CISSUS QUADRANGULARIS PLANT EXTRACT ENHANCES THE DEVELOPMENT OF CORTICAL BONE AND TRABECULAE IN THE FETAL FEMUR

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Summary

Traditional recipes for treatment of physical and mental ailments exist in all major ancient civilizations of the world. One such recipe popular in the Indian subcontinent involves the use of the extract of *Cissus quadrangularis* plant .*Cissus quadrangularis* is a shrub, with thick and fleshy quadrangular stem. The roots and stem are most useful for healing of fracture of the bones. The question whether the plant *Cissus quadrangularis*, which is proved to contain phyto-estrogens will increase the bone mass during fetal growth is not addressed. Accordingly the objective of the present study was to evaluate the effect of alcoholic extract of *Cissus quadrangularis* on the intrauterine growth of trabeculae in the long bones in rats. Pregnant rats were administered with Ethanol extract of *Cissus quadrangularis* (CQ), orally, at the dose of 750 mg/kg body weight from 9th day of gestation till delivery. Femur bone of the newborn pups were collected, decalcified and processed for paraffin sectioning. Thickness of cortical bone at mid shaft level, thickness of individual trabeculae were measured using a calibrated ocular micrometer. Results showed a significantly increase in thickness of the cortical bone at mid shaft level compared to control rats. The thickness of individual trabecula was increased significantly. The present study demonstrates enhanced bone formation during fetal growth by CQ, which may be related to rich content of calcium, phosphorus and phytoestrogenic property of the plant.

Key words: Ossification, C*issus quadrangularis,* Cortical bone, Trabeculae,Femur

Introduction

Traditional recipes for treatment of physical and mental ailments exist in all major ancient civilizations of the world. One such recipe popular in the Indian subcontinent involves the use of the extract of *Cissus quadrangularis* plant (Fig.1). *Cissus quadrangularis* is a shrub, with thick and fleshy quadrangular stem. It is an edible plant found in India, Srilanka, Malaya, Java and West Africa (1). The plant and its medicinal properties have been described in literature as early as 1970 (1). Ethnobotanical uses of the plant have been reported (2). Stem and root extract of this plant possess antioxidant and antimicrobial activity (3). Various organic macromolecules ranging from terpenoids to large stilbene derivatives have been isolated from this plant (4). Stem extract contains a high percentage of calcium ions and phosphorus (5). The plant has been documented in Ayurveda, an alternative system of medicine in India, for its medicinal uses in gout, syphilis, venereal diseases, piles, leucorrhoea (6). The stem juice of the plant is used to treat scurvy and irregular menstruation, otorrhoea and epistaxis (7). The roots and stem are most useful for healing of fracture of the bones (7, 8, 9, 10, 11, 12). Phytoestrogenic steroids have been isolated from *Cissus quadrangularis* plant (13, 14, 15), has been shown to influence early regeneration and quick mineralization of bone fracture healing process.

Fig 1: *Cissus quadrangularis* plant. Note the fleshy quadrangular stem with nodes at intervals. At nodes we can see a leaf and a tendril

Many studies have focused on assessing the role of estrogens deficiency that plays the major role in the onset of postmenopausal osteoporosis (16, 17, 18, 19). Estrogen receptors have been detected in bone cells (Both in osteoblast and osteoclasts (20, 21, 22) suggesting the direct action of estrogens on these bone cells. Several studies have shown that estrogens can modulate bone cell physiology in vitro by direct estrogen receptor mediated mechanism (23, 24, 25). This evidence implicates a direct effect of estrogen on skeleton and alternatively on bone tissue turnover. Natural estrogens such as estradiol were shown to induce permanent changes in the skeleton of adult animals when given as long term treatment (16, 25, 26, 27). Additionally phyto-estrogens which are widely present in our environment seem to have some modulatory effect on bone cells in vitro (28).

The question whether the plant *Cissus quadrangularis*, which is proved to contain phyto-estrogens will increase the bone mass during fetal growth is not addressed. Accordingly the objective of the present study was to evaluate the effect of alcoholic extract of *Cissus quadrangularis* on the intrauterine growth cortical bone,trabeculae in long bones of neonatal rat pups.

