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Evaluation of antiosteoporotic activity of Root extract of *Rubia Cordifolia* in Ovariectomized Rats

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Abstract

The present study was carried out to investigate the antiosteoporotic activity of ethanolic extract from the root of *Rubia cordifolia (RC)* in bilateral-overiectomy induced osteoporotic rats. The study was performed on female Sprague-dawley rats. Two different doses (200 and 400 mg/kg) of ethanolic extract of *RC* root were evaluated by acute oral toxicity test. Raloxifene (5.4 mg/kg) was used as a reference standard. Rats were randomly divided into 5 groups with 10 per group. Bilateral-overiectomy was performed for all the rats except for the rats from group-1, which were sham-operated and served as a basal control. Rats from group-2 were served as an OVX-control and received vehicle. Group-3 was administered with raloxifene (5.4 mg/kg, p.o.) and served as a standard control. Group-4 and 5 were administered with suspension of ethanolic root extract of *RC* (200 and 400 mg/kg, p.o.) respectively. Treatment was given for 90 days starting from the 15th day after surgery. At the end of the study, the blood samples from all the groups were withdrawn to assess biochemical parameters. The animals were then sacrificed and femur bones were isolated for biomechanical and scanning electron microscopy (SEM) studies. Increased biomechanical strength, calcium absorption, minimal osteoclastic activity and enhanced osteoblastic activity were observed in the rats treated with ethanolic extract of *RC* root. SEM data adds a confirmatory note to the findings. All these results demonstrate significantly antiosteoporotic activity of *RC* extract. Further studies are required to determine the active components that are responsible for its antiosteoporotic activity.

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Key words:

Rubia cordifolia; ovariectomy; osteoporosis; plant extract; Biomechanical strength; Tartrate resistant acid phosphatase (TRAP); Alkaline phosphatase (ALP)

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INTRODUCTION:

Bone is a tissue which has got multi functions like it gives shape to body, helps in movement; take care of blood cell production and weight bearing. This is the reason why some of the bone related problems like

osteoporosis, rheumatoid arthritis, and osteomalacia are the area of concern in drug development.

Osteoporosis is the area of interest since it affects the age old people and particularly the postmenopausal women because of estrogen deficiency after cessation of menopause. Osteoporosis is a condition of low bone mass and micro architectural disruption that results in fracture with minimal trauma. Characteristic sites of fracture include vertebral bodies, distal radius, and the proximal femur, but the osteoporotic individuals have generalized skeletal fragility, and fractures at other sites such as ribs and long bones, also are common. The main reason behind the osteoporosis is being depletion of the hormone estrogen. [1]

Osteoporosis is a common disease in older adults. In women, the incidence of vertebral fractures begins to increase near the time of menopause; in men, vertebral fracture incidence increases at older ages but at ratios 2:1 that of women, not the 8:1 ratio previously reported. Hip fracture incidence accelerates approximately 10 years after menopause in women and after age 70 in men. Women have twice as many fractures as men, although over 75,000 hip fractures occur annually in men. The direct health care costs related to osteoporosis are estimated to be 38 million dollars per day; comparable with the costs attributed to congestive heart failure (CHF) or asthma. The disability, mortality, and cost of hip and vertebral fractures are substantial in the rapidly growing, aging population so that prevention of osteoporosis is a major public health concern. [2] Postmenopausal osteoporosis is characterized by an increase in bone resorption relative to bone formation, in conjunction with an increased rate of bone turnover.[3] The progressive decrease in bone mass leads to an increased susceptibility to fractures, which result in morbidity and mortality.[4] Vertebral fractures are important not only because they can cause pain, kyphosis and height loss but also because they predict subsequent, non-vertebral fractures independently of bone mineral density.[5] A sharp decrease in ovarian estrogen production is the predominant cause of rapid, hormone-related bone loss during the first decade after menopause.[6] Menopause, aging and hereditary factors, inadequate calcium intake and absorption, lack of exercise, prolonged steroid administration, excessive alcohol intake, and cigarette smoking are the major risk factors that predispose osteoporosis.[7]

The pharmacological agents used to manage osteoporosis act by decreasing the rate of bone resorption, thereby slowing the rate of bone loss, or by promoting bone formation. Many synthetic agents such as calcium, calcitonin, hormones, bisphosphonates and selective estrogen receptor modulators (SERMs) such as raloxifene and droloxifene have been developed to treat osteoporosis, but are associated with side effects such as hypercalcemia, hypercalciuria, increased risk of endometrial and breast cancer, breast tenderness, menstruation, thromboembolic events, vaginal bleeding, hot flashes, dyspepsia and GI ulcers.[6]

To prevent all undue physical, mental and financial suffering by patients, there is an extreme importance for the better alternative therapeutic management especially from natural resources which are thought to be healthier and safer for the treatment of osteoporosis.

