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Original article

Glycyrrhizin induces reactive oxygen species-dependent apoptosis and cell cycle arrest at G_0/G_1 in HPV18$^+$ human cervical cancer HeLa cell line

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ABSTRACT

Cervical cancer is the fourth most common cancer among women worldwide and is a major cause of morbidity and mortality. High-risk Human Papilloma Virus (mostly type 16 & 18) infection is the primary risk factor for the development of cervical carcinoma. The quest for strong, safe and cost effective natural antiproliferative agents that could reduce cervical cancer have been focussed now a day. Recently, glycyrrhizin, a triterpene glycoside (saponin) from licorice (*Glycyrrhiza glabra* Linn.), has been shown to exhibit potent antiproliferative and anticancer properties in a few preliminary studies. However, potential of this compound in cervical cancer has not been elucidated yet. Therefore the objective of this study was to analyze the antiproliferative and apoptotic properties of glycyrrhizin in human cervical cancer HeLa cells. Our results showed that glycyrrhizin exposure significantly reduced the cell viability of HeLa cells with a concomitant increase in nuclear condensation and DNA fragmentation in a dose dependent manner. The intracellular ROS generation assay showed dose-related increment in ROS production induced by glycyrrhizin. Glycyrrhizin also induced apoptosis in cervical cancer cells by exerting mitochondrial depolarization. Cell cycle study showed that glycyrrhizin induced cell cycle arrest in G_0/G1 phase of cell cycle in a dose dependent manner. Thus, this study confirms the efficacy of glycyrrhizin in cervical cancer cells which could be an adjunct in the better prevention and management of cervical cancer worldwide.

1. Introduction

Cervical cancer is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases in 2012 [1]. There were an estimated 266,000 deaths from cervical cancer worldwide in 2012, accounting for 7.5% of all female cancer deaths [1]. Almost nine out of ten (87%) cervical cancer deaths occur in the less developed regions [2]. The lack of effective screening and treatment strategies is a major reason for the sharply higher cervical cancer rates in developing countries compared with developed countries [2]. Cervical cancer ranks as the second most frequent cancer among women in India, and the second most frequent cancer among women between 15 and 44 years of age [3]. In India, current estimates indicate that every year 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease [3]. About 7.5% to 16.9% of women in the general population are estimated to harbour cervical HPV infection at a given time, and 87.8% to 96.67% of invasive cervical cancers are attributed to Human Papilloma Virus (HPV) type 16 or 18 [3]. With a lack of organized screening programs and with the sheer size of the Indian population, the country shoulders more than one-fourth of the world's burden of the disease [1]. High-risk HPV (mostly type 16 & 18) infection is the primary risk factor for the development of cervical carcinoma and over 90% of cancers of the uterine cervix contain high-risk HPV DNA [4–7]. HPV oncogenes E6 and E7 have been reported to be vital for cervical carcinogenesis, invasion and metastasis. By simultaneous disruption of the functions of key cellular tumor suppressors proteins p53 and pRb, these viral oncoproteins immortalize and transform human cells, setting the stage for cancer progression [6–10].

The standard first line treatment for invasive cervical cancer is usually cisplatin and 5-fluorouracil (5-FU) used in combination and in addition to radiation therapy. Recurrent and late stage cervical cancers are often treated with a combination of cisplatin, bleomycin, methotrexate, and 5-FU. Chemotherapy is accompanied with side effects; hence, the situation demands an alternate, safe and more specific drug, which either independently or in combination with other drugs may be used for the treatment of uterine cervical cancer. There are various epidemiological reports which suggest that plant derived compounds have anticancerous potentials [11].

Several plant phytochemicals are currently being studied for their chemopreventive potential. Many of these have been reported to

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exhibit anticancer properties by modulating various cellular and molecular events occuring in a cancer cell [12–16]. Glycyrrhizin, a triterpene glycoside, is the main active compound of licorice (*Glycyrrhiza glabra* Linn.); which is used as herbal medicine due to its different pharmacological properties. Glycyrrhizin has been shown to possess anti-oxidant, anti-inflammatory and anti-viral properties [17,18]. Recent preliminary studies suggest that glycyrrhizin exhibit potent *in vitro* cytotoxic activity against different human cancer cell lines [19–22]. However, the anti-proliferative and anti-cancer effects of glycyrrhizin on human uterine cervical cancer cells have not been elucidated yet. Therefore, this study was aimed to explore the cytotoxic role of glycyrrhizin and its possible mode of action in human cervical cancer cells.

2. Materials and methods

2.1. Chemicals

Glycyrrhizin, Cispaltin, 5-Fluorouracil (5-FU), Hoechst 33342, DAPI (4′,6-diamidino-2-phenylindole), Rhodamine123, propidium iodide (PI), capase-3, -8 and -9 inhibitors (Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). Minimal Essential Medium (MEM), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and other chemicals 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, -8 and -9 activity assay kits were purchased from BioVision, U.S.A.

2.2. Cell culture

HPV18$^+$ human cervical cancer HeLa cell line and normal colon epithelial cells IEC-6 were obtained from National Centre for Cell Science (NCCS), Pune, India. HeLa cells were grown in MEM and IEC-6 cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution containing penicillin (10,000 IU), streptomycin (10 mg), and amphotericin B (25 μg) (Himedia, India, Ltd., Mumbai, India) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Determination of cell viability by MTT assay

The effect of glycyrrhizin on normal colon epithelial cells IEC-6 and cervical cancer HeLa cells were analyzed by MTT assay as described previously [23]. In brief, IEC-6 and HeLa cells were seeded into 96-well plate at 5×10^3 cells/well followed by incubation at 37 °C for 24 h for attachment and then treated with glycyrrhizin (20, 40, 80, 160, 320 and 640 μM) for additional 24 and 48 h, respectively. Subsequently, 10 μl of 5 mg/ml MTT dye was added to each well and incubated for 4 h at 37 °C. The purple-colored precipitates (formazan) were dissolved in 100 μl of dimethyl sulfoxide (DMSO) by gentle shaking. After complete dissolution, absorbance of each well was measured on a microplate reader (Bio-Rad, USA)at 490 nm and the cell survival was calculated as percentage (%) over the untreated control. The value of IC 50 was calculated by Origin 6.0 Professional. Moreover, to determine and compare the cytotoxicity of standard drugs on cervical cancer cells with respect to glycyrrhizin, cisplatin and 5-FU were used as solo (5–100 μM) and in combination with glycyrrhizin followed by MTT assay as described above.

2.4. Determination of cell death by trypan blue exclusion assay

Glycyrrhizin-induced cytotoxicity in HeLa cells was also determined by trypan blue dye exclusion cell death assay, as described previously [24]. Briefly, 7.0×10^4 cells/ml were seeded in a 12-well plate and allowed to attach overnight. The next day, cells were treated with different concentrations of glycyrrhizin (20, 40, 80, 160, 320 and 640 μM) for an additional 24 and 48 h. At the end of the incubation, both floating and adherent cells were collected and centrifuged at 1500 rpm for 5 min. The cells were resuspended in 10 μl of 0.4% trypan blue solution and counted using a hemocytometer under an inverted microscope. The result was expressed as the percentage of dead cells in each treatment group from three independent experiments.

2.5. Morphological analysis by phase contrast microscopy

Morphological analysis was performed in glycyrrhizin-treated HeLa cells as described previously [25]. The cells were grown overnight at a density of 5×10^3 cells per well in a 96-well plate and then, re-supplemented with fresh medium having various concentrations of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 24 h. Finally, cellular morphology of untreated cells and treated cells were observed under inverted phase contrast microscope (Labomed, U.S.A).

2.6. Detection of nuclear condensation by DAPI staining

The apoptotic effect of glycyrrhizin on HeLa cells was analyzed by fluorescent nuclear dye DAPI as previously described [26]. Briefly, HeLa cells were seeded into 6-well plate at density 5×10^3 cells/ml, incubated at 37 °C for 24 h, and then, treated with different doses of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 24 h. After washing with cold PBS, cells were fixed in chilled methanol for 10 min. Subsequently, the cells were permeabilized with permeabilizing buffer (3% paraformaldehyde and 0.25% Triton X-100) and stained with DAPI. After staining, the images were captured under an inverted fluorescence microscope (Nikon ECLIPSE Ti-S, Japan).

2.7. Determination of nuclear morphology by Hoechst 33342 staining

Morphological changes associated with apoptosis in HeLa cells were further detected by Hoechst 33342 staining [23]. Briefly, HeLa cells (5×10^4 cells/well) were seeded in a 6-well plate and treated with different concentrations of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 12 h. Cells were washed with PBS, incubated with Hoechst 33342 (5 μg/ml) dye, and then, incubated for 10 min at 37 °C in a humidified dark chamber. Fluorescent nuclei were observed with an inverted fluorescence microscope (Nikon ECLIPSE Ti-S, Japan).

2.8. Annexin V-FITC/PI assay to analyze apoptosis

The apoptotic effect of glycyrrhizin on cervical cancer cells was analyzed with annexin V-FITC/PI apoptosis kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, HeLa cells were seeded into 60 mm-culture dish and treated with different doses of glycyrrhizin (20, 40, 80, 160 and 320 μM) for 24 h. The cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. 100 μl of the cell suspension (1×10^5 cells) was transferred to a 5 ml culture tube. Then, 5 μl of Annexin V-FITC conjugate and 5 μl PI were added to the cell suspension. After incubation for 15 min in the dark at room temperature (25 °C), 400 μl of the 1× binding buffer was added to the cell suspension. The stained cells were then immediately analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA).

2.9. Determination of caspase-8, -9 and -3 activities

The caspase activities in cervical cancer cells were determined by Caspase-8, -9 and -3 Colorimetric Assay Kits (BioVision, USA). Briefly, glycyrrhizin-treated (20, 40, 80, 160 and 320 μM) and untreated HeLa cells (3×10^6) were lysed in 50 μl of chilled cell lysis buffer and incubated on ice for 10 min. The cell lysate was centrifuged for 1 min at

10,000 × *g* and the supernatant was collected, and put on ice for instant assay. The lysate (50 µl) was aliquoted into 96-well plate with 50 µl of reaction buffer, containing 10 mM DTT. After that, 5 µl of the 4 mM DEVD-pNA substrate was added in each well and incubated at 37 °C for 1 h. Absorbance was, then, read at 405 nm on a microtiter plate reader. Percent (%) increase in caspase-3 activity was determined by comparing the result with the level of the uninduced control.

