



3, 3'-DIINDOLYLMETHANE AND 5-FLUOROURACIL ACT SYNERGISTICALLY TO PROMOTE APOPTOSIS AND MODIFY OXIDANT-ANTIOXIDANT STATUS ON HUMAN CERVICAL CANCER (HELA) CELLS

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ABSTRACT

Combined regimen is the most effective treatment strategy in malignancy to overcome drug toxicity and drug induced resistance. The effect of 3, 3'-diindolylmethane (DIM) in combination with 5-fluorouracil (5-FU) against the cervical cancer cells is reported here. The antioxidant activity of DIM was studied in some *in-vitro* antioxidant models showed encouraging results. The cell viability was determined by MTT assay to find IC₅₀ value of DIM (3.9 µg/mL), 5-FU (1.95 µg/mL) and in combination of DIM with 5-FU (0.97 µg/mL) against HeLa cells, also conclude scavenging activity, DNA fragmentation, oxidant, antioxidant activity. The interaction between DIM and 5-FU on human cancer HeLa cells showed that the combination index (CI) values ranging from 0.30 to 0.37 which indicates synergistic effect. The combination of DIM with 5-FU inhibited the proliferation of cervical cancer HeLa cells. Moreover, the level of lipid per-oxidation and enzymatic, non-enzymatic antioxidant activity also modulated. These results suggest that the combined regimen of DIM with 5-FU may be potential chemotherapy for cervical cancer.

KEYWORDS: Cervical cancer, 3, 3'-Diindolylmethane, 5-fluorouracil, HeLa cells, Apoptosis.

1. INTRODUCTION

Cancer of the cervix is the second most common female genital cancer in developing countries where every year about 500,000 women acquire the disease according to the WHO report. National cancer institute has estimated that 12,340 new cases and about 4,030 deaths will be attributed to cervical cancer in the United States in 2014.^[1] These alarming statistics highlight the importance of cancer prevention to use novel strategies. As a chemotherapy agent, 5-fluorouracil (5-FU), an active metabolite of capecitabine, is widely used in combination with other agents improves the overall and disease-free survival of patients after surgery.^[2] Cruciferous vegetables contain a precursor phytochemical glucosinolate that undergoes hydrolysis by the plant enzyme myrosinase, yielding a bioactive compound identified as indole-3-carbinol (I3C). I3C is an unstable compound in aqueous and acidic, medium which is rapidly converted to numerous condensation products. A major *in vivo* condensation product of I3C is DIM.^[3] Both I3C and DIM have been shown to protect against many neoplasms, including prostate, colon cancer, cervical and breast cancer.^[1,4-6]

It has now increasingly believed that natural compounds, such as DIM, induce apoptosis in human cancer cells

without causing unwanted toxicity, could be useful in combination with conventional chemotherapeutic agents for the treatment of human malignancies.^[7] In addition to anti-cancer properties, DIM has pleiotropic effects on immune function. Cho et al 2008 have demonstrated the anti-inflammatory effects of DIM, which can suppress pro-induction of LPS-induced pro-inflammatory mediators.

However, 5-FU related toxicity is a serious and common issue for many cancer patients with myelosuppression, mucositis, dermatitis, diarrhea and gastrointestinal toxicity which are the most commonly observed side effects. The combination of 5-FU with nontoxic phytochemical from vegetables improves the efficacy of chemotherapy by reducing its toxicity. Natural products acting in synergy with commercial drugs to bring about an agnostic or enhance effect are well documented.^[8] Therefore, an effort has been made in our study to analyze whether the combination of DIM with 5-FU enhances anticancer activity.

2. MATERIALS AND METHODS

2.1 Materials

3,3'-Diindolylmethane (DIM), 5-fluorouracil (5-FU), thiobarbituric acid (TBA), phenazinemethosulphate

(PMS), nitroblue tetrazolium (NBT), 3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl- tetrazolium bromide (MTT), minimal essential medium (MEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), ethylene diamine tetra acetic acid (EDTA) was purchased from Sigma Aldrich Mumbai, India. All other chemicals used in this study were of analytical grade.

2.2 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) radical cation decolorisation assay

The generation of the ABTS^{•+} radical cation forms the basis of one of the spectrophotometric methods that has been applied for the measurement of the total antioxidant activity of solutions of pure substances.^[9] The assay employs an ABTS^{•+} radical cation generated from ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). ABTS was dissolved in H₂O at the concentration of 7Mm. The stock solution was mixed with 2.5Mm potassium per sulphate. Blue-green colored ABTS radical cation (ABTS^{•+}) was produced by reacting ABTS stock solution with potassium persulfate solution and allowing the mixture to stand in the dark at room temperature for 24 h before use. Cuvettes were incubated at 37°C in a dry bath for 30 min and the percentage (%) decrease in the observance was read at 734 NM using a Bio-Rad Smart Spec 3000 spectrophotometer. A total volume of 5 mL mixture has contained 0.54 mL of ABTS^{•+}, 0.5 mL of phosphate buffer and varying concentration of DIM (10, 20, 30, 40 and 50 µg/mL). The blank has contained water instead of DIM. The absorbance of the sample were compared with standard ascorbic acid as per the formula given below

$$\text{Scavenging activity (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

