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## **Research Article**

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## Acetoxyroyleanone exhibits selective anti-cancer effects and induces apoptosis in human colon carcinoma cells through the mediation of NF- $\kappa$ B and caspase-3 signalling pathways

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### Abstract

The objective of the current study was to evaluate the antiproliferative and apoptotic activities of acetoxyroyleanone against various cancer cells along with studying its effect on chromatin condensation, NF- $\kappa$ B and caspase-3 expressions and cell migration. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used to evaluate cell viability while as fluorescence microscopy revealed the effects of acetoxyroyleanone on cellular morphology of Colo-205 cells. Western blotting revealed effects on NF- $\kappa$ B and caspase-3 expressions. The results revealed that acetoxyroyleanone induced potent and dose-dependent antiproliferative effects against a range of cancer cell lines with Colo-205 being the most susceptible cell line. However, it required six to eight times higher concentration of acetoxyroyleanone to induce 50% cell death in normal epithelial (fR-2) cell line. Further, following acetoxyroyleanone treatment to Colo-205, it was observed that acetoxyroyleanone induced substantial down-regulation of NF- $\kappa$ B and up-regulation of caspase-3 expressions. In addition, acetoxyroyleanone impairs cell migration, chromatin condensation, cell shrinkage and membrane blebbing.

### Introduction

Colorectal carcinoma is a cancer from uncontrolled cell growth in the colon or rectum or in the appendix. The number of colorectal carcinoma cases increased to 1.3 million with more than 7 lakh deaths globally as per latest global cancer statistics (Jemal et al., 2011). Factors like cell proliferation, cancer cell migration, angiogenesis and inflammation play key roles in colorectal carcinoma (Rupnarain et al., 2004; Kinzler and Vogelstein, 1996; Rodrigues et al., 1990). Conventional chemotherapy involving 5-fluorouracil, leucovorin, capecitabine and irinotecan regimens for the treatment of colorectal cancer have

inadequate efficacy and are accompanied with significant severe adverse effects.

Acetoxyroyleanone is an abietane diterpenic natural product usually isolated from various plant species particularly from *Salvia* species such as *Salvia nemorosa* and *S. martiusii* (da Araújo et al., 2006; Vlasova et al., 1969). Acetoxyroyleanone contains a quinone moiety as its key structural feature. It has been reported that abietane diterpenes are often reported to exhibit cytotoxic effects on cancer cell lines. It was reported that the cytotoxic activity of acetoxyroyleanone was related to the inhibition of DNA synthesis and induction of apop-



tosis (da Araújo et al., 2006). Acetoxyroyleanone has also been reported to induce cytotoxic effects in pancreatic cancer cell line (Fronza et al., 2012). Despite this, till now, very little information exists in the literature regarding the cytotoxic molecular mechanism of action of acetoxyroyleanone.

In the present study, the molecular mechanism of action of acetoxyroyleanone is reported by studying its effects on apoptosis induction, NF- $\kappa$ B and caspase-3 signalling pathways.

## Materials and Methods

### *Chemicals and source of antibodies and kits*

Growth medium (MEM/RPMI), fetal calf serum, trypsin, penicillin, streptomycin, dimethyl sulfoxide, RNase, proteinase K, RIPA Buffer, bisacrylamide, SDS, ([3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) dye, acrylamide, ammonium persulfate, 2-mercaptoethanol, and Tris-base were obtained from Hangzhou Sijiqing Biological Products Co. Ltd, China. Chemiluminescent Western blotting kit (Millipore), Quanti Pro BCA assay kit, 96 and 6 well plate (Iwaki), triton X (Hi-Media), Tris-EDTA (Hi-Media), acetic acid (Rankem), ELISA plate reader (Bio-Rad). NF $\kappa$ B (p65), caspase-3 antibodies were purchased from Millipore Pvt. Ltd. Acetoxyroyleanone was purchased from Sigma Chemical Company (USA), and 100 mg/mL solution dissolved in dimethyl sulfoxide was stored at -20°C prior to use.

### *Cell lines, growth medium and treatment conditions*

Human cancer cell lines; prostate (PC-3), leukemia (THP-1), colon (Colo-205, Caco-2), breast (T47D, MCF-7), pancreatic (MiaPaca-2) and normal epithelial (fR-2) were procured from Shanghai Institute of Cell Resource Center of Life Science (China). Cells were grown in Minimum Essential Medium and Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum and 1% penicillin. Penicillin was dissolved in phosphate buffered saline and sterilized by filtering through 0.2  $\mu$ m filter in laminar air flow hood. Cells were cultured in CO<sub>2</sub> incubator (New Brunswick, Galaxy 170R, eppendroff) with an internal atmosphere of 95% air and 5% CO<sub>2</sub> gas and the cell lines were maintained at 37°C. The media was stored at low temperature (2-8°C) and the medium for cryopreservation contained 20% fetal calf serum and 10% dimethyl sulfoxide in the growth medium.

