

BJP

Bangladesh Journal of Pharmacology

Research Article

18 α -Glycyrrhetic acid inhibits the viability of HR5-CL11 cervical carcinoma cells through induction of apoptosis and DNA damage

18 α -Glycyrrhetic acid inhibits the viability of HR5-CL11 cervical carcinoma cells through induction of apoptosis and DNA damage

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Article Info

Received: 25 January 2016
Accepted: 8 March 2016
Available Online: 26 August 2016
DOI: 10.3329/bjp.v11i3.26592

Cite this article:

Zhuang HM, Hu X, Hong SR, Zhou PP, Zhang Y, Li L. 18 α -glycyrrhetic acid inhibits viability of HR5-CL11 cervical carcinoma cells through induction of apoptosis and DNA damage. Bangladesh J Pharmacol. 2016; 11: 750-55.

Abstract

The present study was aimed to investigate the effect of 18 α -glycyrrhetic acid on induction of apoptosis and DNA-damage in HR5-CL11 cervical carcinoma cells. The results revealed 73.6% reduction in HR5-CL11 cell viability on treatment with 5 μ M concentration of 18 α -glycyrrhetic acid for 48 hours. The DNA of 18 α -glycyrrhetic acid-treated cells showed a ladder-like pattern. Fragmentation of DNA in the 18 α -glycyrrhetic acid-treated cells was markedly higher compared to the control cells. Examination of the DNA damage in HR5-CL11 cells after treatment with 18 α -glycyrrhetic acid showed breakage in DNA strands and formation of comet-like structures. The frequency of comet formation in 18 α -glycyrrhetic acid treated cells was found to be 7.8 after 48 hours. The population of cells with more than four γ H2AX foci was increased to 38.6% on treatment with 5 μ M concentration of 18 α -glycyrrhetic acid. Thus, 18 α -glycyrrhetic acid inhibits the viability of HR5-CL11 cervical cancer cells through induction of apoptosis by DNA damage and can be used for the treatment of cervical cancer.

Introduction

Cervical cancer comprises the third most commonly detected cancer in females throughout the world. In the year 2008, more than five lac cases of cervical cancer were detected and more than 2.7 lac deaths were caused by cervical cancer (Ferlay et al., 2010). In most of the cervical cancer patients, contraction papillomavirus is the main cause of mortality (Steben and Duarte-Franco, 2007). It has also been observed that the initial stage of cervical cancer is not associated with the symptoms making its detection difficult (Steben and Duarte-Franco, 2007). However, the development new treatment strategies and discovery of novel molecules have led to a reduction in the rate of cervical cancer deaths. Despite the reduction in the number of deaths, cervical cancer continues to be a challenge for clinicians (Jemal

et al., 2007). Therefore, the development of a novel and efficient treatment for cervical cancer is desired.

Natural products have an advantage of being safe and efficient in the treatment of various diseases over the synthetic compounds (Toh et al., 2011). Glycyrrhiza glabra is a herbaceous plant with a wide range of traditional medicinal applications. The extract of G. glabra, known as licorice, was used in China for the treatment of swelling, injury and detoxification (Wang and Nixon, 2001; Nomura and Fukai, 1998). One of the active compounds present in licorice is glycyrrhizin (Baltina, 2003) which is found to inhibit inflammatory reaction (Rackova et al., 2007), enhances immunity (Takahara et al., 1994), prevents ulcer formation (He et al., 2001) and acts as anti-tumor agent (Thirugnanam et al., 2008; Niwa et al., 2007). 18 α -Glycyrrhetic acid is



another active compound present in the licorice which exists as a trans-isomer (Zeng et al., 2006; Ha et al., 1991).

In the present study, the effect of 18 α -glycyrrhetic acid on cervical cancer cells along with the mechanism was investigated. It was observed that treatment of cervical cancer cells with 18 α -glycyrrhetic acid inhibited cell viability, induced DNA damage and apoptosis.

Materials and Methods

Cell line

HR5-CL11 cervical carcinoma cell line was purchased from American Type Culture Collection (USA). The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The medium was also supplemented with antibiotics like streptomycin and penicillin (1%). The cells were incubated in a 5% CO₂ and 95% air incubator at 37°C.

