Published in final edited form as:

J Cell Physiol. 2019 July ; 234(7): 10300–10314. doi:10.1002/jcp.27707.

Ethyl Acetate and n-Butanol Fraction of *Cissus quadrangularis* Promotes the Mineralization Potential of Murine Pre-Osteoblast Cell Line MC3T3-E1 (Sub-clone 4)

RABAIL HASSAN TOOR¹, RAAZIA TASADDUQ², ACHYUT ADHIKARI³, MUHAMMAD IQBAL CHAUDHARY³, JANE B. LIAN⁴, JANET L. STEIN⁴, GARY S. STEIN⁴, ABDUL RAUF SHAKOORI^{1,5,*}

¹School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore, Pakistan.

²Kinnaird College for Women, Jail Road, Lahore, Pakistan.

³International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

⁴Department of Biochemistry, University of Vermont College of Medicine, E210E Given, 89 Beaumont Ave, Burlington, VT 05405, USA

⁵Faculty of Life Sciences, University of Central Punjab, Lahore, Pakistan

Abstract

In a sequel to investigate osteogenic potential of ethanolic extract of *Cissus quadrangularis* (CQ), the present study reports the osteoblast differentiation and mineralization potential of ethyl acetate (CQ-EA) and butanol (CQ-B) extracts of CQ on mouse pre-osteoblast cell line MC3T3-E1 (sub-clone 4) with an objective to isolate an anti-osteoporotic compound. Growth curve, proliferation and viability assays showed that both the extracts were non-toxic to the cells even at high concentration (100µg/ml). The cell proliferation was enhanced at low concentrations (0.1µg/ml and 1µg/ml) of both the extracts. They also upregulated the osteoblast differentiation and mineralization processes in MC3T3-E1 cells as reflected by expression profile of osteoblast marker genes such as Runx2, Osterix, Collagen, Alkaline Phosphatase, Integrin related Bone Sialoprotein, Osteopontin and Osteocalcin. CQ-EA treatment resulted in early differentiation and mineralization as compared to CQ-B treatment. These findings suggest that low concentrations of CQ-EA and CQ-B have proliferative and osteogenic properties. CQ-EA however, is more potent osteogenic than CQ-B.

Keywords

Osteoblast differentiation; osteoblast differentiation marker genes; Osteocalcin; Runx2 gene expression; Osteopontin gene expression; Von Kossa staining; alizarin red staining

*Address for correspondence: Prof. Dr. A.R. Shakoori, Distinguished National Professor, Professor Emeritus, School of Biological Sciences, University of the Punjab, Lahore, 54590 Pakistan., Tel: +92-42-99231248, Fax: +92-42-99230980, arshakoori.sbs@pu.edu.pk; dr.arshakoori@ucp.edu.pk; arshaksbs@yahoo.com.

Introduction

The use of traditional medicines has increased during the past decades as they are thought to be safer and more compatible to human body than the modern medicine (Robinson and Zhang, 2011; Kamboj, 2000). Presently constituent molecules of traditional medicines are being evaluated for their therapeutic use for drug discovery and development (Sen and Dash, 2012).

Cissus quadrangularis (CQ) commonly known as bone setter is a shrub of Vitaceae family and is found in southeast and far eastern countries including Pakistan (Chanda et al., 2013; Sen and Dash, 2012; Rao et al., 2011; Mishra et al., 2010). Chemically CQ is reported to contain inorganic minerals like calcium, iron, copper, zinc, potassium etc. and many phytochemicals like resveratrol, carotene, phenolic compounds, tannins, ascorbic acid, flavonoids, stilbenoids, saponins, steroids, glycosides, carbohydrates, vitamins, fatty acids and a variety of other compounds (Shah, 2011; Sen and Dash, 2012; Subhashri et al., 2011; Rao et al., 2011; Kalpana, 2013).

Osteoporosis is a health condition affecting more than 200 million people worldwide, in which bone tissue deteriorates resulting in loss of bone mass and weakening of bones leading to high risk of fractures (Mishra et al., 2010; Eijken, 2007). According to an estimate, about 50% females and 20% males worldwide suffer from bone fractures due to fragile bones each year (Sambrook and Cooper, 2010). In Pakistan about 9.91 million people (7.19 million women and 2.71 million men) suffer from osteoporosis. These estimates are likely to rise to 11.3 million in 2020 and 12.91 million in 2050 (Sultan et al., 2006). Khan et al. (2018) have reported high burden of osteoporosis ranging from 5.6 to 17.8% in premenopausal females and 20 to 49.3% in postmenopausal females. Lack of hormones (estrogen in females and androgen in males, imbalance in bone remodeling process (Mishra et al., 2010) and inflammatory disorders (increased oxidative stress or high level of glucocorticoids) usually lead to osteoporosis (Yang et al., 2011). As lack of estrogen causes decrease in bone mineral density, osteoporosis is more prevalent in post-menopausal women (IOF, 2014; Mishra et al., 2010; Weitzmann and Pacifi, 2006).

As osteoporosis and other bone diseases are mainly a result of increased osteoclast activity, the drugs or treatment should target osteoclast activity or differentiation process (Fasipe et al., 2018; Khosla and Hofbauer, 2017; Stapleton et al., 2017; Farr et al., 2017; Chan et al., 2016; Rodan and Martin, 2000). Currently hormone therapy (estrogen treatment), introduction of estrogen receptors (estrogen receptor modulators (SERMS)), and therapeutic agents (bisphosphates and calcitonin) are used for treatment of osteoporosis (Mishra et al., 2010; Eijken, 2007). Hormone therapies target osteoclast differentiation whereas therapeutic agents target osteoclast activity (Eijken, 2007). Other treatment options include hormones that stimulate bone formation i.e. parathyroid hormone (PTH) (Kurland et al., 2000; Reeve et al., 1980; Neer et al., 2001), but continuous treatment with PTH results in loss of bone mass (Murray et al., 2005). All these treatment options have side effects i.e. vaginal bleeding, hypercalciurea, hypercalcemia, and risk of developing breast (Mishra et al., 2010), ovarian or endometrial cancer (Yang et al., 2011).

Various in-vivo studies have been conducted on CQ and its various extracts that report its fracture healing properties in animal models (Stohs and Ray, 2012; Deka et al., 1994; Prasad and Udapa, 1963; Chopra et al., 1976; Pathomwachaiwat et al., 2014) and describe it as anti-osteoporotic agent (Shirwaikar et al., 2003; Potu et al., 2009, 2010, 2011; Aswar et al., 2012, Banu et al., 2012). Effect of various extracts of CQ has been studied on mesenchymal stem cells (Potu et al., 2009; Kumar et al., 2010; Parisuthiman et al., 2009), SaOS-2 cells (Muthusami et al., 2011), MC3T3-E1 (Pathomwachaiwat et al., 2014; Tasadduq et al., 2017) which suggest that CQ has potential to be used as anti-osteoporotic drug. Human trials have been conducted that reported CQ to accelerate fracture healing (Mishra et al., 2011; Singh et al., 2011). Kumar et al. (2010) identified 6-O-trans-cinnamoyl catapol to be the main osteoporotic constituent of CQ. Another study by Pathomwachaiwat et al. (2014) identified 7 of 29 compounds from hexane fraction of CQ to have stimulatory effect on osteoblast differentiation of MC3T3-E1 cell line.