2.3 2, 2-Diphenyl-1-picrylhydrazyl (DPPH[•]) radical scavenging assay

The percentage of antioxidant activity (AA %) of each substance was assessed by DPPH free radical assay. The measurement of the DPPH radical scavenging activity was performed according to the methodology described by Shirwaikar *et al.*^[10] The samples were reacted with the stable DPPH radical in an ethanol solution. The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol and 0.3 mL of DPPH radical solution 0.5 mM in ethanol. When DPPH was reacted with the antioxidant compounds which was reduced due to their

H-donating ability. The reaction mixture containing 1 mL of 0.1 mM DPPH, various concentrations of test compound DIM (10, 20, 30, 40 and 50 µg/mL) were made up to 3 mL with water. The changes in color (from deep violet to light yellow) were read at 517 nm after 10 min of reaction using a UV-VIS spectrophotometer.

$$\text{Scavenging activity (\%)} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

2.4 Cell lines and cell culture

The human cervical cancer cell line (HeLa), were obtained from National Centre for Cell Science (NCCS), India. The cells were cultured in Minimum Essential Medium (MEM), containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The cell were maintained in flasks under standard conditions, incubation at 37°C and 5% CO₂. Duration subculture, cells were detached by trypsinization when they reached 80% confluence and split (1:2) growth medium was changed every 3days.

2.5 Cytotoxicity Assay

MTT assay was first proposed by Mossman *et al.*^[11] MTT assay, the yellow tetrazolium salt was metabolized by NAD-dependent dehydrogenase (in active mitochondria) to form a dark blue formazan product. MTT assay was performed 24 h after transfection. For this purpose, MTT solution was prepared at 1 mg/ml in PBS and was filtered through a 0.2 µm filter. Then, 50 µl of MTT plus 200 µl of DMEM without phenol red were added into each Cuvette. Cells were incubated for 4 h at 37°C with 5% CO₂, 95 % air and complete humidity. After 4 h, the MTT solution was removed and replaced with 200µl of DMSO and 25 µl Sorenson's glycine buffer (glycine 0.1M, NaCl 0.1M, pH: 10.5 with 0.1 NaOH). The Cuvette was further incubated for 5 min at room temperature. The visible cells showed the purple color formation. The optical density (OD) of the wells was determined using a UV-visible spectrophotometer at a wavelength of 570 NM.

The effect of the samples on the proliferation of HeLa was expressed as the percentage cell viability, using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Control optical density} - \text{Test optical density}}{\text{Control optical density}} \times 100$$

Experimental study protocol

Group I	Control (Untreated HeLa cells)
Group II	HeLa cells + DIM (3.9 µg/mL)
Group III	HeLa cells + 5-FU (1.95 µg/mL)
Group IV	HeLa cells + DIM + 5FU (0.48 + 0.48 = 0.97 µg/mL)

2.6 Data analysis for the combination treatment

The interaction of DIM with 5-FU was evaluated by the isobolographic analysis.^[12] The studies were performed over a range of concentration of each individual DIM and also in combination with 5-FU at a fixed

concentration ratio (1:1). Combination studies were based on the IC₅₀ values of the compounds when used alone, as recognized in preliminary experiments. Median effect analysis using the combination index (CI) was employed to determine whether the interaction of the

compounds was attentive, antagonistically or synergistically.^[13] For fifty percent (50%) toxicity the CI values were calculated based on the equation stated below

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$$

(Dx)₁=Dose of drug 1-produce 50% cell kill alone; (D)₁=Dose of drug 1-produce 50% cell kill in combination with (D)₂; (Dx)₂=Dose of drug 2- 50% cell kill alone; (D)₂=Dose of drug 2-produce 50% cell kill in combination with (D)₁. A CI of <1 would indicate synergy and a CI of >1 would mean antagonism.

2.7 Apoptotic DNA fragmentation

Apoptotic DNA fragmentation assay provides a qualitative method for assessing cell death by detecting DNA fragments using agarose gel electrophoresis.^[14] HeLa cells were treated with DIM (3.9 µg/mL), 5-FU (1.95 µg/mL) and DIM with 5-FU (0.48 + 0.48 = 0.97 µg/mL). Untreated and treated cells were centrifuged at 200 Mg at 4°C for 10 min. The supernatant was carefully transferred in new tubes. 0.5 mL of TE buffer with point 0.2% Triton X-100 (TTE) solution was added to the pellet, tubes were vortexed vigorously. This procedure allows the release of fragmented chromatin from nuclei, after cell lysis (due to the presence of Triton X-100 in the TTE solution) and disruption of the nuclear structure. To separate fragmented DNA from intact chromatin, tubes were centrifuged at 20,000 xg for 10 min at 4°C. The supernatant was carefully transferred in a new 0.5 mL of TTE solution was added to the pellet. 0.1 mL of ice-cold 5 M sodium chloride (NaCl) was added and vortexed vigorously. The addition of the salt should be able to remove histones from DNA. 0.7 mL of ice-cold isopropanol was added to each tube and vortexed vigorously. The precipitation was allowed to proceed overnight at -20°C. After drying DNA was dissolved in TE buffer and separated by 2 % agarose gel electrophoresis at 100 V for 50 min and DNA damage was analyzed by gel documentation. The electrophoresis were stopped when the dye reaches about 3 cm from the end of the gel. DNA was visualized by placing the gel on a UV-trans illuminator and photos of the gel was taken.

