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ANTIOSTEOPOROTIC ACTIVITY OF ROOT EXTRACT OF *RUBIA CORDIFOLIA* IN METHYL PREDNISOLONE ACTETATE INDUCED OSTEOPOROTIC RATS

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Abstract:

The main aim of the study was to carry out and investigate the Antiosteoporotic activity of ethanolic root extract of Rubia cordifolia (EERC) in Methylprednisolone acetate (MPA) induced osteoporotic rats. Acute oral toxicity was performed according to OECD guidelines 425 in albino mice and extract was found to be safe upto 2000 mg/kg. All the rats were randomly divided into 5 groups (n=6 each). Group I (normal control) received water for injection s.c., Group II MPA (0.2 mg/kg, s.c) positive control, Group III was MPA (0.2 mg/kg, s.c) + standard drug Alendronate (200 µg/kg, p.o), Group IV& V received EERC (200 mg/kg & 400 mg/kg, p.o) + MPA (0.2 mg/kg, s.c) respectively and treated for 4 weeks. The serum biochemical markers revealed dose dependent restoration of calcium, ALP and TRAP which was significant (P<0.0001) when compared to MPA control. Similarly, the findings from bone biomechanical parameters also found to have significant changes, where bone mechanical strength (P<0.0001) and bone mineral content was also significantly increased (P<0.0001) by improving calcium absorption and enhanced osteoblastic activity nearly to normal levels in rats treated with EERC. Furthermore, SEM data of femur bones of EERC (200 and 400 mg/kg) treated rats showed reduced pore formation and improved bone compactness. All these results significantly indicate a possible antiosteoporotic activity of EERC. In addition, further studies are required to determine the active components that are responsible for its antiosteoporotic activity.

Keywords: *Rubia cordifolia, Methylprednisolone (MPA), Osteoporosis, Biochemical parameters, Ash content, Scanning electron microscopy (SEM).*

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INTRODUCTION:

Osteoporosis is the condition in which there is a decrease in bone mineral density (BMD) and structural changes in the bone tissue that leads to bone brittleness and increased susceptibility to fracture. Many of these fractures are associated with significant morbidity and mortality [1]. It is one of the most predominant metabolic bone diseases in developed countries and the clinical studies in osteoporosis mainly relate to fractures that are associated with lifetime risk of hip and wrist. The clinical data has also been diagnosed where the vertebral fracture was found to be about 40% for white woman and 13% for white men [2]. It can also be considered as rapid loss of mineralised bone tissues, which leads to enhanced bone delicacy and resulting increase in fragility, especially sites rich in cancellous bone such as vertebrae, hip and distal forearm [3]. The utmost common type of osteoporosis are seen in old age people and particularly associated with ovarian hormone deficiency during the first decade after menopause [4]. Most therapies that are presently used for the management of osteoporosis acts mainly by inhibiting the bone resorption [5]. The synthetic pharmacological agents used to manage osteoporosis are calcium, calcitonin, bisphosphonates and hormones, among those a class of selective estrogen receptor modulators (SERMs) such as Raloxifene have been effectively developed in the management of osteoporosis [6]. Alendronate is one of the best and widely studied bisphosphonates in the treatment of osteoporosis. The role of alendronate has successively been emphasized by extending clinical studies for its safe use and combining the effects on fracture risk [5]. The Estradiol, a major estrogen secreted by the ovary is gained importance in maintaining bone mass primarily by retarding osteoclast activity. The increased expression of bone matrix proteins such as osteonectin, collagen and alkaline phosphatase partly promote the bone calcium balance by inducing renal hydroxylase enzyme which generate active form of vitamin D_3, that has a potent effect in increasing calcium absorption from the intestinal tract [7]. The newer class of Glucocorticoids, prednisolone and methylprednisolone were developed in the years 1950–1960 with anti-inflammatory and lesser mineralocorticoid activities, as well as used in a wide variety of disorders including autoimmune, rheumatologic, malignancies and in organ transplantation. Chronic exposure to extreme concentration of cortisol or to pharmacological doses of glucocorticoid (GC) causes multiple deleterious effects on bone and that may leads to altered body structure and function. Osteoporosis and other bone fractures are the main consequences of excessive Glucocorticoid exposure and the main cause of secondary osteoporosis is Glucocorticoid (GC)-induced osteoporosis that are associated with fractures, which are often asymptomatic [8,9,10]. The mechanism of GC action is complex and has not been revealed completely. In addition, the main reasons for bone resorption are inhibition of bone formation with an indirect action on bone by decreasing intestinal Ca^{2+} absorption, vitamin D metabolism and also associated with variable plasma parathyroid hormone (PTH) levels, which finally inhibit the gonadotropic somatotropic axis [11].