In a previous paper we had studied the effect of ethanolic extract of CQ (CQ-E) on osteoblast differentiation of murine pre-osteoblast cell line MC3T3-E1 (Tasadduq et al., 2017). Our findings suggested dose dependent effect of CQ-E with lower concentrations exhibiting anabolic and osteogenic properties as reflected by substantial increase in expression of the early osteoblast marker ALP activity and at later stage by mineralization of extracellular matrix. We have now evaluated the effect of two fractions of CQ viz., ethyl acetate fraction (CQ-EA) and butanol fraction (CQ-B) on growth kinetics, proliferation, metabolic activity, osteoblast differentiation and mineralization of mouse pre-osteoblast cell line MC3T3-E1 (Sub-clone 4). It was found that cell proliferation was enhanced at low concentrations, which also upregulated the expression profile of osteoblast marker genes such as Runx 2, Osterix, Collagen, Alkaline phosphatase, Integrin related bone sialoprotein, Osteopontin and Osteocalcin, The CQ-EA treatment resulted in early differentiation and mineralization compared to CQ-B treatment.

Materials and Methods

Cell culture

Mouse pre-osteoblast cell line MC3T3-E1 (sub-clone 4) (ATCC – 2593™) was purchased from American Type Culture Collection. The cells were maintained in complete growth medium (Alpha MEM Medium (Hyclone) without ascorbic acid, 10% fetal bovine serum (Hyclone – SH30071.03), 1% (2mM) L-glutamine (ThermoFisher Scientific – 21051024), 1% penicillin/ streptomycin (ThermoFisher Scientific – 15140122)) as described by Wang et al. (1999) at density of 3000 cells/ cm² in a 100mm culture plate (Nunc™ – 150350) under standard culture conditions (37°C, 5% CO₂, humidified conditions). The cells were sub-cultured at 70 – 80% confluence, usually after 3 – 4 days. All experiments were carried out in triplicate; the cells of passage 5 – 20 were used for the study.

Preparation of CQ fractions

The ethanolic CQ extract prepared according to Tasadduq et al. (2017) was fractionated by solvent-solvent extraction into ethyl acetate (CQ-EA), butanol (CQ-B), hexane (CQ-H) and dichloromethane (CQ-D) fractions. This study deals with the effect of CQ-EA and CQ-B for

which stock solution (1g/ml) prepared in dimethyl sulfoxide (DMSO) were further diluted (400, 200, 20, 2, 0.2 and 0.02 mg/ml) for preparation of complete growth medium with final concentrations of 200, 100, 10, 1, 0.1 and 0.01 µg/ml of each fraction. For both fractions, DMSO was used as vehicle control. The concentration of DMSO in the cell culture medium was maintained to 0.05% for all experiments. For negative control, the cells were treated with medium containing 0.05% DMSO.

Growth Kinetics, Cell Viability, Proliferation, and Metabolic Activity Assays

For optimizing concentrations of the two organic fractions for cell growth, cell viability, proliferation and metabolic activity, MC3T3-E1 cells were passaged and cultured at 3000 cells/cm² in 24 well culture plates (NuncTM – 142475) in complete growth medium for 24 h under standard culture conditions. Then, in both cases, the medium was replaced with fresh growth medium having different concentrations (0.01, 0.1, 1, 10, 100, 200 µg/ml) of either CQ-EA or CQ-B for 48 h. Cells were fed with fresh medium containing different concentrations of CQ-EA or CQ-B after 48h. In all experiments, the controls were treated with 0.05% DMSO. Percentage of metabolic activity, viability and proliferation were calculated with reference to controls.

To examine the effect of CQ-EA and CQ-B on the growth of pre-osteoblast cell line MC3T3-E1, the cells were treated with above-mentioned concentrations of CQ-EA and CQ-B in independent experiments. On day 1, 3, 5 and 7 of treatment, the cells were harvested, counted using hemocytometer (Improved Neubauer Type, Germany) and growth curve was plotted by taking log10 of average of cell count for each time point against days of treatment. Complete growth medium with 0.05% DMSO was used as control. Doubling time and specific growth rate were determined as explained by Butler (2004).

For cell viability, proliferation and metabolic activity assays, MC3T3-E1 cells were cultured and treated with different concentrations of CQ-EA and CQ-B as mentioned above.

Neutral red assay determines the cell viability by the amount of neutral red dye taken up by cell lysosomes, - more the dye taken up by the cells, more number of viable cells. Non-viable cells will not take up neutral red dye. For this the cells were treated with complete growth medium containing 40µg/ml neutral red (Sigma Aldrich – N4638) for 90 min on day 1 and 3. Then the medium was removed, cells were washed thrice and destained with mixture of absolute ethanol, water and glacial acetic acid (5:4:1) mixture for 10 min with occasional stirring. The contents were transferred to 96-well flat bottomed, ELISA reader plates for CQ-EA and CQ-B separately. Absorbance was taken at 570nm using BioTek ELx808 absorbance reader.

EC₅₀, defined as effective concentration of CQ-EA and CQ-B at which the cells were 50% viable with reference to 0.05% DMSO treated control cells values were also calculated.

Proliferation of MC3T3-E1 cells in response to CQ-EA and CQ-B was examined by BrdU incorporation assay. BrdU is a thymidine analogue that the cell can incorporate in its DNA during replication, so it indicates cell proliferation in terms of DNA synthesis. ELISA based colorimetric assay measures the amount of BrdU incorporated into the DNA of the cells. For

this, ELISA based colorimetric BrdU cell proliferation assay kit (Roche – 11647229001) was used according to instructions provided by the manufacturer on day 1 and day 3, separately for CQ-EA and CQ-B treatments. After adding stop solution, the contents were transferred to 96-well flat-bottomed ELISA reader plates. Absorbance was taken at 450 nm using BioTek ELx808 absorbance reader.

Metabolic activity was determined by MTT assay, the NAD(P)H dependent-oxidoreductase enzyme reduces the tetrazolium salt (MTT) into formazan product (colored product), that directly correlates to the metabolic activity of the cells. For assessing the metabolic activity the cells on day 1 and day 3 of CQ-EA and CQ-B treatments were treated with medium containing 0.1mg/ml MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Thermofisher Scientific – M6494) for 4h. Then acidified isopropanol (0.1N concentrated HCL in isopropanol) was used to solubilize formazan crystals. The contents were transferred to 96 well flat-bottomed ELISA reader plates and absorbance was taken at 570nm using BioTek ELx808 absorbance reader.

The concentrations of CQ-EA and CQ-B that had more than 80% cell viability during log-phase of MC3T3-E1 cells were considered as safe concentrations for the cells.

Osteogenic differentiation and mineralization

MC3T3-E1 cells were cultured in 6-well culture plate (Nunc™ – 140675) at 3000 cells/cm² density. At confluence, osteogenic (mineralization) medium (Alpha MEM without ascorbic acid, 10% fetal bovine serum, 1% (2mM) L-glutamine, 1% penicillin/ streptomycin) and 25µg/ml ascorbic acid (Sigma Aldrich – 94417) was fed to the cells for 48h. The same medium containing 50µg/ml ascorbic acid and 5mM beta-glycerophosphate (ACROS Organics –410991000) was used for the second and subsequent feedings. The media were changed after every 48h. The concentrations of CQ-EA and CQ-B (100, 10, 1, and 0.1 µg/ml) that exhibited 90% or more cell viability were tested for their effect on osteogenesis and mineralization of MC3T3-E1 cells. For negative control, complete growth medium was added to the cells and for positive control osteogenic medium (without herbal fractions) containing 0.05% DMSO only was added to the cells. The cells were incubated at 37°C under 5% CO₂ and humidified conditions for 21 days. Samples were collected on day 7, 14 and 21 of osteogenic induction to represent stages of differentiation process. All experiments were carried out in triplicates.

