ejpmr, 2016,3(6), 488-496

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

ANTIOSTEOPOROTIC ACTIVITY OF ETHANOLIC EXTRACT OF *CARDIOSPERMUM HALICACABUM* L. IN OVARIECTOMIZED RATS

Abin Joy*[1], Chaitra N.[1] and Mukund Handral[2]

[1]PG Scholar, Department of Pharmacology, People's Education Society (PES) College of Pharmacy, Bangalore, Karnataka-560050. India.

[2]Assistant Professor, Department of Pharmacology, People's Education Society (PES) College of Pharmacy, Bangalore, Karnataka-560050. India.

***Corresponding Author: Abin Joy**

PG Scholar, Department of Pharmacology, People's Education Society (PES) College of Pharmacy, Bangalore, Karnataka-560050. India.

Article Received on 14/04/2016 **Article Revised on 04/05/2016** **Article Accepted on 24/05/2016**

ABSTRACT

The main aim of the study was to carry out and investigate the Antiosteoporotic activity of ethanolic extract of *Cardiospermum halicacabum* (EECH) in ovariectomy (OVX) induced osteoporotic rats. All the rats were randomly divided into 5 groups (n=6 each). Group I (Sham operated) received vehicle, p.o., Group II OVX control (vehicle, p.o), Group III was OVX + standard Raloxifene (5.4 mg/kg, p.o) and Group IV& V received OVX + 300 & 600 mg/kg, p.o. of EECH respectively for 90 days. The studies revealed that EECH showed a dose dependent improvement in serum biochemical markers, which were significantly ($P<0.0001$) higher, whereas the urinary excretion profile was reduced in extract treated when compared to OVX control group. Similarly, in biomechanical parameters also EECH showed a significant change in body weight, bone mechanical strength ($P<0.01$), ash content ($P<0.0001$) and BMD ($P<0.05$) when compared with OVX control rats. Further, histopathological sections studied from the femur bone supported protective effect of EECH, as it restored the normal bone architecture by mineralisation with calcified cartilaginous deposit. All these results significantly indicate a remarkable antiosteoporotic activity of EECH. However, further studies are required to explore the exact mechanism of EECH on improving bone mass and qualities as well as its long term effect on maintenance of bone mass.

KEYWORDS: *Cardiospermum halicacabum*, Ovariectomy (OVX), Osteoporosis, Biochemical markers, Ash mineral content and BMD.

INTRODUCTION

Ethnomedicine refers to the study of traditional medical practice which is concerned with the cultural interpretation of health, diseases and illness along with healthcare seeking process and healing practices. The practice of ethnomedicine is a complex multidisciplinary system, which constitutes the use of plants where the natural environment has been the source of healing for people. Since last few decades the research interest and activities in the area of ethnomedicine have increased enormously. However, the scientific research in ethnomedicine has made an important contribution to understand the traditional subsistence, medical knowledge and practice.[1] The spiritual aspects of health and sickness have been an integral component of the ethnomedicinal practice for centuries. In addition, a dimension of herbal medicine uses were ignored by biomedicine practitioners because of the difficulties involved in validating its scientific principles and experiments. Today about 80% of the world's population rely on plants and plant products for healthcare. Ethnomedical practices and theories are part of a total belief system that transcend class, ethnicity and religious beliefs in a such a manner that the terms "folk or traditional" can be used to describe practices that are truly universal.[2]

Osteoporosis, a major worldwide public health issue which is characterized by a progressive loss of bone mineral density, disruption of bone microarchitecture and an increased risk of fractures which causes disability and mortality.[3] Osteoporosis is the area of interest since it affects the age old people and particularly postmenopausal women because of estrogen deficiency after cessation of menopause. Characteristic sites of fracture include vertebral bodies, distal radius and the proximal femur, but the osteoporotic individuals have generalised skeletal fragility and fracture at other sites such as ribs and long bones. The prevalence of osteoporosis increases with age for all sites measured by the WHO and revealed up to 70% of women over age 80 have osteoporosis based on low measurements at the spine, hip or wrist. Older women have much higher fracture rate than younger women with the same bone density because of increasing risk from other factors such as bone quality and tendency to fall.[4] With aging,

an erratic absorption of calcium from gut disturbs the calcium homeostasis leading to an imbalance in the calcium regulating hormones thereby increase bone turnover. A sharp decrease in ovarian estrogen production is the predominant cause of rapid hormone-related bone loss during the first decade after menopause.[5]

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 the usage is limited because of its side effects.[6] Traditional herbal medicines have been the integral part of medical practice. The long tradition of herbal wisdom has employed various herbs to speed fracture healing. Phytopharmacotherapy for bone and fracture healing is expected to be safe when compared with synthetic drugs in terms of side effects.[7] To overcome the wide range of side effects faced by these synthetic drugs, there is an increasing demand for 'green medicines' which are thought to be healthier and safer for the treatment of osteoporosis. The phytoestrogens, which are known to bind to the estrogen receptor sites thereby exhibits estrogenic activity, have a promising role in the treatment of osteoporosis. The Isoflavonoids are the most active phytoestrogens in the flavonoid class. Ipriflavone, a synthetic flavonoid derivative has been found to be effective in preserving bone mass in several models of experimental osteoporosis. The isoflavones found in soybeans such as genistein were found to prevent bone loss in the ovariectomized rat model of osteoporosis.[8,9,10]

