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DIOSGENIN INDUCES OXIDATIVE STRESS AND APOPTOSIS IN HUMAN HEP-2 LARYNGEAL AND KB ORAL CANCER CELLS

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Abstract:

The present study evaluated the apoptotic effect of Diosgenin on oral cancer cells Hep-2 and KB. Cell growth inhibition and apoptosis effects were demonstrated by nuclear DNA cleavage, condensation, change in membrane potential of mitochondria. Further, apoptosis-related proteins expressions such as mutant p53, Bcl-2, Bax and caspase-3 were determined by Western blotting assays. Results were revealed that, Diosgenin treatment significantly inhibited the cell viability and colony formation, inhibited cell proliferation lead to increased generation of reactive oxygen species through induction of MMP depolarization, morphological changes and DNA damage in dose-and time-dependent manner. Moreover, Diosgenin significantly decreased the Bcl-2 and mutant p53 simultaneously up-regulated Bax, caspase-3 expressions suggest that Diosgenin may serve as a potential candidate in the prevention of cell proliferation and enhances apoptosis.

Key Words: Diosgenin, Oral Cancer, p53, Bcl-2 & Caspase-3

Introduction:

Oral squamous cell carcinoma (OSCC) is the most common oral cancer and is presently ranked sixth among malignant human diseases (Gao et al., 2018). The average survival period, following metastatic OSCC, is approximately 5 years and has not improved considerably (Chen et al, 2015). OSCC has been linked with the most established risk factors tobacco and alcohol and the cofactors viruses, immunodeficiency, and diet (Fan et al., 2014; Gupta and Gupta, 2015; Butt et al., 2012; Gellrich et al., 2015). Recent research suggested that evasion of apoptosis is a hallmark of cancer, because, alteration in apoptotic pathway causes tumor development, progression and resistance to chemotherapy (Fernald and Kurokawa, 2013). Hence, inductions of stresses by genotoxic agent, extrinsic and intrinsic signals activators are consider prominent factors to triggered apoptotic pathway (Swift and Golsteyn, 2014). However, most commonly used chemotherapy drugs are difficult to trigger apoptotic signaling pathways for generate programmed cell death in cancer therapy (Wong, 2011). In particular, enhanced expression of Bcl-2 and suppression of Bax and caspase can regulate anti-apoptosis and inhibits the immune system, which causes dramatic oncogenic functions in cancer cells (Reshi et al., 2017). Acquiring of these hallmark capabilities is frequently occur by up-regulation of various mutant protein, among them p53 is the most prominent. Therefore, novel drug that inhibit mutant p53 expression and down-regulate the anti-apoptotic Bcl-2 is a promising strategy to progress the effectiveness of cancer therapy.

Diosgenin (3β,25R)-spirost-5-en-3-ol), a phytosteroid sapogenin, is present in detectable amounts in *Costus speciosus* and *Smilax menispermoidea*, species that possess significant pharmacological activities such as antioxidant, anti-inflammatory, anti-proliferative, renalprotective, and chemopreventive effects (Selim and Al Jaouni, 2016). However, the molecular mechanism and action of Diosgenin on apoptosis in oral cancer cells are not elucidated. Therefore, we assessed the apoptotic efficacy of diosgenin on modulatory expression of Bcl-2, Bax and caspase-3 by down regulating mutant p53 expression in Hep-2 and KB cells.

Materials and Methods:

Materials: Diosgenin and protease inhibitor cocktail, bovine serum albumin, acrylamide, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylene diamine (TEMED), were purchased from Sigma Chemical Company (USA). Dulbecco's Modified Eagles Medium (DMEM), phosphate buffered saline, fetal bovine serum, 0.25% trypsin, EDTA, antibiotics (penicillin, streptomycin), dimethylsulfoxide, 2,7-diacetyl dichlorofluorescein (DCFH-DA), ethidium bromide, rhodamine 123, acridine orange, Hoechst 33342 stain were obtained from Hi-media Lab Ltd., India. The antibodies against p53, Bcl-2, Bax, and caspase-3 were purchased from Santa Cruz Biotechno-logy (USA).

Cell Culture Treatment: Human laryngeal epithelial cancer cells (Hep-2 and KB) were bought from the National Center for Cell Science, Pune, India. Allow the cells to grow in Eagle's minimal essential medium augmented with Fetal bovine serum 10%, with 100 µg/mL of pencillin- streptomycin. The cells were incubated at 37°C and 5% CO_2 atmosphere.