Materials and methods

Animals

Female Wistar rats of 3 months age (200-250 g weight) were housed in the central animal research facility of the Manipal University, Manipal. All animals were allowed to have free access to water and food, and were maintained in 12:12 h dark and light cycle. All experiments were carried out with prior approval from the institutional animal ethical committee. Only the minimum required numbers of rats were used, and they were handled in a humane way. To get pregnant rats of known gestational day, female rats in estrous cycle were mated with young healthy male rats and then, they were subjected for vaginal smear test. Detection of sperms in vaginal smear was considered as day 0 of pregnancy.

Preparation of extract:

CQ stem were collected from Nalgonda district of Andhra Pradesh, India. A specimen was deposited in the Pharmacognosy Laboratory of the Manipal University, Manipal. The fleshy stems were washed, cut into small pieces, air-dried and crushed into powder. Stem powder was exhaustively extracted with 95% ethanol using a Soxhlet apparatus. The total ethanol extract was concentrated in vacuo till a syrupy consistency is obtained.

Experimental groups

Pregnant rats were divided into control and experimental group (n=6 in each group). Pregnant rats in the experimental group were treated with CQ alcoholic extract, 750 mg/kg/day from gestation day 9 till delivery. The control group received equivolume of saline.

Tissue processing and staining

At the end of treatment period, i.e on the day of birth, rat pups born to control and treated mothers was perfused transcardially with 4% paraformaldehyde. Femur bone was dissected and post fixed in the same fixative for 48 hrs. Fixed bone tissue was processed for paraffin sectioning and 5µm thick longitudinal serial paraffin section was taken in a rotary microtome. Sections were mounted on gelatinized slides and air dried. After 24 hr of drying sections were stained with hematoxylin and eosin.

Measurement of cortical bone thickness and trabecular thickness

To measure the thickness of the cortical bone and thickness of the trabeculae,form each rat 8 longitudinal sections from middle of the femur was selected from each femur in all the rat pups. The interval between the adjacent sections was 30 microns (6sections apart).In each section cortical thickness (length between periosteum and endosteum) was

measured in these selected sections using a calibrated ocular micrometer. In each section 2 measurements were taken and mean for that section was calculated. Finally grand mean was calculated for a given rat pup and then group mean was calculated.

To measure the thickness of the trabeculae, sections were selected as above. In each section thickness of 10 trabeculae was measured using the calibrated ocular micrometer. Section mean, mean for animal and finally group mean thickness of individual trabeculae were calculated.

Data was expressed as mean ± SEM (Standard error of means).Control and treated groups were compared using Student's t-test. Probability (P) value less than 0.05 was considered as significant.

Results

The oral administration of alcoholic extract of *Cissus quadrangularis* plant caused neither any mortality nor any signs of clinical abnormality in the treated pregnant rats. At necropsy no gross pathological observation could be made in the target organs. There was no mortality observed in the pups born to the treated pregnant rats. Thus it appears that the drug dose given appears to be safe.

Thickness of Cortical bone:

The bones sections stained with Hematoxylin and Eosin revealed thick cortical bone in the Cissus quadrangularis plant extract treated group compared to control group (Fig.2, 3). The bone was very compact in the treated rats compared to control rats. Thickness of trabeculae was increased by 148% in the treated pups compared to control rats. On quantification, it was found that thickness of the cortical bone was 2770.83 ± 159.74 μm(Mean ± SEM,n=6) in treated animal's bone and it was 1118.33 ± 33 μm (n=6) in control animals($P<0.0001$,Student's t-test, $t=10.160$,DF-10,Fig.4).

