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# Induction of apoptosis by quinine in human laryngeal carcinoma cell line (KB)

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**KEYWORDS**

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**A B S T R A C T**

To investigate the effect of quinine on the apoptosis of laryngeal cancer cells (KB). The growth inhibition rate of KB cells in vitro was measured by MTT assay and apoptosis by levels of reactive oxygen species, mitochondrial membrane potential, morphological changes and flow cytometry. Based on the MTT results, we determined the median inhibition concentration ($IC_{50}$) of quinine as 125.23µM/mL for 24hr and 117.81µM/mL for 48hr. The sequences of events marked by the improvement of programmed cell death by the loss of cell viability, modulation of reactive oxygen species and cell cycle arrest through the induction of G0/G1 phase on KB cells. Our study suggests that, quinine possesses cytotoxic effect on cancer cells and a novel candidate for cancer chemoprevention.

## Introduction

Oral cancer is the one of sixth most cancers worldwide. Consumption of tobacco, abusing alcohol, age, gender and the human papillomavirus are important risk factors in the prognosis of this disease. Cancer of the mouth and pharynx ranks the sixth most common cancer in the world population. Important risk factors in the prognosis of this disease are consumption of tobacco, abusing alcohol, age, gender and the human papillomavirus [1]. Recent reports have shown the use of natural and semi-synthetic compounds for chemotherapy significantly suppresses and inhibit the malignant transformation.

Quinine is a natural white crystalline alkaloid (Fig.1) having antimalarial, analgesic, antipyretic and anti-inflammatory properties. To our information there is no any scientific reports accessible on the literature for in vitro inhibition of growth and induction of apoptosis in human laryngeal epithelial neoplastic cell line KB by quinine. Hence, in the present study, we evaluated the effect of quinine on inhibition of growth induction and apoptosis in KB cancer cells.

## Materials and Methods

### Cell culture and drug treatment

Human laryngeal epithelial cancer cell line (KB) was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were placed into 75 $cm^2$ tissue culture flasks and grown at 37ºC under a humidified, 5% $CO_2$ atmosphere in Minimum essential medium (Eagle) supplemented with 10% Fetal bovine serum (FBS), 1% glutamine and 100µg/ml penicillin-streptomycin at 37ºC in 5% $CO_2$ atmosphere.

### Determination of effective dose of Quinine (MTT assay)

In brief, KB oral cancer cells were seeded in 96 well microtiteplate ($5\times10^3$ cells/well) and incubated for 24 and 48hr, and then the cells were incubated with quinine at different concentrations ranging from 25-250µm for 24 and 48hr. The untreated cells serve as control. After the incubation the cells were washed twice with phosphate buffered saline (PBS). MTT (100µM/0.1ml of PBS) was added to each well. Cells were incubated at 37ºC for 4hr, and DMSO (100µL) was added to dissolve the formazan crystals. Samples were transferred into culture plates and the absorbance was measured colorimetrically at 590nm.[2]

### Determination of Reactive Oxygen Species

2',7'-Dichlorofluorescein diacetate (DCFH-DA), is a probe used for the highly sensitive and quantifiable detection of ROS. The non-fluorescent DCFH-DA diffuses into the cells and is cleaved by cytoplasmic esterases into 2',7'-dichlorodihydrofluorescein ($H_2$DCF) which is unable to diffuse back out of the cells. In the presence of hydrogen peroxide, $H_2$DCF is oxidized to the fluorescent molecule dichlorofluorescein (DCF) by peroxidases. The fluorescent signal emanating from DCF can be measured and providing an indication of intracellular ROS concentration was determined by the method of Rastogi *et al.*,(2010) [3].The levels of oxidative stress were determined quantitatively and qualitatively by the production of ROS by DCFH-DA. Briefly, an aliquot of the KB cells (8 x $10^6$ cells/mL) were treated with with $IC_{50}$ concentration of quinine for 24 and 48hr, then made up to a final volume of 2mL in normal phosphate buffered saline (pH 7.4). An 1ml aliquot of cells were taken, to which 100µL DCFH-DA (10 µM) was added and incubated at 37°C for 30min. We have observed the cells microscopically using blue filter at excitation and emission wavelengths of 480 and 530nm. All images were captured using a Nikon fluorescence microscope.