The samples collected above during differentiation at different time points were washed thrice with 1X cold PBS (Phosphate Buffer Saline), fixed with 10% NBF (Neutral Buffered Formalin) for 15 min, washed with distilled water and then used for von kossa staining, alkaline phosphatase staining and alizarin red staining. For von kossa staining 5% silver nitrate (Sigma Aldrich – S8157) was added to the wells, exposed to the bright light for 1h to detect hydroxyapatite mineral deposits in the cells. The silver nitrate solution was then removed and cells were washed with distilled water thrice, before taking an image.

For alkaline phosphatase staining 200mM Tris Buffer (pH 9.2) was added to the wells for 15 min and then ALP substrate (0.013 g of Naphthol AS MX-PO₄ (Sigma Aldrich – N5000) in 100µl of N, N-dimethylformamide (Sigma Aldrich – D4254), mixed with 0.1% Fast Red AS

TR salt (Sigma Aldrich – F8764) solution in 0.1 M Tris-maleate buffer pH 8.3) was added to the fixed cells for 45 min at room temperature. The plates were washed thrice with distilled water. The areas rich in ALP activity developed reddish pink color.

For alizarin red S staining 1% alizarin red S stain solution (ACROS organics – 400480250) in 1% ammonium hydroxide solution (pH 4.1) was added to the fixed cells for 30 min at room temperature. Then the plates were washed with distilled water to remove excess stain. The calcium rich areas were stained bright red.

Semi-quantification of calcium deposition by Alizarin Red S staining

The mineral deposits were semi-quantified by alizarin red stain based method as described by Gregory et al. (2004). 800µl of 10% acetic acid was added to the stained cells, followed by incubation for 30 min at room temperature. The cells were scrapped from plate by cell scraper (Biolab – SPL 90020) and contents were transferred to microcentrifuge tube and vortexed for 30 sec. Then the contents of microcentrifuge tubes were heated at 85°C for 10 min, cooled on ice and centrifuged for 15 min at 20,000g. 500µl of supernatant was taken in separate microcentrifuge tubes and 200µl of 10% ammonium hydroxide was added to it. pH was set to 4.1 and 150µl of supernatant was read in triplicate in 96-well, flat bottomed, ELISA reader plates at 405nm. Standard curve of alizarin red stain was prepared and concentration of mineral was determined using alizarin red S standard curve.

Relative expression of osteoblast-related genes

1 ml Trizol (ThermoFisher Scientific – 15596018) was added to each well of the sample plates collected on day 0, 7, 14 and 21 of mineralization to isolate total RNA as per manufacturer's instructions. RNA was treated with DNase I (Ambion – AM2222) and purified using PureLink™ RNA mini kit (ThermoFisher Scientific – 12183018A). For RT-PCR, cDNA was synthesized using purified RNA using RevertAid First Strand cDNA synthesis kit (ThermoFisher Scientific – K1622).

For quantitative expression of osteoblast-related genes, Real Time PCR was done using Maxima SYBR Green/ROX qPCR master mix (2X) (ThermoFisher Scientific – K0222), cDNA and 500nM forward and reverse primers each (Table 1). Two step thermal cycle protocol with initial denaturation of 3 min at 95°C followed by 40 cycles each of denaturation at 95°C for 30 sec and annealing and extension at 60°C for 30 sec was used for amplification and quantification of amplicons on PikoReal Real Time PCR system (ThermoFisher Scientific). Melting curve data at 60°C – 90°C with every 0.2°C increment was taken to determine the uniformity of PCR product.

Ct values of target genes (*RUNX2*, *SP7*, *COL1A1*, *ALP*, *IBSP*, *SPP1* and *BGLAP*) were normalized using Ct value of housekeeping gene Hydroxymethylbilane Synthase (*HMBS*). Relative expression (n-folds) of target genes was calculated as ratio of relative expression of target genes (normalized to HMBS) under experimental conditions to the relative expression of target genes (normalized to HMBS) under control conditions.

Statistical analysis

Each experiment was conducted as sets of independent biological triplicates. Data were represented as Mean $\pm$ SD of technical and biological replicates. Statistical analyses of data (where required) were conducted by student's t test, one-way and two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using Graphpad Prism 7.03 software. The results were considered significant if $P < 0.05$.

Results

Effect of CQ-EA and CQ-B on growth parameters of MC3T3-E1 cells

Figures 1A and 1E show the effect of different concentrations of CQ-EA and CQ-B, respectively on the growth curves of MC3T3-E1. In both cases, the cells treated with 200 $\mu\text{g/ml}$ exhibited a significant decrease in the number of cells compared to vehicle control-treatment (0.05% DMSO). The concentration of 1 and 10 $\mu\text{g/ml}$ exhibited an increase in number of cells as compared to 0.05% DMSO treated control cells with 21.1 and 17.2% increase in number of cells respectively for CQ-EA and 20.9 and 14.3% increase in number of cells respectively for CQ-B treated cells.

The doubling time and specific growth rate was calculated for each concentration of CQ-EA and CQ-B. For both fractions, the vehicle-treated control cells had doubling time of 27.7h and specific growth rate was calculated to be 0.012. For both fractions CQ-EA and CQ-B, treatment with 1 $\mu\text{g/ml}$ dose exhibited lowest doubling time of 25.7h with 7.3% decrease compared with vehicle-treated control cells and specific growth rate was 0.013.

Effect of CQ-EA and CQ-B on viability, proliferation and metabolism of MC3T3-E1 cells

Figures 1B and 1F show the effect of different concentrations of CQ-EA and CQ-B on the viability of MC3T3-E1 pre-osteoblast cells. The concentrations 0.1, 1, 10 and 100 $\mu\text{g/ml}$ seemed to be the most suitable for cells with more than 90% survival in the presence of both the fractions. EC_{50} for cell viability was found to be 294 $\mu\text{g/ml}$ for CQ-EA and 207.9 $\mu\text{g/ml}$ for CQ-B.

Figures 1C and 1G show the metabolic activity of cells treated with different concentrations of CQ-EA and CQ-B. The highest increase in metabolic activity (47%) in response to 1 $\mu\text{g/ml}$ of CQ-EA was observed with reference to 0.05% DMSO treated control cells. The 14.8% increase in metabolic activity after 1 $\mu\text{g/ml}$ CQ-B treatment was not found to be significant.

Compared with 0.05% DMSO treated cells, the cells treated with different doses (0.1, 1, 10 and 100 $\mu\text{g/ml}$) of CQ-EA and CQ-B showed no detrimental effect on the proliferation of cells (Fig. 1D and 1H). Treatment with 1 $\mu\text{g/ml}$ of CQ-B showed 17.9% increase in proliferation of cells as compared to 0.05% DMSO treated control cells.

The results for growth curve parameters and cell viability, proliferation and metabolic activity assays in independent experiments for CQ-EA and CQ-B revealed that the concentration range of 0.1, 1, 10 and 100 $\mu\text{g/ml}$ had no detrimental effect on any of these

parameters. The concentration of 1µg/ml of both the fractions exhibited significant mitogenic effect on MC3T3-E1 cells.

