GROWTH INHIBITORY AND ADJUVANT THERAPEUTIC POTENTIAL OF AQUEOUS EXTRACT OF *TRITICUM AESTIVUM* ON MCF-7 AND HeLa CELLS

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Aim: The purpose of the present study is to evaluate the potent growth inhibitory effects of aqueous wheatgrass extract (AWE) alone and in combination with cisplatin on human breast and cervical cancer cells. *Materials and Methods*: The cytotoxic potential of AWE alone and in combination with cisplatin was evaluated on human breast and cervical cancer cells (MCF-7 and HeLa) by cell viability assay. Further, the mode of cell death induced by AWE was determined by nuclear morphological examination and cell cycle analysis. These effects were then correlated with the expression of genes involved in apoptosis and proliferation (cyclin D1 and Bax) by RT-PCR. *Results*: AWE showed dose- and time dependent selective cytotoxicity towards the cancerhighlighting its safe profile. Lower dose combinations of AWE and cisplatin induced increased growth inhibition compared with the individual drugs on both cell lines (combination index < 1) indicating strong synergistic interactions. AWE was found to induce apoptosis and arrested the cells at G_0-G_1 phase of the cell cycle which correlated with the modulation of expression of bax and cyclin D1 in a time-dependent manner in MCF-7 and HeLa cells. *Conclusion*: These results suggest that the anti-cancer potential of AWE may be due to apoptosis induction and its anti-proliferative properties. This study also provides the first evidence demonstrating synergism between AWE and cisplatin, which may enhance the therapeutic index of prevention and/or treatment of human breast and cervical cancer.
Key Words: Bax, cisplatin, combination, cyclin D1, synergistic, wheatgrass.

Cancer is a multistep and multifactorial disorder, involving aberrations in the genetic and epigenetic makeup of the cells, influenced directly and indirectly from the environmental factors which account for almost 90–95% of all cancer cases [1, 2]. Numerous phytonutrients, such as sulforaphane, genistein, resveratrol, curcumin, β-elemene etc. derived from edible plants are the foremost prospective agents reported to interfere with different stages of carcinogenesis and many other health conditions, including cardiovascular diseases, diabetes etc. Mounting scientific reports on these dietary agents corroborate that a diet rich in fruits and vegetables could reduce 7–31% of all cancers worldwide [3–5]. These phytonutrients act through multiple signalling pathways which make these agents important for cancer prevention and therapy. Concurrent therapies, utilizing these nutrients along with other standard cancer treatment modalities like chemotherapy, are one of the current thrusted areas to enhance the therapeutic index by synergistic or additive interactions between these drugs [6, 7].

Wheat (*Triticum aestivum*), one such phytonutrient, is an important part of human diet, and epidemiological studies have suggested its protective role against many chronic diseases including thalassemia, cancer etc [8–10]. A number of studies have used wheat in various forms such as whole grain, wheatgrass etc. (5–10 day old grass of the common wheat plant) [10–12]. Wheatgrass contains selenium, laetrile, vitamins C and E, beta carotene, ferulic acid and vanilic acid, phenolic compounds including flavonoids, the concentrations of which increase with the germination period [11, 12]. It has been shown to inhibit the metabolic activity of carcinogens and has anti-mutagenic and antioxidant activities and can reduce chemotherapy associated side-effects [13–18].

The present study was designed to evaluate the potent growth inhibitory effects of aqueous wheatgrass extract (AWE) alone and in combination with cisplatin on human breast cancer, MCF-7 and human cervical cancer, HeLa cell lines. Further, its effect on the modulation of genes involved in proliferation and apoptosis were analyzed to understand the chemopreventive mechanism of wheatgrass.

MATERIALS AND METHODS

Cancer cell lines. The effect of AWE alone and in combination with cisplatin was studied on human breast carcinoma cell line (MCF-7) and human cervical carcinoma cell line (HeLa) obtained courtesy of Dr. Tahir Rizvi, UAE University, Al Ain, UAE. The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Lymphocytes were isolated from healthy non-smoking donors using HiSep Media (HiMedia, India) as per the manufacturer's instructions and were maintained in RPMI media (Sigma, USA) [6].

Preparation of drug solutions. AWE was prepared as previously described [19]. Briefly, wheat seeds were purchased from the local market, washed with tap water, followed by distilled water. The seeds were soaked in distilled water for 9 h and transferred to containers with soil. The wheatgrass was collected on day 11. Only wheatgrass of uniform size, shape and without injuries were selected. The wheatgrass was washed, wiped and cut into small pieces. A 30% (w/v) aqueous extract was prepared (stock) using a clean

mortar and pestle to make a homogenized paste. The extract was centrifuged at 15,000 rpm for 20 min at 4 °C and filtered using 0.2 μm filter. The purified extract was stored at -20 °C in aliquots until further use. Further dilutions were made from the 30% stock in complete medium to required concentrations between 1–25% for the treatment of MCF-7 cells, HeLa cells and lymphocytes.

