

Cytotoxic Effect of *Syzygium aromaticum* Extract and Gemcitabine on Human Cervical Cancer Cell Line

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Abstract: In fact, cervical cancer is one of the most common carcinomas in the world among women and is highly radio-resistant and chemo-resistant. For locally advanced disease, radiation is mixed with low-dose chemotherapy; however, this modality often results in severe toxicity. Prevention of cancer through dietary intervention recently has received an increasing interest, and dietary agents have not only become significant chemopreventive, but also therapeutic agents. Emerging evidences suggest that cancer preventative agents (such as clove, *Syzygium aromaticum*) might be mixed with chemotherapy or radiotherapy for the more potential treatment of cancer. In this study, we monitored that treatment of human cervical carcinoma cell line, HeLa with ethanolic clove extract in combination with gemcitabine synergistically led to a significant dose-dependent reduction in cell viability. It is noteworthy that application of clove extract increased the efficacy of gemcitabine and interestingly, it was found to be minimally toxic to normal cells. Besides, these results provide new insights into the probable involvement of a novel mechanism in the synergistic impact of this putative combination, ultimately, leading to an effective modality in the treatment of cervical cancer.

Keywords: Clove, cytotoxicity, chemoprevention, chemotherapy, gemcitabine, *Syzygium aromaticum*.

INTRODUCTION

Chemotherapy plays a critical role in the local and regional control of malignant tumors. However, its efficacy can be limited by a number of factors, inclusive of enhanced normal tissue injury, drug resistance, toxicity, and enhanced side effects [1-4]. Recent trials to upgrade the therapeutic index of chemotherapy and minimizing its cytotoxicity on normal tissues have focused on employing conventional chemopreventive agents as biological response modifiers, leading to better survival [5-8].

Employment of certain specific dietary agents has received the importance owing to their relatively safe toxicity profiles and their significance as chemosensitizers [7, 9]. *Syzygium aromaticum* (Clove) has been observed to be a potent chemopreventive agent, used by the traditional Ayurvedic healers of India since ancient times to treat respiratory and digestive ailments [10, 11]. Various studies have reflected that clove has antiviral properties which can be exploited in the treatment of cervical cancer as viruses like herpes simplex virus (HPV) have been implicated in its etiology [11, 12]. Besides, clove also possesses both anti-inflammatory and antioxidant properties [13]. A classic pharmacologic strategy would be the more widespread use of low-dose combinations of chemotherapeutic and chemopreventive agents, with the aims of achieving a therapeutic

synergy between individual drugs [14]. These combinations may exert enhanced antitumor activity through synergic action. The combination treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies because lower doses could be used [7, 15, 16].

Taking into the account the above mentioned facts, we decided to test the potential anti-inflammatory and anti-cancer effects of clove extract in combination with a chemotherapeutic drug, gemcitabine to monitor the synergistic pharmacological effect of the combination to enhance the efficacy of chemotherapy. Hence, further research along these notions and objectives could provide new insights in improving the life pattern of millions of people who undergo intensively painful periods of sufferings with current chemotherapy.

MATERIALS AND METHODS

This study was conducted in the Department of Biotechnology, College of Engineering & Technology, Moradabad, UP, India, during July, 2008 to June, 2009, in collaboration with Physiology & Climatology Division, Indian Veterinary Research Institute, Izatnagar, U.P., India and Halberg Hospital and Research Institute, Moradabad, U.P., India. Human cervical carcinoma cell line, HeLa used in this study was maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The cell line was grown in 5% CO₂ at 37°C in a humidified incubator. Short term culture of lymphocytes (isolated from healthy non-smoking donors), was established to evaluate the effect of clove and gemcitabine. Lymphocytes were isolated using HiSep Media (HiMedia, India) as

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per the manufacturer's instructions) [17]. Briefly, fresh blood was collected in heparinised collection vials and HiSep media was added to blood in the ratio 1:3 (media:blood) and centrifuged at 160 x g for 20 min. The lymphocytes were then separated into fresh tube and equal volume of FBS was added. This was again centrifuged at 140 x g for 15 min for removal of HiSep Media. A second wash in FBS was given followed by centrifugation at 140 x g for 15min. The pellet was resuspended in RPMI media, counted and plated in triplicates in 96-well microplates.

