Research Article

Comparative Analysis of Chemopreventive Efficacy of Cruciferous Phytochemicals in Combination With Etoposide Drug on Lung Cancer Cell Lines

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ABSTRACT

Cancer is a one of the deadliest diseases. Cancer treatment along with phytochemicals is a emerging strategy to treat cancer and decrease toxicity of anticancer drugs. Hence, to analyse the combinational therapy, anticancer drug etopside is combined with dietary phytochemicals rutin, allylisothiocyanate (AITC), benzyl isothiocyanate (BITC) and (phenethyl isothiocyanate) PEITC singly and in combination were treated on human lung cancer cell lines such as A-549 and N-460. Cell viability of lung cancer cells was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay. IC_{50} values of all the phytochemicals and drug etoposide were obtained. Morphological changes of lung cancer cell lines exposed to rutin, AITC, BITC, PEITC and etoposide alone and in combination were examined by light microscopy and by fluorescent microscopy after propidium iodide staining. The extent of DNA (deoxyribonucleic acid) fragmentation, a hall mark of apoptosis was identified by DNA ladder assay by agarose gel electrophoresis and diphenyl amine reaction method. Cell proliferation and cell viability studies by MTT assay showed a combination of the phytochemicals and etoposide at 50% of their IC_{50} values exhibited upto 85% of cell inhibition. Among the four phytochemicals, BITC and PEITC in combination with etoposide exhibited 87%-89% of DNA fragmentation on N-460 cell lines. Changes in nuclear morphology characteristic of apoptosis were observed. Anti-cancer drugs in combination with dietary phytochemicals could exert the same effect to the patient at lower dosages with minimal side effects. In the present study, dietary phytochemicals along with etoposide exerted chemo efficacy on lung cancer cell lines, thereby decreasing the toxic effects of drug etoposide on normal tissues.

Keywords: Lung Cancer, Cruciferous phytochemicals, Combination therapy, Apoptosis.

INTRODUCTION

Lung cancer is one of the leading causes of cancer related deaths worldwide accounting to 19% of cancer deaths [1]. Surgery, chemotherapy and radiotherapy as a single method or in combination are used in treatment of lung cancer [2]. High systemic toxicity and drug resistance limit the successful outcomes of treatment in most cases [3]. New strategies need to be developed to control and treat cancer.

Phytochemicals, plant secondary metabolites are known for their antioxidant and anticancer activities [4]. Cruciferous vegetables are key sources of different phytochemicals. Rutin, a flavonoid found in buckwheat, the buds of the Chinese herb Sophora japonica and propolis, fruits, vegetables especially red grape juice, green tea, soy and many other legumes was reported to have antiproliferative and anticancer properties in various cell lines [5-7]. Isothiocyanates allylisothiocyanate, benzyl isothiocyanate, phenethyl isothiocyanate (AITC, BITC and PEITC) present in substantial quantities in brassicaceae vegetables like mustard, garden cress, water cress, and broccoli (especially broccoli sprouts) are considered to be potential chemo preventive compounds [8-10].

Combinational chemotherapy using phytochemicals with known anticancer properties in combination with chemotherapeutic drug is considered to be one of the new strategies to reduce the systemic toxicity of chemotherapeutic agents and enhance the therapeutic efficacy [11-13].

The present study focuses on developing new combinational cancer therapy strategy to decrease toxicity of the chemitherapeutic drug, Etoposide. The study was conducted to analyzethe chemo preventive efficacy of cruciferous phytochemicals-rutin, allylisothiocyanate (AITC), benzylisothiocyanate (BITC), phenethylisothiocyanate (PEITC) singly and in combination with a conventional semi-synthetic chemotherapeutic drug etoposide human lung cancer cell lines A-549 and N-460.to enhance therapeutic efficacy and reduce toxicity on normal tissues.

MATERIALS AND METHODS

Cell Lines and Culture conditions

N-460 (human large cell carcinoma lung cell line), A549 (human alveolar basal epithelial carcinoma cell line), were obtained from the National Centre for Cell Science, Pune, India. All cell lines were grown in a DMEM culture media supplemented with 2 mM L-glutamine, 10% (v/v) heat inactivated FBS, penicillin (50 IU/ml) and streptomycin (50µg/ml) at 37°C in a humidified incubator with a 5% CO_2 atmosphere and were pass aged weekly twice to maintain a sub confluent state [14].

