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Effects of *Cissus quadrangularis* on the Proliferation, Differentiation and Matrix Mineralization of Human Osteoblast Like SaOS-2 Cells

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ABSTRACT

Osteoporosis is a public health problem which is associated with significant morbidity and mortality. The repair of bone defect is still a big challenge for orthopedic surgeons. Traditional use of *Cissus quadrangularis* (*C. quadrangularis*) in the treatment of bone disorders has been documented. The present study was employed to delineate the effects of ethanolic extract of *C. quadrangularis* on the proliferation, differentiation and matrix mineralization of human osteoblast like SaOS-2 cells. Lactate dehydrogenase assayed in the conditioned medium of control and *C. quadrangularis* treated cells did not differ significantly indicating that ethanolic extract of *C. quadrangularis* is nontoxic to osteoblastic cells. [^{3}H] Thymidine incorporation assay revealed that *C. quadrangularis* treatment has increased the DNA synthesis of human osteoblastic SaOS-2 cells indicating increased proliferation of these cells. The data on alizarin red and ALP staining revealed increased matrix mineralization of human osteoblast like SaOS-2 cells. The study also revealed that the anabolic actions of ethanolic extract of *C. quadrangularis* in human osteoblast like cells are mediated through increased mRNA and protein expression of Runx2, a key transcription factor involved in the regulation of bone matrix proteins. Chromatin immunoprecipitation analysis revealed increased transcriptional activity of Runx2 on the promoter of osteocalcin after *C. quadrangularis* treatment. These results indicate positive regulation of *C. quadrangularis* on the proliferation, differentiation, and matrix mineralization of human osteoblast like SaOS-2 cells. J. Cell. Biochem. 112: 1035–1045, 2011.

KEY WORDS: C. QUADRANGULARIS; SaOS-2 CELLS; MATRIX MINERALIZATION; Runx2

The formation of bone involves a complex series of events that include the proliferation of osteoblasts and their differentiation, eventually resulting in the formation of a mineralized extracellular matrix. The steady state number of osteoblast in vivo is a function of their rate of formation and death, and progression to osteocyte phenotype. Based on the bone nodule formation in vitro, the process has been subdivided into three stages, (i) proliferation, (ii) extracellular matrix (ECM) deposition, and (iii) mineralization.

Initially, proliferating osteoblasts turn on genes associated with the cell cycle and cell growth control (e.g., histone and c-fos) along with genes encoding matrix proteins. However, upon cessation of osteoblast proliferation, there is an upregulation in the expression of genes that support the maturation and organization of the ECM [Klein et al., 1996]. The differentiation processes that follow proliferation are matrix maturation and mineralization. A number of genes including alkaline phosphatase, type I collagen and osteocalcin are highly expressed in differentiation period [Beck, 2003]. The osteogenic cell secretes matrix where calcium phosphate is deposited as hydroxyapatite crystal [Long et al., 1995; Lecanda et al., 1997]. During mineralization, genes for osteopontin and

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osteocalcin are actively expressed [Owen et al., 1991; Stein and Lian, 1993]. These cultures proceed through the deposition of collagenous matrix in the presence of ascorbic acid and β glycerophosphate [Xiao et al., 2002]. The occurrence of osteoporosis is associated with decreased osteoblastic activity and increased osteoclastic activity [Riggs and Melton, 1988]. SaOS-2 cells have been used to assess the effect of herbal compounds on the proliferation, differentiation and matrix mineralization of osteoblastic cells [Kim et al., 2003; Huh et al., 2006; Oh et al., 2008]. Multiple signaling pathways that affect osteoblast function converge to and are being coordinated by Runx2. Growth factors such as BMPs, TGFβ, FGFs, and IGFs, which are known to be local regulators of bone formation targets Runx2 [Yamaguchi et al., 2000]. SaOS-2 cells represents one of the cell lines available to specifically study gene regulation by Runx2 since expression of neither Runx1 nor Runx3 was detected [Sudhakar et al., 2001].

The identification of the herbal components that regulate the proliferation and differentiation of osteoblastic lineage remains one of the challenges in the field of bone biology. Among indigenous medicinal plants, *Cissus quadrangularis* is one of the most widely prescribed herb for patient with bone fractures. Even though, both *C. quadrangularis* and vitamin C promoted fracture healing, *C. quadrangularis* showed faster healing effect than vitamin C. Thus, it appears to be a cooperative effect of the constituents of *C. quadrangularis* extract [Udupa et al., 1961]. A clinical study evaluated in 16 patients revealed that 80% of the cases were excellent in clinical results of reduction in healing time, excellent in 6 cases, good in 9 cases, and poor in 1 case. These results indicate earlier calcification and callus formation in human [Udupa and Prasad, 1962]. *C. quadrangularis* has been shown to prevent ovariectomy induced bone loss in ovariectomized rats and increases osteogenesis during fetal development [Shirwalkar et al., 2003; Rao et al., 2007; Potu et al., 2009]. Intramuscular injection with *C. quadrangularis* in rabbits showed evidence for the relative increase in the levels of mucopolysaccharide, proliferation, and differentiation of osteoblasts in callus bone [Felix and Mary, 2004]. A recent study reported that ethanolic extract of *C. quadrangularis* promoted the ALP activity and mineralization in mouse osteoblastic cells [Parisuthiman et al., 2009]. However, the direct effects of *C. quadrangularis* on the proliferation, differentiation and matrix mineralization in human osteoblastic cells are yet to be explored. Hence, the present study is aimed to delineate the effect of *C. quadrangularis* on the proliferation, differentiation and matrix mineralization of human osteoblastic SaOS-2 cells.

MATERIALS AND METHODS

PLANT EXTRACT PREPARATION

The whole plant of *C. quadrangularis* used in the present experiment was procured locally and authenticated with the help of plant taxonomist of the University of Madras and assigned with a accession number MUCHS-H104. Fresh aerial parts of *C. quadrangularis* were washed with double distilled water, surface sterilized and soaked in ethanol for 48 h. Then, the solvent was separated, vacuum dried and concentrated using speed vac concentrator. This concentrate was dissolved in DMSO (final concentration of DMSO does not exceed 0.05%), diluted in the culture medium and then filtered through negative pressure with a sterile filter (0.2 μm).

