

Original Research Article

Evaluation of Anti Estrogenic activity and Anti-Osteoporotic activity of extracted Quercetin from *Bambusa arundinacea* leaves on ovariectomized rats.

Abstract

Phytoestrogens has now become a emerging era of interest for the researchers . the mimicking effects of phytoestrogens had lead to its wide use in reproductive system. In this study it was aimed to study the estrogenic activity and anti-osteoporotic activity of isolated quercetin female Wistar rats. The estrogenic effect was analyzed by uterotropic assay, vaginal cytology and measurement of vaginal opening in female Wistar rats. The administration of isolated quercetin in ovariectomized immature and mature female Wistar rats in a dose of 30 mg/kg b.w. resulted in significant increase in the uterine wet weight when compared with ovariectomized control rats. The treated rats, showing only Cornified epithelial cells was an indication of the presence of the estrogen and also showed 100% vaginal opening. Thirty six female albino Wistar rats were randomly divided into six groups (n=6). After 60 days of ovariectomy, animals were treated with of isolated quercetin for the next 45 days. Finally femur bone length, weight, bone ash calcium level, and bone mineral density (BMD) were estimated. The levels of serum alkaline phosphatase (ALP), calcium, and phosphorous, and bone histopathology were also evaluated. OVX-induced increased serum ALP, calcium, and phosphorous levels were impaired in of isolated quercetin treated rats. The isolated quercetin which was evident by uterotropic assay, measurement of vaginal opening, and histopathological changes. It also exhibited a significant anti-osteoporotic effect in the experimental model of OVX-induced osteoporosis in rats, indicating advantageous effect in postmenopausal osteoporosis.

Keywords: Quercetin , estrogenic activity, uterotropic assay, vaginal cytology, vaginal opening, Osteoprotective,

Introduction

Compounds with natural origin have been found to have estrogenic activity. They have significant estrogenic compounds that mimic estrogens like biological activity. There are studies

that show the relevance of phytoestrogens in human health (1). The Phytoestrogens are generalized to a wide group of plant known as flavonoids. The competitively binding to estrogenic receptors for instigation of estrogen-responsive genes is prominently seen by Phytoestrogens (2,3). Osteoporosis is said to be a bone disease that leads to decreased bone density making the bones fragile with increased susceptibility to fracture (4). The most common osteoporosis is Postmenopausal osteoporosis and is common in women having estrogen deficiency (5). The postmenopausal osteoporosis is treated with Calcium selective estrogen receptor modulators such as raloxifene and droloxifene, estrogen, bis-phosphonates, fluoride, and calcitonin but has limitations due to side effects (4). Thus the studies have focused on the use of natural remedies for osteoporosis management (5). Quercetin is a flavonol, one of the six subclasses of flavonoid compounds (6). Quercetin is found abundantly occurs in many ethnic plants (7). Quercetin has shown many properties like as its use as antioxidant, anticancer and neuroprotective (8). Many studies have shown the presence of quercetin in *Bambusa arundinacea* (9). Quercetin was isolated from *Bambusa arundinacea*. Thus, the study is focused on investigating and exploring the Anti Estrogenic activity and Anti-Osteoporotic activity anti-osteoporotic activity of isolated Quercetin from *Bambusa arundinacea* Ovariectomy-induced model in rats

Material and method

Anti Estrogenic activity

2. MATERIAL AND METHOD

2.1 Animals

Impuberal female albino wistar rats , 21-day-old, with body weight of 45–50 g, were used . Animals were housed four per cage, in a multiple rat rack. Temperature (21 ± 2 °C) and humidity ($55 \pm 15\%$) were controlled and a 12 h light/dark cycle, was maintained. Water and food were ad libitum. *The experiment protocol was approved by the Institutional Animal Ethical Committee (IAEC) according to the regulation of committee for the purpose of control and supervision of experiments on animals (CPCSEA) and ethical norms was strictly followed during all experimental procedure. (Ref. No. VCTE/07/2016 CPCSEA).* All animals had been acclimatized for three days in the animal room prior the first treatment (10).

