

Synergistic Chemopreventive effect of PAC and Doxorubicin on human Colorectal cancer (HT-29) cells through molecular mechanism

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ABSTRACT: Proanthocyanidin (PAC) is rich in naturally occurring flavonoid; originate from the grape seed product have been exposed high anti-tumor properties of various cancers. Doxorubicin characterized by damaging DNA, is widely used in the chemotherapy of malignancies, including lung, breast, colon and myeloid leukemia cancers, etc. This prompted us to investigate the chemopreventive potential of co-treatment with PAC and Doxorubicin against colon cancers (HT-29). The results showed that PAC and Doxorubicin in combination synergistically inhibited the cell growth of colon cancer cells than the compounds used alone. The GI₅₀ value was found to be 20 μM for 24-h exposure. The linguistic differences, similarly membrane blebbing, cell shrinkage and chromatin condensation were observed in PI staining. The indication of apoptotic simulating were analysis of DNA fragmentation in HT-29 cells. Thus, the study provides an insight into the potential application of PAC and Doxorubicin in a combination for the chemoprevention and treatment of colon cancers.

Keywords: PAC; Doxorubicin; MTT; DNA fragmentation;

1. INTRODUCTION

Globally, cancer is considered as a second most leading major public health issue that causes death [1]. In each year plenty of colon cancer grown in rapidly and the cancer patients are not identified in early symptoms, after it moves critical stage of cancer. Which were diagnosis and treatment for the conventional method such as surgery, radiation, chemotherapy of these treatments can destroy the normal cells along with the cancerous ones [2]. In recent trends chemotherapeutic drugs used treatment for many cancers. These drugs are taking high concentration doses can produce unpleasant side effects. One of the chemotherapeutic drug, cisplatin has putforth to inhibit the cell division. However, it has adverse effects leading to neutropenia and hypersensitivity reactions [3]. In this

scenario, there is an greater concern to reduce the adverse side effects of chemotherapeutic agents that are being used in the treatment of cancer [4]. Also, the often appearance of drug resistance is an burden for the chemotherapeutic agents efficiency. Therefore, the evaluation of new anti-neoplastic drugs are of great importance in cancer chemotherapy. Anticancer drugs are diverse, and are obtained from both synthetic and natural sources [5].

Proanthocyanidin (PAC) is a major flavonoid in grape seed that has potentially numerous benefit of biological applications. The PAC compound rich in dietary polyphenols, may protect against several cancers, especially in colon cancer without toxic effect on normal cells [6]. The PAC can inhibit the cell proliferation, when treated with co-operatively or synergistically increasing anticancer effects could reviewed in recent reports [7]. The most effective and commonly used antitumor drug is Doxorubicin (DOX) were treated with a wide range of cancers [8]. Since it has limited toxic effect such as myelosuppression, nausea, vomiting, cardiotoxicity and solemn undesirable outcome of serious heart damage, were taking this drug for long duration [9]. These chemotherapeutic drugs are known to damage DNA damage and arrest cell cycle arrest leading to apoptosis. However, defects in oncogene activation and/or the deregulation of apoptotic signaling pathways are common in cancer cells, allowing them to evade apoptosis [10]. The current biomedical application, the synergistic response of combined PAC and doxorubicin treatment of colon cancer cells are studied *in vitro*. The molecular mechanisms occurred in PAC induced apoptosis are explored through the suppression of cell growth, proliferation and apoptosis

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Competing interests

The authors have declared that no competing interests exist.

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caused by the combination of PAC with doxorubicin.

2. MATERIALS AND METHODS

2.1 Materials

PAC and Doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Dulbecco modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin, DNA extraction kit were obtained from Himedia. PI and MTT reagent were prepared, standard stock solutions 1mg/ml dissolved in phosphate-buffered saline (PBS) and stored at 4°C for further studies.