A stock solution of 3.3 mM of cisplatin (Cadila Pharmaceuticals Ltd, India) was used to make drug dilutions of varying concentrations (1–200 μM) in complete medium.

Cell viability assay. The anti-proliferative activity of AWE (1–25%) and cisplatin (1–200 μM) alone or in combination (1 and 5% AWE; 1 and 5 μM cisplatin) on MCF-7 cells, HeLa cells and lymphocytes was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described [20]. In short, the cells were plated at a density of ~1 · 10^4 cells/well in 96-well plates in triplicates. Next day the culture medium was removed and replaced by varying concentrations of AWE (1–25%) or cisplatin (1–200 μM) in complete medium or by the combination doses and incubated for 24 and 48 h. The MTT assay was performed and the optical density (OD) was measured at 570 nm. The percent viability was calculated as (OD of the drug-treated sample/OD of the non-treated sample) x 100, considering that the colorimetric signal is directly proportional to the number of viable cells. This assay allows measurement of growth rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The EC$_{50}$ (50% effective concentration) values were calculated from the dose–response curves.

Calculation of combination effects of cisplatin and AWE. Calculations of combination effects were based on the method of Chouand Talalay (1984) and were expressed as a combination index (CI) [21]. CI analysis provides qualitative information on the nature of drug interaction, and CI, a numerical value, was calculated according to the following equation:

$$CI = C_{A,x}/IC_{x,A} + C_{B,x}/IC_{x,B},$$

where, $C_{A,x}$ and $C_{B,x}$ are, respectively, the concentrations of drugs A and B used in combination to achieve x% drug effect. $IC_{x,A}$ and $IC_{x,B}$ are the concentrations for single agents to achieve the same effect. A CI value < 1, =1, or > 1 represents, respectively, synergy, additivity, and antagonism of cisplatin and wheatgrass, respectively.

Detection of apoptosis in MCF-7 and HeLa cells after treatment with AWE

Microscopic examination. Morphological changes in MCF-7 and HeLa cells were observed on treatment with AWE and cisplatin at different concentrations (5, 15 and 25%) for 24 and 48 h using a normal inverted microscope (Labomed, USA). The untreated cells were used as negative control.

Nuclear morphological studies. Apoptosis induction after treatment with AWE at the EC$_{50}$ concentration (15 and 25% for MCF-7 and HeLa cells, respectively) for different time intervals (0, 6 and 24 h) was evaluated by the nuclear morphological changes associated with it using propidium iodide staining [20]. Briefly, ~10^6 cells/ml cells were seeded on glass coverslips and incubated overnight in complete medium at 37 °C. Further, cells were treated with AWE at its EC$_{50}$ for a series of time periods (0, 6 and 24 h). At the end of the desired time interval, cells were fixed in a mixture of acetone: methanol (1:1) at -20 °C for 10 min, washed with 1X PBS (pH 7.4) twice and stained with propidium iodide (10 mg/ml in PBS) for 30 s in dark at RT. The coverslips were thoroughly washed with PBS and placed upturned onto a glass slide with mounting media (DPX). Slides were viewed at 515 nm under the Progress Fluorescent Microscope (Olympus, USA). The images were captured at ×40 magnification.

Quantification of apoptotic cells by flow cytometry. AWE-induced apoptosis in MCF-7 and HeLa cells was determined by flow cytometric analysis as described earlier [20]. After treatment of synchronous cultures of MCF-7 and HeLa cells with AWE at their respective EC$_{50}$ for 0 and 24 h, both adherent and floating cells were harvested, washed with phosphate buffered saline (PBS, pH 7.2) and fixed with ice-cold absolute ethanol at -20 °C overnight. Cells were then washed with PBS prior to resuspending in a buffer containing PI (50 mg/ml), 0.1% sodium citrate, 0.1% Triton X-100 and 100 mg/ml of RNase A. The cells were analyzed using flow cytometry (Beckman Coulter Flow Cytometer FC500, CXP Version 2.2). The data was analyzed using the Beckman Coulter KALUZA 1.1 analysis software.

Expression analysis of various genes targeted by AWE. Reverse transcription-PCR was used to detect the expression of Bax and cyclin D1 in response to treatment with AWE at EC$_{50}$ for varying time points (0, 6 and 24 h). Total RNA extraction from untreated and AWE-treated MCF-7 and HeLa cells was carried out as per the manufacturer's instructions (GenElute Mammalian Genomic Total RNA Kit, Sigma, USA) at various time intervals. Further, total RNA was subjected to first strand synthesis as per manufacturer's protocol (ProtoScript M-MuLVTaq RT-PCR Kit, New England Biolabs, USA) followed by PCR using gene-specific primers [20, 22, 23]. в-Actin was taken as an internal control. The PCR cycle was as follows: initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for в-actin, 56 °C for Bax and 54 °C for cyclin D1 for 30 s, and extension at 72 °C for 45 s), with final extension at 72 °C for 7 min. Amplified products were visualized on a 2% agarose gel containing ethidium bromide.

