Cissus quadrangularis Linn. Extract Reduces High Glucose Induced Inhibition of *In Vitro* Proliferation and Matrix Mineralization in MG-63 Cells

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ABSTRACT: The aim of this study was to evaluate the effect and mechanism of action of ethanolic extract of *Cissus quadrangularis* Linn. stem in an *in vitro* system using MG-63 (osteoblast-like) cells. Cells were pre-exposed to high glucose (45 mmol/l). Insulin was used as positive control. To determine whether *C. quadrangularis (CQ)* extract could prevent hyperglycaemia induced oxidative stress, and apoptosis we performed reactive oxygen species generation using 2',7'-dichlorofluorescin diacetate and propidium iodide staining for apoptosis. For cell proliferation, we carried out cell viability (MTT) assay. Further, alkaline phosphate assay and alizarin staining were done to study the matrix mineralization. High glucose (45 mmol/l) significantly ($P < 0.001$) inhibited the cell viability and matrix mineralization, and induced apoptosis as compared to physiological control. *C. quadrangularis* extract as well as insulin treatment significantly ($p < 0.001$ *versus* high glucose) restored the cell viability, cell differentiation and bone nodule formation. Thus, ethanolic extract of *C. quadrangularis* stem prevents the antiproliferative effect of high glucose.

Key words: High glucose, apoptosis, cell viability, MG-63, *Cissus quadrangularis.*

INTRODUCTION

Diabetes mediated hyperglycemia increases the risk of osteoporosis and other pathological complications in bone.[1,2] Bone loss during type I diabetes mellitus is accompanied by lack of insulin due to the inactivation and destruction of pancreatic beta cells.[3] This in turn is followed by elevated blood glucose which causes osmotic shock, altered proliferation and induced apoptosis of bone cells with reduced bone remodelling.[4] *Cissusquadrangularis* Linn. (*CQ*) (family – Vitaceae) commonly known as Hadjod (bone healer) in Indian subcontinents is well known for its anti-resorptive and bone healing properties[5]. *CQ*is very well demonstrated for its osteogenic activity for estrogen deficient osteoporosis.[6,7] The ethanol extract of stem of *CQ* is a rich source of calcium ion and has the ability to form bioorganic calcite crystals.[8] Effect of *CQ* is yet to be explored on bone cells of diabetic model. So we evaluated *in vitro* efficacy of *CQ* extract and its potential to ameliorate high glucose-induced cell injuries in human osteoblast like MG-63 cells pre-exposed to high glucose. Along with, we examined the mechanism of its action in relation to the involvement of reactive oxygen species, biochemical and cellular markers of proliferation, apoptosis and matrix mineralization.

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MATERIALS AND METHODS

Chemicals and reagents. Minimum Essential Medium (MEM), Foetal Bovine Serum (FBS), sodium pyruvate, non-essential amino acids, sodium bicarbonate, L-glutamine, antibiotic solution (streptomycin/ penicillin), ascorbic acid, alizarin red powder, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) dye and dexamethasone were purchased from HIMEDIA Laboratories Pvt.

Ltd., Mumbai, India. Cetylpyridinium chloride (CPC) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Insulin used was 100 IU/ml of Huminsulin (regular) from Eli Lilly and Company, India. 2',7'-Dichlorofluorescin diacetate (DCFH-DA), Propidium iodide (PI), β-glycerophosphate was from Sigma-Aldrich Inc., St. Louis, USA. Dimethyl sulfoxide (DMSO) was from Merck Specialities Pvt. Ltd., Mumbai, India. All the other reagents were of analytical grade.

Collection of plant and preparation of extract. The stem of *C. quadrangularis (CQ)* was collected by the Department of Botany, University of Lucknow, India in month of Juneand systematically identified by Muhmmad Arif, Assistant Professor, Department of Pharmacognosy, Integral University, Lucknow. Voucher specimen No. IU/PHAR/HRB/ 14/06, was deposited in the same department for reference. Shade dried parts of the plant was finely powdered and then subjected to ethanolic extraction using 95% ethanol in a Soxhlet extractor.

Cell culture. MG-63 cell – a human osteoblast like cell line was procured from National Centre of Cell Science (NCCS), Pune, India. Cells were cultured in MEM medium containing 10% FBS and 1% antibiotic solution (streptomycin and penicillin). Cells were grown at 37°C in a humidified air containing 5% CO_2 till confluence.