Effect of CQ-EA and CQ-B on mineralization of MC3T3-E1 cells

MC3T3-E1 cells were allowed to differentiate in the presence of non-detrimental doses (0.1, 1, 10 and 100µg/ml) of CQ-EA and CQ-B in independent experiments for 21 days. Positive control cells were treated with osteogenic medium only. The vehicle treated cells were taken as control. In both cases, extracellular matrix (ECM) was formed and cells were mineralized by day 14 of treatment with osteogenic medium (with and without herbal concentrations). The cells treated with different doses of CQ-EA and CQ-B exhibited heavily mineralized matrix compared to positive control treated cells in osteogenic medium (Fig. 2A and B). In the case of treatment with CQ-EA, the ECM started disintegrating and by day 21 it became less dense and could not be properly stained. The heavy mineralization may be attributed to the increase in the metabolic activity of the cells in response to CQ-EA treatment.

The activity of alkaline phosphatase (ALP) enzyme corresponds to the formation of hydroxyapatite mineral during the process of bone mineralization. In both cases, by day 7 there was no significant effect on ALP activity as compared to the control cells (vehicle-treated and positive control cells). However, by day 14, ALP activity was found to be highly enhanced in CQ-EA treated cells compared to the controls (Fig. 3A, B). In the case of treatment with different doses of CQ-B, ALP activity was found to be slightly enhanced by day 14 (Fig. 3B-b). For CQ-EA treated cells, the matrix was weak and disintegrated to be stained by day 14 whereas, for CQ-B treated cells (Fig. 3B-b), ALP activity was found to be highly enhanced in CQ-B treated cells as compared to the controls by day 21. All the doses of CQ-EA and CQ-B showed enhanced ALP activity compared to their respective controls.

Mineralized MC3T3 cells treated with different doses (0.1, 1, 10 and 100µg/ml) of CQ-EA and CQ-B respectively were stained for calcium deposition (Alizarin red staining) and phosphate deposition (Von Kossa staining). The CQ-EA treated mineralized cells exhibited enhanced mineral deposition by day 14 for all doses of CQ-EA as compared to the controls (vehicle treated cells and positive control cells) (Fig 3A-a). The cells treated with different doses of CQ-B had mineral deposition in positive control cells, 0.1 and 1µg/ml concentrations on day 14 (Fig. 3B-a). There was almost no mineral deposition at 10 and 100µg/ml CQ-B treated cells. By day 21, the cells treated with all concentrations of CQ-B were mineralized as shown by alizarin red (Fig. 3B-c) and von kossa stained cells (Fig. 3B-a).

Alizarin red method was used for semi quantification of calcium deposition to represent mineral deposition. With reference to the positive control, on day 14, the cells mineralized in presence of 0.1 and 1µg/ml of CQ-EA had higher concentration of mineral deposition, with highest mineral deposition at 0.1µg/ml (Fig. 3A-c). For cells treated with CQ-B, the mineralized cells on day 21 higher mineral deposition in 0.1, 1 and 10µg/ml concentrations of CQ-B with reference to the positive control (Fig. 3B-c). The concentrations 0.1 and 1µg/ml had higher amount of mineral deposition as compared to mineral deposited in response to same concentrations of CQ-EA (Table 2).

The cells treated with CQ-EA were more heavily mineralized on day 14 as compared to cells treated with same doses of CQ-B (Fig. 3C). The cells treated with 1µg/ml CQ-B however, had more mineral deposited on day 21 as the same dose of CQ-EA on day 14 as determined by semi quantitative determination of mineral deposition by alizarin red method (Fig. 3C).

Effect of CQ-EA and CQ-B on relative expression of osteoblast-related genes

The process of differentiation is accompanied by changes in expression of many genes involved in the process. During osteoblast differentiation process, the cells undergo many changes and the process requires spatial and temporal expression of many genes that are specifically related to the process of osteoblast differentiation. To analyze the effect of the mitogenic doses (0.1, 1, 10 and 100µg/ml) of CQ-EA and CQ-B on differentiation of MC3T3-E1 cells into osteoblasts, the expression of osteoblast related genes i.e. Runt-related transcription factor 2 (*RUNX2*), Transcription factor Sp7 or Osterix, Alkaline phosphatase (*ALP*), Procollagen type 1A1 (*COL1A1*), Integrin binding bone sialoprotein (*IBSP*), Osteopontin (*OPN*) or Secreted phosphoprotein 1 (*SPP1*) or Bone sialoprotein 1 (*BSP1*), and Bone gamma-carboxyglutamic acid containing protein (*BGLAP*) or Osteocalcin (*OCN*) was determined.

For this, RNA was isolated from the differentiated cells treated with aforementioned doses of CQ-EA and CQ-B in independent experiments on day 0, 7, 14 and 21 of differentiation along with appropriate controls.

1. In both cases, the expression of transcription factors essential for osteogenic differentiation (*RUNX2* and *SP7*) was enhanced during first week of osteogenic induction to the cells. Mineralized cells treated with 1µg/ml of CQ-EA had enhanced expression of *RUNX2* as compared to the control and other CQ-EA concentrations. Whereas for CQ-B treated cultures, enhanced expression was observed with 10µg/ml CQ-B. In the case of *SP7* expression, on day 7, positive control culture had more expression than the cultures treated with all aforementioned doses of CQ-EA and CQ-B.
2. Collagen is the major component of ECM. The expression of *COL1A1* was observed during the first week of differentiation process. There was no significant enhancement in expression of *COL1A1* when treated with aforementioned doses of CQ-EA and CQ-B.
3. The expression of *ALP* peaked during first week of differentiation when treated with CQ-EA, significant increase in *ALP* expression was observed in culture treated with 100µg/ml CQ-EA. For all other doses, the expression was same as of positive control cultures. For CQ-B treated cells, the positive control culture had increased *ALP* expression during first week but the cultures treated with doses of CQ-B had increased expression of *ALP* during second week of differentiation.
4. The expression profile analysis of *IBSP* revealed that for positive control cells and 0.1, 10, and 100µg/ml CQ-EA treated cultures, the expression of *IBSP* peaked during first week and then the expression was reduced however in the case of 1µg/ml CQ-EA, the increase in expression was observed during first and

second week of differentiation. However, in case of all doses of CQ-EA, the expression was less as compared to positive control cells. For CQ-B treated cells, same trend was observed as in case of treatment with CQ-EA, treatment with 1µg/ml CQ-B showed gradual increase in expression of *IBSP* as differentiation proceeded.

5. The expression of *SPP1* was same for CQ-EA treated cells as of positive control cells having peak expression during second week of differentiation, whereas for CQ-B treated cultures, significant increase was observed in expression of *SPP1* in culture treated with 1µg/ml of CQ-B as compared to positive control cells and other doses of CQ-B.
6. The expression profile of *BGLAP* showed peak during second week of differentiation in case of both CQ-EA and CQ-B respectively. As compared to the positive control cells, treatment with different doses of both fractions had no significant effect on the expression of *BGLAP*.

Figures 4 and 5 show the expression profiles of osteoblast related genes in response to CQ-EA and CQ-B respectively.

The expression profiles of osteoblast related genes indicate that for both fractions, CQ-EA and CQ-B, the expression of osteoblast related genes follow the normal temporal expression during the process of osteoblast differentiation and mineralization of MC3T3-E1 cells. However, the enhancement in differentiation and mineralization of CQ-EA and CQ-B treated cultures in response to the mitogenic doses i.e. 0.1 and 1µg/ml, as shown by semi-quantification analysis (Table 2), is not so evident as the expression profile was determined after intervals of 7 days. In order to reveal the anabolic effect of aforementioned doses of these two fractions, expression profile with narrow time intervals must be studied.

