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Research Paper

C-Phycocyanin Modulates the Cytotoxicity of Platinum Based Anticancer Drugs in Lung Cancer Cell Lines

Biophysics

KEYWORDS : c phycocyanin, cytotoxicity, chemotherapy, lung cancer

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ABSTRACT

The present study has been designed to elucidate the effect of supplementation of C-phycocyanin, a phycobiliprotein present in Spirulina platensis, on the chemotherapeutic potential of the anticancer drugs, cisplatin, carboplatin and oxaliplatin in Type-I (WI-26) and Type-II (A549) lung adenocarcinoma epithelial cells. Our results showed that c-phycocyanin supplementation were effective in enhancing the cytotoxicity when given with carboplatin/ cisplatin but not in case of oxaliplatin. These results were confirmed by ultra-structural changes like cell shrinkage and loss of membrane integrity that are some of the characteristics of cells undergoing apoptosis. Increased reactive oxygen species (ROS) and PI-Annexin staining was found when c-phycocyanin was given alongwith the anticancer drugs in A-549 cells. In WI-26 cells, the co-administration of c-Phycocyanin was observed to cause significant increase in cytotoxicity only with cisplatin. Thus, our results show that the algal protein, c-phycocyanin increased the efficacy of the chemotherapeutic drugs in a selective manner.

Introduction

Chemoprevention is an effective way to reduce cancer risk. Cisplatin, cis-diaminedichloroplatinum (II) (CDDP), is a widely used and effective chemotherapeutic agent that binds to and alkylates DNA and triggers transcription inhibition, cell cycle arrest, and apoptosis [1]. Cisplatin or platinum based compounds have major antineoplastic effect in lung, head and neck, testicular, ovarian and bladder cancers. Adjuvant cisplatin-based chemotherapy improves overall survival in lung cancer [2]. Cytotoxicity of cisplatin is considered to be due to a combination of factors like peroxidation of cell membrane, mitochondrial dysfunction, inhibition of protein synthesis and DNA injury [3]. Introduced to clinical use in 1979, cisplatin has been saving lives since then by effectively destroying tumors and other cancerous growths. However, it has not been the magical panacea to cancer as hoped due to many shortcomings and side effects. Like most of the anticancer drugs, CDDP is not target specific and can affect both normal and malignant cells, which contributes to the side effects observed during chemotherapy. High incidence of toxicities, including progressive and irreversible ototoxicity, neurotoxicity and nephrotoxicity has been reported with CDDP treatment. A large number of patients develop evidence of nephrotoxicity following single dose of cisplatin [4]. This is the major limitation of this compound sometimes requiring a reduction in dose or the discontinuation of treatment. Many other platinum compounds (e.g. carboplatin, oxaliplatin, nedaplatin and the liposomal form lipoplatin) have been tested over the last two decades in order to improve the effectiveness and to reduce the toxicity of cisplatin to normal cells. Not much information (whether beneficial or harmful) is available regarding the use of herbal products along with targeted therapy.Recently, hepatoprotective effect of Spirulina during cisplatin treatment has been demonstrated[5]. A study by Huq et al [6] investigated synergism from the combination of platinum drugs and a number of tumour-active phytochemicals including curcumin, epigallocatechin-3-gallate, thymoquinone, genistein, resveratrol, betulinic acid and ursolic acid in three human ovarian cancer cell lines. They found that binary combinations of platinum drugs with the phytochemicals exert concentration- and sequence-dependent synergism in the cell lines. C-phycocyanin (C-PC), a phycobiliprotein from the microalga Spirulina has been previously shown to have anticancer properties [7]. Shanab et al [8] found 87.25% and 89.4% cytotoxicity by algal water extracts against Ehrlich Ascites Carcinoma Cell (EACC) and Human Hepatocellular cancer cell line (HepG2) respectively. Since aqueous extracts of microalgae exhibit antioxidant and anticancer activities, we suggest that C-PC can potentially improve the efficacy of the currently available anticancer drug. However, it is still unknown how the combination of C-PC with anti cancer drugs effect the apoptosis of malignant cells. It is still not known whether the administration of C-phycocyanin compromise the efficacy of the chemotherapeutic treatment or not. C-phycocyanin may prove to be effective in reducing the dosage of the administered drug which will be beneficial to the patient in long term. The unwanted side effects of the drug may be minimized during the therapy without compromising on the efficacy.

