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ORIGINAL ARTICLE

Quinic Acid Attenuates Oral Cancer Cell Proliferation by Downregulating Cyclin D1 Expression and Akt Signaling

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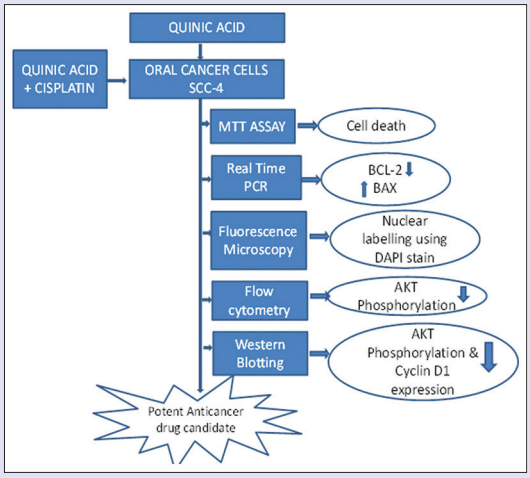
ABSTRACT

Background: Quinic acid (QA), a natural compound found in fruits, for example in apple, berries, and coffee beans, is a neutraceutical chiral compound used to synthesize new pharmaceutically important chemical compounds. Platinum-based drugs such as cisplatin are widely used in the therapy, including oral cancer, one of the most prevalent cancer. Due to their severe side effects and acquired resistance to the treatment, phytotherapies are being explored to find out strong anticancer compounds that can alone and synergistically prevent and cure cancer without side effects. **Objective:** This study aimed to find out the mechanism behind the anticancer property of QA, a natural compound alone and synergistically with cisplatin in oral cancer cells (squamous cell carcinoma-4 [SCC-4]). **Materials and Methods:** Oral cancer cells were treated with QA alone and synergistically with cisplatin. MTT assay was performed to investigate the cytotoxic effect of QA and QA + cisplatin combination. Apoptotic effects of QA and QA + cisplatin combination were analyzed using DAPI and real-time-polymerase chain reaction for the expression of apoptotic marker genes. Effect of QA and QA + cisplatin combination was assessed by flow cytometry and immunoblotting technique. **Results:** Our result shows that QA promotes apoptosis in oral cancer cells by downregulating the expression of anti-apoptotic genes and attenuating the expression of cyclin D1 and Akt signaling pathway. Moreover, QA shows synergistic effect with cisplatin in these cells. **Conclusion:** QA inhibits cell proliferation and promotes apoptosis in oral cancer cells (SCC-4) alone and with cisplatin.

Key words: Cisplatin, head-and-neck cancer, nutraceuticals, oral cancer, quinic acid, squamous cell carcinoma



Abbreviations used: QA: Quinic acid; CDK: Cyclin-dependent kinase; SCC-4: Squamous cell carcinoma-4; FBS: Fetal bovine serum.

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INTRODUCTION

Oral cancer, a type of head and neck cancer, continues to remain among one of the highly prevalent cancer worldwide. Cancer of different origins has distinct features such as oral cancer, a part of head-and-neck cancer. Oral cancer often starts in squamous cells that line lips and the inner part of the mouth, which is called squamous cell carcinoma (SCC).[1] In oral cancer, there is growth on the inner side of mouth and tongue without pain.

Research has shown that people with certain risk factors are more susceptible than others to develop oral cancer. The following are the risk factors for oral cancer: tobacco, alcohol, diet and nutrition, fungal and viral infections, immunodeficiency, habits, and occupational risk. Oral cancer is mainly associated with smoking cigarettes and cigars as well as chewing tobacco. Treatment options include surgical resection, radiation, and/or chemotherapy.[2] Cisplatin is a potent radiosensitizer and the drug most commonly used for chemoradiotherapy in head-and-neck tumors; however, the efficacy of cisplatin is limited by severe side effects, dose dependency, such as renal toxicity, hematologic toxicity, and nausea and vomiting.[3]