2.8 Lipid Peroxidation and anti antioxidant

Addition of lipid peroxides in the cell is associated cellular stress, which leads to cancer cell death. The cells were harvested by trypsinization, the cell pellet obtained from suspended in PBS. The suspension was taken for biochemical estimations. The level of lipid peroxidation in the cancer cells was determined by analyzing thiobarbituric acid reactive substances (TBARS).^[15] 0.5 mL of sample was diluted to 0.5 mL with double distilled water and mixed well and then 2 mL of TBA-TCA-HCL reagent was added. The mixture was kept in a boiling water bath for 15 min, after cooling, the tubes were centrifuged at 1000 xg for 10 min and the supernatant was estimated. In this method, malondialdehyde and

other TBARS react with TBA in an acidic condition to generate a pink color chromosphere which was read at 535 nm. The pink chromosphere and breakdown products formed by the reaction of 2-TBA was measured. The value was expressed as mole/mg of protein for cell line.

The enzymatic and non-enzymatic antioxidant properties were studied by using the following assays:

a. Superoxide dismutase (SOD)

SOD in HeLa cell suspension assayed activity by the method of kakkar et al. SOD in the cancer cells were assayed based on the 50% inhibition of the formation of nicotinamide adenine dinucleotide (NADH) - Phenazine methosulphate (PMS) - Nitroblue tetrazolium (NBT) complex at 520 nm.^[16] 0.5 mL of supernatant was diluted to 1 mL with water followed by the addition of 2.5 mL of ethanol and 1.5 mL of chloroform. This mixture was shaken for 90 Sec at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of phenazinemethosulphate and 0.3 mL of NBT and appropriately diluted enzyme preparation in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH. After incubation at 30°C for 90 sec, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 mL n-butanol. The mixture was allowed to stand for 10 min, centrifuged and n-butanol layer was separated. The color density of the chromogen in n-butanol was measured at 510 nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard condition was taken as 1 unit. The specific activity of the enzyme was expressed as Unit/min/mg of proteins in HeLa cells.

b. Catalase (CAT)

The activity of catalase in the cell suspension was determined by the methods of Sinha. CAT activity was measured as the decrease in hydrogen peroxide H₂O₂ concentration by recording the absorbance at 620 NM.^[17] CAT play a key role in the detoxification of superoxide anion and hydrogen peroxide (H₂O₂), respectively, thereby protecting against oxygen-free radicals (OFR) induced damage.^[18] HeLa cell suspension were prepared by using PBS. To 0.9 mL of phosphate buffer, 0.1 mL of HeLa cell and 0.4 mL of hydrogen peroxide was added. The reaction was arrested after 15, 30, 45 and 60 sec by adding 2 mL of dichromate acetic acid mixture. The tubes were kept in a boiling water bath for 10 min, cooled and the color developed was read at 620 NM. The specific activity was expressed as µmol of H₂O₂ consumed/min/mg of protein in HeLa cells.

Glutathione peroxidase (GPx)

GPx activity was assayed according to the method described by Rotruck et al.^[18] The assay mixture consisted of phosphate buffer, EDTA, sodium azide, GR

(3 units), GSH, NADPH and H₂O₂. Oxidation of NADPH was recorded at 340 nm. Reduced glutathione (GSH) in conjunction with GPx plays a central role in the defense against free radicals, peroxides and a wide range of xenobiotics and carcinogens.^[19] To 0.2 mL of Tris buffer, 0.2 mL of EDTA and 0.1 mL of sodium azide was added to 0.5 mL of HeLa cell supernatant was added. To the mixture, 0.2 mL of GSH followed by 0.1 mL of H₂O₂ was added. The contents were mixed well and incubated at 37°C for 10 min, along with a control containing all reagents except homogenate. After 10 min, the reaction was arrested by the addition of 0.5 mL of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH by the method of Ellman. The activity was expressed as μg of GSH consumed/ min/mg protein for cell line.

d. Glutathione (GSH)

GSH levels were estimated by the method as mentioned by Ellman *et al.*^[20] GSH estimation was based on the development of a yellow color when 5, 5-dithio (2-nitrobenzoic acid) was added to compounds containing sulfhydryl groups. A known amount of enzyme preparation was incubated with H₂O₂ in the presence of GSH for a specified time period. The amount of H₂O₂ utilized was determined by the method of Ellman *et al.*^[20] 0.5 mL of HeLa cells was taken and precipitated with 2 mL of 5% TCA. 2 mL of supernatant was taken after centrifugation and 1 mL of Ellman's reagent and 4 mL of 0.3 M disodium hydrogen phosphate were added. The yellow color developed was measured at 412 nm. A series of standards (20–100 μg) were treated in a similar manner along with a blank containing 1 mL of buffer. The amount of glutathione was expressed as mg/dL protein.

