18*β*-glycyrrhetinic Acid Induces Cell Cycle Arrest and Apoptosis in HPV18^{+} HeLa Cervical Cancer Cells

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Original Research Article

ABSTRACT

Aims: In recent years, natural products have received great attention to cancer prevention owing to their various health benefits, lack of toxicity, and side effects. Accumulating evidence shows that 18*β*-glycyrrhetinic acid (GRA) has antiproliferative and apoptotic activities on many cancer cell lines, while its role in cervical cancer remains unknown. Thus, the current research was conducted to illustrate GRA's cytotoxic effect against the HeLa cell line of HPV18 + human cervical cancer.

Methodology: The effect of GRA on HeLa cell line was tested by MTT and Trypan blue dye exclusion assay. Cell cycle analysis was carried out by flow cytometry after PI staining. Apoptosis was assessed after annexin V / PI double staining by flow cytometry. The Caspase activation assay kit analysed caspase activation. Reactive oxygen species (ROS) generation was measured by fluorimeter after DCFDA dye staining.

Results: Results of the current study have shown that GRA exposure significantly inhibited the cell viability of HeLa cells in a dose- and time-dependent manner. GRA induced growth arrest of HeLa cells at G_0/G_1 phase of the cell cycle. Moreover, GRA's antiproliferative action was mediated through apoptosis, as evident from caspase-3 and -9 activation. Caspase inhibitors blocked the GRA-induced caspase activation and ameliorated the GRA-induced cytotoxicity. This suggested the role of the intrinsic pathway of apoptosis stimulated by GRA. The intracellular ROS generation assay showed a dose-related increment in ROS production induced by GRA. Co-culturing of HeLa cells with *N*-acetyl cysteine (NAC), a ROS inhibitor, completely abrogated GRA-induced cell cycle

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arrest and apoptosis. Thus, the effect of NAC suggested the involvement of intracellular ROS in the GRA-induced cytotoxicity.

Conclusion: In summary, GRA exhibited strong antiproliferative and apoptotic properties and, thus, could act as an adjunct in the prevention and management of cervical cancer.

Keywords: Cervical cancer; GRA; ROS; apoptosis; G_0/G_1.

1. INTRODUCTION

Uterine cervix cancer is the world's second most common cancer in women and is a leading cause of morbidity and mortality [1]. In developing countries, it is the most common cancer in women, accounting for up to 25% of all female cancers [2]. According to the World Health Organisation, morbidity and mortality rates are much higher worldwide, with 4,90,000 new cases and 2,73,000 deaths in 2005, and around 80 percent of such cases occur in developing nations [3]. In India, it is the most active type of cancer occurring in females, presenting a significant public health issue. Epidemiological evidence indicates that the primary risk factor for the development of cervical carcinoma in high-risk human papillomavirus (HPV) (mostly type 16 and 18) infection and over 90% of uterine cervix cancers contain high-risk HPV DNA [4]. It has been documented that expression of high-risk HPV-specific oncogenes E6 and E7 are essential for cervical carcinogenesis, invasion, and metastasis. These viral oncoproteins immortalize and transform cervical keratinocytes by simultaneously disrupting the functions of core cellular tumor suppressor proteins p53 and pRb, setting the stage for initiation and progression of cervical cancer [4,5]. As the expression of HPV E6 and E7 oncoproteins expropriates the cell cycle control in cervical keratinocytes, suppression of these HPV oncogenes may be a strategy for targeting the production and metastasis of cervical cancer. Besides, another possibility that could sensitize cells to therapies is the restoration of regular p53 activity in cervical cancer cells.

Even though various chemotherapeutic agents are available for the treatment of cervical cancer, there is a need for a safe drug that could induce cell death selectively in cancer cells efficiently. It has been shown that many phytochemicals have anticancer activities, and many are used for cancer therapeutics. The 18*β*-glycyrrhetinic acid (GRA), a triterpenoid isolated from the Licorice (*Glycyrrhiza glabra* L.) root, has been found to exhibit many pharmacological properties, such as antimicrobial, anti-inflammatory, antiviral, antiulcer, and hepatoprotective effects. Recently, GRA's antitumor effect has been extensively investigated *In vitro* and *In vivo* in the breast, ovarian cancer, promyelocytic leukemia, hepatoma, and stomach cancer cells [6-9]. GRA has been found to show selective toxicity against cancer cells and significantly affect normal cells [10]. A few preliminary studies have shown GRA's anticancer activity against cervical cancer cells targeting cell cycle regulation and the apoptotic pathway. Nonetheless, the effect of GRA on cervical carcinogenesis has not been studied in detail. Therefore, this study's primary goal was to determine GRA's antiproliferative and apoptotic property against cervical cancer cells.

2. MATERIALS AND METHODS

2.1 Chemicals

Glycyrrhetinic acid, capase-3 and -9 inhibitors (Z-DEVD-FMK and Z-LEHD-FMK), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), RNase A, and *N*-acetyl-*L*-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Trypan Blue dye was from Invitrogen (Carlsbad, CA, U.S.A). Minimal Essential Medium (MEM), and fetal bovine serum (FBS) were purchased from Himedia India, Ltd. (Mumbai, India). FITC Annexin V Apoptosis Detection Kit was obtained from BD Bioscience, PharMingen (San Diego, CA, USA). Caspase-3 and -9 activity assay kits were purchased from BioVision, U.S.A. All other chemicals were of the highest analytical grade available.

2.2 Cell Culture

HPV18$^+$ human cervical cancer HeLa cell line were obtained from the National Centre for Cell Science, Pune, India. HeLa cells were grown in MEM supplemented with 10% heat-inactivated FBS and antibiotic-antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B) in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3 Cell Viability Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium-bromide) assay was used to assess the effects of GRA on HeLa cell viability. Exponentially growing HeLa cells (5×10^4/well) were seeded onto 96-well culture plate and allowed to ,be attached overnight and further treated with 10, 25, 50, and 100 µM of GRA for 24 48 h. 10 µL MTT (5 mg/mL) was added to each well, and the plates were incubated for another 4 h at 37°C. 100 µL Dimethyl sulfoxide (DMSO) was added to each well and thoroughly mixed to dissolve the purple crystals. After complete dissolution, each well's absorbance was measured on a microplate reader (Bio-Rad, USA) at 490 nm, and the cell survival was calculated as a percentage (%) over the untreated control. The value of IC50 was calculated by Prism GraphPad 8.0.

