

Anti-osteoporotic Activity of the Petroleum Ether Extract of *Cissus quadrangularis* Linn. in Ovariectomized Wistar Rats

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Background: The plant *Cissus quadrangularis* (CQ) is used as an osteoprotective agent in Ayurveda, the Indian system of alternative medicine. The present study was done to validate the anti-osteoporotic role of the petroleum ether extract of CQ on ovariectomy-induced osteoporosis in rats.

Methods: Female wistar rats were divided into five groups of six rats each; (1) The Normal control (NC) group (n = 6) received no surgery and no treatment, (2). The Sham control (SHAM) group (n = 6) received sham surgery but no treatment, (3) the Ovariectomized (OVX) group (n = 6) received an ovariectomy and normal saline treatment for 90 days, (4) the Ovariectomized + raloxifene (OVX + RAL) group (n = 6) received an ovariectomy and treatment with raloxifene, a known anti-osteoporotic agent for 90 days commencing from 22nd post ovariectomy day and (5) the Ovariectomy + *Cissus quadrangularis* (OVX + CQ) group (n = 6) received an ovariectomy, and treatment with the petroleum ether extract of CQ 500 mg/kg body weight daily for 90 days commencing from 22nd post ovariectomy day. At the end of the treatment period, rats in all groups were sacrificed and the right femur was used for biomechanical analysis, and the left femur for histomorphometrical analysis.

Results: CQ significantly increased the force required to break the femur ($p < 0.001$) and significantly increased the thickness of both cortical ($p < 0.001$) and trabecular bone ($p < 0.001$). This action of CQ was comparable to the action of raloxifene.

Conclusions: The petroleum ether extract of CQ stem seems to possess anti-osteoporotic activity in rats.

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Key words: anti-osteoporotic activity, ovariectomized rats, biomechanical strength, cortical bone thickness, trabecular bone thickness

Cissus quadrangularis (CQ), a succulent climber (Vitaceae family), is found throughout India, and is the most frequently used medicinal plant in Ayurveda, an alternative system of medicine in

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India. The alcoholic extract of CQ stem has been found to be useful in enhancing bone fracture healing, ossification of fetal bone, and increasing the thickness of trabecular bone.⁽¹⁻⁴⁾ The different fractions (petroleum ether, benzene, chloroform, acetone, diethyl ether, ethyl acetate and aqueous) obtained from the alcoholic extract of CQ have been subjected to osteoblast cultures *in-vitro* to find the potent fraction having osteoblastic proliferating activity.⁽⁵⁾ In this experiment, when the cells were cultured with the petroleum ether fraction, cell proliferation was significantly stimulated and osteoblastogenesis was facilitated compared to other fractions. Although the alcoholic extract of CQ was found to be effective in bone disorders, no further attempts have been made to evaluate the biologically active fractions of CQ. Since the petroleum ether fraction was efficient on osteoblastogenesis *in vitro* compared to other fractions; we aimed to test the biological activity of petroleum ether extract in an ovariectomy-induced osteoporosis model in rats.

METHODS

Plant

The stem of the *Cissus quadrangularis* plant was collected in Rangapuram, Nalgonda District of Andhra Pradesh, India, and identified and authenticated by a botanist. A voucher specimen was deposited in the Pharmacology Department of Manipal University, Manipal, India.

Extraction

The fleshy stems (2.5 Kg) were washed, cut into small pieces, air-dried and crushed into a powder. The powder was exhaustively extracted with 95% ethanol using a Soxhlet apparatus (Optics Technology, India). A yield of 225 g was obtained. The total ethanol extract was concentrated in a vacuum, the extract was dissolved in water and the solution was partitioned with petroleum ether to obtain petroleum ether extract with a yield of 18.2 g.

