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Analyses of In-vitro antioxidant and anticancer activity of *Cissus quadrangularis* stem extract in osteoblastic cell line-UMR-106

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ABSTRACT

Cancer is one of the leading causes of death worldwide with an estimated 7.8 lakh deaths in 2018 in India. Osteosarcoma, a primary tumour of the bone is the second highest cause of bone cancer related death in young people. It is treated with chemotherapy, radiation, and surgery which cause side effects. Certain traditional plants may have antitumour activities. This research aimed to evaluate the invitro antioxidant and apoptosis induced activity of stem extract of *Cissus quadrangularis* (*CQ*) on osteoblastic cell line (UMR-106). Various extracts of CQ was subjected to phytochemical screening and antioxidant studies. Ethanolic extract of CQ showed maximum phytoconstituents and better antioxidant activity for both DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and FRAP(Ferric reducing antioxidant potential) assay. The UMR-106 cell line was treated with different concentrations of the extract ranging from 25μg to 400μg for varying time periods. The dose and time optimization of the ethanolic extract was evaluated in the osteoblastic cell line (UMR-106) using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which showed IC50 value with 200μg/ml at 24 hours. There was a reduction in the percentage of cells by 50% in the treated group by SRB assay showing the cytotoxic potency of the extract. Flow cytometry analysis indicated that the extract of CQ induced apoptosis in UMR-106 cell line. Ethanolic extract of CQ has antioxidant effects, also inducing G0/G1 arrest and apoptosis in osteoblasts, suggesting that this might represent a novel and effective agent against bone cancer.

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INTRODUCTION

Bones are well-organized tissues and catered to work lifelong. It provides a site for attachment of muscles and also protects essential organs like the brain and the bone marrow. They are living tissues that continuously rebuild throughout life. In India, it is estimated that 7.8 lakh cases of death occurred due to cancer in 2018 out of which less than 1% is related to bone cancer (Bray *et al.*, 2018). Osteosarcoma is the common malignant tumour of the bone tissue characterized by the direct formation of immature bone as tumour cells. Tumour

mainly occurs in the tibia, femur or humerus. In about 75% of cases, patients with osteosarcoma are between 15-25 years of age. Male are more frequently affected than female. In general, 80% to 90% of osteosarcoma occurs in the long tubular bone (Picci, 2007).

Adult cancer patients are well known to be at increased risk of developing osteoporosis as a result of complications from their anticancer therapy. Bone loss that occurs during cancer therapy is generally more rapid and severe than a postmenopausal bone loss in women or typical age-related osteoporosis in men. Rates of bone loss occurring with cancer therapy can be up to 10 folds higher than usual. A decrease in bone mineral density has been reported as a consequence of treatment for such cancers in children. Chemotherapy, radiotherapy, and hormone deficiencies are frequently cited as risk factors for osteoporosis in cancer patients (Ahn *et al.*, 2015). Due to the adverse effects of such management of bone cancer, medicinal plants can be added as supplements in preventing osteoporosis due to anticancer therapy.

The plant CQ(handjob in Hindi) belongs to the family of Vitaceae commonly known as pirandai, in Tamil. It is a common perennial climber of the tropics primarily distributed throughout India (Mishra *et al.*, 2010). The whole plant including all parts such as stem, root, leaves are documented to possess medicinal properties, and it's used to treat gastritis, bone fractures, skin infections, constipation, anaemia, asthma, irregular menstruation, burns, and wounds.CQ has potent fracture healing property, along with antimicrobial, anticancer, antiulcer, antioxidative, antiosteoporotic, gastroprotective activities. The stem of the CQ plant contains a high percentage of calcium and phosphorous, which are essential for bone growth (Ghouse and Baig, 2015). So the present study was designed to evaluate the efficacy of CQ on its antioxidant potential and anticancer activity against the osteoblastic cell line (UMR 106).

MATERIALS AND METHODS

Plant collection

The fresh stem of CQ was collected in November 2018 from the local areas of Thanjavur district, Tamilnadu, India. The plant was identified and authenticated by Department of Botany, Arts and science college (Autonomous), Kumbakonam.