2.4 Trypan Blue Dye Exclusion Assay

The effect of GRA on HeLa cells was also determined by Trypan blue dye exclusion assay. HeLa cells (1×10^6) were seeded in a 60 mm tissue culture dish and allowed to attach overnight. After 24 h of GRA treatment (10-100 µM), cells were detached, collected, and counted by a hemocytometer (Shanghai, China) using 0.2% Trypan blue dye (Invitrogen, Carlsbad, CA). A non-treated sample was prepared as a control. The percentage of cell viability was calculated by comparing the sum of non-stained cells from treated samples with the sum from the untreated control.

2.5 Annexin V-FITC/PI Assay to Analyze Apoptosis

GRA's apoptotic effect on cervical cancer cells was analyzed according to the manufacturer's protocol with the annexin V-FITC / PI apoptosis kit (BD Biosciences, San Diego, CA, USA). In short, HeLa cells were seeded into a 60-mm culture dish and treated for 24 hours with GRA (50 and 100 µM). The cells were twice washed with cold PBS and then resuspended at a concentration of 1×10^6 cells/ml in a 1X binding buffer. 100 µL (1×10^5 cells) of the cell suspension was transferred to a 5 mL culture tube. Then, in the cell suspension, 5 µL of Annexin V-FITC conjugate and 5 µL PI were added. After incubation for 15 min in the dark at room temperature (25°C), 400 µL of the 1X binding buffer was added to the cell suspension.

The stained cells were then immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA).

2.6 Determination of Caspase-9 and -3 Activities

Caspase activity was determined by the Caspase-9 and -3 Colorimetric Assay Kits (BioVision, USA) in cervical cancer cells. In short, GRA-treated (10-100 µM) and untreated HeLa (3x106) cells were lysed and incubated on ice for 10 minutes in 50 µL of chilled cell lysis buffer. At 10,000xg, the cell lysate was centrifuged for 1 min, and the supernatant was collected and placed for instant assay on ice. The lysate (50 µL) was allocated to a 96-well plate containing a reaction buffer of 50 µL containing 10 mM DTT. Then, 5 µL of the 4 mM DEVD-pNA or LEHD-pNA substrate was added and incubated for 1 h at 37°C in each well. On a microtiter plate reader, absorbance was then read at 405 nm. By comparing the outcome with that of the uninduced monitor, the percentage (percent) increase in caspase-3 and -9 activity was calculated.

2.7 Analysis of the Effect of Caspase-9 and -3 Inhibitors

HeLa cells were pretreated with 50 µM of Z-VAD-FMK (a general caspase inhibitor), Z-DEVD-FMK (a caspase-3 inhibitor), and Z-LEHD-FMK (a caspase-9 inhibitor) for 2 h to characterize the cytotoxicity of GRA, and then treated with GRA at an indicated concentration (100 µM) for 24 h. Finally, as mentioned above, cell viability was calculated using the MTT assay.

2.8 Measurement of Intracellular ROS Level

Cells (1.5×10^4 per well) were re-seeded into a 96-well black bottom culture plate for the quantitative estimate of ROS generation and incubated at 37°C for 24 hours. The cells were further incubated with 10 µM of DCFH-DA for 30 min at 37°C after treatment with varying doses of GRA (25-100 µM) for 12 h. A multi-wall micro-plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, U.S.A.) measured the fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Relative to control, values were expressed as a percentage of fluorescence intensity.

2.9 Analysis of the Effect of a ROS Inhibitor, *N*-acetyl-*L*-cysteine (NAC)

In GRA-treated cervical cancer cells, N-acetyl-L-cysteine (NAC), a ROS inhibitor, has been used to confirm the generation of intracellular ROS. In short, HeLa cells have been pretreated for 2 h with 1 mM NAC followed by GRA (25-100 µM) for 12 h. The cells were stained with 10 µM DCFH-DA for 30 min at 37°C after washing with PBS. Multi-wall micro-plate readers (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, U.S.A.) measured the fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. We tested the effect of GRA on HeLa cells in the presence of NAC (1 mM), by MTT assay to further investigate the impact of ROS generation on apoptosis in GRA-treated cervical cancer cells.

2.10 Cell Cycle Analysis

Using the intercalating DNA fluorochrome, propidium iodide (PI), cell cycle analysis of GRA-treated HeLa cells has been performed with ethanol fixed cells. HeLa cells (1×10^6 cells) were cultured for 24 h at 37°C and 5 percent CO2 in a 35 mm tissue culture dish and treated with GRA (50 and 100 µM) for another 24 h. Cells were harvested by centrifugation at 600xg for 5 min and washed with PBS, and processed at 37°C with RNase A (200 µg / mL) for 30 min. Eventually, cells were stained at room temperature with PI (25 µg / mL) for 15 min. Cell cycle analysis was performed using a flow cytometer (Becton Dickinson FACS Calibur, San Jose, CA).

2.11 Statistical Analysis

The data were presented as the mean±S.E.M of three independent experiments performed in triplicates. Statistical analysis was performed by one-way ANOVA using Dunnett's multiple comparison test and also by two-tailed, paired Student's t-test (*$p < 0.01$, **$p < 0.001$ represent significant difference compared with control).

3. RESULTS

3.1 GRA Inhibited Proliferation of Cervical Cancer HeLa Cells

Cervical cancer HeLa cells (5×10^4 cells/well) were incubated with increasing GRA (10 to 100 µM) for 24 and 48 h, and their viability was determined by the MTT and Trypan blue dye exclusion assays. In the presence of 50 µM GRA, HeLa cells exhibited about 47.35% inhibition of proliferation after 24 h. At 100 µM GRA, after 24 h, the rate of inhibition reached 56.57% (Fig. 1A). At the dose of 50 and 100 µM, GRA showed marked inhibition of proliferation of about 59.89% and 68.66%, respectively, after 48 h of treatment (Fig 1A). Similarly, we confirmed our above results with Trypan blue dye exclusion assay and obtained almost the same cell survival (Fig. 1B). Thus, these results indicated that GRA inhibited cell growth in HeLa cells due to dose and time.