In ancient system of medicine, a several number of herbal plants have been used for osteoporosis, bone calcification and fracture. In Ayurveda (Sandhaniya) root of *RC* have been reported as bone mender i.e. used to heal fractured bones). [8] Yan-Bin W. et al. has reported the isolated anthraquinones such as physcion, from *Morinda officinalis* having antiosteoporotic activity on osteoblasts and osteoclasts.[9] Since various anthraquinones such as physcion also found in roots of *RC,* the present study was aimed to investigate antiosteporotic activity of

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ethanolic root extract of *RC* using ovariectomized rat model.

MATERIALS AND METHODS:

Chemicals: Raloxifen (Dr.Reddy's Laboratories Pvt. Ltd. Hyderabad, India), ketamine (Neon Laboratories Ltd. Thane, India), xylazine (Indian immunologicals Ltd. Andra Pradesh, India), biochemical estimation kits such as Calcium Estimation Kit, Tartarate resistant acid phoshatase Estimation Kit and Alkaline Phosphatase Estimation Kit (Erba Mannheim, Baddi, Himachal pradesh, india) were used; All the other solvents and chemicals used for the study were of high purity and of analytical grade, purchased from S D fine-chem Ltd, Mumbai, India.

Plant: Dried roots of *RC* were procured from Suganda Kesari pharma depot Pvt Ltd, Bangalore, Karnataka. It was authenticated by Dr. Siddamallayya, reader and taxonomist, Botony, Regional research institute (Ay), Bangalore. A voucher specimen of the collected sample was deposited at museum RRI Bangalore, India.

Extraction procedure: The shade-dried, powdered roots of *RC* were successively extracted in Soxhlet apparatus using ethanol (70%) at 60-80°C. The extracts were then concentrated by distilling the solvent, followed by evaporating the same to dryness at low temperature. The weight and the percentage of extractive value were calculated in terms of air dried weight of the plant material.

Extract pre-treatment: The ethanolic extract was suspended in gum acacia (0.5%) before dosing orally. For every dosing, fresh dilutions of the extracts were done.

Experimental animals: Sprague-Dawley female rats (150-200 g) were purchased from Raghavendra Enterprises, Bangalore. All the animals were acclimatized for seven days under standard husbandry conditions, i.e. room temperature of 25 ± 10°C; relative humidity 45-55% and 12:12h light/ dark cycle. The animals were given free access to standard rat pellet (Amruth Animal Feeds Pvt. Ltd, Bangalore, India), with water *ad libitum* under hygienic conditions. Each group had separate set of animals and care was taken to ensure that animals used in an experiment were not employed elsewhere. Animals were habituated to laboratory conditions for 48 hours prior to experimental protocol to minimize non-specific stress if any. The approval of the Institutional Animal Ethical Committee (IAEC) of P.E.S College of Pharmacy Bangalore (Karnataka) was taken prior to the experimentation. All the protocols and the experiments were conducted in compliance to ethical principles and guidelines as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Govt. of India, New Delhi.

Acute toxicity: The acute toxicity (AOT) of ethanolic root extract of *RC* was determined by using twenty healthy wistar albino female mice, which were randomly divided into two groups of equal size each. All the animals were fasted overnight before the test. The first was given 2000 mg/kg body weight of freshly prepared ethanolic root extract of *RC*, while the control group was given equivolume of 0.5% gum acacia. The animals were observed at 0 min, 30 min, 1 h, 2 h, 4 h, 6 h; thereafter every day for 14 days. At the end of the 14th day the animals were sacrificed by ether anesthesia and dissected for examination of vital organs for pathological changes. [10]

Surgical procedures:

Induction of osteoporosis by ovariectamization: After one week of acclimatization, the rats underwent longitudinal, bilateral skin Incision under anaesthesia anaesthetized with ketamine and xylazine (5 mg/kg i.p.). Ovariectomy was preceded by a bilateral skin incision, 3cm long approximately. Incisions of the muscles were made bilaterally. After peritoneal cavity was accessed, the ovary was found, surrounded by a variable amount of fat. Ligation of the blood vessels

