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Piperine Triggers Apoptosis of Human Oral Squamous Carcinoma Through Cell Cycle Arrest and Mitochondrial Oxidative Stress

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ABSTRACT

Piperine is a nitrogenous pungent substance exhibiting multifunctional pharmacological properties. However, the mechanism underlying its anticancer potential is not well elucidated in human oral squamous carcinoma (KB) cell line. The anticancer potential of piperine was evaluated through potent biomarkers *viz.* reactive oxygen species (ROS), cellular apoptosis, and loss of mitochondrial membrane potential (MMP). In addition, cell cycle kinetics and caspases-3 activity were also carried out to confirm anticancer activity of piperine. Results showed that various concentrations (25–300 μM) of piperine exposure reduced the cell viability of KB cells significantly ($P < 0.01$). Piperine induced significant ($P < 0.01$) dose-related increment in ROS production and nuclear condensation. Moreover, piperine stimulated cell death by inducing loss of MMP, and caspase-3 activation. Cell cycle study revealed that piperine arrested the cells in G2/M phase and decreased the DNA content. Findings of this study suggest the efficacy of piperine in inducing cell death via the decrease in MMP and ROS liberation followed by caspase-3 activation and cell cycle arrest. Further assessment of the anticancer potency of piperine is needed for anticancer drug development.

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Introduction

Cancer is the major public health problem worldwide and is increasing broadly, regardless of intensive drugs and research development. Oral carcinoma is the eighth most common cancer of head and neck malignancies among male patients and its incidence has also been increasing in the developing countries (1,2). Therefore, it is essential to search for chemotherapeutic chemicals or naturally occurring drugs to minimize health problems. Inhibiting proliferation or triggering apoptosis in tumor cells may represent an effective strategy in anticancer therapy. Conventional cancer therapies cause serious side effects, however, plant-derived products are identified possessing antitumor properties and relatively nontoxic in nature (3). Several studies have reported that natural products could improve the sensitivity of resistant cancers to existing chemotherapy drugs (4,5). Black pepper, a flowering vine of the family piperaceae, is produced from unripe but developed fruits. Many spices, including peppers, are a major source of phytochemicals with potential antitumor properties (6). Piperine (1-piperoyl peperdine), an alkaloid pungent substance is present in the fruits of black pepper (Piper nigrum Linn.) and long pepper (Piper longum Linn.). Piperine content of black or white peppercorns generally varies from 3 to 8 g per 100 g (7). Piperine has been used extensively as a condiment and flavoring agent for savory dishes and as a traditional medicine. It exhibits numerous pharmacological and biochemical effects including antioxidant, anticarcinogenic, antimicrobial, antifungal, anti-inflammatory, immunomodulatory and hepato-protective properties (8–10). A study reported that piperine-free *Piper nigrum* extract has been shown to have low toxicity and cancer-preventive effects on N-nitrosomethylurea-induced mammary tumorigenesis in rats (11). Piperine suppresses tumor growth and metastasis of mouse 4T1 mammary carcinoma as revealed by both *in vitro* and *in vivo* studies (12). Piperine has also shown the antimetastatic property against triple-negative breast cancer cells and antiosteosarcoma against human osteoblasts (13,14).

The cytoprotective effect of piperine on B (α)-p (Benzopyrene)-induced experimental lung cancer has been successfully investigated in mice and it has been

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inferred that piperine could exert its chemopreventive effect by modulating lipid peroxidation and augmenting antioxidant defense system (15). Another study has demonstrated that piperine can inhibit breast cancer by targeting the cancer stem cell renewal properties (16). Additionally, piperine also possessed immunomodulating, antioxidative, and chemo-protective ability on murine splenocytes (17,18). Interestingly, a research has shown that natural products inhibit cancer cell progression, proliferation and its metastasis through inducing cell cycle arrest, reactive oxygen species (ROS) generation, by activating both intrinsic and extrinsic apoptosis pathways and downregulating signaling pathways (5). Keeping these views on action mechanism of natural products, the present study has designed to investigate the anticancer effects of piperine on human oral squamous carcinoma (KB) cell line.