2.10. Analysis of the effect of caspase-8, -9 and -3 inhibitors

To characterize the cytotoxicity of glycyrrhizin, HeLa cells were pre-treated with 50 µM of Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor)and Z-LEHD-FMK (caspase-9 inhibitor) for 2 h and then treated with glycyrrhizin at the indicated concentrations (20, 40, 80, 160 and 320 µM) for 24 h. Finally, the cell viability was determined using the MTT assay as described above.

2.11. Assessment of mitochondrial membrane potential (ΔΨ m) by Mito Tracker Red

Mitochondrial membrane potential in glycyrrhizin-treated HeLa cells was analyzed by Mito Tracker Red CMX Ros staining as described previously [27]. Briefly, HeLa cells (2×10^5 cells/well) were grown in a 12-well plate and allowed to adhere overnight. Then, cells were co-cultured with different doses (20, 40, 80, 160 and 320 µM) of glycyrrhizin for 12 h. Cells were washed and fixed with 3.5% paraformaldehyde for 15 min at 37 °C and then permeabilized with 0.05%v/v Triton X-100. Finally, the cell were stained with Mito Tracker Red (25 ng/ml) for 30 min in the dark and images were captured using an inverted fluorescence microscope (Nikon ECLIPSE Ti-S, Japan).

2.12. Measurement of intracellular ROS level

DCFH-DA method was used to study ROS generation in HeLa cells after exposure to different concentrations of glycyrrhizin [28]. In brief, HeLa cells (1.5×10^4 per well) were seeded in a 12-well plate and incubated for 24 h at 37 °C. After exposure to different concentrations of glycyrrhizin (20, 40, 80, 160 and 320 µM) for 12 h, cells were incubated with 10 µM of DCFH-DA for 30 min at 37 °C. Excessive DCFH-DA was removed by washing and images were captured by a fluorescence microscope (Nikon ECLIPSE Ti-S, Japan).

For quantitative estimation of ROS generation, cells (1.5×10^4 per well) were re-seeded in a 96-well black bottom culture plate and incubated for 24 h at 37 °C. After, treating with different doses of glycyrrhizin (20, 40, 80, 160 and 320 µM) for 12 h, cells were further incubated with 10 µM of DCFH-DA for 30 min at 37 °C. Fluorescence intensity was measured by multiwall micro-plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, U.S.A.) at excitation wavelength of 485 nm and emission wavelength of 528 nm. Values were expressed as percentage of fluorescence intensity relative to control.

2.13. Analysis of effect of a ROS inhibitor, N-acetyl-L-cysteine (NAC)

N-acetyl-L-cysteine (NAC), a ROS inhibitor, was used to confirm the generation of intracellular ROS in glycyrrhizin-treated cervical cancer cells. Briefly, HeLa cells were pre-treated with 10 mM NAC for 2 h followed by glycyrrhizin (20, 40, 80, 160 and 320 µM) for 12 h. After washing with PBS, cells were stained with 10 µM DCFH-DA for 30 min at 37 °C. Fluorescence intensity was measured by multiwall micro-plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, U.S.A.) at excitation wavelength of 485 nm and emission wavelength of 528 nm. To further investigate the effect of ROS generation on apoptosis in glycyrrhizin-treated cervical cancer cells, we examined the effect of glycyrrhizin on HeLa cells in the presence of NAC (10 mM), by MTT assay.

2.14. Cell cycle analysis

Cell cycle phase distribution with cellular DNA content was carried out in glycyrrhizin-treated cervical cancer cells by flow cytometry as described earlier [29]. In brief, HeLa cells (5×10^5 cells) were grown in a 60 mm tissue culture dish for 24 h at 37 °C and then, treated with various doses of glycyrrhizin (20, 40, 80, 160 and 320 µM)for an additional 24 h at 37 °C. After 24 h incubation, the cells were harvested, washed with cold PBS, and treated with RNase A (50 µg/ml). Subsequently, cells were fixed with ice cold 70% ethanol and kept overnight at −20 °C, and finally, stained with PI (25 µg/ml) for 30 min at room temperature. Finally, the cell cycle analysis was performed on a flow cytometer (Becton Dickinson FACSCalibur, San Jose, CA).

2.15. 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 ($^*p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$ represent significant difference compared with control).

3. Results

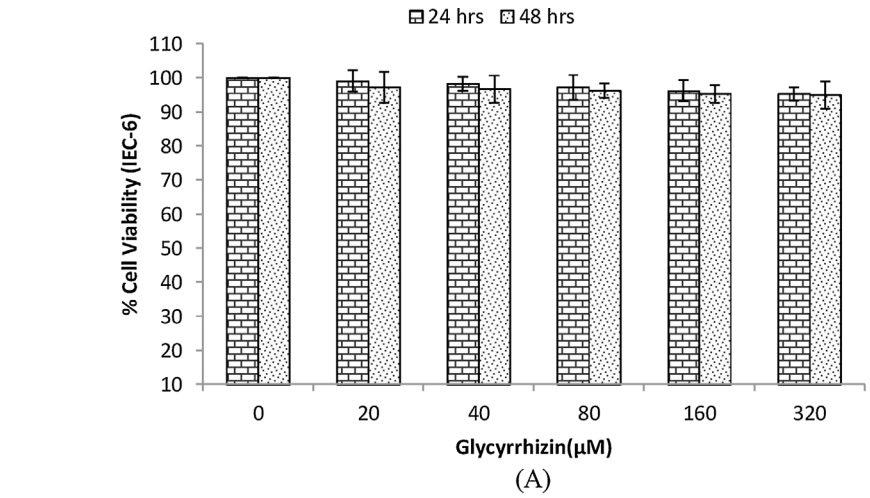
3.1. Glycyrrhizin exhibited insignificant effect on normal colon epithelial IEC-6 cells

To find out the cytotoxicity of glycyrrhizin on normal cells, cell viability assay by MTT dye and morphological analysis by phase contrast microscope were performed on normal colon epithelial IEC-6 cells after treatment with glycyrrhizin. The results showed that, after exposure to various doses of glycyrrhizin (20, 40, 80, 160 and 320 µM) for 24 and 48 h, IEC-6 cells did not show any significant toxicity up to 160 µM dose. The cell viability of normal cells, after 24 h treatment, was found to be around 99.31%, 98.49%, 96.28%, and 94.15% at 20, 40, 80 and 160 µM of glycyrrhizin, respectively; which were statistically insignificant as compared to untreated control (Fig. 1A). Likewise, we could not find any significant cytotoxic effect of different doses of glycyrrhizin (20, 40, 80 and 160 µM) on IEC-6 cells after 48 h of treatment (Fig. 1A). However, at highest concentration of glycyrrhizin (320 µM), a significant decrease in the cell viability was observed and it was found to be 91.15% and 88.91% after 24 and 48 h of treatment (Fig. 1A).

Moreover, morphological analysis suggested that lower and moderate doses of glycyrrhizin (20, 40, 80 and 160 µM) did not exert significant effect on cell morphology after 24 and 48 h of treatment as revealed in Fig. 1B. But, at highest concentration of glycyrrhizin (320 µM), cell morphology was found to be significantly altered, indicating cell death in normal colon cells, after treatment with indicated time periods (Fig. 1B). Thus, the results showed that glycyrrhizin did not exert considerable cytotoxic effect in normal cells.

3.2. Glycyrrhizin inhibited proliferation of cervical cancer HeLa cells

To test the anti-proliferative and cytotoxic potential of glycyrrhizin on cervical cancer cells, cell viability assay was performed on HeLa cells treated with different concentrations of glycyrrhizin (20, 40, 80, 160 and 320 µM) for 24 and 48 h. The results indicated that glycyrrhizin treatment resulted in a significant decrease of cell viability as compared to untreated control (Fig. 2A). Moreover, glycyrrhizin inhibited proliferation of HeLa cells in a concentration and time dependent manner with median inhibitory concentrations (IC_{50}) of around 121.84 µM and 38.53 µM, for 24 h and 48 h, respectively. After 24 h of glycyrrhizin treatment, the cell viability of HeLa cells was found to be significantly reduced to around 88.43%, 67.46%, 54.48%, 47.40%, and 24.63% at



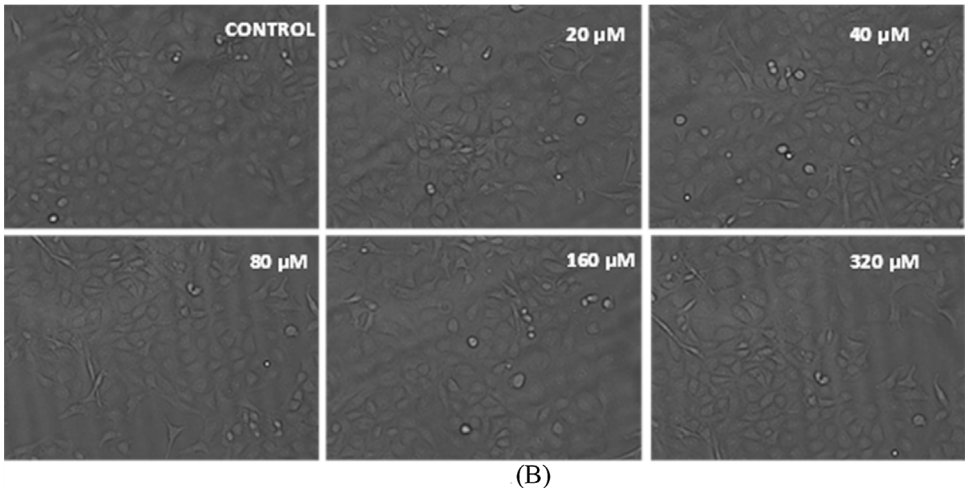


Fig. 1. *In vitro* activity of glycyrrhizin against normal colon epithelium IEC-6 cells (A) Insignificant effect of glycyrrhizin on cell viability of normal IEC-6 cells treated with different doses of Glycyrrhizin (20–320 μM) for 24 and 48 h assessed by MTT assay (B) Phase-contrast photomicrograph of IEC-6 cells treated with either vehicle control or different doses of Glycyrrhizin (20–320 μM) for 24 h. The results represented are the mean ± S.E.M of three independent experiments performed in triplicate (*p < 0.01, **p < 0.001 represent significant difference compared with control).