A stock solution of 133 mM (40 mg/ml) of Gemcitabine (Intas Biopharmaceuticals, India) was prepared in 2% DMEM media and further diluted to desired concentrations in the range 1-100 mM for treatment.

The finely powdered clove was weighed (0.50 g), and extracted in 2 mL of 50% ethanol in water for a week at 4°C. The extract, thus, obtained was centrifuged at 180 x g for 20 min. Supernatant was collected and filtered using 0.2µm filter (Whatmann Inc. UK). Dilutions of crude extract (0.5 mg/ml – 8 mg/ml) were prepared in 2% DMEM [18].

HeLa cells were harvested and counted using hemocytometer (Marienfeld, Germany). ~7000 cells/well were plated (in triplicates) in 96-well microtiter plates. Subsequent to the 24 h incubation, the cells were exposed to different concentrations of gemcitabine (1-100 mM) or clove extract (0.5 -5 mg/ml) /gemcitabine (5 mM) alone, clove extract (2 to 4 mg/ml) alone and their combinations for 24 h. MTT (Sigma-Aldrich) (final concentration 0.5 mg/ml) was added to each well at an appropriate time and incubated for 2 to 4 h. Viable cells have intact mitochondria and dehydrogenases present therein convert the tetrazolium salt to insoluble formazan violet crystals [19]. The formazan crystals were dissolved in 100 µl of DMSO (Sigma, Aldrich). The absorbance was read at 570 nm using an Absorbance Microplate Reader (BioTek, U.S.A). Similarly, lymphocytes were also treated with gemcitabine (5 – 100 mM) or clove extract (0.5-8 mg/ml) for 24 h. MTT assay was then performed as described above. All the experiments were repeated at least three times. The cell viability percentage was calculated as follows:

$$\text{Cell Viability \%} = \frac{\text{Average } A_{570} \text{ of individual test group}}{\text{Average } A_{570} \text{ of controls}} \times 100$$

Average A_{570} of controls

Morphological changes in HeLa cells elicited by gemcitabine and clove extract were documented using normal inverted microscope (Labomed, USA) [20]. The optimized concentration of the respective drugs was used for the morphological studies. HeLa cells were treated with 40 mM gemcitabine or 2 mg/ml clove extract for 24 h. The untreated cells were used as negative control. Morphological alterations were visualized using normal inverted microscope 24 h post-treatment.

RESULTS

In the present study, HeLa cells were observed to show the growth inhibition in a dose-dependent pattern (Fig. 1) when treated with gemcitabine at concentrations ranging from 1-100 mM for 24h. Gemcitabine concentration at/or below 5 mM was not sufficient enough to induce intracellular apoptotic biochemical/molecular factors and ultimately to show antiproliferative effect. The LD₅₀ value of gemcitabine was found to be 35 mM with HeLa cells. The morphological changes observed using the normal inverted microscope show characteristic rounding of dying cells on treatment with 35mM gemcitabine for 24h compared to untreated control cells (Fig. 2a, b). Similar effect on cell viability was observed when lymphocytes were treated with gemcitabine with LD₅₀ at 50mM (Fig. 3). At this moment we can hypothesize that there might be certain biochemical pathway in normal lymphocytes, most probably involving heat shock proteins or the molecules of the similar activity, ultimately leading to potentially combat against antiproliferative effect of gemcitabine at the concentration of about 5 mM. The gemcitabine was slightly more toxic to cancer cells than normal lymphocytes. As cancer cells are highly proliferating, they show more sensitivity towards the cytotoxic drug because the drug directly causes damage at DNA level, impairing their cell cycle and thus causing more cancer cells to die at a lower concentration of the drug.