Since long time, people have relied on nature for their basic needs and for use as medicines that include Indian Ayurveda, an oldest traditional medicinal system that have a long history of use in the treatment of cancer. The World Health Organization has estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care.[4,5] The present study aims to shed further light on a plant-based compound, i.e., quinic acid (QA), a natural sugar compound which is present in several plants, such as carrot leaves, tobacco leaves, peaches, apples, pears, plums, and vegetables. It is a versatile parent chiral compound used for the synthesis of new drugs.[6] QA, a cyclitol,

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a cyclic polyol, and a cyclohexane-carboxylic acid, has been studied as a potent drug for prostate cancer.[7] QA-conjugated nanoparticles act as a drug carrier to solid tumors,[8] but to the best of our knowledge, it has not been explored yet in oral cancer cells. One of the hallmarks of cancer cells is that they evade apoptosis.

The aim of the study is to explore the underlying mechanism of QA, induced apoptosis in human oral cancer cells, and its synergistic effect with cisplatin on apoptosis and cell cycle. To understand how QA induces apoptosis, it is important to know its molecular targets as a function with time. This study will help to understand the facts to develop novel and nontoxic therapy for oral SCC.

MATERIALS AND METHODS

Chemical reagents

QA was purchased from Himedia (catalogue number: RM7448). It was dissolved in water and stored at 4°C for further use. Cisplatin was purchased from Sigma-Aldrich (catalog number: 479306-1G) and was used at a concentration of 20 μM dissolved in water for adherent monolayer SCC-4 cell treatment.

Cell culture and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

SCC-4 cells were cultured in Dulbecco's Modified Eagle Medium, followed by supplementation with 10% fetal bovine serum, 100 mg/mL streptomycin, and 100 U/mL penicillin. The cells were incubated with 5% CO_2 at 37°C. Thereafter, cells were seeded in 96-well plates at a density of 2×10^3 cells/well. Different concentrations of QA were added to each well and incubated for 24 h and 48 h followed by incubation with MTT (0.5 mg/mL, 1 h) and subsequently solubilized in Dimethyl sulfoxide. The absorbance was measured at 560 nm and the percentage cell viability was counted.

Drug combination studies

To evaluate the possible synergistic effect of QA and cisplatin, cells (1 million cells/well) were treated with a constant ratio calculated with respect to the IC_{50} values of compound. The cytotoxicity of the combination was compared with the cytotoxicity of each compound alone using the combination index (CI) as described by Chou and Talalay.[9] If the CI is <0.8, the compounds work synergistically, 0.8 < CI < 1.2 means additive, and a CI >1.2 shows an antagonistic effect. The mean CI was calculated from the data points of QA and cisplatin >0.5, as lower values are not considered relevant for growth inhibition. QA + cisplatin concentration used in experiments was 20 + 10 μM.

Fluorescence microscopy

DAPI (4',6-diamidino-2-phenylindole) staining was used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells were incubated with QA for 48 h, and morphological changes in the cells were observed by phase-contrast microscopy and fluorescent microscopy after staining with DAPI (1 μg/ml). With untreated and cisplatin-treated cells as controls, the QA and QA + cisplatin for 48 h were detected through DAPI staining. Cells stained with DAPI for 15 min were observed fluorometrically with emission at 358 nm and excitation at 461 nm.

Quantification of apoptotic gene expression using quantitative real-time-polymerase chain reaction

Total RNA was extracted from untreated cells, QA alone, and QA + cisplatin-treated cells using genetix RNASure® Mini Kit (catalog number: NP-84105, Genetix, India). Total RNA was quantified with nanodrop and observed on gel electrophoresis followed by cDNA synthesis using protocol of Revert Aid first strand cDNA synthesis Kit (catalog number K1622 Genetix, Genetix, India). SYBR® Green-based real-time polymerase chain reaction (RT-PCR) was used to quantify gene expression (CFX96 quantitative RT-PCR, Biorad). 18sRNA was used as internal control.