Materials and Methods

All the chemicals were purchased from Sigma Aldrich Chemical Co unless otherwise stated. A549 (type-II epithelial) and WI-26 (type-I epithelial) cell line used in the present study were procured from NCCS, Pune, India. All cell culture products were procured from GIBCO-BRL

(Life Technologies). Fluorescence dyes such as 2', 7' dichlorofluorescein diacetate (DCFH-DA), Dihydrorhodamine 123 (DHR-123) and Dihyroethidine will be procured from Molecular Probes, USA.

Cell culturing

Lung cancer cell lines (WI-26 and A549) were maintained in continuous culture at 37^0C temperature and 5% CO_2 in medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml fungizone (Life Technologies, USA). When required for the assays, confluent monolayer of cells were washed with Ca^{2+} and Mg^{2+}-free sterile phosphate buffered saline (PBS) and detached from the culture flask by using Trypsin-EDTA solution (250 mg trypsin, 30 mg EDTA in 100 ml PBS). Cells were collected and centrifuged at 200 x g for 5 min. Cell pellet was washed with Ham's F-12 K medium and about10^4 trypsinized cells/well were dispersed in tissue culture plates. The plates were incubated overnight in the CO_2-incubator till a confluent monolayer (50-60%) was formed. The C-PC /cisplatin /carboplatin /oxaliplatin were filter-sterilized (0.22mm pore size, Millipore) and added to the wells separately. The synergistic effect was studied by adding the combination of C-PC with each drug individually to the cells. The plates were incubated in CO_2-incubator for the requisite time period.

Cell viability assay
Effect of C-PC and each of the chemotherapeutic drug and their co-administration with C-PC, on cell viability will be evaluated by the 3-(4, 5- dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) dye uptake method separately. This assay is based on the reduction of yellow MTT dye into insoluble formazan crystals by dehydrogenase activity in mitochondria, a conversion that occurs only in living cells [9]. Briefly, cells will be seeded in 96- well plates and allowed to grow overnight. After 24 hr of priming, cells will be treated with different concentrations of C-PC/cisplatin/carboplatin/oxaliplatin separately for different time periods in fresh medium. Four hours before the end of desired time interval, 20 µl of MTT solution (2.5 mg/ml) will be added to each well. After 4 hr, resulting formazan crystals will be dissolved in 40 µl of lysis buffer (20%

SDS dissolved in 50% each of DMF and ddH_2O). The developed color will be read at 540 nm on ELISA reader and the relative viability was calculated.

Annexin V-propidium iodide double staining
Phosphatidylserine translocation to the outer leaflet of the plasma membrane was assessed by reaction with Annexin V-FITC and detected by a FACScan flow cytometer. After each treatment, cells were harvested, washed with ice-cold PBS, and resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). These were incubated with Annexin V-FITC for 10 min at room temperature in the dark and counterstained with 5 µg/ml PI. Cells without treatment were used as a negative control. Cells showing up as Annexin V^-/PI^+ were recognized as necrotic, that showing up as Annexin V^+/PI^+ were taken as late apoptotic or secondarily necrotic, whilst Annexin V^+/PI^- cells were recognized as apoptotic cells .

Cellular integrity by FDA uptake method
Cellular injury was determined by the FDA (fluorescein diacetate) and ethidium bromide staining method. FDA is an indicator of membrane integrity and cytoplasmic esterase activity. Ethidium bromide has been shown to penetrate only cells with damaged membranes and form a red complex with nuclear DNA. So the cells with intact membranes fluoresce green and cells with damaged membranes fluoresce red. Cells after each treatment were incubated with 10 µM FDA and 25 µM of ethidium bromide and visualized under fluorescent microscope (Olympus).

Intracellular ROS and estimation by FACS
Intracellular ROS production in the cells after C-PC treatment with or without each chemotherapeutic drug was detected by using fluorescent probe such DCFH-DA (probe for oxygen radical) according to the method of Marchetti et al [10]. Cells were loaded with 1 µM dye and incubated for 15 mins. Cells were then washed and resuspended in complete growth media and treated with different concentrations of C-PC/each chemotherapeutic drug alone/in combination with C-PC for the optimum period separately. Oxidation of dyes was measured by using a FACscan cytometer (Bector and Dickinson).