Our results demonstrated that the anticarcinogenic mechanism of piperine in KB cells is accomplished through the involvement of ROS, cellular apoptosis, loss of mitochondrial membrane potential (MMP), cell cycle kinetics, and caspase-3 activation.

Materials and Methods

Cell Line and Culture

The human oral squamous carcinoma KB cell line was obtained from cell repository-National Centre for Cell Sciences, Pune, India. KB cells were cultured in Eagle's minimal essential medium (MEM, Himedia) with 2.0 mM of L-glutamine, 1.5 g/l of $NaHCO_3$, 0.1 mM of nonessential amino acids, 1.0 mM of sodium pyruvate, and supplemented to contain 10% (v/v) fetal bovine serum (FBS, Himedia). Cells were grown at 37°C and 5% CO_2 in humidified air.

MTT Assay for Cell Viability

The cell viability assay of piperine (Sigma-Aldrich, USA) was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously (19). For 10,000 μM stock solution preparation, approximately 2.85 mg (molecular mass) of piperine was dissolved initially in a least amount (50 μl) of dimethylsulfoxide (DMSO) and diluted in culture media (950 μl) to adjust a final volume of 1 ml solution. The sub-confluent cells were treated with selective doses *viz.* 25, 50, 100, 200, and 300 μM of piperine in 100 μl of complete culture medium and the cells treated only with vehicle (DMSO, 0.2% in media) served as control. After 24 h of exposure, 10 μl of MTT solution (5 mg. mL^{-1} stock solution) was added in each well and re-incubated for 3 h at 37°C until formazan blue crystal developed. Media was discarded from each well and 100 μl of DMSO was added to dissolve formazan crystals for 10 min at 37°C. The absorbance was recorded at 540 nm by microplate reader (BIORAD-680) and relative percent cell viability was evaluated.

Morphological Analysis of Cells

The effect of piperine was analyzed for morphological changes in the cultured cells [20]. The cells were seeded and treated with different concentrations of piperine for 24 h. The cellular morphology was observed under inverted phase contrast microscopy (Nikon ECLIPSE Ti-S, Japan). Three effective doses (50, 100, and 200 μM) of piperine were used for further analysis.

Reactive Oxygen Species (ROS) Activity

Microscopic fluorescence imaging was used to study ROS generation in KB cells after exposure to different concentrations of piperine (20). KB cells (1×10^4 cells per well) were seeded in 96-well culture plate and exposed to 50, 100, and 200 μM concentrations of piperine for 12 h. After exposure, cells were incubated with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 mM) as a fluorescence agent for 30 min at 37°C. The reaction mixture was aspirated and washed with phosphate-buffered saline (PBS) in each well. An inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan) was used to visualize intracellular fluorescence of cells and to capture images. For quantitative fluorometric analysis, cells (1×10^4 per well) were seeded and treated in a 96-well black-bottomed culture plate. After 12 h of exposure, cells were incubated with DCFH-DA (10 mM) for 30 min at 37°C. The reaction mixture was aspirated and replaced by 200 μl of PBS in each well. The plate was kept on a shaker for 10 min at room temperature (RT) in the dark. Fluorescence intensity was measured with a multiwell micro-plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek) at an excitation wavelength 485 nm and emission wavelength 528 nm. Values were expressed as the percent of fluorescence intensity relative to the control wells.

Nuclear Condensation Analysis by DAPI (4′, 6′-Diamidino-2 Phenylindole) Staining

The apoptotic effect of piperine was analyzed by fluorescent nuclear dye DAPI (21). Cells were seeded and treated for 24 h as mentioned earlier. Cells were then washed with PBS and fixed in 4% paraformaldehyde for 10 min. Subsequently, cells were permeabilized with

buffer (3% paraformaldehyde and 0.5% Triton X-100) and stained with DAPI dye. After staining, the images were captured and the quantification of cells was performed in triplicate for each treatment and 100 cells per sample were counted in different fields to score percent apoptotic cells using a fluorescent microscope (Nikon ECLIPSE Ti-S, Japan).