2.9 Statistical analysis

Statistical analysis was performed by SPSS software Version 16 (SPSS Inc., Chicago, IL, USA) and by ANOVA. IC₅₀ values for all the above experiments were determined by linear regression analysis. Results were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 Free radical scavenging effect of DIM

The effects of the duration of interaction of specific antioxidants on the suppression of the absorbance of the ABTS^{•+} radical cation at 734 nm for ascorbic acid, the standard reference compound, compared with DIM (Fig. 1A). The scavenging capacity of the DIM was 54% at 50 μg and 50% at 30 μg in ascorbic acid.

The purple color of the free DPPH radical decays, a change that can be followed spectrophotometrically at 517 nm. Based on the data obtained from this study, DIM was an effective free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which may limit free radical damage occurring in the human body. (Fig. 1B) illustrates that the radical scavenging reaction of ascorbic acid with DPPH was,

essentially, instantaneous. On the other hand, the radical scavenging reaction of DIM with DPPH was also quite fast but slower as compared to that with ascorbic acid. The effect of ascorbic acid at the concentration of 40 $\mu\text{g}/\text{mL}$ shows maximum radical scavenging activity, but DIM at the concentration of 50 $\mu\text{g}/\text{mL}$ shows maximum radical scavenging activity.

3.2 Antiproliferative effect of DIM and 5-FU

The combined regimen effect of DIM and 5-FU on cell proliferation was determined by MTT assay. The proliferation of HeLa cells was significantly inhibited by combination treatment. The inhibitory effect was observed after 48 h incubation. The inhibitory concentration 50 (IC₅₀) value of DIM (Fig. 2A) and 5-FU (Fig. 2B) were 3.9 and 1.95 $\mu\text{g}/\text{mL}$ at 48 h, respectively. However, the maximum inhibition of cell growth was observed in combination therapy of DIM (0.48 $\mu\text{g}/\text{mL}$) with 5-FU (0.48 $\mu\text{g}/\text{mL}$) (IC₅₀ = 0.97 $\mu\text{g}/\text{mL}$) (Fig. 2C) treated HeLa cells in comparison with the single agent therapy groups (DIM or 5-FU). This IC₅₀ value was used for following experiments.

3.3 Analysis of the in vitro cytotoxicity of DIM and 5-FU combination

The combined effects of DIM and 5-FU on cell proliferation of HeLa cells were analyzed using isobologram method. The CI values against HeLa cell line at 3 concentrations of DIM in combination with 5-FU indicated synergistic (CI= 0.30-0.37) interactions. Our findings showed that the combination of DIM and 5-FU revealed the synergistic inhibitory effect on HeLa cells was analyzed using MTT assay. Our findings suggest that DIM and 5-FU combination therapy might be an effective for the inhibition of cervical cancer cell growth owing to their synergistic efficacy.

3.4 DNA fragmentation

Fig 3 shows the DNA fragmentation of HeLa cells on agarose gel electrophoresis was examined. HeLa cells were treated with DIM and 5-FU for 48 h at the concentration of 3.9 $\mu\text{g}/\text{mL}$ and 1.95 $\mu\text{g}/\text{mL}$ respectively. Based on the data obtained from this study, we found that DNA fragmentation was significantly increased with DIM and 5-FU. In combination therapy, DIM with 5-FU (0.97 $\mu\text{g}/\text{mL}$) treated HeLa cells had undergone more DNA fragmentation than individual.

3.5 Changes in the levels of lipid peroxidation and antioxidant status

We measured lipid peroxidation indices (TBARS) in HeLa cell line. DIM with 5-FU (0.97 $\mu\text{g}/\text{mL}$) treatment increased the level of TBARS when compared to individual treatment of DIM (3.9 $\mu\text{g}/\text{mL}$) or 5-FU (1.95 $\mu\text{g}/\text{mL}$) (Fig. 4A). The activities of enzymatic antioxidant such as SOD and CAT (Fig. 4B, C) in normal cells, DIM, 5-FU and DIM+5-FU in HeLa cells. Consequently, DIM+5-FU (0.97 $\mu\text{g}/\text{mL}$) treatment decreased the activities of enzymatic antioxidants in HeLa cells when compared to DIM (3.9 $\mu\text{g}/\text{mL}$) and 5-

5-FU (1.95 µg/mL). DIM + 5-FU (0.97 µg/mL) treatment decreased the levels of GPx and GSH in HeLa cells. DIM with 5-FU enhanced in reducing the level of GPx and GSH in HeLa cells than DIM (3.9 µg/mL) and 5-FU (1.95 µg/mL) (Fig. 5A, B) Table. 1.

TABLE-1

Changes in the levels of lipid peroxidation and antioxidant status.

CAPTIONS FOR FIGURES

FIGURE. 1. Free radical scavenging assay. (A) ABTS free radical scavenging activity of DIM in comparison with ascorbic acid. (B) DPPH radical scavenging activity of DIM in comparison with ascorbic acid.

