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ORIGINAL ARTICLE

Antiresproative Potency of D-carvone on Ovariectomy-Induced Osteoporosis in Rats

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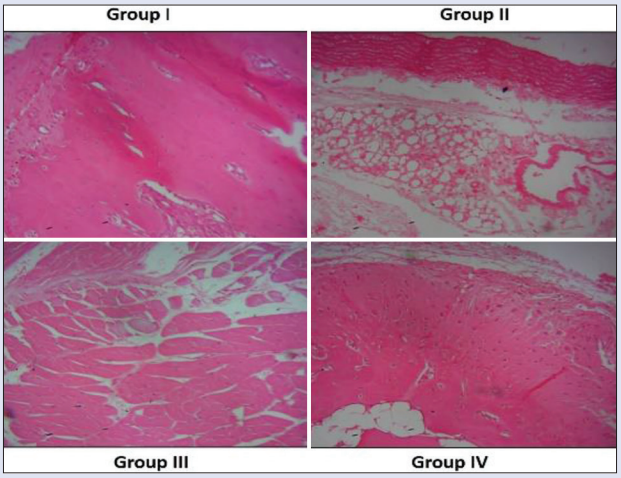
ABSTRACT

Background: Osteoporosis is a quiet disease with a pathological condition of reduced bone mineral density (BMD) leading to weakened bone. In this study, we evaluated the bone formation potency of D-carvone, an unsaturated monoterpenoid ketone phytochemical present in essential oil of aromatic plants with pharmacological prominence against the ovariectomy-induced rats. **Materials and Methods:** Ovariectomy was achieved in Sprague–Dawley rats and was employed for the present study. The rats were clustered into four sham-operated control, ovariectomized, ovariectomized treated with 5 mg/kg b. wt and 10 mg/kg b. wt, respectively. Body weight of rats was observed once a week and after the completion of treatment the rats were euthanized to isolate uterus, vagina, and femur. **Results:** The weight of the uterus, vagina, and femur were restrained to perceive the impact of D-carvone on reproductive organ and bone. The effect of D-carvone treatment in maintaining the BMD was evaluated with dual-energy X-ray absorptiometry scan and the biomechanical properties were measured with three-point bending test. Microcomputed tomography analysis was performed to scrutinize the D-carvone potency in trabecula of ovariectomized rats. Further to confirm D-carvone osteoblastic potency the bone turnover markers levels were enumerated. The bone healing effect of D-carvone in ovariectomized rats was considered with histological examination of femoral metaphysis. The impact of D-carvone on suppressing inflammation in ovariectomized rats was judged by estimating the levels of lipid profile and inflammatory cytokines. The osteoblastic potency of D-carvone was established by quantifying the gene expression osteblastic protein using quantitative polymerase chain reaction analysis. **Conclusion:** In conclusion, our results evidenced that the D-carvone effectively inhibited the osteclastic activity in ovariectomized rats and augmented the expression of osteblastic proteins via suppressing the inflammatory markers. However, the additional experiments in future could endorse the D-carvone as a potential anti-osteoporotic drug.

Key words: Anti-osteoporotic drug, D-carvone, *in vivo* model, inflammation, osteoporosis, ovariectomy

SUMMARY

- D-carvone meritoriously sustained the normal body weight, BMD in ovariectomized rats
- The biomechanical inspection of D-carvone-treated ovariectomized rats shows that D-carvone augmented the bone strength and declined the fragility of bones due ovariectomy
- D-carvone effectively constrains the osteclastic activity in ovariectomized rats and upsurges the expression of osteblastic proteins via suppressing the inflammatory proteins.



Abbreviations used: DEXA: Dual-energy x-ray absorptiometry; Tb. Th: Trabecula thickness; Tb. N: Trabecula number; Tb. Sp: Trabecular separation; Tb. A: Trabecular area; TRAP: Tartrate-resistant acid phosphatase.

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INTRODUCTION

Osteoporosis, a global alarm of the elderly population has a superior influence on the economy of their families and countries. Worldwide about 200 million people have been identified with osteoporosis.[1] Compared to the men population women were more prone to the target of osteoporosis and osteoporotic fractures. Nearly, 40% of women diagnosed with osteoporosis, which are at high risk of osteoporotic fractures and it was stated that 15%–30% of mortality are related to osteoporotic fractures.[2,3] The risk of osteoporosis surges with the age from 2% in women of >50 years to 25% in women >80 years.[4] Since the global life expectancy was gradually increasing, the concern toward treating osteoporosis is also increasing sharply mainly in developing countries. Osteoporosis is a systemic musculoskeletal disease with

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pathological conditions of diminished bone mineral density (BMD) and declined bone microarchitecture causing fragility in bones leading to fractures.[5] Osteoporosis is categorized into three types based on the causative of the diseases as osteoporosis occurring in postmenopausal women, osteoporosis occurring in aged persons and osteoporosis occurring as secondary pathology of some other ailments.[6] The women in postmenopause are frequently exaggerated with osteoporosis this may be due to the hormonal imbalance. The secretion of hormones changes radically during the aging process which leads to various organ dysfunctions.[7] Among that bones were harshly affected in women at the postmenopausal stage since there is a shrill decrease in the synthesis of estrogen.[8] Endogenous estrogen plays an energetic role in maintaining the microarchitecture of bones; hence. the estrogen deficiency hints to fractures in women at the postmenopausal stage.[9]

All over the world for more than a period of 25 years, calcium supplementation is typically recommended to prevent osteoporosis.[10] Even though it blocked the bone turnover and worsening of bone architecture the efficacy of calcium against antifracture in postmenopausal women is uncertain and it also caused various side effects such as renal calculi, gastrointestinal disorders, and cardiovascular diseases.[11] The newly invented potent substitutes of calcium supplementation such as bisphosphates, denosumab (monoclonal antibody), parathyroid hormone, and estrogen treatment also reduce hilarious side effects.[12] Hence, a potent osteoporotic drug, which preserves the bone mass as well efficiently prevents fractures in postmenopausal women needs to be revealed.