Reagents and chemicals

18 α -Glycyrrhetic acid (purity>98%) was purchased from Shanghai Sangon Biotech Co., Ltd. (China). Dimethylsulfoxide, DMEM and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were supplied by Gibco/Life Technologies (USA). Tween 20 and propidium iodide were obtained from Sigma-Aldrich Corp. (USA).

Analysis of cell viability

HR5-CL11 cervical carcinoma cells at a density of 5 x 10⁶ cells per well were put into 96-well plates and allowed to adhere for overnight. The cells were then incubated with various concentrations of 18 α -glycyrrhetic acid (1, 2, 3, 4 and 5 μ M) for 12, 24, 36, 48 and 72 hours. After incubation, 50 μ L of 5 mg/mL MTT was put into each well of the plate and incubated for 4 hours at 37°C. Dimethyl sulfoxide (200 μ L) was added to each well of the plate to dissolve the resulting formazan crystals if formed. Absorbance for each well was measured by a microplate spectrophotometer (Bio-Tek Instruments Inc., USA) at 565 nm. The results were presented by comparison with the control which was assigned arbitrarily a value of 100% viability. The experiments for analysis of viability were performed three times independently for each well.

Analysis of DNA fragmentation

HR5-CL11 cervical carcinoma cells were incubated with 1, 2, 3, 4 and 5 μ M concentrations of 18 α -glycyrrhetic acid for 12, 24, 36, 48 and 72 hours. The nitrosamine derivative, N-methyl-N-nitro-N-nitrosoguanidine (10 μ M) was taken as the positive control. After incubation, the cells were collected and then washed with

phosphate-buffered saline. DNA from the control and ginsenoside-Rg5-treated cells was isolated using commercially available Wizard Genomic DNA purification kit (Gibco/Life Technologies, USA). For the analysis of DNA fragmentation in the cells electrophoresis was performed using urea polyacrylamide gel after staining with silver nitrate solution.

Analysis of cell apoptosis

Analysis of the induction of apoptosis in HR5-CL11 cervical carcinoma cells was performed using the BD Accuri™ C6 flow cytometer (BD Biosciences, USA). Propidium iodide staining (Invitrogen Life Technologies) was used for the quantification of PBS exposure on the extracellular side of the cell membrane. The cells after 24 hours of incubation in six-well plates were treated with 18 α -glycyrrhetic acid for 48 hours. The cells were harvested and then centrifuged to collect the cell pellets which were subjected to PBS washing. Following washing, the cells were incubated with propidium iodide (5 μ L) for a period of 20 min under dark conditions. Then 1x binding buffer (400 μ L) was put into each of the tube and induction of apoptosis was analyzed using flow cytometry.

Alkaline comet assay

HR5-CL11 cervical carcinoma cells were treated with 1, 2, 3, 4 and 5 μ M concentrations of 18 α -glycyrrhetic acid for 48 hours. Then the cells were harvested, subjected to PBS washing and finally put in PBS (pH 7.4). The cells at a density of 2 x 10⁵ were treated with 1% molten low melting point agarose at room temperature. The Olympus BX53 fluorescent microscope (Olympus Corporation, Japan) was used to capture the images of the cells pasted on to microscopic slides.

Analysis of γ H2AX staining

Into the six-well culture plates, HR5-CL11 cells were distributed at a density of 2 x 10⁶ cells per plate. The cells were incubated with 1, 2, 3, 4 and 5 μ M concentrations of 18 α -glycyrrhetic acid for 48 hours. The cells were then subjected to paraformaldehyde fixing, PBS washing and finally 1% Triton-X 100 mediated permeabilization. Incubation with primary monoclonal goat anti- γ H2AX antibody (1:1, 500; Cell Signaling Technology, Inc., USA) for 12 hours was followed by anti-rabbit secondary antibodies (1:360, Cell Signaling Technology, Inc.) incubation for 2 hours. The cells were treated with DAPI (1 mg/mL) for 30 min at room temperature. The Olympus BX53 fluorescent microscope (Olympus Corporation) was used for the capturing of images.