Assessment of Mitochondrial Membrane Potential ($\Delta\Psi$)

Flouroprobe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) is a cationic, lipophilic dye and, therefore, has been extensively used to study the loss of MMP during apoptosis (21). In healthy cells, due to high membrane potential (polarized mitochondria), the dye concentrates in the mitochondrial matrix forming red fluorescent (J-aggregates). In treated cells, it shifts from red (J-aggregates) to green fluorescence (JC-1 monomers). The cells were grown in 24-well plate and treated with increasing concentrations of piperine. After a 24-h exposure time, cells were washed with PBS and stained with 2 μg/ml of JC-1 dye in MEM without phenol red at 37°C in the dark for 30 min. A decrease in mitochondrial depolarization patterns of cells was then examined and photographed using an inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan). Red and green fluorescent images were superimposed to form merged images and mitochondrial depolarization patterns of cells for cell quantification were examined using imaging software NIS-Elements F 4.00.00.

Analysis of Cell Cycle Phase Distribution

Cell cycle phase distribution analysis with cellular DNA contents was carried out using flow cytometry. KB cells were seeded into 6-well plate, each at a density of 1×10^6 cells mL^{-1} and treated with two effective doses, 50 and 100 μM, of piperine for 24 h (22). After 24 h of incubation, the cultured cells were harvested and fixed in 70% ethanol overnight. The fixed cells were then incubated with RNase A (10 $mg.mL^{-1}$) and propidium iodide (PI) dye followed by 30 min of incubation at RT in the dark. The PI fluorescence of individual nuclei was measured using flow cytometer (BD FACS Calibur, Becton Dickinson, USA). Data were analyzed with the Cell Quest Pro V 3.2.1 software (Becton Dickinson, USA).

Analysis of Caspase-3 Activity

The caspase-3 activity was assayed using caspase-3 colorimetric assay kit (BioVision, USA) and caspase-3 immunofluorescence stain (23). For colorimetric assay, approximately 3×10^6 (treated and untreated) cells were resuspended with chilled lysis buffer. The cell lysate was incubated on ice for 10 min prior to centrifugation (10,000 × g for 1 min). The reaction buffer, with 10 mM dithiothreitol (DTT) was added to the supernatant of cell lysate and incubated further for 30 min on ice. The cell lysate (50 μl) was aliquoted into a 96-well microplate and 50 μl of reaction buffer containing 10 mM DTT was then added to the lysate. Approximately, 5 μl of 4 mM DEVD-pNA substrate was added in each well and incubated at 37°C for 2 h. Absorbance at 405 nm was then read in a microplate reader (BIORAD-680). For immunofluorescence stain, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized, and blocked with blocking buffer (PBS containing 1% bovine serum albumin and 0.1% Tween 20) at RT for 1 h. Next, cells were incubated with anticaspase-3-active antibody at 1:400 dilution (C8487, SIGMA), followed by Alexa Fluor 594-conjugated secondary caspase-3 antibody at 1:400 dilution (A-11062, Molecular Probe) for 1 h. Cells were examined under inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan).

Statistical Analysis

Data of the cell viability were expressed as the mean ± standard error of mean (SEM) from three independent experiments. Differences were evaluated by one-way analysis of variance test followed by Dennett's multiple comparison test using Graph Pad prism software (Version 5.01). Differences with P value < 0.05 were considered to be statistically significant. The 50% inhibitory concentration (IC_{50}) was calculated by a nonlinear regression curve with the use of Microsoft Office Excel for Windows.