Cell treatment

The cell lines were incubated with phytochemicals Rutin, Allylisothiocyanate (AITC), Benzylisothiocyanate (BITC), Phenethylisothiocyanate (PEITC) with 10mM concentration singly and in combination with chemotherapeutic agent etoposide at concentrations ranging from 2- 50µg in a final volume of 100µl of DMEM culture media. The cells were incubated for 6hrs, 12h, 24h and 48h. Cells were also treated with etoposide alone. Cells exposed to 0.1% DMSO (dimethylsulfoxide) served as controls.

Cell viability studies-MTT Assay

Cell viability was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay, which is based on the reduction of MTT by mitochondrial dehydrogenase of intact cells to a purple formazan product [15]. 100 µl of 0.4mg/ml MTT in PBS was added to each treated cell line and incubated in the dark for 4h. After incubation period, formed formazan crystals were solubilised by addition of 100µl of DMSO and kept in an incubator for 4h. Amount of formazan was determined by measuring absorbance at 540 nm using an ELISA plate reader. Mean ± SD was calculated and reported as the percentage of growth verses control. The data was presented as percent post treatment recovery (% live cells) whereas absorbance from non-treated control cells was defined as 100% live cells. The percent recovery (% live cells) was plotted (Y-axis) against concentration (X-axis) of phytochemicals, where IC_{50} values could be interpolated from the graph. Cells grown in media containing an equivalent concentration of DMSO served as negative control.

Morphological studies- phase contrast microscopy

Equal number of cells ($1x10^5$) per well were seeded in 60mm dishes and incubated at 37°C in a CO_2 incubator with appropriate concentrations (0-25µg/ml) of phytochemicals (Rutin, AITC, BITC, PEITC) and chemotherapeutic drug Etoposide alone and in combination at concentrations of 50% of their respective IC_{50} values for 24hrs and 48hrs. The morphological differences in the control and treated cells were observed under inverted phase contrast microscope.

Propidium iodide staining fluorescent microscopy.

Propidium iodide (PI) staining method was used to observe apoptotic morphological changes in cells treated with phytochemicals and etoposide. Cells were cultured in 6 wells plate and treated with phytochemicals and etoposide (0-25µg/ml) alone and in combination and were incubated for 24hrs at 37°C in a CO_2 incubator. After incubation period, cells were washed with PBS, fixed in absolute alcohol for 30 minutes at 4°C, rehydrated with PBS and incubated with 100µl of propidium iodide (25µM) at 37°C for 5 minutes. Photomicrographs were taken under a fluorescent microscope.

DNA Fragmentation assay-Diphenylamine method.

Quantity of DNA fragmented in cancer cells was determined by Diphenylamine DNA fragmentation assay [16]. Cells were cultured in a 6-well plate and were treated with different concentrations (0 – 25 µg/ml) of phytochemicals and etoposide alone and in combination for 24 h at 37°C in a CO_2 incubator. Both treated and untreated cells were lyzed and homogenized in a lysis buffer containing 10mM Tris-HCl, 1mM EDTA, 0.5% Triton X-100, pH 8.0. Homogenates were centrifuged at 27,000g for 20 min to separate high molecular weight chromatin from cleavage products. Diphenylamine reagent (DPA 1.5g + 1 ml Conc.H_2SO_4 + 100 ml glacial acetic acid + 0.50 ml 2% acetaldehyde) was added to the collected pellet and supernatant. The reaction mixture was incubated for 16 – 20h at 36°C and read at 600 nm.