CELL CULTURE

Human osteoblast like SaOS-2 cells were procured from National Centre for Cell Sciences (NCCS) Pune, India. The cells were grown in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) till confluence. Then the cells were detached using trypsin EDTA and were used for subculture or treatment.

ISOLATION OF OSTEOBLASTS FROM RAT CALVARIA

Osteoblasts were isolated from calvaria according to the method of Nefussi et al. [1985]. Calvaria from 2 days old rats were excised aseptically, cleansed, and minced into fragments. The bone fragments were rinsed with PBS three times, each for 5 min, and then treated with type I collagenase (1 mg/ml DMEM) at 37°C for 2 h. The supernatant was collected and centrifuged at 1,500 rpm for 5 min. The cell pellet was then resuspended in DMEM supplemented with 10% FBS, and incubated at 37°C under the atmosphere of 5% CO_2 in a 25-cm^2 culture flask.

CELL PROLIFERATION

[^{3}H] Thymidine incorporation assay was performed as described by Terada et al. [1998]. Briefly, cells were seeded in 24-well plates containing DMEM with 10% FBS for 12 h for attachment. Then DMEM with different doses of *C. quadrangularis* were added and maintained for 48 and 72 h. The cells were then pulsed with 3 μCi of [^{3}H] thymidine 6 h before the end of the incubation period. At the end of the incubation period, the cells were washed twice with cold phosphate buffered saline, scraped off with a cell scraper in 5% trichloro acetic acid (TCA) and centrifuged at 400*g* for 20 min at 4°C. One hundred microliters of the supernatant was then blotted on a dry filter paper and the filter paper was placed in a vial containing 3.0 ml scintillation fluid and the radioactivity was counted in a liquid scintillation counter.

ESTIMATION OF ALP AND LDH

Alkaline phosphatase (ALP) activity was determined by the method of Andersch and Szczypinski [1947] using p-nitrophenyl phosphate as the substrate. Control and *C. quadrangularis* treated cells were rinsed with PBS, harvested and processed for determining the alkaline phosphatase activity and OD read at 410 nm. Lactate dehydrogenase (LDH) activity was determined by the colorimetric method as described by King [1965]. After indicated time period, the conditioned media were collected from human osteoblastic SaOS-2 cells and rat calvarial cells and used for the estimation of LDH. The OD was read at 440 nm. The activity of ALP and LDH were normalized to total cellular protein determined by protein assay using crystalline bovine serum albumin (BSA) as the standard [Lowry et al., 1951].

ALP STAINING

The ALP staining in the cultures were performed according to the method followed by Sila-Asna et al. [2007]. The cells were cultured

in the presence or absence of 1 and 10 μg/ml *C. quadrangularis* in a mineralizing medium containing 10 mM β-glycerophosphate, 10 nM dexamethasone and 50 μg/ml ascorbic acid for 21 days. To examine the ALP activity histologically, cells were fixed for 10 min with 70% ethanol at room temperature. After washing with PBS, the cells were fixed for 20 min with a mixture of 0.1 mg/ml napthol AS-MX phosphate, 0.5% N,N-dimethyl formamide, 2 nM magnesium chloride and 0.6 mg/ml fast red salt in 0.1 M Tris–HCl buffer (pH 8.5) at room temperature. Color development was then examined and photographed after counter staining with hematoxylin under a phase contrast microscope.

QUANTIFICATION OF MATRIX MINERALIZATION

Mineralization on the matrix synthesized by the monolayer of osteoblasts was analyzed with alizarin red staining method for calcium deposition [Johnson et al., 2001]. Briefly, the cell monolayer after 21 days culture was washed twice with ice-cold PBS and fixed for 1 h with 10% formalin. Rehydrated with 1 ml of distilled water for 5 min. Then 1% alizarin red in 2% ethanol (pH 4.0) was added and allowed to stand for 5 min at room temperature. The monolayers were then washed five times using distilled water. Then 70% ethanol was added to the monolayer and allowed to stand for 2 min. Then the monolayer was covered with a layer of distilled water. Calcified nodules which appeared as bright red color were identified by light microscopy. After examining under microscope, the mineralized matrix with alizarin red stain was quantified. To quantify matrix mineralization, the alizarin red stained cultures were incubated with 100 mM cetylpyridinium chloride for 1 h to solubilize and release calcium-bound alizarin red into solution. The absorbance of the released alizarin red S was measured at 570 nm using a spectrophotometer.

ANALYSIS OF mRNA EXPRESSION

Total RNA was extracted from control and *C. quadrangularis* treated SaOS-2 cells using the Trizol Reagent (Sigma) at indicated time points. The concentration and purity of total RNA were determined by absorbance at 260/280 nm in a UV-spectrophotometer. If the ratio of A260/280 is 1.8–2.0, then 1.5 μg of total RNA was used for reverse-transcriptase polymerase chain reaction (RT-PCR) analyses. RT-PCR was carried out using one-step RT-PCR kit in a total reaction volume of 50 μl. The first strand synthesis was carried out at 50°C for 30 min using gene-specific oligonucleotide primers for Runx2, ALP, and collagen followed by the initial PCR activation at 95°C for 15 min. The primer sequences used in the present study were listed in Table I. The three step PCR cycles consisted of denaturation at 94°C for 1.5 min, annealing at 55°C for 1.5 min and extension at 72°C for 1.5 min. The PCR amplification was carried out up to 30 cycles and to ensure that the products are extended completely, a final extension at 72°C for 10 min was carried out. Gene-specific oligonucleotide primers for the house-keeping gene β-actin or GAPDH was added to the same PCR reaction vial and co-amplified. RT-PCR product (5 μl) was taken from each reaction tube, mixed with gel loading dye and resolved in a standard 2% agarose gel containing ethidium bromide (0.5 μg/ml) under an electrical field (60 mA and 80 V) for 2.5 h. Molecular weight DNA marker (100 bp ladder) was simultaneously resolved in the first lane. After electrophoresis, the gel was subjected to densitometric scanning and the band intensity of cDNA fragment of each gene of interest was normalized against the band intensity of cDNA fragment of the house-keeping gene, β-actin or GAPDH using Quantity One software (Bio-Rad, USA).

