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A Study on Protective Effect of Quinine against Lipid Peroxidation and Antioxidants Status in Human Oral Cancer Cell Line.

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ABSTRACT

Aim of the present study was undertaken to evaluate the antioxidant effects of quinine against oral cancer cells (KB and HEp-2) as well as its effect on lipid peroxidation. Effective dose was determined for Quinine a plant based product through MTT assay. According to our MTT assay, the IC50 value for the studied compound Quinine was 125.23µm for 24hr, while it was 117.81µm for 48hr with respect to KB cell line. Similarly, the IC50 value of Quinine was found to be 147.58 µm for 24hr and 123.74µm on treatment with Hep2 cell line. Throughout the study, IC50 value of Quinine obtained with each specific cell line was used. Hence, the *in vitro* lipid peroxidation and antioxidant activity were measured in Hep-2 and KB cells at two different time interval i.e 24 and 48hrs through various biochemical parameters such as TBARS, Conjugated Dienes, lipid hydroperoxides, SOD, CAT, GPx and Reduced glutathione. According to our results, lipid peroxides, hydroperoxides were found to be decreased in 24hr incubation, only conjucated dienes showed increased value in 24hr incubation with KB cell line, but the 24hr incubated HEp2 cells showed increased value. Conjucated diene was found to be decreased and lipid peroxides, lipid hydroperoxides were increased in KB cell line treated with IC 50 value of Quinine for 48hr. while, the result was contrary with HEp2 cell line. The results of enzymatic, non enzymatic antioxidants studied shows increased SOD, CAT, GSH with KB cell line treated with IC 50 value of Quinine for 48hr when compared to HEp2 cell line. Among the two cell lines selected for the study, the Quinine was found to be effective on KB cell line. The observed results confirms, that quinine was found to possess anti-lipidperoxidation, antioxidant effect on cancer cells.

Keywords: Antioxidant, cell line, *invitro* study, lipid peroxidation, Quinine.

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INTRODUCTION

Physiological, pathophysiological condition of cells generate ROS which cause lipid peroxidation [1]. Oxidative stress occurs when there is an imbalance between the production of ROS and status of anti-oxidant defense system, leading to cytotoxicity and changes in gene expression that initiate or promote carcinogenesis [2]. Stress induced reactive oxygen species (ROS) was generated from mitochondrial dysfunction, stimulation of oncogenes, abnormal metabolism, and aggravated inflammatory activities, react with membrane lipids, nucleic acids, proteins and enzymes and other small molecules resulting in cellular damage or degradation [3]. Free radicals induced oxidative stress results in the etiopathogenesis of several cancers including oral cancer. Excessive generation of reactive oxygen species in betel quid chewers was reported [4]. Antioxidants counteract to eliminate abnormal ROS accumulation in a physiological state, under pathological condition and thereby inhibiting the development of cancer [5]. Oxidative stress increases the levels of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes [6]. However, human body contains an array of defense mechanism including non-enzymatic (Vitamin E, C and reduced glutathione (GSH) and enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) to protect the deleterious effects of free radical induced oxidative damage [7].

Numerous experimental and epidemiological studies have shown that a wide variety of phytochemicals are able to prevent or slow down oxidative stress-induced damage leading to carcinogenesis by slowing the molecular events in the initiation, promotion or progression. Recent studies demonstrated that the high dietary intake of fruits and vegetables are associated with lower cancer incidence in humans [8]. Current research focus is on natural products and its phytochemicals approximately 60% of drugs applied in the treatment or prevention of cancer are from natural products and their derivatives, of which higher plants contribute around 25%.

Quinine is a bitter, colourless, amorphous powder or crystalline alkaloid, $C_{20}H2_4N_2O_2{\cdot}3H_2O$, derived from certain cinchona barks and used in medicine to treat malaria [10]. Genne et. al [11] works on human leukemic cell line K562/ADM shows the *in vitro* effect of quinine and cinchonine on doxorubicin, mitoxantrone, and vincristine uptake and cytotoxicity. Pharmacokinetic data reported that quinine should be administered before anti-cancer drugs while performing clinical trials [12]. However, there is no scientific report were available on the lipid peroxidation and antioxidants enhancing activity of quinine in human oral cancer cell line. Thus, the present study was designed to study the effect of quinine on lipid peroxidation and enzymatic, non-enzymatic antioxidants in human oral cancer cell line.