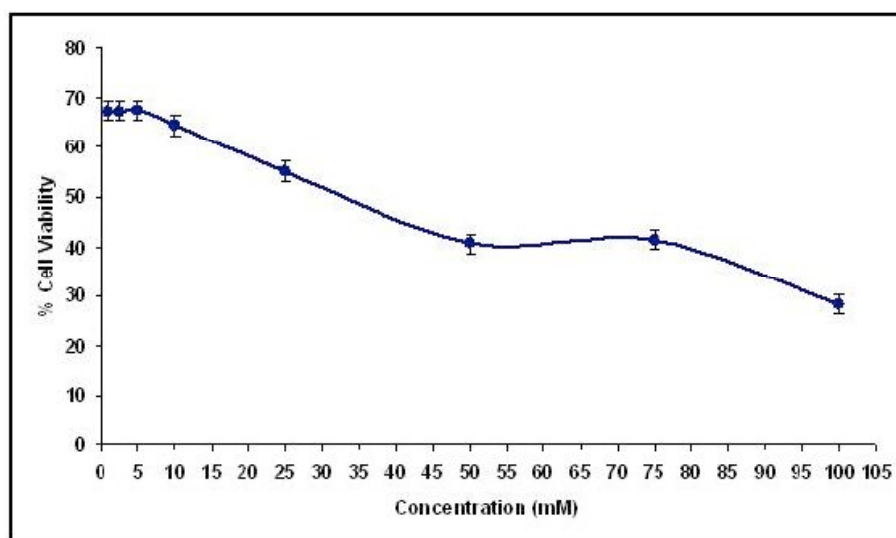


Fig. (1). Dose dependent curves of HeLa cells treated with Gemcitabine (1-100mM) for 24h.

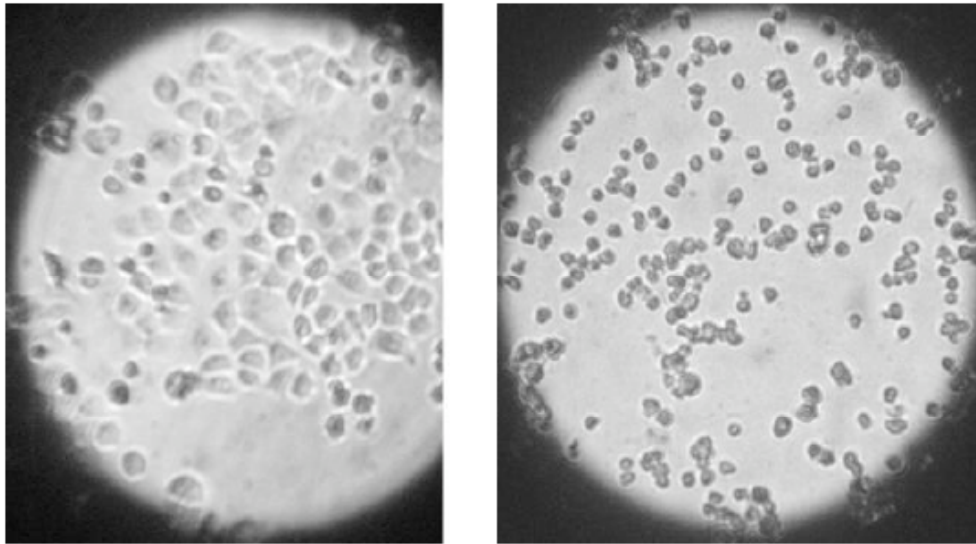


Fig. (2). Microscopic features of HeLa cells (a) before treatment and (b) after treatment with 35mM gemcitabine for 24h (Magnification 100X).