Statistical analysis. All data are expressed as means ± SD of at least 3 experiments. Fisher's exact test was adopted for statistical evaluation of the results. Significant differences were established at $p < 0.05$.

RESULTS

AWE selectively suppresses the viability of MCF-7 and HeLa cells. The growth suppressive effects of different concentrations of AWE on MCF-7 cells, HeLa cells and lymphocytes were evaluated by the MTT assay. AWE treatment at various concentrations ranging from 1–25% for 24 and 48 h induced cell death in MCF-7 and HeLa cells in a dose and time-dependent manner (Fig. 1 *a, b*). The EC_{50} for MCF-7 cells was found to be 15% and 10% whereas for HeLa cells, it was found to be 25 and 15%, respectively, for 24 and 48 h. The experiment was reproduced at least three times.
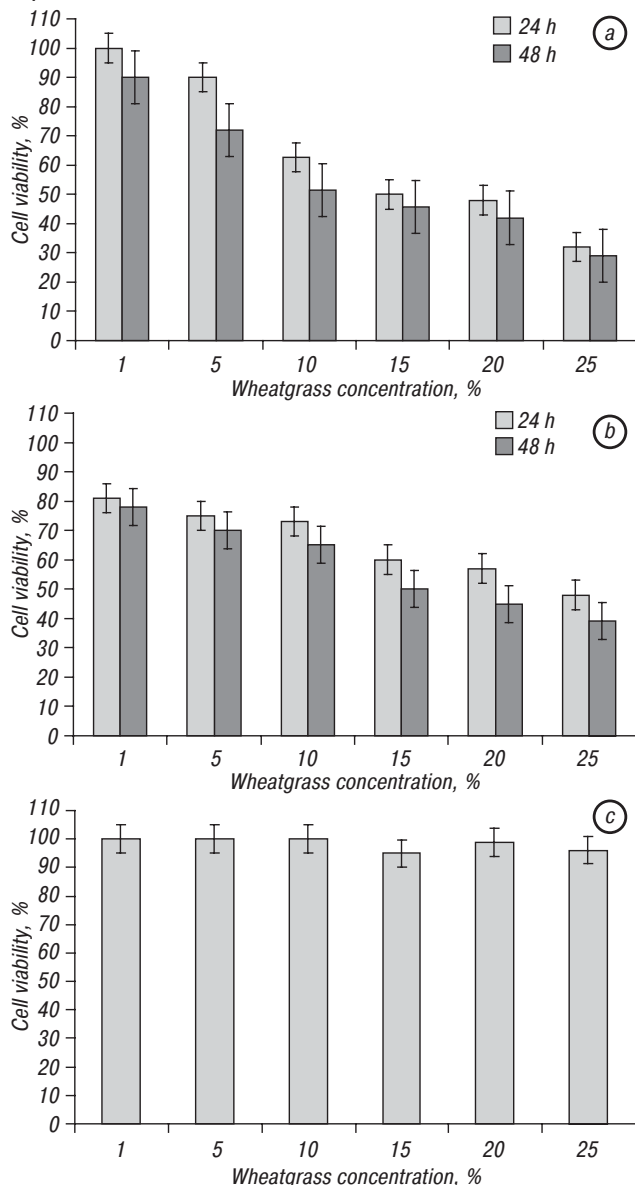

Fig. 1. Selective cytotoxicity of AWE on MCF-7 and HeLa cells (*a, b*). MCF-7 and HeLa cells treated with AWE at varying concentrations (1–25%) results in dose and time dependent growth suppression at 24 h (EC_{50} = 15%, 25%, respectively) and 48 h (EC_{50} = 10%, 15%, respectively) whereas treatment of lymphocytes with AWE at similar concentrations for 24 h was found to be minimally cytotoxic (*c*). Values are means ± SD of three independent experiments. Each value with AWE treatment differs from the control value (p < 0.05)

In order to confirm the safety profile of AWE, its effect was determined on lymphocytes as normal cells. Isolated lymphocytes from a healthy non-smoker adult

were plated in triplicates followed by treatment with AWE at varying doses (1–25%) for 24 h (see Fig. 1 *c*). AWE treatment did not result in a significant decrease in cell viability of lymphocytes thus indicating that AWE is differentially cytotoxic to cancer cells but not to the normal cells. This feature of AWE may provide a platform for its development as a safe drug for treatment for breast and cervical cancer.