FIGURE. 2. Cytotoxicity assay. (A) Cytotoxic effects of DIM treatment on human cervical cancer HeLa cells. (B) Cytotoxic effects of 5-FU treatment on human cervical cancer HeLa cells.(C) Cytotoxic effects of DIM in combination with 5-FU on human cervical cancer HeLa cells.

FIGURE. 3. DNA fragmentation assay. M: Marker DNA, Lane 1: No DNA ladder formation was seen in untreated HeLa cells. Lane 2 and Lane 3: Treatment of HeLa cells with DIM or 5-FU for 48 h resulted in fragmentation of DNA indicates apoptosis. Lane 4: Combination therapy of DIM or 5-FU showed an enhanced cleavage of DNA into inter nucleosome fragments indicates more apoptosis when compared with DIM or 5-FU treated individual agent group.

FIGURE.4. Antioxidant activity in control and treated HeLa cell line. (A)TBARS, (B) SOD, (C) CAT.

FIGURE.5. Antioxidant activity in control and treated HeLa cell line. (A) GPx, (B) GSH.

LEGENDS FOR FIGURE

FIGURE.3

M: Marker DNA, Lane 1: No DNA ladder formation was seen in untreated HeLa cells.

Lane 2 and Lane 3: Treatment of HeLa cells with DIM or 5-FU for 48 h resulted in fragmentation of DNA indicate apoptosis. Lane 4: Combination therapy of DIM or 5-FU showed an enhanced cleavage of DNA into inter nucleosome fragments indicates more apoptosis when compared with DIM or 5-FU treated individual agent group.

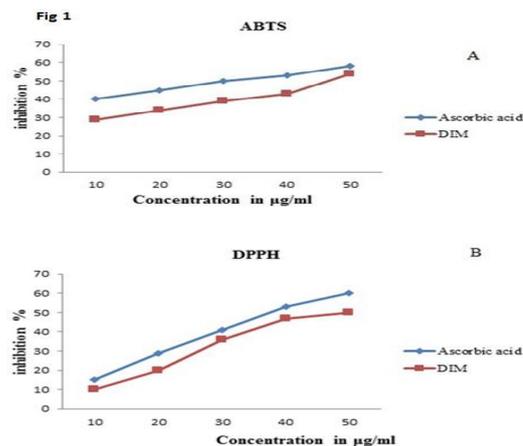
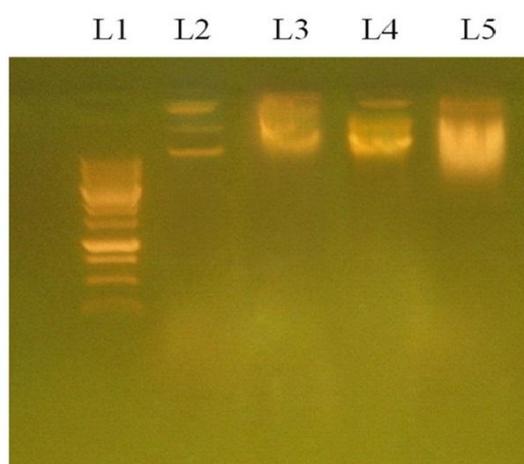
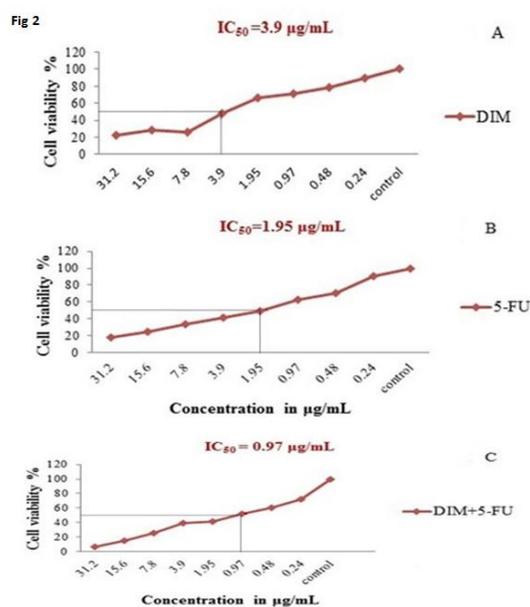


Fig 1



Lane 1 - Marker 1000 bp ladder
Lane 2 - Control
Lane 3 - 5-FU (1.95 µg/ml)
Lane 4 - DIM (3.9 µg/ml)
Lane 5 - DIM + 5FU(0.97 µg/ml)

