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Original Article

Emodin Exhibits Strong Cytotoxic Effect in Cervical Cancer Cells by Activating Intrinsic Pathway of Apoptosis

Amir Saeed[1,2], Ahmed Alharbi[1]

ABSTRACT

Background: Cervical cancer is widely acknowledged as the main cause of mortality and a major obstacle to increasing life expectancy, after breast cancer, in women. Natural products have been proven to be a promising source for the development of potential anticancer drugs. Emodin (1,3,8-trihydroxy-6-methyl-9,10-anthracenedione) is shown to exert wide range of biological effects, including antibacterial, hepatoprotective, anti-inflammatory and anticancer. **Aim:** The major aim of this study was to evaluate the efficacy of emodin against the progression of human cervical cancer. **Materials and Methods:** The cytotoxic effect of emodin on cervical cancer HeLa cells was assessed by cell viability assay and phase contrast microscopy. Moreover, DAPI staining was performed to analyze nuclear condensation. H_2DCFDA staining was done to evaluate ROS generation. Activation of caspases was determined to ascertain mitochondria-mediated apoptosis in cervical cancer cells. **Results:** The results revealed that emodin strongly inhibited the HeLa cell growth and proliferation; which was validated by cell viability assay and phase contrast microscopy. Moreover, DAPI staining authenticated nuclear condensation and fragmentation in HeLa cells indicating initiation of apoptosis. We also observed significant ROS generation and caspases activation in emodin-treated cervical cancer cells. **Conclusion:** Our results suggested strong cytotoxic potential of emodin against cervical cancer cells.

Keywords: Cervical Cancer, Apoptosis, Emodin, HeLa, ROS, DAPI.

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INTRODUCTION

Cervical cancer is the major cause of morbidity and mortality in women despite the existence of effective prophylactic vaccines and approved screening programs for cervical cancer.[1] Globally, around 6,04,000 women were diagnosed and 3,42,000 women died from cervical cancer in the year 2020.[2] The chemotherapeutic drugs, used for the treatment of cervical cancer, could lead to multidrug resistance and serious side effects which needs to be efficiently tackled with the help of new plant based therapeutic approaches with negligible side effects. Numerous phytochemicals, with a wide range of pharmacological activities, have demonstrated promising results for the prevention and management of different malignancies. Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is derived from a natural anthraquinone present in the rhizomes and roots of plant of Rhamnaceae, Polygonaceae, Rubiaceae and Fabaceae family. Emodin show structural similarity with the core of anthracyclines used in cancer chemotherapy such as doxorubicin, daunorubicin etc.[3,4] Emodin is abundantly found in the roots of Chinese rhubarb (*Rheum palmatum*), knotweeds (*Polygonum cuspidatum* and *Polygonum multiflorum*), and knotgrass (*Paspalum distichum*).[5,6] Emodin has been shown to exhibit varied pharmacological properties such as antibacterial, anti-inflammatory and immunomodulatory.[7-9] Moreover, strong antiproliferative and cytotoxic effects of emodin have been observed in many cancer cell lines.[4] Due to its remarkable antineoplastic action on a variety of tumor cells, emodin has recently become the focus of extensive research. In this study, we evaluated the cytotoxic potential of emodin on cervical cancer cells.

MATERIALS AND METHODS

Reagents and chemicals

Emodin, MTT (3-4, 5-dimethylthaizol-2-yl)-2, 5-diphenyl tetrazolium bromide, and H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) were obtained from Sigma, USA. Eagle's minimum essential medium (EMEM), Trypsin–EDTA solution, Fetal bovine serum (FBS), Phosphate buffered saline (PBS), and antibiotic-antimycotic solution were acquired from Gibco, U.S.A. LDH assay kit was purchased from ThermoScientific, U.S.A. Caspase-3, and -9 colorimetric assay kit was purchased from BioVision, USA.

