# ORIGINAL ARTICLE

# Antiproliferative potential of piperine and curcumin in drugresistant human leukemia cancer cells are mediated via autophagy and apoptosis induction, S-phase cell cycle arrest and inhibition of cell invasion and migration

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# Summary

**Purpose:** Leukemia causes annually a significant number of deaths. The main objective of this study was to investigate the anticancer properties of piperine and curcumin against HL60 leukemia cells and to elucidate the underlying mechanism.

**Methods:** Antiproliferative effects were assessed by WST-1 cell viability assay. Cell apoptotic effects were studied by DAPI staining assay. Annexin V/propidium iodide (PI) assay was used to assess apoptosis. Electron microscopy was used for detection of autophagy and flow cytometry for cell cycle analysis, while transwell migration assay was used to study the effects on cell migration and invasion. Protein expression was estimated by western blot.

**Results:** The results showed that both piperine and curcumin inhibited significantly the growth of the HL60 cells and exhibited an IC<sub>50</sub> of 25 and 30  $\mu$ M respectively. Further, it was observed that the anticancer effects of piperine and curcumin were due to the induction of mitochondrial-mediated apoptosis which was also associated with enhancement of the Bax expression. Transmission electron microscopy also showed that both curcumin and piperine induced autophagy in the HL-60 leukemia cells. Flow cytometry showed that piperine and curcumin also caused arrest of the HL-60 cells at the Sphase of the cell cycle. Finally wound healing and transwell assays showed that piperine and curcumin suppressed the migration and invasive potential of the HL60 cells.

**Conclusions:** The present study reveals that piperine and curcumin exhibit significant antitumor activity in human leukemia HL60 cells via multiple mechanisms and may prove promising in the development of systemic therapy for leukemia.

*Key words: curcumin, piperine, acute myeloid leukemia, apoptosis, cell migration, cell invasion* 

# Introduction

Leukemia causes high mortality across the globe and is considered one of the most fatal diseases [1]. In USA alone, more than 0.3 million people are diagnosed with leukemia out of which 0.2 million die [2]. Leukemia constitutes around 3% of all malignancies but it is considered one of the most lethal diseases because of low patient survival rates [3]. The treatment of leukemia is obstructed

by its diagnosis at advanced stages and the adverse effects of chemotherapy [4]. Hence, attempts are being made to develop new and effective drugs with comparatively less or no side effects.

During the course of evolution, plants have developed the potential to synthesize a battery of molecules that have been used for the management of lethal human diseases [5]. Many of these

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chemical compounds have been shown to exhibit anticancer effects [6]. Herein, we examined the anticancer effects of two naturally occurring chemical compounds: piperine and curcumin. Curcumin is a polyphenolic pigment generally obtained from Curcuma longa while piperine is an alkaloid generally isolated from *Piper nigrum* [7,8]. Both of these naturally occurring compounds have been shown to exhibit enormous anticancer potential and have been utilised in the traditional healthcare systems in the management of abnormal human conditions [9,10]. They have also been shown to exhibit potent bioactivities which also include their anticancer properties. Curcumin has been shown to suppress the growth of pancreatic cancer [11]. It has also been shown to cause inhibition of proliferation of prostate cancer cells via induction of apoptosis [12]. Similarly, piperine has been shown to inhibit the growth of colon cancer cells via G1 arrest and apoptosis [13]. Moreover, it has been shown to trigger apoptosis in lung cancer cells [14].

This study was designed to investigate the anticancer effects of these anticancer molecules against leukemia cells.

# Methods

### WST-1 cell proliferation assay

The viability of the human leukemia cells and normal astrocytes was monitored by WST-1 assay. In brief, HL-60 leukemia cells were cultured in 96-well plates at the density of  $2 \times 10^5$  cells /well and treated with 0 to 200  $\mu$ M concentrations of piperine for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C

for another 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation.

#### Analysis of cell death

The HL-60 leukemia cells  $(0.6 \times 10^6)$  were cultured in 6-well plates and treated with piperine and curcumin at 0, 9, 18 and 36 µM concentrations for 24 h at 37°C. Subsequently, 25 µl of cell culture were put onto glass slides and stained with DAPI. The slides were coverslipped and examined under fluorescence microscope. ApoScan kit was used to determine the apoptotic HL-60 cell percentage. In brief, piperine and curcumin-treated HL-60 cells (5×10<sup>5</sup> cells/well) were incubated for 24 h. This was followed by the staining of these cells with annexin V-FITC/propidium iodide (PI). The percentage of apoptotic HL-60 cells at each concentration was then determined by flow cytometry.