Rubia cordifolia L. commonly known as 'Indian Madder' is a vital raw drug for the traditional herbal formulations such as ashwagandharistam, gulguluthikthkarishtam, jaatyaadi ghrita, madhookasavam, etc [12]. In Ayurveda (Sandhaniya) root of *Rubia cordifolia* is been used for the treatment of bone fracture and hence it has been reported as bone mender [13]. As per 'Charaka Samhitaa', the dried roots and fruits powder is consumed internally for the treatment of skin diseases and disorders of spleen. In 'Sushruta samhitaa' preparations based on 'manjistha' are prescribed for the treatment of fractured bone, dysentery and skin burns. It is proven that the root decoction is effective to regulate menstrual cycle and also prescribed to the mother after delivery for cleansing and shrinking of the uterus [13,14]. Since various anthraquinones are also found in roots of *Rubia cordifolia* L., the present study was intended to explore antiosteoporotic activity of ethanolic root extract of *Rubia cordifolia* L. using Methylprednisolone induced osteoporosis in female rats.

MATERIALS AND METHODS:

Drugs and Chemicals

Alendronate (Dr.Reddy's Laboratories Pvt. Ltd. Hyderabad, India), Calcium and Alkaline Phosphatase Kits (Erba Mannheim, Baddi, India), Tartarate resistant acid phosphatase Kit (Accurex Biomedical Pvt. Ltd., Mumbai, India) and all other chemicals and reagents are of analytical grade purchased from S D fine-chem Ltd, Mumbai, India.

Plant material and Extraction

The roots of *Rubia cordifolia* were procured from Suganda Kesari pharma depot Pvt Ltd, Bangalore, Karnataka and was authenticated by Dr. Siddamallayya, taxonomist, Botany, Regional research institute (Ay), Bangalore, Karnataka, India. The collected roots were tray dried for about two weeks and then crushed into powder. Then, the powder was successively extracted in Soxhlet apparatus using ethanol (70%) at 60-70°C for 3 days and the extract was evaporated to dryness at low temperature. The weight and percentage yield were calculated in terms of air dried weight of the plant material.

Extract Pre-Treatment

The weighed quantity of ethanolic extract of *Rubia cordifolia* (EERC) was suspended in gum acacia

(0.5%) and administered orally to rats. The suspension of extract was prepared freshly every day.

Experimental Animals

Sprague-Dawley female rats (150-200 g) were procured from Raghavendra Enterprises, Bangalore. Then all the animals were acclimatized to meet the standard husbandry conditions. All the animals were given free access to standard pellet diet (Amruth Animal Feeds Pvt. Ltd, Bangalore, India) and water *ad libitum* under strict hygienic conditions. Each experimental group were separated with set of animals and utmost care was taken. The approval from the Institutional Animal Ethical Committee (IAEC) of PES College of Pharmacy, Bangalore (Karnataka) was taken (PESCP/IAEC/09/05, Dated: 07/12/09) prior to the experimentation and the experiments were conducted according to CPCSEA guidelines, Govt. of India, New Delhi.

Acute Oral Toxicity

The acute toxicity (AOT) of EERC was determined by using twelve healthy albino female mice, which were randomly divided into two groups (n=6). All the animals were fasted overnight before the test. The first group was given 2000 mg/kg body weight of freshly prepared EERC, while the control group was administered equivolume of 0.5% gum acacia. Subsequently the observations were made at 0min, 30min, 1h, 2h, 4h & 6h for 14 days. At the end of the 14th day the animals were sacrificed by ether anesthesia and vital organs were examined for pathological changes.