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Cell culture

Cervical cancer HeLa cell line was purchased from American Type Culture Collection (ATCC CCL-2), and cultured in EMEM with 10% FBS and 1% antibiotic-antimycotic solution. The cell culture plates were stored under standard culture conditions in a CO_2 incubator humidified with 5% CO_2 and 37°C temperature.

Methods

Cell survival assay

To assess the cytotoxicity of emodin on HeLa cells, the cell survival assay was performed by MTT dye. Briefly, the cells ($5x10^3$ cells/well) were seeded in a 96-well plate and incubated overnight to adhere. Subsequently, cells were treated with various doses (10–50 µM) of emodin for 24 and 48 hr. Further, cells were incubated for 2 hr at 37°C after addition of 10 µl of MTT solution (5 mg/ml) in each well. Finally, the purple formazan crystals were dissolved by supplementing 100 µl of dimethyl sulfoxide (DMSO) in each well. The absorbance was measured at 570 nm on a microplate reader (Bio-Rad, USA). The percent cell survival was calculated relative to untreated vehicle control.

Lactate dehydrogenase (LDH) release assay

Emodin-induced cytotoxicity in HeLa cells was also evaluated using LDH assay kit as per the manufacturer's instructions which detects release of LDH in the incubation medium.

Morphological analysis

Morphological alterations of emodin-treated HeLa cells were examined by phase contrast microscopy. Shortly, $5x10^3$ cells per well were placed in 96-well plate and incubated for 24 hr. Then after, cells were co-culture with various doses of emodin (10-50 µM), followed by incubation for another 24 hr. Thereafter, morphological analysis of cells was performed using FLoid Imaging station (ThermoScientific, USA).

DAPI staining

To qualitatively analyse emodin-mediated apoptosis within HeLa cells, DAPI staining was performed. HeLa cells were co-cultured with various doses of emodin (10-50 µM) and incubated for 24 hr. Cells were washed with the PBS followed by fixation with chilled methanol. Further, cells were permeabilized with Triton X-100 (0.025%) and subsequently stained with DAPI. After incubation of 30 min, nuclear morphology of cells was analyzed using FLoid Imaging station (ThermoScientific, USA).

Qualitative and quantitative evaluation of ROS generation

Qualitative assessment of ROS generation within emodin-treated HeLa cells was performed by H_2DCFDA staining. Briefly, $5x10^3$ cells per well were placed in 96-well plate and incubated for 24 hr at 37°C followed by the treatment of emodin (10-50µM) for another 3 hr. The cells were subsequently stained with 10 µM of H_2DCFDA and further incubated for 30 min at 37°C. The green fluorescence was captured by FLoid imaging station (ThermoScientific, USA).

The intracellular ROS was also quantified by H_2DCFDA staining. Briefly, HeLa cells (1×10^4 cells per well) were incubated for 24 hr at 37°C in a 96-well black bottom plate. The cells were treated with emodin (10-50 µM) for 3 hr and then stained with 10 µM of H_2DCFDA followed by incubation for 30 min at 37°C. Lastly, the fluorescence intensity of each well was measured using a microplate reader (Synergy H1 Hybrid Multi-mode microplate reader, Biotek, USA). Result was shown as percent DCF-fluorescence relative to untreated vehicle control.

Determination of caspase activity

Caspase-9 and -3 colorimetric kits were utilized to assess the caspase activities within emodin-treated cervical cancer cells as per the manufacturer's instruction. Briefly, HeLa cells (3×10^5 cells per well), treated with emodin (10-50 µM) for 24 hr, were lysed using 50µl lysis buffer. The cell suspension was centrifuged for 1 min at 10,000 rpm at 4°C. Afterward, cell lysate and reaction buffer were added into each well in equal ratio (final volume 100 µl). The plate was then subjected to addition of 5µl of caspase-specific substrates (4mM), LEHD-pNA (caspase-9 specific) and DEVD-pNA (caspase-3 specific), into each well and kept at for 1 hr at 37°C. The percent caspase activity was then estimated in comparison to control.