Animals

Three month-old female Wistar rats (n = 30) weighing about 225 g were housed in the central animal research facility of Manipal University in sanitized polypropylene cages containing sterile paddy husks as bedding. The animals were maintained

under controlled conditions of temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$) and a 12-h light-dark cycle. All animals were allowed free access to water and were fed a commercial diet. All the studies conducted were approved by the Institutional Animal Ethical Committee (No.IAEC/KMC/06/2006-2007), Kasturba Medical College, Manipal, according to the prescribed guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Acute toxicity studies

Acute toxicity was determined in fasting rats. Animals were divided into groups of 10 each and 0.5% Carboxymethyl cellulose (CMC) or CQ at a dose of 500, 1000, 1500, 2500, 3000, 3500, 4000, 4500 or 5000 mg/kg body weight was given orally. The rats were observed continuously for 2 h, then, frequently up to 6 hours, and daily thereafter for 30 days, and mortality, if any was recorded.⁽⁶⁾ In the acute toxicity study, administration of the petroleum ether extract of CQ stem up to a dose of 5000 mg/kg body weight did not result in any mortality. In our previous study,⁽³⁾ CQ was used at a dose of 500 mg/kg body weight based on our acute toxicity studies. This dose appears to be safe, as treated animals did not show any adverse effect.

Experimental design

The female rats were divided into five groups of six rats each. (1) The normal control (NC) group (n = 6) remained undisturbed in their cage throughout the experimental period. (2) The sham control (SHAM) group (n = 6) underwent sham surgery, in which the ovaries were exposed but not removed. Osteoporosis was induced in the remaining rats by performing an ovariectomy.⁽⁷⁾ (3) The ovariectomized (OVX) group (n = 6) received an ovariectomy and treatment with normal saline for 90 days commencing from 22nd post ovariectomy day. (4) The ovariectomized + raloxifene (OVX + RAL) group (n = 6) received an ovariectomy and were treated with raloxifene (5.4 mg/kg, Ranbaxy, India), a known anti-osteoporotic agent for 90 days commencing from 22nd post ovariectomy day.⁽⁸⁾ (5) The ovariectomy + *Cissus quadrangularis* (OVX + CQ) group (n = 6) received an ovariectomy, and were treated with the petroleum ether extract of CQ 500 mg/kg body weight daily for 90 days commencing from 22nd post ovariectomy

day. For the ovariectomy, rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.), the abdomen was opened and the ovaries were exposed, excised and removed. The abdomen was sutured and the animals were allowed to recover from anesthesia. At the end of the 3 month treatment period, rats in all groups were sacrificed by cervical dislocation and the femurs were collected. The right femur was used for biomechanical analysis, and the left femur for histomorphometrical analysis.

Biomechanical analysis

The breaking strength of the right femurs was assessed by a three point bending test using a tensile strength testing machine (Gayathri Machine Products, Gujarat, India).⁽⁹⁾ The femur was placed in the material testing machine with two supports separated by a distance of 24 mm. The middle of the femur shaft was compressed by a rounded press until fracture occurred. The force required to break the bone was recorded in newtons (N).

Histomorphometrical analysis

The left femur was removed, dissected free of soft tissue, and fixed with PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution, pH 7.4, stored at 4°C) for 24 hr at 4°C. All femurs were decalcified by a standard as described previously.⁽¹⁰⁾ The bone tissues were dehydrated in a graded series of alcohol and embedded in paraffin wax. The lower end of the femur was sectioned (5- μ m thickness) longitudinally on a rotary microtome and processed for hematoxylin and eosin staining.

Care was taken to ensure that all bones were oriented in the same direction during embedding to minimize differences in the angles at which the bones were sectioned. All bones were processed uniformly to maintain consistency in histomorphometrical analysis. The thickness of individual trabecula and cortical bone was measured using a calibrated ocular micrometer in the middle of the shaft.

Statistical analysis

The results are expressed as the mean \pm SE. All data were analyzed using Graphpad Prism software (Microsoft, San Diego, CA, U.S.A.). One-way ANOVA was first performed to test for any significant differences among groups. When significant, a

post test, Bonferroni's multiple comparison test was applied to determine the specific difference between the groups.