3.2 GRA Induced Cell Cycle Arrest in Cervical Cancer HeLa Cell

We further determined whether suppression of cell proliferation by GRA results from inhibition of cell cycle progression. The result showed that GRA treatment over 24 h caused a significant cell cycle arrest in HeLa cells at G_0/G_1 phase (Fig. 2). The treatment of 50 µM GRA resulted in the reduction of HeLa cells in S (from 26.3% to 20.3%) and G_2/M phase (32.5% to 28.1%) and accumulation in G_0/G_1 phase (41.2% to 51.6%) (Fig. 2). A further increase in GRA concentration to 100 µM resulted in a more pronounced reduction of cells in S (from 20.3% to 18.2%) and G_2/M phase (28.1% to 17.3%) with a concomitant increase in the percentage of cells in the G_0/G_1 (51.6% to 64.5%) (Fig. 2).

3.3 GRA-induced Apoptosis in Cervical Cancer HeLa Cells

We also determined whether the loss of viability in HeLa cells induced by GRA was due to apoptosis. Here, the Annexin V/PI assay results showed that GRA caused a significant amount of apoptosis in HeLa cells of about 17.1% and 31.4% after 24 h of treatment with 50 and 100 µM GRA, respectively (Fig. 3).

3.4 GRA-induced Activation of Caspases during Apoptosis

We again determined whether apoptosis in GRA-treated HeLa cells was due to caspases' activation (caspase-3, caspase-9). Our results showed significant induction of caspase-9 and -3 activities in HeLa cells after GRA treatment at 50 and 100 µM concentrations (Fig. 4A and B). Thus, the caspase activities in GRA treated HeLa cells were found to be dose and time-dependent. Activation of caspase-9 and -3 in GRA-treated HeLa cells clearly indicated the initiation of apoptosis.
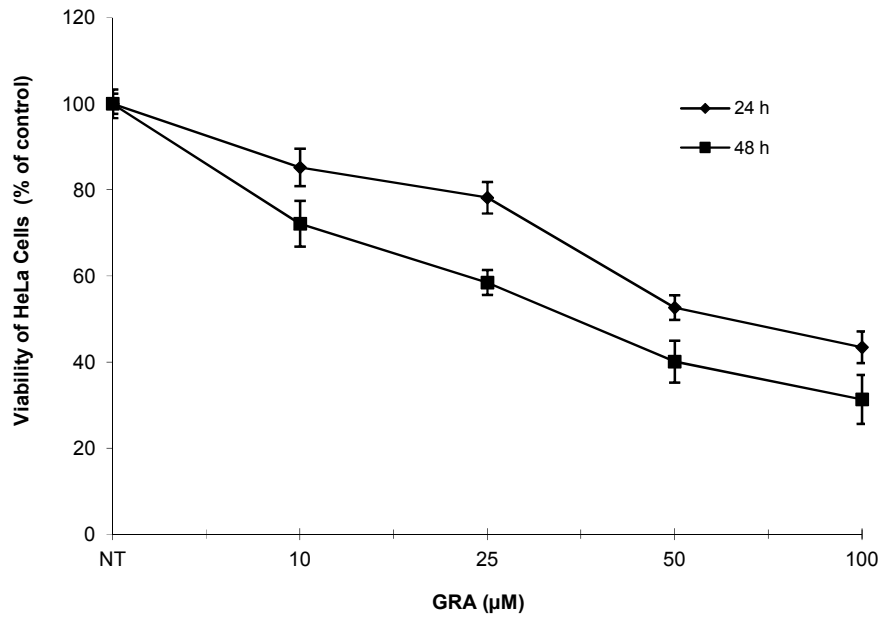
Fig. 1A. Dose-dependent inhibition of growth of cervical cancer HeLa cells treated with GRA (10-100 µM) for 24 and 48 h and assessed by MTT assay: NT denotes no treatment (control)
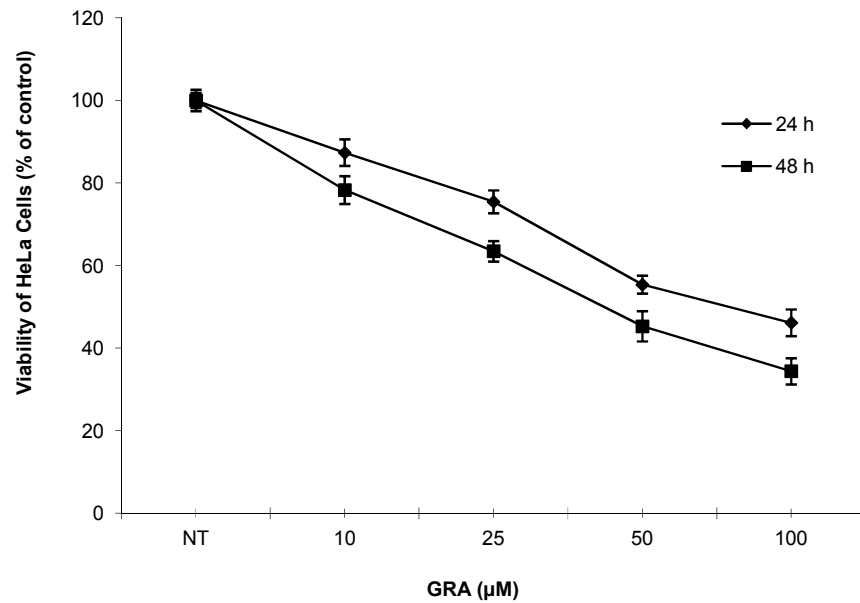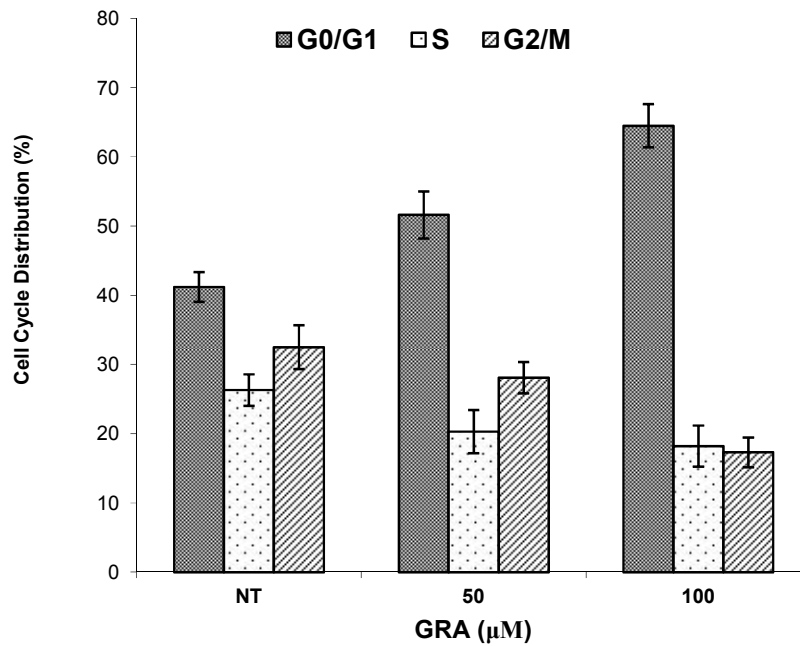*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*