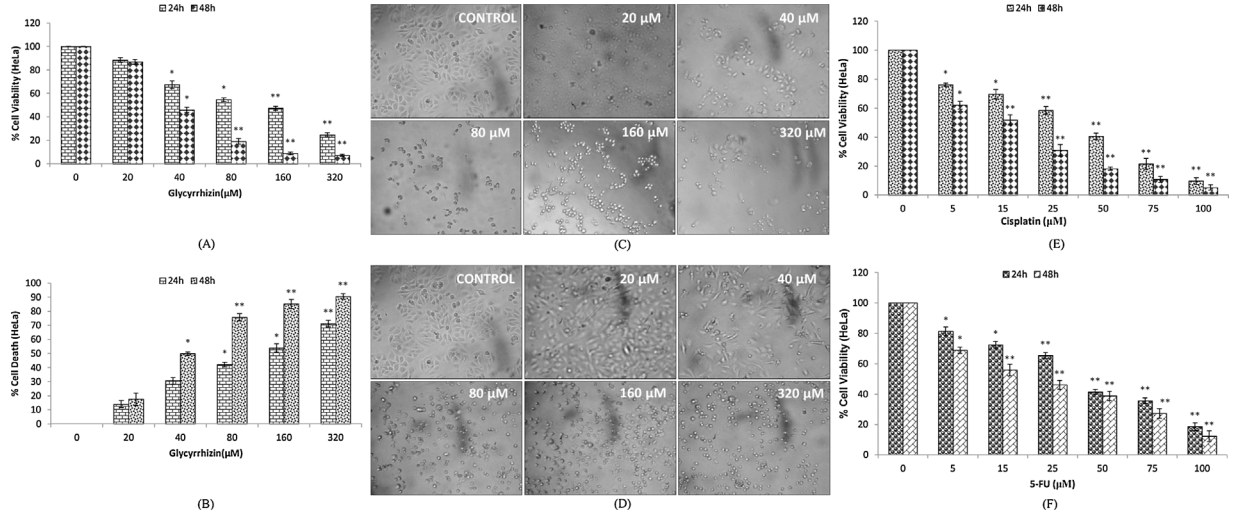


Fig. 2. *In vitro* activity of glycyrrhizin against cervical cancer HeLa cells (A) Percent cell viability of HeLa cells treated with different doses of glycyrrhizin (20–320 μM) for 24 and 48 h assessed by MTT assay (B) Percent cell death of HeLa cells treated with different doses of Glycyrrhizin (20–320 μM) for 24 and 48 h assessed by trypan blue dye exclusion assay. (C) Phase-contrast photomicrograph of HeLa cells treated with either vehicle control or different doses of Glycyrrhizin (20–320 μM) for 24 and (D) For 48 h. The results represented are the mean ± S.E.M of three independent experiments performed in triplicate (*p < 0.01, **p < 0.001 represent significant difference compared with control). (E) Percent cell viability of HeLa cells treated with different doses of cisplatin (0–100 μM) for 24 and 48 h assessed by MTT assay. (F) Percent cell viability of HeLa cells treated with different doses of 5-FU (0–100 μM) for 24 and 48 h assessed by MTT assay. (G) Percent cell viability of IEC-6 cells treated with different doses of cisplatin (0–100 μM) for 24 and 48 h assessed by MTT assay. (H) Percent cell viability of IEC-6 cells treated with different doses of 5-FU (0–100 μM) for 24 and 48 h assessed by MTT assay. (I) Percent cell viability of HeLa cells treated with different doses of glycyrrhizin (20–320 μM) compared to glycyrrhizin treatment (20–320 μM) accompanied with 5 μM cisplatin for 24 by MTT assay. (J) Percent cell viability of HeLa cells treated with different doses of glycyrrhizin (20–320 μM) compared to glycyrrhizin treatment (20–320 μM) accompanied with 5 μM 5-FU for 24 by MTT assay. The results represented are the mean ± S.E.M of three independent experiments performed in triplicate (*p < 0.01, **p < 0.001 represent significant difference compared with control).

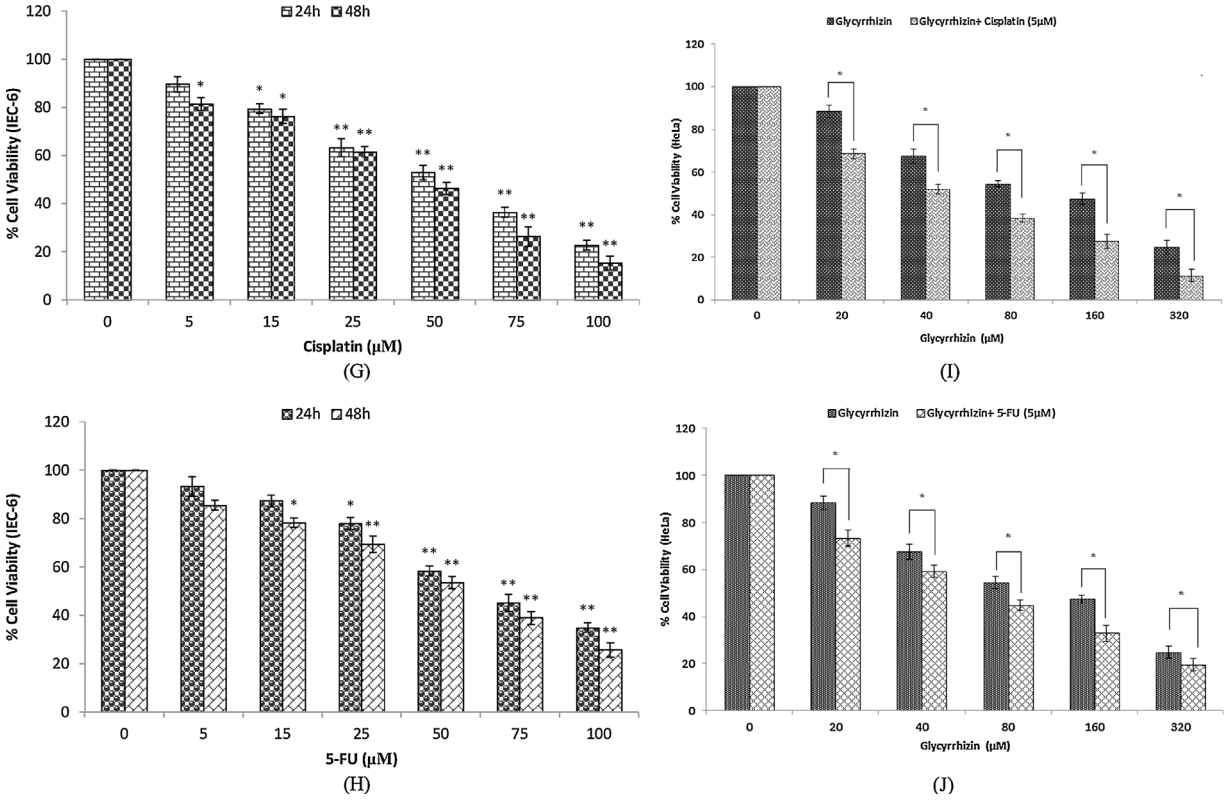


Fig. 2. (*continued*)

20, 40, 80, 160 and 320 μM of glycyrrhizin, respectively, as compared to control (Fig. 2A). The viability of cervical cancer cells was further declined after 48 h of treatment, and was found to be around 86.87%, 45.85%, 19.09%, 8.77% and 7.22% at 20, 40, 80, 160 and 320 μM of glycyrrhizin, respectively, as compared to control (Fig. 2A). Thus, HeLa cells exhibited marked decrease in cell viability at different doses of glycyrrhizin.

Additionally, the cytotoxic effect of glycyrrhizin was also confirmed by trypan blue exclusion assay. A significant cell death in cervical cancer cells was observed after 24 and 48 h of treatment of different doses of glycyrrhizin (20, 40, 80, 160 and 320 μM). The results showed that treatment of HeLa cells with glycyrrhizin for 24 h resulted in significant amount of cell death of around 8.14%, 20.97%, 46.25%, 72.50% and 81.86% at 20, 40, 80, 160 and 320 μM, respectively, when compared to control.

The cell death in HeLa cells was further increased to around 10.16%, 25.31%, 52.15%, 76.75% and 82.45% after 48 h of treatment at 20, 40, 80, 160 and 320 μM, respectively (Fig. 2B). Thus, the above results have established that glycyrrhizin could induce substantial cytotoxic effect on human cervical cancer cells without incurring significant cytotoxic effect on normal cells.

3.3. Cytotoxic effect of cisplatin and 5-fluorouracil (5-FU)

Cisplatin and 5-fluorouracil (5-FU) are chemotherapeutic agents used to treat cervical cancer (paper 2). To evaluate the sensitivity of cervical cancer cells to these drugs, HeLa cells were treated with different doses of cisplatin (5–100 μM) and 5-FU (5–100 μM) for 24 and 48 h followed by MTT assay (Fig. 2E). Our results showed that, after 24 h of treatment, cisplatin at a concentration of 36.36 μM reduced growth of HeLa cells by 50%, while inhibition of 50% viability of HeLa cells was observed at 40.65 μM of 5-FU (Fig. 2F). Similar pattern of results with more pronounced cytotoxic effect was observed after 48 h of treatment with cisplatin (IC50: 16.08 μM) and 5-FU (IC50: 20.12 μM) (Fig. 2E & F). Cisplatin and 5-FU were found to be more antiproliferative and cytotoxic for cervical cancer cells in comparison to glycyrrhizin and the effect was observed to be dose-and time-dependent. Apart from that, both the drugs were much more cytotoxic to normal colon IEC-6 cells than glycyrrhizin (Fig. 2G & H).