### Mitochondrial membrane potential (ΔΨm)

Rhodamine 123 (Rh-123) is a lipophilic cationic dye, highly specific for mitochondria. Rh 123 uptake into mitochondria is driven by mitochondrial membrane potential that allows the determination of cell population with active integrated mitochondrial functions. Loss of ΔΨm leads to depolarization of mitochondrial membranes leading to collapse of mitochondrial functions ensuring cell death. Mitochondrial membrane potential measurement was carried out essentially as described by Scaduto and Grotyohann (1999) [4]. The cells $1x10^6$ cells/mL were cultured in 6-well plate and treated with $IC_{50}$ concentration of quinine for 24 and 48 hr and the untreated cells served as control. The cells were then stained with Rh-123 dye (10µM/mL) and the cells were kept for incubation of 30

minutes in $CO_2$ incubator. The cells were washed by the addition of warm PBS and the mitochondrial depolarization patterns of the cells were observed in the fluorescence microscope using blue filter.

**Apoptosis studies with AO/EB staining method**

The ethidium bromide/acridine orange stain (EBr/AO stain) is a viability stain that detects apoptotic cells. Ethidium bromide is a dye that is only able to pass through the membrane of a dead or dying cell. Acridine orange is a membrane permeable dye that stains all the cells. Each dye that is taken up by a cell fluoresces AO makes a cell green, and EB makes a cell red [5]. Apoptotic studies were performed with a staining method utilizing AO and EBr according to the method of Lakshmi *et al*., (2008) [6]. The KB cells were grown in 6 well plates ($5\times10^3$) for 24 and 48hr, and then treated with $IC_{50}$ concentration of quinine were incubated in $CO_2$ incubator for 24hr, then the cells were trypsinized and stained with 1:1 ratio of AO/EBr. Stained cells were immediately washed again with PBS and viewed under a fluorescence microscope using blue filter with a magnification of 40x.

**Cell cycle analysis by flow cytometry**

Cell cycle analysis was performed by staining the DNA with Propidium iodide (PI) as described by Chakraborty *et al*., (2012) [7]. The PI fluorescent nucleic acid dye is capable of binding and labeling double-stranded nucleic acids, making possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells. Cells were seeded in T25 flask at a density of $1\times10^6$ cells/flask. After, $IC_{50}$ concentrations of quinine were added to each flask they were incubated for 24 and 48hr, and then the cells were trypsinized, harvested and fixed in 70% ice cold ethanol in cell culture tubes and stored at -20°C until use. The cells were centrifuged; the cell pellets were resuspended with PI (40 µM/mL in PBS) solution containing RNase (100µM/mL). The stained cells were analyzed using fluorescence activated cell sorter (FACScan, Becton- Dickinson) with 488nm argon ion laser using MAC Cell-Quest™ Software. The cell cycle distribution was analyzed using PI signals, that was collected using the 585/42 band pass filter. The data acquired were analyzed using quest software.

**Statistical analysis**

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by using statistical package of social science (SPSS) version 12.0 for windows. The values are mean ± S.D. for six samples in each group. $p$ values <0.05 were considered as level of significance.

## Result and Discussion

The $IC_{50}$ value was determined by the concentration of the compound, which reduces the absorbance to half that of the control and the cell viability (50%) was depicted in Figure. 2. Quinine can effectively inhibit the viability of KB cell lines in a dose-dependent manner. The $IC_{50}$ values of quinine were 125.23µM/mL for 24hr and 117.81µM/mL for 48hr, obviously; quinine at a concentration of 125.23µM/mL for 24hr shows higher cytotoxic activity.

KB cells were exposed to various concentrations of Quinine (25-250 µM). The cytotoxicity activity was measured by MTT assay and $IC_{50}$ values were calculated (147.58 µM/mL for 24hr and 123.74 µM/mL for 48hr).

KB cells were treated with $IC_{50}$ concentration of quinine for 24 and 48hr . Generation of ROS levels during quinine treatment was measured spectrofluorimetrically by DCFH-DA staining for 24 and 48hr . Values are given as mean ± SD differ significantly at $P < 0.05$ vs control (DMRT).

Figure 3 shows the fluorescence intensity of DCFH-DA in KB cells after exposure to quinine at $IC_{50}$ concentration for 24 and 48 hr. The ROS level were observed after treatment with quinine 108.32 ± 7.32 for 24hr and 76.41 ± 7.09 for 48hr. The intracellular ROS levels were increased in quinine treated cells as compared to the untreated cells.

