Cissus quadrangularis Linn exerts dose-dependent biphasic effects: osteogenic and anti-proliferative, through modulating ROS, cell cycle and *Runx2* gene expression in primary rat osteoblasts

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Received 21 January 2015; revision accepted 2 April 2015

Abstract

Objectives: This report highlights phytoconstituents present in *Cissus quadrangularis* (CQ) extract and examines biphasic (proliferative and anti-proliferative) effects of its extract on bone cell proliferation, differentiation, mineralization, ROS generation, cell cycle progression and *Runx2* gene expression in primary rat osteoblasts.

Materials and methods: Phytoconstituents were identified using gas chromatography–mass spectrometry (GC-MS). Osteoblasts were exposed to different concentrations (10–100 µg/ml) of CQ extract and cell proliferation and cell differentiation were investigated at different periods of time. Subsequently, intracellular ROS intensity, apoptosis and matrix mineralization of osteoblasts were evaluated. We performed flow cytometry for DNA content and real-time PCR for *Runx2* gene expression analysis.

Results: CQ extract's approximately 40 bioactive compounds of fatty acids, hydrocarbons, vitamins and steroidal derivatives were identified. Osteoblasts exposed to varying concentrations of extract exhibited biphasic variation in cell proliferation and differentiation as a function of dose and time. Moreover, lower concentrations (10–50 µg/ml) of extract slightly reduced ROS intensity, although they enhanced matrix mineralization, DNA content in S phase of the cell cycle, and levels of *Runx2* expression. However, higher concentrations (75–100 µg/ml) considerably

induced the ROS intensity and nuclear condensation in osteoblasts, while it reduced mineralization level, proportion of cells in S phase and *Runx2* level of the osteogenic gene.

Conclusions: These findings suggest that CQ extract revealed concentration-dependent biphasic effects, which would contribute notably to future assessment of pre-clinical efficacy and safety studies.

Introduction

Metabolic bone disorders, including osteoporosis and bone fracture healing, are major public health problems that afflict hundreds of millions people worldwide, importantly post-menopausal women (1). Although many therapeutic systems, such as chemotherapy and radiotherapy, have been developed for treatment of bone disorders, none are free from complications and side effects which limit successful outcomes in many cases (2). Accordingly, we needed to focus our study to develop a novel strategy for control and treatment of disease, free from side effects. Ethno-traditional uses of herbal products have been a major source for discovery of potential osteogenic agents to address these problems (3).

Cissus quadrangularis L. (CQ, Family Vitaceae) is one of the most frequently used medicinal plants widely distributed in India, Africa, Arabia and Indo-Malaysia (4). Recently, various extraction and analytical systems have been developed to study herbal components of its extract as well as its pharmacological activities. Gas chromatography coupled to mass spectrometry (GC-MS) is an ideal technique to identify volatile and semi-volatile components qualitatively as well as quantitatively, for rapid extraction, low solvent consumption and precise analysis

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of herbal components, to obtain enhanced recovery (5). A range of phytochemicals such as iridoids, stilbenes, quercetin, quercitrin, β -sitosterol, β -sitosterol glycoside, triterpenes, fatty acid methyl esters, glycerolipids, steroids, phytols, stigmasterol and cerebrosides have been reported in CQ plants (6,7) and various studies have reported osteogenic activity of CQ extracts in ovariectomized rats (8,9). CQ powder has also been clinically evaluated to reduce the intermaxillary fixation time for patients with fracture of the mandible (4,10). Moreover, phytochemicals present in CQ extracts have demonstrated multifunctional biological effects such as antioxidant capacity, analgesia, being anti-inflammatory, inducing apoptosis in cancer cells yet causing liver dysfunction (11,12).

Proliferation and differentiation of osteoblasts, required for bone formation is mediated by the osteogenic Runt-related transcription factor-2 (*Runx2*) gene. *Runx2* is a key transcription factor regulating premature osteogenesis and delayed mineralization of osteoblasts (13). Previously, *in vitro* studies have revealed that CQ extract has shown proliferative effects in osteoblastic MC3T3-E1 and SaOS-2 cell lines, other than anti-proliferative and apoptotic activities in human skin carcinoma A431 cells (14–16). On the basis of these reports, we speculated that CQ might have biphasic effects (proliferative and anti-proliferative) depending upon dose and time factors. Reactive oxygen species (ROS) also play an essential role in cell proliferation and cell cycle progression, depending upon dose and time of exposure (17,18). Hence, considering the role of ROS and cell cycle kinetics, no reliable studies have been developed so far, to link biphasic roles of CQ extract modulating ROS generation, cell cycle progression and *Runx2* expression, in primary cultures of rat osteoblasts.

Thus, this study was designed to evaluate the phytoconstituents present in CQ extract and to examine its biphasic effects, modulating osteogenic activity using MTT, ALP, mineralized nodule formation, ROS generation, apoptosis, cell cycle kinetics and *Runx2* expression, in osteoblasts. The study illustrated that lower concentrations exerted stimulatory biological effects and higher concentrations resulted in inhibitory effects, which would be beneficial for future assessment of pre-clinical efficacy and safety studies.