2.2 Cell culture and treatment

The colon cancer cell line HT-29 was obtained from National Centre for Cell Science (NCCS) Pune, India. Cells were grown in supplementing media with DMEM, 10% FBS and 1% penicillin/streptomycin and maintained in humidified chamber with a 5% CO₂ at 37°C. The cells (5x10⁴cells/well) seeded into 96-well plates were cultured for 24 hr and treated with various concentrations of PAC and doxorubicin dissolved in DMSO.

2.3 Cell viability assay

The viability of treated cancer cells were checked through MTT assay. Briefly, to the 96-well plates seeded with cells (5x10⁴cells/well) were treated with PAC and doxorubicin. A volume of 50 µl of MTT (10 mg/ml) was added to each well and the cells were incubated for an additional 4 h at 37°C. The purple color formazone crystals were dissolved with 100 µl of di-methyl sulphoxide (DMSO) for 15 – 20 min. The absorbance was measured by ELISA reader (Bio-Rad, Microplate reader, 680) at 570 nm with a reference filter at 655 nm. All experiments were performed in triplicate. The growth inhibition (in percentage) was obtained as: Inhibition concentration (%) = (OD of treated cells / OD of control cells) x 100.

2.4 Morphological analysis

The DMEM supplemented with 10% FBS medium in six well plate were used to culture the HT-29 cells and were treated for 24 hrs with IC₅₀ concentration of PAC, doxorubicin or PAC and doxorubicin in combination. The single cell suspensions was fixed at 1% PBS by placing the cover slip washed (3 times) with PBS. The cell morphological changes were observed under phase contrast microscope (Nikon, Japan) at 400X magnification.

2.5 Nuclear Morphology staining

Using propidium iodide (PI) the nuclear changes of apoptosis cells (5x10⁴cells/well) that are treated with IC₅₀ concentration of PAC, doxorubicin and combinations of both incubated for 24 hr were detected. Prior to staining with 20 µg/mL of PI for 20 min, the cells were washed with PBS and fixed in acetone : methanol (1:3 v/v) for 10 min. The condensed and nuclei fragments was observed using a fluorescent microscope (Nikon, Japan).

2.6 DNA Fragmentation Analysis

DNA fragmentation method was followed by Wang et al. (2007). Briefly, cells (5x10⁴cells) were cultured for 24 hr, followed by treatment of PAC, doxorubicin. At room temperature, the treated cells were suspended in 200 µL of lysis buffer [0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0] for 15 min and centrifuged at 12,000 rpm for 10 min. The DNA was extracted with phenol: chloroform (1:1) and precipitated with ethanol, and resuspended in Tris/EDTA buffer [10 mM Tris-HCl, pH 8.0, and 1 mM EDTA]. The 1.5% agarose gel electrophoresis was used to detect the DNA bands while using ethidium bromide stain and visualized under gel doc unit (Bio Rad).

2.7 Statistical Analysis

The Sigmastat (Version 3.1) statistical package was used in the statistical analysis. The experimental results (in triplicates) are expressed in mean ± standard deviation. The one way analysis of variance (ANOVA) was applied on the parameters under study and p values ($p < 0.05$) was considered as significant.

3. RESULTS AND DISCUSSION

3.1 Antiproliferative effects of PAC and doxorubicin on HT-29 cells

The effect of the PAC and doxorubicin on the proliferation of HT-29 cells was examined by MTT assay. According to both PAC and doxorubicin significantly ($p < 0.05$) inhibited the cell growth of HT-29 cells in a concentration- and time-dependent manner. The cells were treated with various concentrations namely 20, 40, 60, 80 and 100 µM/mL of PAC and doxorubicin, the cell survival was found to be maximum at the incubation time of 24 hrs. The results showed that PAC and doxorubicin treatment alone in a dose-dependent growth inhibition in HT-29 cell, with an IC₅₀ value of (0.51 ± 0.60 and 0.53 ± 0.57µM) with the highest concentration of (60 µM for PAC and 40 µM for doxorubicin). However, co-treatment of the PAC with doxorubicin of 20 µM significantly enhanced the growth inhibition, as indicated by markedly decreased IC₅₀ values of (0.49 ± 0.61µM), respectively. Only the combination of PAC and doxorubicin decreased cell viability by more than 50% for HT-29 cell lines compared with each compound alone (Figure 1). The percentage of cell proliferation was given in the (Table. 1).