In ancient system of medicine, a several number of herbal plants have been used for osteoporosis and fracture healing. In Ayurveda, *Cardiospermum halicacabum* referred as Karnasphota has reported activity against Jwara (fever), Sopha (inflammation), Pandu (anaemia), Sula (pain), Sandhivata (rheumatism), Sarpavisa (snake bite), Kamala (jaundice), Asmari (kidney stone), Indralupta (alopecia), etc. Further studies on *Cardiospermum halicacabum* strongly support the use in lumbago, skeletal fractures, nervous diseases, amenorrhoea, haemorrhoid, erysipelas, emetic, laxative, rubefacient and stomachic.[11,12] The use of mother tincture (ethanolic extract) of *Cardiospermum halicacabum* is also a medicine for animals as referred in homeopathic pharmacopoeias. The mother tincture was reported to have occurrence of esterified fatty acids, pentacyclic triterpinoids, phytosterols and relatively higher amount of flavonoids. In veterinary homeopathy, the mother tincture is intended for oral or parenteral use in all food producing species and also used in human homeopathy as a traditional medicine.[13] In the present study, an attempt was made to investigate the Antiosteoporotic activity of ethanolic extract of *Cardiospermum halicacabum* in ovariectomized rat model.

MATERIALS AND METHODS

Drugs and Chemicals

Raloxifene hydrochloride tablets (Cipla Pvt. Ltd. Bangalore, India), Ketamine hydrochloride injection (Neon Laboratories Ltd.), Xylazine injection (Brilliant Biopharma Pvt. Ltd.), Calcium, Alkaline Phosphatase, Phosphorous and Total cholesterol (Erba Mannheim, Baddi, India), Tartarate resistant acid phosphatase Kit (Accurex Biomedical Pvt. Ltd., Mumbai, India) and all other chemicals and reagents were of analytical grade purchased from S D fine-chem Ltd, Mumbai, India.

Plant collection

Fresh leaves of *Cardiospermum halicacabum* were collected from the rural places of Ernakulam District, Kerala, India in the month of August 2015. The plant was identified by Dr. Shiddamallayya N. and authenticated by Dr. V. Rama Rao, National Ayurvedic Dietetics Research Institute, Bengaluru (Ref. no. SMPU/NADRI/BNG/2015-16/1268).

Extraction

The collected leaves were cleaned and dried under shade at room temperature and crushed into powder. The dried powder was Soxhlet-extracted in absolute ethanol (70%) and the extract was evaporated to dryness at reduced temperature in a rotary evaporator. The percentage yield was 14.57% w/w and the extract was stored in an air tight container.[14]

Extract pre-treatment

The weighed quantity of ethanolic extract of *Cardiospermum halicacabum* (EECH) was suspended in Tween 80 and administered orally to the rats. The suspension of extract was prepared freshly every day.

Experimental animals

Female wistar rats (150-200 g) were purchased from Sri Venkateshwara Enterprises, Bengaluru and were maintained in the animal house of PES College of Pharmacy, Bengaluru (CPCSEA Reg. no. 600/PO/Ere/S/02/CPCSEA). All the animals were acclimatized for seven days under standard husbandry conditions, i.e. room temperature of 25 ± 1°C; relative humidity 45-55% and 12:12 h light/ dark cycle. The animals had free access to standard rat pellet (Amruth Animal Feeds Pvt Ltd, Bengaluru, India), with water supplied *ad libitum* under strict hygienic conditions. The experimental protocols were approved by the Institutional Animal Ethics Committee (PESCP / IAEC/ 01 /2014, Dated: 13/12/2014) and conducted according to CPCSEA guidelines, Govt. of India.

Dose selection

The low dose (300 mg/kg) and the high dose (600 mg/kg) of ethanolic extract of *Cardiospermum halicacabum* (EECH) were selected as per the previous study performed by other researchers and in the pharmacological validation of this plant, the toxicological evaluation of EECH in rats revealed that

the drug was non-toxic and safe upto 2000 mg/kg in rats.[15,16]

Induction of osteoporosis by ovariectomy (OVX)
Surgical procedure[17,18]

After one week of acclimatization, the rats were randomly divided into five groups consisting of six animals each. Then, the rats from group II, III, IV and V were anesthetized with a combination of Ketamine (80 mg/kg i.p.) and Xylazine (10 mg/kg i.p.) and underwent ovariectomy, but the group I was sham operated. The fur on the rat abdomen was completely removed with depilatory cream (Fig. a). The operation was made after placing an animal on its ventral surface, then surgical equipment's used were aseptically cleaned with ethanol. Ovariectomy was preceded by a midline ventral skin incision (bilateral), 3 cm long, approximately half way between the middle of the body and base of the tail (Fig. b & c). After peritoneal cavity was accessed, the adipose tissue was pulled away until the uterine tube and the ovary surrounded by a variable amount of fat were identified (Fig. d & e). Before removal of ovary, a ligation was performed around the area of distal uterine horn with a braided silk suture to avoid bleeding (Fig. f). The connection between fallopian tube and uterine horn was cut and the ovary was excised (Fig. g). Whereas in Sham operated rats, only ovaries were exposed but not excised. The uterine horn was returned to the peritoneal cavity after the removal of ovaries. The muscle incision was sutured with absorbable suture and skin wounds were closed bilaterally with non-absorbable suture (Fig. h & i). Operated animals were given Gentamycin injection (10 mg/kg, i.p.) for 4 days and Povidone-iodine solution was applied locally. After surgery, the rats were housed individually in polyurethane cages for a period of one week to allow recovery and then re-grouped in their home cages.

