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Research Article
Zeylenone from *Uvaria grandiflora* Roxb. Induces Apoptosis in Colon Cancer Cells Through Suppression of NF-κB Signalling

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Abstract

Background and Objective: Plant-derived phytocompounds may be useful in the treatment of cancer, because of their high efficiency, cheap cost and few side effects. Here the inhibitory activity of Zeylenone (Zey) on colon carcinoma (HCT-116) cells and explored the possible underlying mechanisms of cancer cell death. **Materials and Methods:** HCT-116 cells were treated by Zey in various dosages and cell cytotoxicity was studied using MTT analysis. Oxidative-stress mediated cell death was confirmed by the analysis of ROS generation and mitochondrial membrane potential alteration in HCT-116 cells. Furthermore, Zey treatment mediated apoptotic, cell proliferation and metastatic protein expression was measured by western blot analysis. **Results:** Current findings enumerated that Zey treatment effectively reduced HCT-116 when evaluated with control cells by the maximum inhibitory result at a dosage of 15 μM. The apoptotic effect of Zey induced generation of ROS thus leads to loss of mitochondrion membrane potential and nuclear fragmentation was found. Nuclear factor-kappa B (NF-κB) concern as a main transcriptional factor and its overexpression regulate several proliferative, apoptotic and metastatic proteins in response to a cancerous condition. Inhibition of these NF-κB activities has been considered a vital target for suppressing colon cancer cell growth. In this study, Zey treatment suppresses the growth of cell proliferative and metastatic markers such as TNF-α, cyclin D1, PCNA, MMP-2, MMP-9 and Bcl-2 through the downregulation of NF-κB and IκBα in HCT-116. Moreover, Zey mediated inhibition of NF-κB subsequently induces the proapoptotic genes (Bax, cytochrome-c, caspase-9 and caspase-3) expressions in HCT-116 cells. **Conclusion:** Zey treatments considerably inhibit proliferation, metastasis and induces apoptosis, probably by controlling NF-κB signalling in colon cancer cells.

Key words: Zeylenone, colon cancer, inflammation, NF-κB signalling, apoptosis, HCT-116 cells

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Colorectal Carcinoma (CRC) is the foremost important cause of fatality in women and men and position with 3rd most widespread carcinogenesis worldwide. The incidence of CRC is still growing, although improved consideration of pathogenesis of this illness and the concern of better diagnosing approaches for this disease. The previous report informed that about 50% of cases with CRC would grow recurring, representing those presently available treatments cannot manage this lethal illness and there is a necessity for better therapies[1]. Unregulated cell death and cell proliferation are vital to carcinoma growth and resistance to apoptosis. The general characteristics of cancer growth contain genetic alteration and modifications in apoptotic signalling in the pathogenesis and give an approach to curative goals[2]. Treatment approaches planned to demolish carcinoma cells through triggering apoptotic pathways are essential, perfectly treatment that is capable of stimulating cancer cell apoptosis and no dangerous belongings on control cells[3].

It is fine recognized that the human body is proficient in self-curing later than an immediate inflammatory reaction but a chronic inflammation might lead to stimulation of cancer development. Several types of research have revealed that inflammatory mediators (TNF-α and NF-κB) induced inflammation, which leads to DNA damages and eventually instigation of carcinogenesis[4,5]. NF-κB is an essential controller of essential cell tasks, containing cell survival and proliferation[6]. NF-κB is exactingly controlled in control cells, while in tumour cells is constitutively stimulated to elevated levels[7]. More prominently, NF-κB stimulation in tumour cells has been confirmed in several reports to be one of the main culprits of resistance therapy[8].

NF-κB is characteristically a heterodimeric compound formation of Rel family mediators like p65 and p50. It usually resides in the cytoplasm in a dormant appearance due to its involvement with the IκB inhibitor. A variety of extracellular indications can lead to NF-κB stimulation via the phosphorylation and degradation of I κB. The detached NF-κB then translocated to the nucleus, combined with promoters and controls targeted gene expression[9].