Procedure:

Induction of osteoporosis by Methylprednisolone acetate (MPA) in female rats [15,16]

Sprague-Dawley female rats of six months old weighing 150-200 g were used in the study. All the rats were divided into 5 groups (n=6) and treated for 4 weeks.

Group I- Normal control (water for injection, s.c.)
Group II- MPA (0.2 mg/kg, s.c.)
Group III- MPA (0.2 mg/kg, s.c.) + Standard Alendronate (200 µg/kg, p.o.)
Group IV& V- MPA (0.2 mg/kg, s.c.) + EERC, 200 and 400 mg/kg, p.o. respectively.

Evaluation Parameters:

Femur Physical Parameter

Fresh isolated left femurs were weighed using an electronic balance. The length was measured from the proximal tip of the femur head to the distal tip of the medial candyle using a digital caliper [3].

Serum Biochemical Markers

The serum calcium and alkaline phosphatase were carried out by kinetic assay (Erba Mannheim), whereas tartarate resistant acid phosphatase was estimated by kinetic method using commercially available kit (Accurex) [17,18,19].

Bone Mineral Content

The bone mineral content was estimated by preparing left femur bone ash 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) [3,20].

Three-Point Bending Of Femur

The isolated femur bones (right) were assessed for their biomechanical strength by using three- point bending apparatus. The sample was fixed horizontally between the mounting slots of the apparatus (Zwick/Roell 2005) against the cylindrical stoppers of 5 mm diameter where the cylindrical stoppers support the bone at ends which were rigidly fixed to the frame of the apparatus. Load was applied exactly at the centre of the bone by means of a steel wire of 0.5 mm diameter passing horizontally. Load was varied gradually from 5 N to maximum breaking point and the sample values were showed by increments of small steps. Then the corresponding deflection of the sample was evaluated by means of a laser displacement sensor through a data acquisition system [21].

SEM Evaluation

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

Statistical Analysis

All the values were expressed as mean ± SEM. Statistical comparisons were performed by one way ANOVA followed by Tukey's post-test using Graph Pad Prism version 5.0. *P<0.05, **P<0.01, **P<0.001 was considered as significant compared to disease control.

RESULTS AND DISCUSSION:

Acute Oral Toxicity

The EERC did not show any signs and symptoms of toxicity and mortality up to 2000 mg/kg dose. Hence, The LD50 value was found to be more than 2000 mg/kg, p.o.

Femur Bone Physical Parameters

Table-1 shows that the femur length was significantly (P<0.01) reduced in MPA control when compared to normal control, but the standard Alendronate and EERC (400 mg/kg) treated rats revealed significant (P<0.0001) increase in bone length when compared to MPA control. The MPA control rats also showed a reduction in femur weight, but it was less significant (P<0.05) when

compared to normal control. But, the rats treated with EERC (400 mg/kg) exhibited significant increase (P<0.05) in femur weight when compared to MPA control.

Table 1: Effect of EERC on Femur length & weight in MPA induced osteoporotic rats.

Sn	Groups	Femur length (mm)	Femur weight (g)
1	Normal control	35.75±0.289	0.58±0.031
2	MPA control (0.2 mg/kg, s.c.)	35.21±0.151**[a]	0.53±0.014*[a]
3	Alendronate (200 µg/kg)	36.24±0.256***[b]	0.56±0.014
4	EERC (200 mg/kg)	35.20±0.281	0.53±0.031
5	EERC (400 mg/kg)	36.12±0.261***[b]	0.58±0.033*[b]

*Values are expressed as Mean ± SEM (n=6). Statistical analysis was carried out by One way Anova followed by Tukey-Kramar multiple comparison test *P<0.05, **P<0.01, ***P<0.0001. [a] = Normal vs. MPA control, [b] = MPA control vs. treated groups.*