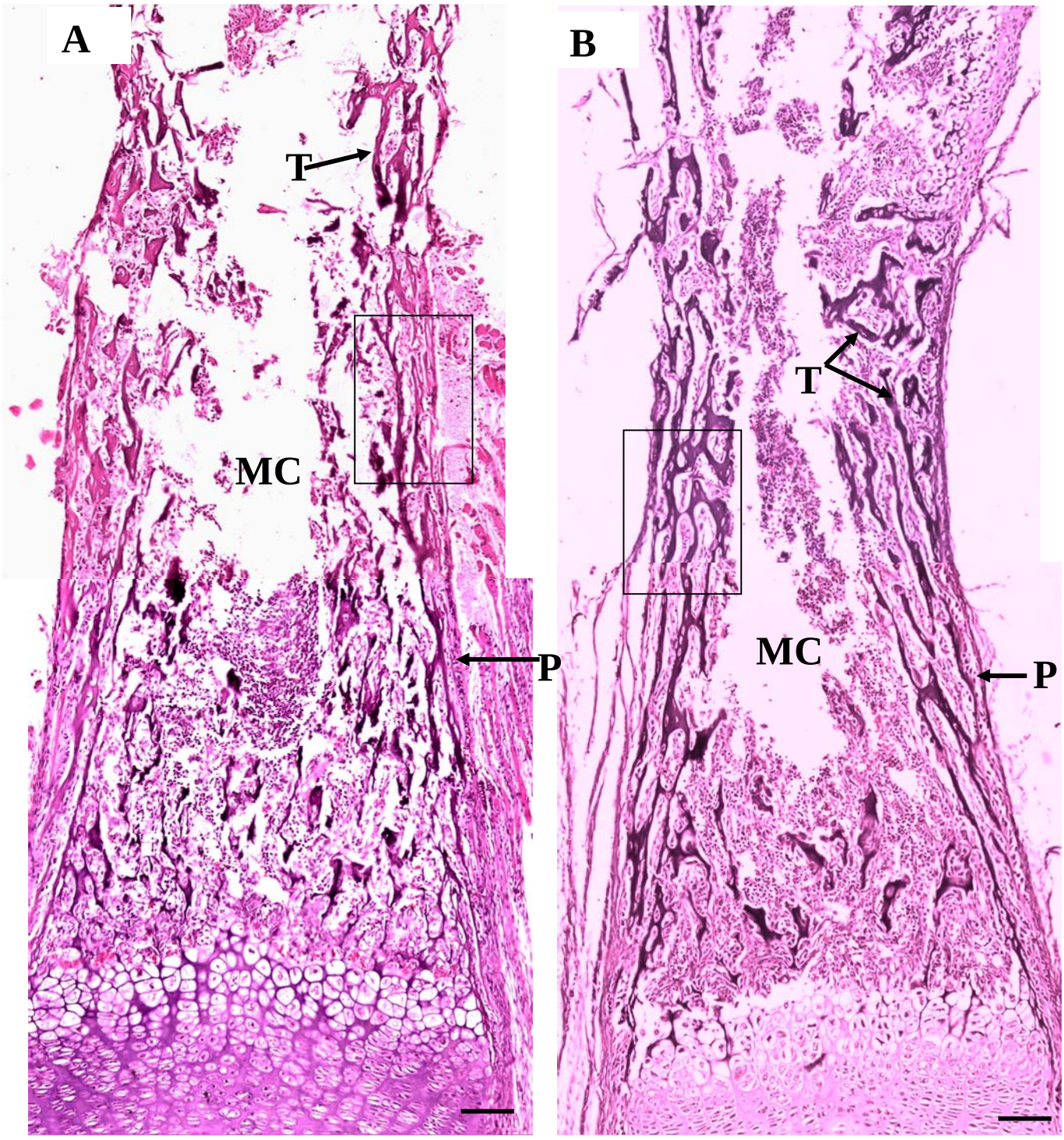

Fig.2.Photomicrograph of lower end of the femur bone of control (A) and Cissus quadrangularis plant extract treated (B) neonatal rat pups. Note the significantly increased thickness of cortical bone and thickness of individual trabeculae in the treated rat pups..MC- medullary cavity,P-periosteum,T-trabeculae. Rectangular area marked is magnified in figure 5. Scale bar=150µm