RESULTS

Biomechanical strength

The mean force required to break the femur in the NC, SHAM, OVX, OVX + RAL and OVX + CQ groups was 68.97 ± 1.32 , 68.17 ± 1.24 , 31.50 ± 3.45 , 70.67 ± 1.25 and 72.83 ± 0.94 newtons, respectively. The biomechanical strength measured in the different groups indicated that the total force required to break the femur was significantly lower in the OVX group of animals than the NC and SHAM groups ($p < 0.001$, Fig. 1). Treatment with raloxifene ($p < 0.001$) and CQ ($p < 0.001$) increased the biomechanical strength of the femur as evident from the greater force required to break them.

Cortical bone thickness

The mean thickness of cortical bone measured from histological sections of the femur in the NC, SHAM, OVX, OVX + RAL and OVX + CQ groups was 504.00 ± 16.86 , 501.5 ± 6.95 , 232.83 ± 10.78 , 398 ± 9.49 and 368.33 ± 6.10 μ m, respec-

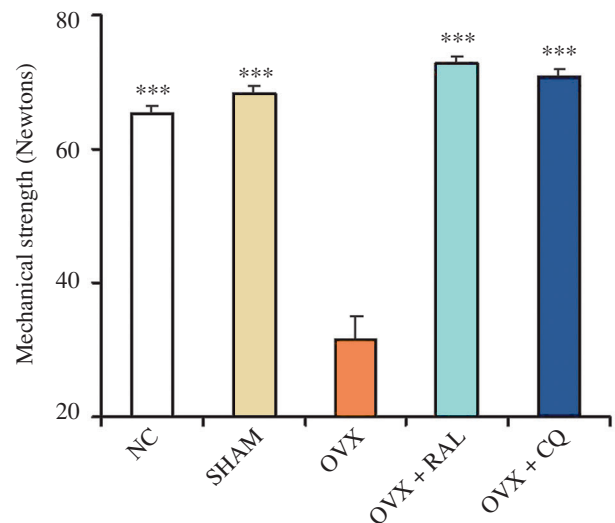


Fig. 1 The mechanical strength of the femur in different groups. Note there is a significant decrease in the mechanical strength in the ovariectomized (OVX) group, compared to the normal control (NC) and sham (SHAM) groups. It is nearly normal in the raloxifene (OVX + RAL) and CQ (OVX + CQ) groups. $p < 0.001$, in comparison to OVX.

tively (Fig. 2). The ovariectomy resulted in a significant reduction in cortical thickness compared to the NC and SHAM groups ($p < 0.001$). Treatment with raloxifene ($p < 0.001$) and CQ ($p < 0.001$) significantly increased the cortical thickness compared to the OVX group.

Thickness of trabecular bone

The mean thickness of trabeculae in the femur in the NC, SHAM, OVX, OVX + RAL and OVX + CQ groups was 190 ± 4.42 , 187 ± 2.86 , 94.66 ± 3.4 , 172.66 ± 3.25 and $171.66 \pm 0.95 \mu\text{m}$, respectively (Fig. 3). The ovariectomy resulted in a significant reduction in trabecular thickness compared to the NC and SHAM groups ($p < 0.001$). Treatment with raloxifene ($p < 0.001$) and CQ ($p < 0.001$) significantly increased the trabecular thickness compared to the OVX group.

DISCUSSION

Estrogen deficiency is a well-known risk factor in the pathogenesis of osteoporosis. Our present study clearly demonstrated the usefulness and beneficial effects of CQ in the treatment of osteoporosis induced by ovariectomy. Estrogen influences bone loss, either directly by binding to the receptor on the bone or indirectly by influencing calcium regulatory hormones (PTH and vitamin D) and cytokines IL-1 and IL-6. Biomechanical data suggest that bone becomes stronger after treatment with the petroleum ether extract of CQ, which may be due to enhanced mineralization of the bone.⁽⁴⁾ Interestingly, CQ shows activity similar to that of a standard antiosteoporotic drug, raloxifene. This suggests that this plant extract is safe in treatment of osteoporosis in elderly women.