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was necessary. The connection between the fallopian tube and the uterine horn was cut and the ovary was moved out. Because of the muscle bleeding, its incision required suturing with absorbable catguts. Three single non-absorbable catgut stitches were placed on the skin. [11] In the sham operation, the ovaries were exposed as above and manipulated gently but not excised. The animals were given prophylactic amoxicillin (25mg/kg, i.p.) for 4 days and Povidone-iodine Solution applied locally. Experimental animals were divided randomly into five groups of ten animals each. All rats were untreated for 15 days after surgery to allow for the development of osteoporosis. [12]

Experimental procedure: Group 1 was sham operated and served as basal control. All the other groups were bilaterally-ovariectomised and received treatment for 3 months starting from the fifteenth day of ovariectomy. Group 2 received vehicle and served as ovariectomized control. Group 3 was orally administered with Raloxifen (5.4 mg/kg). Group 4 and 5 were orally treated with suspension of ethanolic root extract of *RC* at doses of 200 and 400 mg/kg body weight, respectively. At the end of the study period, the blood samples from all the groups were withdrawn by retro-orbital method to assess biochemical parameters. Serum was separated by centrifugation (10,000 rpm X 10 min) and kept at -20°C until further use. The animals were then sacrificed using ethyl ether and femur bones were isolated for biomechanical and scanning electron microscopy (SEM) studies.

Evaluation parameters for anti-osteoporotic activity:

Biochemical parameters:

Serum alkaline phosphatase (ALP): Erba Mannheim diagnostics kit Baddi, Himachal Pradesh, was used for the in vitro estimation of serum ALP. Estimation was carried out by Kinetic Assay. At p^{H} 10.3 ALP catalyses the hydrolysis of colourless p-Nitrophenyl phosphate (pNPP) to yellow coloured p-Nitrophenol and Phosphate. Change in absorbance due to yellow colour formation is measured kinetically at 405 nm using Semi-autoanalyzer (RMS-BCA 201) and is proportional to ALP activity in the sample. [13]

Serum tartrate resistant acid phosphatase (TRAP): In vitro determination of TRAP by Kinetic method was carried out by using Accurex autozyme diagnostic kit (Mumbai). In acidic pH of buffer system, acid phosphatase hydrolyses alpha napthyl phosphate to alpha napthol and phosphate. The alpha napthol is then coupled with diazotized Fast Red TR to form a diazo dye which has strong absorbance at 405nm using Semi-autoanalyzer (RMS-BCA 201). The increase in absorption is directly proportional to the level of acid phosphatase in serum. The addition of L tartarate inhibits Prostatic acid phosphatase (PAP) but does not inhibit other isoenzyme. The activity of prostatic acid phosphatase is obtained by subtracting the result of Non prostatic acid phosphatase (NPAP, Determination with tartarate) from Total acid phosphatase (TAP. without tartarate). [14]

Serum calcium: Serum calcium was estimated by using Erba Mannheim diagnostics kit Baddi, Himachal Pradesh. Estimation was carried out by End Point Assay.In alkaline solution, calcium binds with metal complexing dye O-Cresolphthalein complexone (OCPC) to form a bluish-purple complex, which is measured at 578 nm using Semiautoanalyzer (RMSBCA 201).The intensity of color formed is proportional to calcium concentration in the sample. Hydroxyquinolne will act as a masking agent and eliminate the interference of magnesium. [15]

Biomechanical parameters:

Bone mineral content: Bone (left femur) ash was prepared in a muffle furnace (700°c for 6 h) and dissolved in 0.1 mol/L HCL solution. Bone mineral (calcium) was measured by a UV-visible spectrophotometer (RMS-BCA 201).

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Three-point bending of femur: The isolated bones (right and left femur) were assessed for their biomechanical strength by using the tensile strength testing machine. The sample was mounted horizontally between the mounting slots of the apparatus (Zwick/Roell 2005) against the cylindrical stoppers of 5 mm diameter; the cylindrical stoppers supporting the bone at ends are rigidly fixed to the frame of the apparatus. Load was applied exactly at the center of the sample between the end supports by means of a steel wire of 0.5 mm diameter passing horizontally. Load was varied gradually from 5 N to maximum breaking point value for the sample by increments of small steps and corresponding deflection of the sample was assessed by means of a laser displacement sensor which is set to measure the deflection of the sample at the loading point. The displacement sensor was connected to a computer through a data acquisition system which continuously acquired and stored the displacement data in the computer.