Carvone is an unsaturated monoterpenoid ketone prime phytochemical component present in seeds of *Carum carvi* plant fitting to the family *Apiaceae*. It is also present in the essential oil of aromatic plants such as *Mentha spicata* and *Anethum graveolens*.[13,14] Carvone exists as L-carvone with the sweetish minty smell and D-carvone with spicy aroma. Compared to L-carvone the D-carvone hold numerous pharmacological properties such as immunomodulatory, anticarcinogenic, antimicrobial, antitumor genic, antiinflammatory, antihyperlipidemic, and antihypertensive.[15-17] The efficacy of D-carvone on preventing osteoporosis was not yet explicated; therefore, we scrutinized the osteoblastic property of D-carvone in a rat model induced osteoporosis by ovariectomy.

MATERIALS AND METHODS

Chemicals

D-Carvone and other chemicals were acquired from Sigma-Aldrich Chemicals, USA. All the ELISA assay kits were procured from RandD Systems Inc., USA, Randox Laboratory Ltd., UK, and Abcam, USA, respectively. The RT-PCR kits were obtained from Thermo Fischer Scientific, USA.

Animals

Young healthy female Sprague–Dawley rats weighing about 250 ± 10 g were designated for the present experiment. The detailed protocols to be achieved on rats during the experimental period were elucidated before the institutional ethical committee and gained the approval of rats' procurement. The procured rats were adapted in the laboratory condition with a temperature of 24°C ± 2°C, relative humidity of 60% ± 5% and strict 12 h light and dark cycle for a week. Sterilized paddy husk were employed for bedding and the husk was altered every day and the cage was reformed thrice a week. The rats were fed *ad libitum* with standard hygienic laboratory pellet diet and clean drinking water. The rats were preserved with utmost concern and care, all possible measures were taken to diminish the suffering of rats during the experimental period.

Ovariectomy surgery

The rats were overnight fasted before the surgery day and on the day of surgery, they were anesthetized with 50 mg/kg pentobarbital sodium (intraperitoneal injection). The surgery was achieved in the animal surgical room strict hygiene was sustained throughout the surgery. The anesthetized rats were located on the surgical table and an abdominal incision was made in the middle of the abdomen. For sham-operated rats bilateral laparotomy alone was finished and for ovariectomized rats, bilateral ovariectomy was accomplished. The rats were then positioned in the observation chamber and monitored for 30 min and then transferred to the home cage. No massive hemorrhage or mortality was happened during and after the surgical procedure.

Experimental design

After the recovery period of 4 weeks, the rats were congregated into four: group I rats are sham-operated control rats, group II are ovariectomized rats, group III are ovariectomized rats treated with 5 mg/kg b. wt of D-carvone/day (dissolved in DMSO), and group IV are ovariectomized rats treated with 10 mg/kg b. wt of D-carvone/day for 16 consecutive weeks. The sham operated and ovariectomized alone rats' conventional distilled water through gavage treatment. The weight of the rats was checked weekly once throughout the experimental period. The rats were then euthanized using ether anesthesia. The blood samples were composed for biochemical analyses; the femur, vagina, and uterus were isolated for molecular and histological analyses. The samples were deposited in − 80°C until further analysis.

Measurement of organ coefficient

The body weight was restrained weekly once and the surge in body weight of control and experimental groups were intended every 4 weeks. The femur bone, uterus, and vagina were detached and weighed in digital weighing balance. The organ coefficients were calculated using the formula:

Organ coefficient = Organ weight/Body weight of the animal

Assessment of BMD and biomechanical parameters

The whole femur of control and experimental rats were insulated and the muscles, connective tissues were entirely detached. BMD of femur was evaluated with dual-energy X-ray absorptiometry (DEXA) scans (HOLOGIC discovery WA, USA). The experiment was completed as per the manufacturer's protocol. The small animal scanning mode is used and the BMD values were measured routinely.

Three-point bending flexural test method using CSS-4420 material testing machine (Changchun Research Institute for Testing Machines Co. Ltd., Changchun, China)[18] was finished to guesstimate the biomechanical property of ovariectomized and D-carvone-treated ovariectomized rats. The machine was programmed with stride distance of 20 mm and the loading velocity is preserved at 5 mm/s. The data were routinely verified and the maximum deflection, maximum load, energy, stiffness, young modulus, maximum stress, maximum fracture load to femoral midshaft, and femoral neck were designed.

Microcomputed tomography analysis of trabecular bone

The microarchitecture of trabeculae of femoral metaphysis was judged with SIEMENS Inveon PET.SPECT.CT, Germany). The femurs were isolated from the control and experimental rats and preserved in 70% ethanol until the experiment was executed. About 25–125 slices from the distal femoral growth plate was take at the region of interest and sliced with the thickness of 21 μm and the voxel resolution was maintained at

22 μm^3. The trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp), trabecular area (Tb. A) were dignified and calculated using associated software.

Quantification of bone turnover markers

Bone turnover markers estradiol (Abcam), osteocalcin/bone gla protein (Life Span BioSciences) and acid phosphatase (Life Span BioSciences) were projected using commercially available ELISA kits. The procedures were achieved according to the manufacturer's instruction and the OD was measured at 450 nm using microplate reader. Serum calcium and phosphorous were measured calorimetrically by the method of Brown,[19] creatinine was assessed using Jaffe's method[20] and Tartrate-resistant acid phosphatase (TRAP) was dogged by the method of Godkar.[21]

Quantification of osteoprotegerin and RANKL

The serum levels of osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa β ligand were measured using commercially available sandwich ELISA kit (R and D Systems Inc., USA). The experiment was achieved as per the manufacturer's protocol and the concentration of the test sample was planned based on the standard curve drawn with known concentrations of standard samples.

Histopathological analysis of femoral metaphysis

The femoral bone was fixed with 10% formaldehyde solution for 24 h and then decalcified using 3% Muller solution for 48 h. The bone tissue was then washed with distilled water followed by series of ethanol and xylene wash (50%–100%). The tissue was then entrenched in paraffin and sliced into 4-μ thickness using rotary microtome. The sliced tissue was dewaxed and stained with H and E stain. The stained femoral metaphysis was observed under light microscope and the photograph was taken for further examination.