MATERIALS AND METHODS

Chemicals

Quinine ((R)-(6-Methoxyquinolin-4-yl)((2S,4S,8R)-8-vinylquinuclidin-2-yl)methanol), DMSO (dimethyl sulfoxide), triton-X-100, EDTA (Ethylene diamine tetra acetic acid), pyrogallol, glutathione reduced, NADPH (Nicotinamide adenine dinucleotide phosphate), TCA (Trichloroacetic Acid), TBA (Thiobarbituric acid) were purchased from Sigma-Aldrich-USA. Dulbecco's Modified Eagle's Medium (DMEM), (FCS) fetal calf serums were purchased from Sigma Aldrich. Rest of chemicals was purchased for analytical grade.

Culture medium for the maintenance of oral cancer cell lines

Human oral cancer cell line (KB and HEp-2) was obtained from National Centre for Cell Science (NCCS) Pune. cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% heat inactivated fetal calf serum and antibiotic solution and incubated at 37ºC and 5% CO_2.

Quinine preparation

Quinine was prepared with DMSO and stored at -20°C. The stock solution was diluted to arrive at 25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 µg of quinine.

Experimental study group

The present study was undertaken with the following four groups: Group 1-Control (Untreated KB/HEp-2 cells of 24 hr), Group 2-KB/HEp-2 cells + quinine (IC_{50} Value of 24 hr), Group 3-Control (Untreated KB/HEp-2 cells of 48 hr), Group 4-KB/HEp-2 cells + quinine (IC_{50} Value of 48 hr).

Determination of effective dose of Quinine (MTT cytotoxicity assay)

Oral cancer cells (KB, Hep2) seeded in 96 well microtiter plate (5×10^3 cells/well) and incubated for 24 and 48hr served as control and cells incubated with Quinine at different concentrations ranging from 25-250µm for 24 and 48hr served as experimental study group. 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 at 590nm using colorimeter [13]. The IC50 value was determined by the concentration of the drug which reduce the absorbance to half that of the control.

Cell line cultivation and preparation

KB and HEp-2 human oral cancer cell lines ($2.5x10^6$ cells) were grown for in complete MEM until they reached the post-confluence stage. Cells were incubated with IC_{50} concentration of Qunine for 24 and 48hr. The untreated KB and HEp-2 cells were maintained as control for 24 and 48hr. Then the confluent cells were washed twice with phosphate buffered saline (PBS), pH 7.2 and treated with 0.25% trypsin, 2mM EDTA in PBS for 10 min. The cell suspension was centrifuged for 10 minutes in a centrifuge (600 rpm). Cell pellets were then lysed in 50mM phosphate buffer pH 7.0, followed by sonication for 2min on ice. The mixture was then centrifuged for 10 minutes at 10,000xg and the supernatant was assayed for enzyme activities and protein concentration

Estimation of TBARS

The level of TBARS was evaluated as described by Donnan [14] with modification. In brief cells grown on 6-well plates were washed with 0.01 M PBS, scraped, and resuspended in 1 ml PBS. An aliquot was taken out for a protein assay, and 0.5 ml TBA reagent (100 mg trichloroacetic acid, 3.35 mg thiobarbituric acid) was added to each tube and vortexed. The reaction mixture was incubated at 90°C for 20min and stopped on ice. After cooling to room temperature, TBARS were extracted with 1.0 ml n-butanol and separated at 3000 ×g centrifugation for 5 min. The absorbency of the total TBARS was measured at 532 nm. Tetraethoxypropane in absolute ethanol was used to prepare MDA standards. The measurements were performed and the results were expressed as nmol equivalent of MDA/mg protein.

Estimation of Conjugated Dienes

Conjugated diene level was evaluated as described by Recknagel and Glende [15] with modification. 25µl of cells lysate were extracted with 3 ml chloroform/methanol (2:1, v/v). After centrifugation at 3,000 rpm for 15 min, 2 ml of organic phase was transferred into another tube and dried at 45°C. The dried lipids were dissolved in 2 ml of methanol and absorbance at 233 nm was determined. It corresponds to the maximum absorbance of the extracted compound cell line and culture conditions.

Estimation of lipid hydroperoxide

The levels of lipid hydro peroxide (LOOH) were estimated by the method of Jiang et. Al [16]. Fox reagent, 1.8 ml, was mixed with 0.2 ml of cell line culture and incubated for 30 min at room temperature. The colour developed was read at 560 nm.