SEM evaluation: The right femur of each animal, (one from each group) were preserved in the neutral phosphate formalin solution were trimmed using rotating saw. The trimmed bones were then dried by exposing to heat and mounted on stubs and coated with gold using sputter coater. Then bones were exposed on a JEOL, JSM-840A Scanning Electron Microscope. Scanning electron micrographs of a frontal view of the metaphyseal region of the distal femur was taken at 500X. All samples were examined uniformly at a specific position to minimize the errors.

Statistical analysis:

The values were expressed in terms of Mean ± standard error of mean (SEM) at each dose level. The level of significance was determined by one-way ANOVA followed by Tukey's multiple comparison tests. P value < 0.05 was considered as significant.

RESULTS:

Acute oral toxicity: The acute toxicity study showed that oral administration of the ethanolic extract of *RC* produced neither mortality nor signs of clinical abnormality in either group till 2000 mg/kg. At necropsy, no gross pathological observations could be made in the target organs. Hence, The LD50 value of *RC* was thus found to be more than 2000 mg/kg body wt, p.o.

Body weight: There was no significant difference between initial body weights of the five groups at the beginning of the study. At the end of the study period OVX-control group showed a significant weight gain compared with the sham-control group, raloxifene (5.4 mg/kg) and ethanolic extract of *RC* (200 and 400 mg/kg) inhibited the weight gain and maintained the body weight changes almost near to the body weight range of the untreated group.

Table 1: Effects of ethanolic extract of *RC* on body weight of rats with ovariectomy-induced osteoporosis

Group (n=10)	Change in body weight (g)		% increase in body weight
	Initial	Final	
Sham operated (basal control)	211.7 ± 2.092	240.0 ± 2.366	13.36
OVX-control	219.0 ± 5.721	273.0 ± 5.627^{c}	24.65^{c}
Raloxifene (5.4 mg/kg)	211.7 ± 3.029	238.7 ± 5.627***	12.75***
Ethanolic extract 200mg/kg	210.0 ± 2.129	241.0 ± 4.405***	14.76***
Ethanolic extract 400mg/kg	208.4 ± 1.012	235.7 ± 4.910***	13.09***

All values are expressed as mean ± S.E.M. $^{C}P < 0.001$ vs. sham-control group; $^{***}P < 0.001$ vs. OVX-control group.

Weight and length of femur: The femur weight was considerably decreased in the OVX group when compared with all the other groups, i.e. the sham-control group, standard and extract treated groups. However, none of the group showed significant variation in femur length.

Table 2: Effects of ethanolic extract of *RC* on weight and length of femur in rats with ovariectomy-induced osteoporosis

Group (n=10)	Femur weight (g)	Femur length (mm)
Sham operated (basal control)	0.6587± 0.0033	34.63 ± 0.3597
OVX-control	0.5852±0.03414^{a}	35.35 ± 0.1843
Raloxifene (5.4 mg/kg)	0.6830± 0.0016**	35.71 ± 0.0106
Ethanolic extract 200mg/kg	0.6860± 0.0065**	35.81 ± 0.0532
Ethanolic extract 200mg/kg	0.6825± 0.0041**	35.78 ± 0.0503

All values are expressed as mean ± S.E.M. $^{a}P < 0.05$ vs. sham-control group; $^{**}P < 0.01$ vs. OVX-control group.

Serum biochemical constituents: As compared to sham-control group, OVX-control group rats showed a significant increase in both the serum ALP and TRAP level at the end of the study. The elevated level of serum ALP shown to be decreased following treatment with Standard raloxifene and ethanolic extracts (200 and 400 mg/kg).

The TRAP level of the standard treated group showed a decrease when compared to the OVX group, which is almost similar to sham operated group. Whereas, in the extract treated groups, TRAP level increased drastically, except for the higher dose of ethanolic extract (400 mg/kg), which decreased the serum TRAP level that was comparable to the Standard treated group.

Serum calcium level significantly decreased in OVX-control group compared with sham-control group. Administration of the ethanolic extracts at 200 and 400 mg/kg and raloxifene strongly increased the serum calcium level in OVX rats.