Fig 3

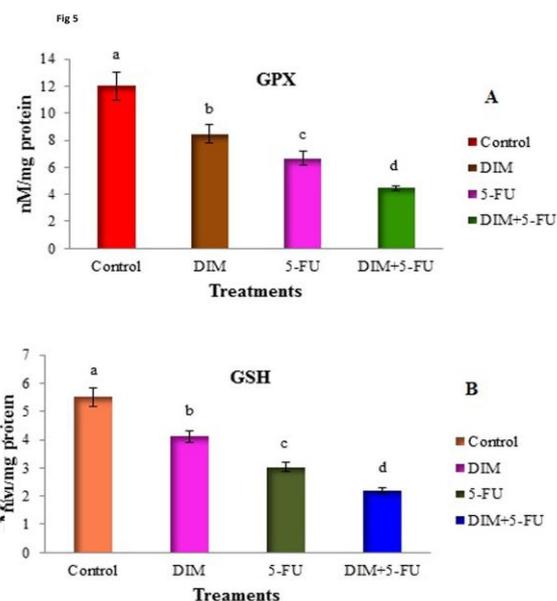
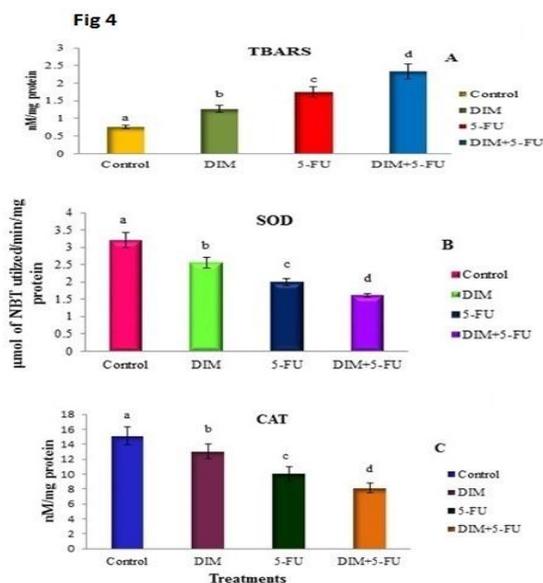


Table 1

Groups	Treatment	TBARS µg/mL	SOD µg/mL	CAT µg/mL	GPx µg/mL	GSH µg/mL
1	Control	0.75±0.05	3.20±0.22	15.09±0.61	12±1.00	5.51 ±0.31
2	DIM (3.9µg/mL)	1.27±0.1	2.55±0.15	13.05±0.99	8.44±0.68	4.11±0.21
3	5-FU(1.95µg/mL)	1.75±0.15	2.00±0.10	10.02±1.00	6.65±0.53	3.03±0.15
4	DIM+5-FU(0.97µg/mL)	2.33±0.21	1.61±0.14	8.14±0.62	4.44±0.21	2.19±0.09

4. DISCUSSION

Combination therapy is the most effective treatment strategy in cancer. The rationale for combination chemotherapy is to use drugs that work by different mechanisms of action, thereby decreasing the likelihood of development of resistant cancer cells. From a clinical perspective, it is desirable to concomitantly target these molecular abnormalities by using a combination therapy or an agent with pleiotropic effects to optimize therapeutic outcomes. Several experimental evidence for all common cancer sites have indicated that intake of fruits and vegetables and a number of other dietary items are associated with decreased cancer incidence.^[21] DIM is a major *in vivo* acid catalysed condensation product of I3C and a plant-derived, non-toxic, dietary agent that selectively kills cervical cancer cells without affecting normal cells.^[22] 5-Fluorouracil belongs to the class of chemotherapy drugs known as anti-metabolites. It interferes with cells making DNA and RNA, which stops the growth of cancer cells but it has associated with various side effects. An alternative strategy, 5-FU can be combined with nontoxic natural compound which may enhance the efficacy of chemotherapy with reduced toxicity to normal cells. For this reasons, we selected DIM to examine its potential as such an agent *in vitro*. *In vitro* results using various methods showed that DIM and 5-FU alone are quite effective in suppressing the proliferation and inducing apoptosis but two agents together are much more effective. ABTS assay is an excellent tool for determining the antioxidant activity.

The ABTS free radical assay can be used to measure the antioxidant activity of broad diversity of substance.^[23] The scavenging activity of the DIM on free radical ABTS, generated by potassium persulfate was compared with a standard. Moreover, the radical scavenging activity was evaluated by the DPPH assay. In the present study, DIM showed a concentration dependent scavenging of DPPH and ABTS radical, which may be attribute to its hydrogen-donating ability. For compound DIM in DPPH model, their IC₅₀ were 50% higher like vitamin C, due to their hydrogen-donating ability with the presence of two N-H group as an H-donating group necessary to react with free radical and slightly less potent than the standard antioxidant. Previous study confirmed that I3C and DIM in the DPPH and β-carotene model were pure antioxidant compared to vitamin C.

Recent studies demonstrated that DIM has reached an important position in the research works aimed at understanding the mechanisms of antiproliferative processes in tumour cell lines, especially in human breast cancer cells.^[24] The cytotoxic effect of DIM and 5-FU was evaluated by MTT assay. In the present study, HeLa cell lines were pre-treated with DIM (3.9 µg/mL), 5-FU (1.95 µg/mL) and in combination with DIM and 5-FU (0.97 µg/mL) in order to test its cytotoxic effect. The decrease in cell proliferation and cell facility at slightly high concentrations of DIM and 5-FU can be interconnected not merely to the toxic consequences on the metabolism of the cells or DNA by direct interaction,

but also to the induction of apoptosis. In combination therapy, our results showed the higher concentration of DIM and 5-FU because of significant in DNA damage and induce apoptosis when compare to individual treatments.