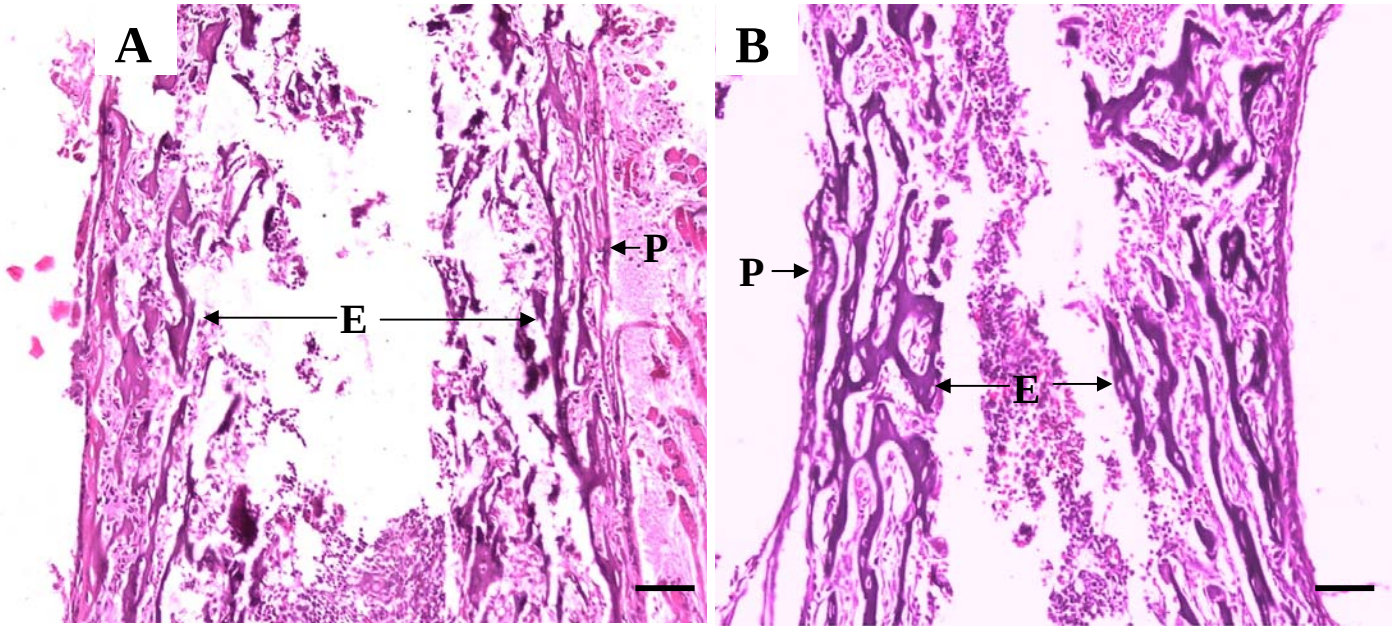

Fig. 3 Photomicrographs of femur bone at mid shaft level in control (A) and plant extract treated (B) neonatal rats. Note the increased thickness of cortical bone (between endosteum and periosteum) in the treated rat.P-Periosteum,E- Endosteum.Scale bar=150 µm