KB cells were treated with $IC_{50}$ concentration of quinine for 24 and 48hr . The reduction of mitochondrial membrane potential in quinine treated KB cells was measured spectrofluorimetrically by rhodamine 123 staining for 24h and 48hr . Values are given as mean ± SD differ significantly at $P <0.05$ vs. control (DMRT).

Figure 4 Shows the accumulation of rhodamine 123 within the active mitochondria of KB cells after exposure to quinine at $IC_{50}$ concentration for 24 and 48hr incubation. The results shows significantly, increase in the progressive loss of red Rh 123 aggregates fluorescence by increasing the incubation time and the concentration of quinine determines the significant increase in the cytoplasm diffusion of green monomer fluorescence. In the $IC_{50}$ concentration; partial or 50% loss of red fluorescence were observed, due to loss of mitochondrial trans-membrane potential in the cancer cells. It has also been observed that there was significant increase in fluorescence intensity (123.76 ± 7.81 for 24hr; 134.78± 9.76) in untreated KB cells.

KB cells were treated with $IC_{50}$ concentration of quinine for 24 and 48hr . The percentage of apoptotic cells were significantly increased compared with the control. The values are expressed as mean ± SD from the six independent experiments $p<0.05$

AO/EB staining was done on KB cells treated with $IC_{50}$ concentration of quinine for 24 and 48hr were shown in figure 5. The changes were determined by the color of individual KB cells. Figure. 5-A & C shows that live KB cells exhibit bright green spots. The stained KB cells with $IC_{50}$ concentration of quinine for 24 and 48hr showed gradual increase in the apoptotic death (orange), whereas necrotic (red) cells were shown in Figure 5-B & D and percentage of apoptosis feature (apoptotic cells and necrotoic cells) also was shown in figure 5. Quinine treated tumor cells characterized by the membrane blebbing, chromatin condensation in cells, thus showing the apoptotic features.

KB cells were treated with $IC_{50}$ concentration of quinine for 24 and 48hr. Cell cycle distribution was determined by PI staining and FACS analysis. The experiments were performed three times, representative results are shown. Quinine treated cells showed a cell cycle arrest in G0/G1 phase at 24 and 48 hr for cells. Values are given as mean ± SD differ significantly at $P < 0.05$ (DMRT).

Figure 6 Shows the effect of quinine on cell cycle processes by flow cytometry with propidium iodide staining. In the treatment of KB cells with $IC_{50}$ concentration of quinine for 24 and 48 hr, the $G_1$/S phase were significantly decreased when compared to control or untreated cells. These results indicate that when treated with quinine, the cells undergo DNA fragmentation, so that their DNA content below that of the $G_2$/M

phase. Quinine treated with KB cells for 24 and 48hr shows cell cycle arrest at the $G_1$/S-phase. The quantitative analysis of the histograms to determine the percentage of cells in the $G_1$,S and $G_2$/M phases for the control and quinine treated cells for 24 and 48hr are shown in figure 6.

Quinine is a characteristic alkaloid mostly present in *Cinchona officinalis* and utilized as numerous medicinal applications. The objective of this study was to investigate the mechanism underlying quinine-impelled cell death in human KB cancer cells. The apoptosis inducing effect of quinine was confirmed by ROS, MMP and double AO/EtBr staining assays and cell cycle analysis. Our results revealed that quinine is more potent to inhibit the cell proliferation and induce apoptotic cell death in KB cell line. The results of the MTT assay conclude that, the quinine caused cytotoxicity in a dose and time dependent manner, the IC50 values obtained after 24hr treatment of different concentrations of quinine (125.23 µM/mL for 24 hr). ROS is critical for the metabolic and signal transduction pathways associated with cell growth and apoptosis [8]. Several anticancer agents, including anthracyclines, cisplatin, bleomycin, and irradiation currently used for cancer treatment have been shown to cause increased intracellular ROS generation. The results of this study showed that the intracellular ROS levels were significantly increased in KB cells treated with $IC_{50}$ concentration of quinine at time and dose dependent manner.

