, 25-OH vitamin D, $1,25(OH)_2$ vitamin D_3 and PTH level of rats injected with GCs showed a very highly significant ($p<0.001$) reduction vitamin D (10,930.539 - 6,302.380 pg/ml) respectively, accompanied with a very highly significant ($p<0.001$) elevation in PTH level (957.139 pg/ml) as compared with negative control group (24,570 - 14, 364 - 277.959 pg/ml) respectively. It reported that rats injected with GCs showed a significant reduction in 25-OH vitamin D. Also, these results are in agreement with that who reported that rats injected with GCs showed a significant reduction in $1,25\ (OH)_2$ vitamin D_3 and elevation in PTH level. This is accordance with our result and a significant reduction in 25-OH vitamin D and $1,25(OH)_2$ vitamin D_3 and elevation in PTH level may be due to hypocalcemic effect induced by GCs is reversed by elevation of PTH [25].Treatment with curcumin and flaxseed oil or combined showed elevate the level of 25-OH vitamin D and $1,25(OH)_2$ vitamin D_3 more than control (+ve) but decrease in PTH level. The effect of curcumin or combined is better than flaxseed oil and estrogen in improvement 25-OH vitamin D, $1,25(OH)_2$ vitamin D_3 and PTH. These results due to curcumin or combined acts as free radicals scavenge and increased vitamin D receptor expression [24].Flaxseed oil and curcumin active ingredient may have positive effect in synthesis of active form of vitamin D.

The OC, bone ALP, and type I procollagen peptide are a good indicator of healthy bone (25). The OC is one of bone markers related to bone metabolism in postmenopausal women and usually used for screening purpose in postmenopausal patients (26).In this study, OC level and ALP activity of rats injected with GCs showed a very highly significant ($p < 0.001$) reduction in OC level (510.838 pg/ml) accompanied with significant ($p<0.05$) decrease in activity of ALP (49.20U/ml) as compared with negative control group (1,789.379 pg/ml-111.6 U/ml) respectively. It was reported that rats injected with GCs showed a significant reduction in OC. Also, these results are in agreement with who reported that rats injected with GCs showed a significant reduction in OC and ALP. This is accordance with our result and significant reduction due to the decrease osteoblasts activity results in a significant reduction in bone formation, and it has been postulated that the loss of osteocytes results in a disrupted osteocyte–canalicular network and failure to respond to bone damage [25-27]. Treatment with curcumin and flaxseed oil or combined showed an elevation in the level of OC and activity of ALP more than control (+ve). The effect of curcumin or combined is better than flaxseed oil and estrogen alone ($p<0.01$).This may be due to the activity of curcumin or combined in reduce the osteoclast number however also increase the osteoblast count [28].The histological examination of both cortical and trabecular bone of female rat femur was support the biochemical analysis by improving the cortical profile, matrix of trabeculae, due to the bioactive ingredients of curcumin synergize with omega-3 fatty acids in improving the bone density.

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REFERENCES

[1] Jung, K. Y., Kim, K. M., Ku, E. J., Kim, Y. J., Lee, D. H., Choi, S. H. and Lim, S. (2015).Age-and sex-specific association of circulating osteocalcin with dynamic measures of glucose homeostasis, Osteoporosis International, doi:10.1007/s00198-015-3315-7

[2] Joshi, A., Reddy, S. V. B., Bhatia, V.,Choudhuri, G., Singh, R. K., Singh, N. and Bhatia, E. (2011) High prevalence of low bone mineral density in patients with tropical calcific pancreatitis, Pancreas, vol.40:762-767.

[3] Jordan, N., Barry, M. and Murphy, E. (2006) Comparative effects of antiresorptive agents on bone mineral density and bone turnover in postmenopausal women, Clinical interventions in aging, vol.1:377-378.

[4] Johnell, O. and Kanis, J. A. (2006) An estimate of the worldwide prevalence and disability associated with osteoporotic fractures, Osteoporosis international,.17:1726-1733.

[5] Johansson, H., Oden, A., Johnell, O., Jonsson, B., De Laet, C., Oglesby, A.and Kanis, J. A. (2004) Optimization of BMD measurements to identify high risk groups for treatment—a test analysis, Journal of bone and mineral research, vol.19:906-913.

[6] Ivanov, S., Rashevskaya, T. and Makhonina, M. (2011) Flaxseed additive application in dairy products production, Procedia Food Science, vol.1:275-280.

[7] Iom, Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride (Washington: National Academy Press, 1997), 699-706.

[8] Ioannou, G. N.and Boyko, E. J. (2013) Effects of menopause and hormone replacement therapy on the associations of hyperuricemia with mortality, Atherosclerosis, vol.226:220-227.