Reactive Oxygen Species (ROS) Assessment: Reactive oxygen species was assessed according to Rastogi et al, (2010). Probe used was 2,7-diacetyl dichlorofluorescein (DCFH-DA). Treat 8 x 106 cells (Hep2 and KB) with IC_{50} concentration of Diosgenin for 24 and 48 hours and made up to 2 mL using phosphate buffered saline (pH 7.4). To 1 mL of cells added 100 µL of DCFH-DA (10 µM), incubated for 30 min at 37°C. Observe the cells under Nikon fluorescence microscope via blue filter at excitation and emission wavelength of 480 nm and 530 nm.

Change in Membrane Potential Analysis of Mitochondria: Change in membrane potential of mitochondria was performed according to Scaduto and Grotyohann (1999). Culture 1 x 106 cells/mL (Hep2, KB) in a 6-well plate along with IC_{50} concentration of Diosgenin for 24 and 48 hours. Control groups were maintained without Diosgenin. Rhodamine 123 at 10 µM/mL was used to stain the cells and incubated at 5% atmospheric CO_2 in a CO_2 incubator for 30 min. Wash the cells with phosphate buffered saline and observe using fluorescence microscope via blue filter.

Programmed Cell Death Assay by Dual Staining: Programmed cell death was assessed by the method of Lakshmi et al, (2008) using dual stain. Culture 5 x 10^3 cells in a 6-well plate with IC_{50} concentration of Diosgenin for 24 and 48 hours. Control groups were treated similarly lacking Diosgenin. The cultures were incubated at 5% CO_2 for 24 hours in a CO_2 incubator. The cells were trypsinized, stained with acridine orange/ ethidium bromide (1:1), then washed with phosphate buffered saline and observe using blue filter at the magnification of 40x under fluorescence microscope.

Western Blotting: SDS-PAGE was performed using equivalent protein extracts (50 µg) from each sample. The resolved proteins were electrophoretically transferred to poly-vinylidene di-fluoride membranes. The blots were incubated in 1 x TBS containing 5% bovine serum albumin for 2 hours to block non-specific binding sites. The blot was incubated with 1:200 dilutions of primary antibodies overnight at 4ºC. After washing, the blots were incubated with 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 45 min at room temperature. After extensive washes with high and low salt buffers, the immunoreactive proteins were visualized using DAB and enhanced chemiluminescence detection reagents.

Statistical Analysis: Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by using Statistical Package of Social Science (SPSS) version 12.0 for windows. The values are mean ± SD for six samples in each group. p values <0.05 were considered as level of significance.

Results:

Generation of Intracellular ROS: The generation of ROS in Hep-2 and KB were studied in control as well as in treatment with Diosgenin (Figure 1). The maximum of ROS generation was observed in the IC_{50} value of Diosgenin among all doses.

Mitochondrial Membrane Potential: Changes of mitochondrial membrane potential in Hep-2 and KB cells were observed when treated with Diosgenin (Figures 2). Rhodamine 123 green fluorescence observed in cells with high membrane potential. Decrease in the fluorescence upon treatment with effective doses of Diosgenin when compared to the untreated control cells evidenced apoptotic properties of Diosgenin.

Apoptotic Morphological Changes: Figure 3 and 4 shows increased apoptotic cell deaths in Hep-2 and KB cells by treatment with Diosgenin. The changes were determined by the color of DNA binding dyes acridine orange/ethidium bromide stain in individual cells. Live cells show bright green spots. The stained cells with IC_{50} concentration of Diosgenin for 24 and 48 hours showed gradual increase in the apoptotic death (orange), whereas necrotic (red) cells were found.

Modulation of p53, Bcl-2, Bax and Caspase-3 Expression: To predict the Diosgenin induces apoptosis, Western blot analysis to determine the protein expressions of p53, bcl-2, bax and caspase-3 (Figure 5) in Hep-2 and KB cells at 24 and 48 hours. Diosgenin treatments significantly down-regulated anti-apoptotic mediator's p53 and Bcl-2, at the same time up-regulated pro-apoptotic markers like Bax and caspsae-3 protein expression in Hep-2 and KB cells as compared to control. Therefore, Western blot analysis result showed that Diosgenin modulates the expression levels of inflammatory and apoptotic mediators expression near to normal.

