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Cytotoxic and pro-apoptotic activities of leaf extract of *Croton bonplandianus* Baill. against lung cancer cell line A549

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The acetone extract (AcE) of the *Croton bonplandianus* Baill., an exotic weed of the Euphorbiaceae family, was studied for cytotoxicity, apoptosis, cell cycle arrest in A549 cell line and antioxidant capacities using MTT assay, acridine orange/ethidium bromide (AO/EB staining), cell cycle analysis and DPPH radical scavenging assay, respectively. Based on the cytotoxic activity, the extract was tested for the apoptotic effect using AO/EB and Hoechst 33258 staining. The apoptosis was characterized by chromatin condensation and DNA fragmentation. Further, to determine the stage of cell death, cell cycle analysis was performed by flow cytometry and AcE was found to arrest G2/M phase in a dose dependent manner. The number of cells in G2/M phase increases with concurrent accumulation of cells in sub G0/G1 phase indicates the induction of apoptosis at G2M phase. The free radical scavenging activity of the AcE against DPPH was considerably significant. The cytotoxic, apoptotic and antioxidant effect of the AcE could be well correlated with the presence of potent free radical scavenging secondary metabolites such as phenols (43±0.05 µg/mL), flavonoids (3.5±0.07 µg/mL) and tannin (0.36±0.1 µg/mL). Our study has shown that A549 cells were more sensitive to AcE with an IC_{50} of 15.68±0.006 µg/mL compared to the standard drug 2.20±0.008 µg/mL (cisplatin). The results suggest that *Croton bonplandianus* could serve as a potential source of alternative therapeutic agent for treating cancer. Further research is required to isolate the active principle compound and determination of its anticancer property.

Keywords: Antioxidants, Apoptosis, Cell cycle arrest, Herbal, Secondary metabolites

Cancer remains the major human health threats worldwide. The incidences of cancer have increased to 14 million new cases and 8.2 million cancer related mortality in 2012[1]. Lung cancer has been the most common cancer worldwide contributing 13% of the total number of new cases diagnosed in 2012. Among men, lung cancer alone contributes nearly 17% of the total number of new cases diagnosed in 2012[2]. In India, NCDIR-NCRP (ICMR) 2016 data reports significant increase of incidence of lung cancer in females from Bangalore, Chennai, Delhi and Mumbai[3]. Lung cancer is one of the malignant tumors that contribute to the highest mortality rate since it is difficult to diagnose until there has been substantial progression of illness. Smoking is the leading cause of lung cancer and the other etiological factors includes exposure to asbestos, silica, diesel exhaust, radiation and air pollution[1]. Recently, Jayashree *et al*.[4] who reviewed Targets in anticancer research have highlighted the role of histone methyl transferases (HMT) in various cancers including lung cancer and the association between histone methylation and tumor development. The current treatment methods for lung cancer include surgery, radiation therapy, chemotherapy which is generally based on the type and stage of the cancer[1.] Chile *et al*.[5] have demonstrated the broad spectrum anticancer potential of AURK-B RNA silencing. Viral gene oncotherapy has been reported to be an emerging non-infectious therapeutic cancer treatment modality[6]. Since the prevalence of the lung cancer is significantly higher, there is an increased need to unravel potential therapies with fewer side effects.

The increasing occurrence of cancer has been associated with increased levels of intracellular free radicals[7] which causes detrimental effects to cellular protein, lipid, and DNA leading to genomic instability and ultimately promotes cancer progression[8]. Therefore, scavenging the free radicals with antioxidant supplement would salvage the cells from oxidative stress and decrease the progression of cancer[9]. Recent developments in cancer research showed promising results in the treatment of cancer at the molecular level with the use of plant compounds with fewer side effects. Since ancient times, medicinal