Quantification of lipid profile

The total lipid profile was assessed in untreated ovariectomized rats and ovariectomized rats treated with different concentrations of D-carvone using commercially available assay kits. The serum concentration of triglycerides was appraised with GPO-PAP kit, total cholesterol high-density lipoprotein cholesterol, and low-density cholesterol were enumerated using assay kit procured from Randox Laboratory Ltd., UK. The assay was done as per the manufacturer's protocol.

Quantification of inflammatory markers

The levels of inflammatory markers-Tumor Necrosis Factor α (TNF-α), interleukin 1 β (IL-1 β), and IL-6 in control and experimental rats were restrained using commercially available ELISA kits (Abcam, USA). The experiment was carried based on the instruction of the manufacturer and the end product was calculated at 450 nm. The inflammatory cytokines levels were considered using the standard curve reference values.

Quantitative polymerase chain reaction analysis of bone turnover markers

mRNA expression of osterix (Osx), Runt-related transcription factor 2 (Runx2), and alkaline phosphatase (ALP) in control and experimental rats were measured using quantitative polymerase chain reaction (qPCR) analysis. The tissue was homogenized with TriZol reagent and the RNA was phase separated with the addition of chloroform and centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous phase was separated and the RNA was precipitated with equal volume of isopropanol. The precipitated RNA was composed by centrifuging at 12,000 rpm for 10 min at 4°C and the RNA was cleansed with ethanol twice by centrifuging at 10,000 rpm for 5 min at 4°C. The pellet was dissolved with Milli-Q water and then subjected to quantification with NanoDrop Spectrophotometer. The RNA was then exposed to cDNA conversion with Cells-to-cDNA™ II Kit, Thermo Fischer Scientific, USA. Then, the cDNA was subjected to qPCR analysis with respective primers Osx, Runx2, ALP and internal control beta-actin using Real-time PCR kit (Takara, USA). The relative gene expression was dignified using the comparative computed tomography (CT) method (2−ΔΔCt) normalized with GAPDH. The mRNA expressions of the samples were intended using CFX Manager Version 2.1 and the results portrayed as fold change (Bio-Rad, USA).

Statistical analysis

The experiments were achieved in triplicates and the values were logged. The results of each and every experiment were statistically examined using Graph Pad PRISM. The results were evaluated with one-way ANOVA and the intra significance was measured with *post hoc* Dunnett test. All the data were articulated as mean ± standard deviation and $P < 0.05$ were measured to be statistically significant.

RESULTS

D-carvone influence on body weight of ovariectomized rats

Figure 1a illustrates the effect of D-carvone effect on the bodyweight of ovariectomized rats. The bodyweight of ovariectomized rats revealed a significant rise compared to sham-operated control rats. The sham-operated rats gained about 50 g weight for 20 weeks, whereas ovariectomized rats gained more than 100 g of weight. Both the doses of D-carvone treatment repressed the rise in body weight of ovariectomized rats. Initially, up to 12 weeks of D-carvone treatment, there is a shrill surge in the bodyweight of rats, whereas it dropped from 16 weeks and during the 20th week of treatment. No substantial change was detected between D-carvone-treated ovariectomized rats and sham-operated control rats.

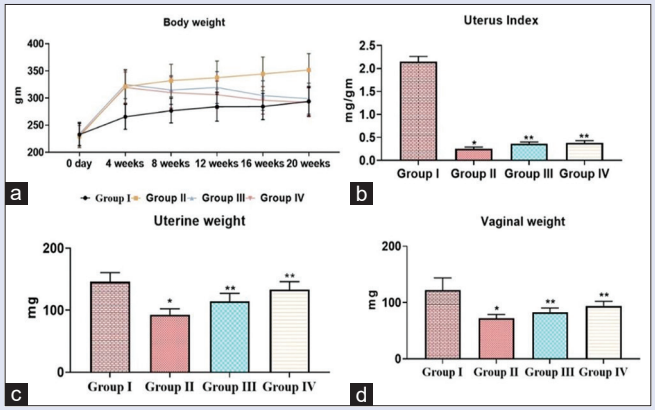

Figure 1: D-carvone influence on body weight and organ weight of ovariectomized rats. (a) Body weight of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats measured for a period of 20 weeks. (b) Uterus index (c) Uterine weight (d) Vaginal weight measured at end of treatment period. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

D-carvone influence on the uterine index of ovariectomized rats

The uterine index of sham-operated control, ovariectomized, and ovariectomized rats treated with D-carvone are portrayed in Figure 1b. The ovariectomized rats were presented a momentous decrease in their uterine index compared to the control rats. D-carvone treatment does not6 shown noteworthy increase in the uterine index compared to sham operated and ovariectomized rats, whereas high-dose D-carvone-treated rats shown small increase in the uterine index compared to the ovariectomized rats. The uterine [Figure 1c] and vaginal weights [Figure 1d] of ovariectomized rats were knowingly abridged than the control rats. High-dose D-carvone-treated rats shown a significant upsurge in the uterine and vaginal index compared to ovariectomized rats.

D-carvone effect on femur weight of ovariectomized rats

Figure 2 demonstrates the D-carvone potency on continuing femur weight of ovariectomized rats. Noteworthy lessening in both dry and wet weight of femur was seen in ovariectomized rats than the control rats. High-dose D-carvone-treated ovariectomized rats exposed important increase in femur weight and wet weight femur coefficient than the low-dose D-carvone-treated ovariectomized rats.

Effect of D-carvone on maintaining the bone quality of ovariectomized rats

The results of femoral length and BMD measured with DEXA scan are represented in Figure 3a and b. Both the femoral length and BMD were ominously reduced in ovariectomized rats, whereas high-dose D-carvone treatment in ovariectomized rats augmented both the femoral length and BMD. Figure 3c and d exemplifies the effect of D-carvone on maximum deflection and the maximum load of femoral bone in ovariectomized rats. The drastic decrease in maximum deflection property and maximum load capacity was realized in ovariectomized rat's bones compared to the control rat's bones. High-dose D-carvone treatment meaningfully augmented the maximum load capacity and the maximum deflection property in ovariectomized rat's bones.