Nowadays, isolated substances from plants play an essential role in managing malignant tumours. Zeylenone (Zey), a commonly presenting cyclohexene oxide, is separated since *Uvaria Grandiflora* Roxb. Although, Zey demonstrates potential toxic effects to cancer cells and is less lethal to control cells[10-12]. Earlier reports have confirmed that Zey reveals a substantial impact on tumours, including gastric and prostate cancer[13,14]. At present, to the finest of our knowledge, no information regarding Zey arbitrated apoptosis, inflammation and cell proliferation in colon carcinoma cells is available. Therefore, present research explored the inhibitory properties of Zey on colon cancer inflammation, apoptosis and cell proliferation and investigated its associated mechanisms by the NF-κB pathway.

MATERIALS AND METHODS

Study area: This study project was performed from 12th April, 2018 to 31st January, 2020 in the Digestive Endoscopy Room in the Outpatient Department, Yantai Yuhuangding Hospital, Shandong and Department of General Surgery, Zhangjiagang Traditional Chinese Medicine Hospital Affiliated to the Nanjing University of Chinese Medicine, Zhangjiagang, China.

Chemicals: Zeylenone (Zey), DMSO, DMEM, FBS, PBS, Rhodamine-123 (Rh-123), Acridine orange (AO), Hoechst-33342 and MTT were obtained from Sigma Aldrich. The entire other reagents were used for diagnostic standards. The primary antibodies for NF-κB, I κBα, TNF-α, cyclin D1, PCNA and β-actin were attained from Santa Cruz Biotechnology.

Cell culture: HCT116 cells were seeded in RPMI medium, including streptomycin (100 mg mL^{-1}), fetal bovine serum (10%) and penicillin (100 U mL^{-1}) in a 5% CO_2 humidified environment at 37°C. In all experiments, cells at the semi-confluence were added 24 hrs with various concentrations of Zey.

Cell viability test: The outcomes of Zey on the viability of HCT116 cells were employed through MTT test[15] at concentrations series of 5, 10, 15, 20, 25 and 30 μM. The IC_{50} was calculated by absorbance at 570 nm with a spectrophotometer. The IC_{50} of Zey against the HCT116 cells was found to be 15 μM.

Determination of mitochondrial ROS generation: The ROS status was evaluated in HCT116 cells using the DCFH-DA staining method[16]. The DCFH-DA is a cell-permeable non-fluorescent probe, which has been concerned as a substrate for an amount of intracellular oxidant formation. Following the treatment (10 and 15 μM) of Zey and stained with 10 μM of DCFH-DA for 30 min (37°C) and then determined at 485/535 nm.

Measurement of MMP levels: A modification of MMP was completed with the earlier explained technique[17]. HCT116 cells were added with various dosages of Zey (10 and 15 μM)

for 24 hrs, subsequently cleansed and suspended in chilly PBS. The 1×10^6 cells were kept with Rh-123 (10 mM) at 37°C for 30 min and observed at 485/530 nm.

Apoptotic morphological changes predicted by hoechst staining: HCT116 cells were cultured in a six-well plate at a density of 3×10^4 per well. The cells were treated with Zey (10 and 15 μM) and the culture plate was placed in a CO_2 incubator for 24 hrs. After incubation, cells were washed and stained Hoechst at an appropriate concentration (100 mg mL^{-1} of PBS) for 5 min. Photomicrographic images were taken under a floid cell imaging station from Invitrogen, USA[18].

Caspase assays: Caspase family proteins are the crucial factors, which activate the apoptosis process. The capability of Zey to trigger apoptosis was evaluated by the apoptotic activity analysis kits method (caspase-8, -3 and -9) obtained from Abcam and measured with a spectrophotometer at 400-405 nm.

Western blot examination: Entirety cell proteins were lysed with proteinase inhibitor (10%) and heated for 5 min (at 95°C). Protein levels were concentrated through the process[19]. The 40 μg of protein samples were alienated through SDS-PAGE and were shifted to a PVDF membrane and blocked for 1 hr. Membranes were kept with different primary antibodies (NF-κB, IκBα, TNF-α, cyclin D1 and PCNA). Later than cleansing, blots were kept with secondary antibodies (HRP labelled) for 2 hrs. The indications of blot were subsequently developed by an ECL method.

Statistical investigation: All investigational data were measured as Mean±SD. Results were considered through one-way ANOVA and Duncan measurement with SPSS. A level of $p<0.05$ was considered statistically significant.