WESTERN BLOT ANALYSIS

Whole cell extracts were prepared as described previously [Selvamurugan et al., 2004]. The proteins were resolved by 12% SDS–PAGE. The proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). After blocking in Tris-buffered saline containing 5% (w/v) nonfat dry milk, the membrane was exposed to Runx2 primary antibody overnight at 4°C. The membrane was washed and exposed to horseradish peroxidase conjugated secondary antibody. The immunoreactive signals were visualized using an enhanced chemiluminescence detection kit (Sigma). The experiments were carried out at least three times.

ESTIMATION OF OSTEOCALCIN

The levels of osteocalcin in the conditioned medium of control and *C. quadrangularis* treated SaOS-2 cells were estimated using immuno radiometric assay (IRMA) kit (DSL, USA; sensitivity, 0.3 ng/ml; intra- and inter-assay CV, 1.4–3.4% and 3.3–5.3%, respectively).

CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) assay was carried out using the ChIP kit from Upstate Biotechnology as described previously [Selvamurugan et al., 2004]. Soluble chromatin was prepared. Aliquots (1/100) of total chromatin DNA before immunoprecipitation were saved (input). Pre-cleared lysates were used for immunoprecipitation experiments with Runx2 antibody overnight at 4°C. The immunocomplexes were eluted by adding a 250-μl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$. Samples were sequentially digested with RNase A (10 mg/ml) at 37°C for 1 h and proteinase K (20 mg/ml) at 42°C for 2 h to remove RNA and protein. The crosslinking reaction was reversed by 4 h incubation of the sample at 68°C, and the DNA was recovered. An

TABLE I. List of Primer Sequences Used in the Study

Gene	Forward primer	Reverse primer	Size	Accession no.
ALP	ACCTCGTTGACACCTGGAAG	CCACCATCTCGGAGAGTGAC	189	NM_000478
Collagen	GGCCCAGAAGAACTGGTAC	CGCTGTTCTTGCAGTGGTAG	200	NM_000088
Runx2	CAGTTCCCAAGCATTTCATCC	TCAATATGGTCGCCAAACAG	444	NM_001015051
β-Actin	CAGAGCAAGAGAGGCATCC	TAGCAGAGCCTGGATAGCAA	246	NM_001101
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC	598	NM_002046

aliquot of each DNA fraction was used for semi-quantitative PCR to detect the presence of specific DNA segments.

The sequences of the human osteocalcin promoter oligonucleotide primer used in this study are as follows:

Sense	5′ GAG CCG GGC AGT CTG ATT GTG 3′
Antisense	5′ GCA TGC CTG ACC ATG GTG CGG 3′

STATISTICAL ANALYSIS

All data reported in this report were generated using in vitro assays. The significance of the observation was estimated by Student's *t*-test, using data from at least three independent replicates. The observation was deemed significant if the probability of accepting null hypothesis is ≤ 0.05 (indicated by "*" in the figures).

RESULTS

NONTOXIC EFFECT OF *C. QUADRANGULARIS*

Cytotoxicity of the drug was assessed by assaying the levels of lactate dehydrogenase which is a cytosolic enzme, upon changes in membrane integrity it is released into the conditioned medium. The activity of LDH in the conditioned media of control and *C. quadrangularis* (0.1, 1, 10, and 100 μg/ml) treated osteoblastic cells (rat calvarial and human osteoblast like cells) did not differ significantly after 72 h and hence it can be stated that the ethanolic extract of *C. quadrangularis* is non toxic to osteoblastic cells (Fig. 1A,B).

C. QUADRANGULARIS INCREASES OSTEOBLAST PROLIFERATION

As an index of cell proliferation, the DNA synthesis rate was evaluated by measuring the incorporation of [^{3}H] thymidine into the DNA. The osteoblastic cells, plated at a density of 6×10^4 cells/well in 24-well plates were treated with serially diluted *C. quadrangularis* for 48 and 72 h under serum free conditions. To test whether the cells had just incorporated [^{3}H] thymidine into DNA or they underwent proliferation, the cells harvested from culture wells were also counted manually using hemocytometer following the method described by Morgan and Darling [1993]. The results of cell counting were consistent with that of [^{3}H] thymidine uptake. Therefore, data on thymidine uptake alone is presented. Finally MTT assay was performed to ascertain the viability and metabolic activity of the cells in control and treated wells. Different doses of ethanolic extract of *C. quadrangularis* (0.1, 1, 10, and 100 μg/ml) were used to test its effect on proliferation of human osteoblastic SaOS-2 cells and rat calvarial cells (Suppl. Figs. 1 and 2). A stimulatory effect on osteoblast proliferation was observed when cells were treated with *C. quadrangularis* and the maximum stimulation was seen at 1 and 10 μg/ml dose levels after 48 and 72 h. The mitogenic effect of *C. quadrangularis* were both time and dose dependent. The percent increase in the proliferation after 48 h was 21%, 42%, 80%, and 36% in human osteoblastic cells and in rat calvarial cells it was 24%, 62%, 90%, and 80%. After 72 h, the percentage increase was 14%, 37%, 65%, and 52% in human osteoblastic cells and in rat calvarial cells it was 19%, 39%, 68%, and 47% at 0.1 μg/ml, 1 μg/ml, 10 μg/ml and 100 μg/ml dose levels, respectively (Figs. 2 and 3).

C. QUADRANGULARIS INDUCES DIFFERENTIATION AND MATRIX MINERALIZATION OF SaOS-2 CELLS

The effects of different doses of *C. quadrangularis* on the specific activity of ALP in SaOS-2 cells were tested. The specific activity of ALP was significantly ($P < 0.05$) increased in 1 and 10 μg/ml treated wells and no change at 100 μg/ml dose levels after 48 h (Fig. 4A). These results suggest that *C. quadrangularis* at 1 and 10 μg/ml induces the early differentiation in human osteosarcoma cells, as ALP is an early differentiation marker of osteoblasts. Thereafter the activity started declining at 100 μg/ml dose levels when compared with 10 μg/ml. The activity of ALP was maximum at 1 and 10 μg/ml, since maximum proliferation was also observed at these doses, these doses were considered effective doses and further studies were carried out at these doses alone. Then the activity of ALP was determined after days 10 and 17. Maximum ALP activity was evidenced at day 17 and *C. quadrangularis* at 1 and 10 μg/ml dose levels promoted the ALP activity after 2, 10 and 17 days of treatment