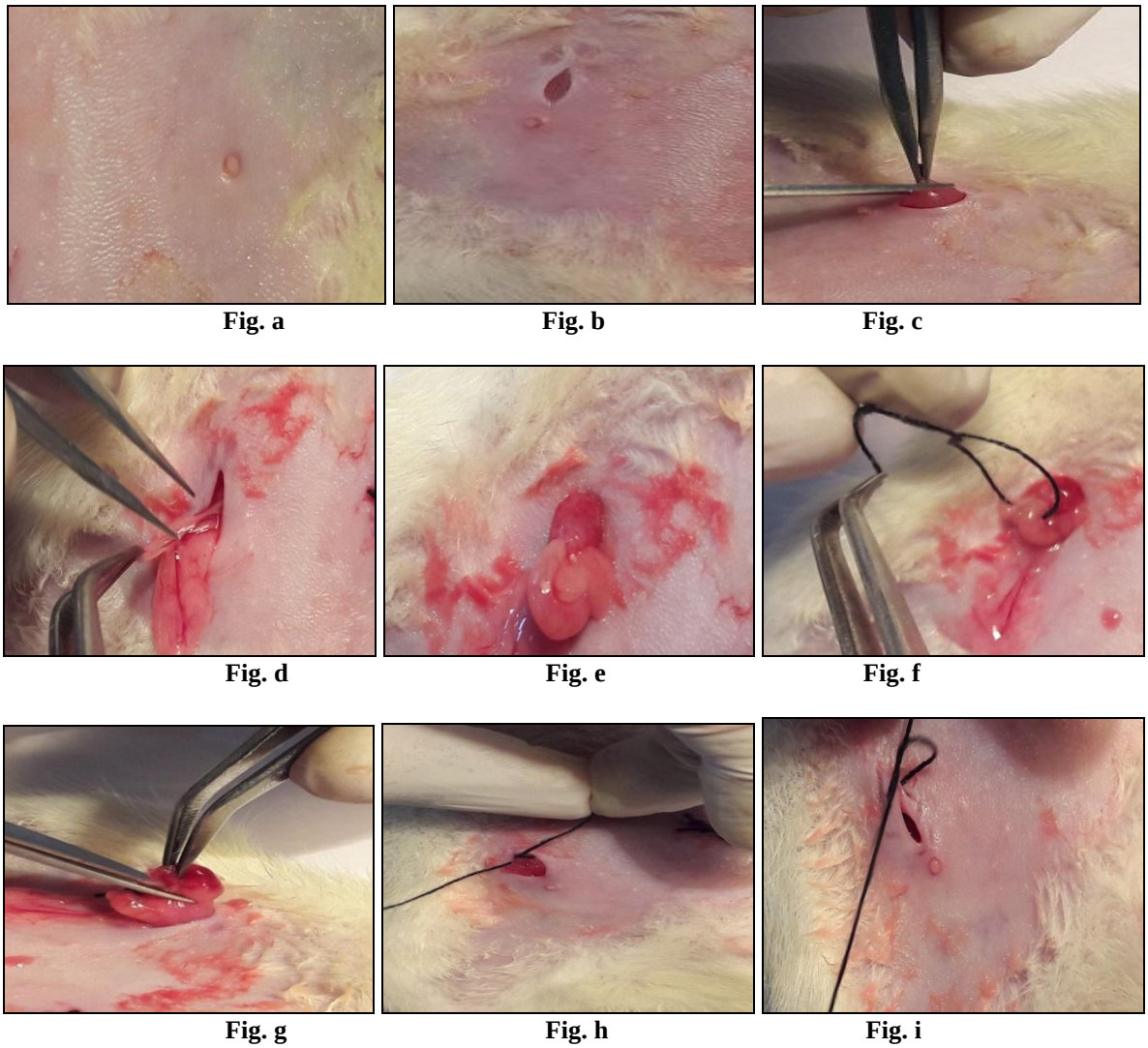


Fig. a **Fig. b** **Fig. c**

Fig. d **Fig. e** **Fig. f**

Fig. g **Fig. h** **Fig. i**

Fig. 1: Steps in ovary removal surgery

a. Shaved rat, **b.** Skin incision, **c.** Muscle incision, **d.** Protrusion of adipose tissue, **e.** Pulling of ovary, **f.** Ligation between uterine horn and ovary, **g.** Removal of ovary, **h.** Muscle suturing, **i.** Skin suturing.

Experimental design

Female wistar rats of six months old weighing 150-200 g was used in the study. All the operated rats were divided into 5 groups (n=6) and treated for 90 days.