Preparation of plant extract

The fleshy stem of *CQ*was washed with tap water, cut into small pieces, and grounded to a fine paste using a blender. Ethanolic extract (100g of sample + 250ml of ethanol), aqueous extract (100g of sample + 250ml of water) and petroleum ether extract (100g of sample + 250ml of petroleum ether) of the stem were done by cold maceration method at room temperature for 72 hours. When the solvent developed maximalcolour, the extract was filtered and evaporated dry. Then, the sample was stored at 4ºC for further analysis.

Cell line and culture

The cell line UMR 106 was purchased from National Centre for Cell Science (NCCS), Pune, India. The cell count was done, and the cell viability was tested by trypan blue dye exclusion test using a haemocytometer. The cells were grown in Hams F12 medium supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin in tissue culture flasks and incubated in a CO_2 incubator at 95% humid atmosphere. Once the cells attained confluent growth, the cells were trypsinized using trypsin-EDTA to dislodge the cells from tissue culture flask and the required number of cells 106, and 103 cells/ml were seeded into 6-well and 96-well plates respectively for carrying out various assays. Cell viability and cytotoxicity assays were carried in 96-well plates, and the flow cytometric tests were performed in 6-well plates, incubated in a CO_2 incubator (Innova CO-170, United States) with 5% CO_2 and 37^0C.

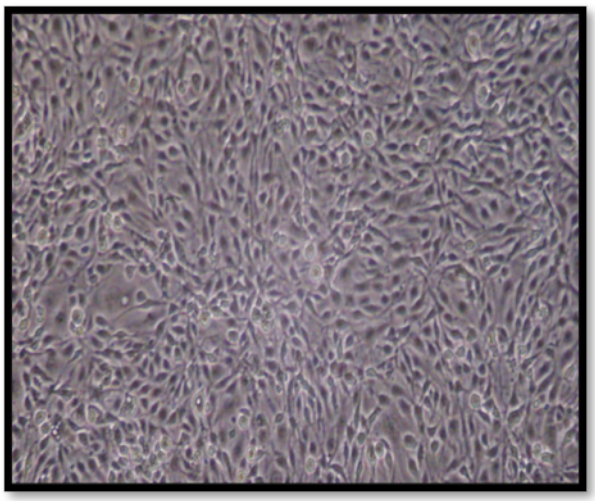


Figure 1: Control (Dose optimisation using MTT assay to calculate the IC50 value Effect of different concentrations of ethanolic stem extract of *Cissus quadrangularis* on UMR 106 cells)

Phytochemical screening

CQ stem extract was screened for the presence of various phytoconstituents using standard procedure (Altemimi *et al.*, 2017).

Table 1: The difference in treated and control cells in apoptosis and cell death

Cell	Live cells %		Late apoptotic cells %		Early apoptotic cells %		Necrotic cells %	
population	Control	Treated	Control	Treated	Control	Treated	Control	Treated
UMR 106	99.93	3.70	0	1.38	0	91.84	0.07	3.08

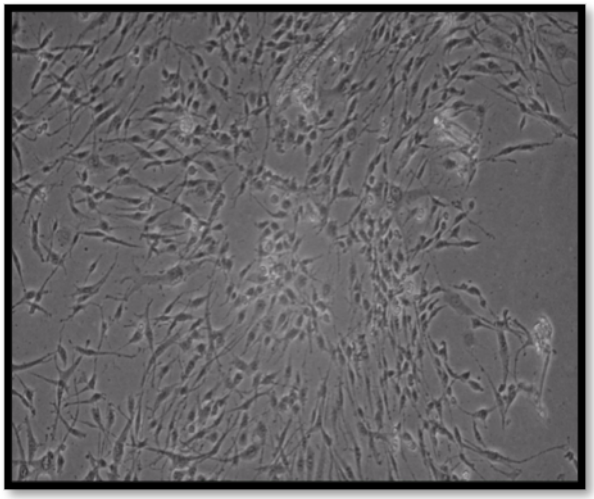


Figure 2: 25 μg (Dose optimisation using MTT assay to calculate the IC50 value Effect of different concentrations of ethanolic stem extract of *Cissus quadrangularis* on UMR 106 cells)