Fig. 1B. Dose-dependent inhibition of growth of cervical cancer HeLa cells treated with GRA (10-100 µM) for 24 and 48 h and assessed by trypan blue dye exclusion assay: NT denotes not treated (Control)
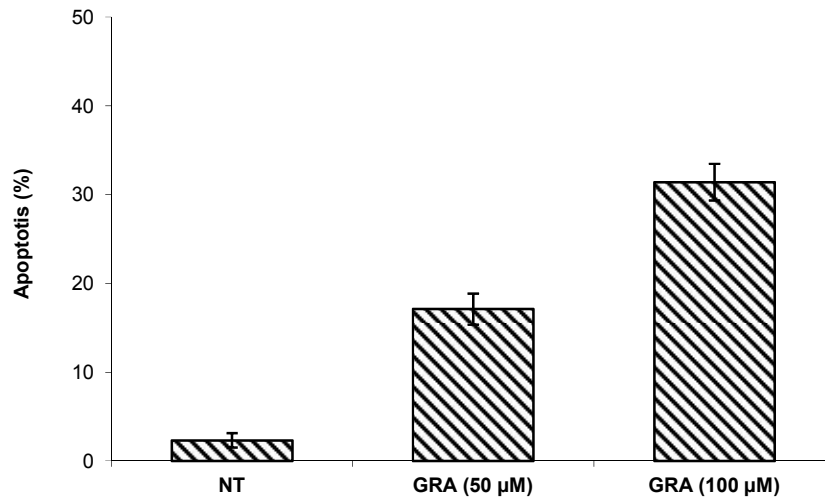*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*

Fig. 2. Percent apoptosis in GRA-treated HeLa cells: Cells were treated with 50 and 100 µM GRA for 24 h and stained with FITC-Annexin V and propidium iodide as described in materials and methods. Percent apoptosis was determined by a flow cytometer. NT denotes no treatment (control)
*Data represent mean ± S.E.M of three independent experiments. *p<0.01, **p<0.001 were considered significant*

Fig. 3. Percent cell cycle distribution of GRA-treated HeLa cells: Cells were treated with 50 and 100 µM GRA for 24 h and stained with propidium iodide in the presence of RNase A as described in materials and methods. Cell cycle analysis was carried out with a flow cytometer. NT denotes no treatment (control)
*Data represent mean ± S.E.M of three independent experiments. *p<0.01, **p<0.001 were considered significant*
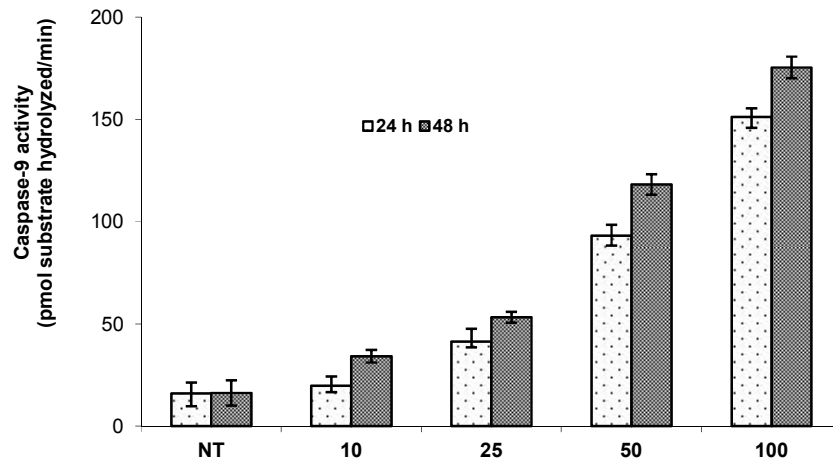
Fig. 4A. Dose and time-dependent activation of caspase-9 in GRA-treated HeLa cells: HeLa cells were treated with different concentrations of GRA (10-100 µM) for 24 and 48 h. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-9 was determined by incubation of 50 µg of total protein with substrate LEHD-pNA for 2 h at 37°C. The release of p-NA was monitored spectrophotometrically at 405 nm. NT denotes no treatment (control)
*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*
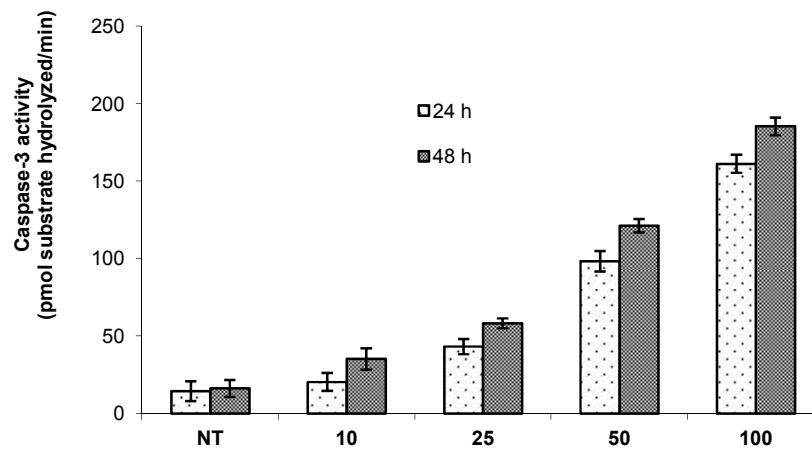