The biomechanical properties of ovariectomized and D-carvone-treated ovariectomized rats were measured using three-point bending test and the outcomes are represented in Figure 4. The energy, stiffness, young modulus, and maximum stress of the bone were severely reduced in ovariectomized rats compared to the sham-operated control rats. Both the doses of D-carvone treatment improved the quality of bone in ovariectomized rats but compared to low-dose D-carvone treatment high-dose D-carvone-treated ovariectomized rats shown knowingly augmented energy, stiffness, young modulus, and maximum stress on bones [Figure 4]. The quality of the bone in D-carvone ovariectomized rats was evaluated by determining the maximum fracture loading capacity of the femoral midshaft and femoral neck in ovariectomized and D-carvone-treated ovariectomized rats [Figure 5]. Compared to ovariectomized rats the quality of femoral bone considerably enlarged in D-carvone treated ovariectomized rats. Sharp diminution was detected in maximum fracture loading capacity of both femoral midshaft and femoral neck of ovariectomized rats, whereas D-carvone treatment augmented the maximum fracture loading capacity.

Effect of D-carvone on the trabecular microarchitecture of ovariectomized rats

Figure 6 proves the results of trabecular thickness Figure 6a, trabecular area Figure 6b, trabecular separation Figure 6c, and trabecular number Figure6d in control, ovariectomized and D-carvone-treated ovariectomized rats. The trabecular thickness, area, and number were meaningfully reduced in ovariectomized rats (52 mm, 13%, 1.8 mm, respectively) compared to the control rats (78 mm, 37%, 4.7 mm, respectively). Both the high (72 mm, 31%, 4.1 mm, respectively) and low-dose D-carvone (68 mm, 24%, 3.6 mm, respectively) treatment knowingly augmented the levels of trabecular thickness, area and number in ovariectomized rats compared to the untreated ovariectomized rats. The trabecular separation was extremely augmented in ovariectomized rats (340 mm) compared to the control rats (180 mm), whereas D-carvone treatment declined the levels in ovariectomized rats (270 mm and 220 mm low and high dose, respectively).

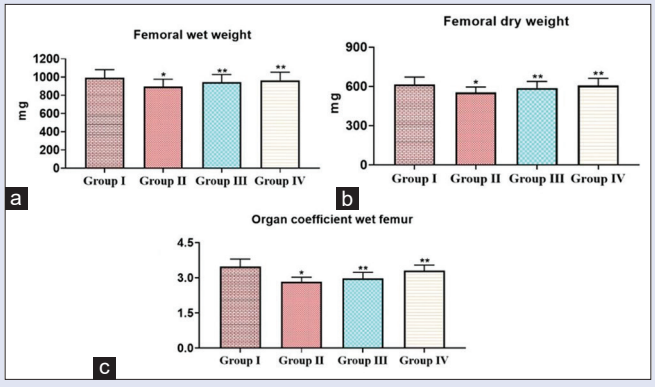

Figure 2: D-carvone effect on femur weight of ovariectomized rats. (a) Wet femur weight, (b) Dry femur weight (c) Organ coefficient of wet femur of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

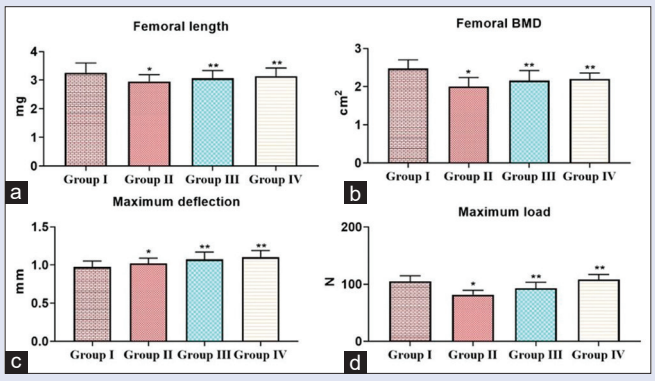

Figure 3: Effect of D-carvone on maintaining bone quality of ovariectomized rats. (a) Femoral length (b) bone mineral density (c) Maximum deflection (d) maximum load capacity of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

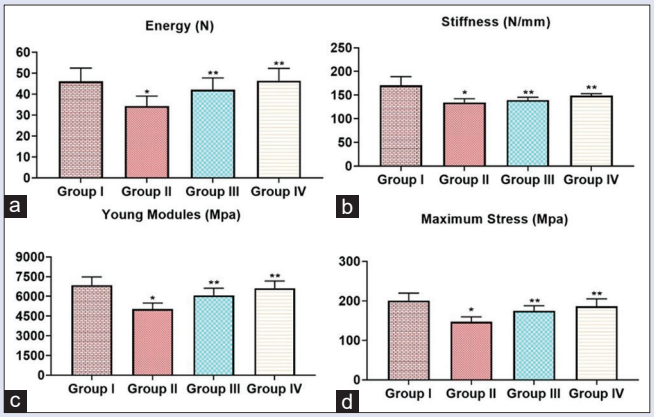

Figure 4: Effect of D-carvone on maintaining biomechanical property ovariectomized rats. (a) Energy (b) Stiffness (c) Young modulus (d) maximum stress bearing capacity of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

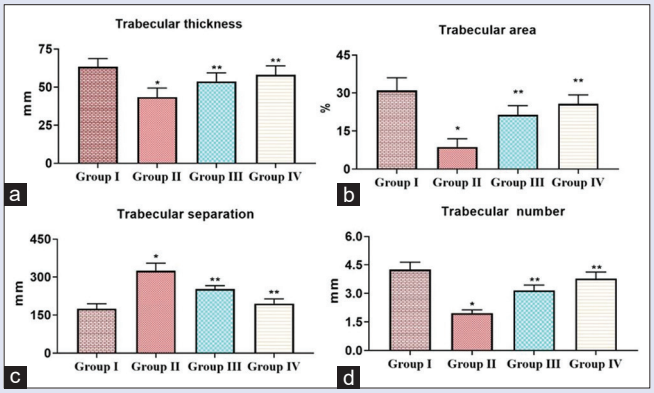

Figure 6: Effect of D-carvone on trabecular microarchitecture of ovariectomized rats. Illustrates the results of (a) Trabecula thickness (b) Trabecular area (c) Trabecular separation (d) Trabecula number in sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