Cisplatin induces cell death in both cancer and normal cells. The effect of increasing concentrations (1–200 μM) of cisplatin on the viability of MCF-7, HeLa cells and lymphocytes was determined by MTT assay. Cisplatin was found to induce dose dependent cytotoxicity in these cells as compared to the untreated controls. The EC_{50} of cisplatin on MCF-7 and HeLa cells was found to be 200 μM and 75 μM, respectively, after 24 h treatment (Fig. 2). Importantly, it was found that cisplatin reduced the viability of lymphocytes (normal cells) (see Fig. 2). Thus this study provides an insight into the observed side-effects of chemotherapy seen in patients which can be extrapolated *in vitro*.
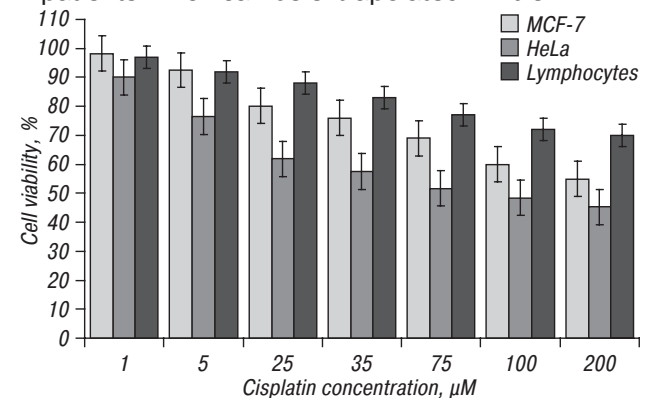

Fig. 2. Cisplatin is cytotoxic to both cancer and normal cells. Cisplatin treatment at varying doses (1–200 μM) for 24 h induced dose-dependent cytotoxicity in MCF-7, HeLa and lymphocytes. The EC_{50} of cisplatin was found to be 200 and 75 μM at 24 h in MCF-7 and HeLa cells. Values are means ± SD of three independent experiments. Each value with cisplatin treatment differs from the control value (p < 0.05)

Combination treatment of wheatgrass and cisplatin on HeLa and MCF-7 cells. Combinational therapy utilizing the currently available chemotherapeutic drugs with the natural dietary agents is a promising treatment strategy to reduce the non-specific cytotoxicity caused by chemotherapeutic agents such as cisplatin. In this study, a combination of wheatgrass and cisplatin was evaluated by cell viability assay to minimize the side effects and potentiate the chemotherapeutic activity of cisplatin.

Cells (MCF-7/ HeLa) were treated with different concentrations of cisplatin and wheatgrass alone, and in combination for 24 h and their viability was evaluated. It was observed that 1 μM of cisplatin (C1) used in combination with 1% (W1) and 5% (W2) wheatgrass resulted in a significant decrease in cell viability (85 and 80%, respectively) of MCF-7 cells as compared to either of the compounds alone (98% for C1, 97% and 90% for W1 and W2). When these combinations were used on HeLa cells, the combinations resulted in 60 and 53% (for C1W1 and C1W2) decrease in cell

viability while individual drugs decreased the cell viability by 84% for C1 and 81 and 75% with W1 and W2, respectively (Fig. 3). Also, treatment of MCF-7 and HeLa cells with 5 µM of cisplatin (C2) combined with W1 and W2 resulted in synergistic decrease in cell viability (81 and 70% for MCF-7; 50 and 45% for HeLa) as compared to individual doses (C2 = 93% and 77% for MCF-7 and HeLa; W1 and W2) (see Fig. 3). Further combinational index was calculated and it was found to be less than 1 for all the combinations indicative of synergistic action of these combinations on both MCF-7 and HeLa cells.
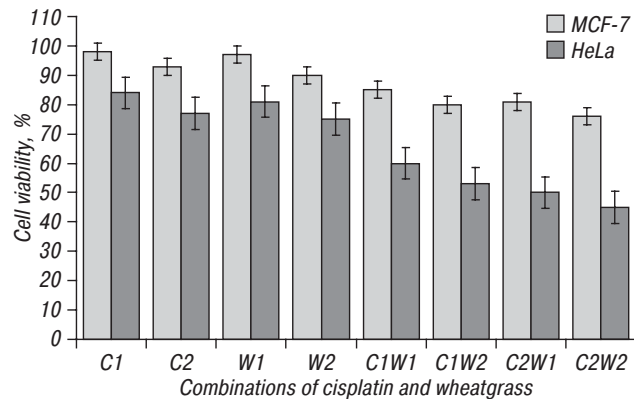
Fig. 3. Concurrent treatment of MCF-7 and HeLa cells with sub-lethal doses of cisplatin (C1 and C2) and AWE (W1 and W2) was found to induce synergistic decrease in cell viability in these cells (combination index (CI < 1). Each value is a ratio of the level in the treated cells to that in the untreated control cells. Values are means ± SD of 3 independent experiments. Each value with cisplatin and AWE treatment differs from the control value (p < 0.05).

AWE induces cell death via apoptosis in MCF-7 & HeLa cells

Morphological changes induced by wheatgrass on MCF-7 & HeLa cells. MCF-7 and HeLa cells treated with increasing concentrations of wheatgrass (5, 15 and 25%) for 24 and 48 h were observed under an inverted microscope and their morphological characteristics were noted. Treated cells, as compared to untreated cells, showed typical rounding off of cells, along with cell shrinkage indicating that wheatgrass induces cell death by apoptosis in these cells. The increase in these characteristics was in a dose and time-dependent manner (Fig. 4 *a, b*).

