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OSTEOPROTECTIVE EFFECT OF DIFFERENT COMPONENTS OF *MORINGA OLIEFERA* IN OVARIECTOMY INDUCED OSTEOPOROSIS MODEL OF WISTAR RATS

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ABSTRACT

Osteoporosis is a bone condition defined by low bone mass, increased fragility, decreased bone quality, and an increased fracture risk. Due to lack of compliance in current pharmacological interventions targeting bone problems like postmenopausal osteoporosis, there is an urge for developing new alternative therapies for osteoporosis. In recent times, interest has been given to phytotherapy due their ease of availability and acquiescence. Hence, the present study demonstrates that supplementing ovariectomized (OVX) animals with *Moringa oliefera* (MO) flower. leaf and fruit extract have positive effect on bone health. Following ovariectomy there was a dramatic decrease in the serum calcium levels, with an increase in the excretion of calcium. Elevated TRAcP and ALP after OVX decreased with MO extract exposure, fall in both the markers clearly suggest that MO plant extract ameliorate the damage caused by estrogen deficiency. Further, phytochemical analysis showed an array of phytochemicals with anti inflammatory as well as antioxidant properties, proving its osteoprotective efficacy. In general, the presence of these Phytochemicals could account for the much touted medicinal properties. Because of the chemical complexity of the MO, one individual phytochemical cannot be given the credit for its pharmacological property. Some compounds may be collectively affecting broad aspects of physiology, detoxification mechanisms, reducing the stress and re-supplementing the lost hormones such as phytoestrogens.

KEYWORDS: *Osteoporosis, Phytochemicals, Ovariectomy, Moringa Oliefera.*

INTRODUCTION

Osteoporosis becomes a serious health threat for aging postmenopausal women by predisposing them to an increased risk of fracture. Osteoporotic fractures are associated with substantial morbidity and mortality in postmenopausal women, especially older women.[1-2-3] The pathogenesis of osteoporosis is associated with increased osteoclastic bone resorption which is due to the increase in the production of pro-inflammatory cytokines like IL-1 and IL-6.[5-6] These pro-inflammatory cytokines prevents the apoptosis of osteoclasts and diminishes the osteoblast proliferation. This may be attributed to sex steroid deficiency as well as increase in glucocorticoids. Identifying the pharmacological agents which can modulate the osteoclasts and osteoblast cells, so as to preserve or enhance bone mass is the main stay of current pharmacological treatments for osteoporosis. Various therapies are in use for the treatment of osteoporosis, including calcium supplementation with Vitamin D_3 and Hormone Replacement Therapy (HRT) with estrogen, selective estrogen receptor modulators, calcitonin, raloxifene, amino-bisphosphonates, teriparatide, parathyroid hormone, strontium ranelate, growth hormone, and IGF -1.[6] However, HRT poses the threat of breast, ovarian and endometrial cancer. Hence, alternative therapies are gaining attendance to discover new remedies for osteoporosis[6] One of such alternative therapy, phytotherapy, is earning the importance these days because of its lesser side effects and compliance. Various plants have the potency to check the activity of osteoclastic cells as well to promote osteoblastic cells. Medicinal plants constitute an effective source of traditional and modern medicines.[7] Phytotherapy is gaining its importance in the new world countries and is considered to be more effective, safe and compliant when compared to classical drugs like strontium renelate or even HRT. *Litsea glutinosa*, an ayurvedic herb, has been been proved to ameliorates calcium metabolism, increases the osteoblastic number as well as activity and prevents the osteoclastic bone resorption in overactomised rats.[8-9] It has been reported by Bureau of plant industry that *Moringa* is an outstanding source nutritional components. Asserting its multi-faceted value, the plant is utilized for its highly nutritive, medicinal, and water purification properties.[10] The plant's nutritive properties are ubiquitous throughout the plant, resulting in the observation that most plant parts attain nutrition and can be eaten: leaves, seeds, bark, roots, exudates, flowers, and pods.[11-12] A list of possible medical applications conferred by M. oleifera plant parts includes, antihypertensive, anticancer, antispasmodic, antitumor, antiulcer, cholesterol lowering, diuretic, hepatoprotective, and hypoglycemic capabilities, as well as treatment of infectious skin and mucosal diseases.[13] Leaf extracts have been used to treat hyperthyroidism and currently fruits of *moringa* have

been proved to have osteoprotective effect on overectomized Wistar rats[14] however the studies were confined to leaves only. Hence The objectives of the present study was to evaluate the effect of ethanolic extract of different parts of *moringa oliefera* (Fruit, Flower and Leaf) and to analyze the phyto components of all the three parts of MO for its usefulness in preventing bone loss in estrogen deficient OVX rats.