Statistical analysis

All 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 (ns> 0.05, $*p < 0.05$, $**p < 0.01$, and $***p< 0.001$ represent significant difference compared with control).

RESULTS

Emodin inhibited the surival of cervical cancer cells

First of all, the cell survival assay was performed to evaluate the cytotoxic potential of emodin on HeLa cells. The results disclosed that treatment of emodin lead to a dose-and time-dependent reduction in HeLa cells. After 24 hr of treatment, cell survival was found to be 88.31±1.97%, 72.07±2.75%, 49.86±2.35%, 27.75±3.12% and 12.14±1.59% as compared to untreated control, at the dose of 10, 20, 30, 40 and 50 µM, respectively (Figure 1A). However, a more profound reduction in cell growth was observed at 48 hr of treatment of HeLa cells by emodin and the cell viability was found to be 78.42±3.35%, 62.76±3.12%, 33.85±2.89%, 12.49±2.17% and 6.38±1.14% at the dose of 10, 20, 30, 40 and 50 µM, respectively (Figure 1A). The IC_{50} of emodin at 24 and 48 hr was 25.43 µM and 20.73 µM, respectively (Figure 1B). Thus, above results illustrated that emodin suppress the growth of cervical cancer cells in dose- and time-dependent manner.

Similarly, LDH release assay was also performed to affirm the cytotoxicity of emodin on cervical cancer cells. LDH release into the culture medium, due to cell membrane damage, was estimated in emodin-treated-HeLa cells. A dose- and time-dependent increase in the cytotoxicity in HeLa cells was noticed after treatment of emodin (Figure 2). The percent cytotoxicity, after 24 hr of treatment, in emodin treated-HeLa cells was found to be 11.95±2.52%, 29.61±2.31%, 51.64±2.41%, 73.48±3.16% and 88.64±3.68% as compared to untreated control, at 10, 20, 30, 40 and 50 µM dose, respectively. Similarly, after 48 hr of treatment of emodin, HeLa cells exhibited percent cytotoxicity of 22.85±3.14%, 38.14±3.07%, 67.62±2.87%, 88.47±2.16% and 95.56±1.23% at 10, 20, 30, 40 and 50 µM dose, respectively. Thus, the above results also suggest that the emodin reduced the growth of cervical cancer cells.

Emodin-mediated morphological alterations in cervical cancer cells

The phase contrast images of emodin-treated HeLa cells showed severe morphological alterations in a dose dependent manner (at 10-50 µM). Moreover, an increase in detachment and cytoplasmic shrinkage of cells were observed in emodin-treated HeLa cells. The untreated cells revealed progressive cell growth with intact cell morphology under microscope (Figure 3). Thus, the result confirmed that emodin exerted cytotoxic effect in cervical cancer cells.