Discussion:

Accumulating scientific evidences shows that Diosgenin possesses significant biological features, including antitumor activity both *in vitro* and *in vivo* (Wang et al., 2002). Present study, Diosgenin treated with Hep-2 and KB cells showed reduced cell viability, MMP and increased ROS. However, immoderate ROS levels with reduced cellular proliferation in the treated cells demonstrates that the cell death induced by Diosgenin. Anti-cancer agents that alternating ROS levels through redox modulation is a way to destruct the cancer cells with less toxicity to normal cells (Peng and Gandhi, 2012). Diosgenin treated Hep-2 and KB cells ROS levels were increased and decreased its antioxidant status, due to, cancer cells possess centrally acidic region demonstrates that Diosgenin act as significant pro-oxidant. Martin-Cordero et al (2012) suggested that numerous saponin agents act as pro-oxidant in acidic environment of cancer cells and induce cellular ROS generation, oxidative stress to primarily killing cancer cells. Hence, pro-oxidant role of Diosgenin induce intracellular ROS generation with subdued antioxidant properties that block key steps in the cell cycle on Hep-2 and KB cells.

In general, DNA fragmentation, chromatin condensation and plasma membrane blebbing are substantial characterization of apoptotic cell death (He et al., 2009). Increased ROS concentrations stimulated higher toxic to cells and change biochemical metabolism like oxidative DNA damage that causes alter purine and pyrimidine bases, resulting break single and double strand of DNA. In our findings, Diosgnin treated Hep-2 and KB cells showed fragmented apoptotic bodies in contrast to the large nucleus in untreated cells that revealed by Hoechst and acridine orange/ethidium bromide staining. Therefore, reduced mitochondrial membrane potential indicates positive activation of apoptotic mechanism and cell death blockage from necrosis (Kuznetsov et al., 2011). These results are similar to other that demonstrated Disogenin modulates apoptosis regulating proteins, damaged DNA and enhanced nuclear fragmentation in different types of cancer cells (Hou et al., 2004; Corbiere et al., 2004; Liu et al 2005).

Apart from ROS generation and mitochondrial inhibitory effects, Diosgenin also induces apoptosis in oral cancer cells by suppressed the expression of Bcl-2 and increase in the expression of Bax. Enhanced Bcl-2 expression modulates the balance in Bcl-2/Bax that privilege cell survival and can stimulate resistance to chemotherapy. These carcinogenic potential of Bcl-2 has been established various in vitro and in vivo models tumors and lymphomas by identifying its overexpression that create resistance against chemotherapy and radiation.

The cysteine protease Caspase-3 plays a vital role in intinsic and extrinsic apoptosis such as activation of PARP amplification of caspase- 8 and 9. However, involvement of a caspase-3 in ROS-induced apoptosis has not been clearly defined. Imbalance between pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins would blockage release of cytochrome C from mitochondria that difficult to activate caspase-3 and subsequent apoptosis has been reported in oral cancer and other kinds of cancer cells including Hep-2 and KB (Kochubei et al., 2015). In our study, we observed Diosgenin treated cells increased expression of Bax, caspase-3 and decrease in the expression level of Bcl-2 protein. Increased expression of Bax might have also triggered caspase-3 expression in Hep-2 and KB. It has been reported that Diosgenin induced apoptosis in human oral cancer cells through down-regulation of Bcl-2 and up-regulation of Bax . A similar study also reported that Diosgenin induced apoptosis in Hep-2 cells through up-regulation of Bax protein , activation of caspase-3, and decrease in Bcl-2 and Bcl-XL levels (Kim et al., 2012).

Moreover, Diosgenin treatment to these oral cancer cells significantly triggers the induction of apoptosis expressions via inhibiting mutant protein activities. Tumor suppressor p53 significantly regulate cell proliferation and programmed cell death through stimulating internal and external stresses to activating apoptosis (Wawryk-Gawda et al., 2014). Mutation and over expression in p53 cause lesions of the oral cavity (Lawall Mde and Crivelini, 2006). Therefore, blocking mutant p53 expression may represent viable targets for treating oral cancer. Diosgenin treated Hep-2 and KB cells were found to decrease mutant p53 activity could account for the protective role of natural saponin. Tin et al., (2007) reports also suggesting that saponin compounds induce growth inhibition and apoptosis via inhibit mutant p53 expression.