MATERIALS AND METHODS

Experimental protocol

The experimental protocol was approved by IAEC (Institutional animal ethical committee). 3 month-old virgin female Wistar rats 30 in number were procured from Sun Pharma Advance Research Center (300±20gm). The animals were acclimatized for 8 days before the onset of the experiment to adapt to laboratory conditions (The room temperature was 22±4 °C with a 12 h/12 h light/dark cycle). Then the rats were ovariectomized (OVX) and sham operated after being anesthetized under intraperitoneal injection of sodium pentobarbital at a dose of 30mg/kg body weight, as described previously.[15] The success of the OVX was assessed through vaginal cytology after five days of surgery as described previously[16]. Rats were given the lag phase of 10 days to recover from the stress of operation and then treatment was started as follows.

Group 1: Sham control and received vehicle.

Group 2: Ovariectomized (OVX) control and received vehicle.

Group 3: Ovariectomized + leaf extract (200 mg/kg b.wt. / day oral)

Group 4: OVX + flower extract (200 mg/kg b.wt./day / oral)

Group 5: OVX + fruit extract (200 mg/kg b.wt. /day /oral). (LD_{50}=400mg/kg B.W).

Preparation of Extract and phytochemical analysis

Fruits, leaves and flowers of MO were obtained following the method of.[9] All the parts of the plants were dry in oven at 50º C until constant weight was attained. They were kept away from direct sunlight to avoid destroying active compounds. They were then minced using automated mincer and fine powder was produced. Dried powder was prepared by drying MO in oven at 50º C. 100 gm dried powder of each component was extracted with 500 ml methanol in Soxhlet's apparatus for 48 hours . Methanolic extract was dried on water bath at 55º C. The percentage yield of the plant was found to be 9.8%, 6.3% and 7.7% for fruits, leaves and flowers respectively. The plant extract was freeze dried and stored at -70º C. Working solution was prepared by dissolving the extract in DMEM and filtered using 0.23 µ

filters. The dry extracts of MO leaves, flower and fruit were used to determine the compounds. The qualitative methods for flavanoids, alkanoids steroids and triterpenoids, saponins, Tanins and Phenols was performed using standered method.[17-22]

Gas chromatographic analysis

GC/MS analysis was carried out using Perkin Elmer autosystem XL with turbo mass system equipped with PE 5 MS 30m X 250 micron silica capillary. Injector and detector temperatures were 250º and 300ºC, respectively. The temperature started from 70º C for 5 min and then rose to 290º C at the rate of 10º C per minute. Helium was used as carrier gas. The MS was taken at 70 eV. Scanning speed was 0.84 scans s^{-1} and the scanning period was from 40 to 550 s. Sample volume was kept 3 µL.

Biochemical analysis

Every alternate day food and water intake was measured, and the body weight was recorded. All experimental designs and procedures had received the approval of the institutional ethics committee. The treatment was continued for 30 days and at the end of experimental period total urine excreted over 24 h period was collected from overnight fasted rats by housing each group individually in a metabolic cage. Animals were euthanized by overdose of ether; blood was collected by orbital sinus puncture. 0.5 ml blood was collected; serum was separated and stored at -80º C for further analysis. Rats were dissected, bone, uterus and liver was immediately removed, washed in PBS (pH 7.4), and stored at -80º C for further analysis. Liver and uterus were blotted and weighed. All the assays were carried out using commercial kits purchased from Reckon Diagnostics.

Statistical Analysis

Data were expressed as mean values and S.E.M. One-way ANOVA was used to compare data from all groups and Bonferroni post test to compare the results ($p < 0.05$ by the statistical software of Graph Pad PRISM (Version 5.0). A p value of less than 0.05 was considered statistically significant.

RESULTS

Body weight and relative organ weight

As shown in Table 1, after 8 weeks of study there was significant increase in the body weight of all the OVX animals, whereas control animals showed a marginal increase in the weight. Exposure of MO extract to OVX resulted into marginal decrease in the body weight, and of

the three parts of MO extracts used, exhibited significant decrease in the body weight (Figure 1). Maximum alterations were observed in relative uterine weight. As expected, ovariectomy caused significant decrease in the weight of the uterus and relative uterine weight (Table 2). However, no changes were observed in the relative uterine weight of any of the treatment groups (Figure 2).