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plants have been in use for various disorders and diseases, the activity of which may be attributed to phenolic compounds such as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids[10-17]. Herbal medicines have shown to be effective approach for lung cancer therapy as they have proven to function in sensitizing conventional agents, prolonging patient survival time, preventing side effects of chemotherapy, and improving quality of life in lung cancer patients. A recent cohort study with 453 lung cancer patients showed that approximately 77% of the patients use herbal medicines in combination with the conventional therapy[1]. Singh *et al*.[18], suggested supplementation of aqueous extract of *Phyllanthus fraternus* Webster to reduce cyclophosphamide (CPA) induced oxidative stress and reproductive toxicity. Similarly, researchers have demonstrated the modulatory effects of ascorbic acid and aqueous extract of *Azadirachta indica* A. Juss. on not only the efficacies of anticancer drugs chlorambucil[19] and doxorubicin[20] but also their induced renal and cardiotoxicity, respectively. Plant derived compounds or their synthetic analogs possess potential activities including free radical scavengers[14-21], cytotoxic and induce apoptosis against malignant tumors but safe in the normal cells[22,23]. Recently, Sharma *et al*.[24], have demonstrated the therapeutic potential of dietary cucurbits in inhibiting colon cancer and inflammatory cytokine. Krishnamurthy and coworkers have successfully isolated and characterized a potent anticancer fraction from the leaf extracts of *Moringa oleifera* L.[25]. Manjegowda *et al*.[26] demonstrated, both *in vitro* and *in vivo*, antineoplastic activity of the green tea polyphenol, epigallocatechin gallate against breast carcinogenesis. They suggested that it acts possibly by influencing the expression of estrogen regulated genes. Although, plant derived products present many challenges, investigations on the understanding and exploration of its biological significance has been raised due their structural diversity[27]. However, there is continuing need to develop new anticancer drugs through methodical scientific experiments.

Croton bonplandianus Baill., an exotic weed of the Euphorbiaceae family distributed in the waste lands of southern India[28]. The leaf extract controls blood pressure, cure skin diseases and shows wound healing activity[29]. The seed cures jaundice, acute constipation, abdominal dropsy and internal abscesses[30]. Here, we investigated the antioxidant and cytotoxic effects of *C. bonplandianus* leaf extract on lung cancer cell line A549.

Materials and Methods

Cell culture

Lung adenocarcinoma cell line (A549) was purchased from National Centre for Cell Sciences (NCCS), Pune. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), incubated at 37ºC in 5% CO_2 and grown to confluence in 25 cm^2 flasks.

Collection, processing &extraction of plant material

The plant was collected from Chennai, Tamilnadu, India and authenticated, (No:PARC/2011/1021) by Dr. Jayaraman, Director, Plant Anatomy Research Center and deposited in the Department of Biomedical Sciences, Sri Ramachandra University, India. The leaves were shade dried and ground into a coarse powder. For extraction, 100 g of the coarsely ground leaves were extracted with acetone for 72 h by cold percolation method, filtered & condensed in a water bath at 60ºC.

Qualitative phytochemical analysis

The AcE was screened for the presence of phytochemical constituents according to the standard protocol[31].

Quantitative analysis of secondary metabolites

Polyphenol, flavonoids and tannin

The AcE (1 mg/mL) was mixed with 20 µL of Folin-Ciocalteau reagent and 50 µL of 2.5% Na_2CO_3 and incubated for one hour and the absorbance was read at 765 nm. Phenol content was determined using gallic acid as standard[32].

The extract was mixed with 0.1 mL of 10% aluminium chloride and 0.1 mL of 1M sodium acetate, incubated at 37°C for 30 min and the absorbance was read at 415 nm. Flavonoid content was determined using quercetin as standard[32.]

About 500 µL of the extract was mixed with 0.5 mL Folin's phenol reagent followed by 5 mL of 35% sodium carbonate, left in room temperature for 5 min and the absorbance was read at 640 nm. Tannins content was determined using Gallic acid as standard[33].

Free radical scavenging activity

Free radical scavenging activity of the plant extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay[34]. Fifty µL of the AcE in the range of 62.5-1000 µg/mL was treated with 150 µL of DPPH and

incubated in the dark for 30 min and absorbance was read at 517 nm using ascorbic acid as positive control. IC_{50} value was calculated from the standard equation. The scavenging activity in terms of percent inhibition was calculated from the formula:

% Inhibition= [Absorbance of control−Absorbance of test sample / Absorbance of control] × 100

MTT assay

Approximately, 5×10^3 cells were seeded in 96-well plate and incubated overnight at 37ºC with 5% CO_2. After 80% confluency, the cells were treated with different concentrations (200 & 400 µg/mL) of the AcE and incubated for 24 h. After incubation, the cells were washed with PBS, and 20 µL of MTT was added. After 2 h of incubation, the formazon crystals were dissolved in 200 µL DMSO and the absorbance was measured spectrophotometrically at 540 nm using cisplatin as positive control[35].

Acridine orange/Ethidium bromide staining (AO/EB)

Morphological changes due to apoptosis was observed using AO/EB fluorescence staining[36]. Two different concentrations 200 and 400 µg of AcE were chosen for further study based on the MTT assay value. Cells were treated with extracts for 24 h and treated with 10 µL of the reaction mixture (1:1 acridine orange-ethidium bromide). After 5 min of incubation, the cells were observed under Nikon Eclipse inverted microscope.