Materials and methods

Reagents and chemicals

Alpha-modified minimum essential medium (α -MEM), foetal bovine serum (FBS), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), *p*-nitrophenyl

phosphate (pNPP), naphthol AS–MX phosphate, fast blue BB salt and ascorbic acid were purchased from Himedia, India. Propidium iodide (PI), 4', 6'-diamidino-2 phenylindole (DAPI), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), β -glycerophosphate, 17 β -estradiol (E2) and RNase A were purchased from Sigma-Aldrich, Saint Louis, MO, USA. RNAiso Plus reagent was procured from Takara, New Delhi, India. cDNA synthesis kit was purchased from Thermo Scientific, Mumbai, Maharashtra and SYBER green kit from Roche, Mumbai, Maharashtra. All the reagents used were of high purity grade.

Ethics statement

Animal experiments were performed according to the protocol approved by the Institutional Animal Ethics Committee (IAEC), Faculty of Pharmacy, Integral University, Lucknow, India. Approval ID for this study was IU/Biotech/Project/CPCSEA/12/19.

Plant materials and extraction

Fresh cultivated CQ plants were collected from the Department of Botany, University of Lucknow, Lucknow, India in June 2012. A reference specimen (voucher No. IU/PHAR/HRB/14/06) was deposited in the herbarium of the Faculty of Pharmacy, Integral University, Lucknow. Collected areal components of plant materials were cut into small pieces, washed in double distilled water, air dried in the shade and pulverized into powder in a mechanical grinder. 95% ethanolic extract of CQ powder (400 g) was prepared with the help of Soxhlet apparatus (Borosil Glass Works Limited, India) at 60 °C using Whatman No. 1 filter paper. Filtrate obtained was concentrated under vacuum at 40 °C using a rotary evaporator (BUCHI Rotavapor R-205, Flawil, Switzerland) to obtain approximately 10% of the final yield. Plant extract was then used for GC-MS analysis and *in vitro* study.

GC-MS analysis

Gas chromatography combined with mass spectroscopy is preferable methodology for identification of compounds. Chemical composition of 95% ethanolic extract of CQ was analysed using a GCMS-QP2010 Plus system (Shimadzu, Tokyo, Japan). Ethanolic extract (1 μ l) of CQ was injected in split ratio 10:0 on to a RTX-5 column (60 m $\times$ 0.25 mm internal diameter, film thickness 0.25 μ m). Helium was used as carrier gas at constant flow rate of 1.21 ml/min at 90.4 kPa inlet pressure. Oven temperature was programmed to 100 °C (isothermal for 3 min) with increase rate of 10 °C/min to 250 °C (isothermal for 6 min), followed by 5 °C/min

increment to final temperature of 310 °C, which was held for 12 min. Injector and ion source temperatures were 260 °C and 230 °C respectively. Mass spectra were taken at 70 eV with a scan interval of 0.5 s and mass range of 40–600 (m/z). Total GC/MS running time was 42.99 min. For compound identification, CQ extract was separated into various constituents with different retention times, by mass spectrophotometry. Chromatograms of intensity against retention time were recorded by software attached to the mass spectrophotometer. Mass spectrum of unknown components was compared to spectra of known components stored in the NIST08 and WILEY8 library. Name, molecular weight and structure of the components of the CQ extract were ascertained.

Primary culture of osteoblasts

Osteoblasts – bone-forming cells, were isolated from neonatal rat pup calvaria following the protocol of sequential digestion, with slight modification (19). In brief, calvaria were dissected from four to five neonatal (1–2 days old) rat pups. After removal of sutures and adherent mesenchymal tissues, calvaria were subjected to five sequential (10–15 min) digestions at 37 °C, in a water bath shaker at 120 rpm containing 0.1% each of dispase and type II collagenase enzyme. Supernatants were pooled from the second to fifth digestions in a tube containing 800 µl foetal bovine serum (FBS). Cells were re-suspended in α -MEM containing 10% FBS and 1% penicillin/streptomycin solution, and plated in T-25 cm² culture flasks. Cells were then incubated at 37 °C in 5% carbon dioxide in a CO₂ incubator (Excella ECO-170, Hamburg, Germany).

Cell viability assay

Cell viability effects of CQ extract was performed using MTT assay, according to a previously used protocol (20). In brief, calvarial osteoblasts were trypsinized and 2×10^3 cells were seeded in each well of 96-well plates in 100 µl α -MEM complete media. Cells were incubated overnight at 37 °C in 5% CO₂. Stock solutions of CQ extract were prepared using α -MEM complete media. Cells were then treated at different concentrations (10, 25, 50, 75 and 100 µg/ml) of extract for different time periods (12, 24, 48 and 72 h). E2 at the concentration of 10^{-9} M was used as a positive control. After each treatment period, 10 µl MTT reagent (5 mg/ml in phosphate-buffered saline–PBS) was added to each well and the plate was further incubated at 37 °C for 3 h until formazan blue crystals developed. Supernatant was discarded from each well and 100 µl of DMSO was added (to solubilize the dark blue formazan crystals), at 37 °C

for 10 min. Absorbance was recorded at 540 nm by a microplate reader (BIORAD-680, Hercules, CA, USA) and percentage cell viability was calculated.

