Project Report

“Cissus Quadrangularis Linn. (Asthisamharaka) as a regulator of bone progenitor cells: Molecular and functional basis for osteogenic regenerative medicine”

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Final report

1. Title of the project:
   Cissus Quadrangularis Linn. (Asthisamharaka) as a regulator of bone progenitor cells: Cellular and functional basis for osteogenic regenerative medicine

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6. Implementing institution and other collaborating institutions
   Kasturba Medical College, Manipal University, Manipal

7. Date of commencement – 9/10/10

8. Duration - One year

9. Date of completion - After request for extension of deadline to submit the final report - 15\textsuperscript{th} December 2011

10. Objectives as approved

   1. Effect of Cissus quadrangularis (CQ) and Emblica officinalis (EO) on
      
      o Proliferation of MSCs
      o Differentiation of MSCs into osteoblast
      o Biological activity of osteoblast
      o Differentiation of osteoclast cells
      o Biological activity of osteoclast

   2. Osteoprotective mechanisms of action of these plant extracts singly or in combination, on osteoporosis and also as a preventive measure for osteoporosis using osteoporotic rat model.
11. Deviation made from the original objectives if any, while implementing the project and reasons thereof.

Experiments related to osteoclast isolation and differentiation, evaluation of CQ and EO on their proliferation, differentiation and biological activities did not give any conclusive results even after four repeated attempts. This may be due to technical error and needs further evaluation.

12. Experimental work giving full details of experimental set up methods adopted, data collected, supported by the necessary tables, charts, diagrams and photographs

a) Isolation and culturing of Rat bone marrow mesenchymal stem cells (BMSCs);

Rat bone marrow was collected after the ethical committee clearance, under aseptic precaution, from the bones of the lower limb. Using either Ficoll method or RBC lysis method, bone marrow mesenchymal stem cells (BMSCs) were obtained and cultured in DMEM with 15% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The culture was allowed to grow for 4 days of initial culture. On 5th day the floating cells were removed and then the cells were allowed to grow and when they reach 80% confluence, these cells were either subcultured or cryopreserved for further experiments.

For osteoclast cells – after 3 hr of the bone marrow cell culture, the floating cells were collected and sub cultured.

b) Differentiation of BMSCs into osteoblast

Once the cells are confluent, the stem cell media in which MSCs are growing then be replaced with osteogenic medium (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (Gibco), 1 µM dexamethasone (Sigma), 10 mM µ-glycerol phosphate (Sigma), 0.05 mM ascorbic acid (Sigma), and 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco). The medium was replaced every 3 days and MSCs were differentiated into osteoblasts after 3 weeks.

c) Differentiation of Bone Marrow haemopoietic stem cells into osteoclasts

After 3 hour initial cell culture, the floating cells were collected and subcultured in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) and 10^-8 M vitamin D₃ and 10^-6 M dexamethasone. The osteoclast media was changed every 3-4 day. After culturing for about 10 days, cells were subjected to TRAP (Tartrate-Resistant Acid Phosphatase) staining. Here, we also test the effect of CQ and EO extract on osteoclast differentiation and its role in altering the biological activity of osteoclast.
d) Plant Extraction:

The dried and powered aerial parts of Cissus quadrangularis Linn was thoroughly extracted with 95% ethanol. The ethanol extract was drained out and concentrated under reduced pressure; the produced black residue was suspended with distilled water and the suspension was successively extracted with petroleum ether (b.p. 60–90), benzene, chloroform, acetone, diethyl ether and ethyl acetate. The organic solvent was evaporated and the residue was weighed. The activity assay was conducted for each of these extracts by co-culture with osteoblasts cells in vitro. The dark brown residue of petroleum ether extract was then dissolved in distilled water containing 0.1% DMSO to get the final concentration of 1µg/µl. After filtering this solution using syringe filter, a homogenous mixture was then used for our experiments.