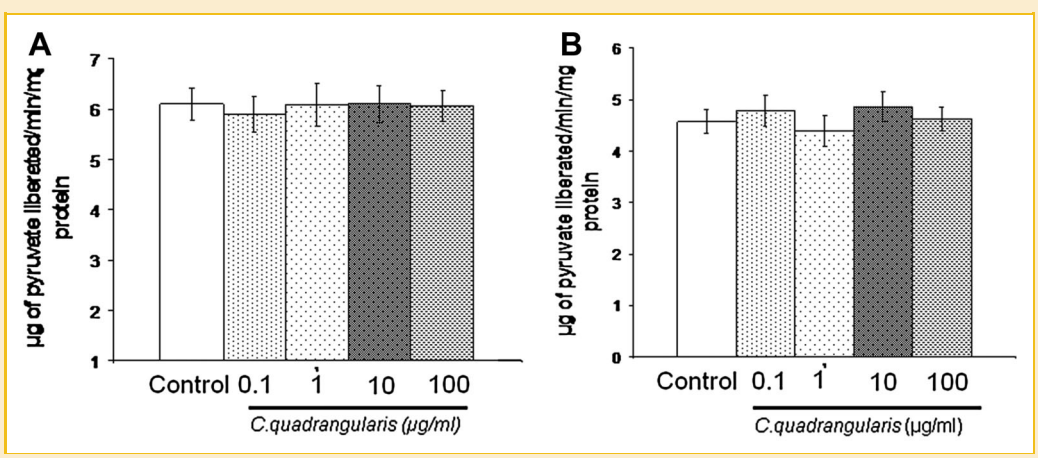

Fig. 1. Effects of ethanolic extract of *C. quadrangularis* on the activity of LDH in the conditioned medium of SaOS-2 (A) and rat calvarial osteoblastic (B)cells after 72 h. Each column and bars represent the mean and standard deviation of three separate experiments for each data point.

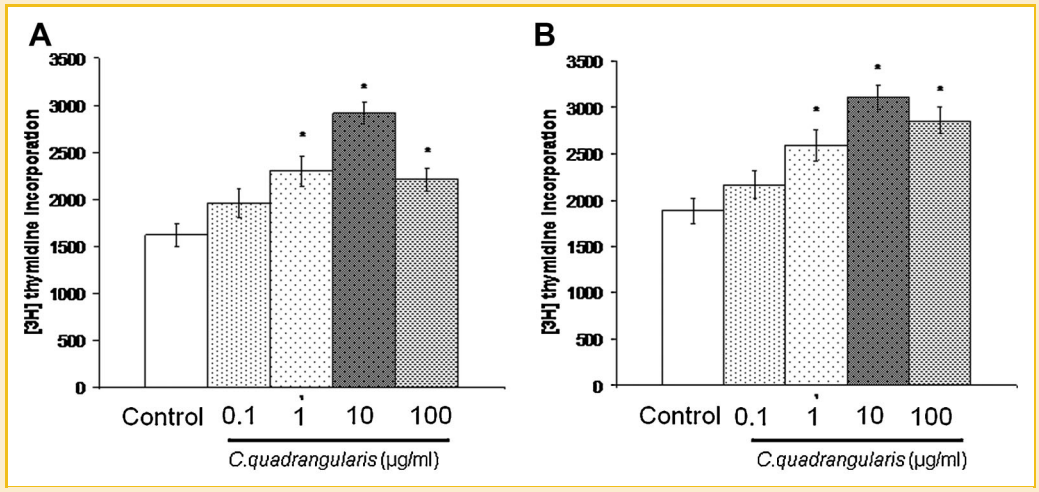

Fig. 2. Effects of ethanolic extract of *C. quadrangularis* on the SaOS-2 osteoblast cell proliferation after 48 h (A) and 72 h (B). [^{3}H] Thymidine incorporation assay was used to measure the cell proliferation as described in the Materials and Methods Section. Each column and bars represent the mean and standard deviation of three separate experiments for each data point. "*" Denotes statistical significance when compared with control.

(Fig. 4B–D) in cells cultured with osteogenic medium with or without *C. quadrangularis*.

In order to identify whether differentiation marker proteins were regulated by *C. quadrangularis*, cells were treated with osteogenic medium (10 nM Dexamethasone + 10 mM β glycerophosphate + 50 μg/ml ascorbic acid) and treated with ethanolic extract of *C. quadrangularis* for 14 days. The cells were then used for the analysis of mRNA expression of ALP and collagen. The cells treated for 21 days are used for the estimation of calcium, collagen, and ALP staining in the mineralized matrix.

A significant increase in the mRNA expression of ALP and collagen was evidenced as revealed by RT PCR analysis. The percentage increase in the mRNA expression of ALP was found to be 53% and 105% at 1 μg/ml and 10 μg/ml dose levels (Fig. 5A). The mRNA expression of collagen was increased significantly in cells treated with *C. quadrangularis* when compared with untreated cells. The percentage increase in the mRNA expression of collagen was 85% and 106% in 1 μg/ml and 10 μg/ml treated cells, respectively (Fig. 5B).

C. QUADRANGULARIS INDUCES BONE NODULE FORMATION

The mineralization of extracellular matrix and the formation of bone nodules are osteoblastic phenotypic markers and represent the final stages of osteoblastic differentiation. Therefore, bone nodule formation and calcium content were assessed in SaOS-2 cell cultures to establish the differentiation end point of these cells. In the mineralization studies, cells treated with *C. quadrangularis* increased the bone nodule formation qualitatively and quantitatively which is revealed by ALP staining and an increase in calcium mineral content, respectively.