Statistical analysis

The data presented are the means \pm standard deviation. Determination of the statistically significant differences was performed by SPSS software, version 20.0 (SPSS,

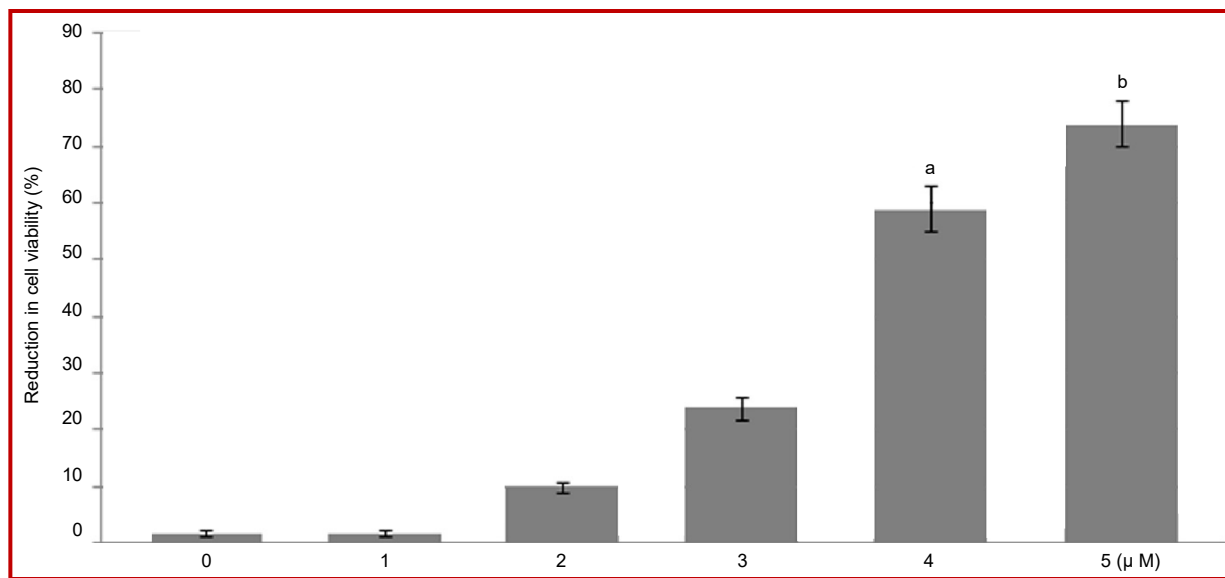


Figure 1: Effect of 18 α -glycyrrhetic acid on inhibition in the viability of HR5-CL11 cervical cancer cells

The cells were treated with 1, 2, 3, 4 and 5 μ M concentration of 18 α -glycyrrhetic acid for 12, 24, 36, 48, and 72 hours. The cell viability was then determined using an MTT assay. The results were compared with control which was assigned 100% viability. The data presented are the mean \pm standard deviation of the experiments performed three times independently. ^a p <0.05, and ^b p <0.01 and vs control cultures

Inc., Chicago, IL, USA). The significant differences were tested using Student's t-test or one-way analysis of variance. p <0.05 was considered to indicate a statistically significant difference.

Results

Growth and viability of cervical carcinoma cells

HR5-CL11 cells were treated with 18 α -glycyrrhetic acid (1, 2, 3, 4 and 5 μ M) for 12, 24, 36, 48, and 72 hours. A concentration- and time-dependent decrease was observed in the cell viability of HR5-CL11 cell cultures (Figure 1). Among the tested concentrations viability was decreased by 73.6% in the cells treated with 5 μ M concentration of 18 α -glycyrrhetic acid compared to control after 48 hours of the treatment (Figure 1).

Induction of apoptosis in cervical carcinoma cells

HR5-CL11 cells were treated with various concentrations of 18 α -glycyrrhetic acid for 48 hours and then examined for DNA fragmentation and apoptosis. DNA of the 18 α -glycyrrhetic acid treated cells showed ladder-like pattern after 48 hours (Figure 2A). Fragmentation of DNA in the 18 α -glycyrrhetic acid treated cells was markedly higher compared to the control cells. Flow cytometry revealed a markedly higher number of apoptotic cells in the cultures treated with 5 μ M concentration of 18 α -glycyrrhetic acid after 48 hours (Figure 2B).