Aqueous extract EO was prepared by dissolving the dried powder in distilled water and decoction was prepared. Decoction was then filtered after 48hours, and to the filtrate 2 to 3 drops of chloroform was added, finally the filtrate was evaporated to dryness. The dried powder was then dissolved in sterile water with appropriate concentration to prepare the stock solution. The diluted stock solutions were used for experiments.

e) Treatment with plant extract for in vitro assay:

a) Control  b) Treated with CQ  c) Treated with EO  d) Treated with CQ+ EO

The undifferentiated (mesenchymal stem cells) cells were treated with different doses (CQ - 100,200,300µg/ml and EO- 25, 50, 100 µg/ml of media) of the plant extract for 1-15 days. These doses were fixed after the acute toxicity studies using MTT assay.

f) Staining methods

f1. Alkaline phosphatase staining - Above said 4 group of cells were grown in 24-well plates, were washed with PBS twice, fixed with 4% paraformaldehyde and air dried. The fixed cells were treated with ALP substrate and counterstained with Methyl Green.

f2. Von Kossa staining (to determine the extent of bone mineralization): The media was aspirated from the cultures (4 group) grown in 24 well plates and frozen at -80°C for 10-15 minutes. Then these cells were rinsed with distilled water and dried completely. Cells were fixed in 95% ethanol and treated with 5% silver nitrate (AgNO₃) and was incubated in a cool and dark place for 10-20 minutes. These cells were then treated with 0.5% hydroquinone (aqueous) and exposed to UV for 5 minutes.
After rinsing with distilled water, 5% sodium thiosulphate was added to these cells and swirled for 1 min. Then the cells were counter stain with methyl green stain for 1 min.

g) Cell count - After trypsinising the cells, the live cells in the suspension were counted using 0.04% of trypan blue and hemocytometer.

h) MTT assay - Ten thousand MSCs were plated in 96 well plates with stem cell media. After cells have attached to the plate, cells are then treated as follows a) only with stem cell media b) stem cell media + different doses of plant extract. After incubation for 24, 48 & 72 hrs MTT stock solution was added to each well and was incubated for 4 hours at 37°C. Then, the formazan crystals were dissolved either in DMSO in lysis solution (1:24 ratio of HCl: isopropanol) and incubated for 1 hr at room temperature. The absorbance of the colored solution was measured using ELISA plate Reader at wavelength 570nm. Readings were analyzed appropriate computer software program.

II. In vivo study

a. Animals - All the studies were conducted with proper approval from the Institutional Animal Ethical Committee, Kasturba Medical College, Manipal, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Virgin female Wistar rats weighing approximately 225 g were housed in the Central Research animal research facility of Manipal University. The rats were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. The animals were maintained under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and a 12 hour light–dark cycle. All animals were allowed free access to distilled water and fed on a commercial diet.

b. Osteoporotic rat model - Overiectomised female rat are known to develop osteoporosis due to lack of Oestrogen. Ovaries were removed from 21 day old female mice using aseptic surgical methods. The onset of osteoporosis was confirmed by histopathological study of the decalcified bones looking for osteoporotic changes in the bone formation.

c. CQ, EO treatment for the osteoporosis rat model - Once osteoporosis is confirmed, rats were treated with different doses of plant extracts for different duration. Here, we also tested the preventive role of CQ on osteoporosis.

To identify the reference dose of CQ and EO, the healthy Wister rats of either sex weighing 150-200 g maintained under standard laboratory conditions were used for acute toxicity test according to
OECD guidelines 425 (OECD guidelines 2000). The animals were treated with single dose of 100 to 1000mg/kg body weight of petroleum ether extract of CQ and 50-500mg/kg body weight of aqueous extract of EO (3 animals per group). Animals were kept overnight fasting prior to treatment. After administration of extract, food was withheld for further 3-4 hrs. Then these animals were observed individually at least once during first 30 min after dosing, periodically during first 24 hrs. (with special attention during the first 4 hrs.) and daily thereafter for a period of 14 days. Observation were done daily for change in skin and fur, eyes and mucus membrane (nasal) respiratory rate, circulatory signs (heart rate and blood pressure), autonomic effects (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremors, and convulsions) changes. Based on the changes observed the reference dose for CQ was fixed as 500mg (per kg body weight/day) and for EO 200mg (per kg body weight/day).