Table 3: Effects of ethanolic extract of *RC* on Serum biochemical parameters in rats with ovariectomy-induced osteoporosis

Group (n=10)	Biochemical parameters		
	Alkaline phosphatase(IU/L)	Tartarate resistant acid phosphatase (IU/L)	Serum calcium (mg/dl)
Sham operated (basal control)	168.6±10.43	1.138 ± 0.5683	6.723 ± 0.2583
OVX-control	284.3± 5.193^{c}	3.775 ± 0.3002^{b}	4.755± 0.4014^{a}
Raloxifene (5.4mg/kg)	226.2± 15.37*	1.327± 0.4187**	6.851± 0.4047**
Ethanolic extract 200mg/kg	206.5 ± 9.382**	3.924± 0.3980	6.378±0.2136*
Ethanolic extract 400mg/kg	195.7 ± 5.560***	1.855± 0.1509*	6.495±0.2781*

All values are expressed as mean ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs. sham-control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. OVX-control group.

Bone mineral content: As compared to sham-control group, OVX-control group exhibit a significant decrease in ash weight and ash calcium. These changes were significantly normalized in rats treated with raloxifene (5.4 mg/kg) and ethanolic extract (200 mg/kg and 400 mg/kg).

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Table 4: Effects of ethanolic extract of *RC* on ash parameters in rats with ovariectomy-induced osteoporosis

Group (n=10)	Ash weight (g)	Ash calcium (mg/dl)
Sham operated (basal control)	0.5323 ± 0.0084	9.887 ± 0.0508
OVX-control	0.3583 ± 0.0057^{c}	6.195 ± 0.0587^{c}
Raloxifene (5.4 mg/kg)	0.6052 ± 0.0133***	10.19 ± 0.0848***
Ethanolic extract 200mg/kg	0.5093 ± 0.0029***	8.559 ± 0.0812***
Ethanolic extract 400mg/kg	0.5435 ± 0.0034***	9.728 ± 0.1131***

All values are expressed as mean ± S.E.M. $^{c}P < 0.001$ vs. sham-control group; $^{***}P < 0.001$, vs. OVX-control group.

Three-point bending (Bone impact test) of femur: The OVX control group showed a decrease in bone strength following ovariectomy as compared to sham-control group. It is observed that the energy required to break the femur of rats of all the treated groups is significantly higher it indicates that all the treated groups significantly restored the ovariectomy-induced altered bone strength in comparison to OVX group. However, raloxifene (5.4 mg/kg) was more potent than ethanolic extract (400 mg/kg) followed by ethanolic extract (200 mg/kg, p. o.) offer significant restoration of ovariectomy-induced altered bone strength.

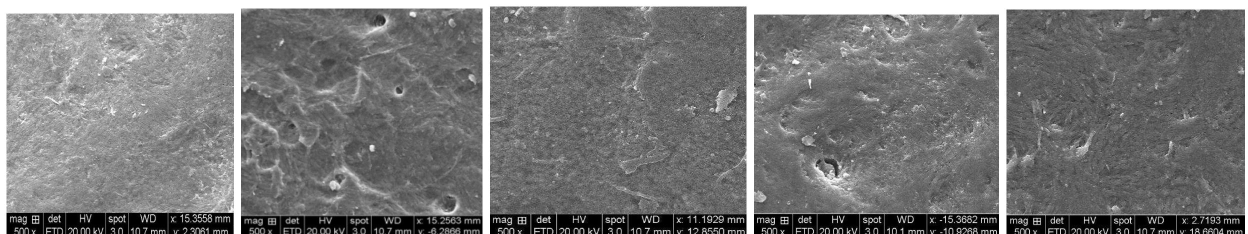
Table 5: Effect of *RC* extract on bone mechanical strength of femur bone as determined by impact test in rats with ovariectomy-induced osteoporosis

Group (n=10)	Force at break (N)
Sham operated (basal control)	108.54 ± 0.1792
OVX-control	75.93 ± 0.6937^{c}
Raloxifene (5.4 mg/kg)	159.3 ± 1.528***
Ethanolic extract 200mg/kg	139.2 ± 1.308***
Ethanolic extract 400mg/kg	156.35 ± 1.263***

All values are expressed as mean ± S.E.M. $^{c}P < 0.001$ vs. sham-control group; $^{***}P < 0.001$, vs. OVX-control group.

SEM evaluation: The SEM of femur bone was taken in all the groups to study the changes in the cortical and cancellous bone loss.