The activity of CQ on the thickness of cortical

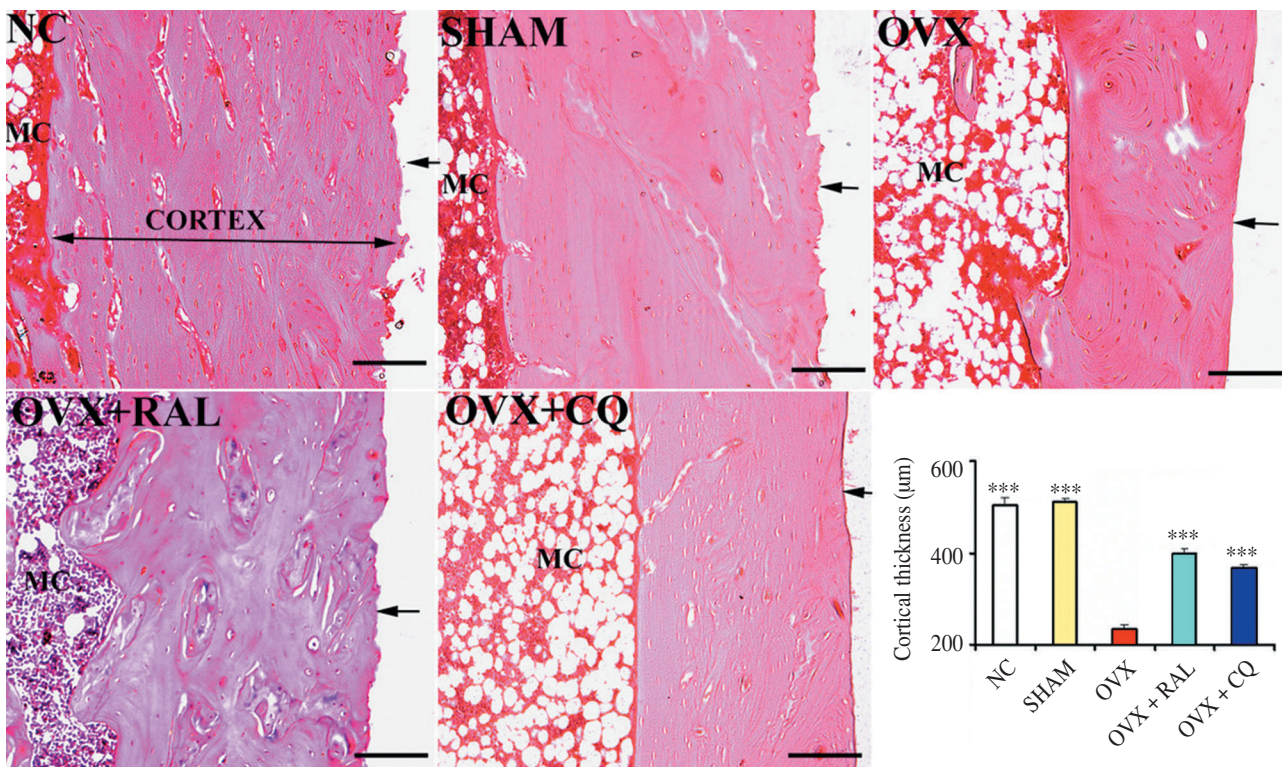


Fig. 2 The cortical thickness in the different groups. These photomicrographs were taken from the lower end of the femur. Note there is a significant decrease in the thickness of cortical bone in the ovariectomized (OVX) group, compared to the normal control (NC) and sham (SHAM) groups. Cortical thickness is nearly normal in rats ovariectomized and treated with raloxifene (OVX + RAL) and CQ (OVX + CQ). MC- medullary cavity, Arrows point to the periosteum. H & E staining; Scale bar = 100 μm . $p < 0.001$, in comparison to OVX.