Flow cytometry: Akt and phosphor-Akt expression

Flow cytometry SCC-4 cells treated with QA, QA + cisplatin, and control group cells (untreated, cisplatin) were stained with Akt monoclonal antibodies, anti-Akt mouse monoclonal antibody (anti-AKT/PKB), PH PC 12-301 (catalog number: 05-591), and anti-phospho-Akt (ser473) (05-1003) in Fluorescence-activated cell sorting-buffer. SCC4 cells were preincubated for 15 min and analyzed with a FACS LSR flow cytometer (Becton Dickinson, Jamia Hamdard, India).

Protein extraction and immunoblotting

Protein extraction was performed using radioimmunoprecipitation assay buffer (RIPA lysis buffer) with 1X protease inhibitor cocktail (1X phosphate-buffered saline [PBS]; 1% NP40; 0.1% sodium dodecyl sulfate (SDS); 5 mm ethylenediaminetetraacetic acid; 0.5% sodium deoxycholate; and 1% phenylmethylsulfonyl fluoride). After washing cells with PBS, 300 μL of RIPA lysis buffer/well was added and incubated at 4°C for 5 min. Then, the cells were scraped, and the cell lysate was centrifuged at 4°C for 10 min. Protein concentration was quantified by the BCA protein estimation kit (catalog number: GX-6410A, Genetix, India). Furthermore, equal amounts of proteins (30 μg/ml) were separated by SDS-polyacrylamide gel electrophoresis (10%) and blotted onto polyvinylidene fluoride membranes. Immunoblotting was performed (primary antibody incubated at 4°C for overnight) according to protocol of anti-Akt/PKB, PH PC 12-301 (catalog number: 05-591) and anti-phospho-Akt (ser473) (05-1003) (Merck Millipore, Germany). Protein band signals were detected with DAB chromogenic kit (Genetix, India). The intensity of antibody-reacted bands was quantified with ImageJ 1.46r software.

RESULTS

Morphological and cytotoxicity assessment

The cytotoxic effects of QA and QA + cisplatin along with controls (untreated, cisplatin) were tested in cultured human oral cancer cells (SCC-4). Through phase-contrast microscopy and using cytotoxicity assay, we had observed that QA alone and in combination with cisplatin markedly induced death in cancer cells, while the viability of untreated cells was affected only minimally at 48 h [Figure 1]. The cells treated with QA and QA + cisplatin had changed, appeared to be highly damaged and bow shaped.

Apoptotic analysis using DAPI staining

DAPI staining showed apoptosis in SCC-4 cells, which were characterized by cytoplasm and nuclear shrinkage, chromatin condensation, and apoptotic body. As shown in Figure 2, cells treated with DAPI, i.e., negative control were normally blue. However, the QA and QA + cisplatin-treated group appeared compact condensed and crescent shaped. The cells exhibited strong blue fluorescence, revealing the typical apoptotic characteristics. These findings demonstrate that QA alone and with cisplatin could induce apoptosis against SCC-4 cell lines, which is consistent with the results for cytotoxicity.