2.2 Dose selection

Doses were selected on the basis of previous toxicity studies carried out (11) for isolated quercetin .

2.3 Preparation of doses:

The plant extracts were prepared in distilled water using CMC as a suspending agent (1%). The standard drug was also prepared in CMC and given as suspension to the animals. Control group (Negative control group/Olive oil control group) here through with the help of orogastric tube 0.2ml of Olive oil was given Orally once in a days in between experimental time. Conjugated Equine Estrogen (CEE 0.2 mg/kg) was purchased from Pharmacy shop, Bijnor, India. CEE (Wyeth Montreal, Canada), prepared in a dosage of 0.2 mg/kg by dissolving it in distilled water and given by i.p route (12) and was used as a positive control for comparing with the Test groups. The isolated quercetin was dissolve in 0.2 ml of Olive oil and with the help of Orogastric tube was given by Oral route (13).

2.4 Treatment protocol

Immature Uterotrophic animals were divided into five groups each containing six animals and one group of normal immature rats. Animals were fasted 18 hrs prior to dosing and 3-4 hours after administration of the plant extracts. The plant extracts were given at a dose of 200 mg/kg, 300 mg/kg and 400 mg/kg Oral routes to the immature female animals for a period of 14 days.

Healthy virgin female rats, Swiss albino, were divided into five groups (n = 6). Anti-estrogenic activity was determined after daily administration of extract and subcutaneous injection of ethinyl estradiol for 7 days. Uterine weight at the end of the experiment was used as a parameter for the anti estrogenic property.

Group	Treatment
Group I	Animal received normal saline (1 ml/kg, p.o.)
Group II	Animal received Ethinyl estradiol as

	suspension in distilled water (0.2 mg/kg, body weight Orally)
Group III	Animal received Quercetin (30 mg/kg) extracted from Ethanolic extract of <i>Bambusa arundinacea</i>

Table: 1 Treatment protocol for estrogenic activity of quercetin using immature uterotrophic model

Osteoporosis Induction

The osteoporosis induction was done in the twenty four female wistar rats (Six animal in each group) through the intramuscular administration of dexamethasone disodium phosphate (Decadron ® 4 mg/ml) at the dose level of 7 mg/Kg of bodyweight, once a week, during five weeks in all groups. Animal received Quercetin (30 mg/kg) extracted from Ethanolic extract of *Bambusa arundinacea* for thirty days by oral route once a day.

S.no	Groups	Treatment
1.	Group A	2% CMC (Carboxy methyl cellulose) solution 5 ml/kg
2.	Group B	2% CMC (Carboxy methyl cellulose) solution 5 ml/kg(ovariectomized rats)
3.	Group C	Raloxifene (5.4 mg/kg i.p.)
4.	Group D	Quercetin (30 mg/kg) extracted from Ethanolic extract of <i>Bambusa arundinacea</i>

Table : 2 Treatment protocol for Osteoporosis Induction of Quercetin using immature uterotrophic model

Blood collection

After 30 days of the treatment period, all the rats euthanized using overdose of Ketamine through intramuscular route, and blood collected from carotid bleeding. The samples were collected in clean polypropylene tubes, left to clot at 37°C for 10 minutes, then centrifuged at 3000 rpm for 20 minutes at 4°C, the resulting supernatant which is serum was transferred to sterile vial and frozen at -20°C until used for analysis of various biochemical parameters (14).

Serum Biochemical Markers

The serum calcium was carried out by kinetic assay, whereas tartarate resistant acid phosphatase was estimated by kinetic method using commercially available kit (15).

Estimation of Serum phosphorous

Reagent composition: Inorganic phosphorous reagent Sulfuric acid 210 mmol/L Ammonium molybdate 650 mmol/L. The 1000µl of reagent was mixed with 20µl of the sample and incubated for 1 minute at 37°C and absorbance read at 340nm with linearity up to 15mg/dL.

Calculation: Phosphorous concentration (mg/dL) = Absorbance of sample / Absorbance of standard x 5. (16)

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 condyle using a digital caliper (17).

Bone Calcium count

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 (18).