Alkaline phosphatase (ALP) assay

ALP activity was measured following a previously described protocol (21). Briefly, calvarial osteoblasts at 2×10^3 cells/well were seeded in 96-well plates. At 70–80% confluence, cells were treated with different concentrations (10–100 µg/ml) of CQ extract for the periods of 12, 24, 48 and 72 h, in osteoblast differentiating medium (α -MEM supplemented with 50 µg/ml ascorbic acid, 10 mM β -glycerophosphate and 10% FBS). E2 at the concentration of 10^{-9} M was used as positive control. At the end of the incubation period, the plate was washed twice in PBS and fixed by retaining at –70 °C, for 20 min and then brought to 37 °C for freeze fracture. Chilled pNPP substrate (50 µl) was added to each well and kept at 37 °C for 30 min for colour development. Activity was measured by the colorimetric method at 405 nm.

Determination of ROS activity

ROS are a key regulator in cell proliferation and apoptosis pathways and hence, intracellular ROS generation in osteoblasts was assessed by 2',7'-dichlorofluorescein diacetate (DCFH-DA) (22). In brief, osteoblasts (2×10^3 cells/well) were seeded in a 96-well black bottom culture plate for 24 h. They were then exposed to 10–100 µg/ml concentrations of CQ extract for 48 h in triplicate. After exposure, cells were incubated in DCFH-DA (10 mM) at 37 °C for 30 min. Then the reaction mixture was aspirated and replaced by 200 µl PBS, in each well, on a shaker for 10 min at room temperature in the dark. Fluorescence intensity was measured using a multiwell plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader; BioTek, Seattle, WA, USA) at excitation wavelength of 485 nm and emission wavelength of 528 nm. Photomicrographs of a further set of cells seeded in 96-well plates were taken using fluorescence microscopy (Nikon ECLIPSE Ti-S, Tokyo, Japan) to analyse ROS intracellular fluorescence intensity.

Assay of apoptosis

Apoptotic effect of CQ extract at different concentrations (10–100 µg/ml) was analysed using nuclear fluorescent DAPI following a previously used protocol (22). Cells were seeded and treated for 48 h in a 96-well plate. After treatment, they were washed in PBS and fixed in 4% paraformaldehyde for 10 min. Subsequently, cells were permeabilized with permeabilization buffer

(3% paraformaldehyde and 0.5% Triton X-100) and stained with DAPI. After staining, images were taken using a fluorescence microscope. Quantification of cells was performed in triplicate for each treatment and 100 cells per sample were counted in different fields (at least 10 random fields per sample) to score percentage apoptotic cells, as reported earlier using a fluorescence microscope (Nikon ECLIPSE Ti-S) (23).

Mineralization assay

Alizarin Red S, an anthraquinone derivative, was used to identify calcium content in the osteoblast preparation, following a previously used protocol (20). Mineralization assay was performed by culturing osteoblasts at 2×10^4 cells/well in a 12-well culture plate in differentiation medium (DM) consisting of α -MEM with 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate. CQ treatment at various concentrations (10–100 μ g/ml) was continued for 21 days and medium was changed on alternate days. At the end of experiments, cells were washed in PBS and fixed in 4% paraformaldehyde in PBS for 15 min. Fixed cells were stained with 40 mM Alizarin Red S (pH 4.5) for 30 min followed by washing in distilled water. Calcified nodules (which appeared bright red in colour) were photographed using an inverted phase contrast microscope. For quantification of staining, 100 mM cetylpyridinium chloride solution was added for 1 h to each well to solubilize and to release calcium-bound Alizarin red into solution. 100 μ l supernatant from each well was transferred to a 96-well plate in triplicate and absorbance was recorded at 570 nm by a microplate reader.

DNA content analysis

Cell cycle phase distribution and DNA content investigation was carried out using flow cytometry (20). Osteoblasts were plated in six-well plates at 1×10^6 cells/ml and treated with different concentrations (10–100 μ g/ml) of CQ extract for 48 h. Cultured cells were then washed in cold PBS and fixed in 70% ethanol at -20°C for 2 h. Fixed cells were treated with RNase A (10 mg/ml) and stained with PI in the dark for 30 min at room temperature. PI fluorescence of individual nuclei was measured using flow cytometry (BD FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA). Data were analysed with the aid of Cell Quest Pro V 3.2.1 software (Becton Dickinson).