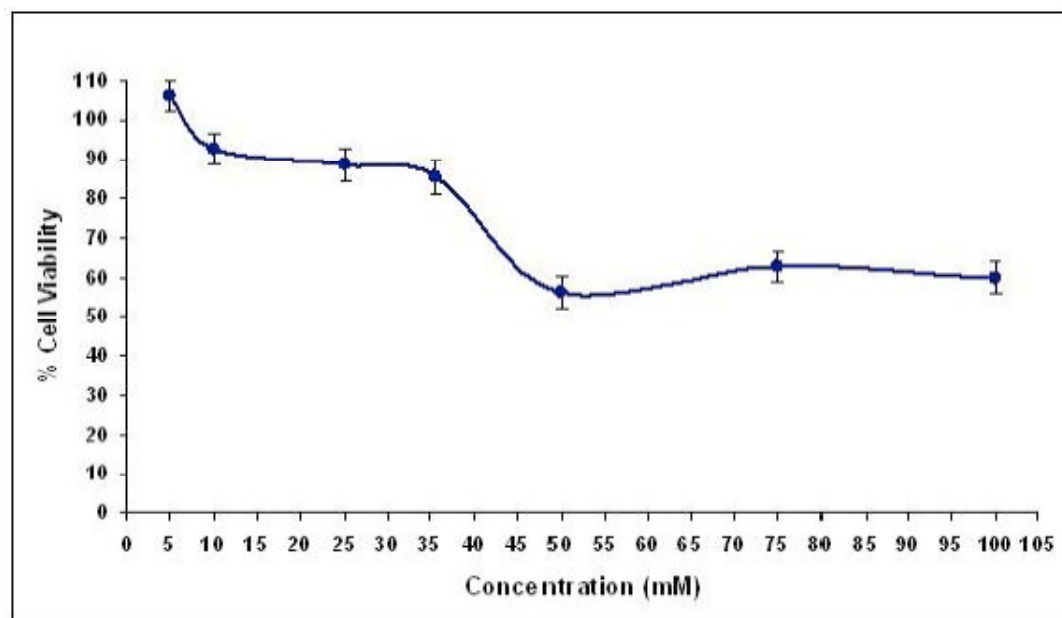


Fig. (3). Dose dependent curve of Gemcitabine (5-100mM) on lymphocytes treated for 24h.

Clove (*Syzygium aromaticum*) is a potent antioxidant, it has been hypothesized that its active components can result in differential cell death of cancer cells. HeLa cells treated with clove extract (0.5-5mg/ml) for 24h showed dose-dependent inhibition of cell growth (Fig. 4). The LD₅₀ of *S.aromaticum* extract on HeLa cells was found to be 2mg/ml for 24h. The morphological changes on treatment with 2mg/ml clove extract (for 24h) were examined using the normal inverted microscope. Clove extract treated cells showed characteristic rounding of dying cells compared to untreated control (Fig. 5a, b). It is well established that chemopreventive drugs have differential effect on cancer cells and normal cells *in vivo* and *in vitro*. In the light of this fact, we assessed the effect of *S.aromaticum* extract

on lymphocytes (as normal). Isolated lymphocytes treated with clove extract at varying doses of 0.5-8mg/ml for 24h (Fig. 6) showed no significant effect on cell viability. Our study re-affirms the cancer preventive properties of clove, and the fact that it has relatively less (or no) toxicity to normal cells.

In view of minimizing these problems concerning with chemotherapy as well as with an increase the efficacy of chemotherapeutic drugs, the authors hypothesized that chemopreventive agent such as clove (*S.aromaticum*) can be used in combination with chemotherapeutic drugs. To confirm this assumption, the combined effect of gemcitabine and *S.aromaticum* extract on HeLa cells was studied. Sub-lethal dose (5mM) of gemcitabine was employed in combi-

nation with increasing concentrations of *S.aromaticum* extract (2, 3 and 4mg/ml) to treat HeLa cells for 24h (Fig. 7). 5mM gemcitabine was used in combination with 2mg/ml of the clove extract resulted in significant decrease in cell viability (35.5%) compared to either of the compounds alone (75% and 62% respectively for gemcitabine and clove extract). Thus, sub-lethal doses of gemcitabine in combination with *S.aromaticum* extract increased effectiveness of the chemotherapeutic drug, hence, reducing its cytotoxicity. Similar experiment was also performed with normal cells (data not shown). There was no significant decrease in cell viability on treatment of lymphocytes with combination of sub-lethal dose of gemcitabine (5mM) and clove extract (2, 3 and 4 mg/ml).