Results

Effect of Piperine on Morphological Changes and Antiproliferative Activity

Cellular morphological changes were significantly observed in KB cells exposed to different concentrations of piperine at 24 h under inverted phase contrast microscopy. The photomicrographs showed that the treated cells drastically changed their morphological shapes into round shape in contrast to spindle or square shape in the control group. The frequencies of cellular morphological changes were dose dependent as characterized by cellular shrinkage (Fig. 1B). Moreover, the cell viability data showed that 25, 50, 100, 200, and 300 μM doses of piperine result in significant reduction in cell viability of approximately 90.14%, 76.59%, 52.39%, 25.26%, and 18.96% ($P < 0.001$), respectively as compared to control

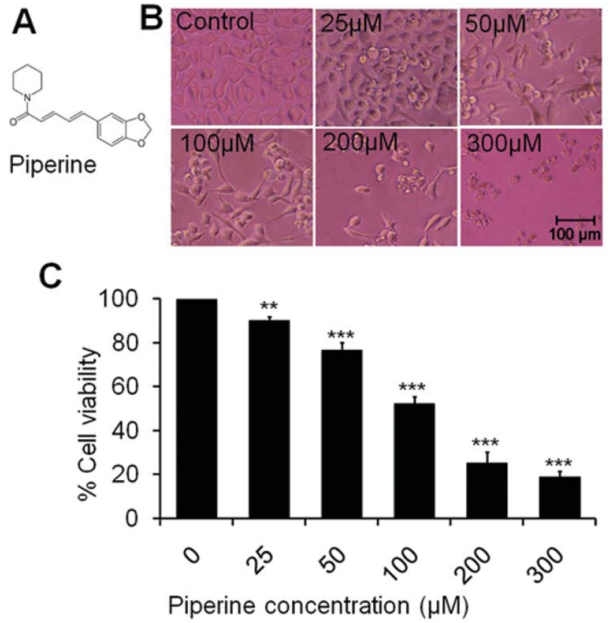

Figure 1. MTT assay of piperine against KB cells. (A) Chemical structure of piperine. (B) Morphological view of live and dead cells of KB cell line treated with 25–300 μM concentrations of piperine. Photomicrographs were taken by inverted phase contrast microscope. (C) The percent cell viability of KB cells measured by a MTT assay at 24 h as described in the experimental section. Values are expressed as means ± SEM of at least three independent experiments, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with their respective control.

(Fig. 1C). The IC_{50} value of piperine was evaluated to be 124 μM in KB cells. These results suggested that piperine significantly reduces the cell viability of KB cancer cell line in a dose-dependent manner.

Piperine Induces Intracellular ROS Generation

Excessive ROS production can lead to oxidation of macromolecules resulting in cellular damage of KB cells. Treatment of KB cells with piperine for 12 h resulted in a dose-dependent enhancement of ROS generation by increasing the intensity of DCF fluorescence as depicted in Fig. 2A. As evident from Fig. 2B, piperine-treated KB cells showed remarkable production of ROS level by 15.17%, 27.37%, and 62.34% ($P < 0.001$) at 50, 100, and 200 μM concentrations, respectively as compared to control. This result provides supportive data for the molecular mechanism of piperine, which is presumably related to induction of early apoptosis caused by oxidative stress.

Piperine Induces Nuclear Condensation

As depicted from photomicrograph of KB cells (Fig. 3A), piperine demonstrated apoptotic cell death in KB cells in a dose-dependent manner as evidenced by the increased permeability of cells to DAPI showing nuclear apoptotic bodies and condensed chromatin. The quantitative measurement of apoptosis showed that 50, 100, and 200 μM of piperine increased the percent apoptotic cells by approximately 14.66%, 27.0%, and 44.3% (Fig. 3B). Fragmented and condensed nuclei in KB cells suggest that piperine caused cell death by an apoptotic process.

Piperine Depolarizes the Mitochondrial Membrane Potential ($\Delta\Psi m$)

The loss of $\Delta\Psi$m of the cells stained with JC-1 dye is indicated by decrease in red fluorescence and increase in green fluorescence. As shown in Fig. 4A, treatment of KB cells with piperine shows a dose-dependent increment in the number of green-fluorescence^{+} cells from 9.33% in untreated cells to 23.33%, 40.33%, and 77.33% at 50, 100, and 200 μM concentrations, respectively