Mitochondria have been shown to play a central role in the apoptotic process, because both the intrinsic pathway and the extrinsic pathway can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization [9]. Most of the conventional anticancer treatments are thought to induce cell death through indirect activation of the mitochondria dependent pathway of cell death, often found altered in drug-resistant cancer cells. In this study, we demonstrated that quinine on the intracellular ROS by increasing its level in the cell; it is possible that considerable increase in intracellular ROS level enhances the cell death by the treatment of quinine. This result is in agreement with a recent study of Ma *et al.,* (2012) reported, that an active natural anthraquinone derivative of emodin induced apoptotic cell death through the associated with loss of mitochondrial membrane potential ($\Delta\Psi m$) [10].

More than 50% of neoplasm undergoes aberrations in the apoptotic pathway, which cause abnormal cell proliferation [11]. Most of the report indicated that the most of chemotherapeutic agents halt tumor cells proliferation via induction of apoptosis [12]. The vast majority of current anti-cancer medication like vincristine, taxol, camptothecin, etoposide and paclitaxel are plant phytochemicals. These bioactive phytochemicals are known to exert their anti-cancer activity through different systems, including altered cancer-causing agent metabolism, prompting of DNA repair systems, and concealment of cell cycle movement and induction of apoptosis [13]. In present study, we examined quinine can induces apoptosis in the KB cell lines in a dose dependent manner confirmed by typical morphological changes as the apoptotic cells e.g. cell shrinkage, membrane blebbing, nuclear fragmentation, chromatin condensation, apoptotic bodies and loss of adhesion were observed in the quinine treated KB cell lines. [14]. Allover results finding and sorted that quinine used in the treatment of cancer have strong apoptotic activities in KB cell lines.

The induction of apoptosis and inhibition of cell proliferation are revealed to the activity

of signal transduction molecules involved in the cell cycle. The premitosis $G_2$/M phase and pre-replication $G_1$/S phase which are specifically regulated by specific genes, are the two check points for DNA damage [15]. It is also reported that phenolic acids found to arrest (specific genes) cell cycle progression either at $G_1$/S phase or $G_2$/M phase boundaries [16]. In our study the cell cycle arrest was analyzed by the flow cytometry techniques. The results of flow cytometry showed that, quinine induce an early $G_1$/S phase arrest in $IC_{50}$ concentration of quinine at 24 and 48hr. A lessened number of S-phase and an increment in the G1 peak were the characteristic for the early stage capture of G1/S phase cell cycle arrest.