Experimental animals were divided randomly into five groups of six animals each. Group 1 was sham operated and served as basal control. All the other groups were ovariectomized and received treatment for 3 months starting from the fifteenth day of ovariectomy. Group 2 received vehicle and served as ovariectomized control. Group 3 was orally administered Raloxifen (5.4 mg/kg body weight/day) (Dr. Reddy’s Laboratory, Hyderabad, India). Groups 4 was treated with 500mg (per kg body weight/day) of CQ, group 5 with 200mg (per kg body weight/day) of EO and group 6 with both reference doses of CQ and EO was treated orally for 30 days. At the end of the treatment blood samples from all the groups were withdrawn by tail vein method to assess biochemical parameters. The animals were then sacrificed using sodium pentathionate and bones were isolated for histopathological studies.

d. Prenatal exposure to CQ - After confirmation of pregnancy, the pregnant rats were treated with the reference dose of plant extracts for 5-16 days of gestation period. Female offspring were collected at the end of gestation and are subjected to ovariectomy at the 8th week of age. Onset of osteoporosis was confirmed by histological studies and effect of prenatal exposure to CQ was assessed.

e. Histopathological evaluation –

The right femur was collected from all groups of animals were 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 and decalcified in EDTA-G solution (14.5 g EDTA, 1.25 g NaOH, and 15 ml glycerol were dissolved in distilled water and the pH was adjusted to pH 7.3) then bone tissues will be dehydrated in a graded series of alcohols and embedded in paraffin.
wax. The 5micron thick sections were stained with H &E, ALP, Van Kosa and Trichrome staining for Collagen fibres. Sections were also evaluated for the trabacular pattern, thickness and collagen architecture and bone density. The bone thickness was measured using Image J software.

f. Biochemical parameters

f1. Serum calcium - The test was carried out by using diagnostic reagent kit (Span diagnostic Ltd., Surat, India) for the in vitro determination of serum calcium. The calcium present in the serum was precipitated with naphthyl hydroxamic acid (calcium reagent). The precipitate will then be dissolved in EDTA reagent and calcium from this solution was complexed with color reagent to give a colored complex that was measured colorimetrically.

f2. Serum alkaline phosphatase (ALP) - In vitro determination of ALP by Kind and King’s method was carried out by using diagnostic reagent kit (Span diagnostic Ltd.). ALP from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in presence of oxidizing agent potassium-ferricyanide and forms an orange red colored complex, which can be measured colorimetrically. The color intensity is proportional to enzyme activity.

f3. Tartrate resistant acid phosphatase (TRAP) - In vitro determination of TRAP by King’s method was carried out by using diagnostic reagent kits (Span Diagnostic Ltd.). Acid phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 4.8. Phenol so formed reacts in alkaline medium with aminoantipyrine in presence of oxidizing agent potassium-ferricyanide and forms an orange red colored complex, which can be measured colorimetrically. The color intensity is proportional to enzyme activity. Since tartrate inhibits the prostatic fraction of the enzyme, the difference in acid phosphatase activity without and with tartrate represents the activity of prostatic fraction.

g. Statistical analysis: All groups were compared using oneway ANOVA and performed Bonferroni’s post test to compare with in the groups.
13. Detailed analyses of results indicating contributions made towards increasing the state of knowledge in the subject

I. Effect of prenatal exposure of CQ on induced osteoporosis in postnatal life

**Figure 1.** Pups born to CQ administered mother (CQ) had longer femur when compared to control (NC) (*p<0.05) indicating the positive effect of CQ on fetal bone growth. However, when osteoporosis was induced in the pups born to the untreated mother (OVX), the length of the femur was slightly higher than that of normal control and sham operated pups due to initial compensatory mechanism ($P<0.05$). Further, induction of osteoporosis in pups born to the CQ administered mother (CQ+OVX) did not show any decrease in the length of the femur. Instead, they had significantly longer femur resulting due to additive effect of initial compensatory mechanism and beneficial role of prenatal exposure to CQ (SHAM vs. CQ+OVX= #P<0.05 and OVX vs. CQ+OVX= βP< 0.05).

**Figure 2.** Total weight of the dried femur was significantly high in the pups born to mother treated with CQ (CQ) in comparison with that of normal control (NC) group (*p<0.05). Further, sham operation (SHAM) did not alter the weight of the femur. However, when osteoporosis was induced in the pups born to the untreated mother (OVX), the weight of the femur was significantly lesser than
that of normal and sham operated pups ($p< 0.05$). Interestingly, when osteoporosis was induced in pups born to the CQ treated mother (CQ+OVX), the weight of the femur was almost near to normal and better than that of pups born to untreated mother ($\beta \ p< 0.05$). These results indicate that the prenatal exposure to CQ significantly facilitate the peak bone mass formation in the fetal life thus preventing the deterioration of bones when these animals were subjected to osteoporosis later in life.