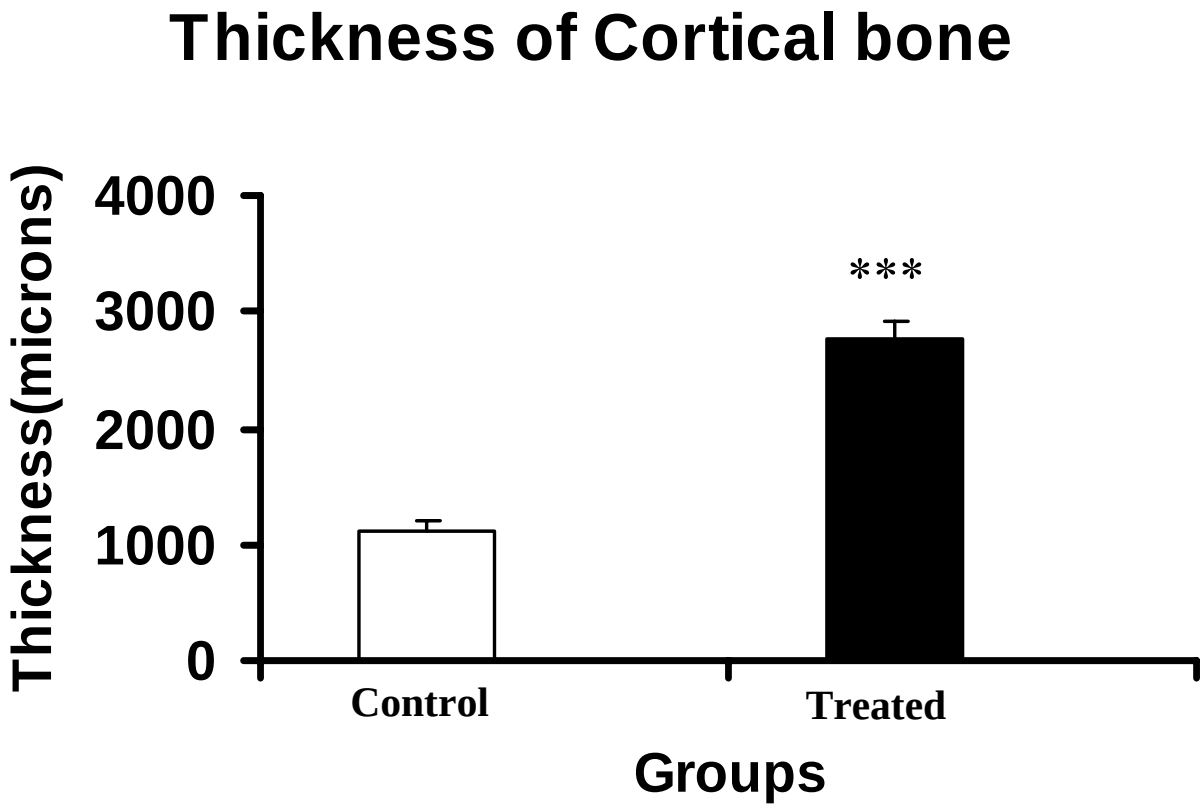

Fig.4.Thickness of the cortex of femur bone at mid shaft level in control and plant extract treated rat pups. Note there is a significant ($p<0.0001$) increase in the thickness of the cortical bone in the treated pups.

Thickness of the individual trabeculae:

On observation, the thick cortical bone in the treated animal it was found that individual trabeculae are much thicker and stronger in the treated rat pups, than the control rat pups. It was also noted that trabeculae are more compactly arranged in the treated animals (Fig.5).Thickness of trabeculae was increased by 104% in the treated pups compared to control rats. On quantification, it was found that thickness of the cortical bone was 177.5 ± 6.67 µm (Mean ± SEM, n=6) in treated animal's bone and it was 87.33 ± 4.13 µm (n=6) in control animals(P<0.0001,Student's t-test, t=11.480,DF-10,Fig.6).