Quantitative real-time PCR (qPCR)

Total RNA was isolated from treated and untreated cultured osteoblasts, with different concentrations (10–100 μ g/ml)

of CQ extract, using RNAiso Plus reagent following the manufacturer's instructions. 2.0 μ g aliquots of total RNA in 10 μ l reaction volume were used to synthesize cDNA using the cDNA synthesis kit. Quantitative real-time PCR was performed in a light cycler PCR system (LightCycler 480; Roche) using SYBER green kit. *Runx2* gene expression in calvarial osteoblasts was determined by qPCR using a standard protocol (3). Sequence of primer pairs of the genes were as follows: *Runx2*-CCACAGAGCTATTAAGTGACAGTG (F), AACAACTAGGTTTAGAGTCATCAAGC (R); GAPDH (housekeeping gene)-CAGCAAGGATAGTGAAGAG (F), GGA TGAATTGTGAGGGAGATG (R). Data were normalized to GAPDH expression as the internal control, to study relative expression of the targeted gene.

Statistical analysis

All results are presented as mean $\pm$ SEM and differences between treatment groups compared to controls was analysed using Student's *t*-test. Probability value of $P < 0.05$ was considered to be statistically significant.

Results

GC-MS analysis

GC-MS chromatogram of the alcoholic extract of native CQ had approximately 40 peaks representing presence of 40 bio-active compounds (Fig. 1). The identified compounds, their retention time, per cent peak area and *m/z* (mass-to-charge ratio) are summarized in Table 1.

Cell viability assay and kinetics

MTT assay showed that at 12 h exposure, viability of the osteoblasts increased significantly ($P < 0.05$) in a dose-dependent manner compared to controls. However, 75 μ g/ml concentration of CQ extract increased the cell viability and 100 μ g/ml extract reduced cell growth at 24 h extract exposure. Similarly, 50 μ g/ml extract resulted in approximately 22.82% cell growth at 48 h, but at higher concentrations (75 and 100 μ g/ml) it showed adverse effects. Greatest cell viability, approximately 28.52%, was observed at 72 h with 50 μ g/ml CQ treatment. As a result, lower concentrations (10–50 μ g/ml) enhanced cell activity for 72 h (Fig. 2a). Exposure of cells with 10^{-9} M of E2 increased the cell viability to 18.66, 20.1 and 23.12% at 24, 48 and 72 h respectively. Figure 2b and 2c represent the kinetic model of cell viability data. Equation $C = C_0 + Kt$ was found to be the most suitable fit for data obtained in cases of lower concentrations (10–50 μ g/ml). However, for higher concentrations (75 and 100 μ g/ml),

Figure 1. GC-MS chromatogram of the 95% ethanolic extract of CQ. Chemical composition of CQ extract with their retention time identified by GC-MS.

the best suitable fit included linear growth followed by exponential decay with equations $C = C_0 + K_1t$ and $C = A \exp [-K_2 (t - t_0)]$ respectively. Here, C_0 and K are initial number and rate constant of cell viability respectively. Corresponding values of growth and decay are shown in Table 2. Regression analysis yields the value of R^2 and is found to vary between 0.999 and 0.997 for lower concentrations (Table 2a), while for higher concentrations, the value of R^2 was found to vary between 0.875 and 0.717 (Table 2b).

Microscopic observation of osteoblasts

Figure 2d reveals comparative cell morphology of osteoblasts treated with different concentrations of CQ extract at 48 h. By phase contrast microscopy untreated primary osteoblasts had typical spindle shapes with fibroblastic appearance. Osteoblasts treated with lower concentrations of CQ extract elicited faster growth compared to untreated cells. At 10 $\mu\text{g/ml}$ of CQ extract, osteoblasts changed their morphology and acquired slightly elongated shapes indicative of cell proliferation. Moreover, at 25 and 50 $\mu\text{g/ml}$ extract treatment, cells had more rapidly fusiform appearance with increase in numbers compared to controls. On the other hand, 75 and 100 $\mu\text{g/ml}$ CQ extract distorted the cells to slightly rounder shapes with loss of membrane integrity, and reduced cell growth.

ALP assay

As observed from the results, concentration range (10–100 $\mu\text{g/ml}$) of extract induced cell differentiation at both time periods of 12 (data not shown) and 24 h in a dose-dependent manner. Higher concentrations (75–100 $\mu\text{g/ml}$) of extract tended to reduce ALP levels at 48 and 72 h

(Fig. 2e). However, lower concentrations (10–50 $\mu\text{g/ml}$) significantly increased ALP levels at both 48 and 72 h ($P < 0.05$) compared to controls. Exposure of cells to 10^{-9} M E2 also significantly increased ALP activity. These data were consistent with the MTT result (Fig. 2a).

As a result of substantial increase in proliferation and differentiation of the osteoblasts, the effect of different concentrations of CQ extract on ROS generation, apoptosis, DNA content and subsequently osteogenic gene *Runx2* expression were further evaluated at 48 h.