Conclusion:

In summary, we revealed that simultaneously inhibiting mutant p53 and Bcl2 expression by Diosgenin can significantly inhibit cell proliferation and induce apoptosis in oral cancer cells cells. These data support further development of Diosgenin for clinical use for the treatment of oral cancer patients expressing higher level of mutant p53 and Bcl-2. By inhibiting both expressions simultaneously, a superior than additive growth inhibitory and antitumor effect will be generated in oral cancers, thereby resulting in improved clinical efficacy.

References:

1. Corbiere C, Liagre B, Terro F, Beneytout JL.Induction of antiproliferative effect by diosgenin through inhibition of mutant p53, release of apoptosis-inducing factor (AIF) and modulation of caspase-3 activity in different human cancer cells. Cell Res. 2004 Jun; 14(3):188-96.
2. Fernald K, Kurokawa M. Evading apoptosis in cancer. Trends Cell Biol. 2013, 23: 620-633.
3. Gao J, Tian G, Han X, Zhu Q. Twenty-four signature genes predict the prognosis of oral squamous cell carcinoma with high accuracy and repeatability. Mol Med Rep. 2018 Feb; 17(2):2982-2990.
4. He B, Lu N, Zhou Z. Cellular and nuclear degradation during apoptosis. Curr Opin Cell Biol. 2009 Dec; 21(6):900-12.
5. Hou R, Zhou QL, Wang BX, Tashiro S, Onodera S, Ikejima T. Diosgenin induces apoptosis in HeLa cells via activation of caspase pathway. Acta Pharmacol Sin. 2004 Aug; 25(8):1077-82.
6. Kim DS, Jeon BK, Lee YE, Woo WH, Mun YJ.Diosgenin Induces Apoptosis in HepG2 Cells through Generation of Reactive Oxygen Species and Mitochondrial Pathway. Evid Based Complement Alternat Med. 2012; 2012:981675.
7. Kochubei T, Kitam V, Maksymchuk O, Piven O, Lukash L. Possible mechanisms of Leukoagglutinin induced apoptosis in human cells in vitro. Cell Biol Int. 2016 Dec; 40(12):1313-1319.
8. Kuznetsov AV, Margreiter R, Amberger A, Saks V, Grimm M. Changes in mitochondrial redox state, membrane potential and calcium precede mitochondrial dysfunction in doxorubicin-induced cell death. Biochim Biophys Acta. 2011 Jun; 1813(6):1144-52.

9. Lawall Mde A, Crivelini MM. PCNA and p53 expression in oral leukoplakia with different degrees of keratinization. J Appl Oral Sci. 2006 Aug; 14(4):276-80.
10. Liu MJ, Wang Z, Ju Y, Wong RN, Wu QY. Diosgenin induces cell cycle arrest and apoptosis in human leukemia K562 cells with the disruption of Ca2+ homeostasis. Cancer Chemother Pharmacol. 2005 Jan; 55(1):79-90.
11. Martin-Cordero C, Leon-Gonzalez AJ, Calderon-Montano JM, Burgos-Moron E, Lopez-Lazaro M. Pro-oxidant natural products as anticancer agents. Curr Drug Targets. 2012 Jul; 13(8):1006-28.
12. Peng X, Gandhi V. ROS-activated anticancer prodrugs: a new strategy for tumor-specific damage. Ther Deliv. 2012 Jul; 3(7):823-33.
13. Rastogi RP, Singh SP, Hader DP, Sinha RP. Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2', 7'-dichlorodihydro-fluorescein diacetate in the cyano- bacterium Anabaena variabilis PCC 7937. Biochem Biophys Res Commun. 2010; 2397: 603-07.
14. Reshi L, Wang HV, Hui CF, Su YC, Hong JR. Anti-apoptotic genes Bcl-2 and Bcl-xL overexpression can block iridovirus serine/threonine kinase-induced Bax/mitochondria-mediated cell death in GF-1 cells. Fish Shellfish Immunol. 2017 Feb; 61:120-129.
15. Selim S, Al Jaouni S. Anti-inflammatory, antioxidant and antiangiogenic activities of diosgenin isolated from traditional medicinal plant, Costus speciosus (Koen ex.Retz.) Sm.Nat Prod Res. 2016 Aug; 30(16):1830-3.
16. Swift LH, Golsteyn RM. Genotoxic anti-cancer agents and their relationship to DNA damage, mitosis, and checkpoint adaptation in proliferating cancer cells. Int J Mol Sci. 2014 Feb 25; 15(3):3403-31.
17. Tin MM, Cho CH, Chan K, James AE, Ko JK. Astragalus saponins induce growth inhibition and apoptosis in human colon cancer cells and tumor xenograft. Carcinogenesis. 2007 Jun; 28(6):1347-55.
18. Wang LJ, Wang Y, Chen SW, Ma JS, Fu Q, Wang BX. The antitumor activity of Diosgenin in vivo and in vitro. Zhongguo Zhong Yao Za Zhi. 2002 Oct; 27(10):777-9.
19. Wawryk-Gawda E, Chylińska-Wrzos P, Lis-Sochocka M, Chłapek K, Bulak K, Jędrych M, Jodłowska-Jędrych B.P53 protein in proliferation, repair and apoptosis of cells. Protoplasma. 2014 May; 251(3):525-33.
20. Wong RS. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res. 2011, 26; 30:87.