Nuclear morphological changes induced by AWE on MCF-7 & HeLa cells. The apoptotic changes induced by AWE were verified by analyzing various nuclear morphological characteristics with or without treatment. Untreated MCF-7 and HeLa cells appeared uniform in chromatin density with an intact nucleus. On the other hand, AWE treatment of MCF-7 (15% AWE) and HeLa (25% AWE) cells for 6 and 24 h resulted in evidences of apoptosis like chromatin fragmentation, accumulation of nuclear debris, apoptotic bodies and nuclear blebbing (Fig. 5 *a, b*).With an increase in duration of AWE exposure, there was a cumulative accrual of the said features consistent with apoptosis (see Fig. 5 *a, b*).
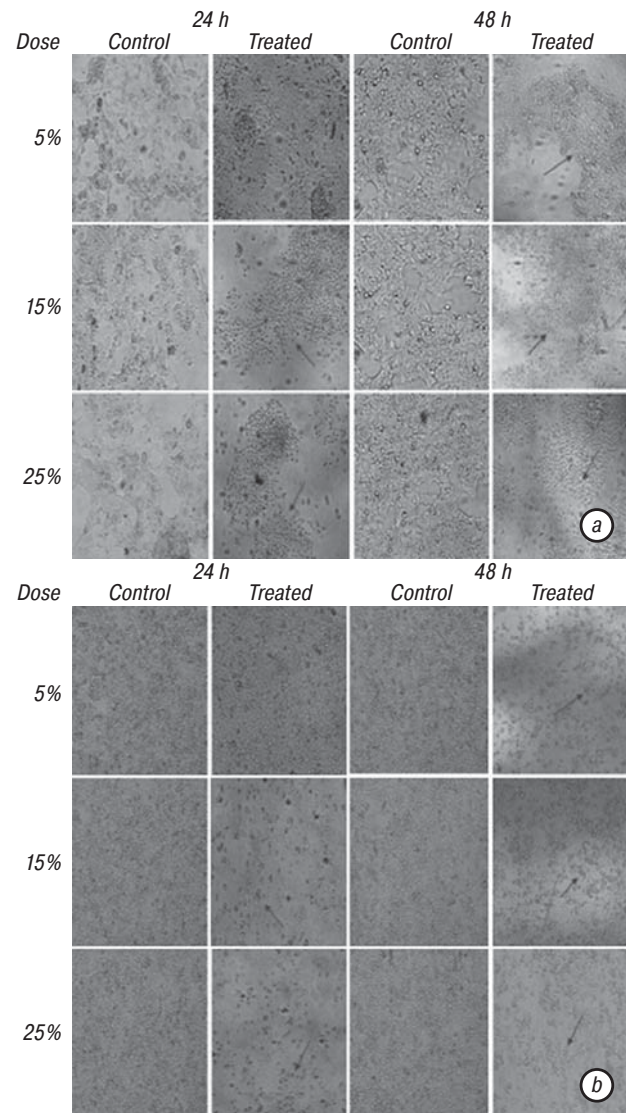
Fig. 4. Morphological changes induced by AWE treatment in MCF-7 (*a*) and HeLa cells (*b*) at varying concentrations. AWE treatment (at 5, 15 and 25% for 24 and 48 h) of MCF-7 (*a*) and HeLa (*b*) cells results in characteristic rounding off (arrows) of these cells compared to untreated cells. These changes accumulated with an increase in the dose and time of AWE treatment (magnification ×100)
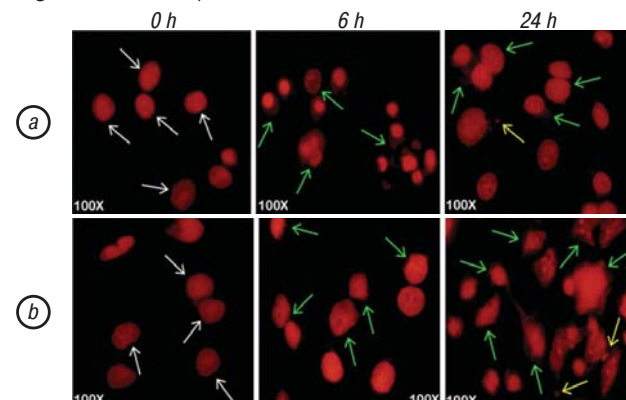
Fig. 5. The nuclear morphological changes induced by AWE treatment at various time intervals (0, 6 and 24 h) on MCF-7 (*a*) and HeLa (*b*) cells. Untreated MCF-7 and HeLa cells (0 h) show large and prominent nuclei indicating no significant characteristics of apoptosis (white arrows). MCF-7 (*a*) and HeLa (*b*) cells treated with AWE at their respective EC_{50} for 6 and 24 h showed time-dependent increase in nuclear morphological changes associated with apoptosis such as nuclear condensation and fragmentation (green arrows) and apoptotic bodies (yellow arrows) (magnification ×400)