Serum Biochemical Markers

Table-2 illustrates that effect of EERC on serum ALP, Calcium and TRAP levels. In MPA control, the ALP activity and TRAP level was found to be significantly (P<0.0001) raised when compared to normal control. Whereas, the groups received standard Alendronate and EERC (200 & 400 mg/kg) showed protective action and they brought down the levels into normal respectively. The serum calcium level in MPA control was significantly decreased (P<0.0001), but the groups supplemented with EERC (200 & 400 mg/kg) exhibited increased level of calcium when compared to MPA control, which was statistically significant (P<0.0001).

Table 2: Effect of EERC on Serum biochemical markers in MPA induced osteoporotic rats.

SN	Groups (n=6)	Alkaline Phosphatase (IU/L)	Tartarate Resistant acid Phosphatase (IU/L)	Serum Calcium (mg/dl)
1	Normal control	114.0 ± 3.449	1.845 ± 0.0665	13.81 ± 0.0722
2	MPA control (0.2 mg/kg, s.c.)	266.6 ± 3.233***[a]	6.410 ± 0.0749***[a]	11.62 ± 0.1696***[a]
3	Alendronate (200 µg/kg)	112.3 ± 5.219***[b]	2.747 ± 0.0615***[b]	15.64 ± 0.1597***[b]
4	EERC (200 mg/kg)	192.1 ± 2.604**[b]	4.543 ± 0.3413***[b]	13.49 ± 0.3568***[b]
5	EERC (400 mg/kg)	208.8 ± 5.073***[b]	1.422 ± 0.2352***[b]	16.12 ± 0.2089***[b]

*Values are expressed as Mean ± SEM (n=6). Statistical analysis was carried out by One way Anova followed by Tukey-Kramar multiple comparison test *P<0.05, **P<0.01, ***P<0.0001. [a] = Normal vs. MPA control, [b] = MPA control vs. treated groups.*

Table 3: Effect of EERC on Ash parameters in MPA-induced osteoporotic rats.

Sn	Groups	Ash weight (g)	Ash calcium (mg/dl)
1	Normal control	0.576±0.004	9.878±0.012
2	MPA control (0.2 mg/kg, s.c.)	0.366±0.005***[a]	7.89±0.105***[a]
3	Alendronate (200 µg/kg)	0.606±0.009***[b]	11.35±0.153***[b]
4	EERC (200 mg/kg)	0.501±0.013***[b]	9.19±0.051***[b]
5	EERC (400 mg/kg)	0.536±0.018***[b]	9.37±0.077***[b]

*Values are expressed as Mean ± SEM (n=6). Statistical analysis was carried out by One way Anova followed by Tukey-Kramar multiple comparison test *P<0.05, **P<0.01, ***P<0.0001. [a] = Normal vs. MPA control, [b] = MPA control vs. treated groups.*

Bone Mineral Content

The bone calcium level and ash weight are shown in Table-3. The rats of MPA control showed a marked decrease (P<0.0001) in bone calcium level as well as ash weight when compared to normal control. But, the rats administered with EERC (200 & 400 mg/kg), the mineral content was considerably normalized when compared to MPA control..

Table 4: Effect of EERC on bone mechanical strength in MPA-induced osteoporotic rats.

Sn	Groups	Force at Break (N)
1	Normal control	105.22±0.056
2	MPA control (0.2 mg/kg, s.c.)	83.52±0.094***[a]
3	Alendronate (200 µg/kg)	127.2±0.027***[b]
4	EERC (200 mg/kg)	100.25±0.11***[b]
5	EERC (400 mg/kg)	118.3±0.035***[b]

*Values are expressed as Mean ± SEM (n=6). Statistical analysis was carried out by One way Anova followed by Tukey-Kramar multiple comparison test *P<0.05, **P<0.01, ***P<0.0001. [a] = Normal vs. MPA control, [b] = MPA control vs. treated groups.*

Three-Point Bending Test of Femur

The Table-4 illustrates, bone mechanical strength of femur bone determined by three point bending test. The Bone mechanical strength was found to be less in MPA control as compared to normal control (P<0.0001). It is perceived that the energy required to break femur bone of rats in all the treated groups is greater, hence significantly restored the altered bone strength in comparison to MPA group. Though, EERC showed a significant (P<0.0001) increase in mechanical strength of the bone when compared to MPA control.