RESULTS
Optimization of time period and platinum compounds concentration for maximal effect of platinum compounds on lung epithelial cells:

Cell viability assay:
MTT assay was done to examine the cytotoxic effect of cisplatin, carboplatin and oxaliplatin on A549 cells and WI-26 cells for 24 and 48 hr of time intervals. Cisplatin, carboplatin and oxaliplatin showed concentration dependent increase in cell toxicity of the cells after 24 and 48 hours in both cell lines. In case of A549 cells 50% cellular viability for cisplatin (Fig.1A), carboplatin (Fig.1B) and oxaliplatin (Fig.1C) were found to be 200µM, 250µM and 450µM respectively after 24 hours and 50µM, 200µM and 175µM respectively after 48 hours. While in WI-26 cells, dose calculated for at least 50 % cell- viability for cisplatin treatment (Fig.2A), carboplatin treatment (Fig.2B) and oxaliplatin treatment (Fig.2C) were 50 µM after 48 hours and 100 µM, 250 µM and 200 µM respectively after 24 hours.

Optimization of c-phycocyanin concentration
The MTT assay was also done on both the cell lines with different doses of c-phycocyanin (0.5-10 mg/ml) at 48 hours post treatment. There was no significant cytotoxicity in the cells treated with c-phycocyanin in either cell lines. At a concentration of 5mg/ml (protein content 40%), cell viability was slightly compromised and no significant decrease in cell viability was observed in both A549 and WI27 cell lines at higher concentrations, so this concentration was selected for further studies (Fig 3).

Effect of co-administration of c-phycocyanin on cisplatin, carboplatin and oxaliplatin induced cell death in lung epithelial type II and type I cells
Light microscopic assessment of the cellular morphology and FDA uptake studies in A549 cell line showed that when c-phycocyanin was given alongwith the chemotherapeutic drugs in the cultured cells, the cytotoxic effect was more pronounced in cisplatin and carboplatin supplemented cells compared to when these drugs were given alone (Fig 4B,E). There was no observable change in the cytotoxic effect of oxaliplatin on supplementation with c-phycocyanin (Fig 4F,G)

In WI26 cell line the supplementation of c-phycocyanin was found to induce maximum cytotoxicity when given with cisplatin (Fig 5B,C) The combination of carboplatin or oxaliplatin with c-phycocyanin did not show any significant change in cell viability compared to when carboplatin or cisplatin were given alone (Fig 5D-G).

The PI- annexin study in A549 cells showed increased apoptosis when c-phycocyanin was supplemented with cisplatin compared to when cisplatin was given alone. There was increased apoptosis when carboplatin or oxaliplatin were supplemented with c-phycocyanin treatment (Fig 6). The WI26 cells also showed increase in apoptosis as evident from PI-annexin study. The co-administration of c-phycocyanin was found to be effective in enhancing the cytotoxic effect. (Fig 7).

It was observed ROS expression was increased on co-administration of cisplatin with c-phycocyanin compared to control or cisplatin alone in WI26 cell line. The ROS expression was raised in both carboplatin and oxaliplatin treated cells compared to the control. The supplementation of c-phycocyanin also increased ROS production however; it did not show any significant effect when compared to carboplatin or oxaliplatin alone in either cell lines (Fig 8).

Discussion
Cisplatin or platinum based compounds also have major antineoplastic effect in lung, head and neck, testicular, ovarian and bladder cancers. Adjuvant cisplatin-based chemotherapy improves overall survival in lung cancer [2]. The platinum based drugs comprise a major family of chemotherapeutic agents but their dose and efficacy is limited due their side effects like nephrotoxicity, hepatotoxicity etc. Numerous human and experimental studies have been performed to understand the mechanism of platinum drug associated toxicity and thereby to minimize it.A recent study by [6] has suggested that combination of selected phytochemicals can overcome the drug resistance in ovarian cancer. Zang et al has hypothesized that combined effect of natural products may improve the treatment effec-

tiveness in combating proliferation of cancer cells[11]. In this context, Spirulina, a planktonic blue - green algae, is gaining increasing attention because of its nutritional and medicinal properties. There are several new peer reviewed