The cells were treated with IC₅₀ concentration of PAC and doxorubicin alone and in combination for 24 h and then analyzed under inverted light microscope revealed the cellular morphology during the cell death. HT-29 cells are polyhedral or irregular shape and grow in a tightly connected manner. The healthy cells are visible with a distinct cytoskeleton in untreated control. When HT-29 cells treated with alone PAC (Figure. 2b) and doxorubicin (Figure. 2c) the cellular morphology of HT-29 cells was severely distorted and some cells turned round in shape when compared with untreated control (Figure. 2a).

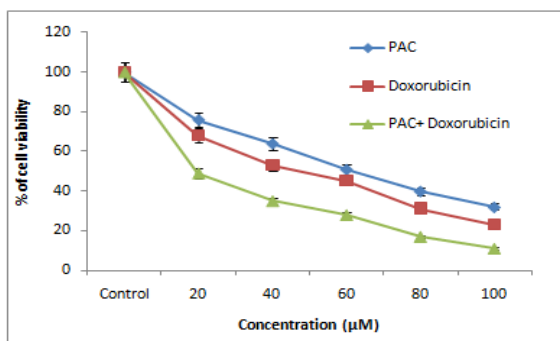


Fig. 1 The cell survival was measured by MTT assay. Effects of PAC and doxorubicin on HT-29 cell were treated with different concentrations of (A) PAC or (B) doxorubicin for 24 hr. Data are represented in the Mean ± Standard deviation

When the HT-29 cells are subjected to combinational treatment with PAC and doxorubicin (Figure. 2d) the majority of these cells had shrunken, and rounded up from their culture surface. The significant synergistic cytotoxic apoptosis are revealed by detached cells from the culture dish and showed the characteristic membrane blebbing and/or bubble formation is found on treatment with a combination of PAC and doxorubicin.

3.2 Combination of PAC and doxorubicin induces apoptosis in HT-29 cells

The synergistic anticancer activity of PAC and doxorubicin in HT-29 cells was treated with PAC and/or doxorubicin is subjected to PI staining to visualize the apoptotic morphological alterations.

Table 1: Effect of PAC and doxorubicin toward HT-29 cells as determined by the MTT assay. The cells were incubated with different concentration of drugs (20 - 100µM/mL) for 24 h.

S.NO	Concentrations (µM/ml)	PAC (% of cell viability)	Doxorubicin (% of cell viability)	PAC+Doxorubicin (% of cell viability)
1	20	0.76 ± 0.62	0.68 ± 0.91	0.49 ± 0.61
2	40	0.64 ± 1.08	0.53 ± 0.57	0.35 ± 0.31
3	60	0.51 ± 0.60	0.45 ± 0.54	0.28 ± 0.42
4	80	0.40 ± 1.48	0.31 ± 0.65	0.17 ± 0.47
5	100	0.32 ± 0.10	0.23 ± 0.93	0.11 ± 1.04
6	Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

*Data are represented in the Mean ± Standard deviation.