DISCUSSION

In fact, cervical cancer is one of the most common cancers in women worldwide. Surgical resection and/or radia-

tion ablation or systemic chemotherapy is the main lines of treatment for cervical cancer, but post-treatment recurrence is quite frequent. All anti-cancer drugs currently in use are highly cytotoxic agents and may be toxic to normal cells specially to rapidly dividing cells like bone marrow, fetal cells, germ cells, hair follicles cells, intestinal cells, etc [3, 4]. Gemcitabine is a pro-drug and is metabolized intracellularly to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides. Gemcitabine exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and also blocking the progression of cells through the G₁/S phase boundary [21]. Owing to its inhibitory effect on DNA replication, gemcitabine has been toxic to normal cells as well. However, the effect of chemotherapeutic drugs on tumor cells is greater than that on normal cells [22]. In view of the previous studies, we attempted to confirm the differential effect of chemotherapeutic drug (Gemcitabine) on cancer and normal cells. We observed that the inhibition

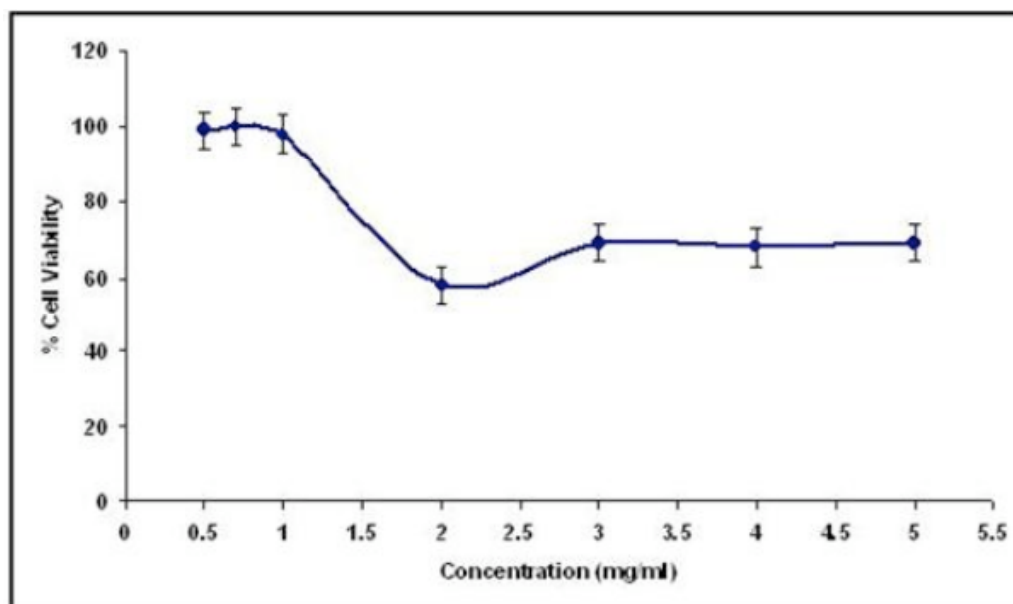


Fig. (4). Dose dependent curve of *S.aromaticum* extract (0.5-5mg/ml) on HeLa cells.