3.4. Synergistic effect of glycyrrhizin with cisplatin and 5-FU on the cervical cancer cell growth inhibition

The inhibitory effect of glycyrrhizin on cervical cancer HeLa cell growth raised the possibility that glycyrrhizin could exert synergistic effect with cisplatin and 5-FU on the cell viability. Briefly, HeLa cells were grown for 24 h in the presence of sub-optimal dose of cisplatin (5 μM) and 5-FU (5 μM) in combination with increasing doses of glycyrrhizin (20–320 μM) followed by MTT assay. The results showed that the proliferation of HeLa cells was suppressed up to around 88.43%, 67.46%, 54.48%, 47.40% and 24.63%, respectively, when glycyrrhizin (20–320 μM) was used alone but an additional administration of cisplatin (5 μM), with each indicated dose of glycyrrhizin (20–320 μM), abrogated cell growth up to around 68.57%, 51.88%, 38.48%, 27.62% and 11.34%, respectively (Fig. 2I). Similarly, glycyrrhizin (20–320 μM) and 5-FU (5 μM) combination caused an additional decrease in the cell viability up to around 73.24%, 59.19%, 44.83%, 32.82% and 19.52%, respectively (Fig. 2J). Thus, the combined treatment of glycyrrhizin and chemotherapeutic drugs (cisplatin & 5-FU) caused an additional decrease in cell viability over that of glycyrrhizin alone. The overall results of this experiment indicated that glycyrrhizin and the above drugs can act synergistically to reduce survival of cervical cancer cells, thus potentially reducing the doses of these drugs required to achieve the same effect.

3.5. Glycyrrhizin induced morphological changes in cervical cancer cells

Morphological analysis of the glycyrrhizin-treated HeLa cells was performed using a phase contrast microscope. A dose-and time-dependent change in the cell morphology was observed in HeLa cells after

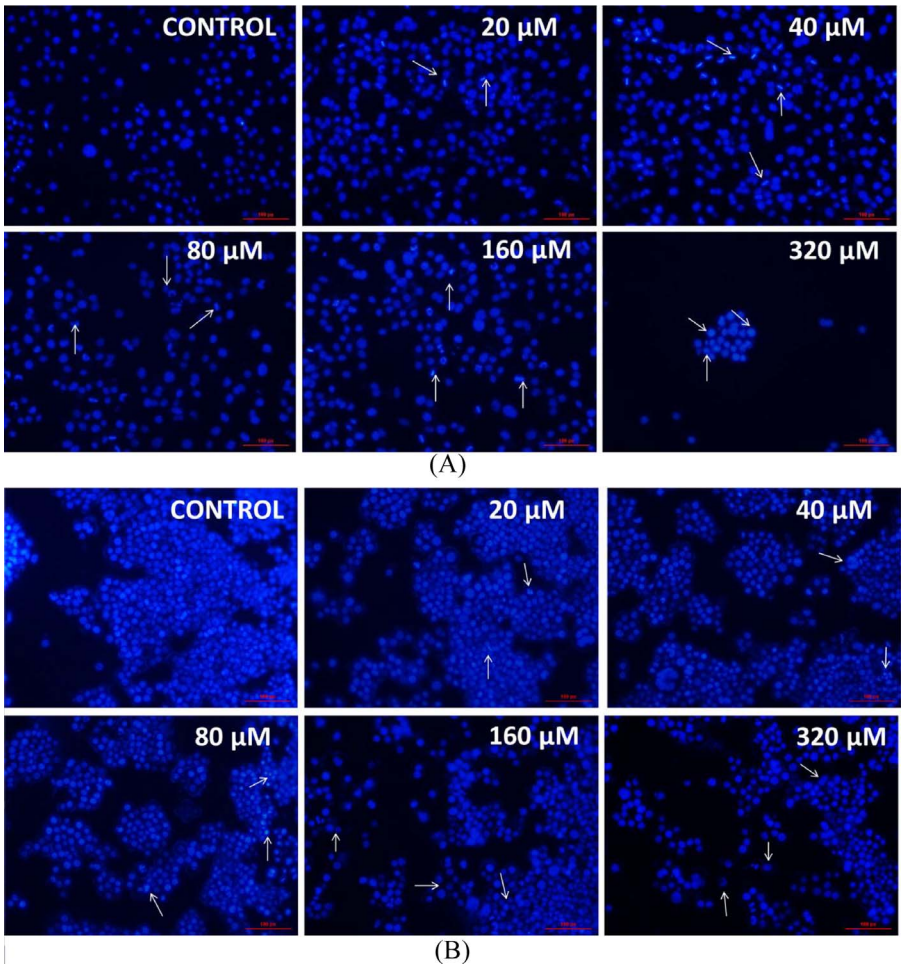


Fig. 3. Glycyrrhizin induced nuclear condensation in human cervical cancer HeLa cells; Nuclear morphology of (A) DAPI-stained and (B) Hoechst-3342 stained nuclei of HeLa cells treated with varying concentrations of glycyrrhizin (20–320 µM) for 24 h analyzed by fluorescence microscopy. Data shown are representative of three independent experiments.

treatment with glycyrrhizin for 24 and 48 h. In the presence of different doses of glycyrrhizin (20, 40, 80, 160 and 320 µM), HeLa cells showed round morphology with small shrinkage and nuclear condensation. A proportion of the cells revealed swelling, cell membrane lysis and disintegration of organelles, suggesting glycyrrhizin-induced cytotoxicity in HeLa cells (Fig. 2C & D). These morphological changes in cervical cancer cells were more evident with the increase in the dose of glycyrrhizin. In contrast, well spread flattened morphology was observed in untreated control cells.

3.6. Glycyrrhizin induced nuclear condensation in HeLa cells

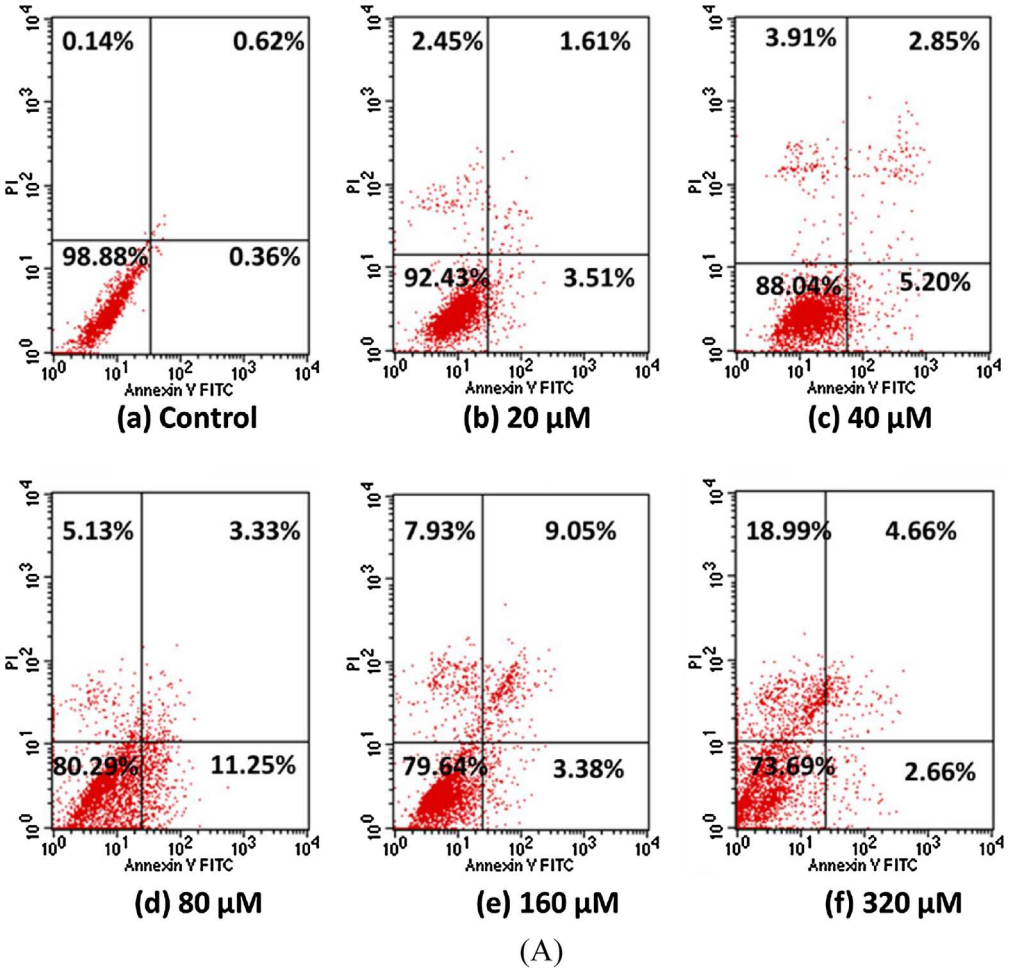
Apoptosis is characterized by prominent nuclear changes in a cell like nuclear fragmentation and condensation. Thus, Hoechst 33342 and DAPI staining were performed to investigate whether inhibition of cell proliferation in glycyrrhizin-treated cervical cancer cells is due to apoptosis. After treatment with glycyrrhizin (20, 40, 80, 160 and 320 µM) for 24 h, significant nuclear changes in HeLa cells were observed as shown in Fig. 3 As apparent from photomicrographs, glycyrrhizin induced nuclear condensation and fragmentation in HeLa cells in a dose dependent manner as indicated by arrow, whereas the control cells exhibited normal cell morphology (Fig. 3A). Similar results, showing condensed and fragmented nuclei, were obtained with Hoechst 33342 dye which also indicated apoptosis in glycyrrhizin-treated HeLa cells; however, untreated HeLa cells showed diffusely stained intact nuclei (Fig. 3B). The results, ultimately, suggested that glycyrrhizin induced apoptosis in cervical cancer cells in a dose-dependent manner.