The SEM photographs are shown in the following figures. The frontal view of metaphyseal region of the femur was studied to see the extent of bone loss. From the figure it is observed that, significant difference in the intactness and architecture of the bone by finding the resorptive pits on the surface of the bone. SEM of group-1 (sham-control) showed normal bone architecture. Whereas group-2 (OVX-control) showed significant morphological changes (Pore formation, disintegrated bone architecture, reduced compactness). Group-3 treated with standard drug Raloxifene showed intact bone architecture, which was comparable to Group-1. Treated group-4 and 5 showed significant restorations compared to OVX-control.



Sham-control OVX-control Raloxifene (5.4 mg/kg) RC extract (200 mg/kg) RC extract (400 mg/kg)

Figure 1: Scanning electron microscopy images (500 x magnifications)

The OVX-control group showed porous perforated and disintegrated bone architecture compared with the sham-control group. *RC* extract (200 mg/kg) treatment showed partial restoration; *RC* extract (400 mg/kg) and raloxifene (5.4 mg/kg) treated groups showed complete restoration of osteoporosis bone changes to a compact firm. OVX: ovariectomized.

DISCUSSION

Osteoporosis is a systemic skeletal disease characterized by the low bone mass and the mechanical deterioration of the bone tissue with a consequent increase in the bone fragility and susceptibility to fracture. Osteoporosis damages mechanical property of the bones and typically exposes elderly people. Ovariectomy leads to decreased trabecular bone volume, bone mineral density and strength in rats. The OVX rat is an excellent preclinical animal model that correctly emulates the important clinical feature of the estrogen depleted human skeleton and the response of therapeutic agents. [16] Thus a model for osteoporosis which could clearly reveal the loss of bone mechanical strength is desirable. Since same effect is seen in the post menopausal women, the ovariectomised rat model was selected for this study. Although the most effective method to reduce the rate of post-menopausal bone loss is Estrogen Replacement Therapy (ERT), but it is accompanied by the side effects. [17]

Previous studies have reported that the plant (Morinda Officinalis) containing anthraquinones have estrogen-like effects. [18] Thus, we inferred that the antiosteoporotic activity of *RC* may be related with its estrogen-like Activity as it contains many anthraquinones. The body weight changes before and after the study support the observations of the other investigators related to increased body weight due to ovarian hormone deficiency. [19] The administration of ethanolic extract of *RC* (200 and 400 mg/kg) or raloxifene (5. 4 mg/kg) significantly normalized the ovariectomy-induced increases in body weight.

Serum ALP and TRAP are biochemical markers of bone formation and bone resorption, respectively. The significant elevated level of serum ALP and TRAP level in OVX-control rats indicates increased bone turnover due to the induction of osteoporosis. Well established drug raloxifene decreased serum ALP and TRAP and inhibited high bone turnover in OVX rats. Administration of ethanolic extracts (200 and 400 mg/kg) significantly decreased serum ALP and TRAP levels. These results showed that *RC* extract strongly increased bone density by enhancing bone formation and inhibiting bone resorption.

Serum calcium level significantly decreased in OVX-control group compared with sham-control group. In the present study, administration of the ethanolic extracts (200 and 400 mg/kg) and raloxifene restored the increased serum calcium concentration to normal levels. As serum calcium level indirectly reflect bone metabolism to some extent, our findings supported that *RC* extract improved the calcium absorption and bone matrix formation.

In the present, three point bending test used for the assessment of biomechanical strength of bone showed that, OVX-control rats have a low biomechanical strength compared to sham-control rats. Administration of *RC* extract at 200 and 400 mg/kg and raloxifene significantly strengthened the bones in OVX-rats.

The SEM photomicrographs of rats treated with *RC* extract (200 and 400 mg/kg) and raloxifene (5.4 mg/kg) showed significant restoration of morphological changes (pore formation, disintegrated bone architecture, reduced compactness) compared with OVX-control rats.

CONCLUSION:

The administration of ethanolic extract of *RC* for 90 days significantly increased biomechanical strength, higher osteoblastic activity and minimal osteoclastic

activity account for bone formation. In addition increased serum calcium absorption, bone ash content and ash calcium in RC extract treated groups supports the observation. SEM data adds a confirmatory note to the findings. All these results demonstrate significantly antiosteoporotic activity of *RC* extract. Further studies are required to determine the active components that are responsible for its antiosteoporotic activity. Followed by, testing the isolated active principle in vitro and in vivo are desirable to understand and establish its mechanism of action.

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