Fig. 4B. Dose and time-dependent activation of caspase-3 in GRA-treated HeLa cells: HeLa cells were treated with different concentrations of GRA (10-100 µM) for 24 and 48 h. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3 was determined by incubation of 50 µg of total protein with substrate DEVD-pNA for 2 h at 37 °C. The release of p-NA was monitored spectrophotometrically at 405 nm. NT denotes no treatment (control)
*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*

3.5 Caspase Inhibitors Disrupted Caspase Activation and Abrogated Apoptosis Induced by GRA

We further investigated whether the activation of caspase-9 and -3 is a key feature during GRA-induced cell death. Our results showed that pretreatment of caspase inhibitors abolished the respective caspase activities in HeLa cells induced by GRA (Fig. 5A and B). Similarly, these caspase inhibitors also abrogated the cell growth inhibition and, in turn, apoptosis in HeLa cells induced by GRA (Fig. 5C). Thus, the results showed that caspases' activation was an important essential event during GRA-induced apoptosis in HeLa cells.

3.6 Intracellular ROS Generation in GRA-treated HeLa Cells

We further explored whether ROS play any role in the GRA-induced apoptosis. Our results showed that GRA induced dose-dependent ROS generation in HeLa cells,, which confirmed ROS involvement in GRA-induced apoptosis (Fig. 6A).

3.7 *N*-acetylcysteine (NAC) Completely Suppressed GRA-Induced ROS Formation in HeLa Cells

We then explored whether enhanced intracellular ROS generation was due to the treatment of GRA. Our results showed that GRA-induced ROS generation was inhibited entirely by NAC, suggesting the role of GRA in ROS formation (Fig. 6B).

3.8 *N*-acetylcysteine (NAC) Ameliorated GRA-induced Inhibition of Cell Proliferation

We again investigated whether an elevated level of ROS is responsible for GRA-induced cytotoxicity in HeLa cells. Our results showed that HeLa cells treated with GRA in NAC's company recovered to typical viability pattern similar to control, suggesting that NAC sequestered GRA's antiproliferative activity in HeLa cells by completely suppressing ROS formation (Fig. 7A).
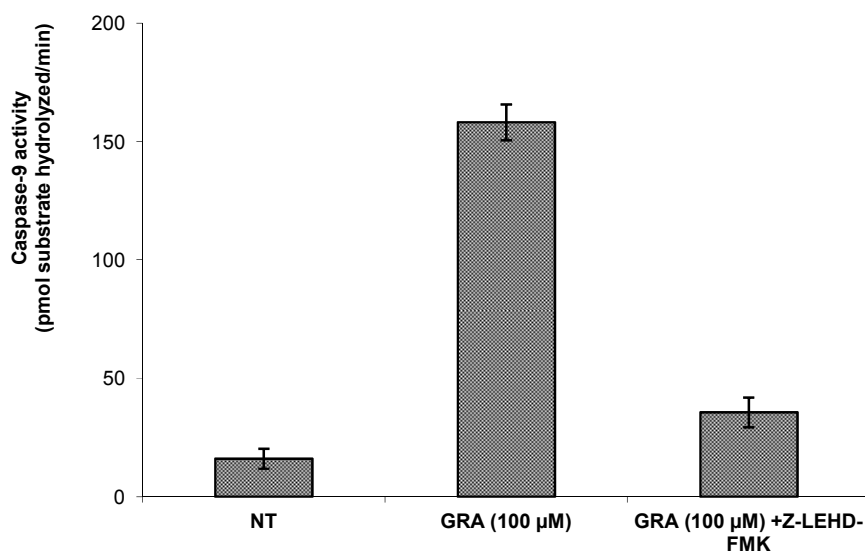

Fig. 5A. Suppression of GRA-induced caspase-9 activation by caspase-9 inhibitor (Z-LEHD-FMK): HeLa cells were pretreated for 2 h with caspase-9 inhibitor (Z-LEHD-FMK) and then treated with 100 µM of GRA for 24 h. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3 was determined by incubation of 50 µg of total protein with substrate LEHD-pNA for 2 h at 37 °C. The release of p-NA was monitored spectrophotometrically at 405 nm

Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant
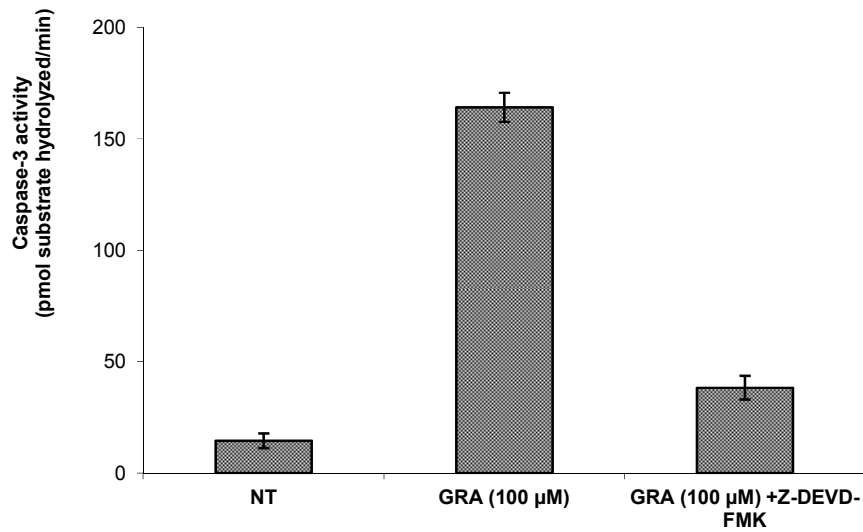
Fig. 5B. Suppression of GRA-induced caspase-3 activation by caspase-3 inhibitor (Z-DEVD-FMK): HeLa cells were pretreated for 2 h with caspase-3 inhibitor (Z-DEVD-FMK) and then treated with 100 µM of GRA for 24 h. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3 was determined by incubation of 50 µg of total protein with substrate DEVD-pNA for 2 h at 37°C. The release of p-NA was monitored spectrophotometrically at 405 nm