scientific studies about Spirulina's ability to inhibit viral replication, strengthen both the cellular and humoral arms of the immune system and cause regression and inhibition of cancers chemopreventive efficacy of a of Cphycocyanin, the major phycobiliprotein present in Spirulina, and piroxicam, a traditional non-steroidal anti-inflammatory drug was observed in 1,2 dimethylhyadrazine (DMH)-induced colon carcinogenesis in rats [12]. In this study, it has been suggested that by combining the phycobiliprotein, c-phycocyanin with these drugs, the chemotherapeutic efficacy can be enhanced without compromising on the dose and hence minimizing the side effects.

For this study two different lineages of human lung epithelial cells were used. Human lung epithelial type-I (WI26) and type-II cells (A549 cells) were procured. Cells were maintained at 37°C temperature and 5% CO_2 in humidified CO_2 incubator. The effect of the chemotherapeutic drugs, cisplatin, carboplatin and oxaliplatin on the cell lines was evaluated separately and the LD_{50} dose was calculated for each compound on each cell line. Concentration of CPC was also standardized. When combined effect of each of the drug was observed on the cell viability, we found that CPC in combination with cisplatin was more effective in inducing cellular apoptosis and probably increased the cytotoxicity of cisplatin at the given dose. A study on the effect of C-PC on growth and multiplication of human chronic myeloid leukemia cell line (K562) has shown a significant decrease in the proliferation, increased apoptosis through release of cytochrome c into the cytosol and poly (ADP) ribose polymerase (PARP) cleavage [13]. C-PC's were also found to induce apoptosis in the doxorubicinresistant human hepatocellular-carcinoma cell line, HepG2

[14]. Pardhasaradhi et al [15] showed C-PC-induced apoptotic death in rat histiocytic tumor cell line, AK-5.The apoptosis was found to be inhibited by Bcl expression through regulation of free radical generation. Wang et al [16] expressed the beta-subunit of C-PC in Escherichia coli and found that the recombinant C-PC/beta showed anti-cancer properties. Zhang et al [11] has shown potentiation of paclitaxel activity by combination with curcumin in human breast cancer cell by modulating apoptosis. However, the effect of C-PC with platinum derived chemotherapeutic agents has not been studied earlier. The platinum based drugs like cisplatin, carboplatin and oxaliplatin are a widely used for treatment of various types of solid tumors. These are effective chemotherapeutic agents that bind to and alkylate DNA and triggers transcription inhibition, cell cycle arrest, and apoptosis. However, high incidence of toxicities has been reported with these

treatments which include progressive and irreversible ototoxicity, neurotoxicity and nephrotoxicity [4]. Our study confirms that the c-phycocyanin have some cytotoxic effect on the cancer cells and supplementation of c-phycocyanin with platinum based drugs like cisplatin, carboplatin and oxaliplatin may increase the efficacy of these drugs. The anticancer effect may be mediated through reactive oxygen species (ROS) production resulting from ER stress and mitochondrial dysfunction. Nutraceuticals like c- phycocyanin are effective because unlike pharmaceutical drugs, these agents modulate multiple targets, including transcription factors, growth factors, tumor cell survival factors, inflammatory pathways, and invasion and angiogenesis linked closely to carcinogenesis [17].

CPC exhibited antiproliferative activity against human cancer cells in combination with chemotherapeutic drugs through apoptosis. This was shown by the PI annexin staining where combination of anticancer drugs with cPhycocyanin increased the percentage of cells undergoing apoptosis. However, the synergistic effect of c-phycocyanin with these drugs may depend on the cell type since in the present study variable results were obtained with A549 and WI26. Thus, more thorough study is needed but c-phycocyanin, which is a natural phycobiliprotein, has chemotherapeutic potential in cancer without any additional side effect. In that respect, we suggest that C-PC can potentially improve the efficacy of the currently available anticancer drug, and therefore diminish its harsh side effects in the patient. This basic knowledge may help to better plan and optimize strategies for chemoprevention or chemotherapy.