DNA of cervical carcinoma cells

In the HR5-CL11 cells treatment with 18 α -glycyrrhetic acid caused breakage of DNA strands which then

assembled to form comet-like structures. However, the DNA of control cells was found to be normal without any breakage and the comet-like structures were absent. The frequency of comet formation in 18 α -glycyrrhetic acid treated cells was found to be 7.8 after 48 hours. The length of tail and its moment was significantly (p <0.05) higher in the 18 α -glycyrrhetic acid treated cells compared to the control cells (Figure 3).

Appearance of γ H2AX foci

Analysis of the effect of 18 α -glycyrrhetic acid showed a significant increase in the population of HR5-CL11 cells with more than four γ H2AX foci compared to the control cells (Figure 4). The population of cells with more than four γ H2AX foci was increased to 38.6% on treatment with 5 μ M concentration of 18 α -glycyrrhetic acid for 48 hours.

Discussion

The present study demonstrates the role of 18 α -glycyrrhetic acid in inhibition of cervical cancer cell growth and viability through induction of DNA damage and apoptosis. Natural products have an advantage of being safe and efficient in the treatment of various diseases over the synthetic compounds (Toh et al., 2001).

Glycyrrhizin treatment in prostate cancer cells leads to the inhibition of growth and reduction in the cell viability (Thirugnanam et al., 2008). The results from the present study demonstrated that 18 α -glycyrrhetic acid treatment induced concentration and time-dependent reduction in the viability of cervical cancer

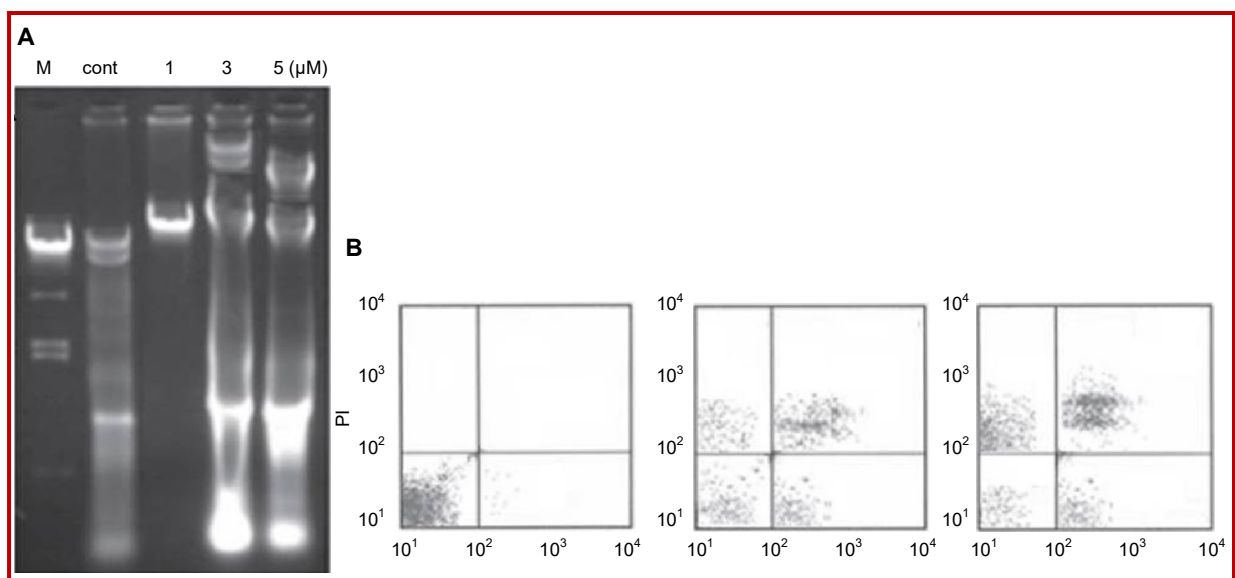


Figure 2: Effect of 18 α -glycyrrhetic acid on induction of apoptosis in HR5-CL11 cells. (A) Fragmentation of DNA in HR5-CL11 cells by 18 α -glycyrrhetic acid treatment at various concentrations. (B) Induction of apoptotic in HR5-CL11 cells on treatment with 18 α -glycyrrhetic acid and nitroso derivative control. The data presented are the mean \pm standard error of the three experiments performed independently. ^a $p < 0.05$ and ^b $p < 0.01$, compared to the control cell cultures