Discussion

Our results revealed that for both fractions, same range of concentrations (0.1, 1, 10 and 100µg/ml) were non-detrimental for the cells. This was supported by analysis of growth parameters, and assays for cell viability, metabolic activity and cell proliferation. For both fractions, the higher and lower concentrations (200 and 0.01µg/ml) were found to have significant detrimental effects on the growth and proliferation of the cells, whereas the concentration 1µg/ml for both fractions was found to be significantly proliferative. Other studies by Tassaduq et al. (2017) and Parisuthiman et al. (2009) have reported dose dependent effect of ethanolic extract of CQ on cell viability with lower concentrations to be more proliferative for the cells. Potu et al. (2009) and Muthusami et al. (2011) also have reported dose dependent effect of petroleum ether extract and ethanolic extract on the mesenchymal stem cells and SaOS-2 cell line. However, in our study, for both fractions, the concentrations 0.1–100µg/ml exhibited mitogenic effect on the cells as compared to other lower and higher concentrations tested. The difference in doubling time and proliferation was not significant but a significant increase in metabolic activity was observed in our study especially when cells were treated with 0.1 – 1µg/ml CQ-EA which may contribute to heavy mineralized matrix observed when cells were differentiated with CQ-EA supplemented

medium. There was no significant difference of the tested concentrations on cell viability. Treatment with 1µg/ml CQ-B had significant increase in the proliferation of the cells that was not observed in case of treatment of cells with same concentration of CQ-EA.

We found that the CQ-EA and CQ-B fractions had significant effect on the differentiation and mineralization of MC3T3-E1 pre-osteoblast cell line. For both CQ-EA and CQ-B concentrations of 0.1 and 1µg/ml had significant mineral deposition compared to the mineral deposited in positive control cultures by day 14 of mineralization. The cultures treated with 10 and 100µg/ml CQ-B, mineralization was delayed. However, the ECM of CQ-B treated cultures was properly mineralized by day 21 and had significantly higher mineral deposition compared to positive control and CQ-EA treated cultures. In case of both fractions, high ALP activity was observed. However, we found no significant difference in the expression of osteoblast-related genes. Tasadduq et al. (2017) reported upregulated ALP activity and enhanced mineralization process of CQ-E during differentiation of MC3T3-E1 cell line after CQ-E treatment. The study reported stimulated expression of osteoblast-related genes (*RUNX2*, *COL1A1* and *BGLAP*) in response to treatment with lower doses of CQ-E. However, Parisuthiman et al. (2009) reported enhanced ALP and mineralization of the cells by p38 mitogen activated protein kinase (MAPK) pathway, but no difference in the expression of osteoblast-related genes (*SP7*, *RUNX2* and *BGLAP*) after treatment of MC3T3-E1 cells with Polyvinylpyrrolidone (PVP) dissolved CQ-E. Our results are supported by results of Parisuthiman et al. (2009). Tasadduq et al. (2017) used crude extract of CQ-E, whereas this study reports the effect of purified fractions (CQ-EA and CQ-B) of same crude fractions on the growth and proliferation of the cells. The differences in the results may be related to the different chemical compositions of the crude extract and the purified fractions (Tables 2 and 3).

Muthusami et al. (2011a) reported upregulated ALP activity and enhanced mineralization of SaOS-2 cell line in response to treatment with CQ-E. They also reported upregulated expression of osteoblast-related genes (*RUNX2*, *ALP*, and *COL1A1*) and found that CQ-E effects osteoblast differentiation process by upregulating Insulin-like growth factor (IGF) system. Potu et al. (2009) have also reported enhanced mineralization and ALP activity of petroleum ether extract of CQ on mesenchymal stem cells.

In this study, the stimulatory effect of these two fractions on the growth and metabolism of cells may be due to the presence of alkaloids, glycosides, proteins and amino acids, flavones and flavonoids and saponins in CQ-EA, and glycosides, tannin-phenolic compounds, proteins and amino acids, flavonoids and flavones, saponins and steroids and sterols in CQ-B (Table 3) (Kalpana, 2013). High level of quercetin have also been reported in QB-EA (Patarapanich et al., 2004). The flavonoids (quercetin, resveratrol and kaempferol) are reported to enhance osteogenic differentiation via ER-dependent ERK pathway (Prouillet et al., 2004; Dia et al., 2007; Gou et al., 2012). Quercetin has been reported to enhance cell proliferation and osteogenic differentiation of mesenchymal stem cells in dose dependent manner by activation of ERK and MAPK pathway (Zhou and Lin, 2014; Kim et al., 2006; Zhou et al., 2015, 2017; Li et al., 2015). Notoya et al. (2004) have also reported inhibitory effect of quercetin on the osteogenic differentiation. Osteogenesis is promoted by activated BMP2 and TGFβ pathway and is inhibited by activated NF-κB pathway. The effect of

quercetin on the differentiation of the cells depend on its net effect on the different pathways involved in the process of osteogenesis (Yamaguchi and Weitzmann, 2011). Resveratrol, another flavonoid, has also been reported to enhance osteogenic activity of mesenchymal stem cells in dose-dependent manner (Park et al., 1997; Mizutani et al., 1998; Miyake et al., 2003; Wong and Rabie, 2008; Peltz et al., 2012). Kumar et al. (2010) have reported many constituents of CQ extracts to enhance osteoblast differentiation of mesenchymal stem cells, they have identified main anti-osteoporotic constituent to be 6-O-trans-cinnamoyl catapol.

The overall effect of these fractions on growth and differentiation of the cells depends on the net effect of the compounds present in these fractions on the various signaling pathways that are involved in the process of osteogenesis. Furthermore, apart from the effect on the process of bone formation, the effect of these fractions on the activity of osteoclasts is also a very interesting study.

Ancient literature claims CQ to be very effective for healing bone fractures that supports our study that CQ can be used as a medicine for fracture healing and for osteoporosis. As the study reveals no cytotoxic or metabolic side effects of the fractions of CQ, these fractions of CQ have a great potential to be used as a medicine for osteoporosis, however, further studies are required to identify the active compounds and to figure out the molecular pathways regulated by those compounds.

Acknowledgements

The project is supported financially by the research grant of Pakistan Academy of Sciences. Work has been done in collaboration with HEJ Karachi.

Literature Cited

- Aswar UM, Mohan V, Bodhankar SL. 2012 Antiosteoporotic activity of phytoestrogen-rich fraction separated from ethanol extract of aerial parts of *Cissus quadrangularis* in ovariectomized rats. *Ind J Pharmacol* 44: 345–350.
- Banu J, Varela E, Bahadur AN, Soomro R, Kazi N, Fernandes G. 2012 Inhibition of bone loss by *Cissus quadrangularis* in mice: a preliminary report. *J Osteoporos* 26: 1–9.
- Butler M 2004 Animal cell culture and technology. Taylor & Francis ISBN: 9781135325381
- Chan CKY, Mason A, Cooper C, Dennison E, 2016 Novel advances in the treatment of osteoporosis. *Br Med Bull* 119: 129–141. [PubMed: 27558130]
- Chanda S, Baravalia Y, Nagani K. 2013 Spectral analysis of methanol extract of *Cissus quadrangularis* L. stem and its Fractions. *J Pharmacogn Phytochem* 2: 149–157.
- Chopra SS, Patel MR, Awadhiya RP. 1976 Studies of *Cissus quadrangularis* in experimental fracture repair: A histopathological study. *Indian J Med Res* 64:1365–1368. [PubMed: 1010630]
- Dai Z, Li Y, Quarles LD, Son T, Pan W, Zhou H, Xiao Z. 2007 Resveratrol enhances proliferation and osteoblastic differentiation in human mesenchymal stem cells via ER dependent ERK1/2 activation. *Phytomedicine* 14:806–814. [PubMed: 17689939]
- Deka DK, Lahon LC, Saikia J, Mukit A. 1994 Effect of *Cissus quadrangularis* in accelerating healing process of experimentally fractured radius-ulna of dog: A preliminary study. *Indian J Pharmacol* 26: 44–45.
- Eijken M 2007 Human osteoblast differentiation and bone formation: growth factors, hormones and regulatory networks. Doctoral dissertation, Erasmus MC University Medical Center, Rotterdam.
- Farr JN, Xu M, Weivoda MM, Monroe DG, Fraser DG, Onken JL, Negley BA, Sfeir JG, Ogrodnik MB, Hachfeld CM, Brasseur NK, Drake MT, Pignolo RJ, Pirtskhalava T, Tchkonina T, Oursler MJ,