Effect of D-carvone on bone turnover markers of ovariectomized rats

The bone turnover markers estradiol [Figure 7a], bone Gla-protein [Figure 7b] and acid phosphatase levels [Figure 7c] were assessed in sham operated, ovariectomized and D-carvone-treated rats. The levels of estradiol, bone Gla-protein, and acid phosphatase were diminished in ovariectomized rats (7.4 pg/ml, 1.8 µg/L, 54 U/L, respectively) compared to the sham-operated rats (9.8 pg/ml, 2.8 µg/L, 97 U/L, respectively). Slight rise in levels of estradiol, bone gla protein, and acid phosphatase was seen in low-dose D-carvone-treated rats (8.7 pg/ml, 2.8 µg/L, 83 U/L, respectively) and significantly amplified

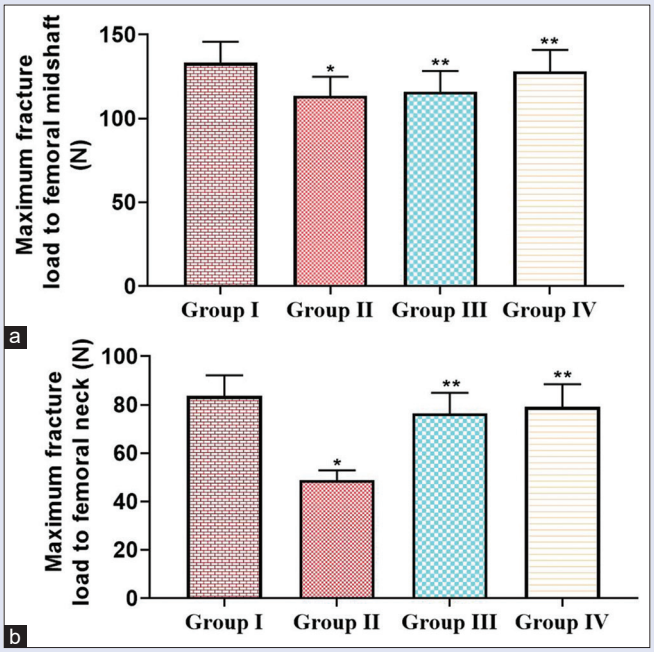

Figure 5: Effect of D-carvone on fracture bearing capacity of ovariectomized rats. Maximum fracture loading capacity of (a) femoral mid shaft (b) femoral neck of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

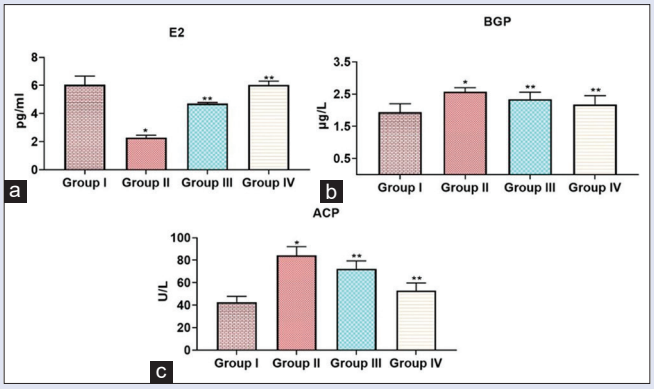

Figure 7: Effect of D-carvone on bone turnover markers of ovariectomized rats. (a) Estradiol (b) Bone gla protein (c) Acid phosphatase levels of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

in high-dose D-carvone-treated rats (8.1 pg/ml, 1.98 µg/L, 72 U/L, respectively) compared to ovariectomized rats.

Figure 8 clarifies the results of serum calcium Figure 8a, phosphorous Figure 8b, creatinine Figure 8c, and TRAP Figure 8d in sham-operated, ovariectomized, and D-carvone-treated rats. The levels of calcium,

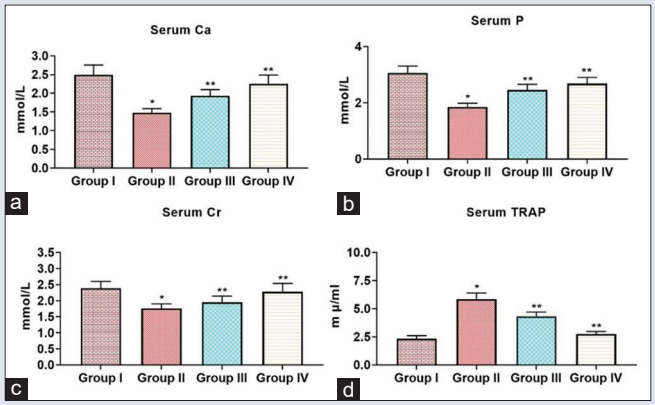

Figure 8: Effect of D-carvone on bone vital markers of ovariectomized rats. (a) Serum Calcium (b) serum phosphorous (c) serum creatinine (d) Tartrate-resistant acid phosphatase levels of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

phosphorous, and creatinine were suggestively lessened in ovariectomized rats (1.44 mmol/L, 1.82 mmol/L, 1.76 mmol/L, respectively) compared to the sham-operated control rats (2.65 mmol/L, 3.2 mmol/L, 2.4 mmol/L, respectively). High-dose D-carvone-treated rats significantly improved the levels of calcium, phosphorous, and creatinine, respectively (2.4 mmol/L, 2.7 mmol/L, 2.4 mmol/L, respectively) compared to the ovariectomized rats. Figure 4d illustrates the results of TRAP levels. The TRAP level severely increased in ovariectomized rats (5.8 mmol/L) compared to the control rats (2.4 mmol/L) and the levels were significantly declined in both high (3.9 mmol/L) and low-dose D-carvone-treated rats (4.2 mmol/L).