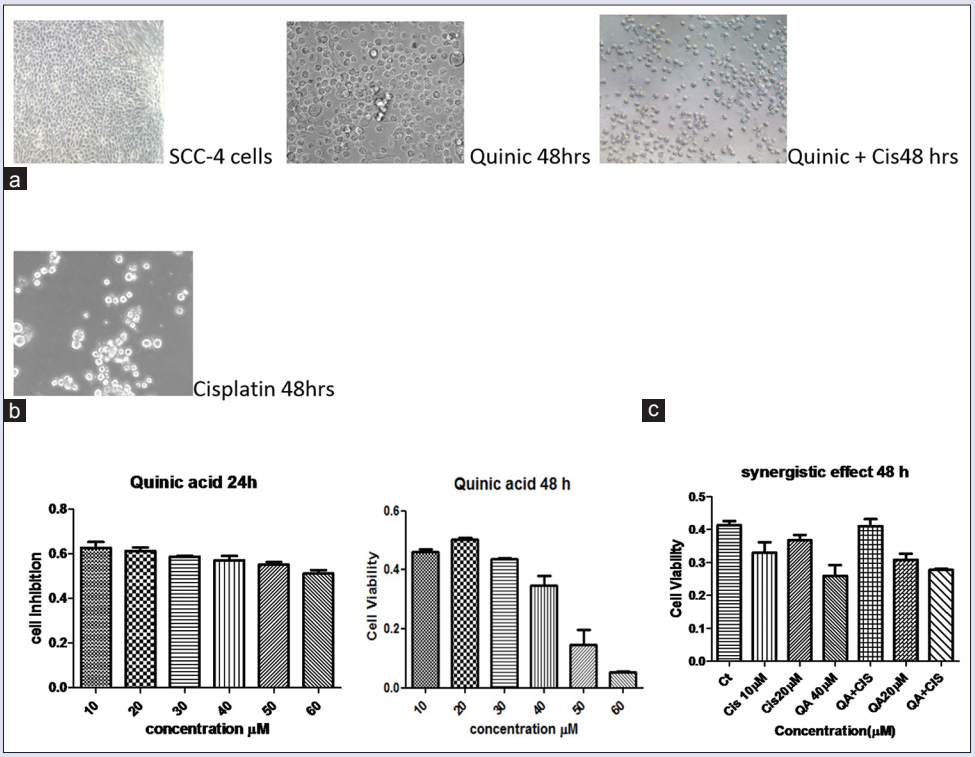

Figure 1: (a) Treatment with QA (40 µM) and QA + cisplatin (20 +10 µM) with control groups, i.e.,, untreated cells and cisplatin-treated cells at 48 h. (b) Cells incubated with QA at 10, 20, 30, 40, 50, and 60 µM concentrations for 24 and 48 h. Results represent the mean ± SEM of three replicates ($P < 0.05$). (c) Synergistic interaction between QA and cisplatin. Cells exposed to compounds QA and QA + cisplatin compared with control groups for 48 h. The combination index at fraction affected of 0.25, 0.5, and 0.75 of SCC-4 cells; values are means ± SEM. QA: Quinic acid; SEM: Standard error of mean; SCC: Squamous cell carcinoma

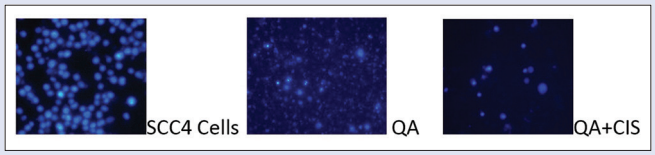

Figure 2: Effects of QA on SCC-4 cells: Cell damage induced by QA (40 µM) and QA + cisplatin (20 + 10 µM) compared with untreated cells and ST-treated cells. Apoptotic cells appear with condensed and fragmented nuclei in QA and QA + cisplatin at 48 h. SCC: Squamous cell carcinoma; QA: Quinic acid

Quantitative gene expression analysis

Further, we studied the effect of QA, which is a polyol on the apoptotic pathway genes Bcl-2 and Bax. It was found that upon addition of QA (40 µM) and QA + cisplatin (20 + 10 µM) at 48 h incubation, expression level of Bcl-2 decreases significantly in oral cancer cells and Bax gene expression increases in SCC-4 cells. We observed that QA shows notably synergistic effect with cisplatin at 48 h treatment in human oral cancer cells.

Quinic acid attenuates phospho-AKT expression in oral carcinoma cells

We then explored the effect of QA and QA + cisplatin and found that QA alone and synergistically with cisplatin could markedly decrease the expression of phosphor-Akt in SCC-4 cells at 48-h incubation. It may be the case that QA kills oral cancer cells by targeting the Akt and phospho-Akt protein and increases the antitumor activity with cisplatin, a first-line chemotherapeutic agent used in head-and-neck cancer therapy.