Experimental setup. The osteoblasts were divided into six groups: physiological control (Ph. Ctrl) in which the cells were maintained with physiological glucose concentration (5.5 mmol/l); a negative control high glucose (HG) group in which the cells were exposed to high glucose (= 45 mmol/l was the concentration at which ~ 50% cell death observed).[9] In groups 1, 10 and 50 means cells were pre-exposed to high glucose for 24 h before treatment and then treated with1µg, 10µg and 50µg of *CQ* extract, respectively and insulin (INS) group in which HG pre-exposed cells were treated with insulin (0.5IU).

MTT assay for cell proliferation. MTT assay was performed to evaluate cell viability of MG-63 in different treatment groups. Cells were cultured in 96-well plates at a density of 1.2×10^6 cells/well according to a previously described protocol.[10] After 44 h of treatments, the cells were exposed to10 µl of MTT solution (5 mg/ml stock solution) in each well and re-incubated for 4 h at 37°C until formazan blue crystal developed. Media was discarded from each well and 100 µl of DMSO was added to dissolve formazan crystals for 10 min at 37ºC. The absorbance was recorded at 540 nm by microplate reader (BIORAD-680) and relative percentage cell viability was evaluated using the formula:

$$\text{Cell viability} = \frac{A_T - A_0}{A_C - A_0} \times 100$$

Where A_T is absorbance of treated, A_0 is absorbance of blank and A_C is absorbance of physiological control.

Propidium Iodide staining for apoptosis detection. This staining was based on selective labelling of cells in very late apoptotic phase or necrotic cells by propidium iodide (PI).[11] Briefly, the monolayer washed thrice with PBS, fixed in 4% paraformeldehyde for 10 min and 100 µl of PBS was added. 1 µl of PI dye (5 mg/ml) was loaded in each well of 96 well plates and incubated for another 10 min. Cells were visualized under fluorescence microscope (Nikon, ECLIPSE T*i*-Series) for determination of morphological changes. For quantification, percentages of apoptotic cells were calculated as the ratio of cells showing bright red fluorescence (PI positive) to the total number of cells (PI positive + PI negative).

Alkaline phosphatase (ALP) Assay. ALP activity was measured according to Faucheux *et al*, (2008). MG- 63 cells were plated at 5×10^3 cells in 100 µl ALP media (MEM containing 10 mmol/l β-glycerophosphate and 50 µg/ml ascorbic acid) per well in a 96 well plate and incubated for 24 h. After incubation, cells were pre-exposed to HG and treated with 1, 10 and 50 µg of *CQ* extract and 0.5 IU of insulin. Beside this a physiological control group was maintained at 5.5 mmol/l of glucose for comparison purpose. After treatment plates were washed with PBS and kept at -70ºC for 20 min. Plates were taken out and then kept at 37ºC for 10 min. 75 µl of chilled pNPP substrate was added to plates (Substrate as 9.4

mg pNPP/5 ml of milliQ water). Cells were incubated at 37°C for half an hour for color development. Finally, absorbance was taken at 405 nm in multi well ELISA plate reader (BIORAD 680).

Mineralization assay. Mineralization assay was performed according to published method. Briefly, upon 80% confluence in 12 well plates, cells were cultured in differentiation medium containing 10 mmol/l β-glycerophosphate, 50 µg/ml of ascorbic acid and 10^{-7}mol/l dexamethasone. Treatment groups were same as in above experiments. Culture media was refreshed every second day till two weeks. On day 15, media was aspirated and cells were washed twice with PBS, fixed in 10% formalin for half an hour, and stained with 40 mmol/l alizarin red stain for 10 min. Excess stain was removed by washing 5 times with distilled water. Cells were analyzed under inverted microscope and images were taken (Nikon, ECLIPSE T*i*-Series). For quantification purpose, alizarin red S-calcium complex then incubated with 10% CPC in 10 mmol/l sodium phosphate for 30 min. The concentration of released alizarin red stain was determined by taking absorbance at 610 nm in a multi-well ELISA plate reader.