Hoechst staining

Condensation of chromatin is the hallmark of apoptosis and studied by Hoechst staining[37]. Approximately, 1×10^5 cells were seeded in 6-well plate and treated with AcE as same as AO/EB staining for 24 h. After incubation, the cells were washed with PBS followed by 10 µL of Hoechst 33258 stain was added. After 30 min of incubation, the cells were observed under Nikon Eclipse inverted microscope at 20X magnification with a DAPI filter.

Flow cytometric analysis

For cell cycle analysis, cells were seeded in 6-well plates with a density of approximately, 2×10^5 cells with and without AcE for 24 h. After incubation, the cells were trypsinized and centrifuged at 1000 rpm for 10 min. Cells were washed with PBS and fixed with 70% ice cold ethanol and stored overnight at 4ºC. Then the cells were washed with PBS and treated with 30 µL of RNase for 2 h then 30 µL of propidium iodide was added and incubated for 30 min at room temperature in dark. Samples were analyzed in a FACS flow cytometer and cell cycle distribution was calculated using software (Flow jow)[38].

Results

Preliminary phytochemical screening and Quantification of secondary metabolites

The preliminary qualitative phytochemical screening confirms the presence of alkaloids, carbohydrates, flavonoids, phenols, tannins, saponins, terpenoids. But anthraquinone was absent in the AcE. The quantitative analysis of AcE was found to contain 43±0.05 µg/mL of polyphenol, 3.2±0.07 µg/mL of flavonoids and 0.36±0.10 µg/mL of tannins.

DPPH radical scavenging assay

Antioxidant activity of the AcE was tested using DPPH as substrate. The concentration dependent DPPH radical scavenging activity was observed (Fig. 1). The extract was found to have the highest activity 55.78±0.003% inhibition (IC_{50} value 26.97±0.001 µg/mL) at the concentration of 1 mg/mL and the ascorbic acid (positive control) showed 89±0.07% inhibition (IC_{50} value 1.28 ± 0.005 µg/mL).

Cytotoxicity of the Acetone Extract

AcE showed comparable cytotoxic activity (IC_{50} 15.68±0.006 µg/mL) against the A549 cancer cells in comparison with the standard cisplatin (IC_{50} 2.20±0.008 µg/mL). The graph showed the dose dependent response of the cells to the extract (Fig. 2). The AcE extract up to 5 mg/mL did not show

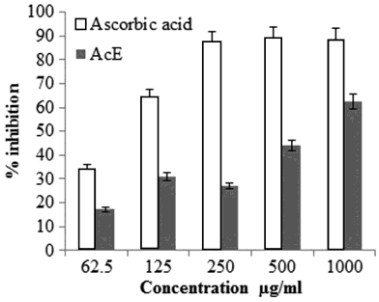

Fig. 1—Free radical scavenging activity at various concentrations (62.5-1000 µg/mL) of AcE of *Croton bonplandianus* as determined by DPPH method.

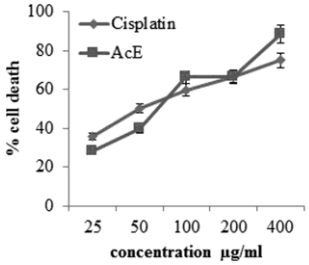

Fig. 2—Cytotoxicity effect of AcE of *C. bonplandianus* as determined by MTT assay.

significant cytotoxicity to the normal Vero cell line. This clearly indicates the safety of the extract in the normal cells.

Acridine orange/ Ethidium bromide staining

AO/EB dual stain was employed to identify the apoptotic and necrotic cells. AO will stain the nuclei green by permeating into the cell membrane and EB will stain the nuclei red when the cytoplasmic membrane integrity is lost. The A549 cells treated with AcE (200 & 400 µg) showed more apoptotic cells than control group (Fig. 3A).

Hoechst staining

Hoechst dye diffuses through intact membranes of cells and binds to the AT rich regions of double stranded DNA and exhibits enhanced fluorescence. The cancer cells treated with AcE showed significant apoptotic morphology (Fig. 3B) apparently visible as small fluorescent masses, whereas, the control cells were uniformly stained.

Effect of AcE on cell cycle

AcE arrested the cell cycle in a phase-specific and concentration-dependent manner (Fig. 4). The extract elicited the maximum activity at 400 µg/mL for the concentration tested such as 200-400 µg/mL. The cells were arrested at G2/M phase with a concurrent accumulation at sub G0-G1 phase indicating the apoptotic cell death (Fig. 5).