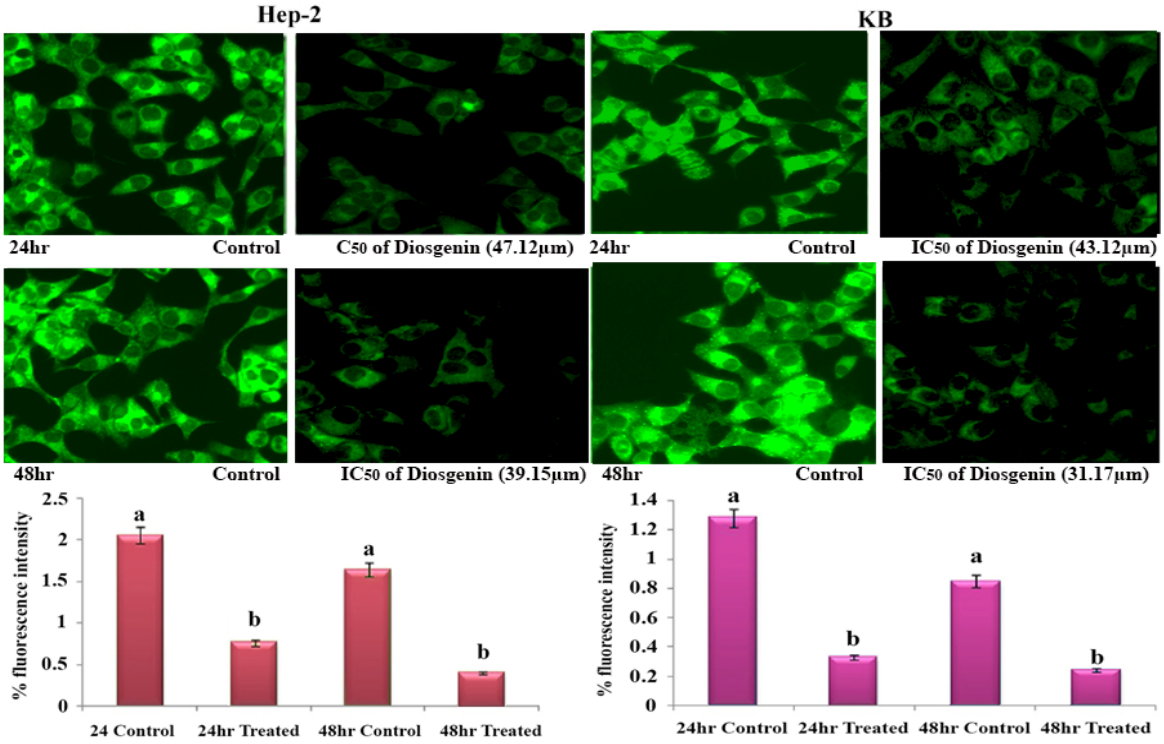

Figure 1: Effect of Diosgenin on intracellular ROS generation was evaluated with Hep-2 and KB cells by using DCFH-DA staining (40X). The photomicrographic image shows control and treated cells. Untreated control (24 h and 48 h) Hep-2 and KB cells show weak fluorescence with DCF. Diosgenin (24 h and 48 h) treated Hep-2 and KB cell shows increased ROS generation, indicating deep DCF fluorescence intensity. Diagrammatic graph shows the percentage of ROS that was detected by spectrofluorometer. All experiments were performed in triplicate and all the values were expressed as Mean ± SD. Statistical significance was determined by a one way ANOVA followed by DMRT. Asterisks indicate significant different from control ($p < 0.05$).