*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*
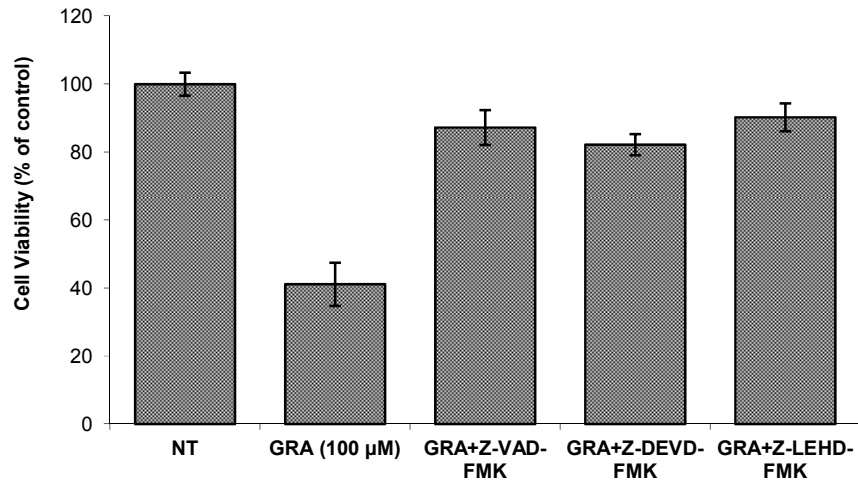

Fig. 5C. Abrogation of GRA-induced growth suppression of HeLa cells by caspase inhibitors: HeLa cells were pretreated for 2 h with a general caspase inhibitor (Z-VAD-FMK), a caspase-9 inhibitor (Z-LEHD-FMK) and a caspase-3 inhibitor (Z-DEVD-FMK) followed by 100 µM of GRA for 24 h and cell viability was determined by MTT assay

*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*
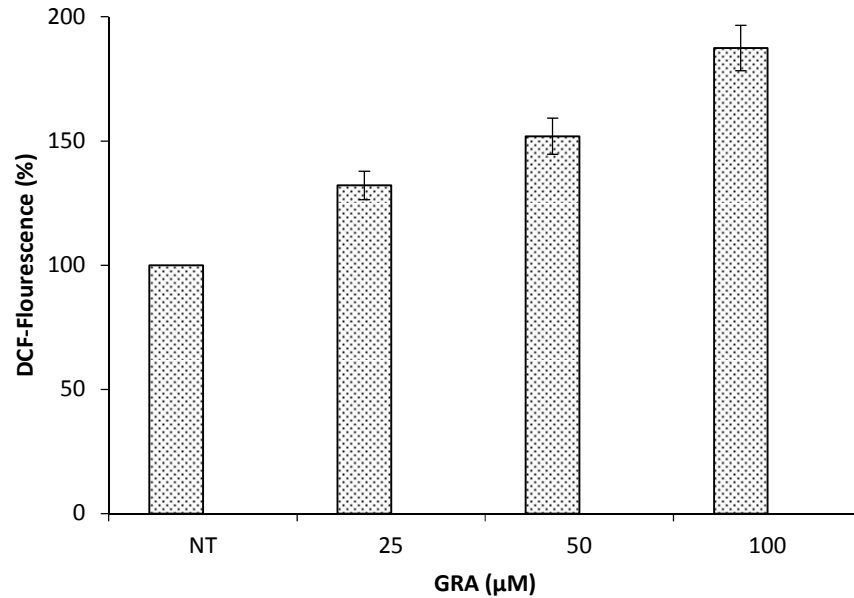
Fig. 6A. Measurement of intracellular ROS generation in GRA-treated HeLa cells by DCFDA method. Cells were treated with GRA (25-100 µM) for 24 h followed by DCFDH staining. Percent DCFDA fluorescence relative to control was calculated which showed the amount of ROS generated

*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*
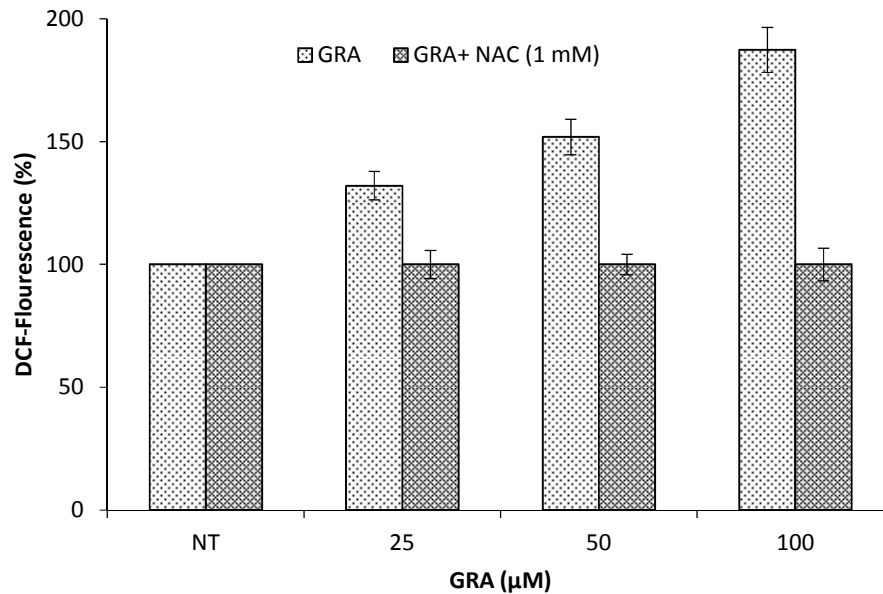

Fig. 6B. Abrogation of intracellular ROS generation in GRA-treated HeLa cells by a ROS inhibitor, NAC. Cells were pretreated with NAC (1 mM) for 2 h followed by GRA (25-100 µM) for 24 h and DCFDH staining. Percent DCFDA fluorescence relative to control was calculated which showed the amount of ROS generated