3.7. Glycyrrhizin induced apoptosis in cervical cancer HeLa cells

To establish whether the anti-proliferative property of glycyrrhizin observed in HeLa cells was due to apoptosis, fluorescence activated cell sorting (FACS) based Annexin-V FITC/PI assay was performed to measure the amount of apoptosis in HeLa cells treated with different doses of glycyrrhizin (20, 40, 80, 160 and 320 µM) for 24 h. As shown in Fig. 4A, the lower left quadrant (LL) showed untreated control cells that were viable and primarily negative for Annexin V-FITC and PI. The lower right quadrant showed early apoptotic cells that were Annexin V-FITC positive and PI negative. The upper right quadrant showed late apoptotic cells (Annexin V-FITC and PI positive) and the upper left showed dead or necrotic cells (Annexin V-FITC negative and PI positive). The results of this assay showed that treatment of glycyrrhizin significantly reduced the amount of viable cells and increased the amount of apoptotic cells in a dose-dependent manner upto 80 µM, which were found to be around 5.12%, 8.05%, and 14.58%, respectively, at 20, 40, and 80 µM doses (Fig. 4B). However, when the dose was increased to 160 µM, amount of dead cells increased considerably which was observed to be around 7.93% along with 12.43% of apoptosis. The amount of dead cells was further increased to 18.99% along with 7.23% of apoptosis after treatment with 320 µM dose of glycyrrhizin (Fig. 4B).

3.8. Glycyrrhizin induced activation of caspases (capsae-8, -9 and -3) cervical cancer HeLa cells

We further investigated whether caspases play any role during glycyrrhizin-induced apoptosis in HeLa cells. Caspases are the members



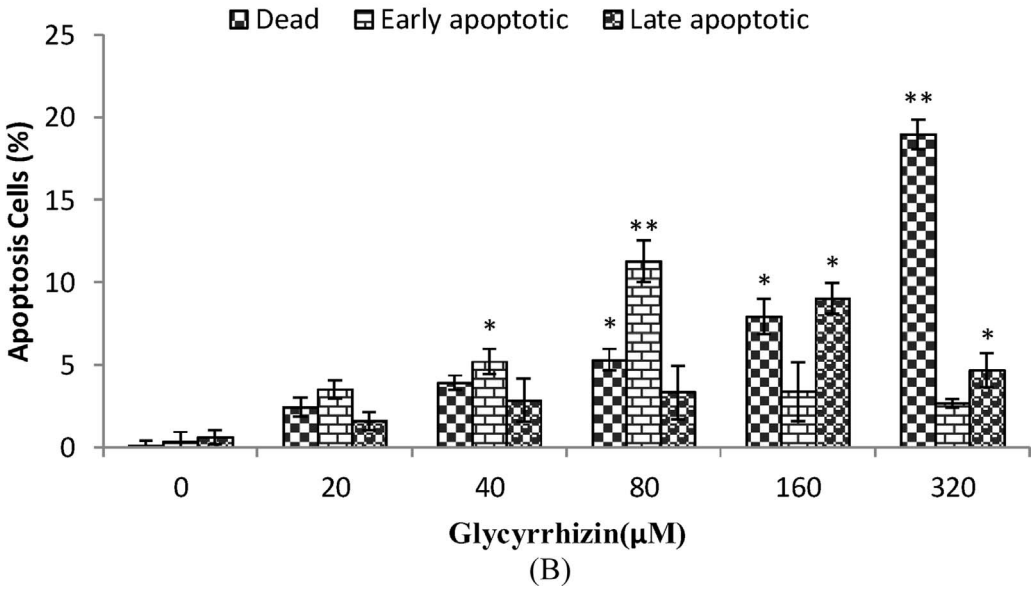


Fig. 4. Glycyrrhizin induced apoptosis in cervical cancer HeLa cells (A) Percent apoptosis in Annexin V-FITC/PI-stained HeLa cells treated with varying concentrations of glycyrrhizin (20–320 µM) for 24 h observed by flow cytometric analysis. The lower right (LR) quadrant of the histograms indicates the early apoptotic cells, and the upper right (UR) quadrant indicates the late apoptotic cells. Data shown are representative of three independent experiments (B) Graphical representation of percent apoptosis as observed by Annexin V-FITC/PI assay. The results represented are the mean ± S.E.M of three independent experiments performed in triplicate (*$p < 0.01$, **$p < 0.001$ represent significant difference compared with control).

of cysteine-aspartate protease family and important executioners of apoptosis which arbitrate the proteolytic degradation of many cellular proteins. Since, caspase-8 and -9 are the initiator caspases of extrinsic and intrinsic apoptotic pathway, respectively; and caspase-3 is the main downstream effecter caspase; hence, we determined their activities in treated and untreated control cells. A significant induction of caspase activities in HeLa cells was observed after treatment with different concentrations of glycyrrhizin (20, 40, 80, 160 and 320 µM) for 24 h (Fig. 5A). Caspase-3 activity was significantly increased by 32.88%, 58.6%, 89.53%, 118.45% and 152.72% as compared to untreated control, at the dose of 20, 40, 80, 160 and 320 µM of glycyrrhizin, respectively (Fig. 5A).

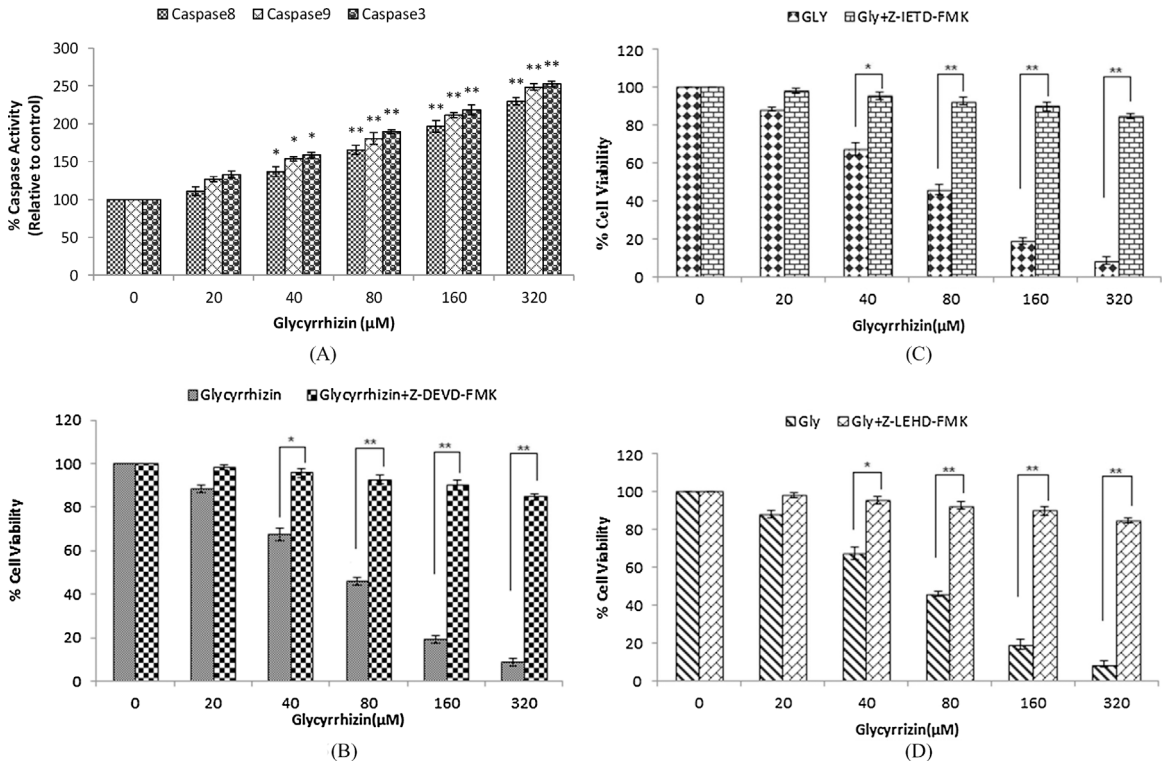


Fig. 5. Activation of caspase in glycyrrhizin-treated HeLa cells (A) Percent caspase-3, 8 and 9 activity in HeLa cells treated with different concentrations of glycyrrhizin (20–320 μM) for 24 h determined by caspase-3, 8 and 9 activity assay (B) Percent cell viability of HeLa cells pre-treated with a caspase-3 inhibitor, Z-DEVD-FMK and then treated with different doses of glycyrrhizin (20–320 μM) for 24 h assessed by MTT assay. (C) Percent cell viability of HeLa cells pre-treated with a caspase-8 inhibitor, Z-IETD-FMK and then treated with different doses of glycyrrhizin (20–320 μM) for 24 h assessed by MTT assay. (D) Percent cell viability of HeLa cells pre-treated with a caspase-8 inhibitor, Z-LEHD-FMK and then treated with different doses of glycyrrhizin (20–320 μM) for 24 h assessed by MTT assay. Data represent mean ± S.E.M of three independent experiments performed in triplicate (*p < 0.01, **p < 0.001 represent significant difference compared with control).