Qualitative Analysis of DNA Fragmentation-DNA Ladder Assay

DNA laddering was detected by isolating fragmented DNA using SDS/Proteinase K/RN ase extraction method which allows the isolation of only fragmented DNA without contaminating genomic DNA [17]. Lung cancer cell line N-460 cells ($5x10^6$ cells) were incubated at 37°C with BITC, PEITC (10µm, 25µm, 50µm) for 24h. Treated cells were washed in cold PBS and lysed in a buffer containing 50mM Tris/Hcl (PH 8.0),1mM EDTA and 0.2% Triton X-100 for 20 min at 4°C and centrifuged at 14000g for 15 minutes. The supernatant collected was treated with proteinase K (0.5mg/ml) and 1% SDS for 1hr at 50^0C. DNA was extracted twice with phenol and precipitated with 140mM NaCl and 2 volumes of ethanol at -20°C overnight. DNA precipitates were washed twice in 70% (v/v) ethanol and dissolved in TE buffer and treated for 1h at 37°C with RNase A. DNA preparations were electrophoresed in 1% agarose gels and visualized under UV light.

Statistical analysis

The results were expressed as mean ± SD (standard deviation). (n=3)

RESULTS & DISCUSSION

Cell Viability studies- MTT Assay

Phytochemicals alone and in combination with etoposide showed a dose and time dependent growth inhibitory effect on two human cancer cells with varying intensities. The compounds showed significant growth inhibitory activity within 24hrs on cell lines and the effect reached maximum at 48hrs. As shown in Table 1 phytochemicals exhibited substantial growth inhibition and percent inhibition was in the range of 40-90 %. IC_{50} values of each compound clearly indicated the chemo preventive efficacy of all phytochemicals. It was observed that a combination of phytochemical + etoposide at 50% of their IC_{50} value has a growth inhibitory activity (80-85%) against all cells as illustrated in Table 2. Phytochemicals in combination with etoposide displayed equal growth inhibition, thus minimizing the side effects of

conventional chemotherapeutic drug. Maximum inhibition of 87% was observed in N-460 cell lines on treatment with BITC+ etoposide. Phytochemicals + etoposide combination at concentration of 50% of their respective IC_{50} values was further used in all experiments for understanding the molecular mechanism leading to growth arrest and cell death. Some isothiocyanates were reported to effect cell proliferation and viability [18]. AITC was compared to be less effective than PEITC with IC 50 values of AITC and PEITC of 5 and 10 µM were reported against A-549 cell lines [19]. Rutin with IC50 values 27.18±0.59 µg/ml exhibited potent cytotoxic effect [20].

Table 1: IC_{50} values in µg/µM concentrations of Phytochemicals on A-549and N-460 cell lines for 24h and 48h.

S.No	Phytochemical	A-549		N-460	
		24hr	48hr	24hr	48hr
1	RUTIN	40.5 ±1.02	18.3 ±2.54	38.8 ±2.35	16.1 ±2.31
2	AITC	42.7 ±1.69	18.8 ±2.69	42.5 ±1.20	18.5 ±2.40
3	BITC	42.4 ±1.41	17.0 ±2.41	24.2 ±1.87	15.6 ±2.10
4	PEITC	43.8 ±1.02	17.7 ±2.0	25.1 ±2.52	16.9 ±1.39
5	ETOPOSIDE	16.7 ±1.84	7.9 ±2.36	18.3 ±2.62	7.6 ±1.32

Each value represented as Mean±SD (n =3); AITC (allylisothiocyanate), BITC (benzylisothiocyanate), PEITC (phenethylisothiocyanate)

Morphological studies -Phase contrast microscopy.

N460 lung cancer cells treated with a combination of phytochemical + etoposide at 50% of their IC_{50} values for 24hrs and 48hrs showed significant morphological changes indicative of cell death and growth inhibition as compared to untreated cells. The results indicated that phytochemical affected the spreading and elongation of N460 cells leading to rounded cell morphology and detachment of cells from the culture plate. N460 cells have undergone changes in nuclear morphological characteristic of apoptosis i.e. loss of adherence, cell shrinkage, apoptotic bodies formation and nuclear fragmentation prior to permeability change of plasma membrane. Microscopic images of control cells and treated cells were shown as Fig 1 (A, B, C&D). Control cells were observed to be viable and healthy.

Table 2: Cell inhibition caused by combination of phytochemicals + etoposide and etoposide on A-549, N-460 human cancer cell lines at 50µg concentration.