- Kirkland JL, Khosla S. 2017 Targeting cellular senescence and prevents age-related bone loss in mice. *Nature Medicine* 23: 1072–1079.
- Fasipe OJ, Ibiyemi OB, Adelosoye AA, Idowu AA. 2018 Recent advances and current trend in the pharmacotherapy of postmenopausal osteoporosis. *J Hlth Res Rev* 5:13–21.
- Gregory CA, Gunn WG, Peister A, Prockop DJ. 2004 An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Analyt Biochem* 329: 77–84.
- Guo AJ, Choi RC, Zheng KY, Chen VP, Dong TT, Wang ZT, Vollmer G, Lau DT, Tsim KW. 2012 Kaempferol as a flavonoid induces osteoblastic differentiation via estrogen receptor signaling. *Chin Med* 7:10. [PubMed: 22546174]
- IOF (International Osteoporosis Foundation). 2014 Facts and statistics. International Osteoporosis Foundation Website. Available at <http://www.iofbonehealth.org/factsstatistics> Accessed on 01–18-2014.
- Kalpana T 2013 HPLC analysis of extract of in vivo medicinally important climber *Cissus quadrangularis* L. *Int Res J Pharm* 4: 140–142.
- Kamboj VP. 2000 Herbal medicine. *Curr Sci* 78: 35–39.
- Khan AH, Jafri L, Ahmed S, Noordin S. 2018 Osteoporosis and its perspective in Pakistan: A review of evidence and issues of addressing fragility fractures. *Annls Med. Surg* 29:19–25.
- Khosla S, Hofbauer LC. 2017 Osteoporosis treatment: recent developments and ongoing challenges. *Lancet Diabetes Endocrinol* 5: 898–907. Doi 10.1016/s2213-8587(17)30188-2. [PubMed: 28689769]
- Kumar M, Rawat P, Dixit P. 2010 Anti-osteoporotic constituents from Indian medicinal plants. *Phytomedicine* 17: 993–999. [PubMed: 20554183]
- Kurland ES, Cosman F, McMahon DJ, Rosen CJ, Lindsay R, Bilezikian JP. 2000 Parathyroid hormone as a therapy for idiopathic osteoporosis in men: effects on bone mineral density and bone markers. *J Clin Endocrinol Metab* 85: 3069–3076. [PubMed: 10999788]
- Li Y, Wang J, Chen G, Feng S, Wang P, Zhu X, Zhang R. 2015 Quercetin promotes the osteogenic differentiation of rat mesenchymal stem cells via mitogen-activated protein kinase signaling. *Exp Ther Med* 9: 2072–2080. [PubMed: 26136938]
- Mishra G, Srivastava S, Nagori BP. 2010 Pharmacological and Therapeutic activity of *Cissus quadrangularis*: An Overview. *Int J PharmTech Res* 2: 135–139.
- Miyake M, Arai N, Ushio S, Iwaki K, Ikeda M, Kurimoto M. 2003 Promoting effect of kaempferol on the differentiation and mineralization of murine pre-osteoblastic cell line MC3T3-E1. *Biosci Biotechnol Biochem* 67:1199–1205. [PubMed: 12843643]
- Mizutani K, Ikeda K, Kawai Y, Yamori Y. 1998 Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun* 253:859–863. [PubMed: 9918820]
- Murray TM, Rao LG, Divieti P, Bringhurst FR. 2005 Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxylterminal region and related biological actions of carboxyl-terminal ligands. *Endocr Rev* 26: 78–113. [PubMed: 15689574]
- Muthusami S, Senthikumar K, Vignesh C. 2011 Effects of *Cissus quadrangularis* on the proliferation, differentiation and matrix mineralization of human osteoblast like SaOS-2 cells. *J Cell Biochem* 112: 1035–1045. [PubMed: 21308732]
- Neer RM, Arnaud CD, Zanchetta JR. 2001 Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 344: 1434–1441. [PubMed: 11346808]
- Notoya M, Tsukamoto Y, Nishimura H, Woo JT, Nagai K, Lee IS, Hagiwara H. 2004 Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts in vitro. *Eur J Pharmacol* 485:89–96. [PubMed: 14757127]
- Parisuthiman D, Singhatanadgit W, Dechatiwongse T, Koontongkaew S. 2009 *Cissus quadrangularis* extract enhances biomineralization through up-regulation of MAPK-dependent alkaline phosphatase activity in osteoblasts. *In Vitro Cell Dev Biol Ani* 45:194–200.
- Park CK, Ishimi Y, Ohmura M, Yamaguchi M, Ikegami S. 1997 Vitamin A and carotenoids stimulate differentiation of mouse osteoblastic cells. *J Nutr Sci Vitaminol* 43:281–296. [PubMed: 9268918]