**Figure 3.** An average thickness of the trabecular bone in the epiphysial end of the femur was nearly 60µm in pups born to untreated mother (NC) and was significantly increased to nearly 100µm in pups born to CQ treated mother (*$p< 0.05$, CQ). However, no change in the thickness of the trabecular bone was found in sham operated pups (SHAM) and remained equal to that of normal group. After the induction of osteoporosis, pups born to untreated mother showed significantly decreased trabecular bones (OVX) when compared to that of sham operated pups ($p< 0.05$). However, such osteoporosis induced destruction of trabecular bone was found to be less in the pups born to the CQ treated mother (CQ+OVX).
Figure 4. An average thickness of the cortical bone in the shaft of the femur was nearly 500µm in pups born to untreated mother (NC) and was significantly increased by nearly 100µm in pups born to CQ treated mother (CQ, *p< 0.05). However, no change in the thickness of the cortical bone was found in sham operated pups (SHAM) when compared to that of normal group. After ovariectomy, pups born to untreated mother (OVX) showed significantly decreased cortical thickness when compared to that of sham operated pups ($p< 0.05$). Further, the cortical bone loss was significantly decreased in the pups born to the mother treated with CQ (CQ+OVX).

Therefore, these results indicate that, the prenatal exposure to CQ increases the bone length, weight and thickness of the cortical and trabecular bones. When the pups born to CQ treated mother were induced osteoporosis in their adult life, the extent of deterioration of bone is significantly decreased when compared with pups born to the untreated mother. This may be because of favorable induction of CQ on optimization of fetal bone growth during osteogenesis period to attain the peak bone mass. This optimization and attaining the peak bone mass during the fetal life slow down the deterioration of bone due to osteoporosis induced in adult life. Therefore, the active ingredients of CQ may cross through the placental barrier and has direct effect on osteogenesis in the fetus. Further evaluation is needed to explore the possible bio-active ingredients of CQ that are crossing the placental barrier and the molecular and cellular mechanisms of osteoprotective actions of these ingredients. Further, the role of CQ on optimization of fetal bone growth induction during osteogenesis period needs to be evaluated.
II. Effect of CQ and EO in prevention of osteoporotic deterioration of the bone in adult osteoporotic model

Figure 5: Representative microphotographs of histological sections of decalcified shaft of the femur in the different experimental groups showing the cortical bone (Indicated by arrow) and medullary cavity (M). Paraffin embedded sections of 5-micron thickness were stained with haematoxylin and eosin. Cortical bone destruction was evident in the ovariectomized (OVX) animals compared to the sham control (SHAM) group. However, when ovariectomized rats were treated with the reference dose of raloxifene (OVX+RAL), CQ (OVX+CQ), EO (OVX+EO) and CQ + EO (OVX+CQ+EO) prevented the cortical bone destruction compared to non-treated ovariectomized (OVX) group. Data presented in graph is Mean ± SEM. The significant difference versus OVX group are indicated as ***P< 0.001.

Figure 7: Representative microphotograph of histological sections of lower end of the femur in different experimental groups showing the trabecular bone (T). Paraffin embedded
sections of 5-micron thickness were stained with haematoxylin and eosin and thickness of trabaculae were measured. Significant reduction in trabecular bone thickness was found in ovariectomized (OVX) group when compared with sham control (SHAM) group. Further, when ovariectomized rats were treated with the reference doses of raloxifene (OVX+RAL), Cissus quadrangularis (OVX + CQ), Emblica officinalis (OVX+EO) and Cissus quadrangularis & Emblica afficinalis together (OVX+CQ+EO), the destruction of the trabecular bone was minimum and the average thickness of trabecular bones were almost similar to that of control group. Data presented in graph as Mean ± SEM. The significant difference versus OVX group are indicated as ***P< 0.001.

