

Original Research Article

Enhancement of Bleomycin Sensitivity in Human Lung Cancer Cell Line using *Centella asiatica* Leaf Extract

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Abstract

Purpose: To demonstrate the effectiveness of *Centella asiatica* aqueous extract in augmenting the cytotoxic effect of bleomycin in the adenocarcinoma human alveolar basal epithelial A549 cell line.

Methods: The inhibitory effect of bleomycin on A549 cells was determined by incubating the cells for 24 h in different concentrations of bleomycin. The effect of *C. asiatica* extract on A549 cells was determined after exposing cells to 5, 10, 15, 20, 25, and 30 % (v/v) bleomycin diluted from an initial 10% stock solution. Based on the 50 % inhibitory (IC_{50}) values of bleomycin, 80, 100, and 120 μ g/mL concentrations were tested with 15, 20, and 25 % (v/v) of *C. asiatica* extract.

Results: IC_{50} value of bleomycin on A549 cells was 100 μ g/mL, and was reduced to 80 μ g/mL when cells were co-incubated with 20 % v/v of *C. asiatica* extract. The results show that *C. asiatica* extract reduces the concentration of bleomycin necessary for inhibition of A549 cell growth (IC_{50}). While the IC_{50} value of bleomycin was 100 μ g/mL, it fell to 80 μ g/mL when used along with 20 % v/v CA extract ($p < 5$)

Conclusions: This study demonstrates that compounds extracted from *C. asiatica* may have potential for use as adjuvants in bleomycin cancer chemotherapy.

Keywords: *Centella asiatica*, A549 cells, Bleomycin, Lung cancer, Increased susceptibility, Cell viability

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INTRODUCTION

Phytochemicals are plant products used in traditional medicine. Several currently used therapeutic drugs are based on phytochemicals. Although plant products have been used for several centuries, the exact mechanisms by which these compounds function remain unknown, and further evidence-based dose effects of the phytochemicals are required.

Centella asiatica is a herb of Asian origin belonging to the Apiaceae family and the Mackinlayoideae subfamily, also commonly

known as the “gotu kola” or “centella.” Grown in wet areas; the leaves of this herb are commonly used in a variety of ways, ranging from preparing juices to porridges and salads. The medicinal uses of *C. asiatica* are well documented in a review by Kashmira *et al* [1]. Plant extracts of *C. asiatica* have been widely used to treat skin diseases, varicose veins, to reduce hypertension, to improve memory, and for wound healing. Its major use is as a brain tonic for improving nervous function [1].

The extracts from the leaves and tender stems of *C. asiatica* confer beneficial effects from

compounds including triterpenoids, saponins, sapogenins, brahmoside, brahminoside, isothankuniside, thankuniside, sterols, flavanoids, indocentelloside, asiaticoside, and tannins. The two most active ingredients are bacoside A and bacoside B. Apart from their wound healing properties the triterpenoid compounds from *C. asiatica* exert beneficial effects primarily on the circulatory and the nervous systems. A comprehensive study of the herb, its natural compounds, the beneficial health effects, and pharmacological aspects of *C. asiatica* are available online from Examine.com, a portal that provides detailed evidence-based, scientific information on food supplements [2].

Cancers are a major global human health care concern with the types and incidence levels increasing at a significant rate. Cancers are complex diseases making them a challenge to understand, manage, and treat. This makes it very important to treat cancer using combinations of approaches. Targeted therapy, gene therapy, and rendering cancer cells more susceptible to therapeutic drugs are currently in use. While the discovery of new drugs is critical, enhancing the effects of currently available chemotherapeutic drugs can also be useful. Modulators that enhance the effectiveness of radiotherapy and chemotherapy can be used as adjuvants and offer several advantages [3].

In this regard, the use of pulsed electromagnetic field treatments includes studies with radiotherapy [4] as well as with chemotherapeutic drugs [5]. Other well-studied modulators for cancer therapy include antibodies used as immunostimulatory agents [6], metal compounds such as arsenic trioxide [7], statins such as simvastatin [8], isoflavones from soy [9], prostaglandin inhibitors [10], and nanoparticles [11].