- Parsad GC, Udupa KN. 1963 Effect of *Cissus quadrangularis* on the healing of cortisone treated fractures. *Ind J Med Res* 51: 667–676.
- Patarapanich C, Thiangtham J, Saifah E, Laungchonlatan S, Janthasoot W. 2004 Determination of antioxidant constituents in the herb *Cissus quadrangularis* Linn. *Iran J Pharm Res* 2: 77.
- Pathomwachaiwat T, Ochareon P, Soonthornchareonnon N, Ali Z, Khan IA, Prathanturug S. 2014 Alkaline phosphatase activity-guided isolation of active compounds and new dammarane-type triterpenes from *Cissus quadrangularis* hexane extract. *J Ethnopharmacol* 160: 52–60. [PubMed: 25449449]
- Peltz L, Gomez J, Marquez M, Alencastro F, Atashpanjeh N, Quang T, Bach T, Zhao Y. 2012 Resveratrol exerts dosage and duration dependent effect on human mesenchymal stem cell development. *PLoS ONE* 7:e37162. [PubMed: 22615926]
- Potu BK, Bhat KM, Rao MS. 2009 Petroleum ether extract of *Cissus quadrangularis* (Linn.) enhances bone marrow mesenchymal stem cell proliferation and facilitates osteoblastogenesis. *Clinics (San Paolo)* 64: 993–998.
- Potu BK, Nampurath GK, Rao MS, Bhat KM. 2011 Effect of *Cissus quadrangularis* Linn on the development of osteopenia induced by ovariectomy in rats. *Clin Ter* 162: 307–312. [PubMed: 21912817]
- Potu BK, Rao MS, Nampurath GK, Chamallamudi MR, Nayak SR, Thomas H. 2010 Anti-osteoporotic activity of the petroleum ether extract of *Cissus quadrangularis* Linn. in ovariectomized wistar rats. *Chang Gung Med J* 33: 252–257. [PubMed: 20584502]
- Prouillet C, Mazi_ere JC, Mazi_ere C, Wattel A, Brazier M, Kamel S. 2004 Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. *Biochem Pharmacol* 67:1307–1313. [PubMed: 15013846]
- Rao GV, Annamalai T, Mukhopadhyay T, Madhavi MSL. 2011 Chemical constituents and melanin promotion activity of *Cissus quadrangularis* Linn. *Res J Chem Sci* 1: 25–29.
- Reeve J, Meunier PJ, Parsons JA. 1980 Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: a multi-centre trial. *Br Med J* 280: 1340–1344. [PubMed: 6992932]
- Robinson MM, Zhang X. 2011 The World Medicine situation 2011: Traditional medicine: Global situation, issues and challenges, WHO Press, Geneva (http://www.who.int/medicines/areas/policy/world_medicines_situation/WMS_ch18_wTraditionalMed.pdf)
- Rodan GA, Martin TJ. 2000 Therapeutic approaches to bone diseases. *Science*. 289: 1508–1514. [PubMed: 10968781]
- Sambrook P, Cooper C. 2006 Osteoporosis. *Lancet* 367: 2010–2018 [PubMed: 16782492]
- Sen MK, Dash BK. 2012 A review on phytochemical and pharmacological aspects of *Cissus quadrangularis* L. *Int J Green* 6:169–73.
- Shah U 2011 *Cissus quadrangularis* L.: Phytochemicals, traditional uses and pharmacological activities- a review. *Int J Pharm Pharm Sci* 3: 41–44.
- Shirwaikar A, Khan S, Malini S. 2003 Antiosteoporotic effect of an ethanol extract of *Cissus quadrangularis* Linn. on ovariectomized rat. *J Ethnopharmacol* 89: 245–250. [PubMed: 14611887]
- Singh V, Singh N, Pal US, Dhasmana S, Mohammad S, Singh N. 2011 Clinical evaluation of *Cissus quadrangularis* and *Moringa oleifera* and Osteoseal as osteogenic agents in mandibular fraction. *Natl J Maxillofac Surg* 2: 132–136. [PubMed: 22639499]
- Stapleton M, Sawamoto K, Almeciga-Diaz CJ, Mackenzie WG, Mason RW, Orii T, Tomatsu S. 2017 *Int J Mol Sci* 18: 1345, doi 10.3390/ijms18071345.
- Stohs SJ, Ray SD. 2012 A review and evaluation of the efficacy and safety of *Cissus quadrangularis* extracts-a review. *Phytother Res* 27:1107–1114. [PubMed: 22976133]
- Subhashri S, Vedha BNH, Ramya DD. 2013 Review- Pharmacological activities based on different extracts of *Cissus quadrangularis*. *IJPPR* 5: 128–133.
- Sultan A, Khan DA, Mushtaq M, Hassan MU. 2006 Frequency of osteoporosis and its associated risk factors in postmenopausal women in clinical practice in Rawalpindi. *Pakistan J Pathol* 17:115–118.

- Tasadduq R, Gordon J, Al-Ghanim K, Lian JB, Van Wijnen AJ, Stein JL, Shakoori A R. 2017 Ethanol extract of *Cissus quadrangularis* enhances osteoblast differentiation and mineralization of murine pre-osteoblastic MC3T3-E1 cells. *J Cell Physiol* 232: 540–547. [PubMed: 27264191]
- Weitzmann MN, Pacifici R. 2006 Estrogen deficiency and bone loss: an inflammatory tale. *J Clin Invest* 116: 1186–1194. [PubMed: 16670759]
- Wong RWK, Rabie ABM. 2008 Effect of quercetin on pre-osteoblasts and bone defects. *Open Orthop J* 2:27–32. [PubMed: 19461927]
- Yamaguchi M, Weitzmann MN. 2011 Quercetin, a potent suppressor of NF- κ B and Smad activation in osteoblasts. *Int J Mol Med* 28:521–525. [PubMed: 21769418]
- Yang L, Chen O, Wang F, Zhang G. 2011 Antiosteoporotic compounds from seeds of *Cuscuta chinensis*. *J Ethnopharmacol* 135: 553–560. [PubMed: 21463675]
- Zhou C, Lin Y. 2014 Osteogenic differentiation of adipose-derived stem cells promoted by quercetin. *Cell Prolif* 47: 124–132. [PubMed: 24617900]
- Zhou Y, Wu Y, Jiang X, Zhang X, Xia L, Lin K, Xu Y. 2015 The effect of quercetin on the osteogenic differentiation and angiogenic factor expression of bone marrow-derived mesenchymal stem cells. *PLoS One* 10: e0129605. [PubMed: 26053266]
- Zhou Y, Wu Y, Ma W, Jiang X, Takemra A, Uemura M, Xu Y. 2017 The effect of quercetin delivery system on osteogenesis and angiogenesis under osteoporotic conditions. *J Mat Chem B* 5: 612–625.

Fig. 1. Effect of different doses of CQ-EA (left panel and CQ-B (right panel) on growth curve (A, E), Cell viability (B, F), Metabolic activity (C, G) and Cell proliferation (D, G). For growth curve, the cells were harvested and counted on day 1, 3, 5 and 7. Cell metabolic activity, viability and cell proliferation was assessed by MTT, Neutral red and BrdU assay on day 1 and 3. In all experiments, the control is treated with 0.05% DMSO. Metabolic activity (%), cell viability (%) and cell proliferation (%) was determined by considering control as 100%. Data has been presented as mean of three technical and biological replicates $\pm$ Standard

deviation. One way ANOVA was used to analyze the statistical significance with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and ns as not significant).

No significant changes were found in cell proliferation and cell viability, except for concentration of 200 $\mu\text{g/ml}$ for cell viability which is consistence with the growth curve.

Fig. 2.

Effect of mitogenic doses of CQ-EA (A) and CQ-B (B) on the differentiation of MC3T3-E1 cell line. The cells were treated with osteogenic medium containing mitogenic doses of CQ-EA and CQ-B. Control cells had 0.05% DMSO in normal complete growth medium whereas positive control had osteogenic medium supplemented by 0.05% DMSO only. In CQ-EA (A) cells were mineralized by second week of differentiation. Due to heavy mineralization, the matrix was disintegrated by day 21. In CQ-B (B) the cells were mineralized by second week of differentiation in positive control, 0.1 and 1 $\mu\text{g/ml}$ CQ-B treated cultures. Less mineral was observed in 10 and 100 $\mu\text{g/ml}$ CQ-B treated cultures. Cells were fully mineralized in all wells by day 21.

Fig. 3.

VonKossa staining (a,d), Alizarin Red S staining (b,e) and ALP staining (c,f) of CQ-EA treated cells (Day14) (top horizontal row) and CQ-B treated cells (Day14 and Day 21) (central horizontal rows). Control (C) well had complete medium supplemented with 0.05% DMSO. Positive Control Medium had complete mineralization medium (50 $\mu\text{g/ml}$ Ascorbic Acid and 5mM β -Glycerophosphate). CQ-EA and CQ-B treated cells had complete mineralization medium supplemented with respective concentrations of CQ-EA and CQ-B.

Bar graph at the bottom Panel C) represents percentage mineral deposition as determined by semi-quantitative method by Alizarin red staining method for quantification of calcium deposition in response to treatment with different doses of CQ-EA (Day 14) (A) and CQ-B (Day 21) (B). The amount of calcium correlated with the amount of mineral deposited (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns= not significant

Fig. 4.