These results indicate that the administration of CQ and EO individually on to osteoporosed adult animals has significant preventive effect on bone destruction. CQ may act as bone protector due to its high content phytosteroids which may mimic the action of oestrogen directly influencing the osteoblast activity. Further, the presence of high level of calcium and vitamin C &D may also have an additive effect on preventing the degenerative changes due to osteoporosis by facilitating the non-cellular organic and inorganic substance deposition in the bone matrix. EO, on the other hand, rich in Vitamin C may help in reinforcing the bone with the addition of new collagen fibers and ground substance by influencing the fibroblast activities in the bone lamella thus slowing the bone deterioration during osteoporosis. When CQ and EO were administered together to the animals with osteoporosis, the additive effect on neo-osteogenesis was observed with increase in the bone mass and fibrous component.
III. Effect of CQ and EO on growth plate of the bones

Figure 9: Representative photomicrographs of histological sections of lower epiphysis of the femur in different experimental groups showing the growth plate (arrow). Paraffin embedded sections of decalcified bones at 5-microns thickness were stained with haematoxylin and eosin. Thin growth plate with less ground and fibrous substance in the matrix and incomplete trabecular formation at the elongation zone was observed in the ovariectomized (OVX) group of animals compared to the sham control (SHAM) group. However, when ovariectomized rats were treated with the reference doses of raloxifene (OVX+RAL), CQ (OVX + CQ), EO (OVX+EO) and CQ+EO together (OVX+CQ+EO) growth plates were found to be more thicker than that of OVX group and showed improved trabecular bone formation at the growing end of the bones. Further, administration of EO has increased the hydroxyprolin /chondroitin sulphate / collagen in the growth plate which is evident by the deep blue stained matrix in the growth plate cartilage.

Therefore, these findings suggest that the CQ not only protects the trabecular bone and cortical bones during osteoporosis but also influences the growth plates at the metaphysical ends of the bones to increase the length of the bone. Administration of EO alone or with CQ influences the content of the cartilaginous matrix by
increasing the ground substances and collagen fibers. Therefore, the combination of CQ and EO can influence on better and stronger bone growth at the growing ends of the bone.

IV. Effect of CQ and EO on collagen content of the trabecular and cortical bone

![ Representative photomicrographs of the lower end of the femur in different experimental groups showing the collagen content (blue) in the trabecular bone.](image)

Figure 10: Masson’s trichrome stained representative photomicrographs of the lower end of the femur in different experimental groups showing the collagen content (blue) in the trabecular bone. Decreased trabecular bone formation and their collagen content were observed in the ovariectomized (OVX) animals in comparison to the sham control (SHAM) group. After induction of osteoporosis, treatment with raloxifene (OVX+RAL) increased the formation of trabecular bone but did not alter the collagen content. However, when CQ (OVX + CQ) was administered, thicker trabaculae with more collagen fibers were observed. Whereas, Emblica officinalis (OVX+EO) only altered the collagen content in the trabecular bones without increasing the trabecular bone formation. Further, when CQ and EO administered together, we observed thicker trabecular bone formation with more collagen content in them.
Figure 10: Masson’s trichrome stained photomicrographs of the cortical bone of shaft of the femur in different experimental groups showing the collagen content (blue). Decreased thickness of the cortical bone and their collagen content was observed in the ovarieectomized (OVX) animals in comparison to the sham control (SHAM) group. After induction of osteoporosis, treatment with raloxifene (OVX+RAL) and CQ (OVX + CQ) could increase the formation of cortical bone with increased collagen content in them. Whereas, Emblica officinalis (OVX+EO) only altered the collagen content in the cortical bone without increasing the new cortical bone formation. Further, when CQ and EO administered together, we observed thicker cortical bone with more collagen content in it.

Therefore, both CQ and EO individually can alter the collagen content in the bone. However, the EO has significant influence on collagen content in the bone. Further investigations are needed to evaluate the influence of CQ and EO on fibroblast activity.
V. Effect of CQ and EO on Serum Calcium, ALP, TRAP and Hydroxyprolin content

![Graph showing serum calcium concentration in different experimental groups.](image1)

**Figure 11. Mean serum calcium concentration in different experimental groups.** Serum calcium is significantly increased in osteoporosed rats (OVX) compared to SHAM group due to increase in the bone resorption. However, when such osteoporotic animals were fed with reference dose of Raloxifine (OVX+RAL), CQ (OVX+CQ), EO (OVX+EO) and both CQ & EO, Serum calcium level was brought back to near normal level. Data represented as Mean ± SEM. The significant difference versus OVX group are indicated as ***P< 0.001. *Therefore, treating with CQ or EO singly or in combination can prevent the active resorption in the osteoporosis rat model thus decreases the free calcium in the blood serum.*