Serum and urine biochemical Markers

Table III represents the alterations in the serum and urine biochemical parameters. OVX resulted into a decrease in serum calcium levels 50% (Table III). Decrease in the calcium was poorly shielded by leaf extracts of MO; however, flower and fruit extracts improved the serum calcium profile (Figure 5). OVX resulted into an enhanced excretion of calcium in the urine, reaching almost 3 fold calcium compared to control (Table III), and treatment with MO components reduced the loss of calcium in the serum, (Figure 8). OVX led to increased urinary excretion which was enhanced to 3 fold at the end of 2 months. MO Leaf extract did not show any significant effect on the calcium excretion, MO flower and fruit extract treatment reduced the calcium excretory rate, (Figure 8). As far as phosphorus is concerned an increase in phosphate level was reported in OVX rats, which seen to be improved by all the three components of MO extract (Figure 6). Serum ALP, the functional markers of osteoblast activity was observed to increase in OVX, MO Plant extracts was successful in improving serum ALP levels (Figure 3). Serum TRAcP, an osteoclast specific marker was found to be high in all OVX rats, and MO extract treatment resulted into lowering the activity (Figure 4).

Tissue biochemical Markers

Bone AlP levels were significantly higher in the OVX group compared to normal and MO plant extract treatment showed lowered AlP levels with all the three components. However, maximum effect was observed with fruit and flower extracts of MO (Figure 9 and 10). Bone TRAcP as anticipated, were higher in OVX animals compared to normal. All the three components of MO plant extract reduced the TRAcP activity in bone .However, liver ALP (Figure 11) didn't show any significant alterations, thus the ALP is Serum can be correlated with that of Bone.

Phytochemical analysis

The phytochemical screening of plants gave positive results for flavonoids, alkaloids, phenols, anthocyonins and saponins. Both flower and fruit was found to be rich in flavonoids,

phenols and saponins compared to leaves. Preliminary tests also confirmed that leaves are having presence of tannins, but we were not able to detect the presence of tannins in flower and fruits (Table 1V). GC MS profile of leaf, flower and fruit extract were studied. Figure 18, 19 and 20 shows the GC scan of leaf, flower and fruit. Relative abundance was measured and the phytochemicals were detected by their comparison of mass spectra with standard mass library. The list of phytochemicals detected using GC MS are listed in Table V.

Table I: Increase in body weight in different treatment groups

	Control	Ovx	Leaf	Flower	Fruit
1	242.500± 5.280	248.330±3.330	240.10± 4.170	240.000±3.330	240.000±3.330
2	250.000± 6.110	260.830± 3.610	245.23±3.890	245.830±3.890	247.830± 3.890
3	262.500±3.330	270.830±5.830	262.43± 3.610	266.23±3.330	252.500±3.330
4	266.670± 3.610	285.830± 4.170	266.15± 3.890	275.000±3.060	268.000± 3.060
5	273.330± 4.170	295.830±5.000	275.45± 4.170	284.170±2.500	276.170±2.500
6	282.500± 3.890	305± 4.170	282.32 ± 5.280	291.830±5.000	283.670± 5.000
7	291.670± 5.000	315 ± 2.780	291.07± 3.610	295.000±2.220	298± 4.170
8	295.830± 5.280	332.500±5.280	305.30± 5.830	307.10±4.170	310 ± 2.780

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

Table II: Relative uterine weight in different experimental groups.

	Control	Ovx	OVX+Leaf	OVX+Flower	OVX+Fruit
Relative uterine weight	0.001± 0.0001	0.00022**±0.000152	0.00024±0.000225	0.000235±0.000205	0.000235±0.000205

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

Table III: Serum/Urine Biochemical markers

Parameters		Control	Ovx	Ovx +Leaf	Ovx +Flower	Ovx +Fruit
Serum Profile	AlP	54.663± 4.57	124.569***± 6.33	118.455± 6.336	79.258***±8.336	68.456***±4.336
	Calcium	8.811± 0.189	5.465***± 0.262	6.608± 0.229*	8.466***±0.293	7.456**±0.233
	Phosphate	7.910±0.569	18.223***±1.892	10.336***±0.689	11.569***±0.881	10.236***±0.233
	TRAcP	6.583±0.556	15.166***± 0.651	9.336*±0.812	7.986**± 0.556	8.456*±1.265
Urinary calcium		4.325± 0.986	12.669***±1.336	11.566±1.256	8.569*± 2.336	7.255* ±1.236
Calcium Excretion rate		0.518±0.110	1.307**± 0.235	0.977± 0.145	0.618*± 0.105	0.655*± 0.129