Estimation of Calcium

Reagent composition: Calcium dye reagent Diethylamine 360 mmol/L Calcium base reagent O-Cresolphthalein complex 0.15 mmol/L 8-Hydroxyquinoline 17.2 mmol/L

Procedure: The 1000 μ l of reagent mixed with 10 μ l of the sample and incubated for 1 minute at 37°C and absorbance read at 578 nm with linearity up to 15mg/dL.

Calculation: Calcium Concentration (mg/dL) = Absorbance of sample/ Absorbance of standard x 10(19).

Histological study

All the animals were sacrificed and the femur was dissected for histopathology study, the bone was collected and immediately fixed in 10% formalin and allowed to remain in it till they were taken up for processing(20).

Fixation

Fixation is the process of preserving the bone from hardening and preventing postmortem changes of the tissues. The bone excised out immediately after sacrificing and cut into pieces of desired thickness, so that the fixative readily penetrated throughout the bone to be fixed, the volume of the fixative was 10:1 ration of fixative to bone. The bone was fixed in 4% formaldehyde solution and allowed to remain in it until they taken up for processing.

Decalcification

Decalcification is the removal of calcium ions from the bone tissue through histological process to make the bone flexible and obtain soft section using microtome for pathological investigation. The right femur was dissected free of soft tissue and fixed in 10% formalin, the bone tissue were then decalcified in formic acid for 10days, tissue were dehydrated in graded alcohols and embedded in paraffin. The 5 μ mm section cut and stained with hematoxylin and eosin Goldner's trichrome. Gooding and Stewart's fluid: • Formic acid - 10ml • Formaldehyde - 05ml • Distilled water - 100ml By using formic acid, it gives a good routine decalcifying fluid and will give reasonable speed and minimum tissue damage(21).

Statistical analysis

All the values were expressed as mean \pm standard error of the mean (S.E.M) of six animals each across the groups. Statistical analysis of data was carried out using one-way analysis of variance (ANOVA) with help of Graph pad Prism software. Dunnett's multiple comparisons test. P value < 0.05 was considered to be statistically significant.

RESULTS

PHARMACOLOGICAL SCREENING OF THE ETHANOLIC EXTRACTS OF THE VARIOUS PLANT EXTRACTS FOR ESTROGENIC ACTIVITY USING IMMATURE RAT SCREENING MODEL

Screening of Isolated Quercetin from Ethanolic Extract of *Bambusa arundinaceae* with the help of Immature Rat screening model (Increase in weight of Uterus of female Rats)

The Isolated Quercetin caused increase in the weight of uterus. The results obtained (Table: 3) showed the estrogenic effect of Isolated when tested in immature ovariectomized rats. In the control animals weight of uterus was changed while the administration of Standard dose of Ethinyl estradiol) (0.2mg/kg) resulted in the significant increase in weight's of uterus. In comparison to the control group isolated quercetin showed promising results (Fig :1) .

Treatment	Wet Weight of the uterus (mg)	Body weight (g)	Uterine/Body weight ratio (%)
Vehicle	34.25 \pm 0.4	68.18 \pm 0.14	50.23
Ethinyl estradiol	44.12 \pm 0.16	69.21 \pm 0.81	63.74
Quercetin (30 mg/kg)	37.18 \pm 0.16	71.34 \pm 0.15	52.11

Table : 3 Effects of isolated Quercetin from *Bambusa arundinacea* leaves on Immature female rats