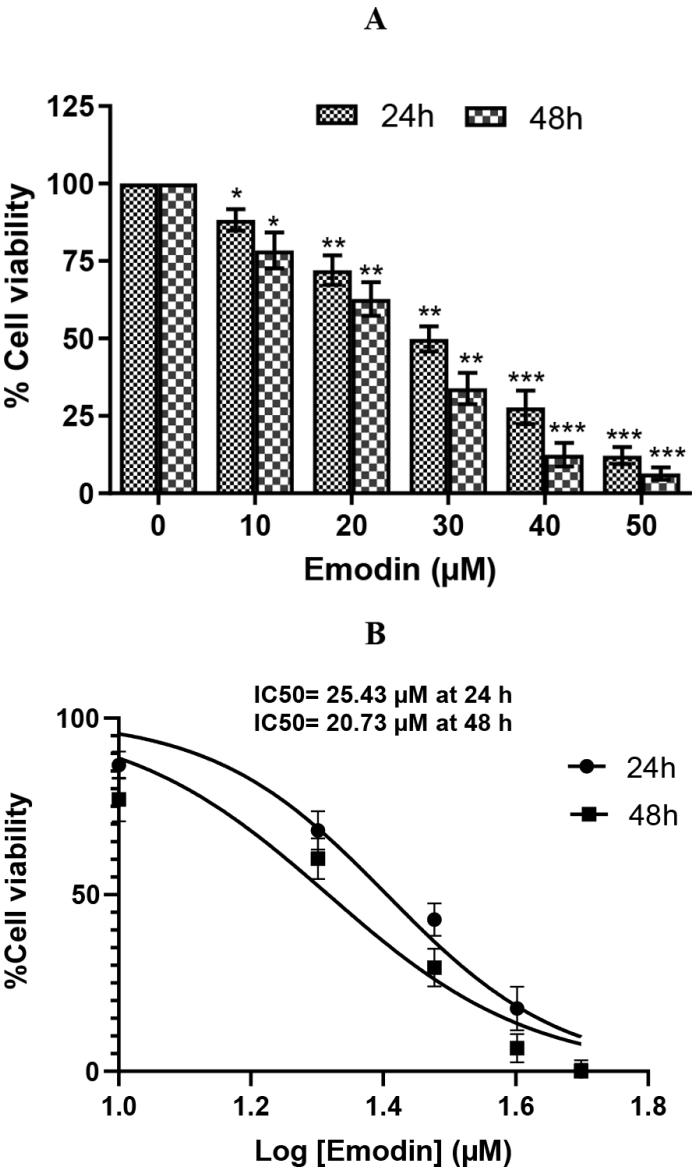

Figure 1: Effect of emodin on HeLa cells. (A) Percent cell viability of HeLa cells treated with different doses of emodin (10-500 μM) for 24 and 48 hr. The results shown are the mean ± SEM of three independent experiments performed in triplicate (ns> 0.01, *p < 0.01, **p < 0.001, and ***p< 0.0001 represent significant difference compared with control) (B) Graph showing IC_{50} of emodin against HeLa cervical cancer cells at 24 and 48 hr.

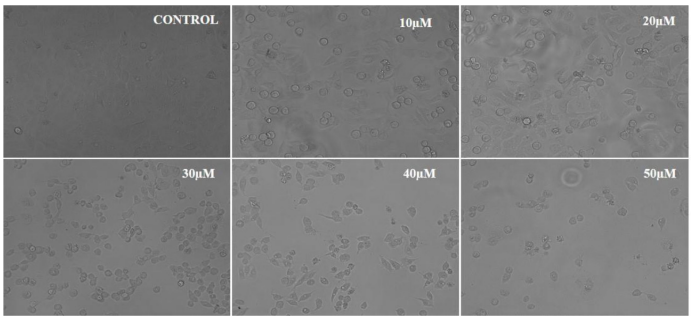

Figure 2: Phase-contrast images of HeLa cells treated with either vehicle or different doses of emodin (10-50 μM) for 24 hr. Photomicrographs shown are the representatives of three independent experiments.

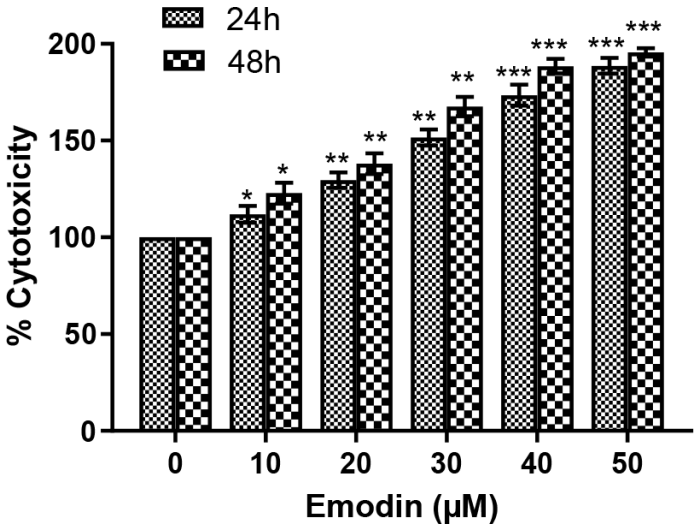

Figure 3: LDH release assay. Percent cytotoxicity in HeLa cells treated with different doses of emodin (10-500 μM) for 24 and 48 hr. The results shown are the mean ± SEM of three independent experiments performed in triplicate (ns> 0.01, *p < 0.01, **p < 0.001, and ***p< 0.0001 represent significant difference compared with control).