Established and well-characterized cancer cell lines are widely used for cancer research. There are many cancer cell lines currently available for research purposes. These continuous cell lines offer unique advantages as model systems. A549 is an adenocarcinoma human alveolar basal epithelial cell line. Bleomycin is a widely used cancer chemotherapeutic drug and several *in vitro* studies have shown the usefulness of bleomycin (a radiomimetic drug) on cancer cell lines used as models for cancer research. The effects of bleomycin on lung epithelial cells are well-known [12–14]. Bleomycin was also used to study the modulatory effects of caveolin-1 on apoptosis and senescence in lung cells, making

this model a reliable choice to determine the modulatory or synergistic effects of supplements [15].

We determined the possible augmentative effects of *C. asiatica* leaf extract on the anticancer cytotoxic effects of bleomycin in A549 cells. The role of *C. asiatica* in radioprotection of A549 cells has been previously studied. An active ingredient of *C. asiatica*, asiatic acid, was shown to have inhibitory effects on A549 cell growth [16]. The documented applications of *C. asiatica* in radioprotection or during radiotherapy also include advantageous psychological effects such as positive behavioural changes [17]. *C. asiatica* extracts were also shown to be advantageous (at 100 mg/kg body weight) in increasing the survival and minimizing body weight loss in Swiss albino mice exposed to Cobalt-60 (60Co) gamma radiation at a sublethal dose of 8 Gy [18].

In this study, we assessed the potential use of *C. asiatica* for cancer therapy as a supplement or as an adjunctive agent. Treatments which increase the sensitivity of cancer cells to a therapeutic drug can result in a decreasing dose, thereby avoiding some of the side effects of such drugs on normal cells and tissues. Further studies should determine the usefulness of commonly available phytochemicals as augmentative cancer therapeutics.

EXPERIMENTAL

C. asiatica were collected from a natural habitat with no apparent pollution and the stems along with the leaves were harvested, cleaned three times with sterile distilled water, and then with phosphate-buffered saline (PBS). The leaves and tender stems were dried in an oven for 84 h at 45 °C and were then milled to obtain a powder. The aqueous extract was obtained from this powder by adding 100 mL of distilled water to every 10 g of the plant powder to obtain a 10 % w/v concentration of the final extract. The mixture was incubated at 4 °C for 1 h, refluxed, vacuum filtered, and then sterilized using 0.4 micron filters followed by a 0.2 micron filter. Ten grams of the plant powder was extracted using 100 mL of distilled water to obtain a final concentration of 10 % v/v. Aliquots of the extract (CA extract) were stored at 4 °C and various concentrations were obtained using the 10 % stock solution.

The A549 cells obtained from American Type Culture Collection (ATCC), USA were maintained in Dulbecco's Minimum Essential Medium

(DMEM) obtained from Gibco BRL, USA supplemented with 10 % fetal bovine serum in T-24 flasks, and were passaged when they reached 90 % confluency. The A549 cells were cultured in 12-well plates to determine the IC_{50} values of bleomycin and the effects of CA extracts. Triplicate cultures in 12-well plates were used for each concentration of bleomycin, CA extract, and the combinations of bleomycin and CA extract. The seeding density for all experiments was 0.16×10^6 cells per well and cells were > 97 % viable, and at 50 % confluency.

The doses used to determine the IC_{50} value of bleomycin-induced cytotoxicity on A549 cells were 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 $\mu\text{g/mL}$. Cells were incubated for 24 h, the monolayers were washed three times with sterile PBS, trypsinized, and cell viability was calculated using trypan blue dye exclusion.

The doses of the CA extract used to study its effects on the A549 cells were 5, 10, 15, 20, 25, and 30 % (v/v) from the stock 10 % solution. Cells were incubated for 24 h with the CA extract and the viability was calculated. Based on the IC_{50} values of bleomycin and the effects of CA extracts on the A549 cells, bleomycin was used at 80, 100, and 120 $\mu\text{g/mL}$, and each of these concentrations were tested with 15, 20, and 25 % (v/v) of CA extract, in triplicate to ascertain the modulatory effects of CA extract on bleomycin inhibitory activity. Cells were incubated in the bleomycin-CA extract combinations for 24 h and viability calculated as described.

Statistical analyses

The percent cytotoxicity at various levels of bleomycin was determined, and the IC_{50} was determined as the concentration of the drug which induced killing of 50 % of the cell population. Samples were in triplicate and results are expressed as mean \pm SD. Statistical analysis included the percent cytotoxicity and the maximum percent toxicity. The association of the cytotoxic drug individually and in combination with the phytochemical extract was shown by graphs using Excel software.