Determination of ROS activity

Fluorescence microscopy images (Fig. 3a) showed that with increasing extract concentrations (10–50 $\mu\text{g/ml}$), ROS intensity was slightly reduced without distorting cell native morphology, compared to controls. However, higher concentrations (75–100 $\mu\text{g/ml}$) induced ROS intensity and deformed morphology. Moreover, quantitative data of ROS generation revealed that 75 and 100 $\mu\text{g/ml}$ extract induced significant ($P < 0.05$) intracellular ROS level approximately 9.57% and 21.64% respectively, compared to controls (Fig. 3b). Little change in ROS level was observed at 10 $\mu\text{g/ml}$ extract, reducing only 0.32% of ROS, while 25 and 50 $\mu\text{g/ml}$ concentrations of extract significantly reduced the ($P < 0.05$) ROS level to approximately 3.48% and 5.56% respectively, compared to controls.

Assay of apoptosis

As observed from photomicrograph (Fig. 3c), untreated and treated cells with lower concentrations of CQ extract (10–50 $\mu\text{g/ml}$) did not show any significant fluorescence or condensed nuclei. However, fragmented

Table 1. Phytoconstituents of 95% ethanolic extract of CQ identified by GC-MS

S. No.	Compounds name	RT (min)	Area (%)	Fragmentation (m/z)
1.	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)	8.903	0.28	43,41,69
2.	Phenol, 2,4-bis(1,1-dimethylethyl)	9.716	0.09	191,57,206
3.	Benzoic acid, 4-ethoxy-, ethyl ester	9.905	0.15	121,149,138
4.	Dodecanoic acid, ethyl ester	10.653	0.25	88,101,41
5.	Estra-1,3,5(10)-trien-17.β-ol	12.648	0.58	43,57,41
6.	2,6,10-trimethyl,14-ethylene-14-pentadecne	13.402	0.20	68,82,95
7.	Pentadecanoic acid	13.723	0.29	73,43,60
8.	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	13.788	0.16	149,57,41
9.	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	13.847	0.12	68,82,43
10.	Pentadecanoic acid, ethyl ester	13.958	0.93	88,101,43
11.	n-Hexadecanoic acid	14.800	8.57	60,73,43
12.	Hexadecanoic acid, ethyl ester	14.973	4.40	88,101,41
13.	Heptadecanoic acid	15.666	0.93	43,57,73
14.	Phytol	16.150	1.02	71,43,57
15.	6-Octadecenoic acid, (Z)	16.488	8.30	55,41,69
16.	n-Propyl 9,12-octadecadienoate	16.558	1.20	67,81,55
17.	Ethyl oleate	16.614	4.76	55,69,41
18.	Octadecanoic acid, ethyl ester	16.822	2.22	88,101,70
19.	4,8,12,16-Tetramethylheptadecan-4-olide	18.271	0.54	99,43,57
20.	Icosanoic acid	18.331	0.76	43,57,73
21.	Docosanoic acid, ethyl ester	19.536	0.70	88,43,57
22.	L-(+)-Ascorbic acid 2,6-dihexadecanoate	20.437	0.53	57,73,43
23.	Heptadecanoic acid, ethyl ester	20.751	1.27	88,101,41
24.	Vitamin E	27.982	0.60	165,430,205
25.	Ergost-5-en-3-ol, (3.β.,24R)	28.789	3.70	107,95,145
26.	Stigmasterol	29.061	0.82	55,83,69
27.	β.-Sitosterol	29.599	6.62	43,57,41
28.	Cholest-4-en-3-one	29.953	0.42	124,43,229
29.	Lup-20(29)-en-3-one	30.099	1.34	205,109,95
30.	4,22-Stigmastadiene-3-one	30.204	0.41	55,69,81
31.	Lupeol	30.340	0.50	43,68,81
32.	Lup-20(29)-en-3-yl acetate	30.602	0.42	43,95,189
33.	Stigmast-4-en-3-one	30.815	4.19	124,43,412
34.	Cholesta-4,6-dien-3-one	31.099	0.28	43,136,382
35.	Friedelin	31.731	0.87	69,109,95
36.	Cyclopropa[5,6]stigmast-22-en-3-one, 3',6-dihydro-, (5.β.,6.α.,22E)-	31.896	0.07	55,43,69
37.	4,4a,6b,8a,11,11,12b,14a-octamethyl-eicosahydro-picen-3-one	32.105	0.26	69,95,55
38.	3.α.,7.β.-Dihydroxy-5.β.,6.β.-epoxycholestane	32.408	0.04	123,95,55
39.	Stigmastane-3,6-dione, (5.α.)	32.637	1.20	55,57,98
40.	Solanesol	34.399	0.13	69,81,93

and condensed nuclei were observed in osteoblasts treated with higher concentrations of CQ extract (75–100 µg/ml). Furthermore, approximately 6.33% and 10.67% apoptotic cells were observed at 75 and 100 µg/ml extract treatment respectively (Fig. 3d). These resulted in deep blue fluorescence that highlighted nuclei with abnormal margins and fragmented chromatin.