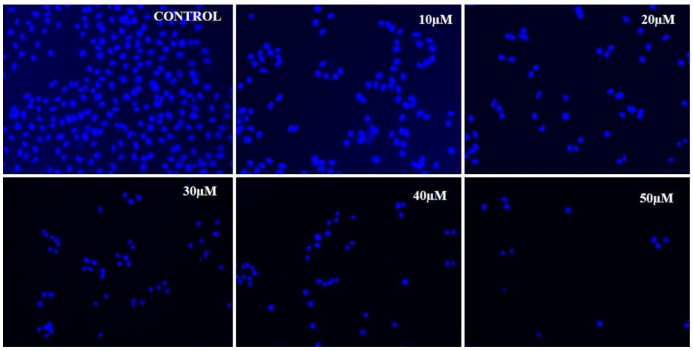

Figure 4: Assessment of apoptosis in emodin-treated HeLa cells. Blue bright nuclei, stained with fluorescent dye DAPI, showed nuclear condensation in HeLa cells treated with emodin at different doses (10-50 μM). Photomicrographs shown are the representatives of three independent experiments.

Emodin elicited nuclear condensation in cervical cancer cells

The results of DAPI staining confirmed that emodin treatment (10-50 μM) led to a dose-dependent nuclear condensation in HeLa cells, whereas a normal nuclear morphology was observed in untreated cells (Figure 4). The result indicated the initiation of apoptosis in HeLa cells induced by emodin.

Emodin induced ROS generation in cervical cancer cells

The ROS generation in emodin-treated HeLa cells was measured using the oxidant-sensitive fluorescent dye H_2DCFDA. In emodin-treated HeLa cells, qualitative assessment of ROS revealed a substantial increase in the intracellular ROS level in comparison to untreated control, which was evident by a dose-dependent increase in the green fluorescence (Figure 5A). Moreover, the quantitative estimation confirmed a significant upsurge in cellular ROS level in emodin-treated HeLa cells, indicated by a dose-dependent increase in the DCF fluorescence. The percent DCF fluorescence was found to be 116.30±2.77%, 136.00±5.98%,