RESULTS

In total, 100 mL of the CA aqueous extract was obtained and was stored as aliquots at 4 °C. There was a dose-dependent increase on the cytotoxic effect of bleomycin on A549 cells with

an IC_{50} value of approximately 100 $\mu\text{g/mL}$ (Figure 1).

Of the six concentrations of the CA leaf extract used, the 5, 10, 15, and 20 % (v/v) concentrations did not have any effects on the A549 cells. However, the cells exposed to 25 % and 30 % (v/v) CA extract showed cell granulation and debris in the culture medium (Figure 2).

The optimal concentration of CA extract was 20 % (v/v) for bleomycin IC_{50} of 80 $\mu\text{g/mL}$. Combinations of bleomycin with 15 and 25 % (v/v) CA extracts did not provide enhancement when compared to cells exposed to 100 or 120 $\mu\text{g/mL}$ bleomycin alone. In summary, supplementation with 20 % (v/v) CA extract provided significant enhancement of bleomycin sensitivity for A549 cells.

DISCUSSION

C. asiatica is well documented in the scientific literature for its beneficial human healthcare effects. Although its applications for various indications such as neurological disorders, vascular disorders, and wound healing are well documented, it was of interest to determine if it has synergistic action on commonly used therapeutic drugs. Bleomycin is a radiomimetic cancer chemotherapeutic drug widely used with cell cultures *in vitro* to study therapeutics.

A549 cells exposed to bleomycin for 24 h exhibited an IC_{50} of 100 $\mu\text{g/mL}$. Exposure to CA extracts did not have apparent effects, although the cells had aberrant morphology when supplemented with 25 % and 30 % (v/v) CA. This might be due to the changes in the medium characteristics and composition when supplemented with the additional volumes from the given extract. Three concentrations of bleomycin were used for combination experiments based on the IC_{50} value of bleomycin on the A549 cells. In addition, three concentrations of the CA extract that did not cause aberrant cell morphology were used for the combination exposure studies. While 15 % and 25 % (v/v) of CA extract did not show modulatory effects alone, the 20 % (v/v) CA enhanced the inhibitory effect of bleomycin on the A549 cells. The IC_{50} of 100 $\mu\text{g/mL}$ bleomycin alone was reduced to 80 $\mu\text{g/mL}$ when cells were supplemented with 20 % (v/v) CA extract. In.