Discussion

Cytotoxicity induced cell death can be divided morphologically and biochemically into apoptosis and necrosis[39]. Apoptosis is the programmed cell death

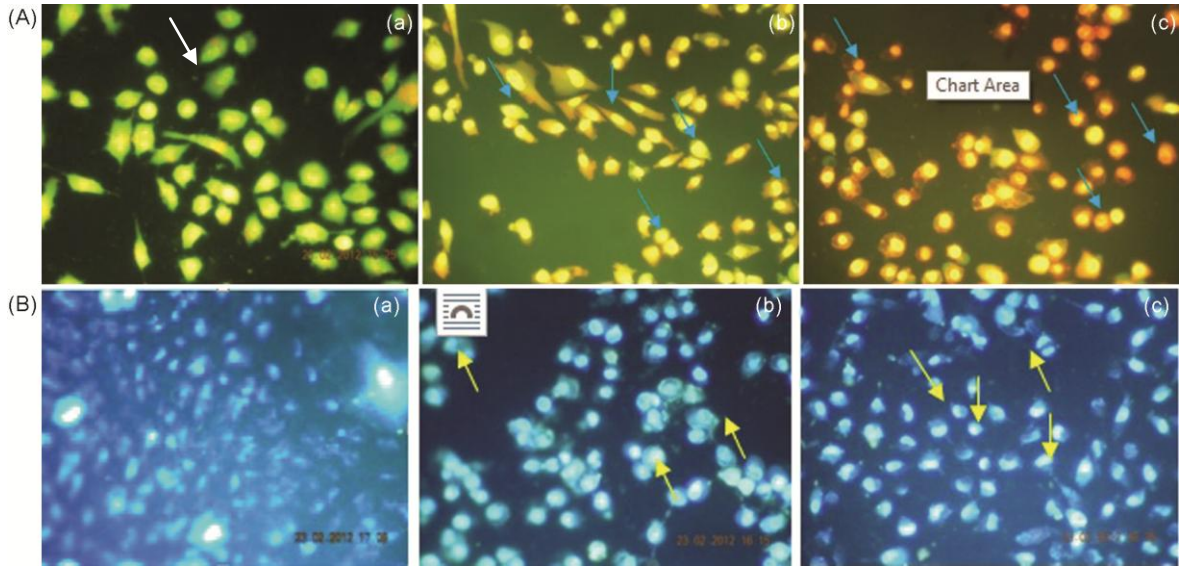

Fig. 3—(A) Nuclei morphological changes seen in lung cancer cells A549 detected by AO/EB staining. (a) Control, Intact cells exhibit homogeneous green nuclei; and (b & c) Treated groups, apoptotic cells with nuclear fragmentation and chromatin condensation exhibit reddish orange fluorescence of AO were visible. White arrow indicates early apoptosis (yellow color cells), Blue arrow indicates late apoptosis (orange color cells); (B) Hoechst 33258 fluorescent staining for detection of apoptotic morphology in A549 cells treated with AcE. (a) Negative control, Homogenous faint light blue nuclei shows absence of nuclear condensation; and (b & c) apoptotic cells exhibit bright blue fluorescence indicating nuclear condensation. Yellow arrow indicates chromatin condensation

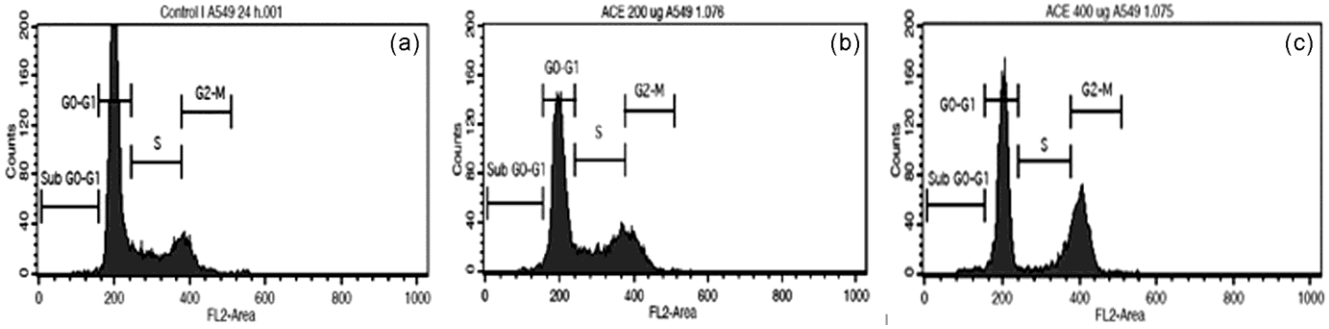

Fig. 4—Flow cytometric cell cycle distribution of AcE treated A549 cells with increasing dose.