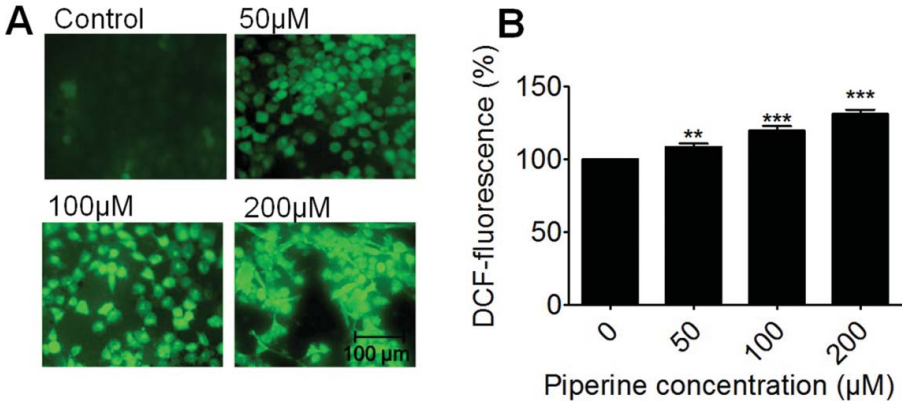

Figure 2. ROS generation assay of piperine against KB cells. (A) Photomicrographs showing intracellular ROS generation induced by 50, 100, and 200 μM of piperine in KB cell line. Photomicrographs were taken by fluorescence phase contrast microscope. (B) Quantitative data were expressed as the percentage of fluorescence intensity relative to the control in KB cells. Values are expressed as means ± SEM of at least three independent experiments, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with their respective control.

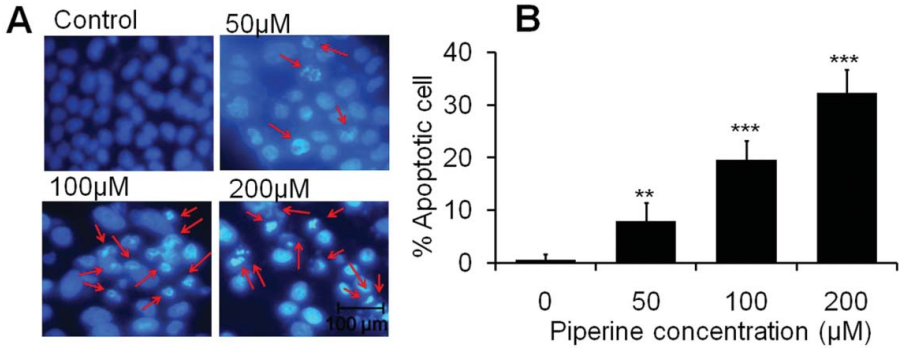

Figure 3. Chromatin condensation of KB cells stained with DAPI after piperine treatment. Cells were treated with 50, 100, and 200 μM of piperine. (A) Photomicrographs showing fragmented and condensed nuclei as indicated by arrow of KB cells. Photographs were taken by fluorescence phase contrast microscope. (B) Numerical data expressed as percent apoptotic cells respective to their control of KB cells. Data are representative of three independent experiments and expressed as means ± SEM, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with their respective control.