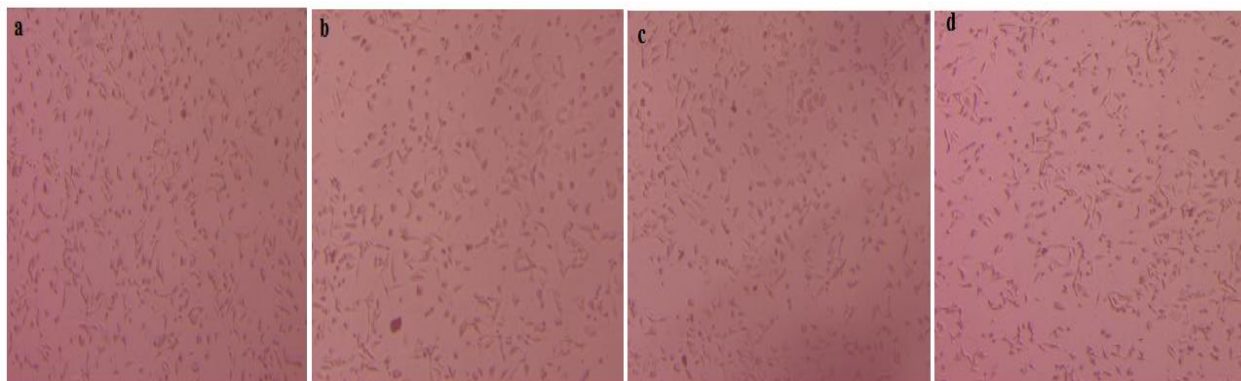


Fig. 2. Antiproliferation effect of PAC and doxorubicin in HT-29 cell line. (a) Control, (b) IC₅₀ concentration of PAC (60µM/mL), (c) IC₅₀ concentration of doxorubicin (40µM/mL) and (d) IC₅₀ concentration of combination in PAC and doxorubicin at (60+40µM/mL).

The number of apoptotic and non- apoptotic cells were counted under phase contrast microscope (Fig. 3), following treatment with PAC and/or doxorubicin for 24 h, a portion of HT-29 cells exhibited nuclear shrinkage, rounding and fragmentation, indicating the occurrence of apoptotic processes. The cells treated PAC and doxorubicin was significantly enhanced.

3.3 DNA fragmentation Assay

The basic results of cytotoxicity and linguistic differences show that PAC induce apoptosis, and they were proposing to compare with the fragmentation pattern of DNA and observed the biochemical features of archetypal dysfunction is involved in DNA fragmentation. The PAC or doxorubicin treated HT-29 cells were used to isolate the

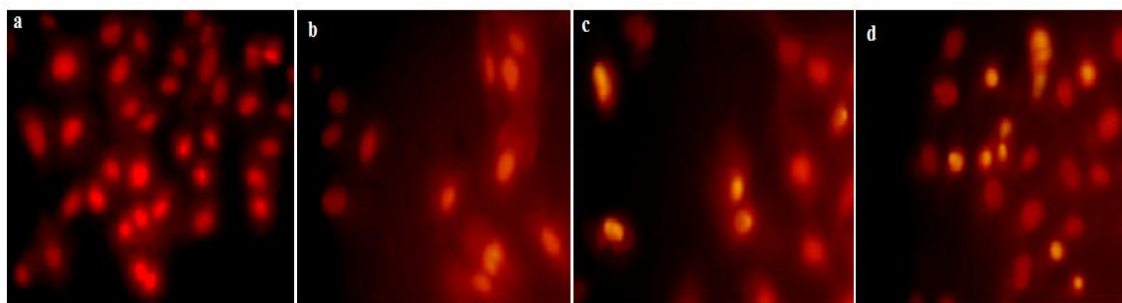


Fig. 3. Apoptosis in HT-29 cells was evaluated by fluorescent microscopy measurements. (a) Untreated (Control), (b) PAC treated cell line, (c) doxorubicin treated cell line, and (d) combination of PAC and doxorubicin treated cell line.

nuclear DNA and subjected to agarose gel electrophoresis. In PAC or doxorubicin treated cells, DNA fragmentation were observed when compared to control cell line (Fig. 4). Interestingly, the fragmentation of DNA changes in the cells treated with a combination of both PAC and doxorubicin are more significant than to control cells.