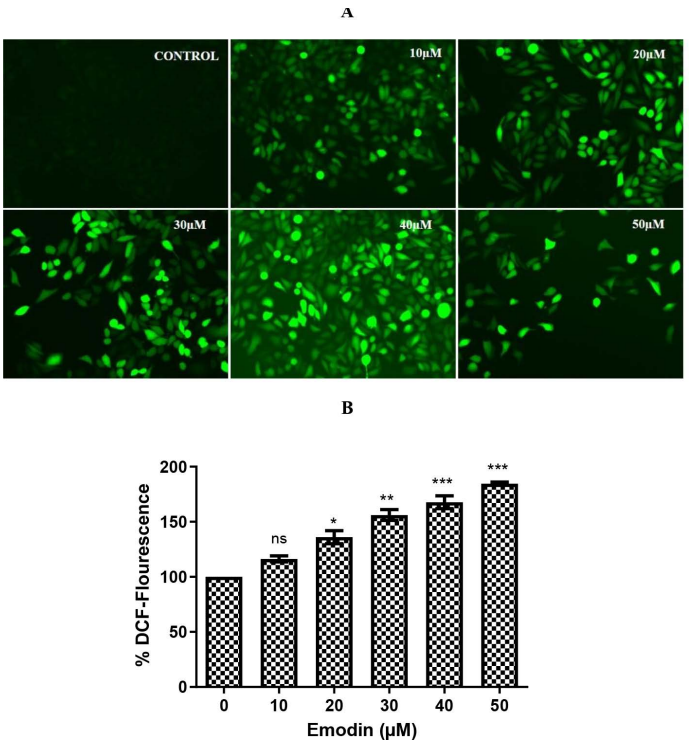

Figure 5: ROS generation in HeLa cells treated with emodin (10-50 μM) for 3 hr. (A) Green fluorescent images show increased ROS level in HeLa cells treated with emodin. (B) Quantification of ROS level is shown in terms of percent DCF fluorescence in HeLa cells. Data represent mean ± SEM of three independent experiments performed in triplicate (ns> 0.01, *p < 0.01, **p < 0.001, and ***p< 0.0001 represent significant difference compared with control.

156.16±4.85%, 167.68±5.83% and 184.63±1.38% as compared to control, at the dose of 10, 20, 30, 40 and 50 μM, respectively (Figure 5B).

Emodin stimulated activation of caspases in cervical cancer cells

The activation of caspases was measured in emodin-treated HeLa cells to reaffirm the induction of apoptosis. A significant dose-dependent activation of caspase-3 and -9 was observed in HeLa cells treated with emodin (10-50 μM). Emodin caused notable activity of executioner caspase-3, which was found to be increased by 12.27±1.90%, 25.84±2.96%, 48.14±3.01%, 72.16±4.02% and 90.50±2.83% as compared to control, at the dose of 10, 20, 30, 40 and 50 μM, respectively (Figure 6). Moreover, initiator caspase-9 activity was also found to be increased by 8.90±2.26%, 21.39±2.85%, 37.45±4.14%, 67.14±3.30% and 77.75±4.18% at the dose of 10, 20, 30, 40 and 50 μM of emodin, respectively (Figure 6). Thus, the result showed induction of mitochondrial pathway of apoptosis in emodin-treated cervical cancer cells.

DISCUSSION

There has been an upsurge in the study of natural phytochemicals, for their anticancer potential, over the last two decades.[10] Recently, emodin has been shown to exhibit growth inhibitory property against different cancer cell lines in many *in vitro* studies.[10] However, only few studies have explored the efficacy of emodin in cervical cancer cells.

In this study, cytotoxic activity of emodin was, first of all, explored via MTT assay on HeLa cervical cancer cell lines which showed that emodin was able to suppress the survival of HeLa cells in a dose- and time-

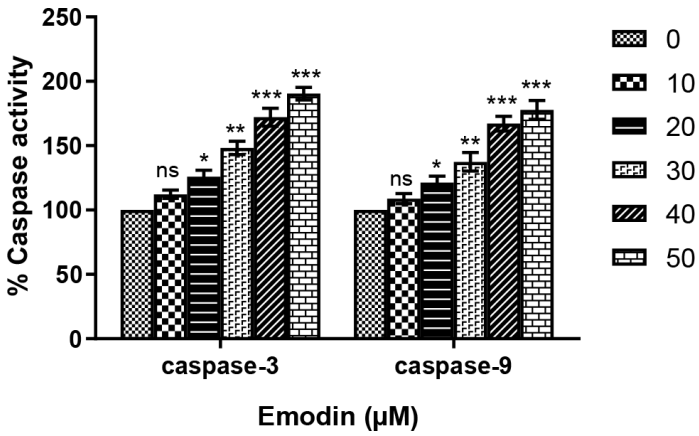

Figure 6: Activation of caspase-3 and -9 in emodin-treated cervical cancer cells. Percent caspase-3 and -9 activity in HeLa cells treated with different concentrations of emodin (10-50 μM) for 24 h. Data represent mean ± SEM of three independent experiments performed in triplicate (ns> 0.01, *p < 0.01, **p < 0.001, and ***p< 0.0001 represent significant difference compared with control).