RESULTS

Effect of Zey on HCT116 cell viability: To appraise the outcome of Zey on HCT116 cell viability was performed by MTT analysis. Evaluated with control cells, there is a reduction in cell viability with consideration to the augmented dosage of Zey (5, 10, 15, 20, 25 and 30 μM) management. It has been revealed that the dose-dependent growth inhibition was observed in Zey treated HCT116 cells and the cell viability was significantly reduced by 24 hrs incubation of Zey. Only 50% of the cells were living in cells added with 15 μM of Zey, thus for further work, the dosage 10 and 15 μM were chosen for further studies in Fig. 1.

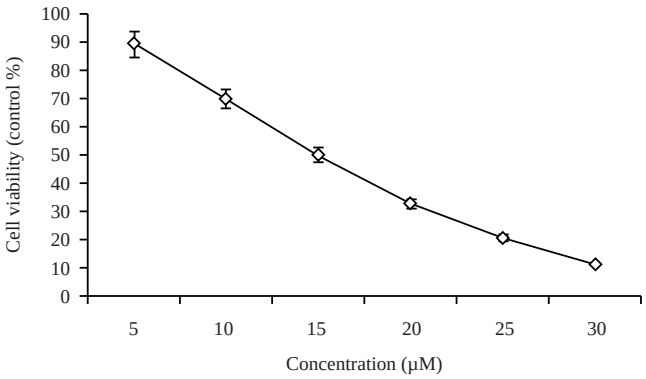

Fig. 1: Cytotoxic effect of Zey on HCT116 cells was determined by MTT assay
Data represent Mean±SD of triplicate, *p<0.05 as compared with the control group

Effect of Zey on DCFH-DA staining: The intracellular formation of ROS was detected using DCFH-DA to reveal the effect of Zey on the proliferation and apoptosis mechanism, which is interconnected with the deregulation of cellular redox status. In this study, control cells were exposed to a low quantity of ROS whereas, Zey (10 and 15 μM) treated cells considerably enhanced ROS production in HCT116 cells in Fig. 2a. Besides, the intracellular ROS generation was quantitatively measured by a fluorescence microplate reader in Fig. 2b. There is no apparent green fluorescence was detected in the untreated control group (506.49±23.78). Nevertheless, HCT116 cells treated with different concentrations of Zey showed heightened intracellular green fluorescence, especially, the 15μm of Zey exhibited the enhancing the intracellular fluorescence intensity (2459.13±96.05). As shown in the figure, Zey treated cells showed a dose-dependent increase in green fluorescence intensity ($p<0.05$) indicating that Zey increases intracellular ROS generation in HCT116 cells.

Effect of Zey on MMP staining: One of the possible approaches concerned with altering mitochondrion membrane potential resulted in apoptosis. The Mitochondrial Membrane Potential (MMP) was investigated using a cationic fluorescent stain of Rhodamine 123. It is used to determine the role of mitochondria mitochondrial membrane potential in Zey treated HCT116 cells. Rhodamine 123 stimulated by Zey predicted the membrane potential was the loss of MMP in Fig. 3a. In this study, control cells demonstrated an improved level of MMP, while Zey (10 and 15 μM) treatment was the loss of MMP in HCT116 cell lines. It was evidenced by the increased ratio of green fluorescence in Zey treated cells. The