Acknowledgement:
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LEGENDS

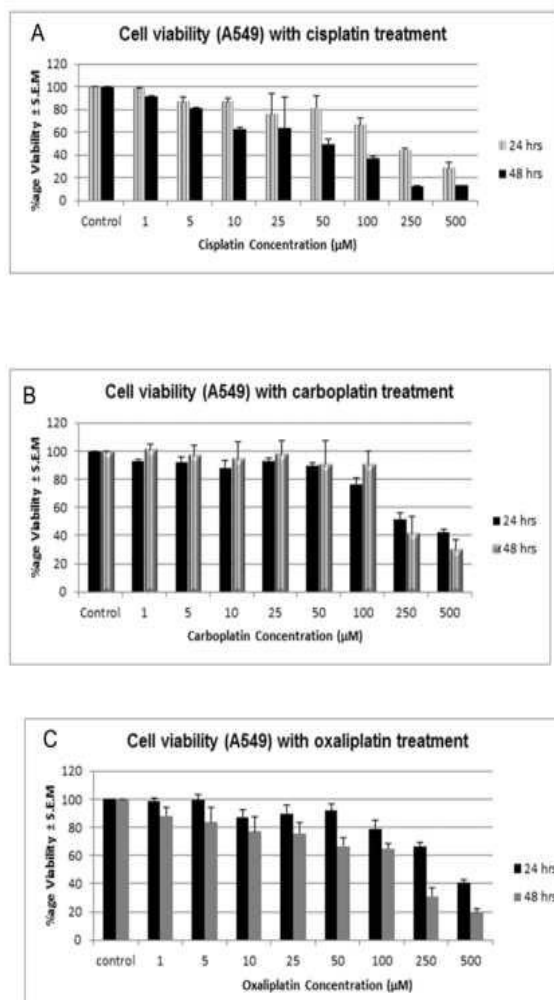

Fig 1. Viability of A549 cells on treatment with A) cisplatin B) carboplatin C) oxaliplatin. The cells were treated with the chemotherapeutic drugs at the indicated concentrations for 24 and 48 hours and assayed by MTT as described in materials and methods section.

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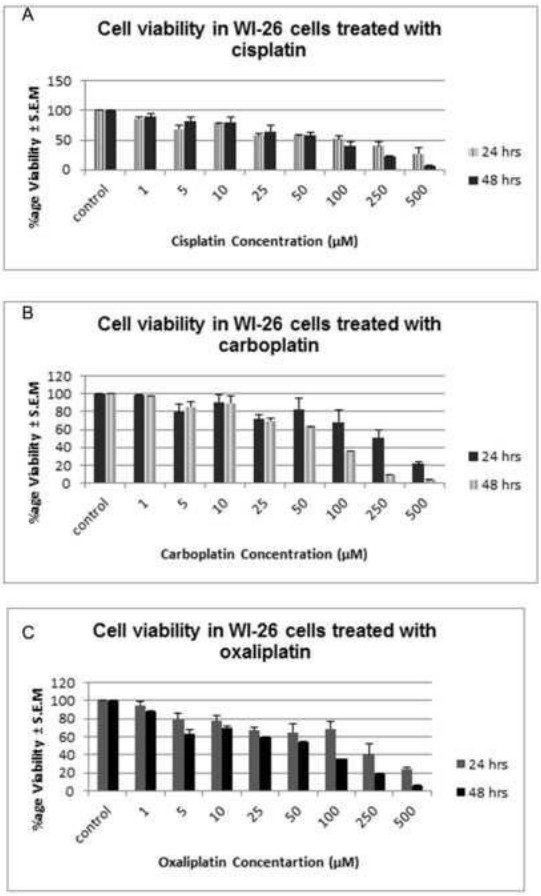


Fig 2. Viability of WI26 cells on treatment with A) cisplatin B) carboplatin C) oxaliplatin. The percentage viability was measured after the cells were treated with indicated chemotherapeutic drugs for 24 and 48 hours and assayed by MTT.