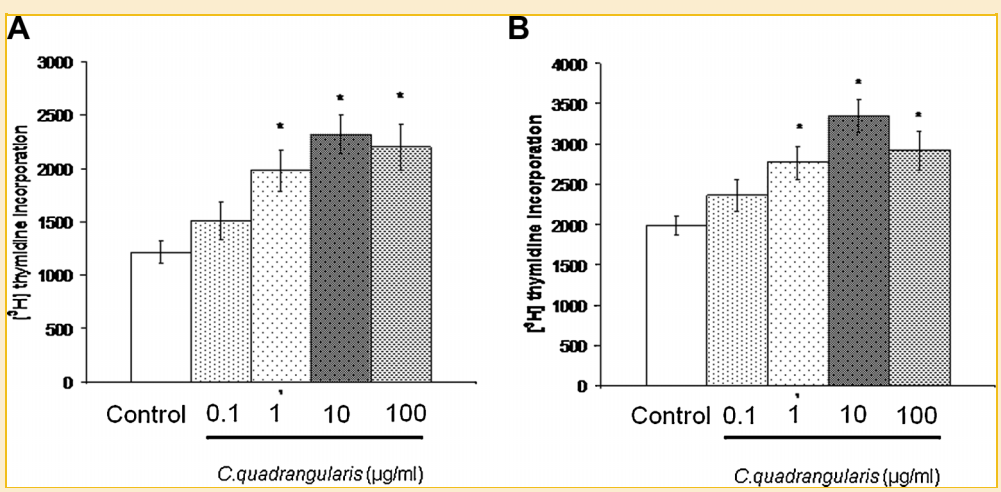

Fig. 3. Effects of ethanolic extract of *C. quadrangularis* on the proliferation of osteoblasts derived from rat calvaria after 48 h (A) and 72 h (B). [^{3}H] Thymidine incorporation assay was used to measure the cell proliferation as described in the Materials and Methods Section. Each column and bars represent the mean and standard deviation of three separate experiments for each data point. "*" Denotes statistical significance when compared with control.

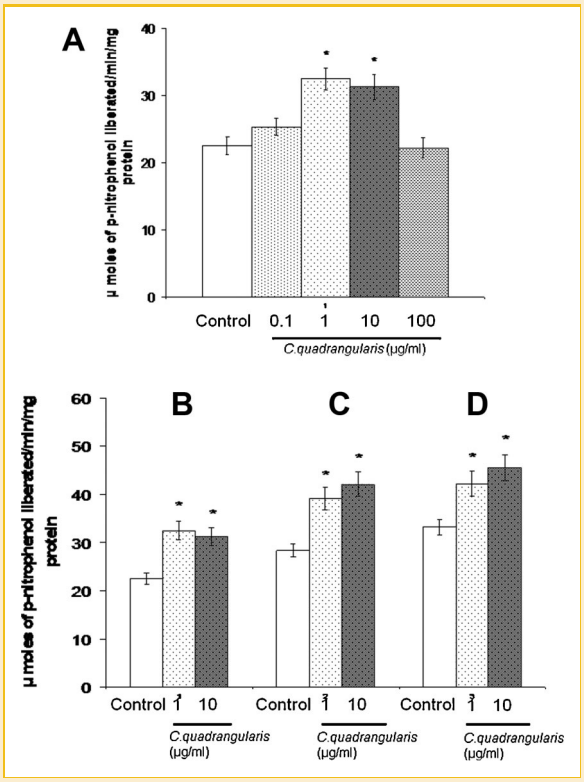

Fig. 4. Effects of ethanolic extract of *C. quadrangularis* on the differentiation of ALP activity of SaOS-2 cell for 48 h at 0.1, 1, 10, and 100 μg/ml dose levels (A). The ALP activity was then measured at 1 μg/ml and 10 μg/ml dose levels after day 2 (B), day 10 (C), and day 17 (D). Each column and bars represent the mean and standard deviation of three separate experiments for each data point. "*" Denotes statistical significance when compared with control.

The mineralized bone nodule formation in SaOS-2 cells cultured for 21 days in osteogenic medium with *C. quadrangularis* is shown in Figure 6. The mineralized nodules appear as red patches depending upon their status of matrix maturation and mineral deposition. The osteoblasts which are positive for ALP appear in red color as the fast red BB salt-ASMX-phosphate complex is acted upon by the activity of ALP. More ALP positive colonies were observed in *C. quadrangularis* treated osteoblastic cells. The amount of calcium deposited in the extracellular was determined using alizarin S red staining, treatment with *C. quadrangularis* has increased ($P < 0.05$) the levels of calcium in the matrix in a dose dependent manner (Fig. 7A,B). The bone nodule formation induced by *C. quadrangularis* in rat calvarial cells is shown in Suppl. Figure 3.

EFFECT OF *C. QUADRANGULARIS* ON THE EXPRESSION AND DNA BINDING ACTIVITY OF Runx2

Runx2 is a transcription factor that has been described as an important master regulatory gene controlling osteogenesis. This molecule acts through the activation of several downstream bone associated genes such as Osterix and OCN to promote osteogenic differentiation and subsequent matrix mineralization. In the present study, the effects of *C. quadrangularis* on the expression and transcriptional activity of Runx2 were studied. In this regard, SaOS-2 cells were treated with *C. quadrangularis* for 48 h and the monolayer was used for RT-PCR, Western blotting, and ChIP assay analyses of Runx2.

C QUADRANGULARIS INCREASES Runx2 mRNA AND PROTEIN EXPRESSION

C. quadrangularis significantly increased the Runx2 mRNA expression in SaOS-2 cells by 65.9% and 118% at 1 and 10 μg/ml respectively when compared with control after 48 h (Fig. 8A). The increase in the mRNA expression is 32.6% and 47.8% at 1 and 10 μg/ml respectively when compared with control after day 7 (Fig. 8B). The increase was dose dependent. The mRNA expression did not vary significantly after day 14 (Fig. 8C). Western blot analysis was carried out to investigate the effects of *C. quadrangularis* on Runx2 protein expression. The representative blot is shown in Figure 8D–F. *C. quadrangularis* significantly increased the Runx2 protein expression by 55.8% and 137% at 1 and 10 μg/ml dose levels respectively, when compared with control (Fig. 8D). The increase in the protein expression is 64.2% and 94.7% at 1 and 10 μg/ml respectively when compared with control SaOS-2 cells after day 7 (Fig. 8E). The protein expression did not vary significantly after day 14 (Fig. 8F).