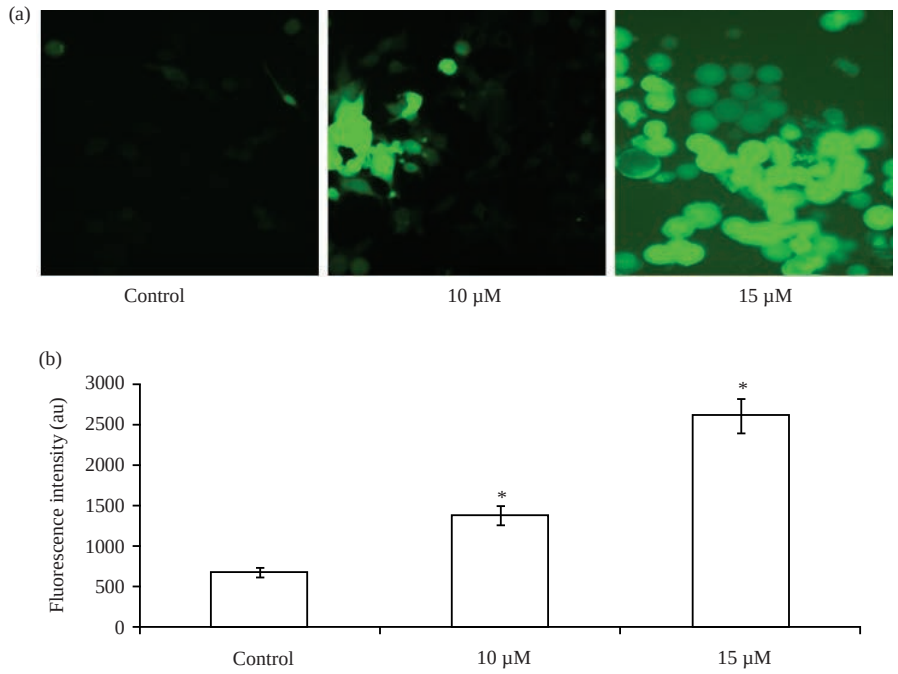

Fig. 2(a-b): Effect of Zey on intracellular ROS production was analysed with HCT116 cells by using DCFH-DA, (a) Fluorescence microscopic (20X) images represent Zey treatment mediated ROS production observed by increased the level of fluorescence in HCT-116 cells and (b) Spectrofluorimetric analysis of Zey treatment mediated ROS generation in HCT-116 cells

Data represent Mean±SD of triplicate, *p<0.05 as compared with the control group

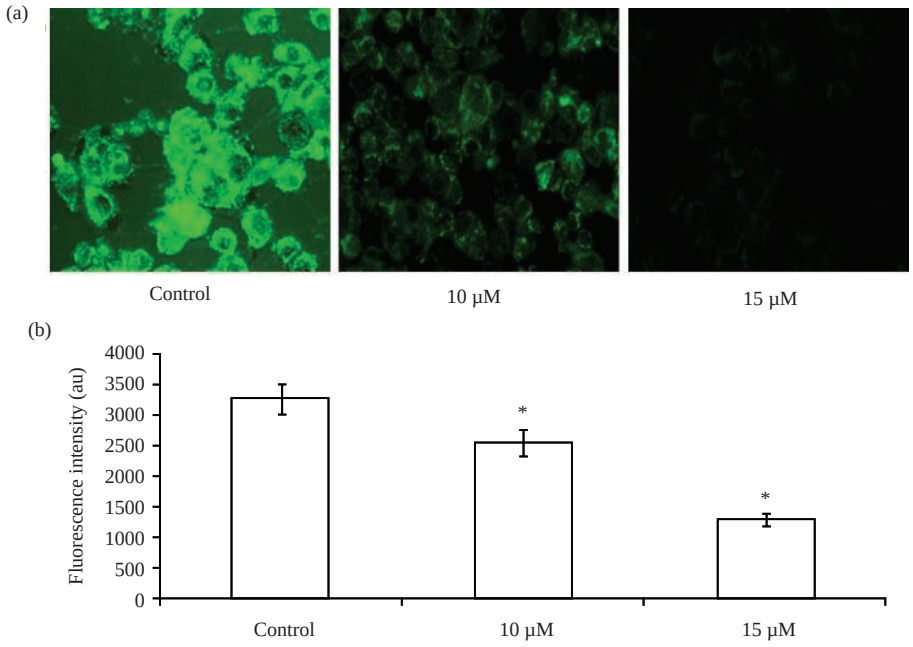

Fig. 3(a-b): Effect of Zey on MMP levels was analyzed with HCT116 cells using the Rh-123, (a) Fluorescence microscopic (20X) images represent Zey treatment mediated alteration of MMP observed by decreased the level of fluorescence in HCT-116 cells and (b) Spectrofluorimetric analysis of Zey treatment mediated alteration of MMPs in HCT-116 cells