Mineralization assay

Study using inverted phase contrast microscopy revealed that proliferative osteoblasts exhibited fibroblastic monolayer morphology. Cells appeared to form mosaic-like multiple layers at 50 µg/ml extract, but this was inhibited at 75 and 100 µg/ml (Fig. 4a). Quantitative data of

mineralized bone nodule formation of osteoblasts treated with CQ extract at 21 days are shown in Fig. 4b. As observed from the graph, lower concentrations (10–50 µg/ml) increased mineralized nodules of cultured osteoblasts, showing significant osteogenic effect ($P < 0.05$) in a dose-dependent manner compared to control osteoblastic media (OBM) and DM. However, higher concentrations (75–100 µg/ml) appeared to reduce the level of mineralization.

DNA content analysis

As observed from the results (Fig. 5a,b), mean proportion of S phase cells of controls sharply increased from 27.5% to 34.8% at 10 µg/ml of CQ treatment. Maximal

Figure 2. Per cent cell viability, morphology and ALP activity of primary osteoblasts. (a) Per cent cell viability of osteoblasts treated with 10–100 µg/ml of CQ extract for the period of 12–72 h. Cells were stained with MTT dye and the absorbance was recorded at 540 nm by a microplate reader. (b, c) Kinetic model of per cent cell viability of osteoblasts treated with lower concentrations (10–50 µg/ml) and higher concentrations (75–100 µg/ml) of CQ extract respectively, (d) Morphology of osteoblasts under inverted phase contrast microscope treated with different concentrations of CQ extract at 48 h (magnification = 20x and scale bar = 0.1 mm) and (e) ALP activity of osteoblasts treated with increasing concentrations (10–100 µg/ml) of CQ extract for 24–72 h. *p*-nitrophenyl phosphate (pNPP) substrate was added for colour development and values were measured by a microplate reader at 405 nm. Values were obtained from three independent experiments and expressed as mean $\pm$ SEM. * $P < 0.05$ compared with control.

Table 2. Comparison of decay times of cell viability at different concentrations of CQ extract (A and B)

CQ concentration (µg/ml)	Value of K (h^{-1})	Time constant (h)	R^{2a}
(A)			
10	0.13197	7.577479730	0.99998
25	0.29894	3.345152873	0.99952
50	0.45543	2.195727115	0.99787
CQ concentration (µg/ml)	Time constant t_1 (h)	Time constant t_2 (h)	R^{2b}
(B)			
75	0.901111972	2.034339653	0.87570
100	1.072248075	1.363029196	0.71753

^aEquation fitted: $C = C_0 + Kt$, $C_0 = 100$.

^bEquation fitted: $C = C_0 + K_1t$, $C = A \exp [-K_2(t - t_0)]$.

cell expansion (DNA content) in S phase was found to be 47.4% at 50 µg/ml of extract. Interestingly, no apoptotic cells were observed at lower concentrations of extract treatment. However, mean proportion of cells in S phase dramatically dropped to approximately 32.3% at 75 µg/ml extract and subsequently it was reduced at 100 µg/ml CQ treatment. Apoptotic cells also markedly increased at higher CQ concentrations. On the other hand, lower concentrations of CQ extract reduced percentage of cells in G2/M phase and higher concentrations increased those in G2/M. Little variation in G0/G1 phases was found between concentrations of CQ extract.

Osteogenic gene *Runx2* expression analysis

Effects of different concentrations of CQ extract on *Runx2* expression were observed by qPCR at 48 h. As

Figure 3. Measurement of intracellular ROS generation and nuclear condensation in primary osteoblasts. (a) Fluorescence microscopy images of osteoblasts showing intracellular ROS generation induced by different concentrations (10–100 µg/ml) of CQ extract, (b) Quantitative data of ROS generation expressed as the percentage of fluorescence intensity relative to the controls, (c) Fluorescence microscopy images showing nuclear condensation in osteoblasts treated with different concentrations (10–100 µg/ml) of CQ extract (magnification = 20x and scale bar = 0.1 mm) and (d) Numerical data were expressed as % apoptotic cells relative to their controls. The cells were counted manually under a fluorescence microscope in at least 10 random fields and per cent apoptotic cells were calculated as detailed under Materials and Methods. Values are obtained from three independent experiments and expressed as mean $\pm$ SEM. * $P < 0.05$ compared with control.

Figure 4. Mineralization assay of osteoblasts. (a) Photomicrographs showing increased formation of mineralized nodules of osteoblastic cells in osteoblast medium (OBM, control group, no induction), osteoblast differentiation media (DM) and osteoblast differentiation media with increasing concentrations (10–100 µg/ml) of CQ extract for 21 days (magnification = 10x and scale bar = 0.2 mm) and (b) Quantitative data of Alizarin Red S extraction expressed in the form of OD. Data are represented as mean $\pm$ SEM of three independent experiments. * $P < 0.05$ versus OBM and DM.

is clear from the results (Fig. 6), lower concentrations (10–50 µg/ml) of CQ extract significantly ($P < 0.05$) elevated *Runx2* expression level compared to control. However, concentrations (75–100 µg/ml) of CQ extract reduced *Runx2* level in a dose-dependent manner compared to controls ($P < 0.05$).