Osteoblastic effect of D-carvone on ovariectomized rats

Figure 9 signifies the levels of OPG and RANL protein in sham-operated, ovariectomized, and D-carvone-treated rats. Ovariectomy drastically lessened the levels of osteoblastic protein OPG (470 pg/ml) and augmented the levels of osteclastic protein RANKL (76 pg/ml) compared to the sham-operated control rats (1020 pg/ml, 18 pg/ml, respectively). Compared to the low-dose D-carvone-treated rats high-dose D-carvone-treated rats shown an expressively augmented level of OPG (740 pg/ml) and reduced level of RANKL (52 pg/ml).

Effect of D-carvone on femoral metaphysic histomorphometry of ovariectomized rats

Figure 10 portrays the H and E stained images of the femoral metaphysic of sham-operated control, ovariectomized and D-carvone-treated ovariectomized rats. Ovariectomized rats exposed broadly separated and disconnected thin trabecula with the increased number of adipose cells along the bone marrow spaces [Figure 10b] compared to the control group [Figure 10a], which revealed the normal architecture of femoral metaphysic. Both high- and low-dose of D-carvone treatment decreased the number of adipose cells and increased the trabecular thickness upturned the deleterious effect of ovariectomy [Figure 10c and d].

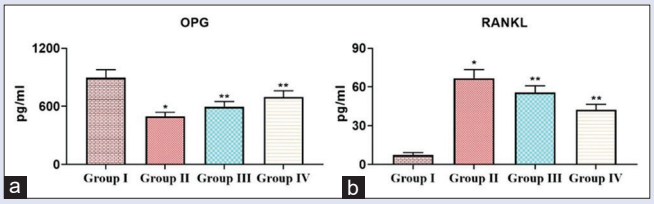

Figure 9: Osteoblastic effect of D-carvone on ovariectomized rats. (a) Osteoprotegerin (b) RANKL protein levels of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

D-carvone influence on lipid profile of ovariectomized rats

The total lipid profile of sham-operated control, ovariectomized, D-carvone-treated ovariectomized rats were measured and the values are described in Figure 11. The total cholesterol HDL cholesterol and LDL cholesterol levels were augmented in ovariectomized rats (1.75 mmol/L, 1.32 mmol/L, and 0.47 mmol/L, respectively) compared to sham-operated control rats (1.25 mmol/L, 0.87 mmol/L, and 0.22 mmol/L, respectively). No noteworthy alteration was detected between ovariectomized and low-dose D-carvone-treated rats (1.62 mmol/L, 1.22 mmol/L, 0.42 mmol/L). High-dose D-carvone-treated ovariectomized rats shown a substantial decrease in the levels of total cholesterol (1.4 mmol/L), HDL cholesterol (1.02 mmol/L), and LDL cholesterol (0.36 mmol/L) compared to ovariectomized rats. The triglycerides levels were diminished in ovariectomized rats (0.52 mmol/L), low-dose D-carvone-treated rats (0.35 mmol/L) and high-dose D-carvone-treated rats (0.24 mmol/L) compared to sham-operated control (0.7 mmol/L).

Influence of D-carvone treatment on inflammatory markers of ovariectomized rats

The inflammatory markers TNF-α, IL-1 β, and IL-6 were quantified in sham-operated control, ovariectomized and D-carvone-treated ovariectomized rats and the fallouts are exemplified in Figure 12. The inflammatory markers TNF-α, IL-1 β, and IL-6 were pointedly augmented in ovariectomized rats (21 ng/ml, 93 pg/ml, and 174 pg/ml) compared to the control rats (13 ng/ml, 73 pg/ml, and 105 pg/ml). High-dose D-carvone effectively declined the levels of TNFα, IL-1 β, and IL-6 (15 ng/ml, 75 pg/ml, and 120 pg/ml) compared to low-dose D-carvone treated ovariectomized rats (18 ng/ml, 84 pg/ml, and 130 pg/ml).

Effect of D-carvone on bone turnover markers gene expression of ovariectomized rats

Figure 13 signifies the results of bone turnover markers gene expression of sham-operated control, ovariectomized and D-carvone-treated ovariectomized rats judged with qPCR analysis. The gene expressions of bone turnover markers OSX Figure 13b, RUNX2 Figure 13c were severely diminished and ALP Figure 13a in ovariectomized compared to the control rats. Both low- and high-dose D-carvone treatment reformed the levels of bone turnover markers gene expression in ovariectomized rats compared to low-dose D-carvone treatment high-dose D-carvone treatment effectively augmented the expression of bone turn over marker proteins.

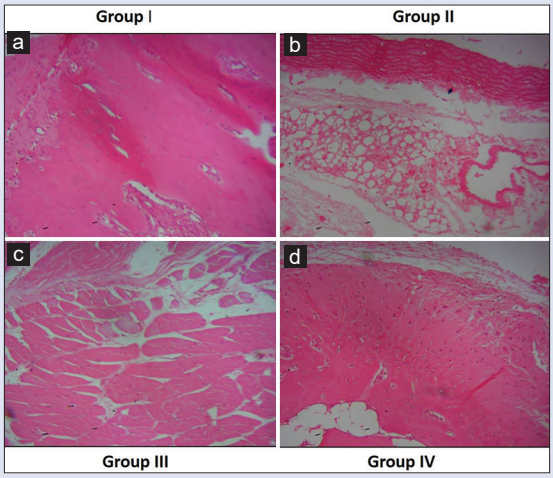

Figure 10: Effect of D-carvone on femoral metaphysic histomorphometry of ovariectomized rats. H and E stained representative photomicrographs of (a) Sham operated control (b) Ovariectomized (c) low dose D-carvone treated ovariectomized (d) high dose D-carvone treated ovariectomized rats