Group I- Sham operated (vehicle)
Group II- OVX control (vehicle)
Group III- OVX + Standard Raloxifene (5.4 mg/kg, p.o.)
Group IV& V- OVX + EECH, 300 mg/kg and 600 mg/kg, p.o., respectively.

Evaluation parameters

Measurement of length, thickness and weight of femur bone[18]

The length was measured as the distance between greater trochanter and medial condyle and thickness at the femoral midshaft was also measured using digital caliper (Bliss classic, Yamayo, Japan). Then the bones were kept in an oven and dried at 100^{o}C and weights of the dried bones were determined by using a digital weighing balance (Sartorius AG).

Measurement of body weight and uterine index

The body weight of each rat from all the groups were monitored in a weekly bases for 90 days. At the end of the study all the rats were sacrificed by anaesthetic overdose and uterus was removed and weighed.

Serum and urine biochemical markers[19-23]

The calcium and phosphorous (serum & urine) and alkaline phosphatase (serum), Total cholesterol (serum) were estimated using Erba diagnostic kits, whereas, TRAP (serum) was estimated using Accurex diagnostic kit.

Analysis of Bone mineral density (BMD)[18]

Bone volume and densities were measured by fluid displacement method using Archimede's principle.

Femur bone Biomechanical strength[24]

This experiment was conducted on the right femur of the rats. The fracture energy for the given femur sample was measured by using a digital hardness tester. The right femur was isolated and the surrounding tissues were cleaned. The fresh bone was placed in digital hardness tester, then compress until it gets fractured and the reading was recorded in Newton's (kg/cm^2).

Determination of femoral ash weight, ash percentage and mineral content[25-27]

The soft tissues were cleaned from the femur bones which were then broken into small fragments, placed into a container of ethanol and soaked overnight before being extracted with ethanol in a Soxhlet extractor for 24 h and further extracted with anhydrous ether. After the second extraction, bones were dried at room temperature for 24 h. and then placed in tarred fused silica crucibles, kept in muffle furnace, ashed at 600^{o}C for 24 hrs, then ash weight and percentage ash was determined. Further the ash was used for the calcium assay by titrimetric method using AOAC standard procedures.

Histopathology

The right femur was used for the histopathological observation. In brief, Bone was fixed (10% phosphate formalin buffer) for 24 h, decalcified in EDTA (15%) for 4 days, dehydrated in alcohol, cleared in xylol and embedded in paraffin wax (56-58^{o}C mp). Sections (6 mm) were cut with rotary microtome, stained with haematoxylin-eosin, in a jar for 2-20 minutes. The blue staining of the haematoxylin is changed to red by the action of acid. Blue colour is regained by washing in alkaline, running tap water for at least 5 minutes. Then stained in 1% aqueous eosin for 1-3 minutes excess stain was washed under running tap water for 2-3 minutes and observed under microscope for histopathological changes.

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

Table No. 1: Effect of EECH on femur weight, length and thickness in OVX-induced osteoporotic rats.

Sr. No.	Groups (n = 6)	Weight (per 100 g b/w)	Length (mm)	Thickness (mm)
1.	Sham operated	0.41±0.145	34.31±0.668	2.94±0.310
2.	OVX control	0.29±0.045	34.29±1.344	2.75±0.219
3.	OVX + Raloxifene (5.4 mg/kg)	0.41±0.105	35.04±0.306	2.93±0.266
4.	OVX + EECH (300 mg/kg)	0.34±0.079	35.23±0.436	2.74±0.296
5.	OVX + EECH (600 mg/kg)	0.39±0.079	35.40±0.347	3.10±0.193

*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. OVX control, [b] = OVX control vs. treated groups.*

The results of weight, length and thickness of rat femur bone in animals of different groups are shown in table 1. The femur weight was considerably decreased in OVX control group compared to normal. The EECH and Raloxifene supplemented rats showed considerable increase in femur weight respectively when compared to OVX control. However, the bone length and thickness

were not statistically significant in treated groups, but were higher than OVX control.

Table No. 2: Effect of EECH on uterine weight and body weight in OVX-induced osteoporotic rats.

Sr. No.	Groups (n = 6)	Uterine wet weight (g) (per 100 g b/w)	Change in body weight (g)		% increase in body weight
			1st day	90th day	
1.	Sham operated	0.36±0.183	178.33±24.01	213.33±17.51	16.21
2.	OVX control	0.09±0.058*[a]	201.66±24.83	231.66±46.65	16.72
3.	OVX + Raloxifene (5.4 mg/kg)	0.22±0.332	198.33±33.11	206.66±31.41	10.36
4.	OVX + EECH (300 mg/kg)	0.09±0.0426	215.00±10.48	256.66±19.66	15.83
5.	OVX + EECH (600 mg/kg)	0.10±0.0147	215.00±27.38	251.66±19.40	14.60

*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. OVX control, [b] = OVX control vs. treated groups.*

In the present study, wet weight of uterus was determined. As expected, the uterus weight was significantly (P<0.05) reduced in OVX control as compared to sham control rats. But, the EECH (300 & 600 mg/kg) and Raloxifene treated groups did not show any significant changes in the uterus weight. The body weights of the animals were also monitored in weekly bases and the results are shown in table 2. Initially, animals of all the groups were more or less of similar mean body weight. After three months duration, the OVX control group had 16.72% weight gains as compared to sham control. Upon administration of EECH (300 & 600 mg/kg) and Raloxifene (5.4 mg/kg) to the OVX animals showed a % increase of 15.83%, 14.60% and 10.36% respectively. However the difference in body weight did not reach statistical significance.

