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 in vitro 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 25 to 400 g/ml 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 200 g/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.

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 CQwas 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 4°C 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 CO₂ 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 CO₂ incubator (Innova C0-170, United States) with 5% CO₂ and 37 °C.

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

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Live cells %</th>
<th>Late apoptotic cells %</th>
<th>Early apoptotic cells %</th>
<th>Necrotic cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>UMR 106</td>
<td>99.93</td>
<td>3.70</td>
<td>0</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>91.84</td>
<td>0.07</td>
<td>91.84</td>
<td>3.08</td>
</tr>
</tbody>
</table>

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 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 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 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)

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 incu-
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

The IC50 value was found to be 200 μg at 24 hours

Figure 8: Cytotoxicity of ethanolic stem extract of Cissus quadrangularis on UMR 106 cells by SRB assay

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

Figure 10: Cissus quadrangularis stem extract in UMR 106 cells

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 of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K₃Fe(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 mixture 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.

% of scavenging activity = \frac{OD \ value \ of \ control - OD \ value \ of \ test \ sample}{OD \ value \ of \ control} \times 100
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 (Vijayalkshmi 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 48 h to 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 100 μL of the MTT [3-(4,5- dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well and incubated at 37°C 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.

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

**Cytotoxicity assay- SRB**

350 μl of ice-cold 40% TCA was layered on top of the treated cells and incubated at 4°C 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 350 μl portions of 1% acetic acid. Then 10mM Tris (350 μl) 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 492 nm. 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 -20°C and resuspended in PBS. The cells were incubated at 37 °C for 1 h. 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
G₀/G₁, S and G₂/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 IC₅₀ value of CQ stem extract was 200 μg/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 presents 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 ± 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 reductoneshown 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 IC₅₀ value was found to be 200 μg/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 (Rajesh et al., 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 200 μg/ml dosage of C. quadrangularis stem extract. The cells treated with ethanolic stem extract of C. quadrangularis were arrested in G0-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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Conflict of Interest

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

REFERENCES