Effect of Quinic acid on phospho-AKT and Cyclin D1 expression using Western blot analysis

Western blot analysis showed that QA significantly decreases the expression of Akt and phospho-Akt in SCC-4 cells. QA also decreases the levels of cell cycle regulatory proteins, i.e., cyclin D1 in oral SCCs. This suggests that QA selectively induces cancer cell death by modulating the expression of Akt, phospho-Akt, and cell cycle pathway.

DISCUSSION

QA, a sugar compound found from nature, is a versatile chiral-starting material for the synthesis of new pharmaceuticals.[10] This nontoxic, bioavailable natural polyol has been found to be a potent antioxidant, antiviral, antimicrobial, antivascular, anti-inflammatory, and anticancer agent.[11-14] With that in concern, we studied the future use of QA for oral cancer prevention and tumor cure. To further increase the efficacy of a promising neutraceutical, i.e., QA, we studied its synergistic effect with cisplatin. With the molecular targets of QA being known, it may be possible to develop more refined therapy that specifically targets SCCs. The focus of our study is to explore the mechanism behind the anticancer properties of QA in oral cancer cells (SCC-4).

First of all, we have studied the morphological changes induced by QA in SCC-4 cells and found that QA induces a significant cell death in SCC-4 cells alone and QA in combination with cisplatin at 48 h. Cytotoxic concentrations were calculated using MTT assay and synergistically with cisplatin that were used throughout further experiments [Figure 1].

It is known that the ability to trigger apoptosis in tumor cells is an important strategy for cancer therapeutics. Considering the fact, we have