***Effect of AWE on the cell cycle distribution.*** MCF-7 and HeLa cells were exposed to AWE at their respective IC_{50} concentrations (15 and 25%, respectively) for 0 and 24 h and their cell cycle distribution was examined by flow cytometry. Flow cytometric analysis of control and AWE-treated cells showed proper distribution of cells in the different phases of cell cycle in untreated cells (0 h), while on treatment with AWE for 24 h resulted in a significant increase in the number of cells in the $G_0–G_1$ phase of the cell cycle (80% for MCF-7 and 76% for HeLa). Furthermore, AWE treatment caused a significant inhibition in the movement of cells into the S-phase (9% for MCF-7 and 11.5% for HeLa) in comparison to the untreated cells, respectively (Fig. 6).
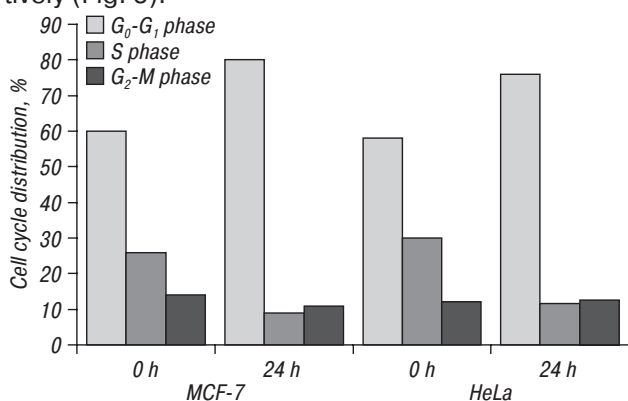
Fig. 6. Cell cycle arrest by AWE in MCF-7 and HeLa cells. DNA content in different phases of the cell cycle was measured using propidium iodide by flow cytometry. MCF-7 and HeLa cells($\sim 1 \cdot 10^6$) treated at their respective EC_{50} doses of AWE for 24 h showed a significant decrease in the proportion of cells in the G_2/M and S phases of the cell cycle while an increase in the number of cells in the $G_0–G_1$ phase indicating that AWE treatment results in cell cycle arrest in this phase in MCF-7 and HeLa cells. The histogram shows % analysis of cells in the different phases of the cell cycle from a representative experiment (out of three individual experiments)

AWE treatment significantly modulates the expression of Bax and cyclin D1. In order to determine the molecular targets of AWE on MCF-7 and HeLa cells, we analyzed the expression of Bax and cyclin D1 before and after treatment with AWE. β-Actin was used as an internal control for comparison of samples.

Bax, a pro-apoptotic Bcl-2-family protein, resides in the cytosol and translocates to mitochondria upon induction of apoptosis. The expression of Bax was found to be low in both untreated MCF-7 and HeLa cells. Upon treatment with AWE, we observed that there was a significant rise in the expression of Bax in a time dependant manner in MCF-7 and HeLa cells (Fig. 7 *a, b*). Another important gene involved in cell cycle regulation, cyclin D1, functions in the progression of the cell from G_1 to S phase, and is over-expressed in cancerous cells. In this study, it was also found to be expressed in high levels in both the untreated cell lines (see Fig. 7 *a, b*). Interestingly, the expression of cyclin D1 significantly decreased in these cells after treatment with AWE for 6 and 24 h compared to untreated cells (see Fig. 7 *a, b*).
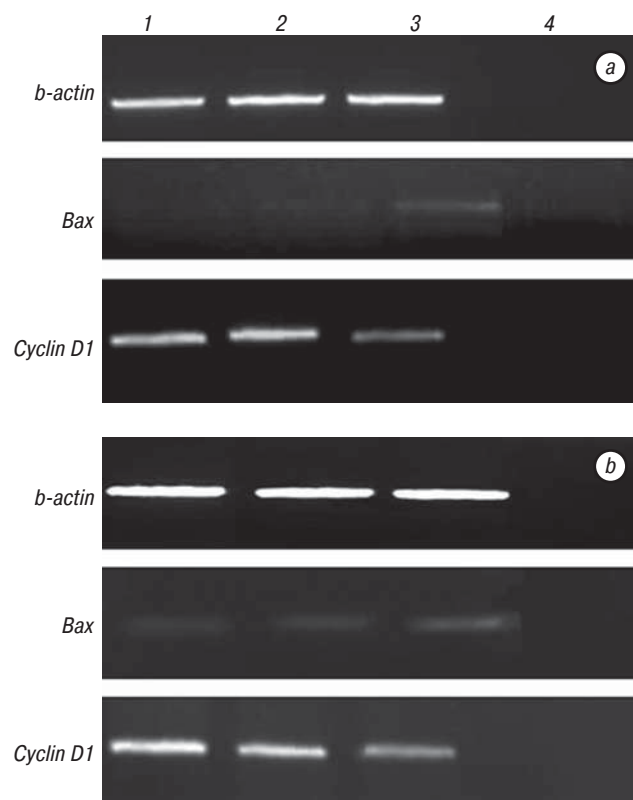
Fig. 7. MCF-7 (*a*) and HeLa (*b*) cells treated with AWE at their respective EC50 doses for 6 and 24 h show a significant decrease in the expression of cyclin D1 while a significant upregulation in the expression of Bax in a time-dependent manner compared to untreated cells. Lanes 1–4 represent untreated cells, cells treated with AWE for 6 and 24 h, and negative control for RT-PCR respectively. β-Actin was used as an internal control