DCFH-DA staining for reactive oxygen species (ROS). Percentage of cells generating ROS can be assessed by staining cells with DCFH-DA.[11] The principle includes diffusion of DCFH-DA, deacetylation into DCFH and its oxidation into highly fluorescent DCF in the presence of ROS. Freshly prepared10 mmol/L DCFH-DA was loaded in 24 well plates and incubated for 30 minutes at 37°C. After cells were visualized under fluorescence microscope (Nikon, ECLIPSE T*i*-Series) percentage of ROS generating cells to that of total number of cells was calculated.

Statistical analysis. All the data were presented as the means ± S.D. of results achieved after experiment. Statistical analysis was performed by one way ANOVA method followed by the Tukey's test of significance using Graph Pad Prism 5.0 software. Probability values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

When data from cell proliferation assay (Figure 1) and apoptosis (Figure 2) were compared together, an inverse relationship between proliferation and percent apoptotic cells was found. The rate of proliferation was significantly ($P < 0.001$) decreased to 38.0% in HG group as compared to physiological control (Figure 1). *CQ* extract in all its groups normalized the effect of HG and restored the proliferation rate near to the physiological control. Cell viability was found to increase to 158.0% in the group treated with 50 µg of *CQ* extract, suggesting that the high dose is more effective in reducing hyperglycaemia-induced inhibition of cell proliferation. When compared with insulin-treated group, *CQ* extract at 50 µg dose was found to be more effective ($p < 0.05$ *versus* insulin). In HG group, increased rate of apoptosis was observed (55.1%; $p < 0.001$). While in all the treatment groups, percentage of apoptotic cells was reduced ($p < 0.001$) (Figure 2).

The ALP activity was significantly ($p < 0.001$) lowered in HG group as compared to physiological control. The protective effect of insulin and *CQ* extract was evident with significant increase ($p < 0.001$) in ALP activity in all of these groups (Figure 3). A significant 33.0%, 48.0% and 56.0% increase in ALP activity in *CQ* treated groups at 1, 10 and 50 µg respectively and 48% increase in insulin treated group was observed when compared with HG.

Mineralization nodule formation was significantly ($p < 0.001$) reduced in HG by 84.5% as compared to physiological control. Rate of mineralization was significantly ($p < 0.001$) increased in 1, 10, 50 µg*CQ* and insulin treated groups by 73.8%, 84.7%, 85.9%, and 80.0%, respectively when compared to HG group (Figure 4). The ROS generation was assessed by DCF fluorescence (Figure 5). The percentage of cells showing fluorescence was quite high in HG (73.0%) than rest of the groups ($p < 0.001$). The percentage of ROS generating cells was reduced to 1.1% in 50 µg*CQ* treated group showing the least number of fluorescence generating cells.

High glucose attenuates osteoblastic proliferation *via* three mechanism- (i) osmotic shock[4,12] (ii) enhanced ROS generation thus causing oxidative stress[13], and (iii) inducing apoptosis.[12] *CQ* treated groups showed dose dependent enhancement in cell proliferation thus reversing the deleterious effects of high glucose. *CQ* at high dose (50 µg) showed marked increase in cell proliferation with enhanced

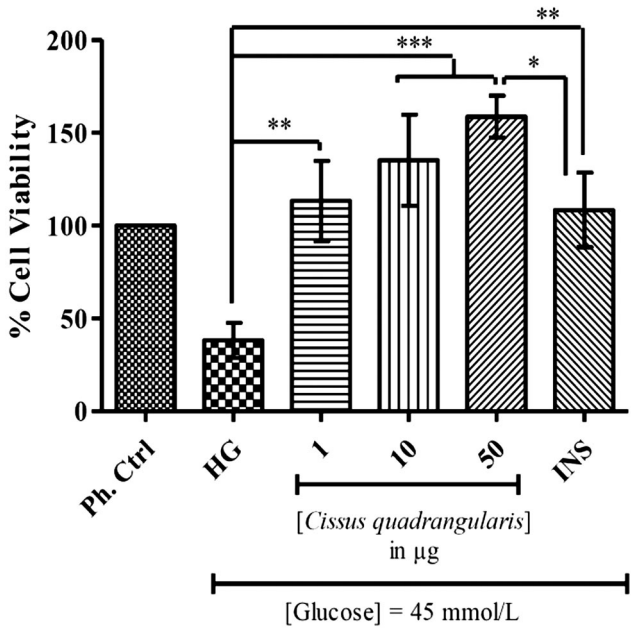

Figure 1.Anabolic action of *Cissus quadrangularis* extract (*CQ*) on osteoblast like MG-63 cells. Cells were preincubated with high glucose (HG) = 45 mmol/L and then treated with insulin (INS) = 0.5 IU and 1, 10, 50 µg concentrations of *CQ* extract Ph. Ctrl simply represent physiological control containing 5.5 mmol/l glucose. MTT assay was performed as mentioned in material and method section. Data represents average of three independent experiments and are expressed as mean ± SD, ***p < 0.001; **p < 0.01; *p < 0.05.