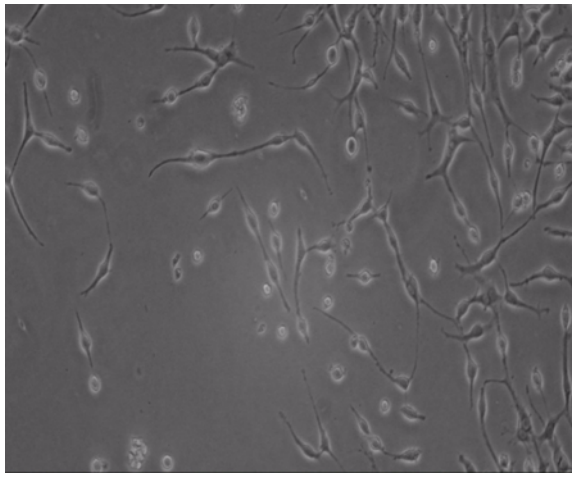


Figure 4: 100 μg (Dose optimisation using MTT assay to calculate the IC50 value Effect of different concentrations of ethanolic stem extract of *Cissus quadrangularis* on UMR 106 cells)

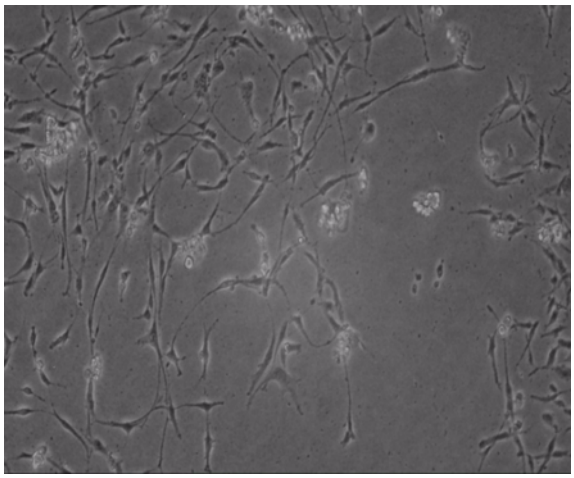


Figure 3: 50 μg (Dose optimisation using MTT assay to calculate the IC50 value Effect of different concentrations of ethanolic stem extract of *Cissus quadrangularis* on UMR 106 cells)

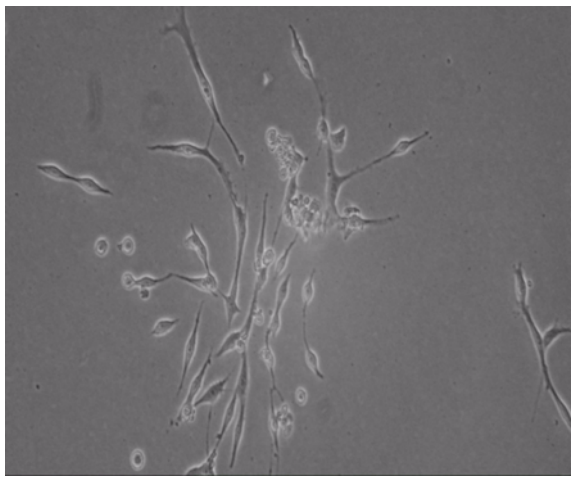


Figure 5: 200 μg (Dose optimisation using MTT assay to calculate the IC50 value Effect of different concentrations of ethanolic stem extract of *Cissus quadrangularis* on UMR 106 cells)

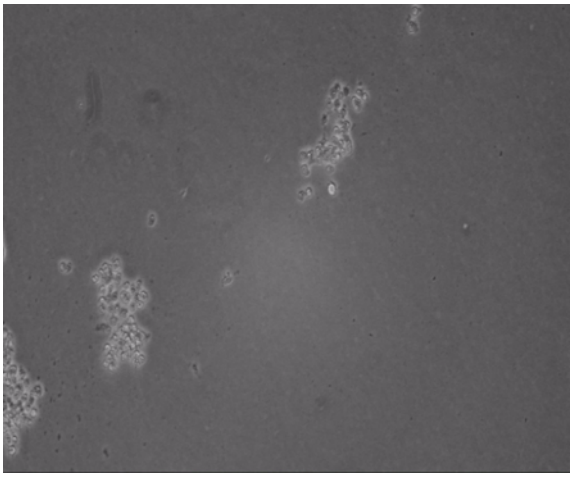


Figure 6: 400 μg (Dose optimisation using MTT assay to calculate the IC50 value Effect of different concentrations of ethanolic stem extract of *Cissus quadrangularis* on UMR 106 cells)