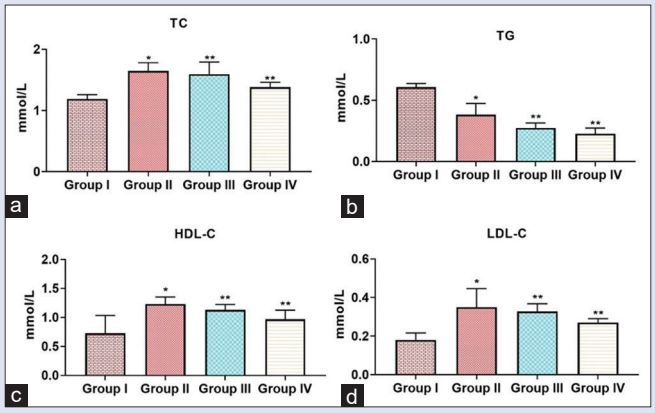

Figure 11: D-carvone influence on lipid profile of ovariectomized rats. (a) Total cholesterol (b) Triglycerides (c) high density lipoprotein cholesterol (d) low density lipo protein cholesterol levels of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

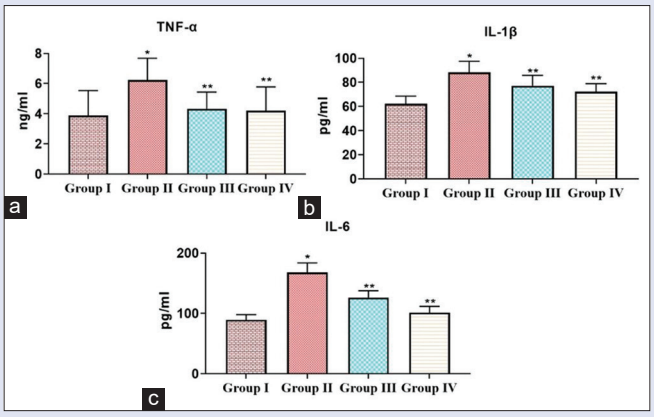

Figure 12: Influence of D-carvone treatment on inflammatory markers of ovariectomized rats. The levels of inflammatory cytokines (a) TNF-α, (b) IL-1β and (c) IL-6 of sham operated control, ovariectomized, low dose D-carvone treated ovariectomized and high dose D-carvone treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low and high dose D-carvone treated ovariectomized rats

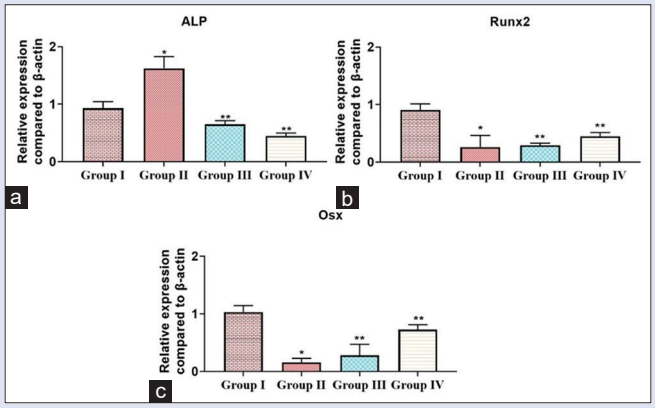

Figure 13: Effect of D-carvone on bone turnover markers gene expression of ovariectomized rats. Quantitative polymerase chain reaction analysis was performed to determine the gene expression of (a) Alkaline phosphatase (b) OSX (c) RUNX2 in sham-operated control, ovariectomized, low-dose D-carvone-treated ovariectomized and high-dose D-carvone-treated ovariectomized rats. Data were statistically analyzed with Graphpad PRISM software and the statistical significance between groups was considered to be $P < 0.05$ were considered to be statistically significant. *Control versus Others, **Ovariectomized group versus low- and high-dose D-carvone-treated ovariectomized rats

DISCUSSION

In the present study, we evaluated the potency of D-carvone on increasing the bone mass and inhibiting osteoporosis induction in *in vivo* conditions. Various animal models were recognized to be employed in assessing both the pathogenesis of osteoporosis and the effect of antiresorptive drugs[22,23] We have selected ovariectomy-induced osteoporosis model since estrogen depletion plays an energetic role in osteoporosis induction in postmenopausal women. Ovariectomized rat model mimics the characteristics of postmenopausal women with estrogen depletion and augmented bone lost.[24,25] Weight gain is main in most of menopausal women due to diminished estrogen synthesis.[26] In our study, ovariectomized rats also shown extreme increase in their body weight and decrease in uterine and vaginal weight which shows the depletion of estrogen. The decrease in uterine and vaginal weight may be due to the regression that ensued due to the ovariectomy. D-carvone has not stated any known toxicity and according to the data of the National Library of Medicine HSDB Database (2018),[27] the LD50 of D-carvone in rats was 5400 mg/kg. In our study, minimal dose of 5 and 10 mg/kg b. wt were employed to treat osteoporosis in ovariectomized rats. Both the doses efficiently suppressed the weight gain and increased the uterine and vaginal weight in ovariectomized rats. This may be due to the phytoestrogenic property of D-carvone.[28]

Metabolic disorder osteoporosis is largely considered with diminished BMD, bone mass and fragile bone, prone to fractures. Investigation of

BMD is a key detector of osteoporosis induction.[29,30] Therefore, in the present study, we scrutinized the weight, length and BMD of the femur bone in ovariectomized rats and D-carvone-treated ovariectomized rats [Figure 2c]. D-carvone significantly augmented the femoral length, weight, and BMD in ovariectomized rats. This associates with the earlier studies where supplementation of calcium along with Vitamin D increased the femur BMD.[31,32] Even though BMD quantification is ideal non-invasive test to evaluate osteoporosis induction the fracture risk and the bone quality cannot be detected with BMD values.[33-35] Hence, biomechanical analysis of bone quality such as bone strength, stiffness, elastic modulus, maximum deflection, maximum load, energy, and fracture bearing capacity should also be studied to assess the potency of an antiresorptive drug.[36] The biomechanical analysis can be achieved with various tests such as tensile strength test, compression test, torsion strength test. In the present study, we used three-point bending extensively to analyze the biomechanical properties of bone in ovariectomized and D-carvone-treated ovariectomized rats [Figures 3-5]. D-carvone treatment improved the maximum load, maximum stress and elastic module of bone in ovariectomized representing it increased the integral strength, capability of resisting deformation and diminished the fragility of bone.[37]