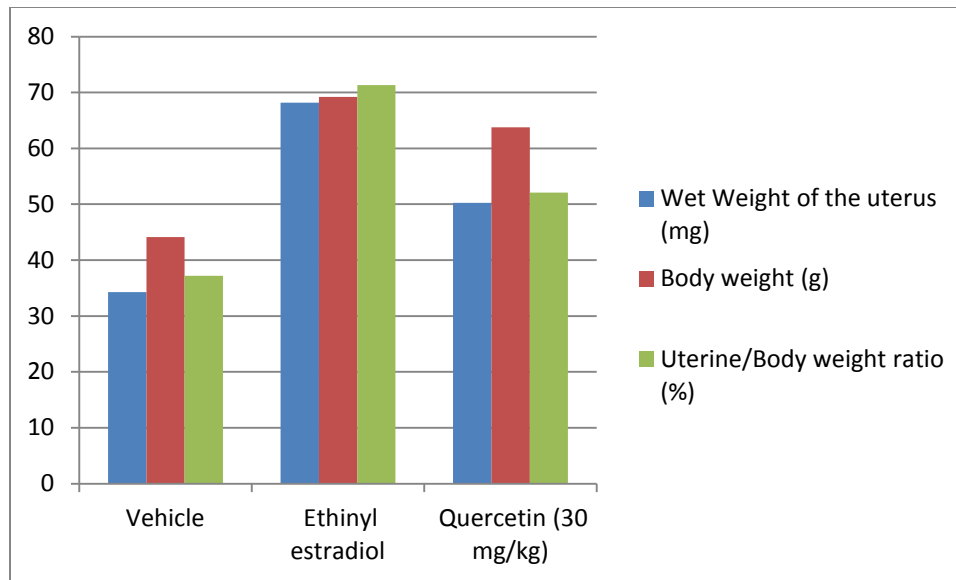


Figure .1 Effects of isolated Quercetin from *Bambusa arundinacea* leaves on Immature female rats

The statistical significance of difference between means was calculated by Analysis of variance (ANOVA) followed by post hoc test for paired comparison. Values are expressed as Mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N=6 animal in each group

Osteoporosis Activity

Serum Biochemical Markers

The effect of isolated quercetin on serum ALP, calcium, phosphorous, Weight femoral bone ,Length of femoral bone (mm), Ash of bone (gm) and Ash calcium (mg/dl) is shown in Fig 2 . The activity of serum ALP was elevated ($P < 0.001$) in comparison to the normal control. Groups treated with ($P < 0.01$) significantly suppressed the rise in serum ALP levels. Serum calcium levels were also found to be significantly increased ($P < 0.01$) in the OVX control as compared to the normal control. Serum calcium levels were also found to be significantly increased ($P < 0.01$) in the OVX control as compared to the normal control. There was significant increase in serum calcium levels as compared to the control. Serum phosphorous levels were also found to be significantly increased ($P < 0.01$) in control as compared to the control. However, quercetin significantly attenuated serum phosphorous levels when compared with the control. Weight femoral bone ,Length of femoral bone (mm), Ash of bone (gm) and Ash calcium (mg/dl) were also altered when compared to control.