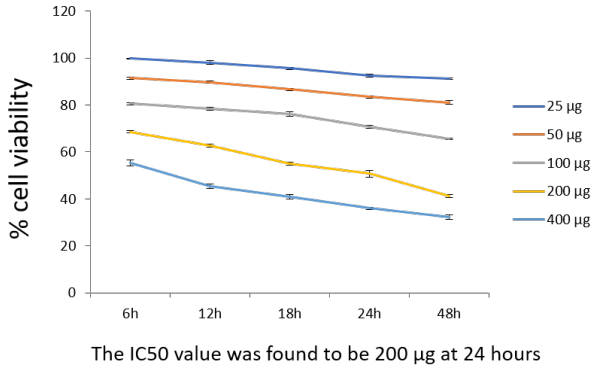


Figure 7: Dose and time Optimization of ethanolic *Cissus quadrangularis* stem extract on UMR 106 cells

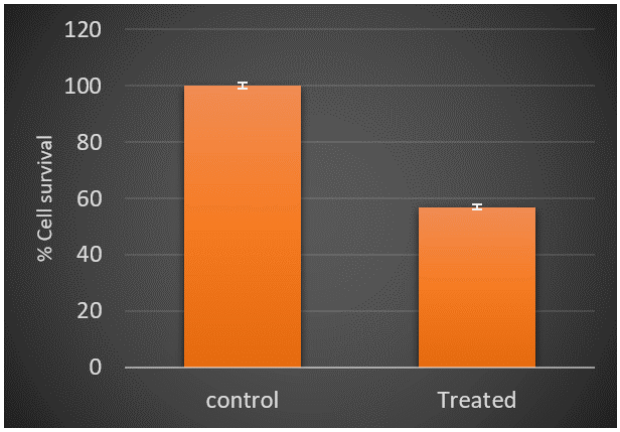
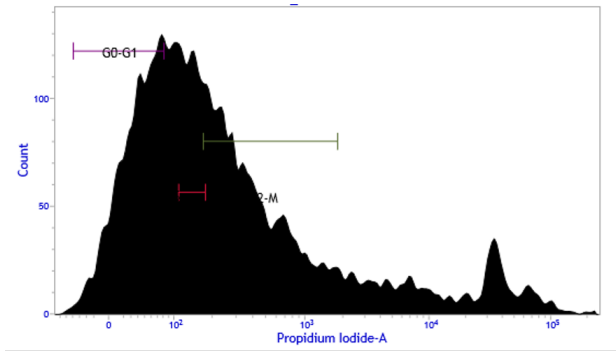
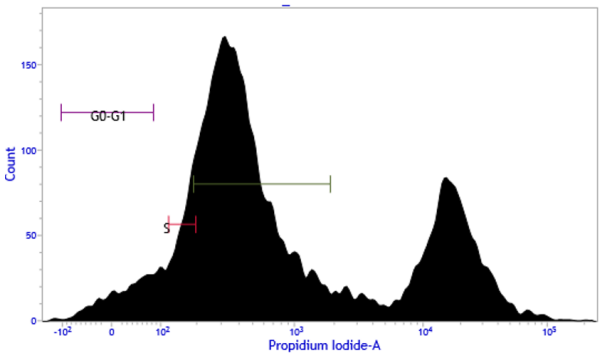


Figure 8: Cytotoxicity of ethanolic stem extract of *Cissus quadrangularis* on UMR 106 cells bySRB assay



In control group the cells were evenly distributed in all phases of cell cycle

Figure 9: Effect of etanolic stem extract of *Cissus quadrangularis* on the different stages of cell cycle



In *Cissus quadrangularis* extract treated group the cells were arrested in G0-G1 phase