#### Detection of autophagy

Autophagy in curcumin and piperine-treated leukemia cells was evaluated by electron microscopy. In brief, the HL-60 leukemia cells were treated with 0, 9, 18 and 36  $\mu$ M curcumin and piperine for 24 h. The cells were collected by trypsinization and washed with phosphate buffered saline (PBS) which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). Then, the cells were post-fixed in osmium tetroxide (1%). This was followed by the treatment of the cells with ethanol and embedding in resin. Thin sections were then cut with an ultramicrotome and subjected to electron microscopy.

#### Cell cycle analysis

The cultured human leukemia HL-60 cells were firstly treated with varying concentrations of piperine and curcumin for 24 h at 37°C. The cells were then



**Figure 1.** Chemical structure of **(A)** Curcumin **(B)** Piperine and **(C)** Effect of curcumin and piperine on the viability of the HL-60 leukemia cells. The experiments were repeated thrice and shown as mean ± SD (\*p<0.05).

washed with phosphate buffered saline (PBS). Afterwards, the HL-60 cells were stained with PI and the cell distribution in cell cycle phases was assessed by FACS flow cytometer.

#### Wound healing assay

The piperine and curcumin-treated cells were cultured till 80% confluence. This was followed by removal of Dulbecco's modified Eagle's medium (DMEM) and subsequent washing with PBS. Afterwards, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 24 h at 37°C and then a picture was taken again under an inverted microscope.

#### Cell invasion assay

The effects of piperine and curcumin on the invasion ability of HL-60 cells was determined by transwell assay with Matrigel. Around 200 ml cell cultures were placed onto the upper chambers and only DMEM was placed in the bottom wells. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200X magnification.

#### Western blot analysis

The HL-60 cells were then lysed in lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were subjected to separation (10%) SDS-PAGE which was followed by transferring it to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4 °C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

### Statistics

The experiments were performed in triplicate. The values presented are the mean of three repeats ± SD. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 were considered statistically significant. Student's t-test using GraphPad prism 7 software was employed for statistical analyses.

# Results

*Curcumin and piperine inhibit the growth of leukemia cells* 

The proliferation rate of the HL-60 cells following treatment with various concentrations of curcumin (Figure 1A) and piperine (Figure 1B) were determined by WST-1 assay. The results revealed that curcumin and piperine caused a significant decrease in the proliferation rate of the HL-60 cells. The effects of curcumin and piperine on the proliferation rate of the HL-60 cells were concentration-dependent and IC<sub>50</sub> of 30  $\mu$ M and 25  $\mu$ M was reported for curcumin and piperine against the HL-60 cells (Figure 1C). These results suggest that piperine exerts more significant anticancer effects on the HL-60 cells than curcumin.



**Figure 2.** Effects of **(A)** piperine and **(B)** curcumin on the nuclear morphology of the HL-60 cells as depicted by DAPI staining. The results of this Figure show that both piperine and curcumin induce apoptosis in HL-60 cells (arrows). The experiments were repeated thrice.



**Figure 3.** Effects of **(A)** piperine and **(B)** curcumin on the percentage autophagy in HL-60 cells as depicted by annexin V/PI staining. The Figure shows that piperine and curcumin induce apoptosis in HL-60 cells at 30  $\mu$ M concentration. The experiments were performed in triplicate.

Curcumin and piperine induce apoptosis and autophagy in HL-60 leukemia cells

HL-60 cells were treated with  $IC_{50}$  concentrations of curcumin and piperine and then stained with DAPI to ascertain if curcumin and piperine cause apoptosis in the HL-60 cells. The results of DAPI assay showed that both curcumin and piperine caused nuclear fragmentation of the HL-60 cells which is the hallmark of apoptosis (Figure 2). Annexin V/PI staining was also carried out and the apoptotic HL-60 cell percentage was determined at IC<sub>50</sub> concentrations of curcumin and piperine. The apoptotic cell percentage was found to be 21.2% in curcumin-treated cells as compared to 1.3% in untreated cells. Similarly, the apoptosis percentage was found to be 37.1% in piperine-treated cells as compared to 3.7% in control cells (Figure 3). Western blot analysis showed that curcumin and piperine caused considerable increase in the expression of Bax and caspase-3 and depletion of Bcl-2 expression at IC<sub>50</sub> concentration (Figure 4). Next, electron microscopic analysis showed that both of these molecules caused development of au-



**Figure 4.** Effects of piperine and curcumin on the expression of Bax and Bcl-2 in the HL-60 cells as depicted by western blot analysis. The Figure shows that both molecules increase Bax and decrease Bcl-2 expression in HL-60 cells. The experiments were repeated thrice.