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

Table IV: Bone and Liver biochemical markers

Treatment groups		control	Ovx	Ovx +Leaf	Ovx +Flower	Ovx +Fruit
Bone	AlP	140.5±5.1	168.6**±4.2	155.6*±8.3	146.6**±7.3	144.6**±6.3
	TRAcP	6.583± 0.556	15.166***±0.651	9.336*± 2.669	7.986**± 0.669	8.456**± 1.365
Liver	AlP	79.081± 12.367	82.1±20.593	79.335±18.844	77.176± 19.883	78.127± 24.734

Values were expressed as Mean ± S.E.M. * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$

Table V: Phytochemical analysis of MO

Fraction		Test	Leaf	Flower	Fruit
Flavonoids		N-lead acetate	+	+++	+++
		Zinc dust	+	+++	+++
		NaOH	++	+++	+++
		H2SO4	+	+++	+++
Terpenoids		Chl+H2so4	+	+	+
Phenols		N-FeCl3	+	++	+++
		FeSO4	++	+++	+++
Alkaloids		Mayers	++	+	+
		Wagners	-	+	+
		Dragondorff's	++	+	+
Anthocyanins		Na acetate	-	+	+
		Na2CO3	-	+	+
Tannins		Gelatin	+	-	-
		K2Cr2O7	+	-	-
		Iodine	+	-	-
		Lead acetate	+	-	-
Saponin		Water	+	+++	+++
		Lead acetate	+	+++	+++
Amines		Na-Nitro	-	-	-
		Dragondorff's	-	-	
		Ehlrich	-	-	-
Glycosides	cynogenic	Molisch	+	-	+
		Cold H_2SO_4	-	+	+
	cardiotonic	Kedde	-	-	-
		Keller	-	+	+

Key: + Low concentration; ++ Moderate concentration; +++ High concentration; - absent (negative)

Table VI : Phytochemicals detected using GC MS scan in different components of MO

LEAF	FLOWER	FRUIT
Adenosine derivatives	Pyridine carbonitrile	Stearic acid derivatives
Ergolin	Thiazolamine	Decanoic acid derivatives
Tertrahydroquinoline	Tertra hydroquinoline	Piperizine derviatives
Cinnamic acid	Phenanthridone	Olean derivatives
Oleic acid derivatives	Cinnoline	Glucobrassicin
Piperizinie derivatives	Cinnamic acid	Octadecane
Heneicosane	Coumarin	Palmitic acid derivatives
Eicosane	Ribitol	Quinolizine derivatives
Quebrachamine	Olien derivatives	Triprolidine
Curan	Ergolin	Ibogamine
Thebaine	Androstan	Yohimbane
Aspidospermidine	Ibogamine	
Carnegine	Nonahexacontanoic acid	
	Arabinitol	
	Aristolocholic acid	

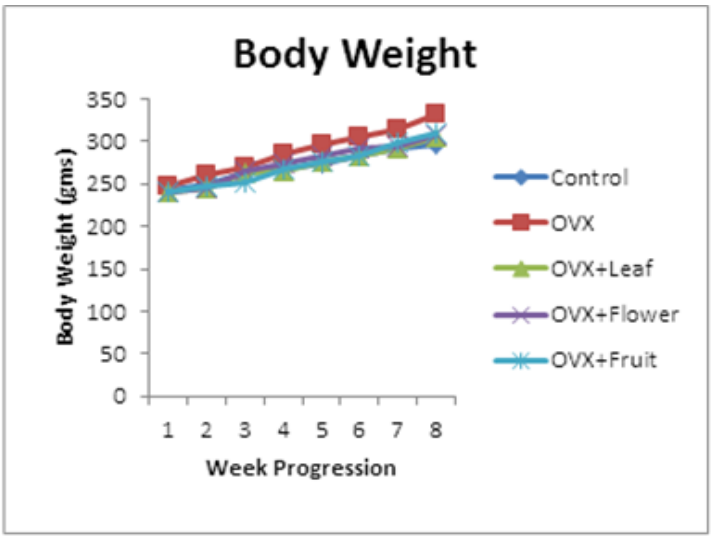

Figure1:Comparision of Body weight of Rats treated with MO components with Control