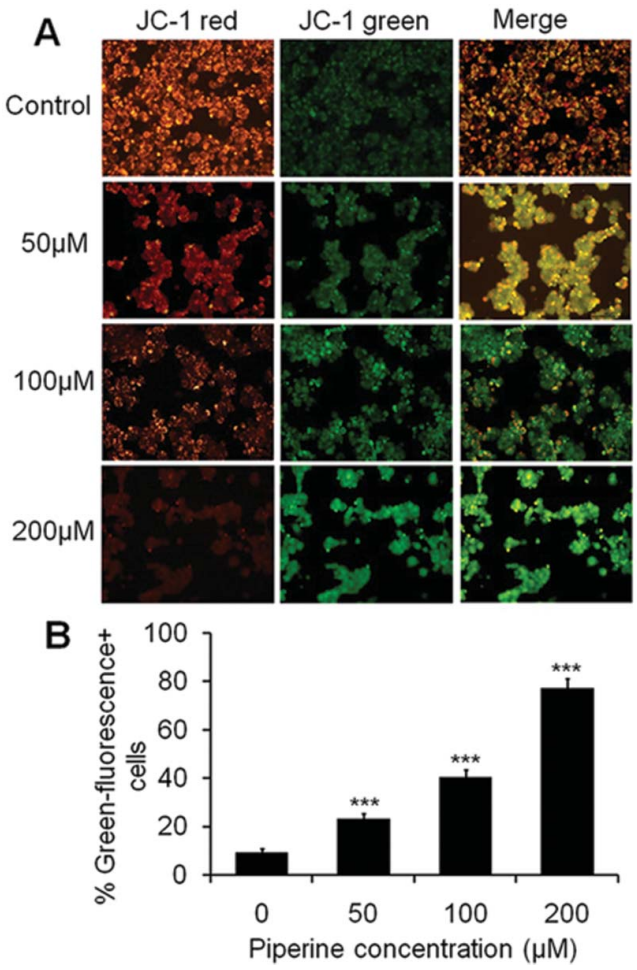

Figure 4. Fluorescence image of KB cells stained with JC-1 with different concentrations of piperine. (A) Photograph showing JC-1 red, JC-1 green, and merge image of KB cells. (B) The percentage of the green-fluorescence^{+} cells that emit only green fluorescence indicates a decrease in mitochondrial membrane potential ($\Delta\Psi$), an early event in apoptosis. The JC-1 green fluorescence increased concentrations of piperine attenuated the loss of ($\Delta\Psi$). Data are representative of three independent experiments expressed as means ± SEM, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with their respective control.

(Fig. 4B). The results indicate that all the sub-fractions of piperine possess promising apoptotic potential.

Piperine Modulates Cellular DNA Content and Induces G2/M Phase Arrest

Cell cycle phase analysis of KB cells treated at 100 and 200 μM of piperine was performed by flow cytometry. Apoptotic cells were estimated by calculating the number of sub-diploid cells in the cell cycle histogram. As depicted in Fig. 5, KB cells exposed to 100 μM of piperine, apoptotic cells were reported to 15.92% while in untreated cells, it was found to 5.41%. However, at 200 μM concentration of piperine, apoptotic cells were markedly increased to 37.54% in KB cells. Furthermore, untreated cells displayed only 7.92% of cells in the G2/M phase, interestingly; piperine increased the cell cycle checkpoints by 15.75% and 37.79% at 100 and 200 μM concentrations, respectively. On the other hand, the increase in the number of cells of G2/M phase was accompanied by a decrease in the cell populations in the S phase of cell cycle. These results indicated that piperine inhibited cell growth by inducing cell cycle checkpoints in G2/M phase and by reducing DNA contents in S phase of cell cycle.

Piperine Induces Caspase-3 Activity

The activity of caspase-3 (cysteine-aspartic acid protease), an important biochemical feature in apoptotic signaling, was further investigated to determine whether the apoptosis was induced by caspase-3 liberation. Fig. 6A shows the distribution of cells detected by the antibodies to active caspase-3 in KB cells. The result from colorimetric assay showed that caspase-3 activity was dramatically increased to 26.92%, 72.13%, and 103.38% ($P < 0.001$) at concentrations 50, 100, and 200 μM of piperine in a dose-dependent manner