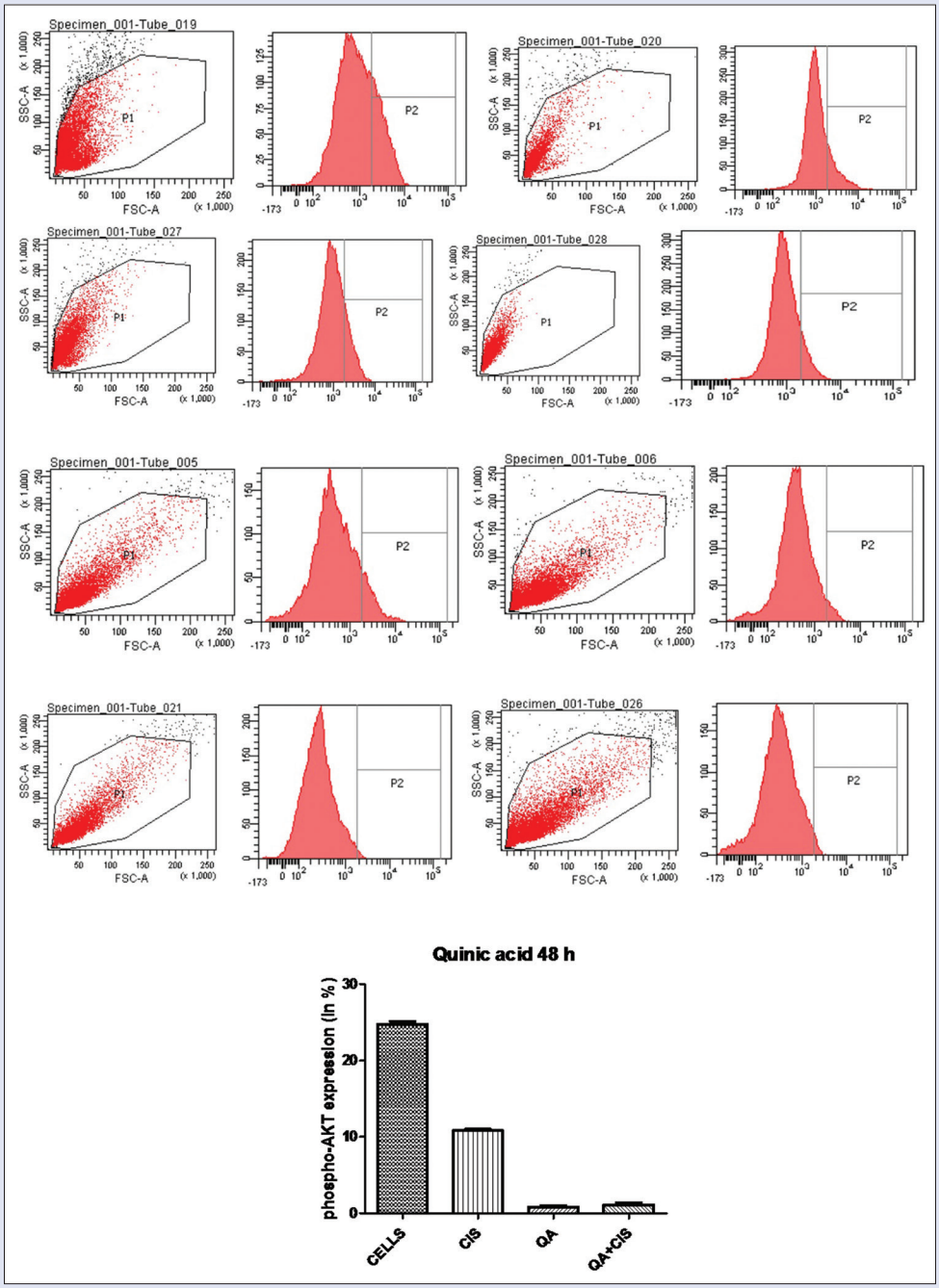

Figure 3: Flow cytometry analysis. (a) Expression of Akt and phosphor-Akt in SCC-4 cells treated with QA (40 μM) and QA + cisplatin (20 +10 μM) at 48 h assessed by flow cytometry. QA: Quinic acid; SCC: Squamous cell carcinoma

studied apoptosis using DAPI fluorescence technique, and results had shown that QA-treated cells and synergistically with cisplatin-treated SCC4 cells appeared to be damaged with ruptured cell membranes and fragmented cellular parts [Figure 3], this indicated that QA can induce apoptosis in oral squamous cells. Further we have investigated induction of apoptosis using Fluorescence technique that shows induction of apoptosis as compared to control. We also found that apoptotic cells (late phase) have a separate nucleus composed of clusters of blue dots that appear blabbing [Figure 2]. Our results confirmed that QA alone and synergistically with cisplatin may induce apoptosis in oral cancer cells.

As oral cancer is a multistep process that requires the accumulation of several mutations and mutations in intrinsic pathway of apoptosis are much more common than alterations in the extrinsic pathway, we have studied the expression of Bcl-2 and BAX using quantitative-PCR technique. Bcl-2 is overexpressed in a broad range of tumors including oral carcinoma cells (SCC-4).[15-18] Inhibition or reduction of its expression by QA is one strategy that has been investigated to find out the potential of QA as a new anticancer neutraceutical. We found that QA significantly reduces the expression of Bcl-2 and increases Bax expression within 48 h in oral squamous carcinoma cells (SCC-4) [Figure 4].

We further investigated the effect of QA on Akt and phospho-Akt expression. Numerous studies have proved the overexpression of Akt in several tumor cell lines and malignancies such as colon, pancreatic, gastric, breast, and oral cancers.[19-22] We have found a marked decrease