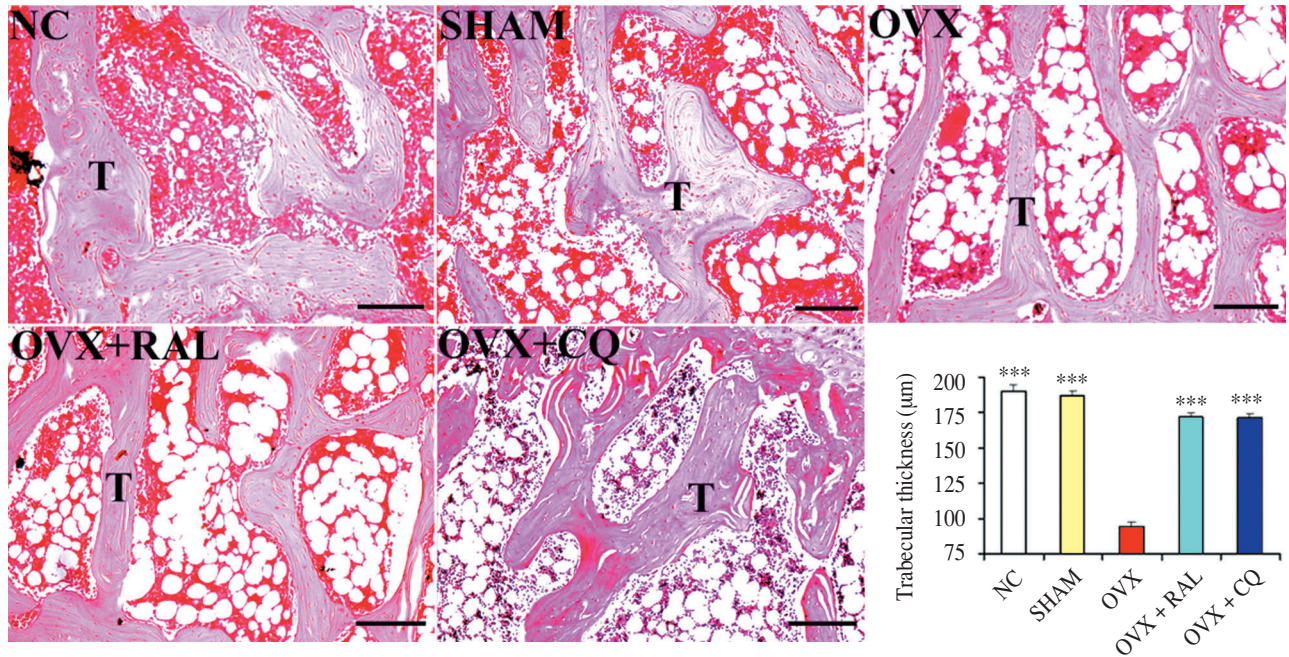


Fig. 3 The trabecular thickness in the different groups. These photomicrographs were taken from the lower end of the femur. Note there is a significant decrease in the thickness of trabeculae (T) in the ovariectomized (OVX) group compared to the normal control (NC) and sham (SHAM) groups. Trabecular thickness was nearly normal in the raloxifene (OVX + RAL) and CQ (OVX + CQ) groups. H & E staining; Scale bar = 100 µm. $p < 0.001$, in comparison to OVX.

bone and trabecular bone is indicative of the anti-osteoporotic activity of the petroleum ether extract of CQ. This activity is similar to the action of the alcoholic extract of CQ.⁽⁴⁾ This suggests that the petroleum ether extract of CQ is as potent as the alcoholic extract. The observed osteoprotective role may be attributed to its phytochemical, steroid-like components. Studies on fracture healing have suggested that the unidentified anabolic steroid isolated by Sen acts on the estrogen receptors of bone cells.⁽¹¹⁾ It also contains flavonoids, triterpenoids stilbene derivatives and other constituents,⁽¹²⁻¹⁵⁾ such as quercetin, resveratrol, piceatannol, pallidol perthenocissin and phytosterols.⁽¹⁶⁻¹⁹⁾ The anti-osteoporotic activity of petroleum ether extract of CQ may be justifiably attributed to its steroid components, which probably act as phytoestrogens to prevent bone loss.⁽²⁰⁾

Conclusions

Our present study clearly demonstrated the usefulness and beneficial effects of CQ in the treatment of osteoporosis induced by ovariectomy.

On the basis of results obtained in this study, it

can be concluded that the petroleum ether extract of CQ stem seems to possess anti-osteoporotic activity in rats. The results of biomechanical and histomorphometrical analysis of the femur seem to support the traditional use of this plant in bone-related disorders.

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