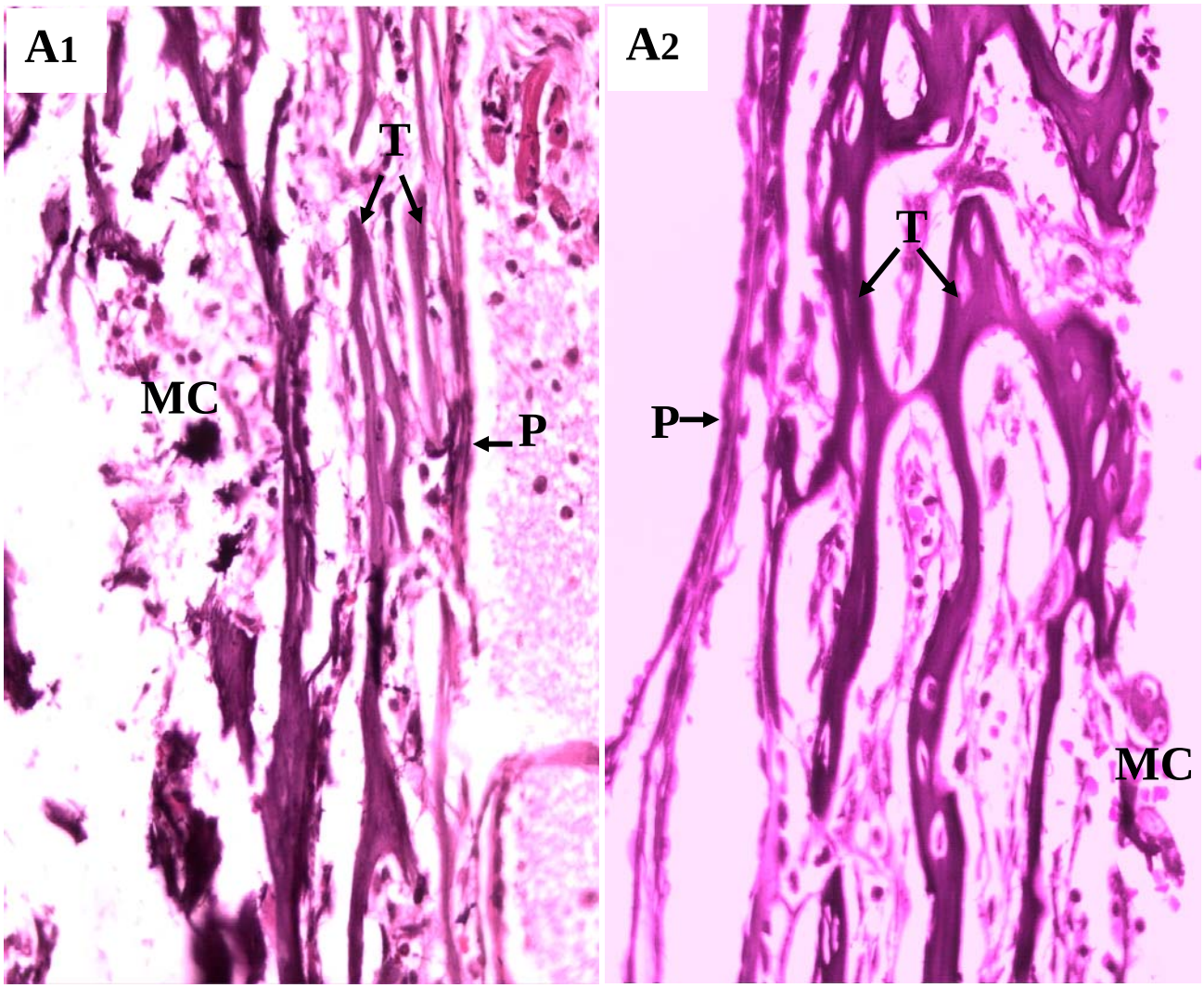

Fig. 5 Photomicrographs of femur bone at mid shaft level in control (A) and *Cissus quadrangularis* plant extract treated (B) neonatal rats. Note the increased thickness of trabeculae in the treated rat pups.P-Periosteum,E- Endosteum. T- Trabeculae .Scale bar=100 µm