Scanning Electron Microscopy (SEM) of Femur Bone

The scanning electron microscopy (SEM) of femur bone was taken to study the changes in the cortical and cancellous bone loss. The SEM photographs are shown in Figure 3. The SEM images of MPA-controlled rats showed changes in bone architecture and morphology with pits on the surface of the bone when compared with normal control. The standard Alendronate (200 µg/kg) showed intact bone architecture, which was comparable to normal. Whereas, EERC (200 & 400 mg/kg) treated groups showed partial restoration of femur bone along with improved bone architecture and increased compactness to normal levels.

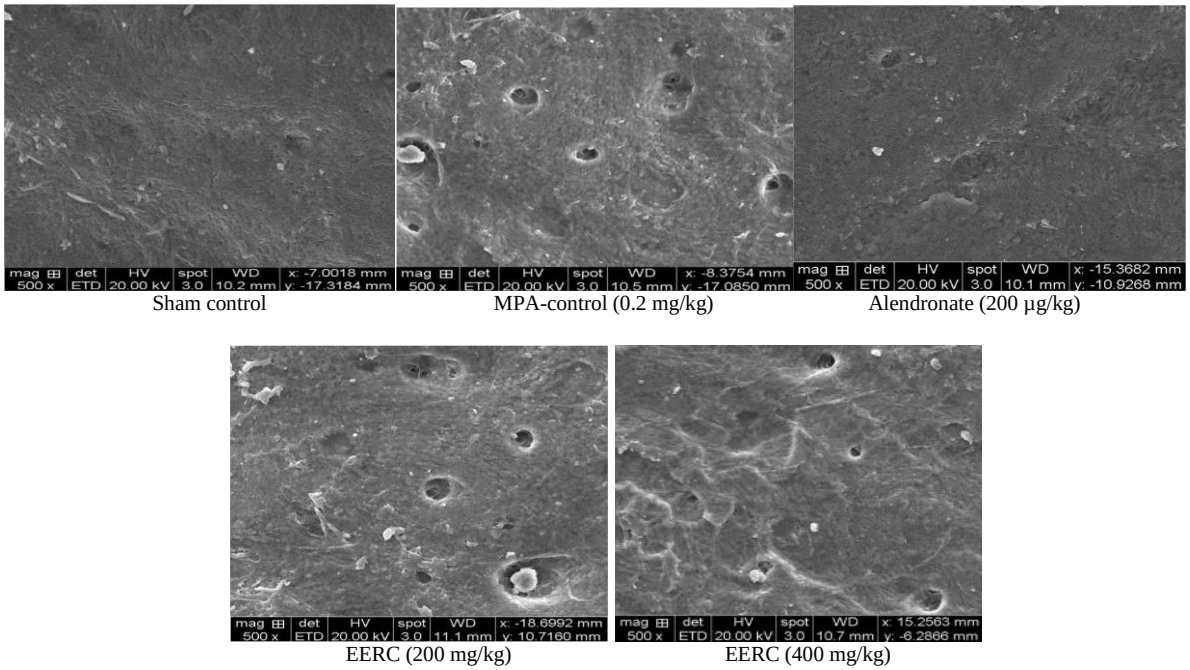

Fig 3: Scanning electron photomicrographs of rat femur bone of different groups.

CONCLUSION:

The present study was concluded that the oral administration of ethanolic root extract of *Rubia cordifolia* was showed significant findings in the biochemical and bone biomechanical parameters in MPA - induced osteoporotic rats. So this drug could be suggested for its bone healing activity especially in postmenopausal women. However further studies are necessary to isolate and characterise active constituents to understand the complete molecular mechanism involved in the osteoporosis.

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