[9] Iliopoulos, C., Zouloumis, L. and Lazaridou, M. (2010) Physiology of bone turnover and its application in contemporary maxillofacial surgery, A review.Hippokratia, vol. 14:244-248.

[10] Ikpeama, A., Onwuka, G. I. and Nwankwo, C.(2014) Nutritional Composition of Tumeric (Curcuma longa) and its Antimicrobial Properties, International Journal of Scientific and Engineering Research, vol.5:1085-1089.

[11] Huybers, S., Naber, T. H., Bindels, R. J. and Hoenderop, J. G. (2007) Prednisolone-induced Ca^{2+} malabsorption is caused by diminished expression of the epithelial Ca^{2+} channel TRPV6, American Journal of Physiology-Gastrointestinal and Liver Physiology, vol.292: 92-97.

[12] Hussan, F., Ibraheem, N. G., Kamarudin, T. A., Shuid, A. N., Soelaiman, I. N. and Othman, F. (2012) Curcumin protects against ovariectomy-induced bone changes in rat model, Evidence-Based Complementary and Alternative Medicine, vol.2012:1-7.

[13] Holick, M. F. (2007) Vitamin D deficiency, New England Journal of Medicine, vol. 357:266-281.

[14] Holick, M. F. (2004) Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease, The American journal of clinical nutrition, vol.80:1678-1688.

[15] Henriksen, K., Bollerslev, J., Everts, V. and Karsdal, M. A. (2010) Osteoclast Activity and Subtypes as a Function of Physiology and Pathology??? Implications for Future Treatments of Osteoporosis, Endocrine reviews, vol.32:31-63.

[16] Heaney, R. P. (2004) Phosphorus nutrition and the treatment of osteoporosis, In Mayo Clinic Proceedings, vol.79:91-97.

[17] Hayakawa, N. and Suzuki, A. (2013) Secondary osteoporosis or secondary contributors to bone loss in fracture. Therapeutic strategy for glucocorticoid-induced osteoporosis, Clinical calcium, vol.23:1337-1344.

[18] Hatcher, H., Planalp, R., Cho, J., Torti, F. M. and Torti, S. V. (2008) Curcumin: from ancient medicine to current clinical trials,Cellular and Molecular Life Sciences, vol.65:1631-1652.

[19] Hall, D. (2001) Nutritional influences on estrogen metabolism, Applied nutritional science reports, vol.1:1-8.

[20] Hahn, T. J., Halstead, L. R., Teitelbaum, S. L. and Hahn, B. H. (1979) Altered mineral metabolism in glucocorticoid-induced osteopenia.Effect of 25-hydroxyvitamin D administration, Journal of Clinical Investigation, vol.64:655-665.

[21] Guyton, A. C. and Hall, J. E., Textbook of Medical Physiology, Seventh Edition (Philadelphia: Philadelphia University, 1986), 875-1007.

[22] Guarda, D. S., Lisboa, P. C., de Oliveira, E., Nogueira-Neto, J. F., de Moura, E. G. and Figueiredo, M. S. (2014) Flaxseed oil during lactation changes milk and body composition in male and female suckling pups rats,Food and Chemical Toxicology, vol.69:69-75.

[23] Griel, A. E., Kris-Etherton, P. M., Hilpert, K. F., Zhao, G., West, S. G. and Corwin, R. L. (2007) An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans, Nutrition Journal, vol.6:1-8.

[24] Greer, W., Ahmed, M., Rifai, A. and Sandridge, A. L. (2008) Exploring the extent of postmenopausal osteoporosis among Saudi Arabian women using Dynamic simulation, Journal of Clinical Densitometry, vol.11:543-554.

[25] Gram, J., Junker, P., Nielsen, H. K. and Bollerslev, J. (1998) Effects of short-term treatment with prednisolone and calcitriol on bone and mineral metabolism in normal men, Bone, vol.23:297-302.

[26] Gracia-Marco, L., Vicente-Rodriguez, G., Valtuena, J., Rey-Lopez, J. P., Diaz Martinez, A. E., Mesana, M. I. and Moreno, L. A. (2010) Bone mass and bone metabolism markers during adolescence: The HELENA Study, Hormone research in paediatrics.74:339-350.

[27] Govindarajan, V. S. and Stahl, W. H. (1980) Turmeric—chemistry, technology, and quality,Critical Reviews in Food Science and Nutrition.12:199-301.

[28] Goltzman, D., Henderson, B. and Loveridge, N. (1980) Cytochemical bioassay of parathyroid hormone: characteristics of the assay and analysis of circulating hormonal forms, Journal of Clinical Investigation.65:1309-1317.