**Figure 5.** Electron microscopic analysis showing **(A)** piperine and **(B)** curcumin on the induction of autophagy in HL-60 cells (arrows depict autophagosomes). The experiments were repeated thrice.

tophagosomes in the HL-60 cells at  $IC_{50}$ , indicative of autophagy (Figure 5A and 5B).

# *Curcumin and piperine cause S-phase arrest of leukemia cells*

The HL-60 leukemia cells were treated with various concentrations of curcumin and piperine and the distribution of HL-60 cells at each phase of the cell cycle was determined by flow cytometry. The results showed that the S-phase cells increased considerably upon curcumin and piperine treatment. However, it was found that the effects of piperine were more profound than curcumin (Figure 6A and 6B).

### Curcumin and piperine inhibit the migration and invasion of the leukemia cells

The effects of curcumin and piperine were also investigated on the invasion and migration of the HL-60 leukemia cells by transwell and wound healing assay. The results showed that curcumin and piperine caused remarkable decrease in the migration of the HL-60 leukemia cells in a concentration-



**Figure 6.** Effect of **(A)** piperine and **(B)** curcumin on the cell cycle distribution of the HL-60 cells as depicted by flow cytometry. The Figures show that both piperine and curcumin cause S-phase arrest of HL-60 cells. The experiments were repeated thrice and shown as mean±SD (\*p<0.05).



**Figure 7.** Effect of **(A)** piperine and **(B)** curcumin on the cell migration as depicted by wound healing assay. The Figure shows that both molecules inhibit the migration of HL-60 cells. The experiments were repeated thrice.

dependent manner as evidenced from the wound width (Figure 7A and 7B). Moreover, transwell assay showed that the invasion of HL-60 cells was also decreased in a concentration-dependent manner by both curcumin and piperine (Figure 8A and 8B).

### Discussion

Leukemia is one of the most fatal malignant diseases and responsible for tremendous mortality. Because of the late diagnosis and the severe adverse effects of chemotherapy, the treatment of the leukemia patients becomes very complicated [15]. Herein, were we examined the anticancer effects of two potent anticancer molecules curcumin and piperine. Both of these two molecules significantly suppressed the growth of leukemia cells, indicative of their potential to be used for the development of leukemia systemic therapy. One of the benefits of using these two molecules in anticancer drug development is their lower toxicity. Because both of these compounds (curcumin and piperine) are edible in the form of spices, they are considered to



Control

30 µM



**Figure 8.** Effect of **(A)** piperine and **(B)** curcumin on the cell invasion of HL-60 cells as depicted by transwell assay. The Figure shows that both piperine and curcumin inhibit the invasion of HL-60 cells. The experiments were repeated thrice.

be safer. The investigation of the underlying mechanisms revealed that both curcumin and piperine induced autophagy and apoptosis in the leukemia cells. These are considered to be important mechanisms to eliminate malignant cells from the body [16,17]. These apoptotic and autophagic-inducing properties have also been reported previously and support our findings. For example, curcumin has been reported to promote apoptosis of breast cancer cells [18] and also to induce autophagy in such cells. Piperine on the other hand has been shown to induce apoptosis in melanoma cells [19]. It has also been reported to trigger autophagic cell death in prostate cancer cells [20]. Both curcumin and piperine have also been shown to cause arrest of cancer cells by inducing cell cycle arrest. For example piperine induces cell cycle arrest in cancer cells [19,21]. Therefore, we also examined the effects of curcumin and piperine on the cell cycle distribution of the HL-60 cells and found that both of these naturally occurring molecules caused increase in the S-phase cells, indicative of S-phase cell cycle arrest. The effects of curcumin and piperine were also investigated on the migration and invasion of the leukemia HL-60 cells by wound healing and transwell assay and it was found that  $IC_{50}$  of both molecules suppressed the migration and invasion of the HL-60 leukemia cells, suggestive of their

# Conclusion

The findings of the present study indicate that the naturally occurring molecules curcumin and piperine suppressed the growth of leukemia cells via induction of autophagy, apoptosis and cell cycle arrest. These molecules also inhibited the migration and invasion of the leukemia cells, indicative of their anticancer potential.

# **Conflict of interests**

The authors declare no conflict of interests.

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