Fig 1: Structure of Quinine

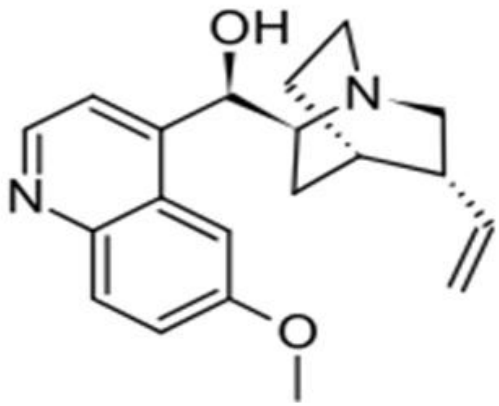



**Fig.2** MTT Assay on Quinine treated KB cells

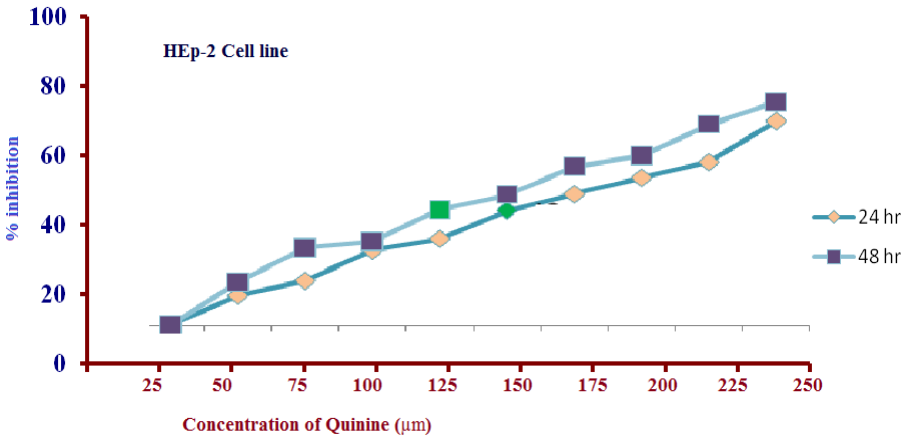
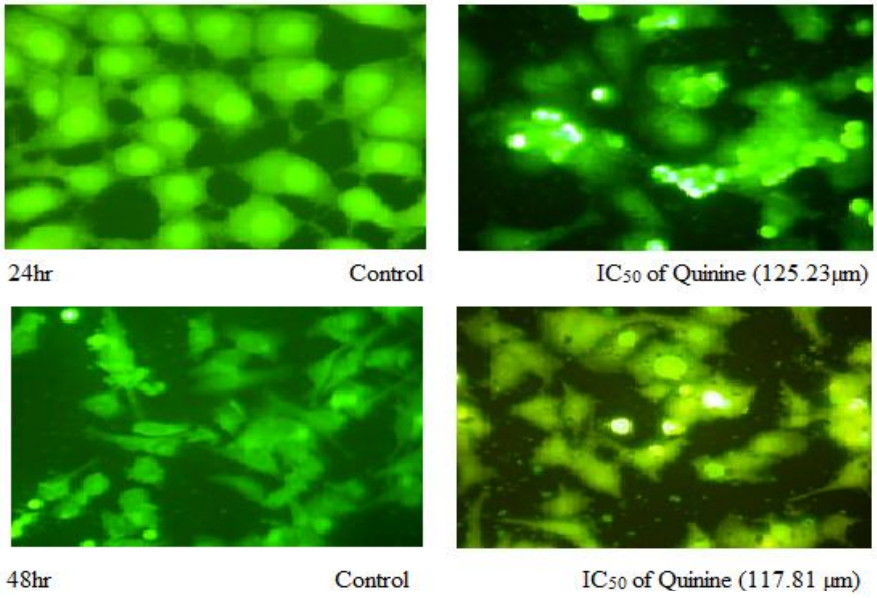


**Fig.3** The levels of intracellular ROS in quinine treated KB cells using DCFH-DA staining for 24 and 48hr



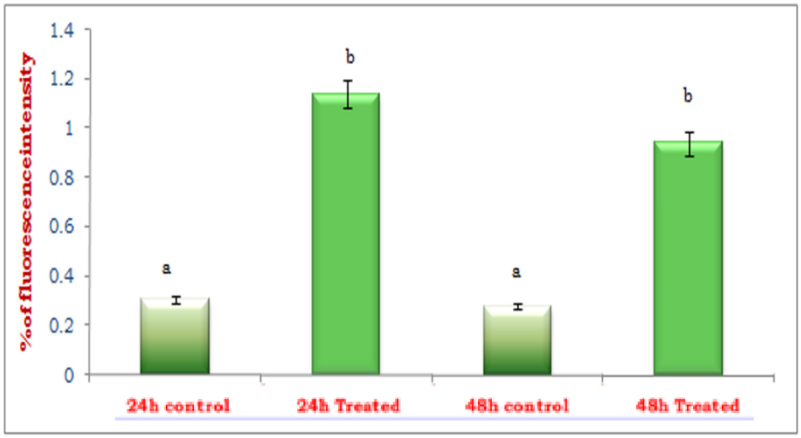
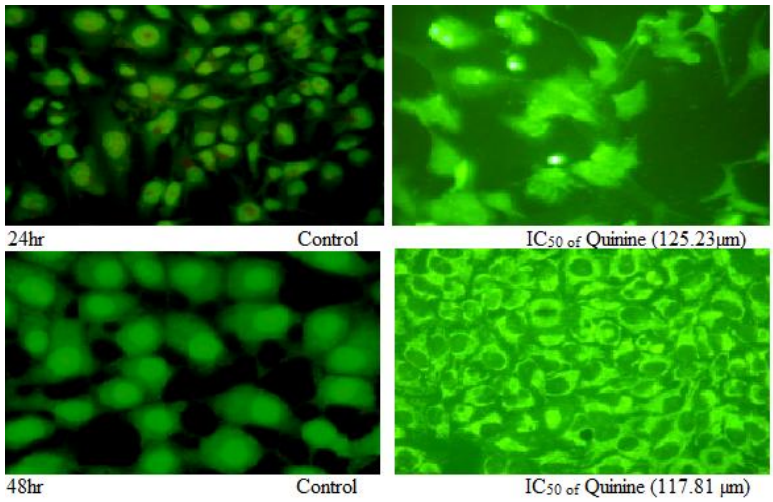