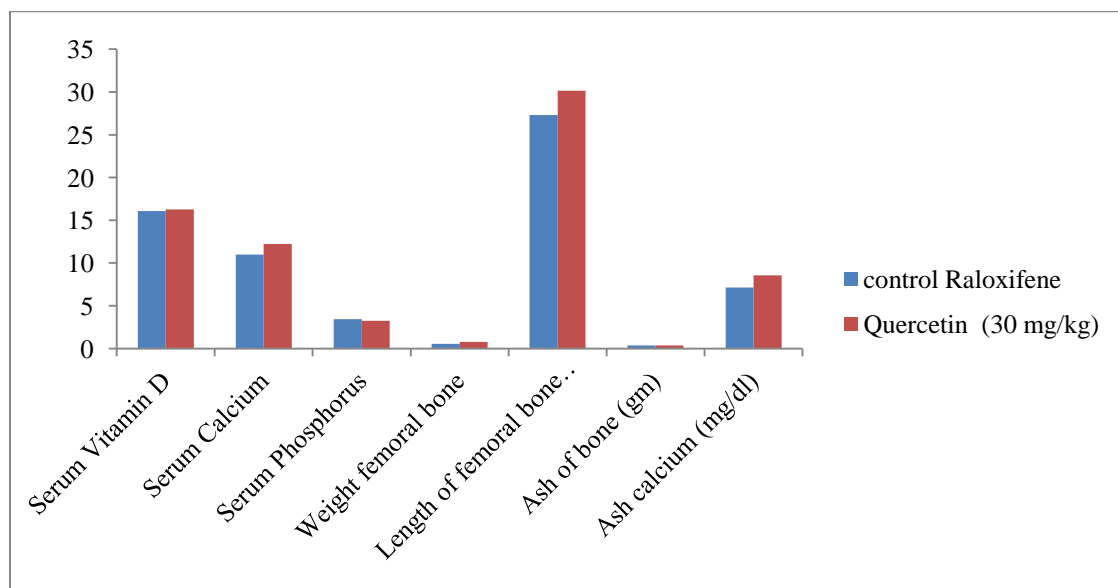
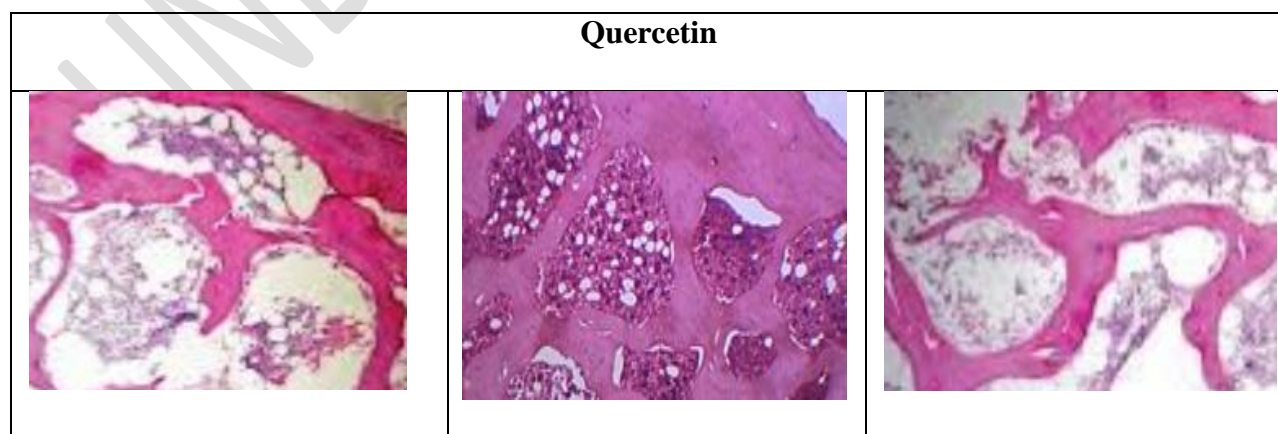


Figure 2 Effects of isolated Quercetin from *Bambusa arundinacea* leaves on Immature female rats on various Serum Biochemical Markers

The statistical significance of difference between means was calculated by Analysis of variance (ANOVA) followed by post hoc test for paired comparison. Values are expressed as Mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N=6 animal in each group

Bone Histology

The Photomicrograph of trabecular pattern of epiphyseal end of femur in control and quercetin treated group showed impressive results when compared to normal (Fig:3).