S. No	Photochemical+Etoposide (concentrations at 50% of their IC_{50} values	Percentage of cell inhibition at 50µg concentration			
		A-549		N-460	
		24hr	48hr	24h	48hr
1	Rutin +Etoposide	56.7 ±2.30	79.2±2.04	64.8±1.45	81.6±2.68
2	AITC +Etoposide	64.9±1.26	80.3±2.87	68.5±1.69	83.5±2.65
3	BITC +Etoposide	71.1±2.69	81.7±1.64	72.3±1.20	87.6±2.10
4	PEITC +Etoposide	69.7±1.05	80.8±1.22	69.6±1.47	85.3±1.60
5	Etoposide	74.6±1.69	84.3±1.37	72.1±2.85	86.5±1.0

Each value represented as Mean±SD (n =3); AITC (allylisothiocyanate), BITC (benzylisothiocyanate), PEITC (phenethylisothiocyanate)

Propidium iodide staining flourescent microscopy.

N-460 cells incubated with combination of phytochemical + etoposide at 50% of their IC_{50} concentrations for 24hrs and 48hrs were stained with propidium iodide and observed under fluorescent microscopy. Fragmentation and condensation of chromatin was observed in treated cells. Untreated cells showed normal nuclear morphology with diffused chromatin structure as shown in Fig 2. The normal untreated cells have a clear and uniform nucleus while apoptotic cells have a dense chromatin [21]. Dietary phytochemicals were reported to induce apoptosis in cancer cell lines [22, 23]. Earlier report showed that the combination of chemotherapy drugs with antioxidants restores the natural antioxidants in the body which are often depleted by chemotherapy [24].

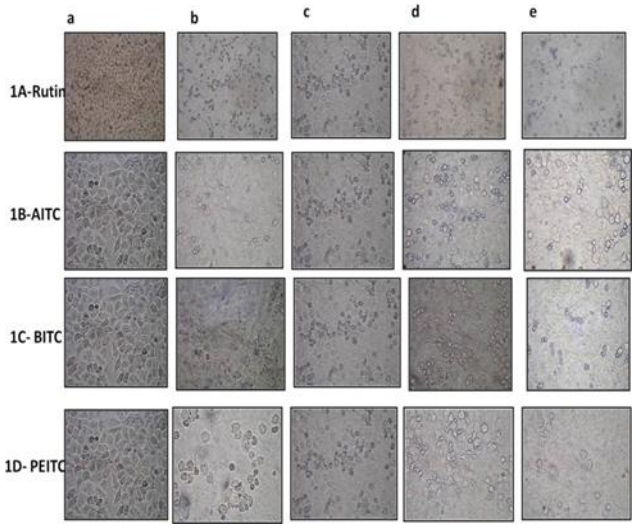

Figure 1: A, B, C&D:

Morphological changes, characteristics of apoptosis, induced in N-460 cells following treatment with- **1A**-Rutin, **1B**- AITC (allylisothiocyanate),, **1C**- BITC (benzylisothiocyanate), and **1D** PEITC (phenethylisothiocyanate).**a**) control cells (treated with 0.1% DMSO); **b**) 48 hrs Rutin/AITC/BITC/PEITC treated cells; **c**) 48hrs Etoposide treated cells; **d)** 24hrs Rutin/AITC/BITC/PEITC + Etoposide treated cells; **e**) 48hrs Rutin/AITC/BITC/PEITC + Etoposide treated cells. (Magnification x40)

The results of Phase contrast and fluorescent microscopy showed the same characteristic features of apoptosis like formation of apoptotic bodies, nuclear condensation and detachment proving the cytotoxic effect of the phytochemicals rutin, AITC, BITC and PEITC singly and in combination with drug etoposide in time dependent manner.

DNA fragmentation assay by diphenylamine method.