*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*
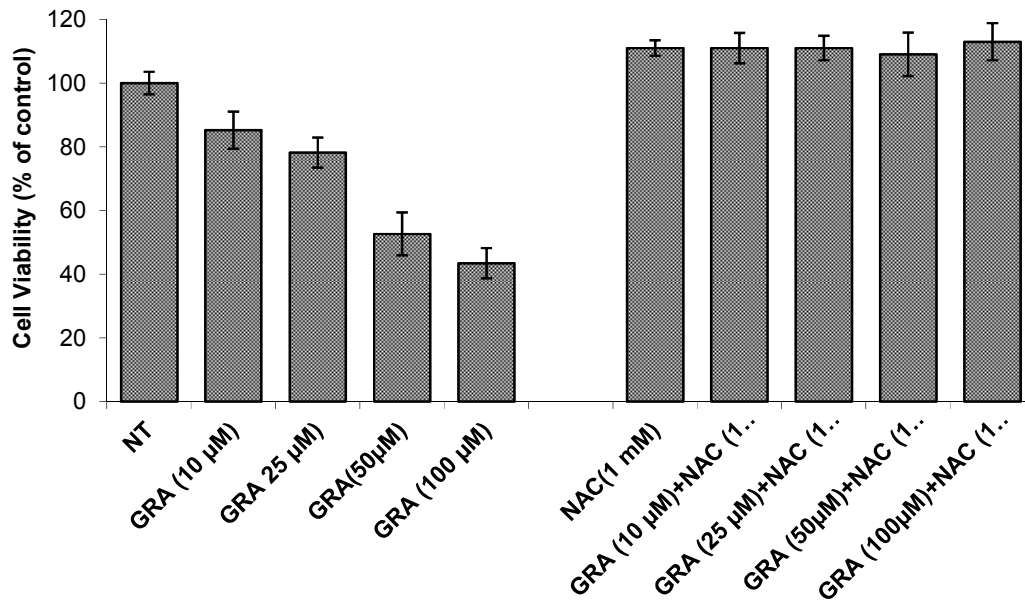
Fig. 7A. Amelioration of cell viability by NAC in GRA-treated HeLa cells: Cells were treated with GRA (10-100µM) for 24 h in the absence or presence of 1 mM NAC and cell viability was measured by the MTT assay. NT denotes no treatment (control)
*Data represent mean ± S.E.M. of three independent experiments. *p<0.01, **p<0.001 were considered significant*
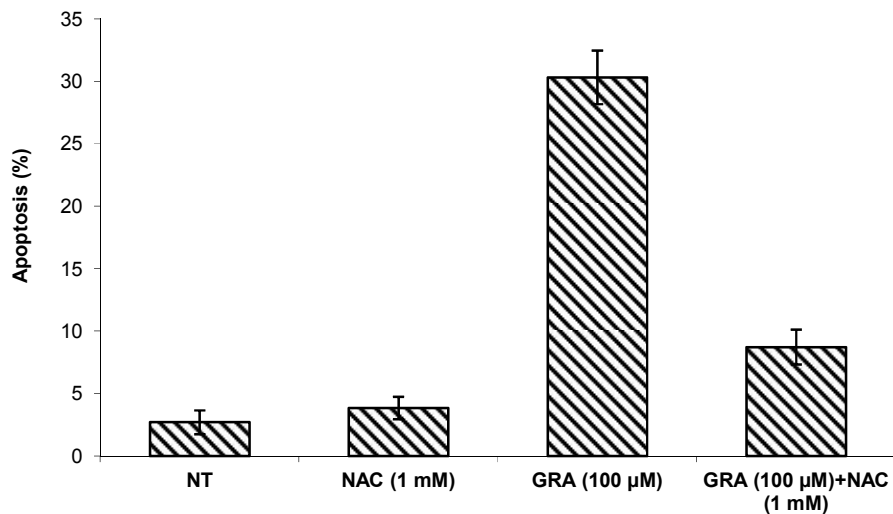

Fig. 7B. Alleviation of GRA-induced apoptosis in HeLa cells by NAC: Cells with or without NAC pretreatment (1 mM) were incubated in absence or presence of 100 µM GRA for 24 h and stained with FITC-Annexin V and propidium iodide as described in materials and methods. Percent apoptosis was determined by a flow cytometer. NT denotes no treatment (control)
*Data represent mean ± S.E.M of three independent experiments. *p<0.01, **p<0.001 were considered significant*

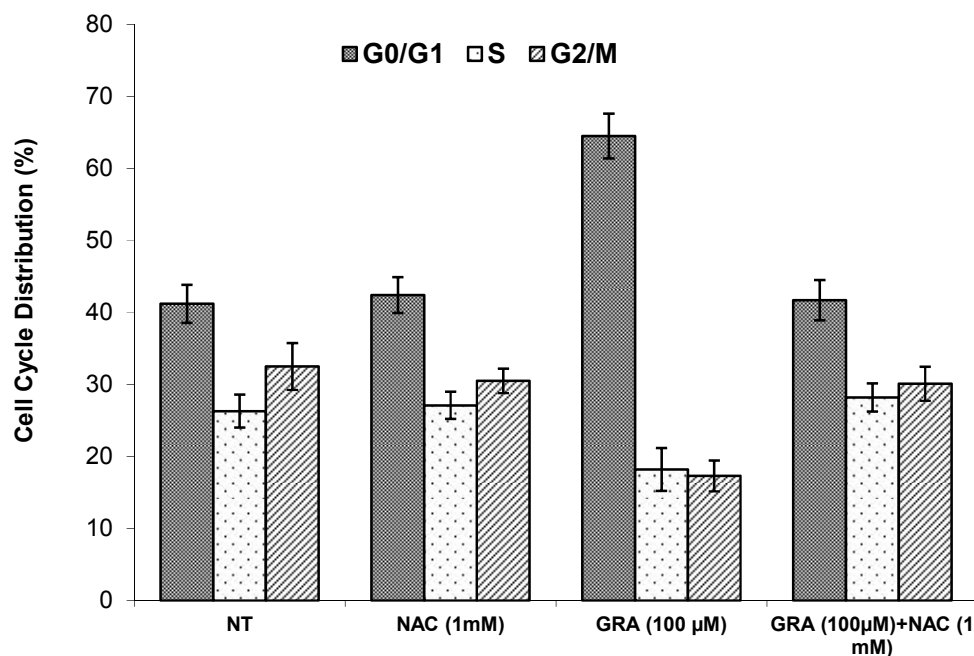*Saeed; JPRI, 32(25): 52-66, 2020; Article no.JPRI.62382*