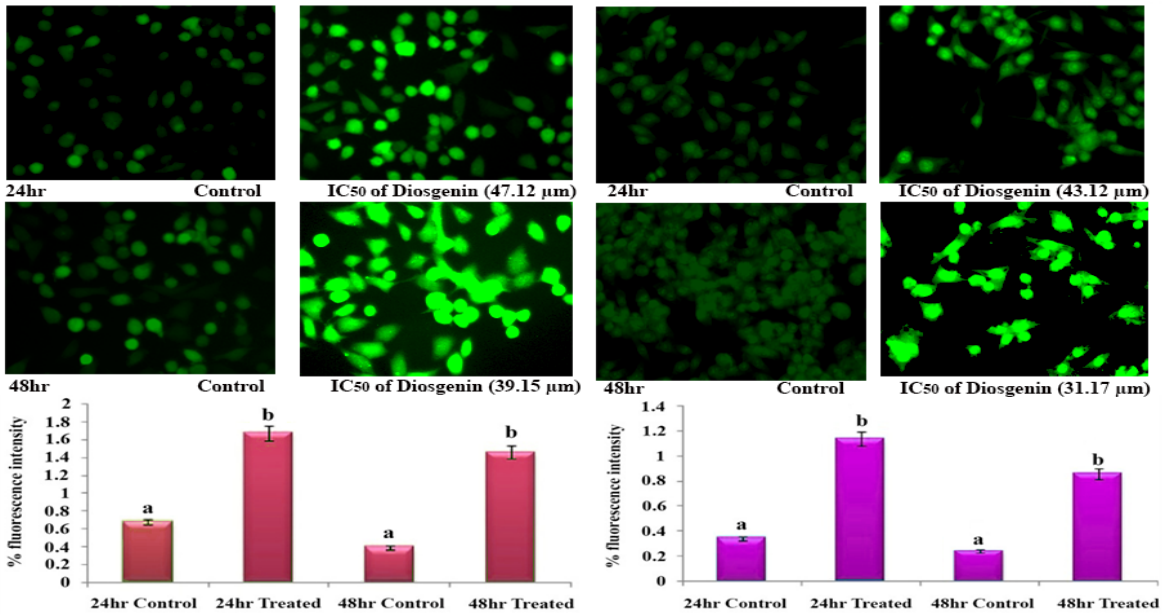

Figure 2: Effect of Diosgenin on mitochondrial membrane potential (ΔΨM) was evaluated with Hep-2 and KB cells by Rh-123 staining (40X). Untreated Hep-2 and KB cells show high fluorescence indicating polarized mitochondrial membrane. Diosgenin treated 24 h and 48 h Hep-2 and KB cells show decreased fluorescence intensity indicating depolarized mitochondrial matrix. Diagrammatic graph shows fluorescence intensity detected by spectrofluorometer. All experiments were performed in triplicate and the values were expressed as Mean ± SD. Statistical significance was determined by a one way ANOVA followed by DMRT. Asterisks indicate significant different from control ($p < 0.05$).