Table No. 3: Effect of EECH on serum ALP, calcium, phosphorous, TRAP and Total cholesterol levels in OVX-induced osteoporotic rats.

Sr. No.	Groups (n = 6)	Alkaline phosphatase (IU/l)	Calcium (mg/dl)	Phosphorous (mmol/l)	TRAP (IU/l)	Total Cholesterol (mg/dl)
1.	Sham operated	115.29± 25.66	9.02± 1.226	10.6± 1.03	2.09± 0.464	45.24± 1.733
2.	OVX control	281.69± 35.59***[a]	4.58± 0.81***[a]	7.05± 0.84***[a]	4.90± 0.395***[a]	74.58± 8.934***[a]
3.	OVX + Raloxifene (5.4 mg/kg)	141.52± 25.24***[b]	10.02± 0.59***[b]	10.21± 0.87***[b]	2.40± 0.202***[b]	49.87± 3.785***[b]
4.	OVX + EECH (300 mg/kg)	181.76± 20.37***[b]	8.89± 0.41***[b]	8.96± 0.42**[b]	2.96± 0.327***[b]	58.99± 2.408***[b]
5.	OVX + EECH (600 mg/kg)	148.31± 23.87***[b]	9.73± 0.35***[b]	9.72± 0.66***[b]	2.46± 0.289***[b]	51.58± 3.607***[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. OVX control, [b] = OVX control vs. treated groups.*

The effect of EECH on serum ALP, calcium, phosphorous, TRAP and total cholesterol are illustrated in table 3. The activity of serum ALP was significantly (P<0.0001) increased in OVX control when compared with sham control. Whereas, groups which received EECH (300 & 600 mg/kg) and Raloxifene (5.4 mg/kg) treatment significantly (P<0.0001) supressed the rise in ALP level respectively. In this study, the serum calcium and phosphorous levels were found to be significantly decreased (P<0.0001) in OVX control compared to sham operated. But, the EECH supplemented groups significantly (P<0.0001) increased both calcium and phosphorous levels when compared with OVX control respectively.

The tartarate resistant acid phosphatase (TRAP), a marker for bone resorption was determined in this study. The activity in OVX control group was elevated to a significant (P<0.0001) level when compared to sham operated. Further the EECH (300 & 600 mg/kg) treated rats bring down the levels into normal range when compared to OVX control. The Raloxifene supplemented group was also significantly (P<0.0001) reduced the TRAP levels.

In this study an attempt was made to correlate possible association between lipid profile and osteoporosis, so the estimation of total cholesterol in OVX control rat showed significant (P<0.0001) rise in the cholesterol level when compared to sham operated. Whereas, the EECH and Raloxifene, dose dependently reduced the levels into normal, which was significant (P<0.0001) when compared with OVX control.

Table No. 4: Effect of EECH on urinary biochemical markers in OVX-induced osteoporotic rats.

Sr. No.	Groups (n = 6)	Calcium (mg/dL)	Phosphorous (mg/dL)
1.	Sham operated	1.40±0.155	4.72±0.143
2.	OVX control	4.74±0.906***[a]	7.65±0.502***[a]
3.	OVX + Raloxifene (5.4 mg/kg)	2.33±0.405***[b]	4.23±0.331***[b]
4.	OVX + EECH (300 mg/kg)	2.93±0.314***[b]	5.05±1.166***[b]
5.	OVX + EECH (600 mg/kg)	2.61±0.143***[b]	4.81±0.614***[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. OVX control, [b] = OVX control vs. treated groups.*

The table 4 illustrates the urinary calcium and phosphorous excretion profile of the rats. There was significantly increased (P<0.0001) excretion of minerals in OVX group than sham operated. Where the rats administered with 300 & 600 mg/kg of EECH showed statistically significant (P<0.0001) reduction in the urinary excretion profile when compared to OVX control. In addition, the Raloxifene (5.4 mg/kg) treated groups were also highly significant when compared to OVX control.

Table No. 5: Effect of EECH on bone mineral density and mechanical strength of femoral bone in OVX-induced osteoporotic rats.

Sr. No.	Groups (n = 6)	BMD (wt/ml)	Force at break (kg/cm^2)
1.	Sham control	0.91±0.212	14.21±3.116
2.	OVX control	0.62±0.090**[a]	8.48±0.691***[a]
3.	OVX + Raloxifene (5.4 mg/kg)	0.89±0.119**[b]	13.76±1.202***[b]
4.	OVX + EECH (300 mg/kg)	0.74±0.128	11.50±1.213*[b]
5.	OVX + EECH (600 mg/kg)	0.83±0.092*[b]	12.30±1.520**[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. OVX control, [b] = OVX control vs. treated groups.*

Table 5 illustrates the bone density and bone mechanical strength on femur of the rats. The OVX group had significantly (P<0.01) lower density when compared to sham operated. The Raloxifene treated OVX rats significantly (P<0.01) increased the bone density. Whereas, the EECH supplemented groups were seen to recover the density of the femur bone which was statistically significant (P<0.05).