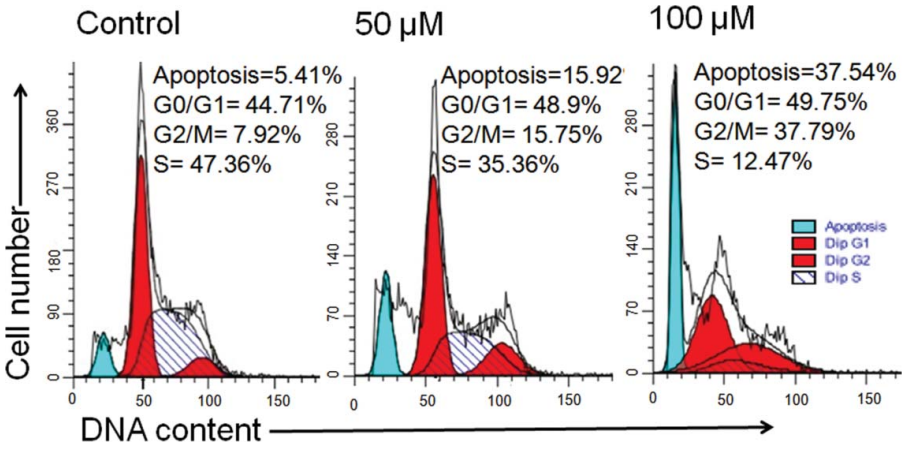

Figure 5. Effect of piperine on different phases of cell cycle. Representative photomicrographs showing the apoptosis and phase distribution of cell population in KB cells. Cells were treated with 50 and 100 μM of piperine for 24 h, stained with propidium iodide, and measured by flow cytometry.

(Fig. 6B). Qualitative data of caspase-3 in KB cells were consistent with the quantitative data.

Discussion

Piperine is a potent anticancer compound proven in various cell types by several studies. Recently, increasing evidence has indicated that piperine suppressed the growth of numerous cancer cells (12,24). However, the antiproliferative effect of piperine and its underlying mechanism on KB cells are not well studied. In this study, results indicated that piperine possessed significant cytotoxic activity against KB cells. The activity of piperine was dose dependent and its nature was found apoptotic rather than necrosis as suggested by nuclear condensation, cell cycle checkpoints, and caspase-3 activation. Based on morphological characteristics, typical morphological features

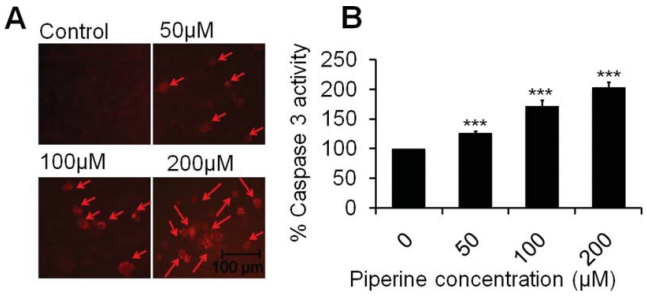

Figure 6. Activation of caspases-3 by piperine against KB cells. Cells were incubated with piperine at 50, 100, and 200 μM for 24 h. (A) Immunofluorescence stain of KB cells and photographs were taken by inverted fluorescence phase contrast microscope. (B) Quantitative data of caspases-3 level in KB cells measured by caspases-3 colorimetric assay kit. Values are expressed as means $\pm$ SEM of at least three independent experiments, $^{***}P < 0.001$ as compared with their respective control.

of apoptosis and the reduction of the cancer cells were observed under inverted phase-contrast microscopy (Fig. 1B). Previous studies have reported the cytotoxic activity of piperine at different concentrations on various cancer cell lines. In a study, piperine inhibited cell growth of both androgen-dependent and androgen-independent prostate cancer cells with IC_{50} values ranging from 60 to 160 μM (25). Further, piperine isolated from *Piper nigrum* also inhibited human cervical carcinoma HeLa cells at the IC_{50} dose of 61.94 μg/ml (26). However, in the respect of μM concentration, this dose was found to be about 2 times greater than the IC_{50} dose of piperine evaluated in our present study. The variation in the cytotoxic doses might be varied owing to the origin and type of cells. Moreover, piperine also reduced the tumor growth and metastasis of mouse 4T1 mammary carcinoma model and significantly inhibited lung metastasis (12).

Various ROS including superoxide, hydrogen peroxide, and hydroxyl radicals are involved in oxidative stresses which regulate both extrinsic and intrinsic apoptosis pathways for cell survival and cell death (27). Oxidative stress is linked with damage to a variety of micro and macromolecular species *viz.* nucleic acids, proteins, and lipids, hence inducing peroxidation of lipids, formation of protein carbonyls, and accretion of DNA damage (28,29). An earlier study suggested that piperine impairs rectal cancer cells and induces apoptosis *via* ROS -dependent pathway (24). Interestingly, our data showed that piperine significantly induced ROS generation in KB cells which trigger ultimately cellular apoptosis and cell death (Fig. 2). Other researchers documented the chemopreventive efficacy of the piperine is probably due to their antilipid peroxidative and antioxidant potential as