dependent manner. Moreover, morphological changes also confirmed cytotoxicity and cell death in emodin-treated HeLa cells when observed under phase contrast microscope. Overall, the above results showed the cytotoxic efficacy of emodin against cervical cancer cell lines. Many previous studies have reported the strong antitumor effects of emodin in many cancers which substantiate our results.[11-13]

Morphological changes like impaired membrane integrity, chromatin fragmentation and condensation, caspases activation, expression of pro- and anti-apoptotic proteins are associated with the onset of apoptosis induction. Our result showed significant nuclear condensation in emodin-treated HeLa when stained with DAPI, a fluorescent dye. Previous reports showed that emodin can induce cell death in certain cancer cells through apoptosis.[11-13]

Reactive oxygen species (ROS) play a critical role in the instigation of apoptosis and cell aging and death. Alteration in cellular homeostasis is correlated with ROS generation which ultimately leads to decrease in mitochondrial membrane potential, DNA damage, and eventually apoptosis. The quantitative estimation of cellular ROS in emodin-treated HeLa cells revealed a dose-dependent increment in the cellular ROS which could led to depletion of cellular and mitochondrial glutathione, NADH and NADPH level, and dissipation of mitochondrial membrane potential, eventually causing apoptosis. Quinones can react with intracellular molecular oxygen to become superoxide anions, which could be the reason for the escalated ROS generation in emodin treated HeLa cells.[10] Emodin has been shown to induce intracellular ROS in cancer cells in previous studies.[11-13]

Recent studies have suggested that emodin could induce apoptosis by targeting mitochondria.[14] Escalated Caspase-3 activation, associated with altered mitochondrial signal leading to proteolytic degradation of cellular proteins, is an important hallmark of programmed cell death or apoptosis. Our results disclosed that emodin treatment led to activation of caspase-3 and -9 in cervical cancer cells. This finding affirmed the activation of intrinsic pathway of apoptosis in emodin treated-HeLa cells. Emodin mediated induction of caspase -9 and -3 activation is also reported by previous studies.[11-13] These studies have corroborated our results that emodin could induce mitochondrial pathway of apoptosis in cervical cancer cells.

Previously, emodin has been shown to exhibit dose-dependent reduction of cell survival in cervical cancer cells (Bu25TK, CaSki, HeLa, and

ME-180) which was found to be associated with nuclear condensation and DNA fragmentation. Moreover, emodin-induced growth inhibition was found to be associated with activation of caspase-3 and -9.[11] Recently, emodin has been shown to induce increased intracellular ROS along with DNA damage in SiHa and C33A cervical cancer cells.[12] In another report, emodin has been demonstrated to induce apoptosis in HeLa cells by intrinsic as well as extrinsic pathways.[13] Overall, above studies corroborated our findings that emodin exhibits strong cytotoxic potential against cervical cancer cells via inducing apoptosis.

CONCLUSION

To sum up, our investigation showed that emodin had outstanding *in vitro* apoptotic and cytotoxic activity in cervical cancer cells. Morphological alterations, nuclear condensation, ROS production, and caspase activation demonstrated emodin's strong anticancer potential. Our results, thus, illustrated emodin's candidature as a potent chemopreventive agent against cervical cancer cells.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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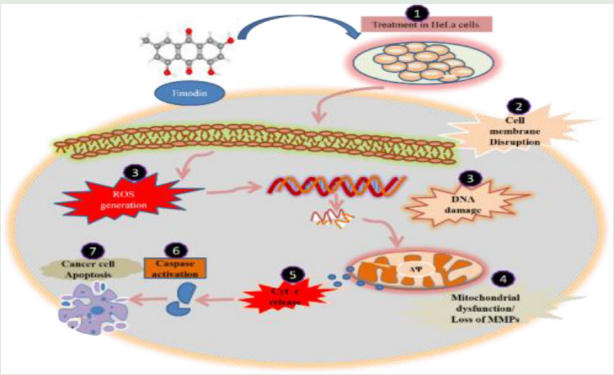
GRAPHICAL ABSTRACT



Mechanism of emodin induced cytotoxicity in cervical cancer cells.

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