The results of bone mechanical strength showed the OVX control group statistically reduced (P<0.0001) the bone strength when compared to sham operated. Treatment with EECH at a dose of 600 mg/kg and standard Raloxifene (5.4 mg/kg) significantly (P<0.001) improved the mechanical strength of the femur respectively when compared to OVX control. Whereas the EECH 300 mg/kg had a moderate significance (P<0.05).

Table No. 6: Effect of EECH on ash content of femoral bone in OVX-induced osteoporotic rats.

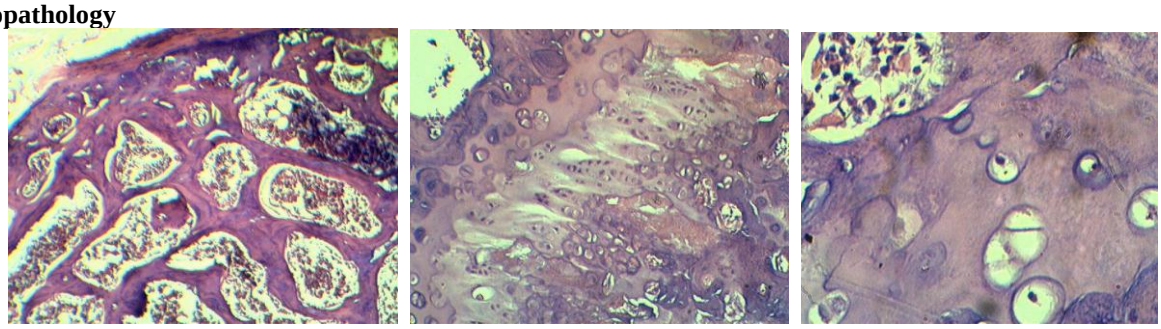
Sr. No.	Groups (n = 6)	Ash weight (g)	Ash (%)	Calcium (mg/dL)
1.	Sham operated	0.63±0.079	84.16±1.584	100.59±8.042
2.	OVX control	0.44±0.028***[a]	64.81±2.355***[a]	43.03±13.253***[a]
3.	OVX + Raloxifene (5.4 mg/kg)	0.64±0.031***[b]	78.65±1.537***[b]	85.49±15.027***[b]
4.	OVX + EECH (300 mg/kg)	0.56±0.040**[b]	66.38±2.308	76.03±5.432***[b]
5.	OVX + EECH (600 mg/kg)	0.58±0.029***[b]	76.12±2.055***[b]	82.67±13.320***[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. OVX control, [b] = OVX control vs. treated groups.*

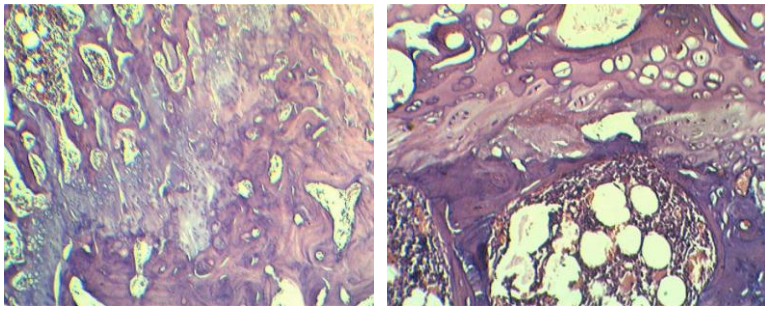
In this study, the total weight of ash in OVX control group significantly (P<0.0001) reduced as compared to sham operated, but there was a significant (P<0.0001) increase in total ash weight in EECH (300 & 600 mg/kg) and Raloxifene treated animals when compared to OVX control.

As shown in table 6, the bone calcium content of the femur was 64.81% in OVX control rats when compared to sham operated (P<0.0001) respectively. Administration of EECH (300 & 600 mg/kg) significantly increased (P<0.0001) the calcium content by 76.12% when compared to OVX control. Raloxifene also significantly (P<0.0001) improved the ash weight respectively.

Histopathology



a. Sham operated **b. OVX control** **c. RLX (5.4 mg/kg)**



d. EECH (300 mg/kg) **e. EECH (600 mg/kg)**

Fig. 2: Histopathology of rat femur bone (H & E, 100X)

The histopathological section studied from the femur bone revealed, **a.** Sham operated showed typical osteoblasts, normal and compact trabeculae, mineral salts and osteon, **b.** OVX control rat revealed poor osteoblastic lining with absence of mineral salts, bone matrix with thinning of trabeculae and loss of connectivity, **c.** Standard RLX group exhibited bone restoration with thick trabeculae and appearance of mineral salts, **d.** EECH (300 mg/kg) revealed partial restoration of trabeculae, **e.** EECH (600 mg/kg) showed maximum restoration of trabeculae into normal with improved osteoblastic activity.