DISCUSSION

Chemoprevention is a promising interventional approach utilizing mainly phytochemicals that possess many health benefits mediated directly or indirectly by modulating signal transduction pathways [7, 24]. Ambit reports indicate their important role in cancer prevention and treatment owing to their relatively safe cytotoxicity profile. Standard cancer therapies include surgery, radiotherapy, chemotherapy etc., but their successful therapeutic outcome is limited due to various side effects and development of multidrug resistance thus necessitating a need for alternate or adjuvant therapies. Hence, phytochemical based therapeutic strategies may help in minimizing these side effects as well as prevent the transformation of precancerous lesions or development of secondary or second primary tumors. Studies are underway to harness the potential of combinational approaches utilizing one or more synthetic or natural phytochemicals along with an effective drug such as chemotherapy to enhance the therapeutic effects of conventional cancer therapy [7, 24, 25].

The present focused on unraveling the chemopreventive and therapeutic action of wheatgrass (AWE) and decipher its molecular targets on human cancer cells. In order to determine the differential cytotoxicity towards cancer cells, the effect of varying concentrations of AWE was evaluated on MCF-7, HeLa cells and lymphocytes. It was found that AWE inhibited the cell

viability of the cancer cells in a dose and time dependant manner and its EC_{50} (effective concentration, the dose which reduces the viability of cells by 50%) was determined to be 15 and 10% on MCF-7 cells, and 25 and 15% on HeLa cells after 24 and 48 h treatment, respectively (see Fig. 1 *a, b*). These results are in line with other *in vitro* studies that have shown the growth inhibitory effects of wheatgrass on several cancer cells including leukemia, skin, prostate cancer cells [17, 19, 26, 27]. To qualify that AWE can be used as a safe chemopreventive agent, its effect on normal cells (lymphocytes) at various concentrations was determined for the first time. It was found that AWE treatment of lymphocytes for 24 h did not result in a significant toxicity towards these cells (see Fig. 1 *c*). This property of selectively cytotoxicity of AWE provides evidence that it can be used as a safe modality for cancer treatment. This is consistent with the previous studies which found that many phytochemicals such as curcumin, sulforaphane etc. are minimally toxic to the normal cells [28, 29].

As mentioned before, the combinational strategies for cancer treatment are considered more favourable due to their higher efficacy, resulting in better survival rates. Cisplatin is widely used chemotherapeutic drug but its efficacy is limited by the associated many side-effects like nausea and vomiting, nephrotoxicity, alopecia, etc. because of its high toxicity to normal cells [30]. In this study, first we analyzed the effect of cisplatin on MCF-7, HeLa cells and lymphocytes for 24 h. It was found that cisplatin inhibited the growth of cancer cells in a dose dependant manner with the EC_{50} at 200 µM and 75 µM for MCF-7 and HeLa, respectively (see Fig. 2). Moreover, cisplatin also induced significant decrease in cell viability of normal lymphocytes though at higher doses compared to the cancer cells, thus confirming that its non-specific cytotoxicity towards cancer and normal cells which may account for its observed side-effects in clinical settings (see Fig. 2). Previous studies have shown that cisplatin is an effective cytotoxic drug used in the treatment of several tumour types including lung, bladder, testicular, and ovarian cancer with associated side-effects. In addition some patients develop resistance on prolonged exposure to cisplatin [30–32].

Taking into account the observed non-specific cytotoxicity of cisplatin, low-dose combination treatment using cisplatin and AWE was analyzed on MCF-7 and HeLa cells. Our results showed that AWE potentiated the growth inhibitory effects of cisplatin at sub-lethal doses. Fig. 3 shows that after treatment of MCF-7 cells with 1 µM of cisplatin (C1) in conjunction with 1% AWE (W1), there was a synergistic (CI < 1) decrease in the cell viability (85%) in combination treatment (C1W1) than achieved with individual doses (98 and 97% for cisplatin and AWE alone respectively). Also, the other lower dose combinations of cisplatin and AWE i.e., C1W2, C2W1 and C2W2 (where C2 = 5 µM, W2 = 5%) showed combination index less than 1 indicating a synergistic action of these drugs in combination at the doses used (see Fig. 3). Similar results were obtained on treatment of HeLa cells with the same combinations (C1W1, C1W2, C2W1 and C2W2) (see Fig. 3). This implies that AWE enhances the efficacy of cisplatin in killing cancer cells, especially at lower doses, thereby minimizing the cytotoxicity to normal cells. Similar studies on various cancer cells using cisplatin in combination with other agents such as EGCG, β-elemene, AT-101, honey bee venom, etc. have shown cisplatin to act in a synergistic manner [33–37]. Thus, combination treatment approaches of cisplatin with phytochemicals such as wheatgrass may have immense prospects for development of therapeutic strategies to overcome cisplatin associated resistance and side-effects in human cancers.