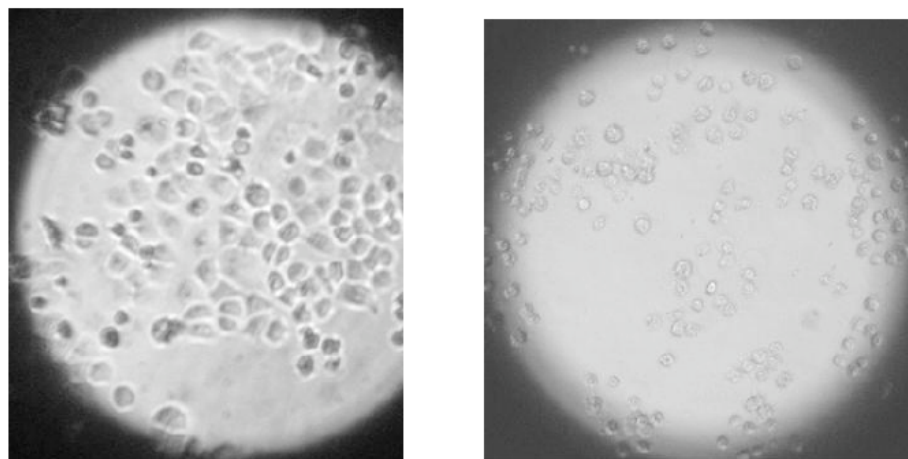


Fig. (5). Microscopic features of HeLa cells (a) before treatment and (b) after treatment with 2mg/ml clove extract for 24h (Magnification 100X).

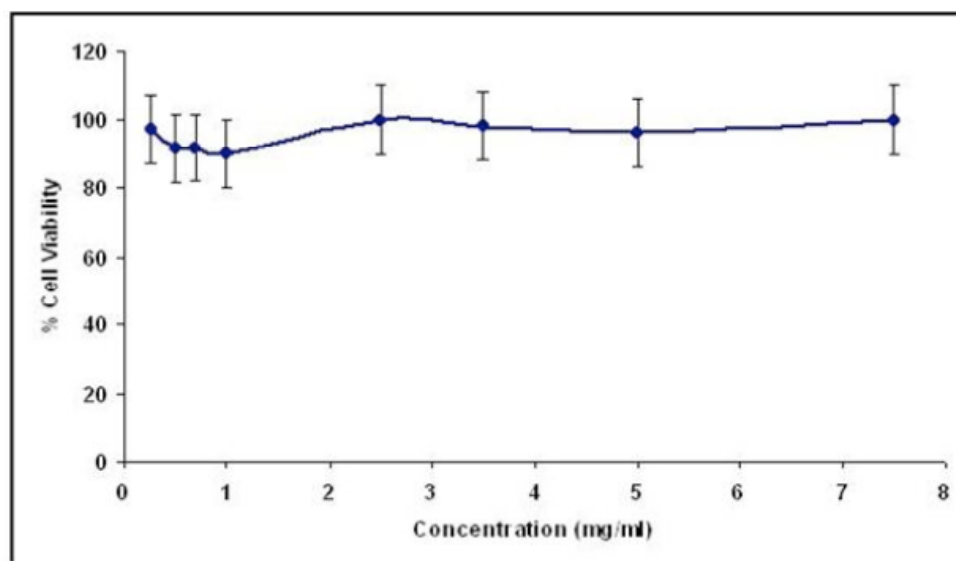


Fig. (6). Dose dependent curve of *S.aromaticum* extract (0.5-8mg/ml) treatment on lymphocytes for 24h.