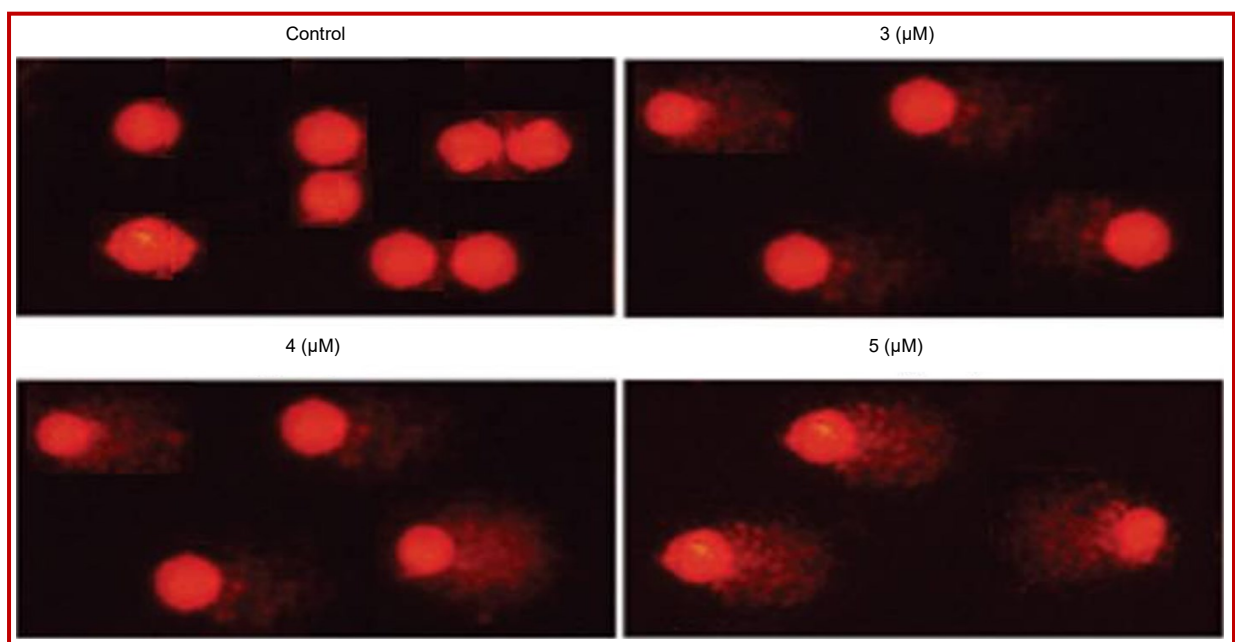


Figure 3: Images of the cells obtained during alkaline gel electrophoresis following 18 α -glycyrrhetic acid treatment. The positive control used in the experiment was a derivative of nitosoamine

cells. It reduced the viability of HR5-CL11 cells after 36 hours of the treatment. Cell death in cancer tissue can be induced by various mechanisms like necrosis, mitotic catastrophe, apoptosis etc. but the commonly observed one is apoptosis (Bras et al., 2005; Edinger and Thompson, 2004). The process of cell apoptosis is caused by various processes which lead to DNA damage and fragmentation (Robertson and Orrenius, 2002).

In the present study, treatment of HR5-CL11 cells with various concentrations of 18 α -glycyrrhetic acid led to a ladder-like pattern of DNA after 48 hours. DNA fragmentation was markedly higher in the 18 α -glycyrrhetic acid treated cells compared to the control cells. The 18 α -glycyrrhetic acid treatment caused induction of apoptosis in markedly higher proportion of cells compared to the control cells.