Figure 10: *Cissus quadrangularis* stem extract in UMR 106 cells

DPPH radical scavenging activity

Different concentrations of plant extract (20-100 μg/ml) were made up with distilled water to 1 ml. 2 ml of 0.1mM methanolic solutions of DPPH (0.39mg in 10 ml methanol) were mixed. 3ml of DPPH was taken as control. Ascorbic acid was used as a reference standard. The reaction mixtures were incubated at room temperature for 30 mins. After the incubation, the absorbance of the mixtures was read at 517nm. The experiment was performed in triplicates. The radical scavenging activity (Singh *et al.*, 2012) was calculated as follows

$$\%\ of\ scavenging\ activity = \frac{OD\ value\ of\ control - OD\ value\ of\ test\ sample}{OD\ value\ of\ control} \times 100$$

Ferric reducing antioxidant potential- FRAP

Different concentrations of the ethanolic and aqueous extract of CQ stem (20-100 μg/ml) was added to 2.5 ml of0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution. The reaction mixture was mixed well and then incubated at 50°C for 20 min using vortex shaker. At the end of the incubation, 2.5 ml of 10% trichloroacetic acid was added to the mix-

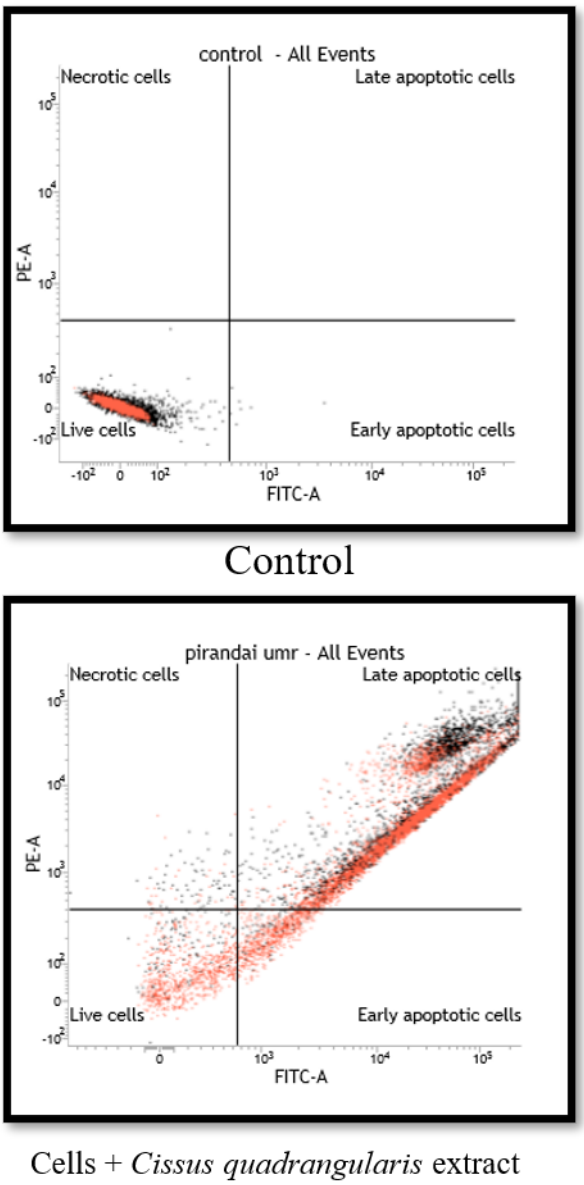


Figure 11: Effect of ethanolic extract of the stem of *Cissus quadrangularis* on the cell death in UMR 106 cells

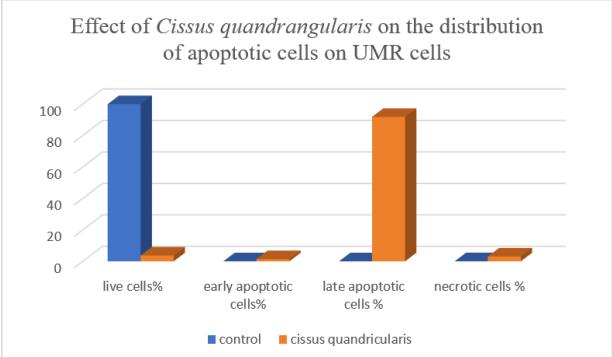


Figure 12: Effect of Cissus quadramgularis ethanolic extracton the distribution of apoptotic cells in UMR 106 cell line

ture and centrifuged at 3,000 rpm for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Ascorbic acid was used as a reference standard. The reducing power of the samples was compared with the reference standard. The coloured solution was read at 700 nm by UV-Spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power ability. Tests were run in triplicate. All data are expressed as the mean ± SD by measuring three independent replicates (Vijayalakshmi and Ruckmani, 2016).