C. QUADRANGULARIS INCREASES THE TRANSCRIPTIONAL ACTIVITY OF Runx2

C. quadrangularis treatment has significantly increased both the mRNA and protein expression of Runx2 in SaOS-2 cells. Therefore, an attempt has been made to check the significance of this increase and functional activity of Runx2. In this regard, the DNA binding activity of Runx2 to OSE2 elements present in the osteocalcin promoter region was studied using ChIP assay in SaOS-2 cells after 48 h treatment with/without *C. quadrangularis*. The results revealed the existence of Runx2 interaction with OC promoter region even at basal levels and *C. quadrangularis* treatment increased the interaction at 1 and 10 μg/ml dose levels respectively, when compared with control (Fig. 8C).

The conditioned medium of the 21 days cultures were used for the analysis of osteocalcin. A significant dose dependent increase in osteocalcin was observed on day 21. The increase in the level of osteocalcin after *C. quadrangularis* treatment was dose dependent. The percentage increase ($P < 0.05$) in the levels of osteocalcin after treatment with 1 and 10 μg/ml of *C. quadrangularis* was 55% and 94%, respectively (Fig. 8D).

DISCUSSION

Promotion of osteoblast activity with drugs that lack toxicity can be a better approach for drug development to treat osteoporosis. In the current study the ethanolic extract of *C. quadrangularis* was considered to evaluate its anabolic activity in osteoblasts. The extract of *C. quadrangularis* is a good source of β-sitosterol, phytoestrogens, β-carotene, flavonoids, calcium, and ascorbic acid [Singh et al., 2007]. The observed increase in the proliferation of

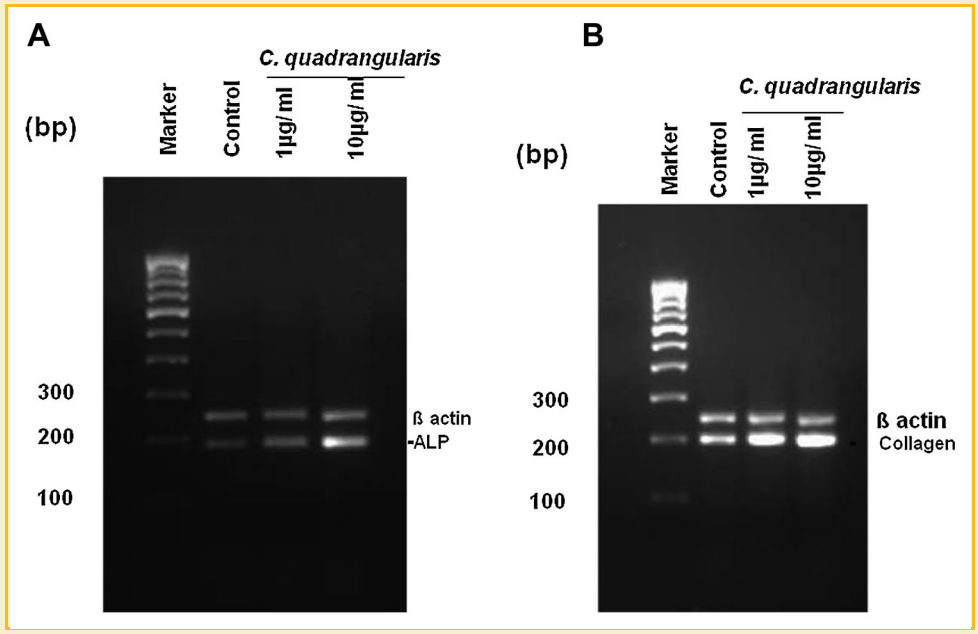

Fig. 5. Cells were lysed using TRIZOL and used for RNA isolation. The isolated RNA were then subjected to RT-PCR analysis. Representative agarose gel electrophoresis pattern of RT-PCR products showing the effects of ethanolic extract of *C. quadrangularis* on ALP (A) and collagen (B) mRNA expression in SaOS-2 cells. Cells were treated with *C. quadrangularis* at specified concentration for 14 days. Expression of ALP and collagen mRNA were determined by RT-PCR analysis. β-Actin is used as internal control. M, DNA marker; bp, base pairs.

osteoblast cells derived from rat calvaria and SaOS-2 osteoblast like cells may be due to the stimulatory effects of β-sitosterol and ascorbic acid as they were reported to increase the proliferation of MC3T3-E1 murine pre-osteoblastic cells [Carinci et al., 2005], human osteoblasts [Oh et al., 2003] and rat osteoblasts [Schmid et al., 2005].

The increase in ALP activity due to *C. quadrangularis* treatment is consistent with a recent observation made in MC3T3-E1 cells [Parisuthiman et al., 2009]. The plant extract has been shown to possess β-carotene (267 U/100 g), a precursor of retinoic acid. Retinoic acid has been shown to increase the mRNA expression of ALP in MG-63, U2OS, and SaOS-2 cells [Gianni et al., 1991; Orimo and Shimada, 2005]. The flavonoid component quercetin identified in *C. quadrangularis* has been shown to stimulate ALP activity in MG-63 human osteoblastic cell line and primary human osteoblasts [Prouillet et al., 2004]. In addition to these in vitro observations, the positive effects of *C. quadrangularis* on ALP activity has also been reported during fracture healing in adult dogs [Chopra et al., 1975]. Taken together, the increase in ALP activity due to *C. quadrangularis* supports the earlier views on the anabolic effects of *C. quadrangularis* on fracture healing and bone formation.

Ascorbic acid stimulates the formation of collagen matrix at multiple levels that include gene expression, hydroxylation of proline and lysine in collagen during post-translational modification [Franceschi et al., 1994]. Further, ascorbic acid is an essential

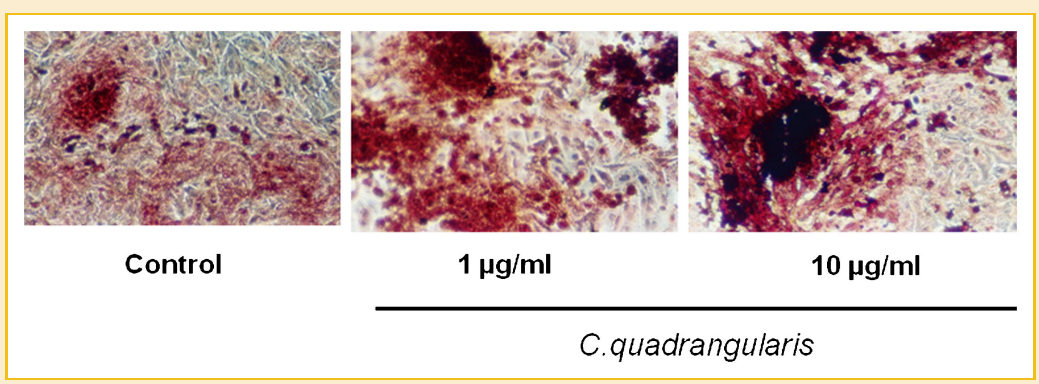

Fig. 6. Effects of ethanolic extract of *C. quadrangularis* on ALP staining in the mineralized matrix of SaOS-2 cells. ALP staining was performed using napthol ASMX as substrate. The osteoblasts which are positive for ALP appear in red color as the fast red BB salt-ASMX-phosphate complex is acted upon by the activity of ALP. The mineralized nodules appear as red patches depending upon their status of matrix maturation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