![Graph showing mean serum alkaline phosphatase concentration in different experimental groups.](image2)

**Figure 12. Mean serum alkaline phosphatase concentration in different experimental groups.** Serum alkaline phosphatase was significantly increased in osteoporosed rats (OVX) compared to SHAM group as an indication of temporary compensatory mechanism to overcome
the osteoporotic changes. However, serum ALP concentration was further increased in osteoporotic animals treated with reference doses of Raloxifine (OVX+RAL), CQ (OVX+CQ), EO (OVX+EO) and both CQ & EO (OVX+CQ+EO) compared to that of OVX group. Further, the Raloxifine and CQ+ EO treated groups showed high concentration of serum ALP level indicating the increased activity of the osteoblasts in these animals. Data presented as Mean ± SEM. The significant difference versus OVX group are indicated as ***P< 0.001 and *P< 0.05.

These results indicates that, administration of CQ, EO or RAL to the osteoporotic animals increases the activity of the existing osteoblast and/or facilitates the differentiation of stem cells into new osteoblasts resulting in increased serum ALP.

Figure 13. Mean serum tartate resistant acid phosphatase (TRAP) concentration in different experimental groups. Serum TRAP was significantly increased in osteoporosed rats (OVX) compared to SHAM group indicating the active resorption of bone. However, the serum TRAP concentration was decreased in Raloxifine treated (OVX+RAL), CQ treated (OVX+CQ), EO treated (OVX+EO) and both CQ & EO treated (OVX+CQ+EO) groups compared to OVX group. Data presented as Mean ± SEM. The significant difference versus OVX group are indicated as ***P< 0.001. However, decrease in the TRAP concentration in these groups was not significantly differing from that of control group.

Therefore, the active ingredients of CQ or EO can also influence negatively on the osteoclast activity in osteoporotic condition thus slowing the bone degenerative changes. However, further validation is warranted.
Figure 14. Mean serum hydroxyprolin concentration in different experimental groups.
Serum hydroxyprolin was significantly increased in osteoporosed rats (OVX) compared to SHAM group due to active destruction of collagen content of the bone. Serum hydroxyprolin concentration was decreased in Raloxifine treated (OVX+RAL), CQ treated (OVX+CQ), EO treated (OVX+EO) and both CQ & EO treated (OVX+CQ+EO) osteoporotic rat groups compared to OVX group and almost similar to that of SHAM control. Data presented as Mean ± SEM. The significant difference versus OVX group are indicated as ***P< 0.001.

Even though the serum hydroxyprolin levels are not direct indicator of bone metabolism in normal condition, in osteoporosis condition, it is an indicator of bone metabolism. As seen in the result, the serum hydroxyprolin content in CQ, EO and RAL administered group is near to that of control group and significantly lesser than that of osteoporotic animal group indicating the lesser bone destruction in these animals.
VI. Effect of CQ and EO on proliferation of bone marrow MSCs

Figure 15. Results of MTT assay when bone marrow mesenchymal stem cells (BMSC) are grown in different concentration of CQ for 24, 48 and 72 hours. Cells grown in basal media with 100µg and 200µg/ml of CQ showed higher proliferation rate (3 to 4 fold) compared to that of cells with vehicle control indicating the stimulatory influence on BMSCs. Due to large internal variations these data was not statistically significant. Further cells, with 300µg/ml of CQ showed nearly 2 fold growth in comparison with that of control group.

Figure 16. Results of MTT assay when BMSC were grown in different concentration of EO for 24, 48 and 72 hours. Results indicate that EO in all different concentration (25, 50, 75 µg/ml) showed higher proliferation rate compared to cells grown in vehicle control alone. Therefore, EO can alter BMSCs proliferation rate. However, due to large internal variations the statistical analysis did not show any significance.
Figure 17. Results of MTT assay when BMSCs grown in different concentration of CQ and EO together for 24, 48 and 72 hours. Cells grown in different combination/concentration of EO and CQ together showed marked increase in the proliferation rate at 48 and 72 hr of exposure. Even though noticeable influence of EO and CQ on cell proliferation is seen, due to large internal variations the statistical analysis did not show any significance differences.