MTT assay

The inhibitory concentration (IC_{50}) value was evaluated using an MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cancer cells were grown (1×10^4 cells/well) in a 96-well plate for 48hto 75% confluence. The medium was replaced with fresh medium containing serially diluted plant extracts, and the cells were further incubated for 48 h. The culture medium was removed, and 100μL of the MTT [3-(4,5-dimethylthiozol-2-yl)-3,5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well and incubated at 37ºC for four h. After removal of the supernatant, 50 μL of DMSO was added to each well and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at 620 nm in an ELISA multiwell plate reader (ThermoMultiskan EX, USA) (Skehan *et al.*, 1990). The OD value was used to calculate the percentage of viability using the following formula.

$$\%\ of\ viability\ =\ \frac{OD\ value\ of\ experimental\ sample}{OD\ value\ of\ experimental\ control}\times 100$$

Cytotoxicity assay-SRB

350μl of ice-cold 40% TCA was layered on top of the treated cells and incubated at 4ºC for one hour. The cells were then washed five times with 200 μl of cold PBS.

The buffer was removed, and 350 μl of SRB stain was added to each well and left in contact with the cells for 30 minutes at room temperature.

The unbound dye was removed by washing four times with 350μl portions of 1% acetic acid. Then 10mM Tris (350μl) was added to each tube, to stabilize the protein-bound dye and plate was shaken gently for 20 minutes.

The tris layer in each tube was transferred to a 96-well plate, and the absorbance was read in a microtitre plate reader (Anthos 2020, Austria) at 492nm. The cell survival was calculated as the per cent absorbance compared to the control (untreated) cells (Krishan, 1975).

Assay Using Flowcytometry

Cell cycle analysis

The treated cells were fixed with 75% ethanol at -20ºC and resuspended in PBS. The cells were incubated at 37 ºC for 1h. The cells were then stained with propidium iodide for 30 minutes at room temperature in the dark. The DNA content of the stained cells was assessed using flow cytometer (BD FACS Verse - BD Bioscience). The population of cells in sub G_0/G_1, S and G_2/M were quantitated (Darzynkiewicz *et al.*, 1987).

Detection of apoptosis

Detection and quantification of apoptosis were done by flow cytometry, staining with Annexin V-FITC and Propidium Iodide (PI). UMR 106 cells were placed in a T25 cultured flask and after 48 hrs incubation, collect the supernatant and trypsinize the adherent cells from each T25 flask. Wash the collected cell twice with PBS and centrifuge. Cells were trypsinized for 5 minutes and collected by centrifugation. One hundred six cells were labelled with one μg Annexin V-FITC and two μg/mL PI, using Annexin V FITC kit. All the experiments were done in triplicates, and the experiments were repeated at least thrice. The statistical software SPSS version 17.0 was used for the analysis.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical analysis shows the presence of many medicinally critical secondary metabolites like alkaloids, coumarin, flavones, saponins, triterpenes, which indicates that the plant possesses high profile values and can be used to treat various kinds of diseases. The qualitative phytochemical investigation gave valuable information about the different phytoconstituents present in the extracts (Yadav, 2016). The ethanolic extract of CQ showed maximum positive results for the presence of various phytoconstituents which correlated with various previous observations. Thus the presence of these phytoconstituents contributes to the antioxidant power of this perennial herb. These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS and therefore enhances the immune defence and lowers the risk of osteoporosis (Prabhavathi *et al.*, 2016).

Cell viability assay- MTT

Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, Figure 7 represents the result of dose and time optimization of the extract, which was evaluated using an MTT assay. By adding various concentrations of ethanolic stem extract of C. *quadrangularis* at different intervals, the percentage of cells was reduced from 99.93% to 32.22% in a dose and time-dependent manner. The IC_{50}value of CQ stem extract was200μg/mL at 24 hours, where the percentage of cells was reduced to 50% from its initial concentration. A similar observation was shown in an in-vitro cytotoxicity study with chloroform extract of 60.65% and ethanol extract of 56.50% against EAC cell line (Kumar *et al.*, 2014).