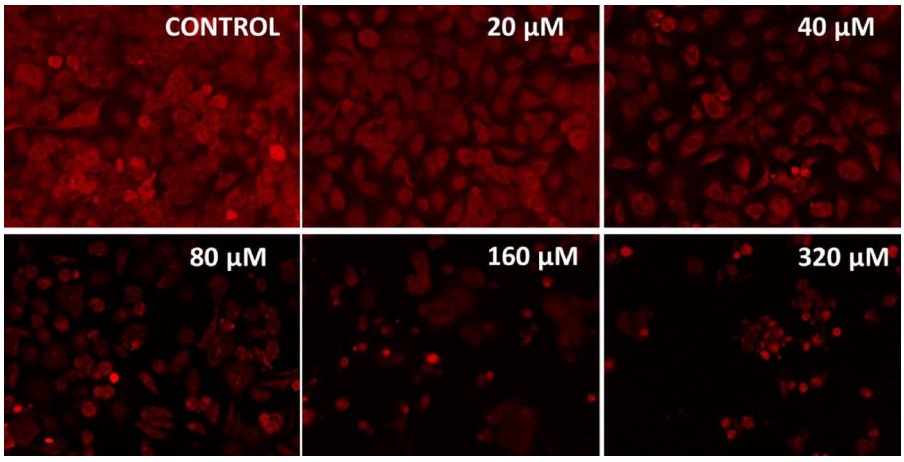


Fig. 6. Mitochondrial depolarization in glycyrrhizin treated HeLa cells (A) Disruption of the mitochondrial membrane potential (ΔΨm) of HeLa cells treated with 20–320 μM of andrographolide observed by staining with a Mitotracker Red CMXROS. Values are expressed as mean ± SEM of three independent experiments (*p < 0.01 and **p < 0.001 as compared with control).

Similarly, a considerable dose dependent augmentation in caspase-8 and -9 activities were found in HeLa cells treated with various doses of glycyrrhizin (20, 40, 80, 160 and 320 μM) as shown in Figure. Thus, the results of caspase activity assay signified the involvement of both extrinsic and intrinsic pathway of apoptosis in glycyrrhizin treated cervical cancer cells.

3.9. Attenuation of glycyrrhizin-induced apoptosis by caspase inhibitors

To characterize whether activation of caspases played role in glycyrrhizin-induced cytotoxicity in cervical cancer cells, cell viability assay was performed in HeLa cells pre-treated with 50 μM of Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK (caspase-9 inhibitor) for 2 h prior to glycyrrhizin (20, 40, 80, 160 and 320 μM) treatment for an additional 24 h. The results showed that pre-treatment of caspase inhibitors resulted in a significant reduction in the cytotoxicity of glycyrrhizin in cervical cancer cells (Fig. 5B–D). Thus, caspase activation in cervical cancer cells played a vital role in glycyrrhizin-induced apoptosis. However, pre-treatment of caspase inhibitors did not result in the complete amelioration of the inhibitory effect of glycyrrhizin on the cell viability of HeLa cells which indicated towards the role of an additional caspase-independent pathway of apoptosis induced by glycyrrhizin.

3.10. Glycyrrhizin disrupted mitochondrial membrane potential in HeLa cells

The characteristic feature of the mitochondrial apoptotic pathway is

the loss of mitochondrial membrane potential (ΔΨm). To examine whether glycyrrhizin could disrupt the ΔΨm, mitochondria-specific and potential-dependent dye Mito Tracker Red CMX Ros was used to detect change in ΔΨm in glycyrrhizin-treated HeLa cells. After treatment with glycyrrhizin (20, 40, 80, 160 and 320 μM), the fluorescence intensity in cells was decreased, indicating a decrease in ΔΨm as shown in Fig. 6. As the concentration of glycyrrhizin increased, ΔΨm correspondingly decreased (Fig. 6). Therefore, the result suggested a dose dependent reduction in the ΔΨm of glycyrrhizin-treated HeLa cell line (Fig. 6).

3.11. Glycyrrhizin augmented intracellular ROS generation in cervical cancer cells

Both caspase-dependent and caspase-independent forms of cell death are known to be activated by an increased production of intracellular reactive oxygen species (ROS) [30,31]. Thus, to explicate the mechanism of action of glycyrrhizin, we further determined the intracellular ROS level in glycyrrhizin treated and untreated cervical cancer cells by DCFH-DA method. Briefly, HeLa cells were stained with DCFH-DA to analyze the changes in intracellular ROS level after 12 h of glycyrrhizin treatment. The photomicrograph depicted that, as compared to control cells, treatment with different doses of glycyrrhizin (20, 40, 80, 160 and 320 μM) resulted in stronger DCF-fluorescence intensity in HeLa cells, which indicated an enhanced intracellular ROS level (Fig. 7A). The above observations were further verified by the quantitative measurement of ROS generation which showed that intracellular ROS level was elevated by around 16.18% as compared to control when HeLa cells were treated with 20 μM of glycyrrhizin. However, ROS generation was further augmented to approximately 41.05%, 73.97%, 134.48% and 184.38% at the concentrations of40, 80, 160 and 320 μM glycyrrhizin, respectively (Fig. 7B). Thus, both the above results suggested that glycyrrhizin augmented ROS formation in HeLa cells in a dose-dependent manner (Fig. 7A & B).Additionally, in order to confirm the glycyrrhizin-mediated augmentation of ROS formation, quantitative estimation of ROS level was done in cervical cancer HeLa cells, pre-treated with a known ROS inhibitor, NAC, prior to glycyrrhizin treatment. The results indicated complete attenuation of increased ROS level by pre-treatment with 10 mM NAC. This substantiated our finding that glycyrrhizin could enhance ROS generation in cervical cancer cells (Fig. 7C).

3.12. Attenuation of glycyrrhizin-induced cytotoxicity by N-acetyl-L-cysteine (NAC)

To further confirm the role of enhanced ROS generation in glycyrrhizin-induced cytotoxicity in cervical cancer cells, we examined the effect of glycyrrhizin (20, 40, 80, 160 and 320 μM) in HeLa cells in the presence of NAC by MTT assay. In brief, HeLa cells were treated with a ROS

scavenger NAC at 10 mM for 2 h prior to glycyrrhizin treatment for an additional 24 h. Pre-treatment with ROS inhibitor NAC significantly reduced the amount of cytotoxicity caused by glycyrrhizin as evident in Fig. 7D. Thus the results indicated that the augmented intracellular ROS production is crucial for glycyrrhizin-induced apoptosis. However, NAC did not completely attenuate the inhibitory effect of glycyrrhizin on the cell viability of HeLa cells which suggested the additional role of ROS-independent pathway of apoptosis in glycyrrhizin-treated cervical cancer cells.

3.13. Glycyrrhizin induced significant cell cycle arrest at G_0/G_1 in cervical cancer cells

Cell cycle analysis was carried out *via* a flow cytometer to investigate whether inhibition of cervical cancer cell growth by glycyrrhizin was due to disruption of cell cycle progression. Briefly, HeLa cells were subjected to flow cytometry analysis after treatment with

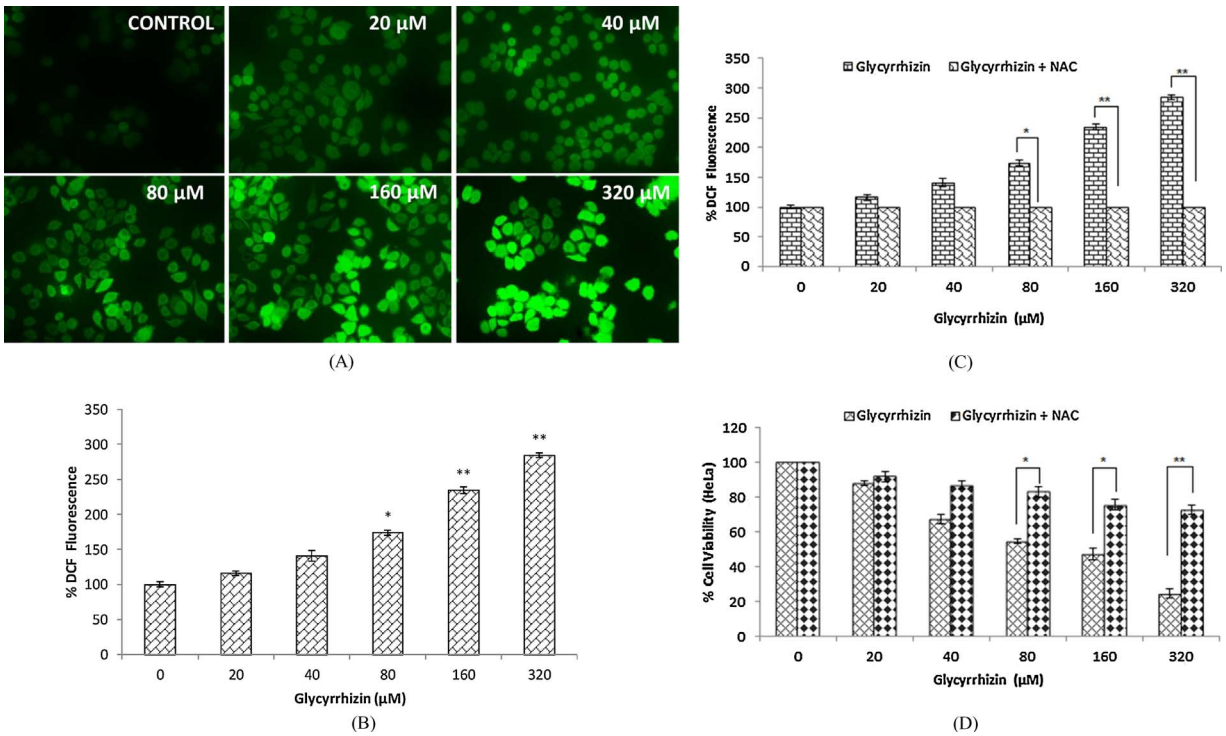
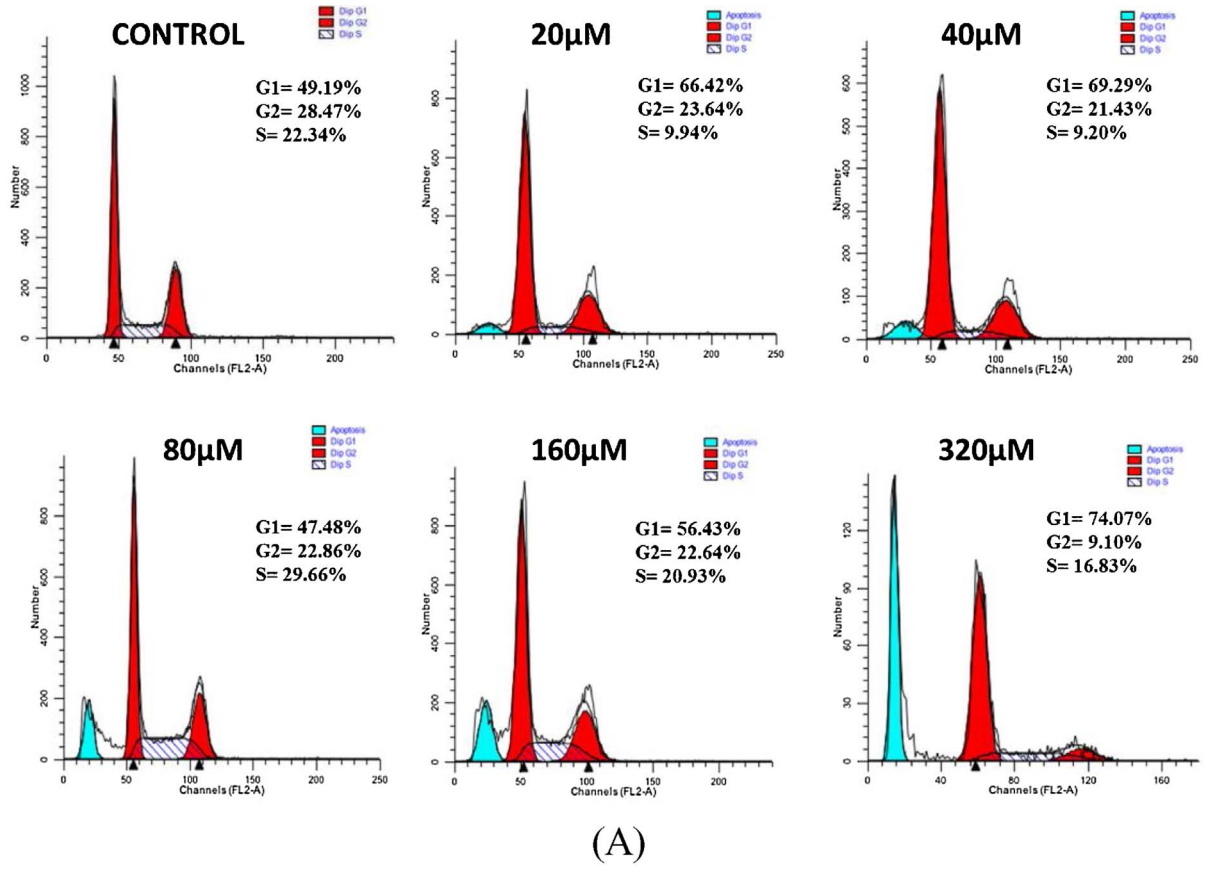