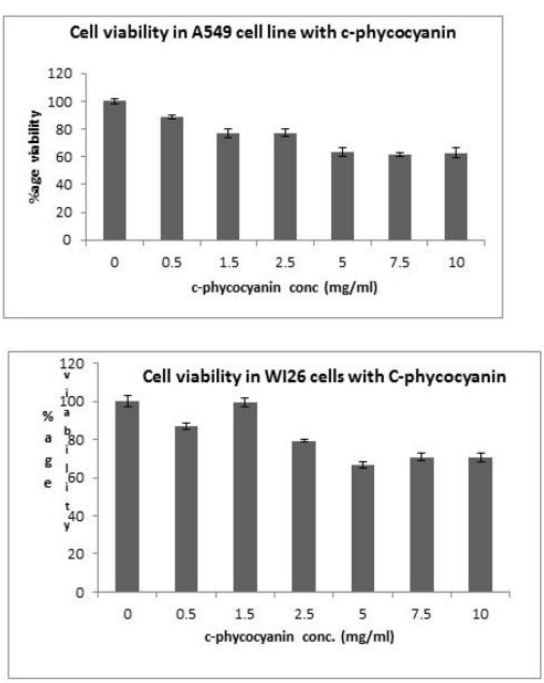


Fig 3. The effect of different concentrations of c-Phycocyanin (0.5-10mg) on viability of A549 cells (upper panel) and WI26 (lower panel).

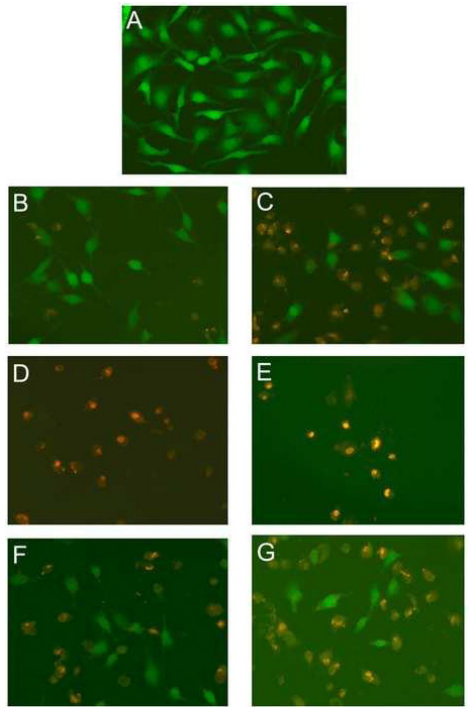


Fig 4. Light microscopy (20X) images of cellular morphology of A549 cells with green fluorescence indicating intact membrane and orange fluorescence due to ethidium bromide show loss of membrane integrity. A) untreated cells showing normal morphology. B) cells treated with cisplatin alone. C) cells treated with combination of cisplatin and c-phycocyanin.D) cells treated with carboplatin alone E) cells treated with carboplatin and c-phycocyanin. F) cells treated with oxaliplatin F) cells treated with both oxaliplatin and c-phycocyanin.

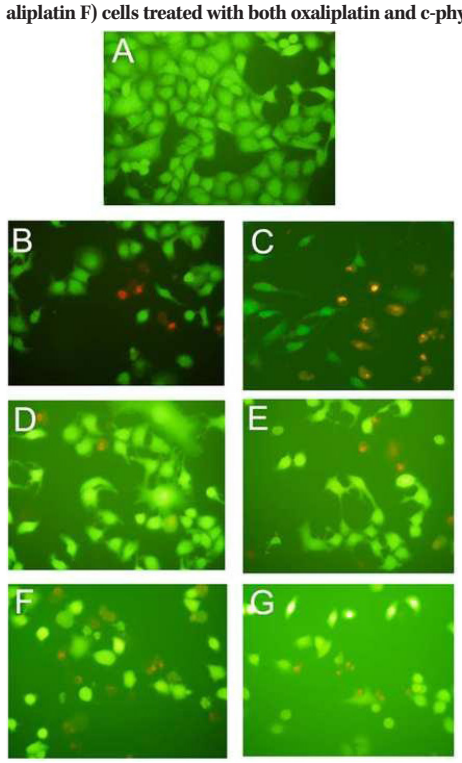
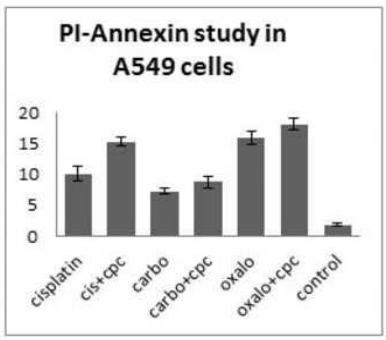


Fig 5. Light microscopy (20X) images of WI26 cells stained with FDA and ethidium bromide. A) untreated cells showing normal morphology. B) cells treated with cisplatin alone. C) cells treated with combination of cisplatin and c-phycocyanin. D) cells treated with carboplatin alone E) cells treated

with carboplatin and c-phycocyanin. F) cells treated with oxaliplatin only. F) cells treated with both oxaliplatin and c-phycocyanin.