well as its modulating effect on the carcinogen detoxification procedure (30,31). An earlier study suggested that the mechanism of ROS generation is varied both in normal and cancerous cells and hence a working system of antioxidants might be different in both types of cells (32). The increase in basal ROS generation renders a biochemical basis to selectively kill cancer cells which disable glutathione antioxidant system and inhibit the important redox-modulating enzymes such as glutathione peroxidase. Inhibition of the antioxidant system in cancer cells could cause a massive accumulation of ROS due to its high basal output in these cells and trigger cell death (32).

The study reported that induction of apoptosis and cell cycle arrest are the major process of aging, which could be important targets for cancer chemotherapy (33). Piperine has also been studied to induce apoptosis and cell cycle arrest in different cancer cells (12,24). However, none of the studies have investigated the effect of piperine on cell cycle distribution and apoptosis induction in KB cells. Our findings indicated that the inhibitory effect of piperine on the growth of KB cells might be contributed by the induction of cell cycle arrest at G2/M phase (Fig. 6). Cell cycle results revealed that exposure of KB cells to piperine resulted in a significant increase in the G2/M phase accompanied by an increase in percent distribution of cells in the apoptotic (sub G0) phase depending upon dose. A similar result has also been reported in a previous study that piperine induced apoptosis by arresting the cell cycle at G2/M phase in lung epithelial A549 carcinoma cells (34). A recent study has reported that pipernonaline, a piperine derived from *P. Longum* Linn., induced G0/G1 phase arrest in PCA cells (35). Interestingly, one study suggested that piperine inducing upregulation of p21Cip1 protein and downregulation of the cyclins might be also responsible for the induction of cell cycle arrest in PC3 (36). Another report showed that piperine causes G1 phase cell cycle arrest and apoptosis in melanoma cells through checkpoint kinase-1 activation (37). Study has shown that cyclin-dependent kinases and p53 gene are valuable factors regulating the cell cycle checkpoints which initiate the programmed cell death (38). Hence, we might postulate that these factors would be accountable for cell cycle checkpoints in KB cells and lead to cellular apoptosis.

Researchers have investigated that disruption of mitochondrial membrane integrity leads to depolarization of the MMP, which is crucial for cell death and apoptosis. (39,40). The evaluation of the effects of piperine on the MMP in KB cells demonstrated the increase of % green fluorescence-positive cells in a dose-dependent manner (Fig. 4). The increase in green fluorescence confirmed the death of cancer cells through the depolarization of their MMP. Caspase-3 is the key downstream effector caspase in apoptotic pathway and plays an important role in the execution of apoptotic cell death by cleaving the cellular substrates (41). Hence, we compared its activity in treated and untreated control cells. Caspase-3 colorimetric assay confirms the efficacy of piperine promoting caspase-3 level (Fig. 6B) that was also supported by immunofluorescence stain (Fig. 6A). Apoptotic pathway can be divided into caspase-dependent and -independent signal pathways (42). However, our study demonstrated that piperine promoted apoptosis *via* the caspase-dependent pathway. In a study, the anticancer activity of piperine *in vitro* was reported possibly due to increase in caspase-3 activity and downregulating expression of cyclin B1 (12). Similarly, other research has shown that piperine induced apoptosis of lung cancer A549 cells *via* activation of caspase-3 and caspase-9 cascades (34). According to some authors, the antitumor activity of piperine may be related to its immunomodulatory properties, which involve the activation of cellular and humoral immune responses (17, 43).

Conclusion

In conclusion, this study demonstrated that piperine can inhibit tumor growth by inducing cell apoptosis, depolarization of MMP, cell cycle blockage, and caspase-3 activation. Moreover, piperine suppresses KB tumor growth by inducing nuclear condensation and cell shrinkage *in vitro*. Hence, our present study suggested that the piperine could be an attractive and promising candidate to develop anticancer drugs and oral cancer therapy.

Declaration of interests

The authors declare that they have no potential conflicts of interest.

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