Data represent Mean±SD of triplicate, *p<0.05 as compared with the control group

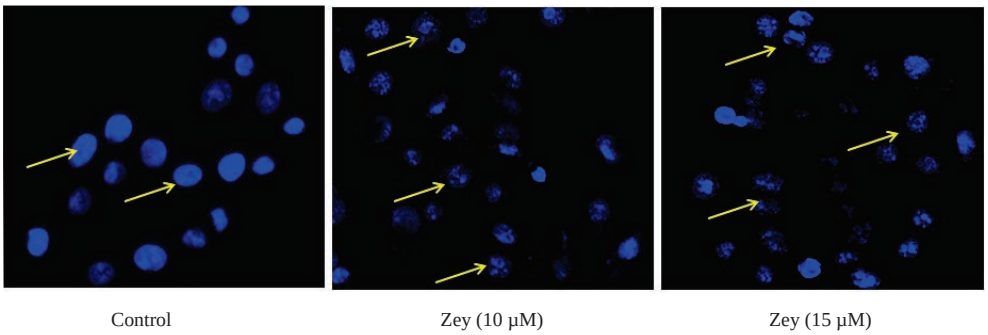

Fig. 4: Effect of Zey on apoptotic morphological changes were Hoechst dye in HCT116 cells
Control cells show normal nucleus, Zey (10 and 15 µM) treatment shows well-fragmented nucleus and apoptotic cells

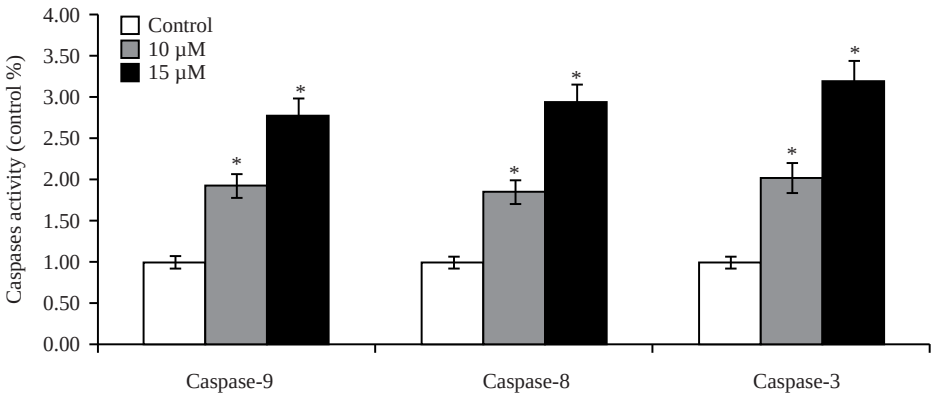

Fig. 5: Zey induces apoptosis through caspase family proteins in HCT116 cells
Zey induced expressions of caspase-8, caspase-3 and caspase-9. The data represent Mean±SD of triplicate, *p<0.05 as compared with the control group

fluorescence intensity (a.u) of Rhodamine 123 was quantitatively measured by a fluorescence microplate reader in Fig. 3b. There was an increased fluorescence intensity was observed in the control group (3263.87±51.47). Nevertheless, due to the loss of mitochondrial membrane potential, Zey treated HCT116 cells were showed decrease green fluorescence. Among the whole, 15 µm of Zey showing a decreasing fluorescence intensity (1406.83±60.36).

Effect of Zey on apoptotic morphological features: Hoechst 33342 is a nuclear-specific fluorescent probe that specifically binds to the double-stranded DNA to signify the nuclear morphology. In this context, Zey treated HCT116 cell's nuclear morphology was studied by using Hoechst-33342 staining. The photomicrographic results explored that increased nuclear fragmentation and condensed nuclei of apoptotic cells were observed in Zey (10 and 15 µM) for 24 hrs of exposure in Fig. 4. In contrast, the untreated control cells have appeared as less fluorescence intensity regular nuclear morphology with flattened and smooth nature.

Effect of Zey on apoptotic markers: To reveal the possible role in inducing the apoptotic associated cell death mechanisms, the expression of caspases 3, 8 and 9 were studied in Zey treated HCT116 cells using western blot analysis as demonstrated in Fig. 5. In untreated control, cells show decreased caspase activity. In contrast, HCT116 cells were treated with Zey (10 and 15 µM) for 24 hrs showed an increased level of caspase 3, 8 and 9 expressions. From the results, it is clear that Zey notably activates the apoptotic signalling cascade in HCT116 cells.