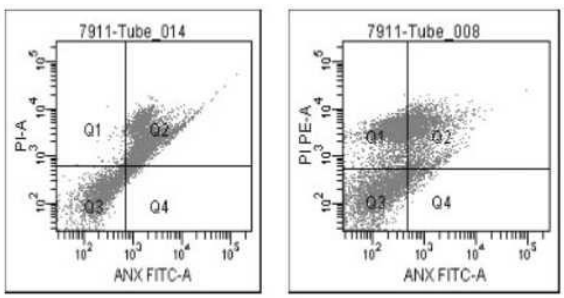
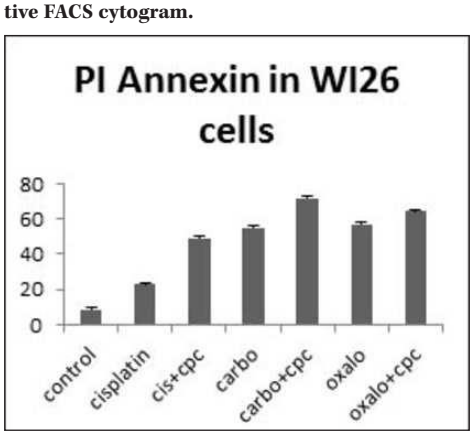


Fig 6. Apoptosis induced by chemotherapeutic drugs in A 549 cells. Upper panel shows the apoptosis induced by cisplatin/carboplatin/oxaliplatin alone or in combination with c-phycocyanin in these cells. Lower panel show representative FACS cytogram.



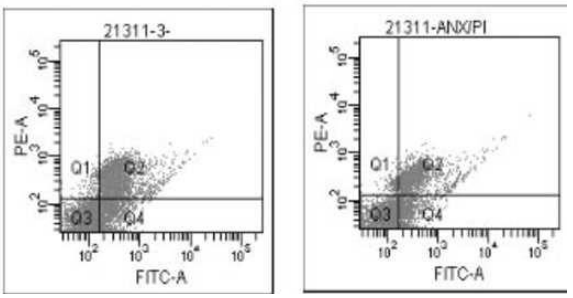
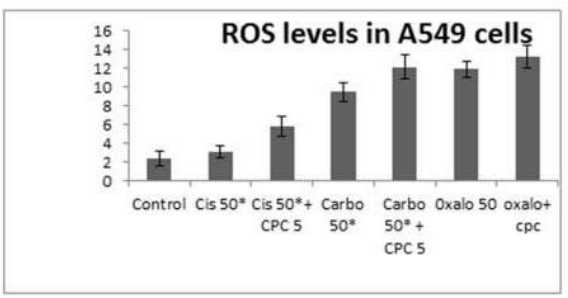


Fig 7. Study showing apoptosis induced by chemotherapeutic drugs in WI26 cells. Upper panel shows the apoptosis induced by cisplatin/carboplatin/oxaliplatin alone or in combination with c-phycocyanin in these cells. Lower panel show representative FACS images



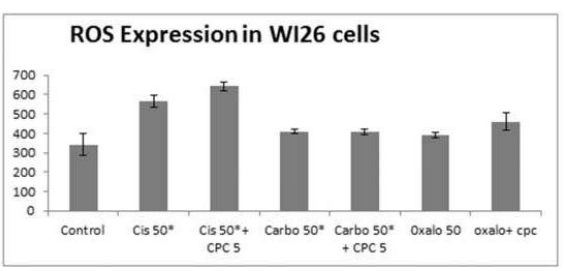


Fig 8. Mean fluorescence Intensity for ROS expression in A549 cells (upper panel) and WI26 cells (lower panel) observed after DCHFDA staining.

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