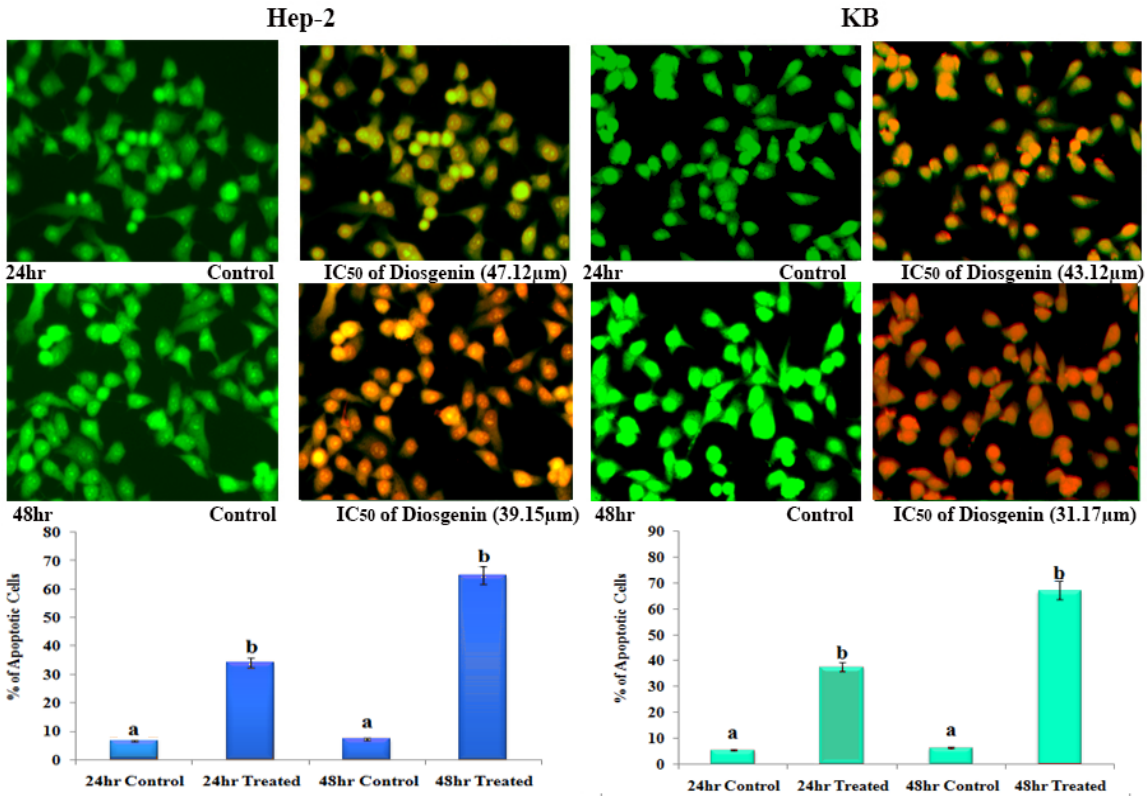

Figure 3: The effects of Diosgenin on morphological changes in the Hep-2 and KB cells were observed on dual staining with AO/EtBr in 24 and 48hr treatment(40X). The percentage of apoptotic cells were significantly increased on comparison with the control. The values are expressed as Mean ± SD from the three independent experiments. $p < 0.05$.Diagrammatic graph shows fluorescence intensity detected by spectrofluorometer. All experiments were performed in triplicate and the values were expressed as Mean ± SD. Statistical significance was determined by a one way ANOVA followed by DMRT. Asterisks indicate significant different from control ($p < 0.05$).