Therefore, the petroleum ether extract of CQ and aqueous extract of EO has stimulatory influence on proliferation of BMSCs when added to cell culture media at different concentrations and with different combinations. This may be the one of the reasons for the osteoprotective properties of CQ and EO by increasing the availability of BMSCs for differentiation into new bone forming cells – osteoblasts and thus increasing the formation of new bone and preventing the osteoporotic changes in the bone. However, further cellular and molecular evaluation is required to explore the role of CQ and EO on BMSCs.
VII. Effect of CQ and EO on osteoblast

Figure 18. Alkaline phosphatase staining of MSCs. Rat bone marrow mesenchymal stem cells grown in basal media (A) treated with 300µg/ml of CQ (B) showed moderate level of differentiation into osteoblast which is evident by weakly expressing ALP after treating with CQ for 17 days. Further, MSCs grown in osteogenic media with 200µg/ml of CQ (C) and 300µg/ml of CQ (D) showed much stronger ALP staining with 15 days of treatment indicating significant level of differentiation into osteoblast. Normally, BMSCs takes nearly 21 days to differentiate into osteoblast in the osteogenic media. However, cells grown in either basal media or in osteogenic media treated with 100µg/ml of CQ, EO alone and other combination of CQ and EO did not show any differentiation indications.

This result indicates that CQ alone can induce the differentiation of BMSCs grown in basal media (without any additional stimulator) into osteoblasts. Further, CQ can facilitate the early differentiation of osteoblast when BMSCs grown in osteogenic media which additionally contains other stimulants of differentiation. Further, the exact molecular mechanisms involved in CQ mediated differentiation of BMSCs into osteoblast and their activity needs to be evaluated.

VIII. Effect of CQ and EO on osteoclast

Administration of CQ and EO singly or in combinations did not show any differences in differentiation at the end of 20 days of treatment with these plant extracts. All cells in all the groups were stained for TRAP (representative picture is given below). This is may be due to technical error which we could not rectify even after 4 attempts. Therefore, further evaluation is required. However, the increased serum TRAP level in the osteoporotic condition was brought down near to normal level when osteoporotic animals were treated with reference dose of RAL,
CQ and EO. This indicates the negative influence of these drugs/plant extracts on osteoclast activity.

**Figure 19.** Representative photograph of TRAP stained Osteoclast cells.

**IX. Effect of CQ and EO on osteoblast activity (extracellular mineralization)**

**Figure 20.** Rat bone marrow mesenchymal stem cells grown in osteogenic media (A) with addition of CQ at 100 (B), 200 (C) and 300µg/ml (C) showed deposition(black) of minerals in their extracellular matrix when stained at the end of 15 days of treatment. However, most significant deposition was found only in the cells grown in osteogenic media with 300µg/ml of CQ (D). Normally, nearly 28 days are required to differentiate the mesenchymal stem cells in to functional osteoblast. The addition of CQ in to osteogenic media with or without EO shown to be additively facilitating the functional ability of osteoblast cells by increasing the extracellular secretions in which calcium was deposited. No such changes were observed in cells treated with CQ or EO in basal media and cells treated with EO alone. These preliminary results indicate that, the CQ not only influences the proliferation and differentiation of BMSCs but also influences
the biological activities of differentiated osteoblast. However, the results are not satisfactory and needs further evaluation.