Like 18 α -glycyrrhetic acid, tanshinone IIA also causes

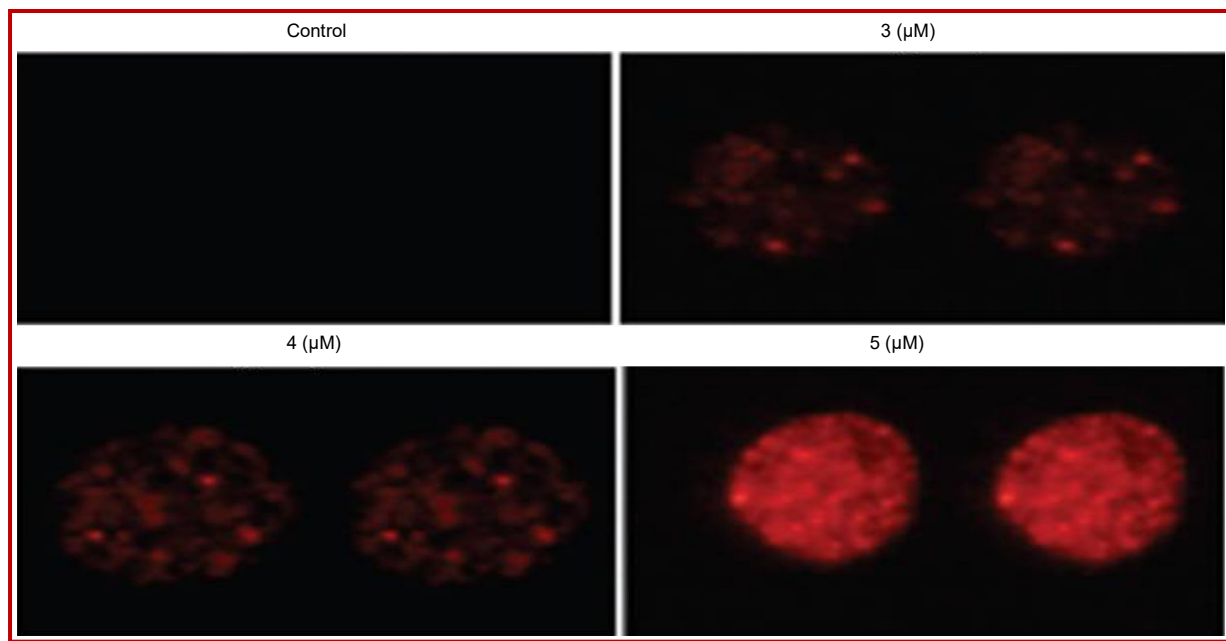


Figure 4: Effect of 18 α -glycyrrhetic acid on structure of DNA in HR5-CL11 cells

The cells were treated with 18 α -glycyrrhetic acid and nitro amine derivative for 48 hours and then analyzed using γ H2AX foci assay. The data presented are the mean \pm standard error of the three experiments performed independently. ^ap<0.05 and ^bp<0.01, vis control cell cultures

death in cervical cancer cells (Li et al., 2015). The mechanism of cell death is different. Tanshinone IIA inactivates Akt and induces caspase-dependent death via the mitochondrial pathway. Anticancer activity of oleanolic acid methyl ester derivative in HeLa cervical cancer cells is mediated through apoptosis induction and reactive oxygen species production (Song et al., 2015).

Induction of DNA damage can be determined by the analysis of γ H2AX accumulation in the nucleus (Yu et al., 2006). The results from the present study demonstrated that 18 α -glycyrrhetic acid significantly increased the population of HR5-CL11 cells possessing more than four γ H2AX foci. Treatment with 5 μ M concentration of 18 α -glycyrrhetic acid for 48 hours increased the population of cells with more than four γ H2AX foci to 38.6%.

Conclusion

18 α -Glycyrrhetic acid reduces the viability of cervical cancer cells through induction of DNA damage. Thus 18 α -glycyrrhetic acid can be used for the treatment of cervical cancer.