Estimation of super oxide dismutase

Superoxide dismutase (SOD) activity was determined as per the method of Kakar et.al [17]. The final volume of 3 ml contained 0.052 M sodium pyrophosphate buffer (pH 8.3), 186µM phenozine metho-

sulphate(PMS), 300Mm nitrobluetetrazolium(NBT), 780µMNADH, cell lysate containing 50µg of protein. The reaction was initiated by adding NADH followed by incubation at 37°C for 90s. After the incubation period, the reaction was stopped by adding 1.0 ml of glacial acetic acid and the content was shaken well with 4.0 ml n-butanol. The mixture was allowed to stand for 10min, centrifuged, and butanol layer was separated. The colour intensity of chromogen in butanol was measured at 560nm against butanol using a spectrophotometer. The enzyme activity was expressed as U/mg protein.

Estimation of Catalase

The activity of catalase levels were measured as per the method of Sinha [18]. An appropriate volume of cell lysate containing 50µg protein was mixed with 1 ml of 50 mM phosphate buffer (pH 7.0) containing 10 mM H_2O_2 in 1 ml quartz cuvette. The decrease in absorbance of H_2O_2 was followed at 240 nm for 4 min. Catalase activity was calculated from the slope of the H_2O_2 absorbance curve and normalized to protein concentration.

Estimation of Glutathione peroxidase

GPx activity was measured by method described by Rotruck et.al [19]. The reaction mixture containing 0.2ml 0.4M Phosphate buffer (pH7), 0.1ml 10Mm sodium azide, 50µg of protein of the cell lysate, 0.2ml glutathione, and 0.1ml 0.2Mm H_2O_2. The contents were incubated for 10min at 37^{o}C, 0.4ml 10% TCA was added to stop the reaction and centrifuged at 3200 rpm for 20min.The supernatant was assayed for glutathione content using Ellman's reagent(19.5g 5,5'-dithiobisnitrobenzoic acid (DTNB) in 100ml 0.1% sodium citrate). The activity was expressed as µMol of GSH consumed/min/mg protein.

Estimation of reduced glutathione

Reduced glutathione was measured according to the method of Ellman [20]. Equal quantity of cell lysate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithio-bis-(2-nitrobenzoic acid) and 0.4 ml double distilled water was added. Mixture was vortexes and the absorbance read at 412nm. The concentration of reduced glutathione was expressed as µg/mg of protein

Estimation of protein

The protein was estimated by Lowry's method [21]. Stock solution was prepared using BSA 100mg dissolved in 100ml distilled water. Diluted working standard ranging from 10-100µg was taken and to this added 4.5ml of alkaline copper reagent , incubated for 10min. After the incubation period 0.5ml of Folin ciocalteau reagent was added and kept for 30min incubation. The absorbance was measured at 660nm.

Statistical analysis

All quantitative measurements were expressed as Mean ± SD for untreated and quinine treated cells of six. The data were analysed using one way analysis of variance (ANOVA) on SPSS (statistical package for social sciences) and the group means were compared by Duncan's Multiple Range Test (DMRT).The results were considered statistically significant if the $p<0.05$.

RESULTS AND DISCUSSION

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay measures the invitro cytotoxic effect of compound Quinine on cell lines in order to monitor the health status of cells after incubation with Quinine. The results of IC50 value of Quinine for the treatment/incubation period of 24, 48hr on KB, HEp2 cell line. The KB cell line showed IC50 value of 125.23µm (24hr), 117.81µm(48hr). Similarly, the Hep2 cell line showed 147.58 µm and 123.74 µm. The obtained IC50 concentration was used for further lipid peroxidation and antioxidant studies. Tetrazolium dye reduction in MTT assay is dependent on cytosolic NAD(P)H-dependent oxidoreductase enzymes as this reaction takes place only in viable, live cells which are metabolically active because it is not only dependent on enzyme activity but also on ATP production,