Fig. 7C. Abrogation of cell cycle arrest in GRA-treated HeLa cells by NAC: Cells with or without NAC pretreatment (1 mM) were incubated in absence or presence of GRA (100 µM) for 24 h and stained with propidium iodide in the presence of RNase A as described in materials and methods. NT denotes no treatment (control)
Data represent mean ± S.E.M of three independent experiments. *p<0.01, **p<0.001 were considered significant

3.9 NAC also Abrogated GRA-induced Cell Cycle Arrest and Apoptosis

We also investigated the effect of pretreatment of NAC on GRA-induced cell cycle arrest and apoptosis of HeLa cells. Here, the results showed that pretreatment of the HeLa cells with 1 mM NAC reduced the apoptosis induced by 100 µM GRA to 8.73% in contrary to the apoptosis induced by GRA alone (30.31%) (Fig. 7B). Likewise, 100 µM GRA caused significant G_0/G_1 arrest (64.5% cells in G_0/G_1 phase) (Fig. 2). However, pretreatment of NAC (1 mM) completely abrogated the cell cycle arrest induced by GRA and the percentage of G_0/G_1 cells recovered to a level similar to the control (41.7%) (Fig. 7C).

4. DISCUSSION

Drugs, which affect mitochondrial function, may inhibit cell survival and proliferation in cancer cells (Armstrong, 2006). Major bioactive compounds from *Glycyrrhiza glabra* L. have been reported to induce programmed cell death and antiproliferative effect in different cancer cells, such as promyelocytic leukaemia HL-60 cells, human stomach cancer cells, and hepatoma cells [11]. Treatment with GRA inactivates antiapoptotic cellular Bcl-2 and induces apoptosis in Kaposi sarcoma-associated herpes virus-infected B lymphocytes [12]. GRA has also been shown to exhibit antitumor activity against human breast cancer [9] and lung cancer cell lines [13]. However, GRA's toxic effect against cervical cancer HeLa cell line has not been elucidated in detail. Therefore, the current study's objective was to evaluate the toxic effect of GRA against HeLa cells. The results showed that GRA strongly suppressed the growth of HeLa cells in a function of dose and time. In addition, we also observed G0 / G1 phase cell cycle arrest in HeLa cells and GRA-induced apoptosis, indicating that the antiproliferative action of GRA in HeLa cells may be due to programmed cell death and arrest of the cell cycle, and so on. However, the percentage of apoptotic cells did not align with the number of viable cells. This difference may be due

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to GRA-induced cell cycle arrest at the G0 / G1 level.

For cancer cell growth regulation, a balance between cancer cell proliferation and apoptosis is necessary [10,14]. Insensitive cancer cells, anticancer drugs also operate by inducing apoptosis [11,15]. Bcl-2 family proteins include both proapoptotic members such as Bax, Bak, and BNIP3, and antiapoptotic members such as Bcl-2 and Bcl-xL-xL, primarily mediate the intrinsic mechanism of programmed cell death. Tumor suppressor protein p53, which simultaneously suppresses Bcl-2 and activates Bax, is another essential factor. For cell survival, the ratio of Bax to Bcl-2 is important, which decides whether a cell undergoes apoptosis [12,16]. Bax, through oligomerization, forms a protein channel in response to undefined apoptotic signals. This putative Bax channel inserts and releases apoptotic factors, such as cytochrome c [17], into the outer mitochondrial membrane. Upon leakage, cytochrome c induces the formation of an apoptosome complex by binding to the activating factor-1 apoptotic protease (Apaf-1), which activates procaspase-9 (a zymogen) in the active caspase-9. To initiate apoptosis, Caspase-9 activates caspase-3 [18]. Caspases are synthesized as inactive proenzymes, and cleavage at unique aspartate cleavage sites results from their activation during apoptosis [19]. While initiator caspase-9 undergoes autocatalytic activation, initiator caspase is processed through executioner procaspase-3. Caspase-3, responsible for several key cellular proteins' proteolytic cleavage, is one of the major executioners of apoptosis [20]. In the current research, HeLa cells treated with GRA displayed increased caspase-9 and -3 behaviors that were found to be dose-dependent. Full abrogation of GRA-induced caspase activation and cell proliferation by pretreating HeLa cells with caspase inhibitors Z-VAD-FMK, Z-DEVD-FMK (caspase-9 inhibitor), and Z-LEHD-FMK (caspase-3 inhibitor). Therefore, the participation of the mitochondrial or intrinsic pathway in GRA-induced apoptosis was confirmed by our above-combined results.

The formation of reactive oxygen species [21] is caused by inhibition of the mitochondrial respiratory chain due to toxic substances exposure. Therefore, we investigated whether enhanced oxidative stress-mediated GRA-induced cell death. Our findings showed that in HeLa cells, GRA induced substantial ROS generation. ROS acts on mitochondria, causing the mitochondrial membrane to be disrupted and cytochrome c to be released. Increased ROS formation indicates that GRA causes ROS formation, which may be involved in cell death and mitochondrial dysfunction. By increasing ROS development , GRA has been shown to induce apoptosis of HL60 cells [22]. Changes in the levels of intracellular antioxidants such as GSH, NADH, or NADPH that affect mitochondrial function are caused by an increase in ROS development [21]. It is suggested that mitochondrial GSH depletion activates the apoptotic pathway. The primary role of N-acetylcysteine (NAC) *in vivo* is to supply and replenish cysteine which are essential for GSH synthesis [23]. The present study results showed that the antiproliferative and apoptotic function of GRA was probably abrogated by blocking the formation of ROS by pretreatment of HeLa cells with NAC. In addition, the GRA-induced cell cycle arrest at the G0 / G1 level was also shown to be abolished by NAC. The observed results may be due to NAC's leading role in supplying and replenishing cysteine for GSH synthesis. In other words, NAC could play an essential role in preserving the GRA-destroyed cellular redox environment.

5. CONCLUSION

In short, the combined results of this study showed that GRA has good antiproliferative and apoptotic properties against cervical cancer cells and could, therefore, be a potential chemotherapeutic agent for cervical cancer prevention and management.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company; rather it was funded by the personal efforts of the authors.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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