The current in vitro results show that the combination of DIM with 5-FU is highly synergistic against HeLa. Previous studies demonstrated that the DIM is found to enhance the cytotoxic effect of paclitaxel against human breast cancer cells.^[25] In agreement with their earlier studies, the interaction between DIM and 5-FU on human cancer HeLa cells shows that the combination index (CI) values ranging from 0.30 to 0.37 which indicates synergistic effect. Treatment of HeLa cells with DIM or 5-FU for 48 hours resulted in fragmentation of DNA. Combination therapy showed an enhanced cleavage of DNA into inter nucleosome fragments indicates more apoptosis when compared with individual treatment of DIM or 5-FU group. However, there was no DNA ladder formation seen in untreated HeLa cancer cells. Our present findings also support the recent study that DIM caused accumulation of DNA strand breaks in cervical cancer cell lines.^[26,27]

The marker of lipid peroxidation, TBARS was measured in treatment with DIM and 5-FU alone and also DIM in combination with 5-FU treated HeLa cells. The lipid peroxidation levels were significantly increased on treatment with both DIM and 5-FU treated HeLa cells. Significant increases in lipid per-oxidation indices in combination of both drugs treated cancer cells were observed. Combined therapy of two drugs enhanced the resistant to lymphocyte DNA damage caused by an oxidant challenge with H₂O₂ which suggests that combination regimen possess potential pro-oxidant action when treated on cancer cells.^[28] SOD is the first antioxidant enzyme to deal with oxy radicals by accelerating the dismutation of superoxide radicals (O₂^{•-}) to hydrogen peroxide (H₂O₂). CAT acts as a preventive antioxidant and plays an important role in protection against the deleterious effects of lipid peroxidation. Similarly, GPx has a well-established role in protecting cells against oxidative stress and this in turn requires glutathione as a cofactor. GPx catalyses the oxidation of GSH to GSSG at the expense of H₂O₂.^[29] In the current study, we observed a significantly decreased in the activity of enzymatic antioxidants that is SOD, CAT and GPx in DIM, 5-FU and combination of both DIM and 5-FU treated cancer cells. Many studies suggested that antioxidant enzymes were critical in protecting against tumour promoting agents. Interestingly, cell malignancy or transformation is often accompanied by a decrease in activity of antioxidants like SOD, CAT and GPx which increases the cellular sensitivity to pro-oxidant compounds.^[30] A non enzymatic antioxidant system such as GSH is considered as the second line of defence against free radicals. GSH a major non-protein thiol, presumed to be an important endogenous defence against per oxidative destruction of cellular membranes.

Glutathione reacts directly with ROS and electrophilic metabolites, protects essential thiol groups from oxidation and serves as a substrate for several enzymes including GPx.^[31] These findings can lead to new treatment strategies and could also pave the way in the reduction of the amount of anticancer agents required as a therapeutic dose.

5. CONCLUSIONS

This proof of our work summarizes, that the combined treatment of DIM and 5-FU initiated the cancer cell death by inducing apoptosis, modulate antioxidant status and DNA fragmentation in HeLa cervical cancer cell line. When compared to individual treatment combination therapy is the most effective treatment strategy in cancer. The rationale for combination chemotherapy is to use drugs that work by different mechanisms of action, thereby decreasing the likelihood of development of resistant cancer cells. 5-FU is associated with side effects such as myelosuppression, mucositis, resistance, dermatitis, cardiac toxicity and is ineffective in subjects with dihydropyrimidine dehydrogenase deficiency. As an alternative strategy, 5-FU can be combined with relatively nontoxic DIM a natural phytochemical which may enhance the efficacy of chemotherapy with reduced toxicity to normal cells also DIM has pleiotropic effects on immune function, therefore a combined treatment of DIM with 5-FU against cancer cell line was found to be effective to overcome the side effect of 5-FU. DIM with 5-FU in contradiction of cervical cancer by exciting chemical protecting effect along with reduced toxicity and drug induced resistance. Thus the present study strengthens our hypothesis and hope that this novel formulation could possibly overcome the current limitations of 5-FU and can open a new avenue for cervical cancer therapy.

REFERENCE

1. American Cancer Society (ACS). (2013, Apr.-Last revised).Cervical cancer: detailed guide. Accessed Aug,20, from <http://www.cancer.org/cancer/cervicalcancer/detailedguide>.
2. Mach over D. A comprehensive review of 5-fluorouracil and leucovorin in patients with metastatic colorectal carcinoma. *Cancer*, 1997; 80: 1179–87.
3. Sarkar FH, Li Y, Wang Z, Kong D.The role of nutraceuticals in the regulation of Wnt and Hedgehog signalling in cancer. *Cancer Metastasis Rev.*, 2010; 29: 383-94.
4. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH. Indole-3-carbinol (I3C) induced cell growth inhibition G1 cell cycle arrest and apoptosis in prostate cancer cells.*Oncogene*, 2001; 20: 2927-2936.
5. Kim EJ, Park SY, Shin HK, Kwon DY, Surh YJ, Park JH. Activation of caspase-8 contributes to 3, 3'-Diindolylmethane-induced apoptosis in colon cancer cells. *J Nutr*, 2007; 137: 31-36.