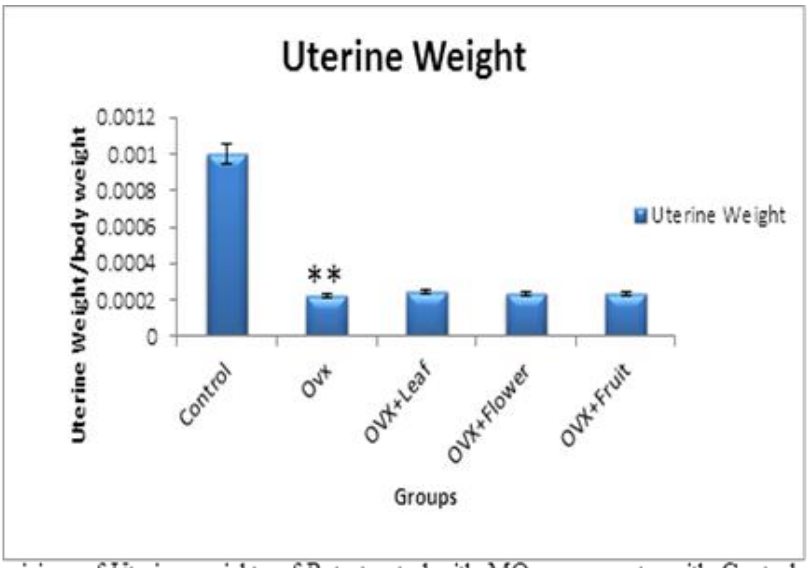

Figure 2:Comparision of Uterine weight of Rats treated with MO components with Control

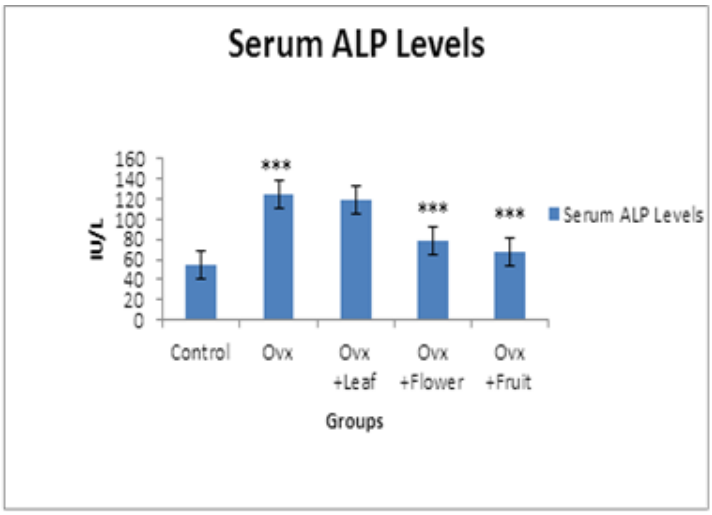

Figure 3:Alteraion in Serum AlP Levels in different groups treated MO extracts

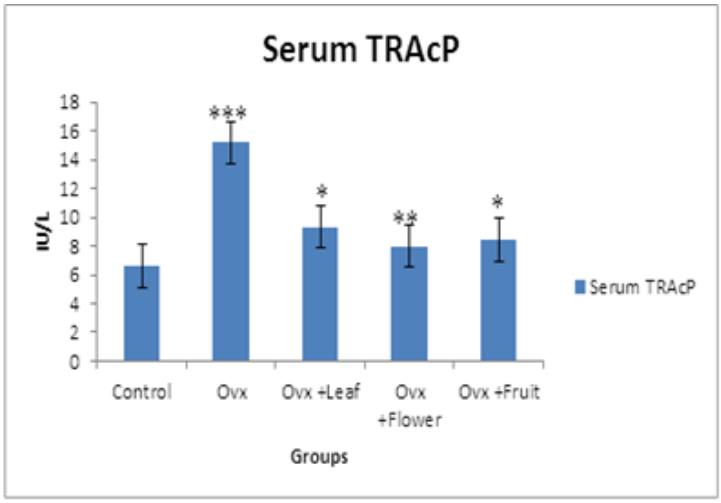

Figure 4:Alteraion in Serum TRAcP Levels in different groups treated MO extracts.

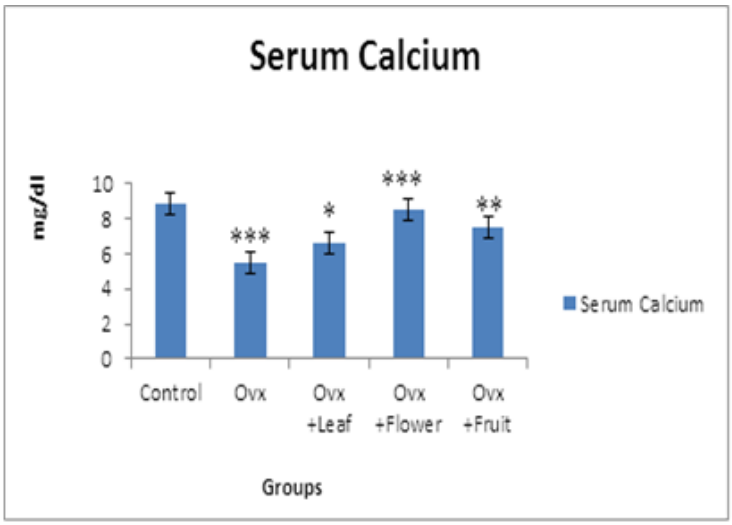

Figure 5:Alteraion in Serum Calcium Levels in different groups treated MO extracts.