Importantly, preventive and therapeutic interventions ought to induce cell death via induction of apoptosis and not necrosis which is associated with extensive tissue damage resulting in an inflammatory response. The mode of cell death induced by AWE was firstly observed by microscopic examination of AWE treated MCF-7 and HeLa cells at various concentrations (5, 15 and 25%) for 24 and 48 h. AWE treated cancer cells showed characteristic rounding off, cell shrinkage and detachment from the matrix, indicating that cell death induced by AWE is through apoptotic pathway as compared to untreated cells in which these morphological changes were absent (see Fig. 4 *a, b*).

Further, to verify the apoptosis induced by AWE in MCF-7 and HeLa cells, changes in the nuclear morphology were examined. Treatment of these cells with AWE (15 and 25% for MCF-7 and HeLa respectively) for varying time points (6 and 24 h) showed accumulation of characteristics changes in the nuclear morphology viz., nuclear condensation and fragmentation, extensive blebbing and presence of apoptotic bodies in these cells which increased in a time-dependent manner in comparison to untreated cells marked by uniform and intact nucleus (see Fig. 5 *a, b*).

Cell cycle analysis by flow cytometry was performed to confirm the mode of cell death. It was observed that AWE treatment of MCF-7 and HeLa cells at their respective EC_{50} concentrations for 24 h resulted in increased proportion of cells in the G_0/G_1 phase of the cell cycle indicating that AWE causes growth arrest of these cells at G_0/G_1 phase of the cell cycle in comparison to the untreated controls (see Fig. 6). Our studies confirm that AWE induces cell death in these cells mediated by the apoptotic pathway. Another study also indicated that wheatgrass induces apoptosis in human acute promyelocytic leukemia cells [26]. Other chemopreventive agents have also been found to induce apoptosis in various cancer cells [20, 38–42].

The mechanism of apoptosis involves expression of several genes and pathways. To authenticate the cell cycle inhibitory and apoptosis-inducing activity of AWE, the expression of cyclin D1, an oncogenic G1 cyclin and Bax, a pro-apoptotic gene were analyzed. Cyclin D1, a key regulator of cell cycle progression, forms a complex with CDK 4 and 6 (cy-

clin dependant kinases) which phosphorylates the retinoblastoma (Rb) protein, thus allowing the progression of cells from G_1 to S phase in the cell cycle [43]. The overexpression of cyclin D1 has been linked to the development and progression of cancer [44, 45]. Treatment of MCF-7 and HeLa cells with 15 and 25% AWE for 6 and 24 h resulted in significant downregulation in the expression of cyclin D1 in a time dependant manner in comparison to untreated cells which showed a high level of expression of cyclin D1 (Fig. 7 *a, b*). Consequently the cell cycle arrest mediated by AWE in MCF-7 and HeLa cells might be through the modulation of cyclin D1 expression. These results are consistent with other studies which showed that many chemopreventive agents act by downregulating the expression of cyclin D1 [46–48]. Thus, cyclin D1 may be an important target for cancer chemoprevention and therapy.

Bax, a member of the bcl-2 family, plays a major role in apoptosis. Gamut reports suggest that evasion of apoptosis in cancer cells may be correlated with the low expression of Bax [49–51]. Untreated MCF-7 and HeLa cells showed no or feeble expression of Bax while on treatment of these cells with AWE for 6 and 24 h, the expression of Bax was significantly upregulated in a time-dependent manner (see Fig. 7 *a, b*). Studies have implicated the role of Bax in induction of sensitization towards therapy as well as in cell cycle arrest and programmed cell death [52, 53]. Various chemopreventive agents have been shown to induce aforementioned activities which have been correlated with increased expression of Bax [53–57]. Taken together, the pro-apoptotic and anti-proliferative activity of AWE may be related to its effect of the expression of Bax.

Conclusively, wheatgrass exhibits tumoricidal effects and acts as a biological response modifier in cancer treatment by inducing apoptosis and cell cycle arrest. These results provide new insights that wheatgrass may serve as an alternative approach for cancer prevention and therapy by potentiating available treatments. Additional *in vitro* and *in vivo* studies are needed to assess the application of wheatgrass for therapeutic purposes.

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CONFLICT OF INTEREST

There is no conflict of interest.

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