DISCUSSION

Osteoporosis is considered a major public health problem which is characterized by decrease bone density, resulting in skeletal fractures. Osteoporosis is three times more common in women than in men, reason being the hormonal changes occur at the menopause. In addition, it is well known that in human females, estrogen deficiency caused by ovariectomy as well as menopause leads to acceleration of bone resorption and rapid bone loss, resulting in development of osteoporosis. It also negatively affects the quality of life of elderly patients. 10% percent of women 55-64% years old and 22.5% of women 65 years were reported that osteoporosis interfered with their daily activities. This disease is also influenced by diet, adequate nutrition; especially calcium intake plays a major role in preventing bone loss and osteoporotic fractures in later life. The research interests towards agents that inhibit bone resorption such as estrogen and calcitonin have emphasized the traditional therapies for postmenopausal osteoporosis. Although the most effective method to reduce the rate of postmenopausal bone loss is estrogen replacement therapy, it may be accompanied by side effects. It is recommended only for woman who are at high risk of osteoporosis and have no contraindications for estrogen.[28]

The mechanism by which ovarian hormone deficiency results in bone loss remains uncertain. However, in human estrogen deficiency, it has been proposed to augment plasma calcium levels as result of increased bone resorption. Thus alternative approaches for managing osteoporosis are needed and in this regard present study evaluated the ethanolic leaf extract of *Cardiospermum halicacabum* for its Antiosteoporotic activity in female Ovariectomized rats. The Ovariectomized rats exhibit most of the characteristics of human postmenopausal osteoporosis.[24,29]

Our study clearly indicated that EECH could suppress bone resorption at a high rate of bone turnover induced by estrogen deficiency. The ALP is an important bone formation marker and levels of those markers are increased in osteoporosis. In this study, serum ALP also supports the observations from other investigators that elevated serum ALP levels are due to ovarian hormone deficiency.[24,30] The levels of calcium and phosphorous were also found to be decreased in OVX rats and to some extent the EECH treatment prevented the fall in minerals. The decreased intestinal absorption of calcium and phosphorous in ovariectomized rat report is similar to postmenopausal women.[31] The correlation between cholesterol levels and ovariectomy was assessed and the results exhibited increased cholesterol in OVX rats, where EECH supplemented rats lowered the serum cholesterol, hence protect from cardiovascular diseases.[32,33]

The abnormal increase in excretion of urinary calcium and phosphorous are supporting bone loss in ovariectomized rats. The extract significantly reduced the urinary excretion profile of the rats, where these findings remained in accordance with previous studies.[34,35]

Bone Mineral Density has been described as a surrogate measure of bone strength and as a primary contributor to bone quality which is markedly decreased due to an increase in bone turnover in the ovariectomized rats; thereby it reduced the mechanical strength of femur bone. The oral administration of EECH further supported BMD findings and bone hardness test evidenced by improving bone mechanical properties. The decreased bone mineral content was observed with ovariectomized rats, but further extract treatment was evidenced by improving the ash weight, ash percent and ash calcium content.[36,37]

Further, histopathological sections studied from the femur bone supported protective effect of EECH, as it restored the normal bone architecture by mineralisation with calcified cartilaginous deposit.

CONCLUSION

The present study evaluated ethanolic leaf extract of *Cardiospermum halicacabum* for its Antiosteoporotic activity in female ovariectomized rat model of postmenopausal osteoporosis. In conclusion, EECH treatment showed a remarkable antiosteoporotic activity in OVX rats, which may result from the enhancement of bone formation and suppression of bone resorption through estrogen effect. Therefore, EECH has promising therapeutic usefulness in prevention of osteoporosis in humans causing from estrogen deficiency. However, further studies are required to explore the true mechanisms of this extract on improving bone mass and qualities as well as its long term effect on maintenance of bone mass.

ACKNOWLEDGEMENT

The authors are thankful to Dr. J Saravanan, Principal, Dr. S Mohan, Director and Dr. Shivalinge Gowda KP, Associate Professor & Head, Department of Pharmacology, PES College of Pharmacy, Bangalore for funding this project and their valuable support to complete this study.