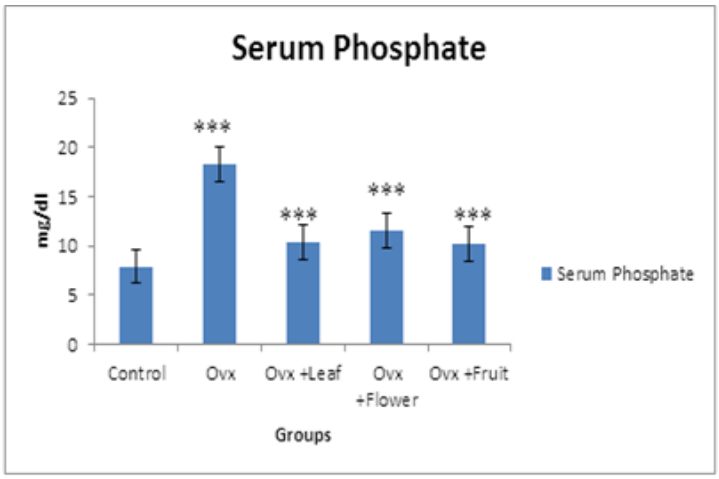

Figure 6:Alteraion in Serum Phosphate Levels in different groups treated MO extracts.

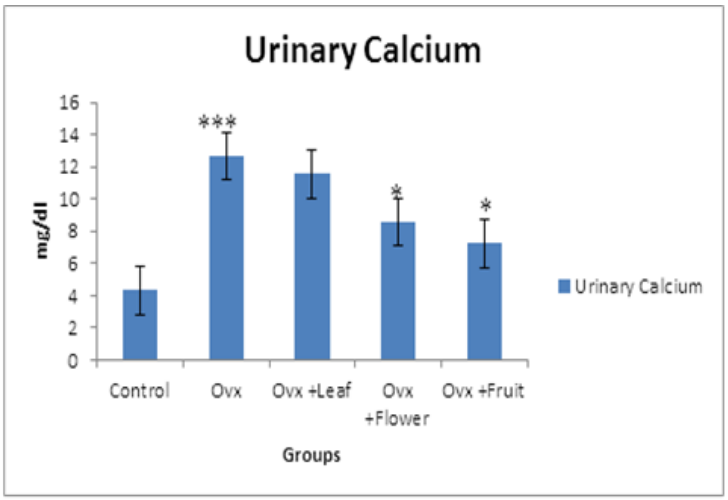

Figure 7:Alteraion in Urinary Calcium Levels in different groups treated MO extracts.

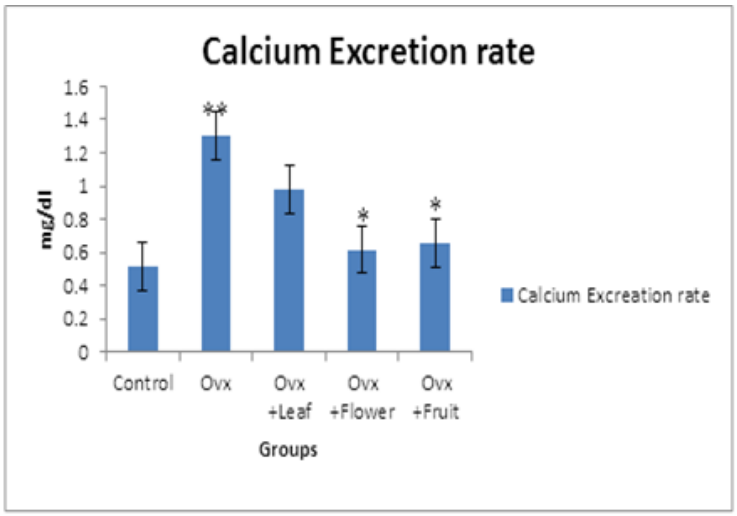

Figure 8:Alteraion in Serum Phosphate Levels in different groups treated MO extracts.