14. Conclusions summarizing the achievements and indication of the scope for future work

These preliminary studies show that treating the osteoporotic animals with reference doses of CQ can ameliorate the osteoporotic degenerative changes by increasing the formation of both trabecular and cortical bones. CQ can also influence the growing end of the bones by facilitating the proliferation of growth plate cartilage and differentiation of cartilage in to tabacular bone at differentiation zone of growth plate. These changes are likely due to the stimulatory effect of active ingredients of CQ on the proliferation of bone marrow mesenchymal stem cells (BMSCs) and differentiation of these cells into active osteoblast cells. As seen in the result, addition of CQ to growth medium can stimulate differentiation of BMSCs into active osteoblast cells. The Mesenchymal stem cells grown in osteogenic condition media with different does of CQ showed mineral deposition in their extracellular matrix indicating the increased biological activity of the differentiated osteoblast cells. Further, CQ may also negatively influence the osteoclast cells differentiation and their activities thus slowing the bone resorption in osteoporotic condition. However, the role of CQ on osteoclast activity could not be established with the current sets of experiments and needs further investigations. CQ is known to contain Phytosterol, flavonoids and triterpenoids, vitamin C, quercitin, quercitrin, β-sitosterol, lupeol and freidalin. Therefore, any of these phytosterol, flavonoids along with rich Vitamin C and D can elucidate such osteoprotective effect. However, further evaluation is required to identify the active compounds and their osteoprotective mechanisms. In addition, the offspring born to mother treated with CQ showed less intense degenerative changes when they are subjected to osteoporosis in their adult life. Therefore, the results indicate that the active ingredients of the CQ can cross the placental barrier and has positive effect on optimizing the bone growth in fetal life. Therefore, prenatal exposure to CQ can facilitate to attain the peak bone mass during osteogenesis thus preventing the degenerative changes resulting from osteoporosis in their adult life. However, further research is needed to identify such active ingredients in these plants which crosses the placental barriers and are responsible for osteoprotective action. On the other hand, the EO can alone increase the proliferation of mesenchymal stem cells and influences the collagen content in the bone. Therefore, EO may not directly differentiate the stem cells into osteoblast but helps in
reinforcing the exiting bones by increasing the collagen contents. Further, the role of EO on fibroblast activity needs to be evaluated. When cells administered together with CQ to the osteoporosed rat, additive effect on both bone mass and its collagen contents were observed. Therefore, the combination of CQ and EO can be the best treatment strategies for preventing the osteoporotic changes thus providing the additional quality life span. Osteoporosed animals treated with both CQ and EO singly or in combination showed healthier growth plate with proliferative and differentiation zones indicating the beneficial effect of these two plant extracts on osteogenesis at the growing end of the bone which results in elongation of the bone. Additionally, administering the EO resulted in increase in ground substance and fibrous component in the growth plate thus forming the healthier hyaline cartilage with active growth and differentiation.

The exact mechanism of actions of CQ and EO on osteoblast, osteoclast and fibroblast cells, signaling cascade involved in CQ and EO mediated osteoprotection and finding the bioactive ingredients of these two medicinal plants are needs to be further evaluated.

15. Procurement / usage of equipment - NONE

16. Manuscript for publication (300 words for possible publication in council’s bulletin)

Repair of bone defects secondary to trauma, osteoporosis, osteomyelitis and fracture nonunion, poses a significant problem for many clinicians. Several strategies have been employed to increase the bone healing process in various clinical conditions. As a part of our continuing screening of biologically active natural anti-osteoporotic agents, we have evaluated the efficacy of petroleum ether extract of Cissus quadrangularis Linn. (CQ) and Emblica officinalis (EO) on the osteomodulation. These plants and their medicinal properties have been described in ancient medicinal system such as Ayurveda. Extract of CQ reported to contain phytoestrogenic steroids, ascorbic acid, carotene, calcium, and anabolic steroids and shown to heal the bone fractures in several in vivo studies. Fruits of EO are known to contain vitamin C and antioxidants. Therefore, the present study is aimed to evaluate osteoprotective effect of CQ and EO by assessing the structural and biochemical alteration using an osteoporotic rat model.

Our results show that the prenatal exposure to CQ can prevent the degenerative changes secondary to osteoporosis in their adult life. Therefore, active ingredients of the CQ may cross
the placental barrier and results in optimizing peak bone formation in fetal life which results in less intense degenerative changes when subjected to osteoporosis in their adult life. Additionally, we also found that the administration of CQ can restore the destructed bone mass after the induction of osteoporosis in adult life by inducing the formation of trabecular and cortical bones. In addition, administration of aqueous extract of EO can increase the collagen and ground substance content in the osteoporosed bones and thus strengthening the bones. When tested on bone marrow mesenchymal stem cells (BMSCs), both the plant extracts increased the proliferation rate and CQ in addition, facilitate the differentiation of these stem cells into active osteoblast cells. However, further investigations are required to evaluate the exact mechanisms of action and osteoprotective bio-active ingredients in these plants.

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