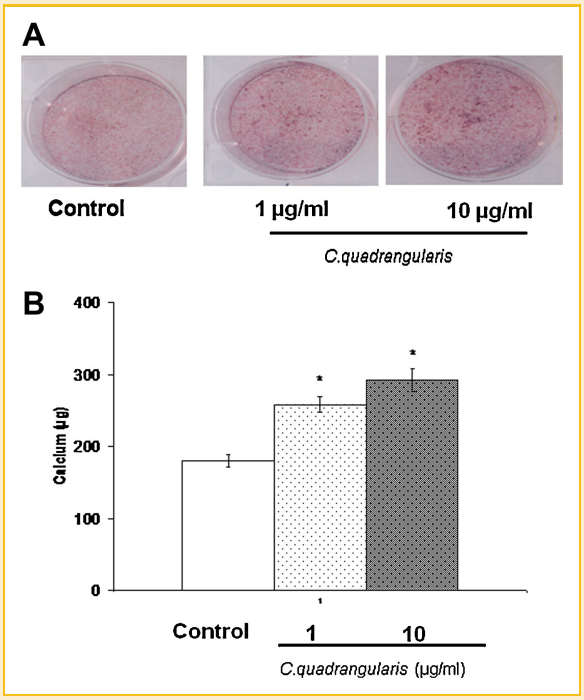

Fig. 7. Effects of ethanolic extract of *C. quadrangularis* on the levels of calcium in the mineralized matrix of SaOS-2 cells. The mineralized matrix was stained with alizarin red and representative well were shown (A) as described in the Materials and Methods Section. After staining, the dye was extracted using cetylpyridinium chloride and quantified (B). Each column and bars represent the mean and standard deviation of three separate experiments for each data point. "*" Denotes statistical significance when compared with control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

constituent of osteogenic medium used to induce differentiation of osteoblasts and mineralization of matrix secreted by osteoblasts [Franceschi and Iyer, 1992; Franceschi et al., 1994; Takeuchi et al., 1996, 1997]. In the present study, the increase in collagen expression after *C. quadrangularis* treatment is in correlation with these earlier observations.

Osteocalcin is a late marker of osteoblastic differentiation that is closely related to osteoblastic maturation and matrix mineralization [Franceschi and Iyer, 1992; Pockwinse et al., 1992; Aubin et al., 1995]. Data from osteocalcin deficient mice suggest that osteocalcin may limit in vivo bone formation [Ducy et al., 1996]. The present study demonstrated a dose dependent increase in the levels of osteocalcin in the conditioned medium in response to *C. quadrangularis* at 21 days of SaOS-2 cell culture. Luteolin, a flavonoid present in *C. quadrangularis* has been shown to increase the production of osteocalcin, collagen, and ALP in mouse osteoblastic cells [Choi, 2007]. Osteocalcin synthesis in osteoblasts is mediated by Runx2. Runx2 binds to OSE2 elements present in the promoter region of osteocalcin and increases its expression. *C. quadrangularis* treatment has increased the DNA binding activity of Runx2 to OSE2 elements present in the promoter region of osteocalcin. The increase in osteocalcin levels has supported increased mineralization as evidenced by quantification of Alizarin red staining in the present study.

The ability to form an extracellular matrix that can undergo regulated mineralization is the ultimate phenotypic expression of an osteogenic tissue. *C. quadrangularis* increased the mineralized nodule formation at 21 days of SaOS-2 cells and rat calvarial cells when compared with untreated cells. *C. quadrangularis* treatment has significantly increased the calcium content in the mineralized matrix. The plant possesses high calcium content, another suspected component as the bone stimulator. Yamaguchi et al. [2005] have suggested the stimulatory effect of calcium (2.8–3.8 mM) in the mineralization of MC3T3-E1 cells. Calcium being one of the key substrate involved in the formation of mineralized nodules, presence of calcium in the plant extract could have motivated the matrix mineralization in long term culture. Even though the amount of calcium present in the extract is in micro molar concentration (1 and 0.1 µM in 10 and 1 µg/ml extract) which is very low than the above mentioned studies, the possibility of calcium influence on matrix mineralization cannot be ruled out.

Runx2 binds to the osteoblast specific *cis* acting element (OSE) which is found in the promoter region of all major osteoblast specific genes like osteocalcin, type-I collagen, BSP, OPN, ALP and control their expression [Ducy et al., 1999]. SaOS-2 represents one of the cell lines available to specifically study gene regulation by Runx2 [Bertaux et al., 2006] since expression of neither Runx1 nor Runx3 was detected in these cells [Sudhakar et al., 2001]. The rise in Runx2 mRNA abundance, its protein expression and DNA binding activity clearly demonstrates that *C. quadrangularis* induces the functional activity of osteoblasts. Runx2 mRNA expression is regulated by two promoters, namely the distal P1 promoter and the proximal P2 promoter [Stock and Otto, 2005]. Sequence analysis failed to reveal any ERE promoter motifs in the human *Runx2* promoter but identified several AP 1 sites that could mediate association of c-fos/c-jun heterodimers suggesting that the upregulation of Runx2 gene transcription in osteoblast by SERM may be under the control of AP 1 promoter activity [Tou et al., 2001]. Osteoblast specific *cis* acting elements OSEs termed OSE1 and OSE2 were also identified in human *Runx2* promoter. Hence, it is reasonable to speculate that the estrogenic compounds such as β-sitosterol present in *C. quadrangularis* could have acted in a manner and increased the binding of *Runx2* to OSE 2 elements in the promoter region of osteocalcin gene in osteoblastic cells. The increase in *Runx2* transcriptional activity might have contributed for the increased mRNA expression of ALP, collagen, and their protein levels. Ethanolic extract of *Rehmannia glutinosa*, a plant which has β-sitosterol as a major constituent has been shown to increase Runx2 expression in human osteoblastic cells [Oh et al., 2003]. Further, flavonoids also have been shown to increase the expression of Runx2 [Chen et al., 2005; Qian et al., 2006]. Thus, β sitosterol and flavonoids present in *C. quadrangularis* may be causal for the increased expression and transcriptional activity of Runx2.