Financial Support

Self-funded

Conflict of Interest

Authors declare no conflict of interest

References

- Baltina LA. Chemical modification of glycyrrhizic acid as a route to new bioactive compounds for medicine. *Curr Med Chem.* 2003; 10: 155-71.
- Bras M, Queenan B, Susin SA. Programmed cell death via mitochondria: Different modes of dying. *Biochemistry (Mosc)* 2005; 70: 231-39.
- Edinger AL, Thompson CB. Death by design: Apoptosis, necrosis and autophagy. *Curr Opin Cell Biol.* 2004; 16: 663-69.
- Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin D. GLOBOCAN 2008 v2.0, cancer incidence and mortality worldwide: IARC cancer Base No. 10. International agency for research on cancer, Lyon, 2010. Available from: <http://globocan.iarc.fr>.
- Ha YM, Cheung AP, Lim P. Chiral separation of glycyrrhetic acid by high-performance liquid chromatography. *J Pharm Biomed Anal.* 1991; 9: 805-09.
- He JX, Akao T, Nishino T, Tani T. The influence of commonly prescribed synthetic drugs for peptic ulcer on the pharmacokinetic fate of glycyrrhizin from Shaoyao-Gancaotang. *Biol Pharm Bull.* 2001; 24: 1395-99.
- Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin.* 2007; 57: 43-66.
- Li H, Xu Y, Yang L, He L. Tanshinone IIA inactivates Akt and induces caspase-dependent death in cervical cancer cells via the mitochondrial pathway. *Bangladesh J Pharmacol.* 2015; 10: 483-88.
- Niwa K, Lian Z, Onogi K, et al. Preventive effects of glycyrrhizin on estrogen-related endometrial carcinogenesis in mice. *Oncol Rep.* 2007; 17: 617-22.

- Nomura T, Fukai T. Phenolic constituents of licorice (*Glycyrrhiza* species). *Fortschr Chem Org Naturst*. 1998; 73: 1-158.
- Rackova L, Jancinova V, Petrikova M, et al. Mechanism of anti-inflammatory action of liquorice extract and glycyrrhizin. *Nat Prod Res*. 2007; 21: 1234-41.
- Robertson JD, Orrenius S. Role of mitochondria in toxic cell death. *Toxicology* 2002; 181-82: 491-96.
- Steben M, Duarte-Franco E. Human papillomavirus infection: Epidemiology and pathophysiology. *Gynecol Oncol*. 2007; 107: S2-S5.
- Song X, Liu C, Hong Y, Zhu X. Anticancer activity of novel oleanolic acid methyl ester derivative in HeLa cervical cancer cells is mediated through apoptosis induction and reactive oxygen species production. *Bangladesh J Pharmacol*. 2015; 10: 896-902.
- Takahara T, Watanabe A, Shiraki K. Effects of glycyrrhizin on hepatitis B surface antigen: A biochemical and morphological study. *J Hepatol*. 1994; 21: 601-09.
- Toh DF, Patel DN, Chan EC, Teo A, Neo SY, Koh HL. Antiproliferative effects of raw and steamed extracts of *Panax notoginseng* and its ginsenoside constituents on human liver cancer cells. *Chin Med*. 2011; 6: 4.
- Thirugnanam S, Xu L, Ramaswamy K, Gnanasekar M. Glycyrrhizin induces apoptosis in prostate cancer cell lines DU-145 and LNCaP. *Oncol Rep*. 2008; 20: 1387-92.
- Wang ZY, Nixon DW. Licorice and cancer. *Nutr Cancer*. 2001; 39: 1-11.
- Yu Y, Zhu W, Diao H, Zhou C, Chen FF, Yang J. A comparative study of using comet assay and gamma H2AX foci formation in the detection of N-methyl-N'-nitro-N-nitrosoguanidine-induced DNA damage. *Toxicol In Vitro* 2006; 20: 959-65.
- Zeng CX, Yang Q, Hu Q. A comparison of the distribution of two glycyrrhizic acid epimers in rat tissues. *Eur J Drug Metab Pharmacokinet*. 2006; 31: 253-58.
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