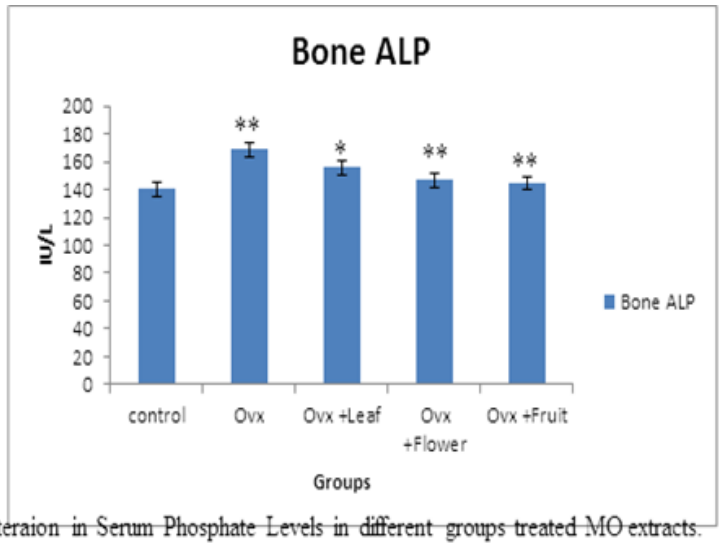

Figure 9:Alteraion in Serum Phosphate Levels in different groups treated MO extracts.

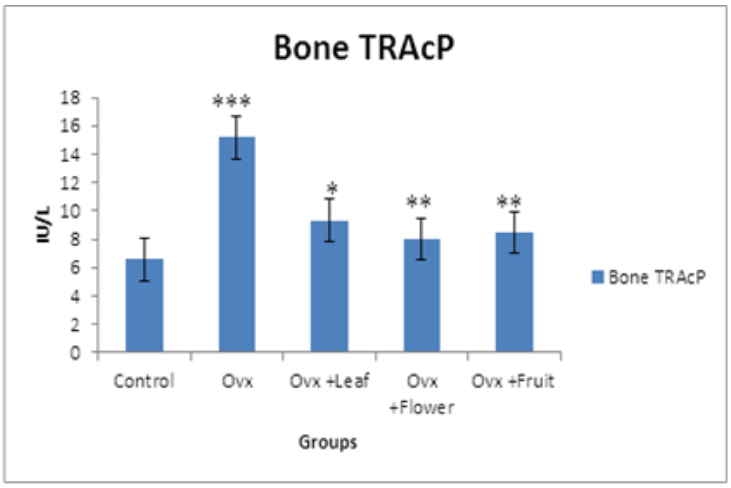

Figure 10:Alteraion in Bone TRAcP Levels in different groups treated MO extracts.

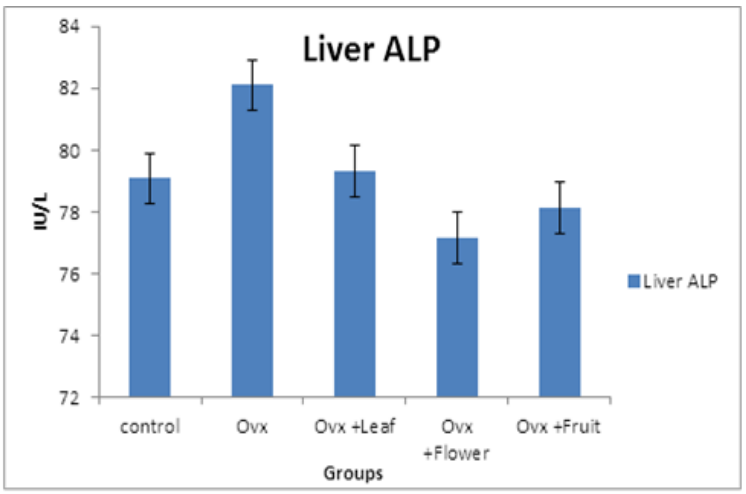

Figure 11:Alteraion in Bone ALP Levels in different groups treated MO extracts.

DISCUSSION

The present study demonstrated that supplementing OVX animals with MO flower, leaf and fruit extracts can have positive effect on bone health. OVX animals showed weight gain which may be due to lack of estrogen. MO flower and fruit had non-significant effect in reducing the weight gain. Maximum weight reduction was observed with leaf extract treatment, which may be due to presence of tannins in leaf of MO. Tannins have been reported to be responsible for decreases in feed intake, growth rate, feed efficiency, net metabolizable energy, and protein digestibility in experimental animals[23] tannins present in the extract could potentially inhibit the activity of lipases found in rats, thereby lowering their fat content.[24-26] The results indicate that the rats treated with MO leaf extract have significantly attenuated the body weight proving the hypolipidemic and thermogenic property.[27-28]

Ovariectomy induced absence of sex hormone initially affects the uterus, leading to uterine atrophy and decrease in the uterine weight, similar results was observed, where the uterine weight was decreased in OVX animals. MO treatment had no significant effects on the uterine. Our observations are in accordance to with the work done by[29-30] who have opined that plants ameliorates OVX induced changes on the bone without affecting the uterus. In the present study the MO extract also did not have the effect on the uterus but potentially inhibits the progression of post menopausal symptoms.