Fig. 7. Intracellular ROS generation in HeLa cells treated with Glycyrrhizin (A) Enhanced ROS generation in DCFH-DA stained HeLa cells treated with different concentrations of Glycyrrhizin (20–320 μM) analyzed by fluorescence microscopy. Data shown are representative of three independent experiments (B) Quantification of ROS level in terms of percent DCFDA fluorescence in HeLa cells treated with different concentrations of Glycyrrhizin (20–320 μM) (C) ROS level in HeLa cells pre-treated with a ROS inhibitor, NAC and then treated with different doses of Glycyrrhizin (20–320 μM) (D) Percent cell viability of HeLa cells pre-treated with NAC and then treated with different doses of Glycyrrhizin (20–320 μM) for 24 h assessed by MTT assay. Values are expressed as mean ± SEM of three independent experiments (**p < 0.01 and **p < 0.001 as compared with control).



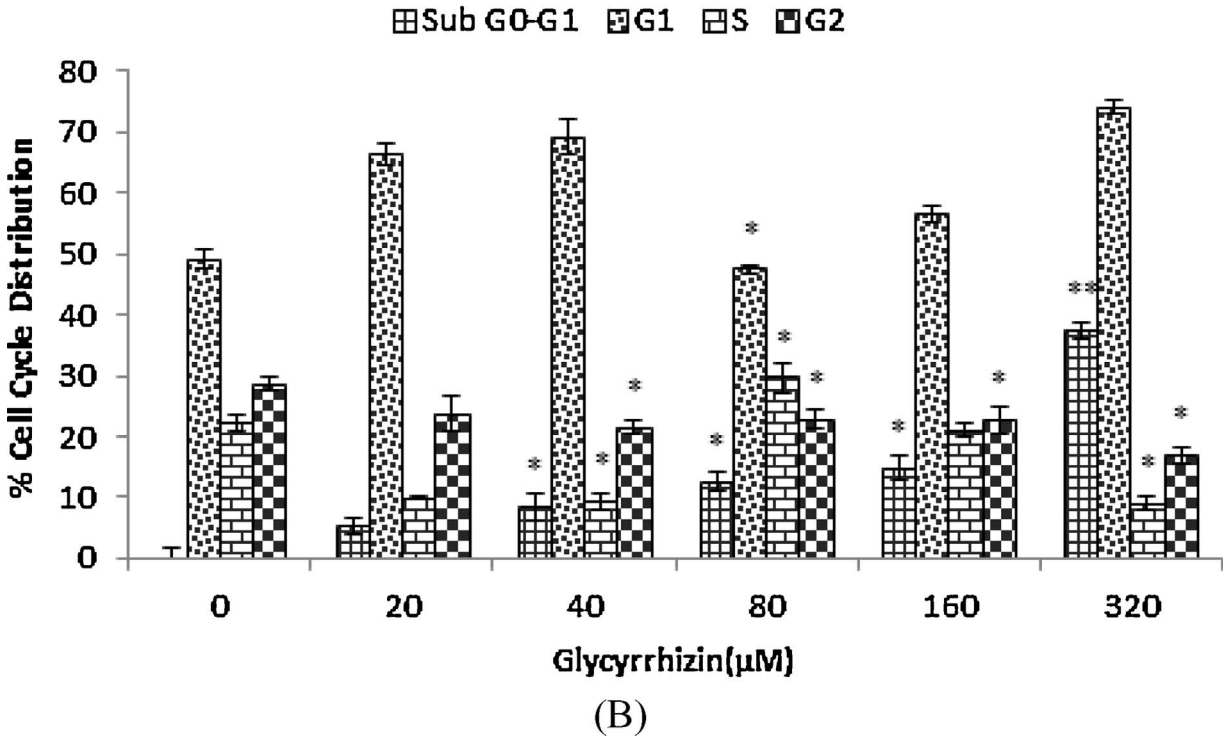


Fig. 8. Effect of Glycyrrhizin on different phases of cell cycle in a dose-dependent manner (A) Cell cycle distribution of propidium iodide-stained HeLa cells treated with 20–320 µM Glycyrrhizin for 24 h observed by flow cytometric analysis. The data shown here is from a representative experiment repeated three times (B) Percent cell cycle distribution as determined by flow cytometric analysis. Data represent mean ± S.E.M of three independent experimentsperformed in triplicate (*P < 0.05 and **P < 0.01 as compared to control).

glycyrrhizin (20, 40, 80, 160 and 320 μM) for 24 h followed by propidium iodide (PI) staining. The result showed that glycyrrhizin treatment over 24 h resulted in a significant cell cycle arrest in HeLa cells at G_0/G_1 phase (Fig. 8A & B). The cell cycle distribution in untreated control was found to be 49.19%, 22.34% and 28.47% for G_0/G_1, S and G_2/M phase, respectively. The treatment with lower dose of glycyrrhizin (20 μM) caused a reduction in S (9.94%) phase cell population and increase in G_0/G_1 (66.42%) phase population (Fig. 8A). A further increase in glycyrrhizin concentration to 40 μM resulted in more accumulation of cells in G_0/G_1 (69.29%) phase (Fig. 8B). Surprisingly, the G_0/G_1 phase cell population was dropped to be 47.48% when the dose of glycyrrhizin was further increased to 80 μM (Fig. 8B). Interestingly, the cells again began to accumulate in G_0/G_1 phase (56.43%) when they were treated with 160 μM glycyrrhizin. At the highest dose of 320 μM, the G_0/G_1 phase cell population was found to be 74.07% (Fig. 8B). Thus, the results showed that glycyrrhizin caused a significant cell cycle arrest in cervical cancer cells at G_0/G_1phase. It is noteworthy that increase in the dose of glycyrrhizin resulted in appearance of sub-G_0/G_1 population of cells (sub-diploid or hypo-diploid cells) in the cell cycle histogram indicating apoptosis (Fig. 8B). Approximate amount of apoptosis was denoted by percentage of sub-diploid cells as shown in cell cycle histogram (Fig. 8B). When cells were exposed to 20 μM of glycyrrhizin for 24 h, 5.26% apoptotic cells were observed as compared to control. However, at 40, 80, 160 and 320 μM of glycyrrhizin treatment, apoptotic cells were markedly increased to approximately 8.40%, 12.62%, 14.79% and 37.37% respectively. The result of sub-G_0/G_1 phase was in agreement with the result of annexin-V FITC/PI apoptosis assay where a considerable amount of cells were observed to be dead at 160 and 320 μM concentration. Thus, the intriguing results suggested that glycyrrhizin could induce cell cycle arrest in G_0/G_1 phase along with a considerable amount of apoptosis, which might be associated with the inhibitory effect of glycyrrhizin on cervical cancer HeLa cells.

4. Discussion

Search for new chemopreventive and antitumor agents that are more effective but less toxic has kindled great interest in phytochemicals. The high cost, side effects and therapeutic limitations of conventional medications are key factors that are driving the revival of herbal remedies.