The extent of DNA fragmentation which is a hall mark of apoptosis was quantified by diphenylamine reaction on N-460 lung cancer cells. This assay was employed on ovarian cancer cells after treatment with a cisplatin and taxol [25]. Results of Diphenylamine fragmentation assay were given in Table 3 indicate DNA fragmentation

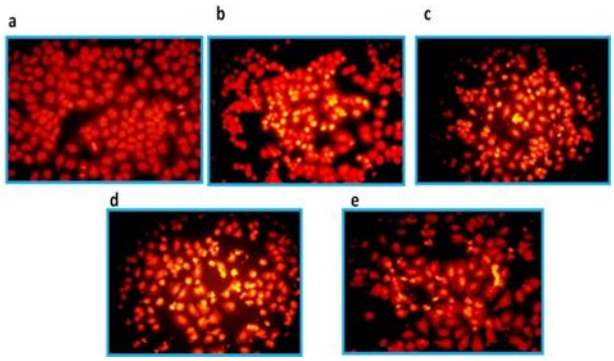

Figure 2: Propidium Iodide Staining Fluorescent Microscopy

induced in a concentration dependent manner. The combination treatment of phytochemical + etoposide at concentrations of 50% of their IC_{50} values was observed to be highly effective on N-460 cells causing upto 89% DNA fragmentation or oxidative damage. Etoposide treated cells were taken as a positive control.

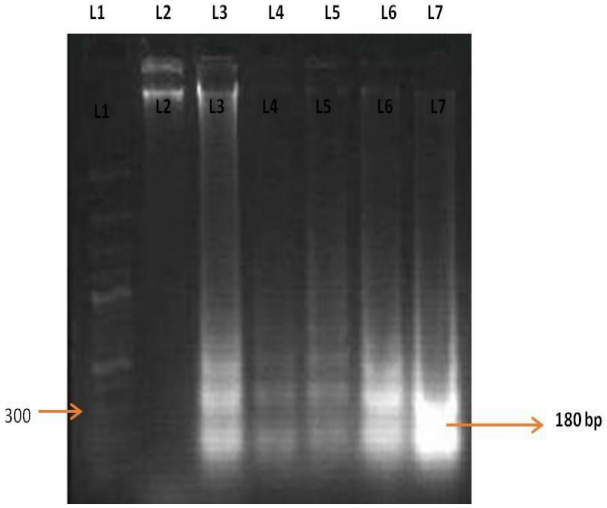

Figure 3: DNA ladder assay of N-460 cells showing the fragmentation of DNA. Lane1-Marker; Lane2-Control; Lane3- etoposide (15µM) treated cells as positive control; Lane4- BITC, benzylisothiocyanate (40µM) treated cells; Lane5-PEITC, phenethylisothiocyanate (40µM) treated cells; Lane6- (20 µM) BITC + (7.5 µM) etoposide; Lane7- (20 µM) PEITC + (7.5 µM) etoposide treated cells showing fragmented DNA due to Apoptosis.

Comparative Analysis of Phytochemicals.

The results of cell viability assays and DNA fragmentation assays of all the four phytochemicals in single and in different combinations with etoposide showed that the phytochemicals have chemo preventive efficacy on two cancer cell lines. Of all the combinations, phytochemical + etoposide combination at 50% of their IC_{50} values exhibited maximum cell proliferation inhibition and DNA fragmentation (Table 4).

Morphological alterations, characteristics of apoptosis, induced in N-460 cells following treatment with phytochemicals and Etoposide in combination at concentration of 50% of their IC_{50} values **a**, control cells (treated with 0.1% DMSO); **b**, cells treated with Rutin + Etoposide for 24 hr; **c,** cells treated with AITC (allylisothiocyanate) + Etoposide for 24 h; **d,** cells treated with BITC (benzylisothiocyanate) + Etoposide for 24 h; **e,** cells treated with PEITC (phenethylisothiocyanate) + Etoposide for 24 hr. Cells were visualized under a fluorescent microscope after propidium iodide staining (magnification x 200).