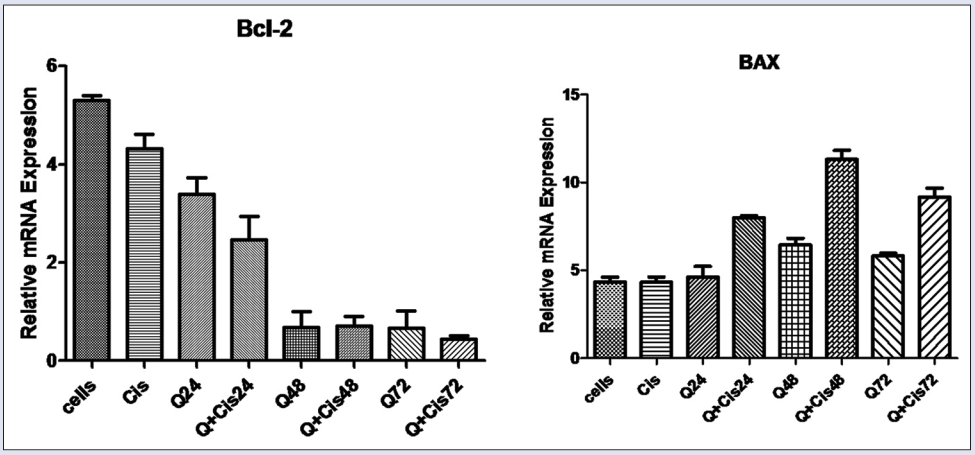

Figure 4: Quantification of mRNA expressions in experimental sets, i.e., SCC-4 cells, cisplatin, QA, and QA + cisplatin at 48 h using the quantitative polymerase chain reaction technique. The results represent the mean ± SEM of three replicates with significant differences ($P < 0.05$). SEM: Standard error of mean; QA: Quinic acid; SCC: Squamous cell carcinoma

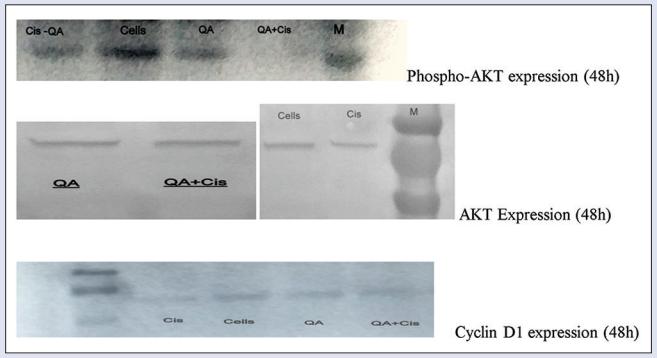

Figure 5: Effect of QA (40 μM) on SCC-4 cells and in combination with cisplatin (20 +10 μM) on Akt protein expression and cyclin D1 at 48 h. SCC: Squamous cell carcinoma; QA: Quinic acid

in Akt/phospho-Akt expression in QA and QA + cisplatin-treated oral squamous cells as compared to controls. Our results reporting that QA decreased the Akt/phospho-Akt expression of flow cytometry data were consistent with immunoblotting assay results [Figures 3 and 5].

In addition to this study, we have examined cyclin D1, a regulatory subunit, that plays a role in the regulation of cell cycle and an important subunit for maintaining the integrity of genome. It is the first cyclin to be synthesized that together with cyclin-dependent kinase 4/6 drives cell cycle progression through G1 phase. Several studies suggest that cyclin D1 is found to overexpress in squamous carcinoma cells.[23] Therefore, we have studied the effect of QA and synergistically with cisplatin on cyclin D1 expression in oral squamous cancer cells. Analysis of results showed that QA alone and in combination with cisplatin notably decreased the expression of cyclin D1 at 48 h in SCC-4 cells [Figure 5].

Our finding strongly supports the fact that QA is a potent anticancer natural compound that may be used for more effective chemotherapeutic strategies.

CONCLUSION

Our result shows that QA alone and synergistically with cisplatin could strongly exert cytotoxic effect and induces apoptosis in oral carcinoma cells. Our results suggest that QA is a chemopreventive agent that inhibits uncontrolled oral cancer cell growth and induces apoptosis through Bcl-2 reduction and increasing Bax expression. Our findings suggest that QA is a potent anticancer agent which synergistically with cisplatin may enhance its antitumor effects through reducing Akt/phospho-Akt and cyclin D1 expression.

Our findings offer a new perspective for the development of QA as an anticancer compound.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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