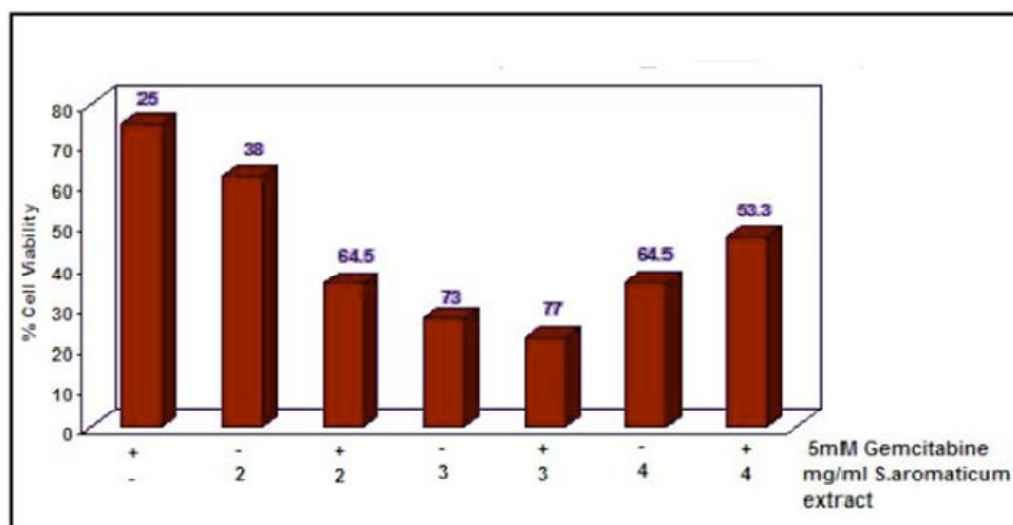


Fig. (7). Combination study of gemcitabine and *S.aromaticum*. HeLa cells were treated with *S.aromaticum* (2, 3 and 4 mg/ml) and gemcitabine (5mM) alone and in combination for 24h.

of growth of HeLa cells was in a dose-dependent manner; on treatment with same concentrations of Gemcitabine, growth inhibition was higher in cervical cancer cells than normal cells (Fig. 1). The LD₅₀ value of gemcitabine for HeLa (cervical cancer cell line) was determined to be 40 mM and for lymphocytes LD₅₀ was 50 mM. Moreover, microscopic examination also confirmed the cytotoxic effect of 40 mM gemcitabine on HeLa cells. The LD₅₀ value of gemcitabine was found to be 40 mM on HeLa cells. The morphological changes observed using the normal inverted microscope showed characteristic rounding of dying cells on treatment with 40 mM gemcitabine for 24 h compared to untreated control (Fig. 2a, b). Rather lesser antiproliferative effect on cell viability was observed when lymphocytes were treated with gemcitabine with LD₅₀ at 50 mM (Fig. 3). This study thus confirms that in addition to targeting the cancer cells, the chemotherapeutic drugs also affect the normal proliferat-

ing cells. Williams and coworkers have shown that tumor cells are more sensitive to cell death by chemotherapeutic drugs than normal cells [23]. This differential effect of gemcitabine on cervical cancer and normal lymphocytes can be attributed to their biochemical differences. Also, tumor drug selectivity may be based on differences in the cell kinetics of normal and cancer cells [21, 22, 24-26].

In addition to their therapeutic effects, chemotherapeutic drugs also have side-effects at physiological and cellular levels. Therefore, another approach that focuses on the minimizing the toxic effects and enhanced efficacy of the chemotherapeutic drugs is gaining a firm base to work on. An alternative and novel approach for the management of cancer is chemoprevention through the recommended intake of health protective food especially those present in vegetables, fruits, beverages and spices in daily diet [11, 27-31]. Various studies have shown that components of clove like

eugenol have apoptosis-inducing effects [7, 11, 32-34]. Clove (*Syzygium aromaticum*) is a potent antioxidant, it has been hypothesized that its active components can result in differential cell death of cancer cells. In the present study we observed a dose dependent decrease in cell viability of HeLa cells treated with 50% ethanolic clove extract at dose ranging from 0.5 -8 mg/ml for duration of 24h (Fig. 4). The LD₅₀ of *S.aromaticum* extract on HeLa cells was found to be 2 mg/ml for 24h. The morphological changes on treatment with 2mg/ml clove extract (for 24 h) were examined using the normal inverted microscope. Clove extract treated cells showed characteristic rounding of dying cells compared to untreated control (Fig. 5a, b). This is consistent with previous studies which also showed that the dose of 1.5 mg/ml was cytotoxic to the cancer cells [18]. This is the first time that the chemopreventive effect of clove extract has been studied in cervical cancer. In the light of this fact, we assessed the effect of *S.aromaticum* extract on lymphocytes (as normal). Isolated lymphocytes treated with clove extract at varying doses of 0.5-8 mg/ml for 24 h (Fig. 6) showed no significant effect on cell viability. However, when observed microscopically, more cell death was visible at these doses than doses below and at 2 mg/ml. Hence, the increase in absorbance (at 570nm) observed could be attributed to the color of the clove extract that interferes with absorbance. Our study re-affirms the cancer preventive properties of clove, and the fact that it has relatively less (or no) toxicity to normal cells.