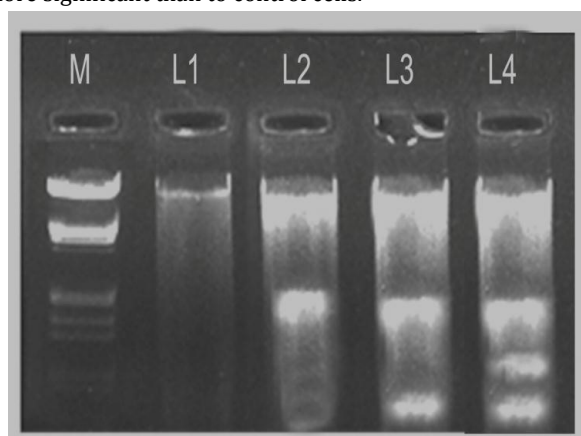


Fig. 4. DNA fragmentation analysis by HT-29 cells. (a) Untreated (Control), (b) PAC treated cell line, (c) doxorubicin treated cell line, and (d) combination of PAC and doxorubicin treated cell line.

4. DISCUSSION

The naturally occurring plants with known anti-tumor activity reported, that combination chemotherapy was significantly reduced the toxicity to normal cells not a cancerous one. [11]. PAC and doxorubicin with inhibit the cell proliferation of different types of cancers, but also its structure is similar to drugs that can reverse chemoresistance [12]. It has been proposed the several mechanisms of doxorubicin i) CYP24 enzyme directly inhibits the apoptosis and enhanced. Similarly the previous report was suggested, genistin isoflavonoids compound would inhibiting the p-EGFR expression and promoting caspase-3 enzymes [13]. PAC is a food sources which are derived from grape seeds and is less expensive than conventional chemotherapeutic drugs [14]. This reduces the economic pressure of cancer patients. A conventional doxorubicin, which is widely used chemotherapeutic drug is highly toxic in normal tissues [15].

It is observed that there is an rapid decrease in doxorubicin plasma concentrations within few hours of patients administrated with doxorubicin. However, the drug exhibits a prologed antitumor effect. Doxorubicin has adverse effects on health and it also induces primary and secondary drug resistance, making the failure of cancer chemotherapy [16].

In this study, the HT-29 human colon cancer cells are treated with PAC and doxorubicin separately and also in combination for 24 hr. The concentration of PAC and doxorubicin are according to the first reporting of achievable peak in plasma concentrations. The MTT assay (Fig. 1) implied that both PAC and doxorubicin can significantly inhibit the growth of HT-29 cells on a time and concentration dependent manner. After 24 hrs of traterment the IC_{50} of the PAC ($0.51 \pm 0.60 \mu M$) doxorubicin ($0.53 \pm 0.57 \mu M$) and combinations ($0.49 \pm 0.61 \mu M$) were observed. Also, the results demonstrated that in 24 treatment with both PAC and doxorubicin could lead to apoptosis and necrotic cell death (Fig. 2). However, it is clear that the apoptosis induced by doxorubicin was higher than that of PAC. On the other hand, the combination of PAC and doxorubicin has caused higher apoptosis and necrosis of cells in 24 hr when compared to their individual treatments. The linguistic differences, similarly nuclear fragmentation, cell shrinkage and rounding in the HT-29 cells were examined in both combinations of PAC and doxorubicin. PAC compound induces the hallmark of caspase-dependent apoptosis and the release of mitochondrial cytochrome c. Furthermore, PAC -induced ROS generation that can lead to induction of proapoptotic protein including p53, p21 and cell cycle regulatory proteins like Rb, cyclin D1, and D3 [17].

5. CONCLUSION

In the present study it has been demonstrated that the combination of PAC and doxorubicin can more apparently possess *in vitro* anti-cancer activity when compared to their individual applications. The MTT assay has significantly envisaged that both PAC and doxorubicin can significantly inhibit the growth of HT-29 cells on a time and concentration dependent manner. Also, the DNA fragmentation in the cells treated with a combination of

both PAC and doxorubicin are more significant than to control cells. Thus, the study explores the synergistic anti-cancer effects of PAC and doxorubicin in tumor chemotherapy. However, the need for clinical trials are needed to schedule its use against various cancers.

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