Effect of Zey on protein expression of inflammatory and cell proliferative markers: In line with previous studies, inflammatory associated cell proliferation was assessed by measuring the NF-κB, IκBα, TNF-α, cyclin D1 and PCNA expression in HCT116 treated Zey cells. The results of western blot analysis confirmed that Zey (10 and 15 µM) treatment reduced NF-κB, TNF-α, cyclin D1 and PCNA expression and increased the expression of IκBα in HCT116 cells in a concentration-dependent approach in Fig. 6a. These results cast a new light on that the treatment of Zey can suppress the

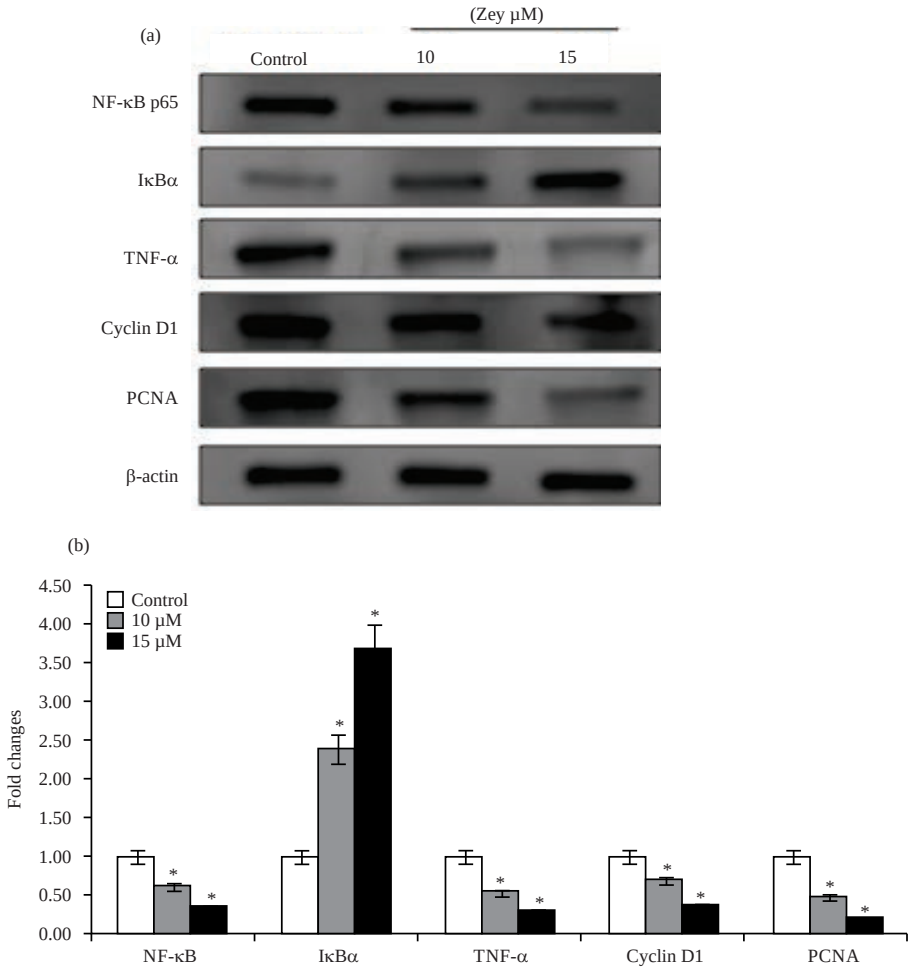

Fig. 6(a-b): Effect of Zey on NF-κB mediated signalling proteins in HCT116 cell lines were explored by the western blotting method, (a) Western blotting analysis on Zey treatment mediated NF-κB, IκBa, TNF-α, Cyclin-D1, PCNA in HCT-116 cells and (b) Densitometric analysis by image-J software and it was normalized by β-actin (NF-κB, IκBa, TNF-α, Cyclin-D1, PCNA in HCT-116 cells)

Data represent Mean±SD of triplicate, *p<0.05 as compared with the control group

inflammatory associated HCT116 cell proliferation. The protein expression was qualitatively expressed by fold changes and it was measured by image-J software. The expression level of NF-κB, I κBα, TNF-α, cyclin D1 and PCNA was normalized by β-actin was shown in Fig. 6a. The densitometric analysis revealed that the dose-dependent fold changes were observed in Zey treated HCT116 cells in Fig. 6b and highest in IκBα at 15 μM.