Discussion

The present study explored identification of phytoconstituents of native CQ and ability of CQ extract to

modulate osteogenic induction through ROS generation, cell cycle kinetics and *Runx2* expression in a primary rat osteoblast model system. In the CQ extract, approximately 40 bioactive compounds were identified by using GC-MS, whereas approximately 3/4 of the extract was primarily composed of fatty acid, esters, phenolic compounds and phytoosteroids (Fig. 1 and Table 1). The major components, n-hexadecanoic acid and its derivatives hexadecanoic acid ethyl ester and octadecanoic acids (rich fatty acids) play an important role in multifunctional biological processes such as being

Figure 5. DNA content analysis by flow cytometry. (a) Pictorial graph showing the mean proportion of cells in S phase treated with different concentrations (10–100 µg/ml) of CQ extract at 48 h. Results expressed as the % of S phase population and (b) Quantitative distribution of percentage cells in different phases of cell cycle treated with different concentrations of CQ extract. Data are representative of three independent experiments and expressed as mean ± SEM. * $P < 0.05$ compared with control.

Figure 6. Analysis of mRNA levels of Runx2 gene by qPCR. Primary osteoblasts were treated with (10–100 µg/ml) of CQ extract for 48 h and Runx2 mRNA expression level was analysed by qPCR. Fold changes in mRNA levels are indicated by the numbers derived after normalizing with GAPDH mRNA levels used as an internal control. Values are obtained from three independent experiments in triplicate and expressed as mean ± SEM; * $P < 0.05$ as compared to control.

anti-inflammatory, causing apoptotic induction of cancer cells and causing liver dysfunction (24,25). In addition to bone healing and anti-osteoporotic activities (4,26), flavonoids and polyphenolic compounds are also valuable for antioxidant and anti-microbial activities (27). Phenolic content of CQ extract has been shown to have

apoptotic activity in the human skin carcinoma A431 cell line (16). Other components such as lupeol have been reported to be osteogenic compounds; however, friedelin exhibits significant loss of osteoblast viability (28). Vitamin E (α -tocopherol) reduces tumour cell growth and viability as a result of its antioxidant activity (29).

Regarding biphasic effects of CQ extract on cell proliferation, we composed the data using a kinetic model and suggest that at lower concentrations of CQ extract, growth and percentage cell viability followed zero-order kinetics. This means that growth was independent of concentration of cells present at any particular time. At higher concentrations >50 µg/ml, initial growth was observed, followed by a gradual decrease in cell viability. This indicated that decay followed first-order kinetics depending on the concentration of the cells. Similar kinetic models have been seen earlier in proliferation and apoptosis of mast cells (30). Viability of lymphocytes has also been examined on activated carbon fibre/carbon nanofibre (ACFs/CNFs) where exponential decay curve followed first-order kinetics (31). The kinetic model in our study also proved that the effect of CQ

extract on cell viability depended upon dose, time and number of cells present (Table 2). Morphological data showed elongated and fusiform appearance of osteoblasts at lower concentrations (10–50 µg/ml) of CQ treatment suggesting its osteogenic activity, while spherical shapes with loss of membrane integrity at higher concentrations (75–100 µg/ml) showed its anti-proliferative activity.

Moreover, ALP data clearly illustrated that lower concentrations of CQ extract not only stimulated proliferation but also induced differentiation of osteoblasts at all time points tested. Increase in ALP activity could be explained by the CQ extract possessing vitamin C (an antioxidant) initiating collagenous extracellular matrix formation (32). CQ and vitamin C both stimulate fracture healing, but the healing effect of CQ has been found to be faster than vitamin C (33). It appears to be a cooperative effect of other antioxidants such as phytoestrogens and polyphenolic compounds present in CQ extract. One recent study has reported that glycerolipids and squalene present in CQ extract stimulate ALP activity (7). On the other hand, decreased cell viability and ALP activity here, were observed at higher CQ concentrations (Fig. 2). In agreement with previous reports, our study also indicated that higher concentrations of CQ extract decreased osteoblast proliferation over longer exposure, suggesting that higher concentration probably reduced bone formation by reducing osteoblast differentiation, accompanied by cell proliferation (14,15). Mineralized nodule formation is a characteristic feature of late-stage osteoblastic differentiation (28). The mineralization data also support our findings that lower concentrations of CQ extract induce mineralization in osteoblasts at 21 days exposure (Fig. 4). A number of studies have reported concentration- and time-dependent effects of *Cajanus cajan* and *Tinospora cordifolia* extract on proliferation, osteogenic differentiation and mineralization of osteoblast model systems *in vitro* (34,35). Interestingly, our study also showed that effects of CQ extract on proliferation and differentiation of osteoblasts are both concentration- and time-dependent.