**Fig.4** The effects of quinine on mitochondrial membrane potential in KB cells were observed on rhodamine 123 stain in 24 and 48hr treatments



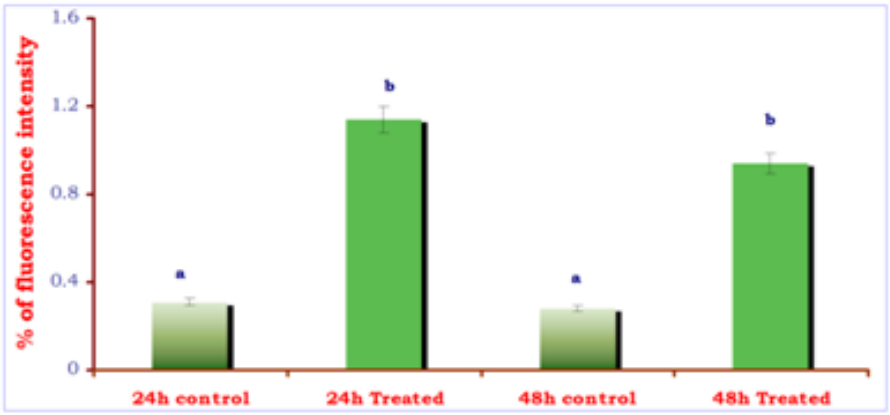
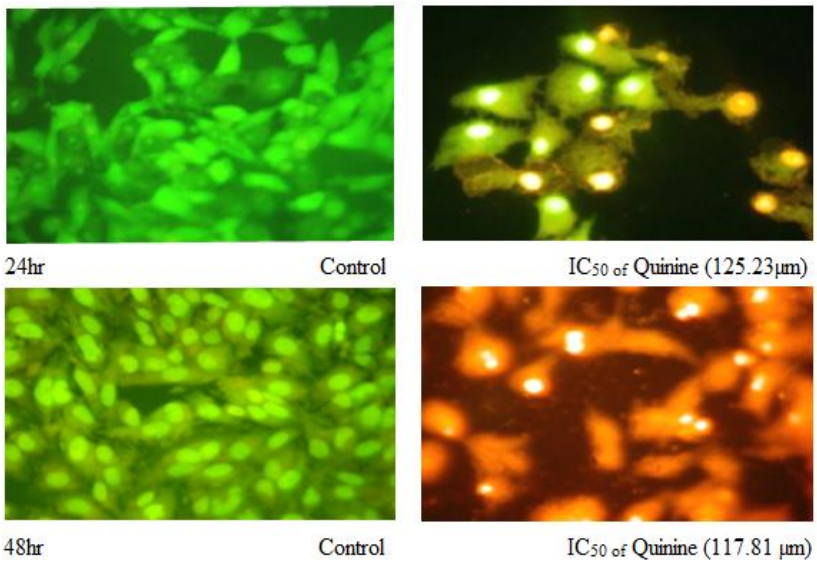
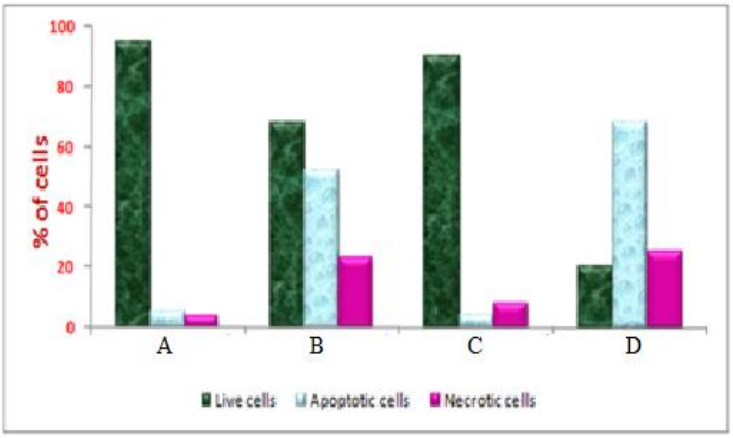