Decrease in serum calcium and increase in urinary calcium post ovariectomy is an established fact and one of the possible reasons for excess bone resorption.[31] Our study also showed similar results and further when excretory rate was considered, it was observed that calcium excretory rate was very high in OVX animals. Ovariectomy in the rat leads to an increase in intestinal calcium secretion, leading to impaired calcium balance, probably this high excretory rate affects the overall calcium metabolism and negative calcium metabolism leads to further progression of the disease[32] Of the three components of MO, leaf was having significant effect on improving the fall in serum calcium Similar observation were seen in case of flower and fruit extracts which were having more significant effects on boosting serum calcium compare to leaf extracts. When we considered the calcium excretory rate, it was clear that MO plant extract, had significant effects on calcium excretion, where, urine calcium excretion as well as the calcium excretory rated were seen to be decreasing suggesting that the components of the extracts were helping in keeping up the calcium load required by the OVX rats. Furthermore, MO plant extracts in OVX rats did not only restored the decreased serum calcium levels but also the phosphorus levels were seen to be near to normal, suggesting that MO flower and fruit extracts are effective in inhibiting bone resorption and in increasing bone formation. Numerous studies have shown the health-promoting properties of polyphenols, through which they promote skeletal health by reducing resorption caused by high oxidative stress.[33-35] The presence of polyphenols in MO plant thus suggests its role is reducing resorption. The antioxidant properties of polyphenols have also been widely studied and reported in the literature.[36-38] They strongly support the role of polyphenols in the delayed onset or reduction in the progression of osteoporosis. The protective effects of polyphenols against diseases, including osteoporosis, have generated new expectations for improvements in health. Our findings are also in agreement with the findings[39-41] who reported that plants containing polyunsaturated fatty acids increases serum

calcium and phosphorus concentrations and reduce urinary calcium and phosphorous excretion, thus enhanced bone formation.

Previous studies in our lab have established that bone is maintained through coordinated activities of osteoblasts and osteoclasts and ovariectomy disrupts this balance, leading to over-activity of osteoclasts, followed by osteoblasts.[8] After a lag phase, osteoblasts are not able to keep up with the pace of osteoclasts, leading to an imbalance, where the bone is resorbed much more then it can be synthesized. Two functional markers of this process are AlP and TRAcP, which are secreted by osteoblasts and osteoclasts respectively.[42,9] In the present study we observed that following ovariectomy, there was an increase in serum AlP and TRAcP. MO extract exposure led to a fall in ALP as well as TRAcP levels, possibly due to the presence of flavonoids as proposed by[43] that certain flavonoids can have positive effect on bone nodule formation. All the three parts of MO plant was found to be rich in flavonoids which might be the one playing a key role in stimulating osteoblast/osteoclast cells. Of the three components maximum effects was seen with the flower, proving its beneficial role in preventing further progression of osteoporosis in OVX rats. Parallel fall in both the markers clearly suggest that fruit, flower and leaf extracts have improved the damage caused by estrogen deficiency. To confirm that the elevation in serum AlP levels is because of bone remodeling only, we estimated liver AlP levels also, which showed no significant variation between the MO extract treated group and control group.

Thus the present study has established the osteoprotective efficacy of leaf, fruit and flower of MO plant. The phytochemical profile of MO leaf, flower and fruits are different and they exhibit different pharmacological property. Studies have proved that[44] and cinnamic acid[45] are osteoprotective. As MO components are rich in cinnamic acid, probably its osteoprotective efficacy is enhanced by the presence of it. Presence of PUFA and flavonoids also participate in expression of the beneficial effect on bone health.[46] Apart from this, another botanical agent Coumarin which is found in flower of MO is also attributed to promote bone health (Tang *et al.,* 2008).The Proliferation and maturation of Osteoclasts follows a pathway that involves variety of inflammatory cytokines. As all the parts of the MO plant shows the presence of flavonoids and hence, the robust anti inflammatory activity might be directly affecting the inflammatory pathways of development of Osteoclasts.[47-48]

CONCLUSION

Our Study is the first to prove the effect of MO Components on OVX induced Rats and showed that among the entire component tested, Flower and fruit gave most promising results. Further, the elucidation of phytochemical compositions from MO extracts, it validated the presence of osteoprotective compounds, which upon isolation can be use as herbal therapeutics for this type of bone disease.

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