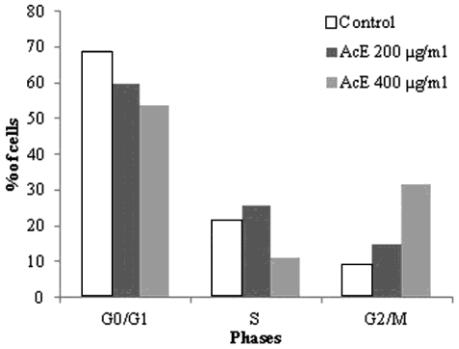

Fig. 5—Graphical representation of control and treated cell population in different cell cycle phases. [Distribution of cell cycle phases of A549 cell line after treatment with AcE was analyzed. DNA content was evaluated with propidium iodide and fluorescence emitted was measured after 24 h incubation with 200 & 400 µg/mL extract. The number of cells arrested significantly (P <0.001) in G2/M phase and accumulated at G0/G1 phase among the groups]

which is characterized by morphological changes such as membrane blebbing, cell shrinkage, chromatin condensation and DNA degradation[40]. It plays a vital role in regulating growth, development and in recent times it has been an important target for the development of effective anticancer drugs[40].

In the present study, acetone leaf extract of *C. bonplandianus* showed cytotoxicity and cell proliferation inhibition in lung carcinoma cells A549 in a dose-dependent manner (MTT assay) without affecting the normal cells with IC_{50} 15.68±0.006 µg/mL. Since the AcE showed potent cytotoxicity, the study was extended to confirm the apoptosis through AO/EB staining. To our confirmation, the AO/EB and Hoechst staining showed apoptosis induced morphological changes in the treated cells but these changes were not observed in untreated cells. The AO/EB staining showed normal green nucleus in the control cells (Fig. 4a), but AcE treated cells showed nuclear fragmentation and chromatin condensation with yellow and orange nucleus exhibiting early and late apoptosis (Fig. 4 b & c), respectively.

Since the extract showed apoptosis induced cytotoxic effect, cell cycle analysis was done by flow cytometry to study the stage of cell cycle arrest. The cell cycle analysis showed an increase in percentage of cells in the G2/M phase compared to control which indicates the AcE induced G2/M phase arrest. The cell cycle arrest at G2/M phase is regulated by the cell cycle check points that prevent the entry of cells into M phase when the DNA is damaged[41]. Concurrently, the p53 is upregulated in response to DNA damage which inhibits the activation of cyclin B and also increases the transcription of genes associated with apoptosis[42]. In the present study, the cell cycle arrest in G2/M phase leads to accumulation of cells at sub G0/G1 phase which indicates the induction of apoptosis in the cells at G2/M phase[43]. Thus, the AcE induces cell cycle arrest as well as cell death through apoptosis. The cell cycle arrest at G2/M phase provides a potential target for developing cancer therapy as it prevents the entry of cell with DNA damage in to mitosis.

In general, increased free radicals and imbalance in redox status has been well correlated with the development and progression of cancer[44]. Thus, scavenging the free radicals using antioxidant sources would be an alternative strategy to treat cancer cells. The present study showed that A549 cells were more sensitive to AcE with an IC_{50} of 15.68±0.006 µg/mL compared to the standard drug 2.20±0.008 µg/mL (cisplatin). The anticancer activity of the AcE against lung cancer cell line A549 might be due to the presence of these remarkable antioxidant components such as (43±0.05 µg/mL of phenols, 3.5±0.07 µg/mL flavonoids and 0.36 ±0.1 µg/mL of tannin) in the *C. bonplandianus*. The antioxidant activity is well correlated with anticancer activity, since the free radicals are one of the major contributing factor for development of cancer[16,19,24,25]. This antioxidant activity of AcE could be attributed to the active components like polyphenols and tannin[45]. Further, flavonoids have anticancer activity in inhibiting cell proliferation and angiogenesis through their effect on signal transduction[46]. Similarly, polyphenolic compounds possess anticancer activity by instigating apoptosis in cancer cells by acting on series of signaling pathways linked to apoptosis[16,47]. These findings are in agreement with the previous studies that phenolic compounds like phenolic acids, flavonoids and tannins have shown antioxidant nature[10-12,14,48], anti-angiogenesis[49] and anti-tumor properties[20,50] through caspase-mediated apoptosis and scavenging free radicals. However, further experimental research is required to isolate the principal active compound present in the AcE and to study their anticancer activities.

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