DPPH radical scavenging

The DPPH radical had been used widely as a model system to investigate the scavenging activities of several natural components such as phenolic compounds. DPPH is stable nitrogen centred free radical the colour of which changes from violet to yellow on electron donation. The colour change can be quantified by its decrease in absorbance at wavelength 570nm. Different concentrations of aqueous and ethanol extracts of CQ stem on radical scavenging activity (DPPH) was examined. Figure 8 represents the scavenging activity of CQ stem extracts in percentage. The aqueous extract showed 53.43% of scavenging effect, whereas the ethanol extract showed 77.42% scavenging activity. Ascorbic acid was used as standard. The result indicated that the ethanolic extract showed maximum scavenging effect when compared to aqueous extract. Similar studies have reported that the ethanolic extract of CQ has high radical scavenging activity of 73.67$\pm$ 0.35. The present study correlates with previous reports suggesting that ethanolic stem extract of CQ has potential DPPH free radical scavenging activity (Mallikajainu and Devi, 2005).

FRAP assay

The ethanolic extract of CQ has a higher reducing activity of 88.34% compared to aqueous extract (73.62%). The assay measures the antioxidant potential of the plant through the reduction of ferric ion to ferrous ion. The presence of reductoneshave shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Various studies confirm that the ethanolic extract has more reducing power of free radicals than the aqueous extract (Bagul *et al.*, 2005).

Cytotoxicity assay- SRB

Figure 8 represents SRB assay which evaluates the cytotoxic effect of ethanolic stem extract of CQ on UMR 106 cell line. The cell was treated with an ethanolic stem extract of C. *quadrangularis* and then incubated for 6h, 12h, 24h, and 48h. The IC_{50} value was found to be 200μg/mL at 24 hours in the treated group. This indicates that the ethanolic stem extract

of C. *quadrangularis* showed a cytotoxic effect in the UMR 106 cell line. The untreated cell was used as the control. This report was similar to the cytotoxicity study in a dose-dependant manner in human breast adenocarcinoma (MCF-7)cells (Monga *et al.*, 2013). Artocarpusheterophyllus also showed the cytotoxic effect in a dose-dependent manner against Hela cell line (Patel and Patel, 2011).

Detection of apoptosis by flow cytometry

After the cells were treated with or without the ethanolic stem extract for 48 hours, it was subjected to cell death detection by staining with Annexin V and propidium iodide, which was analyzed by flow cytometry. Untreated cells were used as control. Figure 9 represents the apoptotic detection of UMR 106 cell line at 24 hours with exposure of 200μg/ml dosage of C. *quadrangularis* stem extract. The cells treated with ethanolic stem extract of C. *quadrangularis* were arrested in G_{0-}G1. Figure 10 represents the early apoptosis. The percentage of cell death was slightly increased in the treated group when compared to the control group. (Table 1) In late apoptosis, the percentage (91.84%) of cell death was significantly increased when compared to the control group. The results indicated that the ethanolic stem extract of CQ induced apoptosis than necrosis in UMR 106 cell line. (Figure 11, Figure 12) The present study correlated with earlier reports that showed 64% arrest of HeLa cell(cervical cancer) growth at G0-G1 phase with 46% apoptosis at 24 h exposure of 200 μg/ml dose of CQ extract. This was accompanied by a decrease in both S phase (35.17%) and G2-M phase cells (0.02 %). The CQ extract-treated normal cells were in the regular phase of the cell cycle. Healthy skin HaCaT cells treated with the same concentration of CQ extract did not show any cell cycle arrest (Sheikh *et al.*, 2015).

CONCLUSION

In this study, it is evident that the ethanolic extract of CQ possesses effective antioxidant and anticancer activities. The extract also induced G0/G1 arrest and apoptosis in osteoblasts, suggesting that this might represent a novel and active agent against bone cancer. Hence extracts of CQ may throw light on inventing novel drugs to counter bone cancer.

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The authors declare that they have no funding support for this study.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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