REFERENCES

1. Williams LAD. Ethnomedicine. NPU. West Indian Med J, 2006; 55(4): 215.
2. George MF. Disease etiologies in non-western medical systems. Am Anthropol, 1976; 78: 772-83.
3. Shan P, Cory JX, Li M, Xiang G, Yuan L. Osteoporosis. Int J Endocrinol, 2013.
4. Nelson HD. Hormone replacement therapy and osteoporosis. AHRQ, 2002; 1-55.
5. Singh U, Singh S. Post-menopausal osteoporosis: treatment with nutraceutical. JDDT, 2013; 1(5): 65-67.
6. Shivakumar K, Mukund H, Rabin P. Evaluation of antiosteoporotic activity of root extract of *Rubia cordifolia* in ovariectomized rats. Int J Drug Dev & Res, 2012; 4(3): 163-72.
7. Chhavi S, Sushma D, Ravinder V, Anju D, Asha S. Recent update on proficient bone fracture revivifying herbs. IRJP, 2011; 2(11): 3-5.
8. Shirwaikar A, Khan S, Kamariya YH, Patel BD, Gajera FP. Medicinal plants for the management of post-menopausal osteoporosis: a review. The Open Bone Journal, 2010; 2: 1-13.
9. Mark P. Bone health & osteoporosis. ANSR, 1999; 5(4): 1-6.
10. Bahram H, Lee A, Bruce WM., Daxa A, Maria S, Peilin QUO, et al. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr, 1996; 126: 161-67.
11. The Ayurvedic Pharmacopoeia of India. Ministry of health and family welfare, department of ayush.
12. Varghese VK, Anila J, Nagalekshmi R, Resiya S, Sonu J. Dasapushpam: the traditional uses and the therapeutic potential of ten sacred plants of kerala state in India. IJPSR, 2010; 1(10): 50-59.
13. Summary report. Committee for veterinary medicinal products. EMA, 1999.
14. Asha VV, Pushpangadan P. Antipyretic activity of *Cardiospermum halicacabum*. Indian J Exp Biol, 1999; 37: 411-14.
15. Ravichandran S, Panneerselvam P. Anti-nociceptive activities of combined extract of *Cardiospermum halicacabum* L. and *Delonix elata* L. leaves. IJBPR, 2012; 3(6): 762-66.
16. Pugazhendy K, Revathi A. Hepatoprotective effect of *Pisonia alba* and *Cardiospermum halicacabum* in atrazine toxicity on biochemical parameters in the liver tissue of albino wister rat rattus norvegicus. Int J Adv Res Biol Sci, 2014; 1(9): 244–48.
17. Khajuria DK, Razdan R, Mahapatra DR. Description of a new method of ovariectomy in female rats. Rev Bras Reumatol, 2012; 52(3): 462-70.
18. Srikanta P, Nagarajappa SH, Viswanatha GL, Handral M, Subbanna R, Srinath R, et al. Antiosteoporotic activity of methanolic extract of an Indian herbal formula NR/CAL/06 in ovariectomized rats. JCIM, 2011; 9(10): 1125-32.
19. Wikinson JH, winsten S. Clin Chem, 1969; 15: 487.
20. Mooorehead WR, Briggs HC, 1974; 20: 1458.
21. Miller GW. Mineral and metabolism. Cin Chem. 3rd ed, 1994; 1395-1457.
22. Seiler D, Nagel, Tritschler, Losser S. Clin Chem Clin Biochem, 1983; 21: 519.
23. Searcy RL. Diagnostic Biochemistry. McGraw-Hill. New York, NY, 1969.
24. Trivedi A, Katti HR, Shalavadi MH, Ramkishan A, Chandrashekhar VM. Anti-osteoporotic activity of ethanol extract of *Terminalia arjuna* (Roxb.) Wight & Arn. on ovariectomized rats. IJNPR, 2015; 6(2): 98-105.
25. Hall LE, Shirley RB, Bakalli RI, Aggrey SE, Pesti GM, Edwards HM. Power of two methods for the

estimation of bone ash of broilers. Poult Sci, 2003; 82: 414-18.
26. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of ash in biomass. NREL, 2005; 1-5.
27. Kramer B, Howland J. The quantitative estimation of calcium, magnesium, phosphate and carbonate in bone. J Biol Chem, 1926; 68: 711-19.
28. Aswar UM, Mohan V, Bodhankar SL. Antiosteoporotic activity of phytoestrogen-rich fraction separated from ethanol extract of aerial parts of *Cissus quadrangularis* in ovariectomized rats. Indian J Pharmacol, 2012; 44(3): 345-50.
29. Hui SL, Slemenda CW, Johnston CC. Age and bone mass as predictors of fracture in a prospective study. J Clin Invest, 1988; 81: 1804-09.
30. Yogesh HS, Chandhrashekar VM, Katti HR, Ganapathy S, Raghavendra HL, Gowda GK, et al. Anti-osteoporotic activity of aqueous-methanol extract of *Berberis aristata* in ovariectomised rats. J Ethnopharmacol, 2011; 134: 334-38.
31. Peter D, Morris HA. Oestrogen deficiency impairs intestinal calcium absorption in the rat. J Physiol, 1998; 511(1): 313-22.
32. Delmas PD, Bjarnason NH, Mitlak BH, Ravoux A, Shah AS, Huster WJ, et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med, 1997; 337: 1641-47.
33. Jeong TD, Lee W, Choi S, Kim JS, Kim H, Bae SJ, et al. Relationship between serum total cholesterol level and serum biochemical bone turnover markers in healthy pre- and postmenopausal women. BioMed Res Int, 2013; 1-7.
34. Reddy NP, Lakshmana M. Prevention of bone loss in calcium deficient ovariectomized rats by OST-6, a herbal preparation. J Ethnopharmacol, 2003; 84: 259-64.
35. Gupta R, Singh M, Kumar M, Kumar S, Singh SP. Anti-osteoporotic effect of *Urtica dioica* on ovariectomised rat. IJRPB, 2014; 1015-19.
36. Sheu Y, Zmuda JM, Boudreau RM, Petit MA, Ensrud KE, Bauer DC, et al. Bone strength measured by peripheral quantitative computed tomography and the risk of nonvertebral fractures: the osteoporotic fractures in men (MrOS) study. J Bone Miner Res, 2011; 26(1): 63-71.
37. Arjmahdi BH, Alekel L, Mollis BW, Amin D, Maria S, Quo P, et al. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr, 1996; 126: 161-67.