Glycyrrhizin, a licorice derived compound, is known for its wide range of biological activities, such as anti-inflammatory, antioxidant, antiviral as well as antitumor properties [18,32]. It has been reported to metabolize into 18β-glycyrrhetinic acid, another active phytochemical of licorice [33]. Both, glycyrrhizin and 18β-glycyrrhetinic acid have been shown to inhibit cell growth and induce apoptosis in many cancer cells, including human hepatoma cells, promyelotic leukemia HL-60 cells and stomach cancer cells [34–36]. However, glycyrrhizin did not showed cytotoxic effect on normal cells up to a concentration of 12 mM, suggesting its efficacy as a safe and non-toxic plant product [22,34,35]. Meanwhile, 18β-glycyrrhetinic acid has recently been shown to induce apoptosis in cervical cancer SiHa cells [36]. But, the cytotoxic effect of glycyrrhizin against human cervical cancer cells has not been elucidated yet. Therefore, the present study was undertaken to examine the efficacy of glycyrrhizin as an anti-proliferative and anti-cancer agent against cervical cancer.

In the current study, we systematically attempted to delineate the effects of the glycyrrhizin on cervical cancer HeLa cell line. The cell viability assay demonstrated that exposure of HeLa cells to glycyrrhizin significantly decreased the viability of cervical cancer cells in a time- and dose-dependent manner. Further morphological analysis showed that the shapes of the glycyrrhizin-treated cells were drastically changed and characterized by cellular shrinkage and detachment from surface, forming clusters. These results suggested that glycyrrhizin could exert significant cytotoxic effect in cervical cancer cell line. In addition, glycyrrhizin showed insignificant cytotoxic effect on normal colon epithelial cells at lower doses; but, a substantial decrease in the cell viability was observed only at the highest concentration of glycyrrhizin used in the experiment. Thus, glycyrrhizin was shown to exhibit cytotoxic effect in cervical cancer cells without significant effect on normal cells. Results from previous studies have shown similar effects of glycyrrhizin on different cancer cells [34,35]. Morevoer, cisplatin and 5-FU were found to be more antiproliferative and cytotoxic for cervical cancer cells in comparison to glycyrrhizin and the effect was observed to be dose-and time-dependent. But at the same time, both the drugs were much more cytotoxic to normal colon IEC-6 cells than glycyrrhizin which strengthened our notion that glycyrrhizin would possibly come out as a safer chemopreventive agent against cervical cancer cells. Apart from that, the combined treatment of glycyrrhizin and chemotherapeutic drugs (cisplatin & 5-FU) caused an additional decrease in the cell viability of cervical cancer cells over that of glycyrrhizin alone. The overall results of this experiment indicated that glycyrrhizin and the above drugs can act synergistically to reduce survival of cervical cancer cells, thus potentially reducing the doses of these drugs required to achieve the same effect. Thus, the above findings potentiate the use of glycyrrhizin as solo or in combination with standard drugs against cervical cancer cells to minimize the cytotoxic effect of these drugs with reduced doses.

Fragmented and condensed nucleus in the cells is a hallmark of apoptotic induction [37]. Our result was supported by above fact where fragmented and condensed nuclei HeLa cells, observed with DAPI and Hoechst 33342 staining, suggested that glycyrrhizin caused cell death *via* apoptosis. Moreover, the cytotoxicity of glycyrrhizin was further characterized by probing the appearance of the early apoptotic marker phosphatidylserine at the cell surface by FITC-Annexin V/PI assay which detected early and late apoptotic changes in cells. Accordingly, a dose-dependent increase in the Annexin V positive cells was observed which suggested induction of apoptosis in HeLa cells by glycyrrhizin.

Caspase-8 and -9 can promote program cell death in response to mitochondria-associated signaling events and death-inducing signaling from cell surface receptors, respectively, *via* catalyzing the activation of caspase-3 [38]. Caspase-3, one of the key executioners of apoptosis, has been shown to mediate the proteolytic degradation of many cellular proteins [39]. A dose-dependent activation of caspase-8, -9 and -3 in HeLa cells treated with glycyrrhizin suggested that glycyrrhizin could induce apoptotic cell death through cell surface receptor as well as mitochondria-mediated pathway. Moreover, the glycyrrhizin-induced cytotoxicity in cervical cancer cells was considerably reduced by a caspase-8, -9 and -3 inhibitors (Z-IETD-FMK, ZLEHD-FMK and Z-DEVD-FMK), indicating key role of caspase-3 activation during glycyrrhizin-induced apoptosis. This result was also in accordance with the earlier report of glycyrrhetinic acid [36]. However, pre-treatment of caspase inhibitors did not completely attenuated the cell viability in cervical cancer cells which indicated the probable additional role of caspase-independent pathway of apoptosis in glycyrrhizin-treated HeLa cells. Thus, it is reasonable that glycyrrhizin might induce apoptosis in HeLa cells *via* both caspase-dependent and independent pathways. However, the role of caspase-independent pathway, in the current study, remains to be evaluated.

The loss of mitochondrial membrane potential ($\Delta\Psi m$) is an early event in apoptotic process as shown by earlier workers [40,41]. Disruption of the mitochondrial transmembrane potential leads to release of cytochrome c in to cytosol and activation of caspase-3, resulting in loss of cell viability [42]. A significant dose-dependent decrease in the mitochondrial membrane potential was observed in glycyrrhizin-treated cervical cancer cells which indicated towards an early event of apoptosis, induced by glycyrrhizin. Thus, the results suggested that glycyrrhizin caused programmed cell death in HeLa cells by disrupting the mitochondrial membrane potential, which resulted in activation of caspase-3.

Excessive ROS production can lead to oxidation of macromolecules and play an important role in ageing and cell death [43]. Augmented

intracellular ROS level has been attributed to cause mitochondrial membrane damage, ultimately releasing apoptotic factors in the cytosol. We scrutinized whether the glycyrrhizin-induced disruption of mitochondrial membrane potential and apoptosis were mediated by enhanced ROS production. Our results of the qualitative and quantitative analysis of intracellular ROS formation suggested that glycyrrhizin induced the excessive generation of ROS which might be implicated in mitochondrial dysfunction and apoptosis. Moreover, the glycyrrhizin mediated augmentation of intracellular ROS production in cervical cancer cells was attenuated by a ROS inhibitor, NAC; it further confirmed the generation of ROS due to glycyrrhizin treatment. In addition, NAC considerably reduced the amount of cytotoxicity caused by the treatment of glycyrrhizin which indicated that the excessive ROS level is critical for the glycyrrhizin-induced apoptosis. Previously, augmented ROS formation has been shown to be attributed to 18β-glycyrrhetinic acid-induced apoptosis of promyelotic leukemia HL60 cells [44]. However, pre-treatment of NAC did not completely abrogate the inhibitory effect of glycyrrhizin on the cell viability of cervical cancer cells, which indicated towards the contribution of additional ROS-independent pathway of apoptosis in glycyrrhizin-treated HeLa cells. Thus, it might be possible that glycyrrhizin could induce apoptosis in cervical cancer cells *via* both ROS-dependent and ROS-independent pathway. However, the role of ROS-independent pathway of apoptosis, in the current study, remains to be investigated.

An increased ROS level could alter the intracellular concentrations of antioxidants, such as glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), or reduced nicotinamide adenine dinucleotide phosphate (NADPH), resulting in mitochondrial dysfunction [36,42]. The depletion of mitochondrial GSH, due to any oxidative insult, has been shown to activate the apoptotic cascade [45].Thus, the findings of the study suggested that the glycyrrhizin-induced disruption of mitochondrial membrane potential and apoptosis could be accredited to the enhanced ROS level and depletion of cellular and mitochondrial GSH. Previously, 18β-glycyrrhetinic acid-induced apoptosis in cervical cancer SiHa cells has been shown to be linked with the depletion in cellular GSH level [36].

It is well known that carcinogenesis is closely associated with uncontrolled cell cycle. G_0/G_1 and G_2/M phases of the cell cycle are the major check points and have an important role in cell cycle progression. In order to elucidate the effect of glycyrrhizin on cell cycle progression of cervical cancer cells, the percentage of cells in the G_0/G_1, S, G_2/M and sub-G_0/G_1phases was determined by flow cytometric analysis using PI staining. Through cell cycle analysis, we observed an interesting phenomenon that glycyrrhizin arrested cell cycle progression of HeLa cells in G_0/G_1phase by blocking the transition from G_0/G_1 to S phase and consequently lead to apoptosis as observed by appearance of sub-G_0/G_1 cells. Thus, the results suggested that the growth inhibitory effect of glycyrrhizin on cervical cancer cells was due to both apoptosis and cell cycle inhibition. Previously, glycyrrhizin have been shown to have antitumor actions in different cancer cell lines *via* causing the cell cycle arrest, induction of apoptosis or a combination of these mechanisms [46,47]. In summary, the results of our study have established that glycyrrhizin exhibited antiproliferative and apoptotic property against cervical cancer cells by inducing the disruption of mitochondrial membrane potential, augmentation of ROS, activation of caspases of both extrinsic and intrinsic pathway of cell death and induction of cell cycle arrest at G_0/G_1 phase. Thus, on the basis of the results of this study, we propose that glycyrrhizin could induce apoptosis in cervical cancer cells *via* activating both intrinsic and extrinsic pathway of apoptosis. However, a caspase-independent pathway of apoptosis along with caspase-dependent pathway is expected to play role in glycyrrhizin-induced program cell death. Furthermore, both ROS-dependent and ROS-independent pathway activated by glycyrrhizin are suspected to follow the demise of cervical cancers cells. Lastly, an early cell cycle arrest of cervical cancer cells induced by glycyrrhizin depicted a strong antiproliferative and a versatile nature of this molecule targeting multiple pathways at a time.

5. Conclusion

Based on our present results we speculated that glycyrrhizin endorsed the excellent cytotoxic potential as well as exhibited the anticancer efficacy through the induction of apoptosis and cell cycle arrest in cervical cancer HeLa cells *in vitro*. Although, understanding the exact underlying molecular mechanisms of glycyrrhizin-induced apoptosis and cell cycle arrest in cervical cancer cells remain far from conclusive, but our study provided new insights to further investigate the molecular mechanism of action of glycyrrhizin against cervical cancer. Therefore, glycyrrhizin is proposed to be a potent anticancer drug candidate for the future chemopreventive and therapeutic studies in cervical cancer.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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