Runx2 is a non-collagenous, highly conserved transcription factor involved in regulation of mineralized matrix of bone. Our study suggests that low concentrations of CQ extract induced Runx2 expression, but higher concentrations reduced it (Fig. 6). Runx2 binds to osteoblast-specific cis-acting elements found in the promoter region of several osteoblast marker genes in osteoblasts and induces expression of osteocalcin, collagen type I alpha 1 (Col1α1), bone sialoprotein (BSP), ALP, and regulates their expression (36). Some study has reported that the flavonoid including quercetin and β-sitosterol present in CQ extract increased transcriptional activity of Runx2

and hence induced ALP mRNA expression, collagen mRNA, and their protein levels (37,38). In addition to matrix mineralization, Runx2 activity is functionally linked to mechanisms of the cell cycle that control proliferation and differentiation of osteoblasts (39). In a recent study, bovine collagen peptide compounds stimulated MC3T3-E1 cell proliferation by promoting DNA synthesis in S phase of cell cycle, which led to increase in osteoblast differentiation by up-regulating level of Runx2 expression (40). Interestingly, our study also supported results that lower concentrations of CQ extract induced cell cycle arrest at S phase, promoting cell proliferation and increasing Runx2 expression level and *vice versa*.

ROS also play a crucial role in cell proliferation and cell cycle progression (17,18). Hence, we attempted to clarify cellular mechanisms modulating osteogenic effects of CQ extract, by employing ROS modulation and cell cycle progression in osteoblasts. Our data demonstrated that lower concentrations of CQ extract inhibited ROS generation and consequently, lead to encourage cell proliferation and differentiation *via* promoting cell cycle progression in S phase (Fig. 5). These findings support reports that low levels of ROS play a pivotal role in regulating several key physiological mechanisms, including cell proliferation, cell differentiation, apoptosis, cell cycle regulation and various signal transduction pathways such as the mitogen-activated protein kinase (MAPK) pathway (41,42). On the other hand, higher concentrations tended to induce excessive production of ROS, indicating that higher concentrations suppress its own promoting effect in S phase of the cell cycle, and lead to loss of cell function by inducing an apoptotic pathway (Fig. 3). One previous *in vitro* study agrees with our results that quercetin, a flavonoid, induced cytochrome-c release and ROS accumulation to promote apoptosis and arrest the cell cycle in G2/M in HeLa cervical carcinoma cells (43). Antioxidants present in CQ extract at higher doses are relatively greater, thereby resulting in enhanced cytotoxicity.

Briefly, data on cell viability, ALP activity, DNA content, mineralization and Runx2 level of osteoblasts, revealed a biphasic pattern, increasing bioactivity at lower but decreasing at higher concentrations of CQ extract. In contrast, data of ROS generation and apoptosis show its reverse effect. Similar biphasic effects have also been reported on bone cells from various other plant extracts (44). Although no definitive explanation for such effect is available, our study demonstrated that these biphasic effects seem to be due to presence of more phytoconstituents (flavonoids, polyphenols) at higher concentrations resulting in inhibitory/cytotoxicity counteracting stimulatory effects of the extract when used at lower concentrations. In general, flavonoids are phenolic and pro-oxidant

or antioxidant activity intimately depends on pH and concentration (45). At low concentration, they act as antioxidants but at higher concentration they can generate free radicals and be pro-oxidants. Interestingly, one study has shown that quercetin had antioxidant activity at low doses (0.1–20 μM), while higher concentrations (>50 μM) reduced cell survival and viability (46).

In conclusion, for the first time, the present study provided corroborative evidence that CQ extract showed biphasic effects – osteogenic and cytotoxic, by employing ROS modulation, cell cycle kinetics and *Runx2* gene expression in primary osteoblasts. These analytical studies have been established to determine dose of CQ extract and time, will contribute notably to quality control of the extract for further assessment of pre-clinical efficacy and safety studies of CQ extract in bone healing and cancer treatments.

Acknowledgements

The authors acknowledge support from University Grant Commission (UGC), New Delhi (No. 37/436-2009 SR) in the form of a research grant. We express our thanks to Advanced Instrumentation Research Facility (JNU, New Delhi) for the GC-MS analysis and to Mr. A.L. Vishwakarma, SAIF-Division, CSIR-CDRI for technical assistance for flow cytometry. Authors Sahabjada Siddiqui and Madu Gupta are thankful to Indian Council of Medical Research (ICMR) and Council of Scientific and Industrial Research (CSIR), New Delhi, India for the award of Senior Research Fellowship (No. 45/26/2013/BMS/TRM) and (No. 09/107(0350)/2010 EMRI) respectively.

Conflict of interest

Authors have no conflicts of interest.

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