Deterioration of trabecular microarchitecture is a hall mark event in osteoporosis induction which decreases the bone quality and eventually increases the fragility fracture occurrences.[38,39] Therefore, we investigated the effect D-carvone on maintaining trabecular microarchitecture using micro CT analysis. D-carvone significantly augmented the trabecular thickness, area and number and declined the trabecular separation, thereby preventing the deterioration of trabecular microarchitecture [Figure 5]. This specifies D-carvone possess osteoplastic property which prevented the worsening of trabecular bone. Therefore, further, we evaluated the impact of D-carvone on bone turnover markers estrogen, bone Gla-protein, and acid phosphatase [Figure 6]. Estrogen deficiency plays a key role in the induction of osteoporosis in menopausal women and hormone replacement therapy appears to be an effective treatment to prevent fragility fractures in postmenopausal women.[9,40] Bone Gla-protein displays high affinity towards calcium and it surges the absorption of hydroxyapatite in the bone matrix thereby promotes bone mineralization.[41] In the present study, D-carvone effectively augmented the estrogen levels in ovariectomized rats and it also enlarged the levels of osteocalcin or bone-Gla protein. This may be the reason for the condensed deterioration of trabecular microarchitecture in D-carvone-treated ovariectomized rats.

Calcium and phosphorous are the two key substrates of bone mineralization controlled by various hormones.[42] Diminished BMD in postmenopausal women is typically related to lessened calcium levels. Decreased calcium levels augment the secretion of parathyroid hormone thereby increases the osteoclastic activity in bones.[43] The rate of creatinine clearance was pointedly decreased in osteoporotic patients and the incidence rate of fractures was reported more in these types of patients.[44] Therefore, in the present study, we studied the efficacy of D-carvone in maintaining the levels of calcium, phosphorous, and creatinine in ovariectomized rats. D-carvone effectively enlarged the levels of calcium, phosphorous, and creatinine levels which in turn would have augmented BMD in ovariectomized rats [Figure 8].

Even though various hormones control bone remodeling the decisive executors are OPG and RANKL. OPG belongs to the family of TNF receptors. They are synthesized by osteoblastic cells which prevent the binding of nuclear factor-κB ligand (RANKL) with receptor activator of nuclear factor-κB (RANK) and thereby inhibit the osteoclastogenic activity and endorse osteoblastic activity.[45-47] D-carvone treatment efficiently amplified OPG and lessened RANKL protein levels which evidence the osteoblastic activity of D-carvone in ovariectomized rats [Figure 9]. The osteoblastic activity of D-carvone was further established with histopathological analysis of femur diaphysis in ovariectomized rats. D-carvone significantly abridged the histopathological fluctuations in ovariectomized rats compared to the untreated rats [Figure 10]. Our results linked with previous studies, where the ovariectomized rats treated with strontium fructose 1,6-diphosphate,[8] 17 β-estradiol,[48] prenylflavonoid xanthohumol[49] effectively reduced the histopathological changes.

Hyperlipidemia is another malefactor which lessens the bone density via inhibiting the osteoblastic cells differentiation and promotes the osteoclastic cells differentiation.[50,51] Therefore, we assessed the levels of lipids in D-carvone-treated ovariectomized rats. D-carvone-treated rats knowingly declined the levels of total cholesterol, triglycerides, low-density lipoprotein and augmented the levels of high-density lipoproteins, thereby preventing hyperlipidemic conditions in ovariectomized rats [Figure 11]. This may be due to the antihyperlipidemic property of D-carvone which is described in earlier study scrutinized in hypertensive rats.[52] Chronic inflammation upsets the balance between osteoblasts and osteoclasts thereby provoking the bone disorders such as osteoporosis.[53] Increased levels of inflammatory cytokines were reported during estrogen deficiency which in turn augmented the osteoplastic activity in the bone marrow.[54,55] D-carvone possess immunomodulatory and anti-inflammatory properties.[17] In the present study also D-carvone treatment significantly decreased the levels of inflammatory cytokines in ovariectomized rats, thereby precluding bone deterioration [Figure 12].

To authorize, the molecular mechanism behind the D-carvone osteoblastic activity in ovariectomized rat's gene expression of osteoblastic transcription factors RunX2 and Osx were enumerated. RUNX2[56] and OSX,[57] play key role in the variation of preosteoblast to osteoblast cells. Abnormal expression of these transcription factors has been stated to cause osteopenia, bone deformation due to low bone turnover.[58-60] D-carvone treatment reduced both osteoblastic transcription factors RUNX2 and OSX expressions [Figure 13]. It also diminished ALP an early differentiation marker of osteoblastic proteins[61] and dwindled the levels of TRAP osteoclastic protein in ovariectomized rats [Figure 8].[62] Thus, it realistically attests that D-carvone persuasively inhibits the osteoclastic activity and promotes osteoblastic activity in ovariectomized rats.

CONCLUSION

Our results from the present study demonstrated the potency of D-carvone as an antiresorptive agent in ovariectomized rats. D-carvone effectively preserved the body weight, BMD in ovariectomized rats. The results proved that the D-carvone-treated ovariectomized rats presented augmented bone strength and dwindled the fragility of bone due to the ovariectomy. D-carvone had prevented bone deterioration and increased osteoblastic proteins and decreased the levels of osteoclastic protein expressions. It also diminished the inflammatory cytokines and increased the osteoblastic transcription factors OSX, RUNX2, and differentiation marker ALP which validly checks D-carvone as a potent antiresorptive drug. Further clinical trials in future could endorse D-carvone as antiresorptive drug to treat osteoporosis in postmenopausal women.

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Conflicts of interest

There are no conflicts of interest.

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