Table 3: Diphenylamine DNA fragmentation assay analysis phytochemicals –Rutin, AITC, BITC, PEITC, Etoposide and PEITC + etoposide of on A-549 and N-460

Cell lines	DNA fragmentation (%)					
	Phytochemical-Rutin DNA fragmentation (%)					
	Rutin25µg	Rutin50µg	Etoposide25µg	Etoposide50µg	(R+E) 25µg	(R+E) 50µg
A549	44.32±1.78	49.31±2.90	63.60 ± 1.41	71.53 ± 1.87	66.56 ± 2.24	72.84 ± 3.13
N460	47.25±3.10	51.95±1.63	62.92 ± 2.39	70.80 ± 2.14	67.20 ± 3.02	73.75 ± 1.97
Phytochemical-AITC DNA fragmentation (%)						
	AITC 25µg	AITC 50µg	Etoposide25µg	Etoposide50µg	(A+E) 25µg	(A+E) 50µg
A549	39.25±2.18	46.83±3.21	63.60 ± 1.41	71.53 ± 1.87	60.27 ± 1.85	70.37 ± 2.15
N460	45.59±1.85	51.28±2.37	62.92 ± 2.39	70.80 ± 2.14	65.18 ± 2.17	73.05 ± 1.42
Phytochemical-BITC DNA fragmentation (%)						
	BITC 25µg	BITC 50µg	Etoposide25µg	Etoposide50µg	(B+E) 25µg	(B+E)50µg
A549	39.13±1.23	52.34±1.58	63.60 ± 1.41	71.53 ± 1.87	68.37 ± 2.13	80.22 ± 1.68
N460	39.20±2.35	55.10±2.11	68.50± 1.49	76.4 ± 1.15	71.90 ± 2.19	89.41 ± 1.76
Phytochemical-PEITC DNA fragmentation (%)						
	PEITC25µg	PEITC50µg	Etoposide25µg	Etoposide50µg	(P+E) 25µg	(P+E) 50µg
A549	39.13±1.72	50.79±2.01	63.60 ± 1.41	71.53 ± 1.87	67.12 ± 1.39	78.95 ± 2.13
N460	38.62±2.10	54.71±2.58	68.50± 1.49	76.40 ± 1.15	70.63 ± 1.73	87.35 ± 1.59

Each value represented as Mean±SD (n =3); Each value represented as Mean±SD (n =3); AITC (allylisothiocyanate), BITC (benzylisothiocyanate), PEITC (phenethylisothiocyanate)

Table 4: Comparative Analysis of combination treatment of phytochemicals and etoposide on A-549and N460 cell lines.

Cell Lines	Phytochemicals+ Etoposide			
	Rutin +Etoposide	AITC + Etoposide	BITC + Etoposide	PEITC + Etoposide
Cell Viability Assay (IC 50 in µg/µM)				
N-460	7.2	7.1	6.6	6.6
A-549	7.6	7.6	6.8	7.0
DNA Fragmentation Assay (%)				
N-460	73.7	73	89	87
A-549	72.8	70	80	78

AITC (allylisothiocyanate), BITC (benzylisothiocyanate), PEITC (phenethylisothiocyanate)

Among the four phytochemicals, BITC and PEITC in combination with etoposide exhibited 87%-89% of DNA fragmentation on N-460 cell lines with 13.3µM and 13.7µM IC_{50} values. Ally isothiocyanates are desirable chemo preventive agents and also higher dosages exhibit lower cytotoxic effects [32].

Rutin (810µM) was reported to induce cell damage after 28 hours on treatment on hepatic cells [33]. Studies on multiple cancer cell lines have reported isothiocyanates inhibit cell proliferation and induce apoptosis [34]. Etoposide, an anti-cancer drug induce DNA damage thereby promote apoptosis of cancer cell lines, hence a combination of etoposide and isothiocyanates exhibited

decrease in cell proliferation thereby enhancing apoptosis of lung cancer cell line. Therefore, dietary phytochemicals given in combination with conventional drugs like etoposide might act synergistically in increasing the efficacy of cancer treatment by minimising adverse effects.

CONCLUSION

Combinational therapy will prove to be less expensive than monotherapy due to its significant savings i.e., lower treatment failure, slower development of resistance and consequently less money needed for the development of new drugs. In the present study, phytochemicals rutin, AITC, BITC and PEITC alone and in combination with etoposide drug showed chemo efficacy on lung cancer cell line N460 and at the same time decreases the toxic effects of drug on normal tissues and can become a cancer treatment strategy for multi-drug resistant cancers. These combinations can be further explored to understand its mechanism of action at molecular level in animal models followed by clinical trials.

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