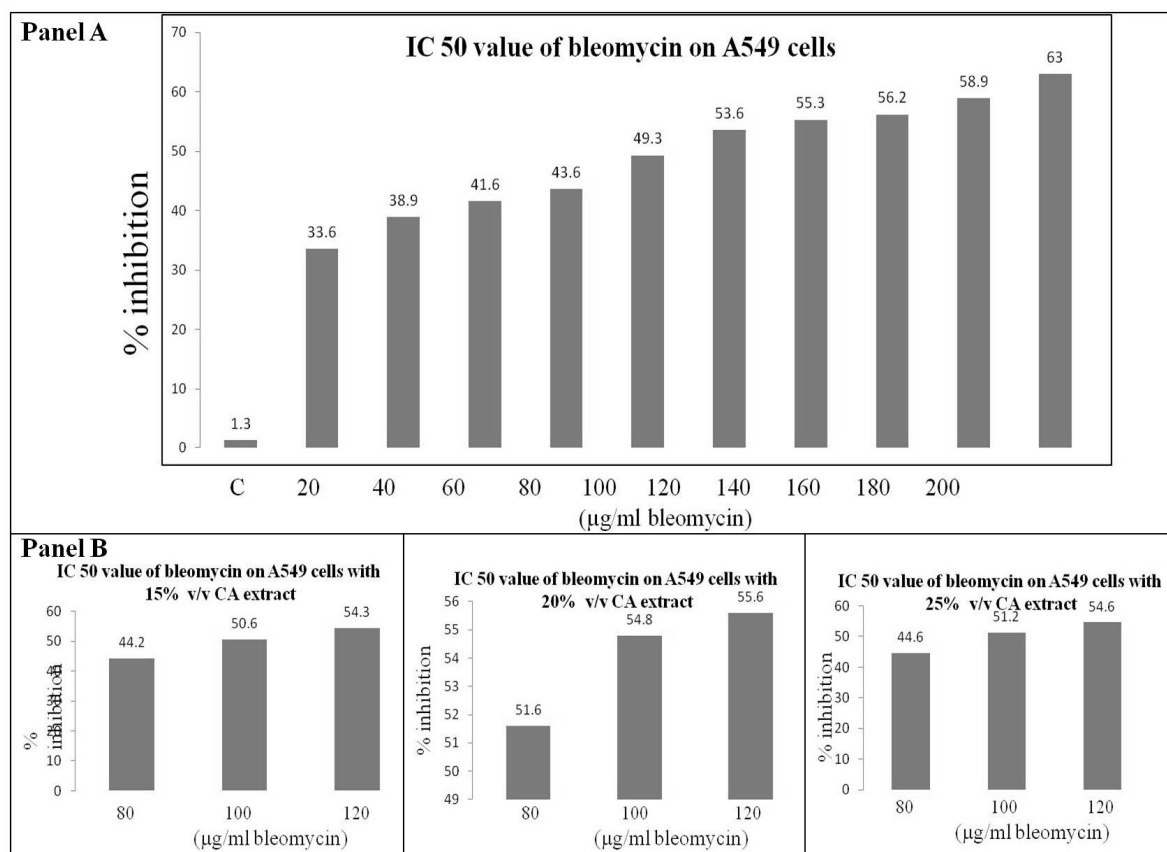


Figure 1: Panel A. Bleomycin induced dose-dependent cytotoxicity on A549 cells when given for 24 h. The IC₅₀ value of bleomycin was 100 µg/mL. **Panel B.** A549 cells incubated with 80, 100, and 120 µg/mL bleomycin combined with 15, 20, and 25 % (v/v) CA extract demonstrated that the combination of 80 µg/mL bleomycin and 20 % (v/v) CA extract gave a synergistic enhancement of the inhibitory effects of bleomycin. This combination resulted in a reduction of the IC₅₀ value of bleomycin to 80 µg/mL, compared to 100 µg/mL with bleomycin alone. However, there was no significant change in the IC₅₀ values in cultures with a combination with either 15 % or 25 % (v/v) CA extract

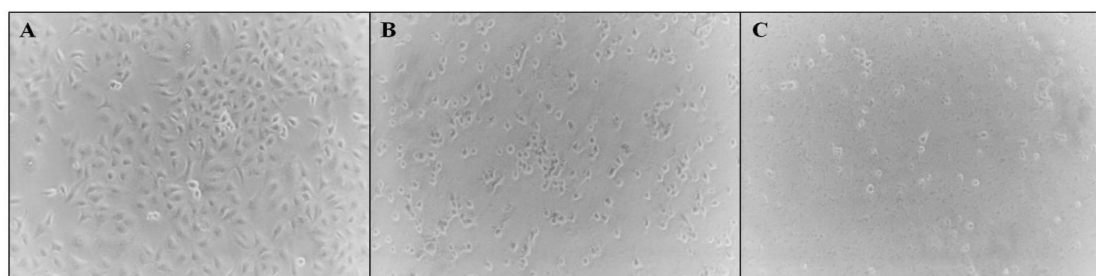


Figure 2: (A) A549 cells exposed to 5, 10, 15, and 20 % (v/v) from the 10 % initial stock solution of CA extract did not exhibit any morphological changes; however, the cultures exposed to 25 % and 30 % (v/v) CA extract from the initial 10 % stock solution of CA extract did show morphological changes **(B) and (C)**, perhaps due to increased culture volumes from the CA extracts. Cell morphology was markedly altered as seen in panel 3 with less adhered spindle-shaped healthy cells. Also, granulation and debris were seen to increase in cultures supplemented with 30 % (v/v) CA extract

summary, our study demonstrates synergistic and modulatory effects of CA extract, and suggests the importance of choosing the correct combination doses for maximizing the effectiveness of chemotherapeutic drugs

CONCLUSION

Phytochemicals have been in use in human health care for several centuries. However, additional data are required for their acceptance and wide utilization. *C. asiatica* is well

documented for its human health care applications and its mechanisms of action have been studied. The findings of this study indicate the usefulness of *C. asiatica* as an adjuvant for augmenting bleomycin in lung cancer therapy.

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