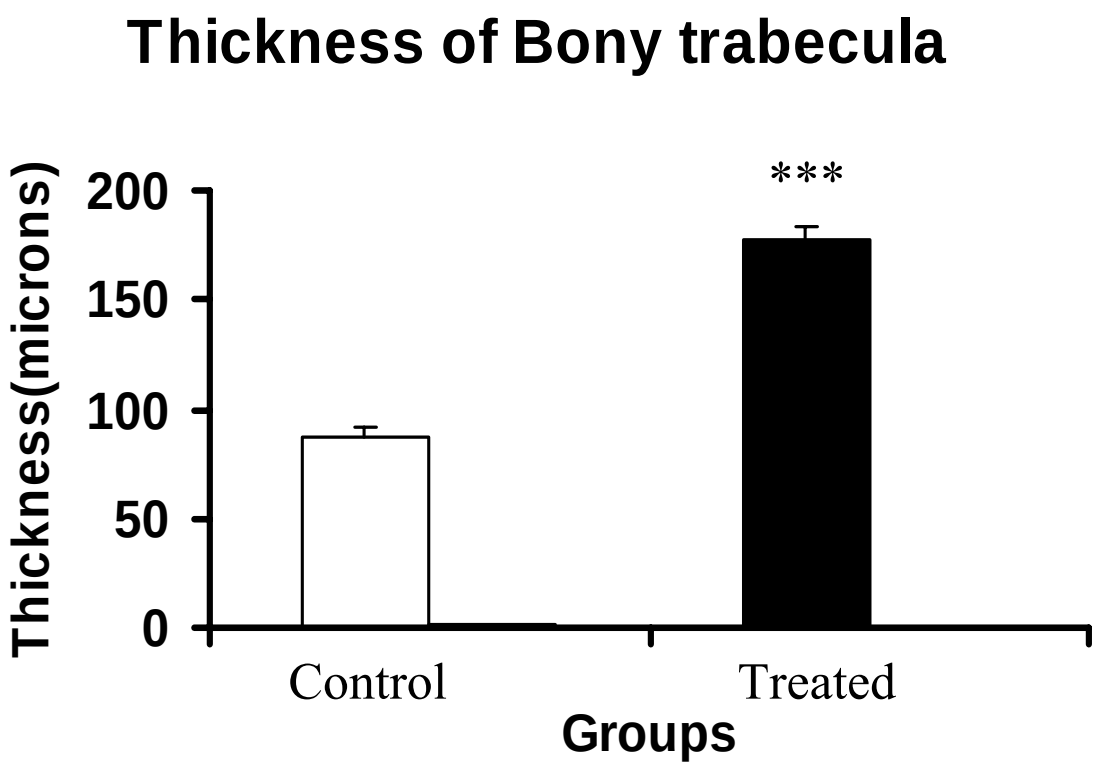

Fig.6.Thickness of individual trabecular bone at mid shaft level in control and plant extract treated rat pups. Note there is a significant ($p<0.0001$) increase in the thickness of the trabeculae in the treated pups.

Discussion

The present study results demonstrate for the first time that maternal treatment with *Cissus quadrangularis* during gestation can dramatically influence the skeletal tissue of developing fetus. In the present study the pups born to the pregnant rats fed with *Cissus quadrangularis* plant extract showed an increased bone formation. In these pups (born to treated pregnant rats) the thickness of the cortical bone and thickness of individual trabeculae in the cortical bobe was significantly greater compared to that in pups born to control rats.

It is well known that changes in estrogen levels can dramatically modify bone tissue turnover in both postmenopausal women (27) and adult experimentally induced postmenopausal animals (16, 17). In addition, long term exposure of adult animals to estradiol or diethylstilbestrol induces hyperplasia of bone tissue (17, 28). These data strongly suggest that bone tissue directly respond to estrogens as other estrogen target tissue. Further that transient neonatal exposure of female mice to estrogens have shown to increase the bone mass in the animals during adulthood (25). In addition, sex steroids can influence the development of dimorphism in the female innominate bone (29). Thus these data strongly suggest that alterations of estrogen levels before puberty, in early phase of development can dramatically influence skeletal maturation including final peak bone density. Perturbation in the maternal estrogen level during pregnancy, shown to alter the developing skeleton. (30).

In the present experimental study we have reported an increased bone mass in the pups born to the treated pregnant rats. This may be attributed to the phytoestrogenic compounds isolated from this plant (13) which might have altered the maternal estrogen level. The plant extract increased the trabecular density of bone the effect was identical on both male and female pups. Hence data was analyzed together.

Alternatively, the phyto-estrogenic compounds or other unidentified chemical compounds might have altered the steroidal hormonal levels in the pregnant rats, there by influencing the skeleton of the fetus/neonatal pups.

Another possibility is that the phytoestrogens present in the plant extract might have crossed the placental barrier and reached the developing fetus and influenced the skeletal system. This is indeed true in case of study by Magliaccio et al (30), where they have altered the maternal estrogen levels by giving external steroidal hormones and showed alterations in the skeleton of neonatal pups.

Alternatively, the increased bone formation in the *Cissus quadrangularis* plant extract treated pups may be due to rich calcium and phosphorous present in this plant (5).

The stem extract of this plant contains a high percentage of calcium ions (4% by weight) and phosphorus, both essential for bone growth (5). In deed using the extract of this plant, high quality calcite crystal has been synthesized as this plant contains high amount of calcium (31). It has been reported earlier that the extract of this plant is very useful in bone fracture healing process (7, 8, 9, 10, 11, 12, 14, 15), which is a process of bone formation in adults. The calcium ions, phosphorous and phytoestrogens present in this plant extract may be made use in the process of ossification and fracture healing.

Thus the plant *Cissus quadrangularis* appears to be very useful in treating the diseases involving deficiency in the bone formation and fracture healing. The active ingredients need to be isolated, characterized and studied further to formulate the therapeutic drugs.

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