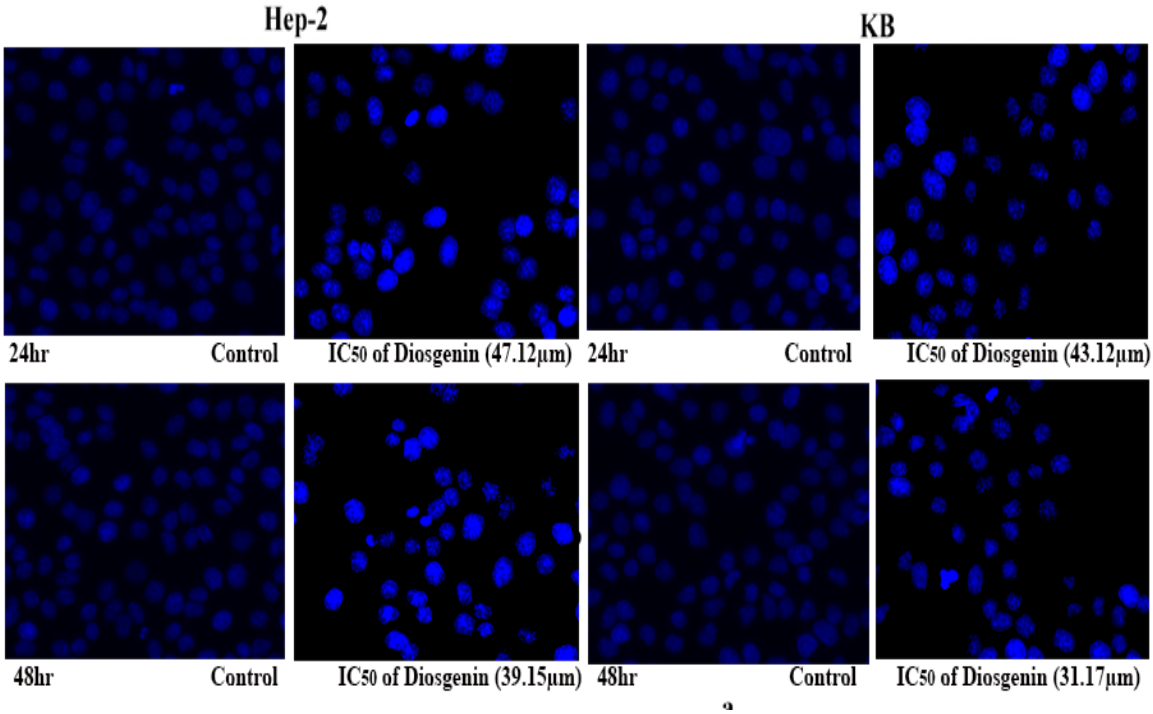

Figure 4: Apoptotic nuclear morphological changes (Hoechst staining) were observed in control and Diosgenin treated Hep2 and KB cells (40X). Untreated control cells (Compact nuclei), Diosgenin treated cells at 24 and 48 h showing chromatin disagreement, nuclear fragmentation, nuclear swelling, and apoptotic bodies.

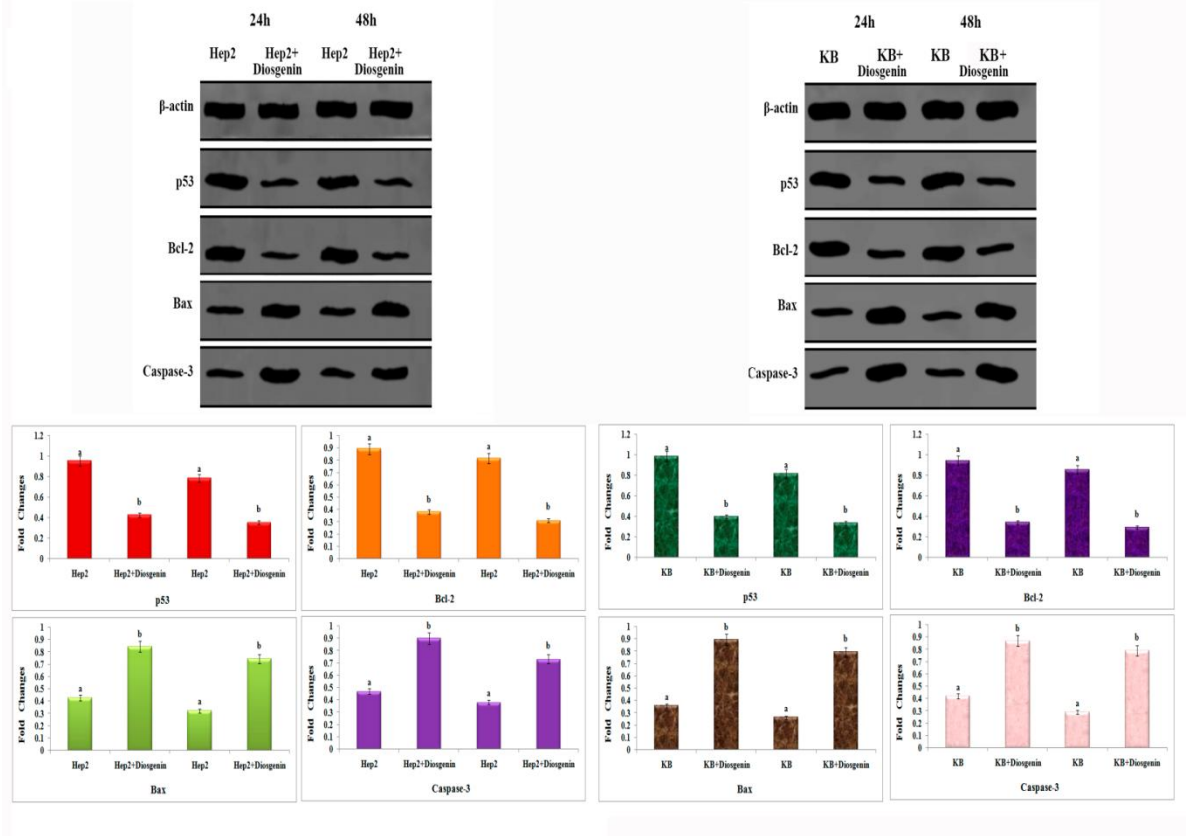

Figure 5: Western blotting analysis of apoptotic markers of p53, Bcl-2, Bax, caspase-3 in Hep-2 and KB cells. The band intensities were quantified by densitometry and normalized to respective β-actin loading control (a). The representative graph shows the relative protein expression of fold changes in western blots (b). Values are expressed as Mean ± Standard Deviation for three experiments. Values that do not share a common superscript letter (a, b and c) between groups differ significantly at $p < 0.05$ (DMRT).