6. Wang Z, Yu BW, Rahman KM, Ahmad F, Sarkar FH. Induction of growth arrest and apoptosis in human breast cancer cells by 3, 3'-diindolylmethane is associated with induction and nuclear localization of p27 kip. *Mol Cancer Ther*, 2008; 7: 341-9.
7. Sarkar FH, Li Y. Harnessing the fruits of nature for the development of multi-targeted Cancer therapeutics. *Cancer Treat Rev.*, 2009; 35: 597-607.
8. Ma J, Shi M, Li G, Wang N, Wei J, Wang T. Regulation of Id1 expression by epigallocatechin-3-gallate and its effect on the proliferation and apoptosis of poorly differentiated AGS gastric cancer cells. *Int J Oncol*, 2013; 43: 1052-8.
9. Benabadji SH, Wen R, Zheng JB, Dong XC, Yuan SG. Anticarcinogenic and antioxidant activity of diindolylmethane derivatives. *Acta Pharmacol sin*, 2004; 25: 666-71.
10. Shirwaikar A, Prabhu KA, Punitha IS. In vitro antioxidant studies of *Sphaeranthus indicus* (Linn). *Indian J Exp Biol*, 2006; 44: 993-6.
11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Method*, 1983; 65: 55-63.
12. Menendez JA, del Mar Barbacid M, Montero S, Sevilla E, Escrich E, Solanas M. Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells. *Eur J Cancer*, 2001; 37: 402-13.
13. Hemaiswarya S, Doble M. Combination of phenylpropanoids with 5-fluorouracil as anti-cancer agents against human cervical cancer (HeLa) cell line. *Phytomedicine*, 2013; 20: 151-8.
14. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, 1980; 284: 555-6.
15. Rosarin FS, Arulmozhi V, Nagarajan S, Mirunalini S. Antiproliferative effect of silver nanoparticles synthesis using amla on Hep2 cell line. *Asian Pac J Trop Med*. 2013; 6: 1-10.
16. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys*, 1984; 21: 130-2.
17. Sinha AK. "Colorimetric assay of catalase". *Anal Biochem*, 1972; 47: 389-94.
18. Rotruck JT, Pope A.L, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WC. Selenium biochemical role as component of glutathione peroxidase. *Science*, 1973; 179: 588-90.
19. Rajneesh CP, Manimaran A, Sasikala KR, Adaikappan P. Lipid Peroxidation and antioxidant status in patients with breast cancer. *Singapore Med J*, 2008; 49: 640-643.
20. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*, 1959; 82: 70-7.
21. Keck AS, Finley JW. Cruciferous vegetables: cancer protective mechanisms of glucosinolate hydrolysis products and selenium. *Integr Cancer Ther*, 2004; 3: 5-12.
22. Del Priore G, Gudipudi DK, Montemaroo N, Restivo AM, Malanowska-Stega J, Arslan AA. Oral 3,3'-diindolylmethane (DIM): pilot evaluation of a nonsurgical treatment for cervical dysplasia. *Gynecol Oncol*, 2010; 116: 464-7.
23. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med*, 1999; 26: 1231-7.
24. Maciejewska D, Rasztawicka M, Wolska I, Anuszevska E, Gruber B. Novel 3,3'-diindolylmethane derivatives: synthesis and cytotoxicity, structural characterization in solid state. *Eur J Med Chem*, 2009; 44: 4136-4147.
25. McGuire KP, Ngoubilly N, Neavyn M, Lanza-Jacoby S. 3,3'-Diindolylmethane and Paclitaxel Act Synergistically to Promote Apoptosis in HER2/Neu human breast cancer cells. *J Surg Res.*, 2004; 132: 208-13.
26. Banerjee S, Wang Z, Kong D, Sarkar FH. 3, 3'-Diindolylmethane enhances Chemosensitivity of multiple chemotherapeutic agents in pancreatic cancer. *Cancer Res.*, 1999; 69: 5592-600.
27. Maruthanila VL, Poornima J, Mirunalini S. Attenuation of Carcinogenesis and the Mechanism Underlying by the Influence of Indole-3-carbinol and Its Metabolite 3, 3'-Diindolylmethane: A Therapeutic Marvel. *Adv Pharmacol sciences*, 2014; 2014: 832161.
28. Choi EJ, Kim GH. 5-Fluorouracil combined with apigenin enhances anticancer activity through induction of apoptosis in human breast cancer MDA-MB-453 cells. *Oncol Rep.*, 2009; 22: 1533-7.
29. Cerutti P, Ghosh R, Oya Y, Amstad P. The role of the cellular antioxidant defence in oxidant carcinogenesis. *Environ Health Perspect*, 1994; 102: 123-129.
30. Sergediene E, Jonsson K, Szymusiak H, Tyrakowska B, Rietiens IM, Cenas N. Prooxidant toxicity of polyphenolic antioxidants to HL-60 cells: description of quantitative structure-activity relationships. *FEBS Lett*, 1999; 462: 392-6.
31. Saravanan N, Nalini N. Antioxidant effect of *Hemidesmus indicus* on ethanol induced hepatotoxicity in rats. *J Med Food*, 2007; 10: 675-82.