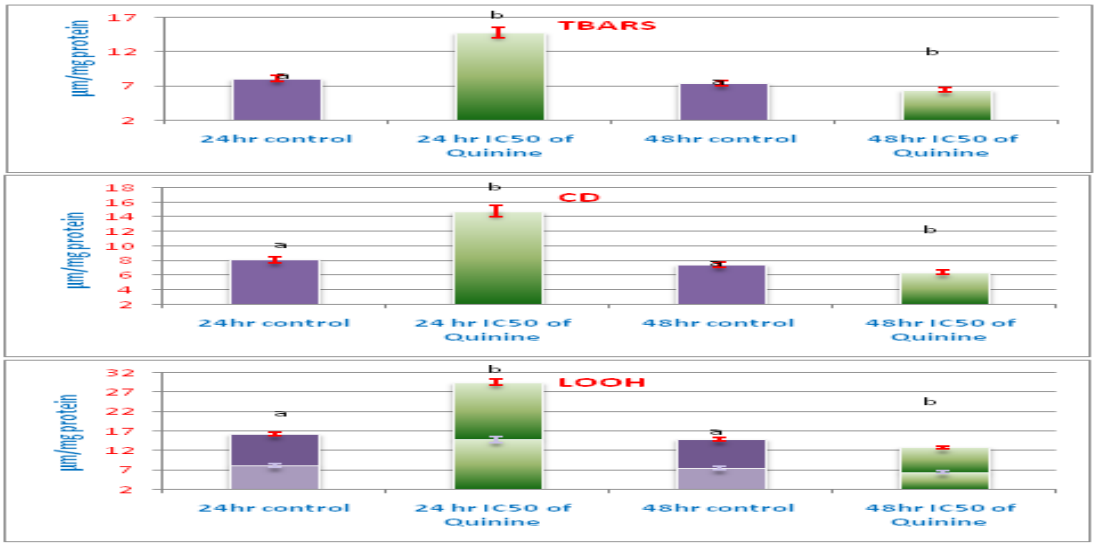
adherence of cell, permeability of membrane, uptake of nucleotide. Otherwise, it indirectly measures the activity of mitochondria, hence, this test serves as a marker for cell metabolism/function.

The results of enzymatic, non-enzymatic antioxidants studied are shown in Fig1, Fig2, Fig3, Fig4.

Figure 1: The status of lipidperoxidation in control and Quinine treated KB Cancer cell line.

Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

Figure 2: The status of lipidperoxidation in control and Quinine treated HEp-2 Cancer cell line.



Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

Figure 1 and 2 represents the status of the lipid peroxidation indices i.e. TBARS, CD and LOOH in KB and HEp-2 cells with and without treatment of quinine at a concentration of IC_{50} value for 24 and 48hr incubation. Activities of lipidperoxidation were tested at IC_{50} concentration (24 and 48hr both KB and HEp-2 cells).

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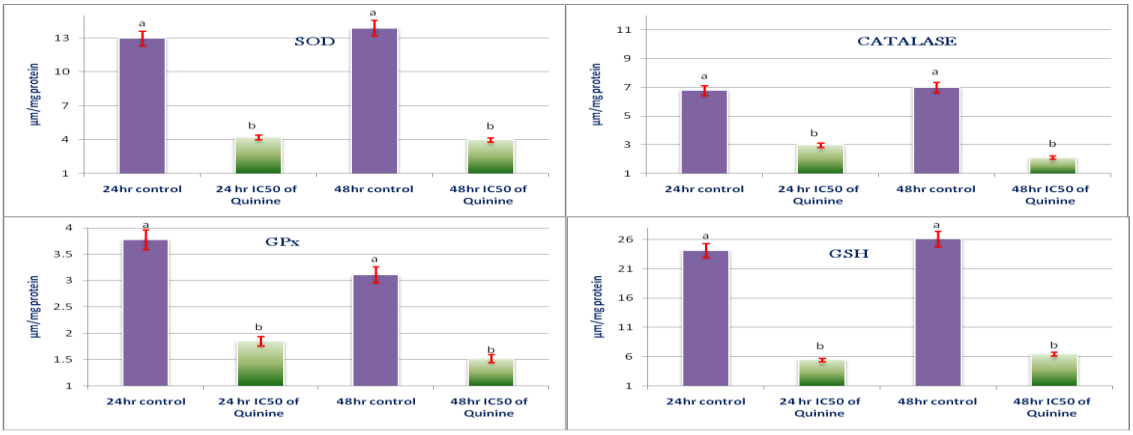
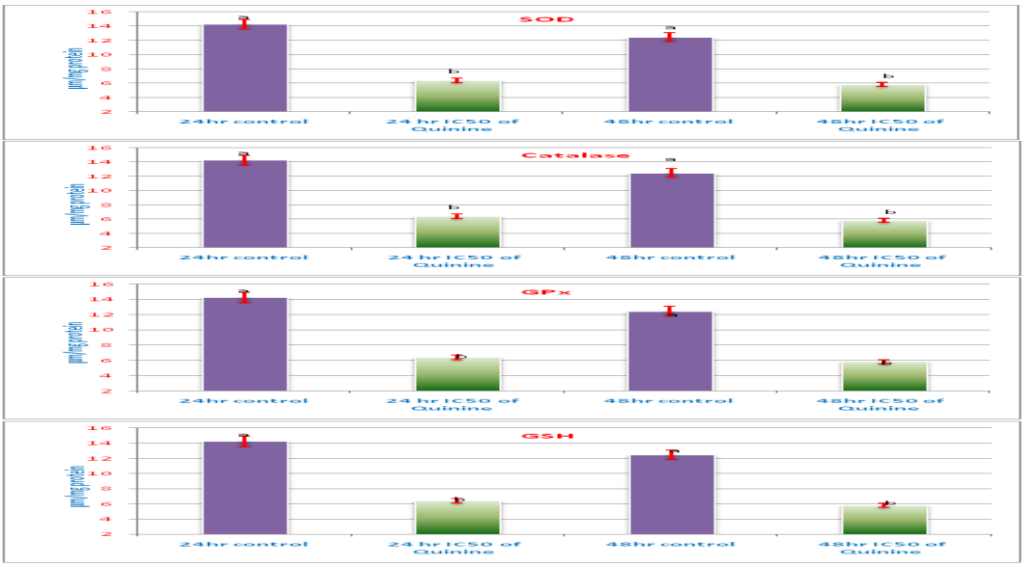
Figure 3: The status of SOD, Catalase, GPx and GSH Levels in control and Quinine treated KB Cancer cell line.



Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

Figure 4: The status of SOD, Catalase, GPx and GSH Levels in control and Quinine treated HEp-2 Cancer cell line.



Values are given as mean ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at P < 0.05 (DMRT).

Figure 3 and 4 represents the status of enzymatic antioxidants such as SOD, CAT and GPx and non enzymatic antioxidant, GSH in KB and HEp-2 cells with and without treatment of quinine at a concentration of IC_{50} value for 24 and 48hr incubation. Activities of enzymatic antioxidants were tested at IC_{50} concentration (24 and 48hr both KB and HEp-2 cells).

In this study, we have investigated *in vitro* antioxidants and anti-lipidperoxidative effects of Quinine on the human oral cancer cell lines (KB) and HEp-2. The results of the present study on Quinine against KB and HEp-2 cells showed cytotoxic activity in time and dose dependent manner. Lipid peroxidation is one of the most investigated consequences of reactive oxygen species' (ROS) on membrane structure and function leading to cancer. Peroxidation of lipids in mitochondrial membrane and its effect along with cross-linking of proteins with amino groups of phospholipids, nucleic acids induced by MDA causes altered membrane fluidity [22].

In an attempt to elucidate the baseline levels of antioxidant enzyme status in KB and HEp-2 cell lines, we measured the activities of SOD, GPx, CAT, and GSH and MDA contents. We had also explored the

antioxidant effects of Quinine, it has been highly suggested that in the process of carcinogenesis excessive accumulation of reactive oxygen species may play an important role in causing oxidative damage. In an attempt to defend the situation, antioxidant enzymes (SOD, GPx, and CAT) may be elevated or reduced in these cells [23]. Due to lipid peroxidation there is a reduction in SOD, CAT, and GSH which counteract the damaging effects of reactive oxygen species. It has been reported that a decrease in SOD, CAT, GSH and GPx were observed in KB and HEp-2 cells ina time and dose dependent manner.

We also found that the quinine reduced the activities of antioxidant enzymes, SOD, CAT and GPX in KB, Hep 2 cell line ina time and dose dependent manner which was found to be higher in untreated KB and HEp-2 cells. This is comparable to the study of Bozinovski et al[24]. A recent study showed that, antioxidant enzymes including SOD, CAT, GPx find major role in the regulation of redox status. Therefore in the present study, activity of SOD, CAT and GPx, and GSH levels were decreased upon exposure of Quinine to KB and HEp-2 cells at 24, 48hr when compared to untreated cells. Thus the data of the results obtained in the *in-vitro* studies conducted against KB and HEp-2 cell lines depicted that the quinine has the significant anti-oxidant activity against tested cell lines in a dose and time dependent manner.

CONCLUSION

The present study has demonstrated the cancer preventive potential of quinine on major oral cancer cells (KB and HEp-2). The results suggest that Quinine might have been playing a major role in bringing a balance between antioxidants and reactive oxygen species generated within the system. It gives a scientific support for Quinine as vital antioxidant or as therapeutic agents in suppressing the progress of oxidative stress mediated degenerative diseases. Quinine is medicinally important due to its non-toxicity, as it is a plant based compound it could be taken regularly and its substantial use in the treatment of malignancy will be of valuable to our society after performing proper clinical trials.

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