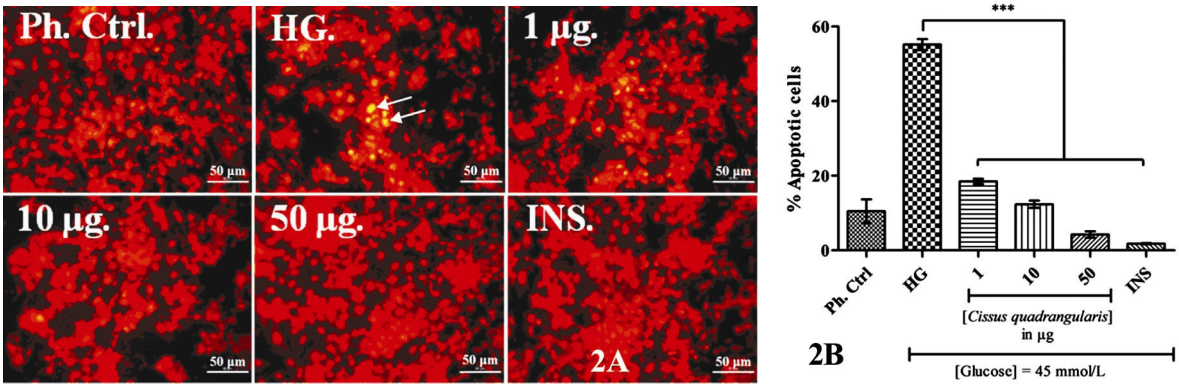

Figure 2. *Cissus quadrangularis* extract (*CQ*) rescue MG-63 cells from high glucose induced apoptosis. (A) After treatment cells were stained with propidium iodide (PI) which is permeable only to cells in late apoptotic phase or dead cells. White arrows indicate the cells showing bright red fluorescence of PI are actually either cells in late apoptosis or dead cells. Images were snapped with Nikon phase contrast with a fluorescence microscope (original magnification: 20X; Scale bar: 50 µm). (B) Results are represented as percentage of apoptotic cells and values are expressed as mean ± SD of three independent experiments, ***p < 0.001.

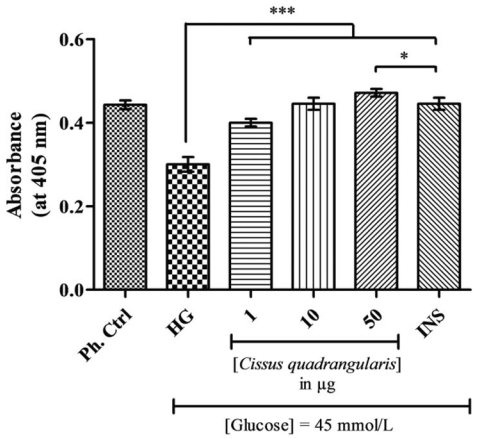

Figure 3. Effect of *Cissus quadrangularis* extract (*CQ*) on ALP activity in MG-63 cells. Data was quantified spectrophotometrically at 405 nm. Values are obtained from three independent experiments and expressed as mean ± SD, ***P< 0.001; *P < 0.05.