Expression profile of osteoblast-related genes (*RUNX2*, Osterix or *SP7*, Collages (*COL1A1*) Alkaline phosphatase (*ALP*), *IBSP*, Osteopontin or *SPP1* and Osteocalcin or *BGLAP*) during the process of differentiation of MC3T3-E1 into osteoblasts in response to CQ-EA. The control culture was treated with 0.05% DMSO in normal complete medium. Positive control cultures were treated with osteogenic medium supplemented with 0.05% DMSO only. Osteogenic medium supplemented with different doses of CQ-EA was used to analyze the effect of CQ-EA on differentiation of MC3T3-E1 cells. The cells were allowed to

differentiate for 21 days with medium change on alternate days. Total RNA was isolated and cDNA was synthesized. Real time PCR was used to analyze the relative expression profile of osteoblast-related genes with time during the process of osteoblastogenesis. The target genes were normalized using Ct values of HMBS as housekeeping gene. Relative expression (n-folds) of target genes was calculated as ratio of relative expression of target genes (normalized to HMBS) under experimental conditions to the relative expression of target genes (normalized to HMBS) under control conditions.

Fig. 5. Expression profile of osteoblast-related genes (*RUNX2*, Osterix or *SP7*, Collages (*COL1A1*), Alkaline phosphatase (*ALP*), *IBSP*, Osteopontin or *SPP1* and Osteocalcin or *BGLAP*) during the process of differentiation of MC3T3-E1 into osteoblasts in response to CQ-B. The control culture was treated with 0.05% DMSO in normal complete medium. Positive control cultures were treated with osteogenic medium supplemented with 0.05% DMSO only. Osteogenic medium supplemented with different doses of CQ-B was used to analyze the effect of CQ-B on differentiation of MC3T3-E1 cells. The cells were allowed to

differentiate for 21 days with medium change on alternate days. Total RNA was isolated and cDNA was synthesized. Real time PCR was used to analyze the relative expression profile of osteoblast-related genes with time during the process of osteoblastogenesis. The target genes were normalized using Ct values of HMBS as housekeeping gene. Relative expression (n-folds) of target genes was calculated as ratio of relative expression of target genes (normalized to HMBS) under experimental conditions to the relative expression of target genes (normalized to HMBS) under control conditions.

Details of primers used for quantitative determination of relative expression of osteoblast related genes by Real Time PCR.

Table 1.

Primer ID	Gene	Sequence 5' – 3'
RColl1a1F	Pro-collagen type 1 alpha 1	GCTCCTCTTAGGGGCCACT
RColl1a1R		CCACGTCTCACCATTGGGG
RIBSPF	Integrin Binding Bone Sialpprotein	ATGGAGACGGCGATAGTTCC
RIBSPR		CTAGCTGTTACACCCGAGAGT
RRUNX2F	Runt-related transcription factor 2	CGGCCCTCCCTGAACTCT
RRUNX2R		TGCCTGCC'TGGGATCTGTA
ROCN2F	Osteocalcin	CTGACAAAAGCCTTCATGTCCAA
ROCN2R		GCGCCGGAGTCTGTTCACATA
RSP7F	Osterix	TCCCTGGATATGACTCATCCCT
RSP7R		CCAAGGAGTAGGTGTGTGCC
RSPP1F	Osteopontin	ATCTCACCATTCGGATGAGTCT
RSPP1R		TGTAGGGACGATTGGAGTGAAA
RALPF	Alkaline Phosphatase	CCAACTCTTTTGTGCCAGAGA
RALPR		GGTACATTGGTGTGAGCTTTT
RHMBSF	Hydroxymethylbilane Synthase	AAGGGCTTTTCTGAGGCACC
RHMBSR		AGTTGCCCATCTTTCATCACTG

Table 2.

Comparison of growth, metabolic, proliferative parameters of MC3T3-E1 cells when treated with mitogenic doses of crude ethanolic extract of CQ (CQ-E), Ethyl acetate fraction (CQ-EA) and n-Butanol fraction of CQ (CQ-B). The control cell were treated with 0.05% DMSO supplemented complete growth medium. CQ treated cells had complete growth medium supplemented with respective concentrations of respective CQ extract/fractions. The positive and negative sign indicate the percentage increase or decrease in the tested parameters in response to CQ treatment.

Parameters Tested		CQ-E (Crude Extract) (Tassaduq et al., 2017)		CQ-EA		CQ-B	
EC ₅₀		109 µg/ml		294 µg/ml		207.9 µg/ml	
Growth and Proliferation Parameters	Doubling Time (h)	Control	21	Control	27.7	Control	27.7
		0.1 µg/ml	22.3	0.1 µg/ml	27.8	0.1 µg/ml	28.3
				1 µg/ml	25.7	1 µg/ml	25.7
		1 µg/ml	22	10 µg/ml	25.9	10 µg/ml	26.3
	Cell Viability (%)			100 µg/ml	28	100 µg/ml	28.3
		0.1 µg/ml	N/A	0.1 µg/ml	-6.8%	0.1 µg/ml	-6.8%
				1 µg/ml	+1.9%	1 µg/ml	+3.1%
		1 µg/ml	N/A	10 µg/ml	-1.4%	10 µg/ml	-1%
	Metabolic Activity (%)			100 µg/ml	-9%	100 µg/ml	-8.5%
		0.1 µg/ml	+18.5%	0.1 µg/ml	+38%	0.1 µg/ml	+3.9%
				1 µg/ml	+47.4%	1 µg/ml	+14.8%
		1 µg/ml	+14%	10 µg/ml	+28.7%	10 µg/ml	+1.1%
	Proliferation (% BrdU Incorporation)			100 µg/ml	-6.7%	100 µg/ml	-3.1%
		0.1 µg/ml	+34.5%	0.1 µg/ml	-8.5%	0.1 µg/ml	-8.2%
				1 µg/ml	+3.3%	1 µg/ml	+17.4%
		1 µg/ml	+30%	10 µg/ml	-14.1%	10 µg/ml	-13.2%
				100 µg/ml	-15.2%	100 µg/ml	-14.8%

Comparison of outset of mineralization process in MC3T3-E1 cells and the amount of mineral deposited determined by alizarin red s semi-quantification method after treatment with mitogenic doses of crude ethanolic extract of CQ (CQ-E), Ethyl acetate fraction (CQ-EA) and n-Butanol fraction of CQ (CQ-B). The positive and negative sign indicate percentage increase or decrease in mineral deposition compared to positive control (PC). Positive control cells were treated with osteogenic induction medium only. CQ-E, CQ-EA and CQ-B treated cultures had osteogenic induction medium supplemented with respective concentrations of the CQ.

Table 3.

Parameters	CQ-E (Crude Extract) (Tassadduq et al., 2017)		CQ-EA	CQ-B
Mineralization Outset	PC	Day 18	PC	PC
			0.1µg/ml	0.1µg/ml
			1µg/ml	1µg/ml
		Day 16	10µg/ml	10µg/ml
	1µg/ml		100µg/ml	100µg/ml
Differentiation and Mineralization				
Alizarin Red S Quantification	PC	6.9 mM	PC	8.2mM
	0.1µg/ml	N/A	0.1µg/ml	(11.4mM) +39%
			1µg/ml	(17.6mM) +114.6%
			10µg/ml	(7.2mM) -12.2%
	1µg/ml	N/A	100µg/ml	(0.6mM) -92.7%