DISCUSSION

The cytotoxic effect of Zey in certain varieties of carcinoma cells is fine identified with several information highlighting the anti-cell proliferative activity of Zey on cells. The findings of the study demonstrated that Zey drastically suppressed cell proliferation and viability and provoked apoptosis of HCT116 cells, consistent with earlier studies. Zey suppresses cell proliferation and enhances apoptosis of cervical carcinoma cells via dropping the expression amount of MAPK/ERK and PI3K/AKT/mTOR signaling[11]. The apoptotic stimulation of Zey was confirmed by the generation of ROS and further substantiated the MMP distraction in the HCT116 cells. Similarly, Annamalai *et al.*[20] reported that the phenolic substance of shogaol could enhance ROS generation, which leads to a change in mitochondrial function and activity. Thus, the biological process is responsible for the induction of oxidative cell death. Evaluated with normal cells, there is an augmented quantity of late and early apoptotic cells in Zey treatment, owing to the activation of apoptosis, which is consistent, reported earlier[21]. Apoptosis is a general regulatory

system employed to regulate cell development. Once equilibrium between cell death and survival is broken, it will lead to carcinogenesis[22]. The mitochondrial (intrinsic) pathway is one of the most critical apoptosis machineries, though carcinoma cells can prevaricate these tightly controlled cell death mechanisms using the control of pro-apoptotic or anti-apoptotic mediators[23]. Thus, activating carcinoma cell apoptosis is an imperative approach for cancer treatment. Caspase protein members are vital molecules associated with apoptosis. Mainly, caspase-8, caspase-9 and caspase-3 are essential molecules implicated in the instigation of mitochondrial-reliant apoptosis signaling[24]. Upregulation of Bax may activate liberate of mitochondrial molecules that trigger caspase-9 subsequently catalyze the stimulation of caspase-3, which finally leads to apoptosis[25]. Xu *et al*.[26] informed that provoked cell apoptosis of ovarian carcinoma cells, recommending that inhibition of cancer growth via Zey could occur through the induction of apoptosis. In the present report, Zey treatment improves cell apoptosis of HCT116 cells through the augmented expression of caspase-3, -9 and -8. Therefore, the results explained above pointed to that Zey activates cell apoptosis through mitochondrial signalling during the regulation of caspase cascade in colon cancer cells.

NF-κB is a ubiquitous transcriptional mediator that controls various proteins concerned with inflammation, growth regulation, apoptosis and carcinogenesis[27]. NF-κB has been recommended to be a goal for multiple biologically energetic plant substances separated from natural sources, for example, epigallocatechin gallate, curcumin and resveratrol[28,29]. However, up to now, the molecular system by which Zey inhibited activation of NF-κB cascades is not entirely known. This current work had confirmed that Zey revealed anti-inflammatory activity via inhibits the phosphorylation of IκBα and suppression of proteasomal degradation of I κBα with an ensuing reduction in p65 nuclear translocation and NF-κB transcriptional stimulation, is agreeing with earlier reports on 6-Shogaol suppress NF-κB signalling in breast carcinoma cells[30]. NF-κB also acts as a central task in malignant alteration. Cell proliferation in various cells, combined with the DNA target is homo or heterodimer to persuade downstream gene regulation[31]. Suppression of NF-κB in cancer cells blocks proliferation and leads to apoptosis, recommending a vital function for this transcriptional mediator in survival and cell proliferation[32]. Present study, Zey management inhibits cell proliferation via the reduced expression of PCNA and cyclin D1 in HCT116 cells. Previous studies informed that curcumin and 5-fluorouracil also affected NF-κB controlled gene products implicated in proliferation, thus inhibiting cell proliferation in HCT116 cells[33].

CONCLUSION

Overall, the finding demonstrated that Zey potentially suppressed HCT116 cell proliferation, inflammatory response markers and enhances apoptosis. Moreover, Zey stimulates oxidative stress to indicate augmented ROS generation, loss of MMP and HCT116 cell morphological changes, thus leads to enhanced apoptosis of HCT116 cells in a dose-dependent approach. Based on the overall findings, Zey might be a novel anti-cancer agent for the treatment of colon cancer.

SIGNIFICANCE STATEMENT

This study discovered the Zey potentially suppressed HCT116 cell proliferation that can be beneficial for anti-cancer therapy. These findings suggest that Zey may have a significant role in preventing colorectal cancer via enhancing apoptosis and suppress cell proliferation. This study will help the researchers to uncover the critical areas of phytomedicine that many researchers were not able to explore in near future.

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