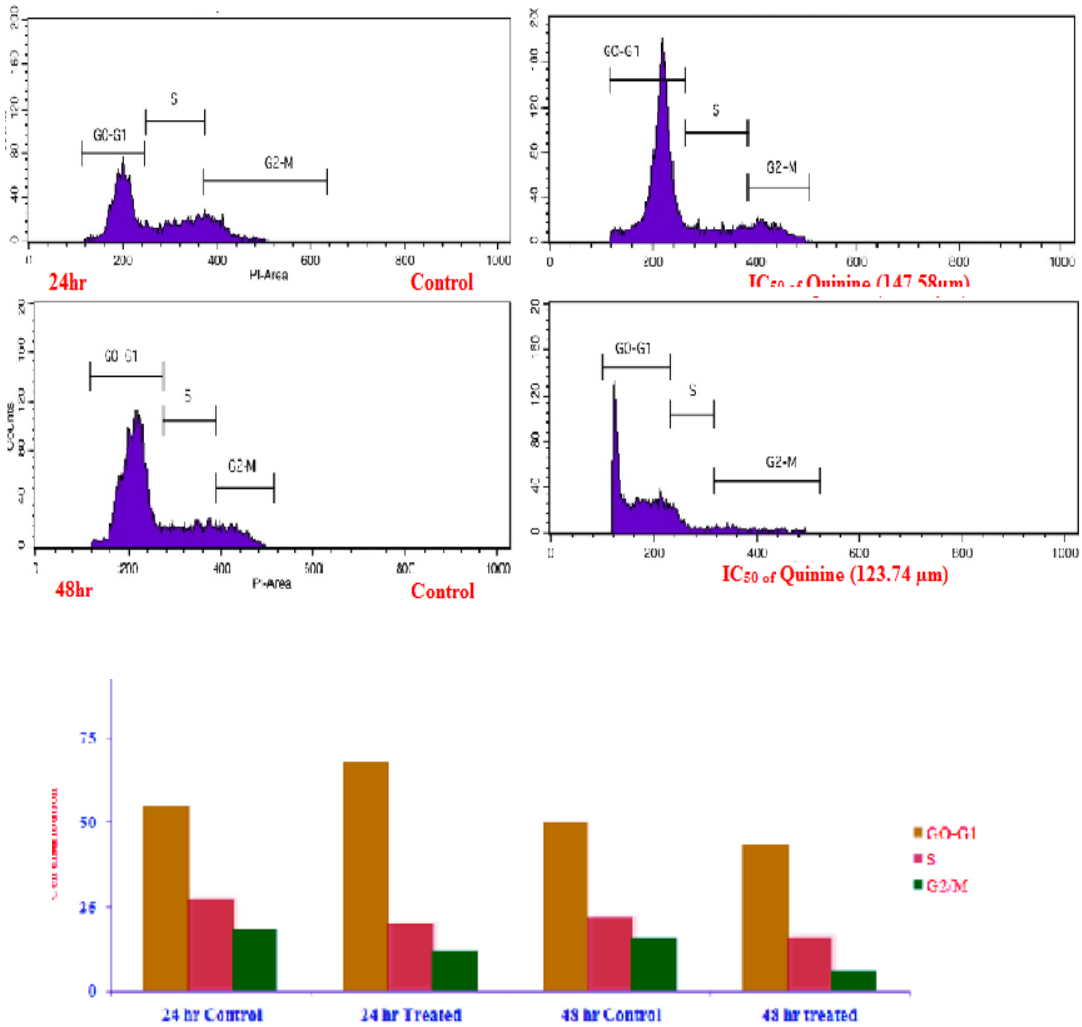

**Fig.5** The effects of quinine on morphological changes in the KB cells were observed on dual staining with AO/EBr in 24 and 48 hr treatments




**A: 24hr Control** **B: $IC_{50\ of}$ Quinine (147.58 µm)**
**C: 48hr Control** **D: $IC_{50\ of}$ Quinine (123.74 µm)**

**Fig.6** The effect of quinine on cell cycle checkpoint potential on KB cells in 24 h and 48 hr as revealed in the FACS analysis




These results showed that the quinine might show potent cytotoxicity effects on KB cancer cells, by inhibiting the growth of the cancer cells, through the apoptotic and cell cycle arrest. Thus, the present study suggests that quinine might be a promising anticancer therapeutic agent for oral cancer cell lines.

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