To minimize these problems related to chemotherapy as well as to increase the efficacy of chemotherapeutic drugs, we hypothesized that chemopreventive agent such as clove (*S. aromaticum*) can be used in combination with chemotherapeutic drugs. To confirm this assumption, the combined effect of gemcitabine and *S.aromaticum* extract on HeLa cells was studied. Sub-lethal dose (5mM) of gemcitabine was used in combination with increasing concentrations of *S.aromaticum* extract (2, 3 and 4 mg/ml) to treat HeLa cells for 24h (Fig. 7). Gemcitabine at the concentration of 5 mM was used in combination with 2mg/ml of the clove extract resulted in significant decrease in cell viability (about 38%) compared to either of the compounds alone (approximately 75% and 60%, respectively, for gemcitabine and clove extract). Thus, sub-lethal doses of gemcitabine in combination with *S.aromaticum* extract increased effectiveness of the chemotherapeutic drug, hence, reducing its cytotoxicity. Similar experiment was also performed with normal cells (data not shown). There was no significant decrease in cell viability on treatment of lymphocytes with combination of sub-lethal dose of gemcitabine (5mM) and clove extract (2, 3 and 4 mg/ml). This finding strengthens our hypothesis to develop this property for augmenting the efficacy of cancer therapy while simultaneously reducing the toxicity on normal cells. Chemopreventive compounds exert the antitumor activities through regulation of different cell signaling pathways. Therefore, common cancer therapies combined with these dietary compounds may exert enhanced antitumor activity through synergistic action or compensation of inverse properties. The combination treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies because lower doses could be used [11, 12, 35, 36]. Studies have reported that genistein (a chemopreventive) *in vitro* potentiated growth inhibition and apoptotic

cell death caused by cisplatin, docetaxel, doxorubicin, and gemcitabine (chemotherapeutic drugs) in prostate, breast, pancreas, and lung cancers [7, 11, 37]. At this juncture, we report for the first time the proliferation-inhibiting and apoptosis-inducing effects of gemcitabine in combination with *S. aromaticum* extract *in vitro* on HeLa cells and lymphocytes. Our results showed that *S.aromaticum* enhanced the growth inhibitory effects of gemcitabine at sub lethal doses. The percentage cell death using combination of 5mM gemcitabine and 2mg/ml of *S.aromaticum* extract was significantly higher (about 65%) than the cell death when either of the compounds was used alone. A similar effect was observed with the combination of 3 mg/ml *S.aromaticum* extract and 5mM gemcitabine. This combination resulted in about 80% cell death which was more than the cell death caused by either of them alone. This *in vitro* study suggests that clove may serve as potent agent for enhancing the therapeutic effects of chemotherapy for the treatment of cervical cancer. These results are in line with the previously discussed studies which observed a synergistic crosstalk between these two probable therapies [7, 11]. However, on increasing the concentration of *S.aromaticum* extract to 4mg/ml, the absorbance was found to increase. Here again cell death was visible on microscopic observation and hence as discussed above, the increase in absorbance by MTT could be attributed to colour of the clove extract. Thus, we can infer that combination of gemcitabine with clove can be used effectively for overcoming the problems associated with chemotherapy. Conclusively, the major findings from the present study provide new insights into the probable involvement of a novel mechanism in the synergistic impact of this putative combination, ultimately, leading to an effective modality in the treatment of cervical cancer.

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