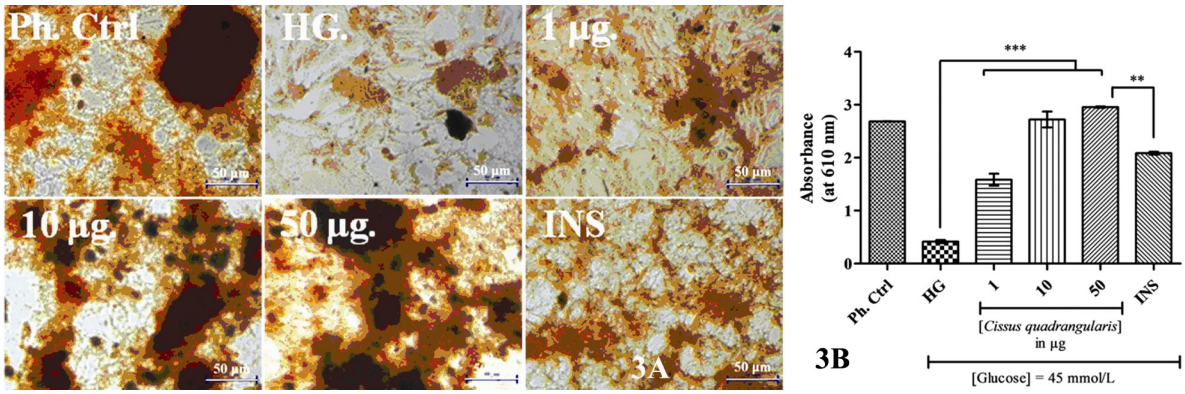

Figure 4. Extract of *Cissus quadrangularis* extract (*CQ*) induces extracellular matrix mineralization. (A) Photographs showing extracellular matrix mineralization and calcium deposition upon staining with alizarin red S which form complex with calcium. (B) After 14 days of incubation, stain was extracted with CPC and absorbance measured spectrophotometrically. Data represented as mean ± SD of three independent experiments, ***p < 0.001.

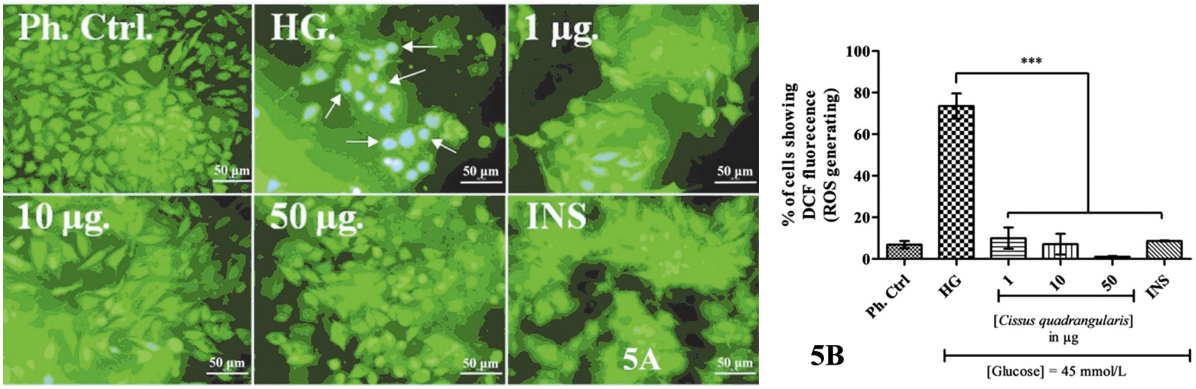

Figure 5. Antioxidative properties of *Cissus quadrangularis* extract (*CQ*). (A) Photomicrographs showing intracellular ROS generation induced by high glucose are restored to the normal level after treatment. Red arrows indicated cells generating DCF which fluoresce green after reaction with ROS. Images were snapped with Nikon phase contrast with a fluorescence microscope (original magnification: 20X; Scale bar: 50µm). (B) Percentage of ROS generating cells calculated as DCF positive cells to that of total number of cells. The experiment was repeated thrice and cell counting was done using NIS Elements Documentation software provided with the microscope. Values are obtained as mean ± SD, ***p < 0.001.

rate of ALP activity which in-turn leads to increased calcium deposition and mineralization nodule formation (Figure 3 and 4). The high dose of *CQ* markedly reduced the percentage of apoptotic cells. A reduction in percentage of DCF fluorescent cells is suggestive of antioxidative effect of CQ extract. High dose of *CQ* was most efficient in protecting the cells from HG induced injury. *CQ* extracts have been shown to possess both osteoblastogenic[14] as well as antiosteoporotic properties.[6] The restorative effect of insulin was similar to physiological control group in all experiments. In the present investigation we reported that the *CQ* extract is effective in reducing high glucose-induced severity in osteoblast like MG-63 cells by increasing the proliferation and matrix mineralization of cells and simultaneously reducing oxidative stress and thereby controlling apoptosis. Therefore, it is concluded that *CQ* could be a better alternative medicine for diabetes and related osteopathy.

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