In conclusion, the present study suggests that *C. quadrangularis* regulates osteoblast proliferation, differentiation, matrix mineralization and osteoblast specific transcription factor Runx2. In view of these, *C. quadrangularis* extract could be an valuable nutritional/therapeutic agent in the prevention/treatment of osteoporosis. In the future investigations, inclusive or exclusive protocols for the major

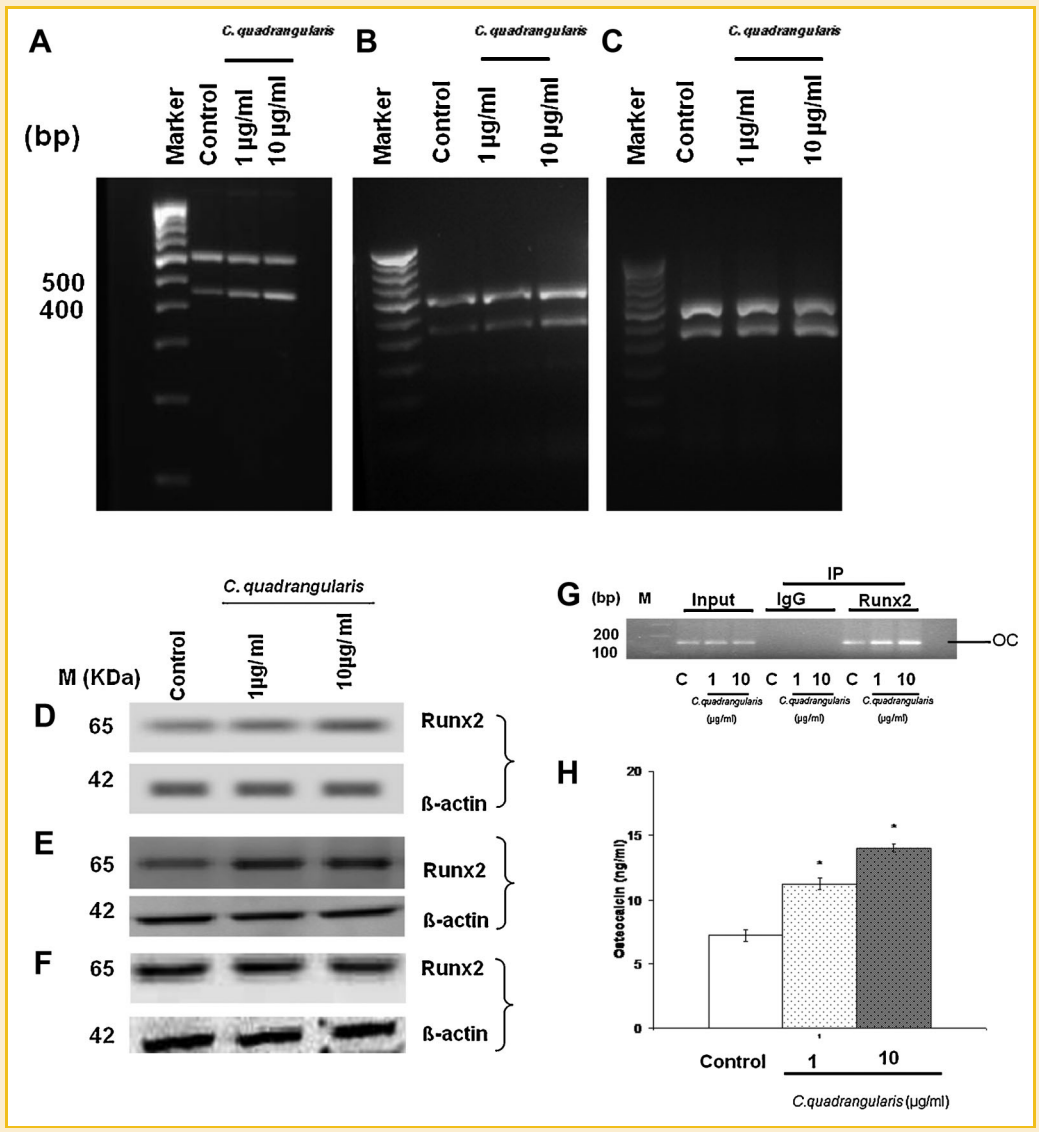

Fig. 8. Cells were lysed using TRIZOL or radio immunoprecipitation assay buffer and used for RNA isolation or Western blot analysis. The isolated RNA were then subjected to RT-PCR analysis. Representative agarose gel electrophoresis pattern of RT-PCR products showing the effects of ethanolic extract of *C. quadrangularis* on Runx2 mRNA expression after days 2, 7, and 14 (A), (B), (C) and protein expression after days 2, 7, and 14 (D), (E), (F) in SaOS-2 cells. GAPDH was used as internal control for mRNA expression and β actin was used as internal control for Western blotting. Cells were fixed with formaldehyde and lysates were prepared as described in the Materials and Methods Section. After immunoprecipitation of the cross linked lysates with either IgG or Runx2 antibody, the DNA was subjected to PCR with primers that amplify the OSE2 region of human osteocalcin promoter (G). Input DNA is the positive control. M, DNA marker; bp, base pairs. Effects of *C. quadrangularis* on the levels of osteocalcin in the conditioned medium of SaOS-2 cells (H). Levels of osteocalcin were determined using Immuno radiometric assay. Each column and bars represent the mean and standard deviation of three separate experiments for each data point. "*" Denotes statistical significance when compared with control.

constituents of *C. quadrangularis* have to be adopted to identify the active principle(s) responsible for the bone anabolic activities.

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