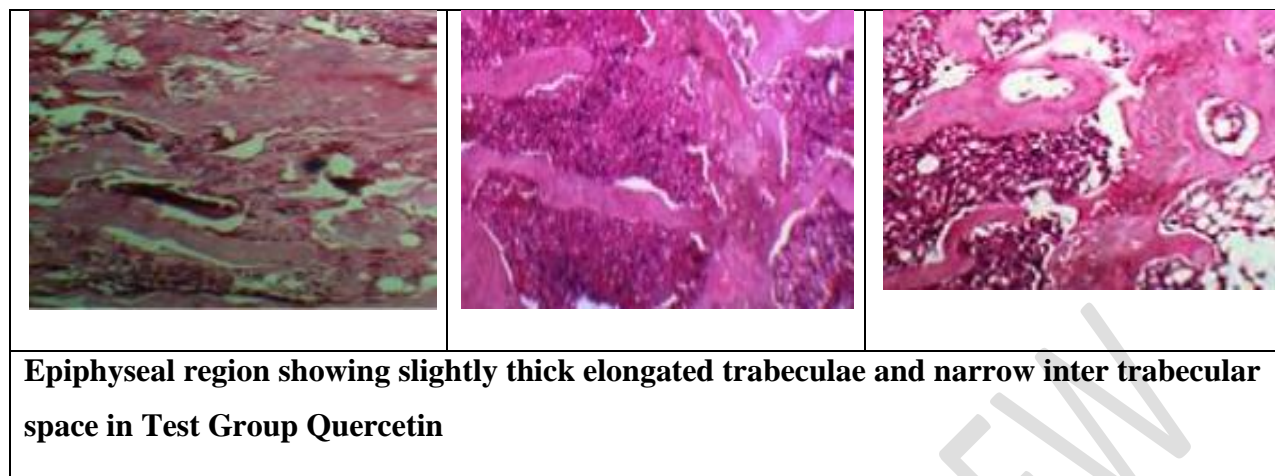


Fig : 3 Histopathology of Various Groups

Discussion

The present study evaluated the effect of isolated quercetin on anti estrogenic activity and Corticosteroid induced osteoporosis. Corticosteroid as they alter skeletal integrity by affecting bone metabolism, reduce the life span of osteoblasts and inhibit osteoblastogenesis in female rats (22). The misbalance of any of the hormones in the body results to unwanted results. Increase in the levels of excess of estrogen results in breast, endometrial, ovarian, and prostate cancer whereas the decrease in the level may cause menopausal symptoms, cardiovascular disease, and osteoporosis. Ovariectomy menopause is one of these reasons of estrogen deficiency in females (23). The growing interest of researchers towards naturopathy has resulted in the discovery of many hidden potentials of plant originated drugs. The promising effect of estrogens has lead to use of novel line of therapy which is completely plant based (24). The various effects of phytoestrogens on human reproductive organs have widespread the area of discovery. These have a negative impact on male fertility but are primarily beneficial to the female health unlike xenobiotic estrogens (environmental pollutants with estrogenic activity), they are believed to have primarily beneficial effects on the health (25). The results obtained for uterotrophic assay showed the dose-related increase in uterine wet weight after administration of quercetin in both in immature and mature ovariectomized rats. The isolated quercetin at higher dose showed only cornified epithelial cells. The vaginal opening also showed significant estrogenic activity. Corticosteroid creates an imbalance in the rhythm between bone formation and bone reabsorption (25). Osteoporosis was induced by intraperitoneal administration of dexamethasone for 5 week and test samples for 30 days. Serum Vitamin D, Serum Calcium, Serum Phosphorus, Weight

femoral bone, Length of femoral bone, Ash of bone, Ash calcium was analyzed and was found that after administration of test samples serum calcium was increased in test groups. It's found statistically significant. The hardness and rigidity of a bone is due to the presence of mineral salts in the osteoid matrix, which is the crystalline complex of calcium and phosphate (27)(hydroxyapatite) in the group B, the loss of mineral in the osteoid matrix due to osteoporosis induction was marked by decreased total ash calcium levels (28), whereas the prevention of bone loss and restructuring of bone with isolated quercetin was evident by increased ash calcium content and ash weight. BMD has been described as a surrogate measure of bone strength and a primary contributor to bone quality (29) and was observed to be markedly decreased in the OVX group due to increased bone turnover (30). A significant increase in BMD on treatment with isolated quercetin, on the other hand, confirmed the remodeling of bones and the prevention of osteoporosis. Histology of Osteoporosis rats bone was found that the Epiphyseal region showing sparse, thinning of trabeculae and loss of connectivity and widening of inter trabecular space found in group A (Negative Control) and after treatment with test samples thickening of trabecule in epiphyseal region in each test groups. But test group D found significant cellular changes in bone microscopy. Test Group D was found more therapeutic active than other test samples. (31). The anti-osteoporotic effect shown by isolated quercetin was promising in comparison is to that Raloxifene which is the standard drug for the treatment of postmenopausal osteoporosis. Hence to conclude it can be said that in this study that the effect of quercetin can be used clinically for their estrogenic effect and can also be used in the management of postmenopausal osteoporosis.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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