### *MTT assay for cell viability evaluation*

Cell viability was measured using [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT) assay. Different cell lines were seeded in 200  $\mu$ L of Roswell Park Memorial Institute medium-1640 medium into 96-well plates, and cultured overnight. Then the

medium was replaced with fresh RPMI-1640 or the same media containing different concentrations of acetoxyroyleanone. After a further incubation for 24 hours, 30  $\mu$ L of MTT (2 mg/mL) was added to each well followed by 3 hours incubation. The medium was discarded and 170  $\mu$ L of dimethyl sulfoxide was added to each well, and incubated for 30 min. The OD<sub>490</sub> nm was measured. The cell viability index was calculated according to the formula:

$$(\text{Experimental OD value}/\text{control OD value}) \times 100\%$$

Cytotoxicity was expressed as the concentration of acetoxyroyleanone inhibiting cell growth by 50% (IC<sub>50</sub> value).

### *Preparation of whole cell lysates and Western blot analysis*

Colo-205 (1  $\times$  10<sup>5</sup> cells/mL/well) cells were treated with acetoxyroyleanone at 0, 5, 10, 20 and 30  $\mu$ M were suspended in cold RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1%SDS, 50 mM Tris, PH 8.0) for 30 min on ice. The lysates were vortexed and centrifuged at 12,000  $\times$ g for 10 min. Supernatant thus obtained was whole cell lysate and was stored at -20°C for further use. Protein content was measured using bovine serum albumin as standard. 1 mg/mL protein standard was taken and samples with unknown concentrations were plotted in a linear range of 0.5 to 30  $\mu$ g/mL of the protein concentration and absorbance measured at 562 nm. The above protein lysates were subjected to discontinuous SDS-PAGE at 100 V and electro transferred topolyvinylidene difluoride membrane (Millipore) for 2.5 hours at 120 V at 4°C. The membrane was blocked with 2% skimmed milk in phosphate buffered serum for 1 hour. After blocking, the membrane was probed with specific primary antibody for overnight at 4°C followed by 2 times washing with tris-buffered saline for 5 min each. A dilution of secondary antibody (mouse and rabbit) conjugate was added for 1 hour of incubation and signals were detected using Millipore chemiluminescent Western blotting kit and analyzed using X-ray film.

### *Cell migration assay*

Cell monolayer (90% confluent) was allowed to become quiescent in medium with 0.1% dialyzed fetal bovine serum for 24 hours. Then cells were scraped to make a straight line wound and treated with acetoxyroyleanone for 48 hours. Photographs were taken at 48 hours and lengths of the wound were determined by Image J (version 1.46) software.

### *Statistical evaluation*

The results of three independent experiments were expressed as the mean  $\pm$  SD. Statistical evaluation was performed using an un-paired t-test.

Table I				
IC <sub>50</sub> value of acetoxyroleanone against different cancer cell lines including normal epithelial cell line				
Tissue	Cell Line	IC <sub>50</sub> (μM) (Acetoxyroleanone)	IC <sub>50</sub> (nM) (BEZ-235)	
Leukemia	THP-1	27	-	
Prostate	PC-3	>50	-	
Breast	T47D	24	10	
Breast	MCF-7	28	-	
Pancreas	MiaPaCa-2	25	10	
Colon	Colo-205	12	10	
Colon	Caco-2	>50	-	
Normal	fR-2	>50	-	

## Results

### *In vitro* cytotoxic effect of acetoxyroleanone against different human cancer cell lines

In this study, *in vitro* inhibitory effect of acetoxyroleanone was evaluated using the MTT viability assay against a range of cancer cell lines and IC<sub>50</sub> after 48 hours was calculated. Initially, acetoxyroleanone was screened (Table I) against leukemia (THP-1), prostate (PC-3), breast (MCF-7, T47D), pancreas (MiaPaca-2), colon (COLO-205, Caco-2) cancer cell lines including normal epithelial cell (fR-2) at indicated concentration (5, 10, 20, 30 and 50 μM) for 48 hours. Acetoxyroleanone (Figure 1) shows concentration-dependent inhibitory effect on cell proliferation against THP-1, T47D, MiaPaca-2 MCF-7 and Colo-205 cancer cell lines and most potent inhibition against Colo-205. The cell viability assay revealed that in Colo-205, acetoxyroleanone (5–50 μM) increased growth inhibition of 96, 82 and 79% at a concentration of 50, 30 and 20 μM (Figure 2), with the calculated IC<sub>50</sub> value of 12 μM and 10 nM for BEZ-235 which were used as positive control (Table I). However, it required six to eight times higher concentration of acetoxyroleanone to induce 50% cell death in normal epithelial (fR-2) cell line. Interestingly, these results also depicted that acetoxyroleanone showed more efficiency against Colo-205 as revealed by their relative IC<sub>50</sub> values.

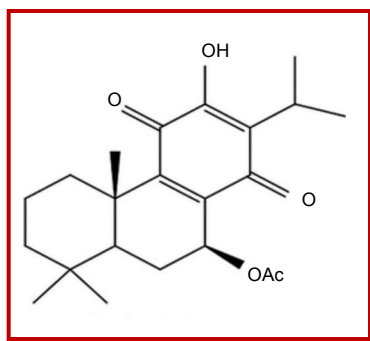


Figure 1: Chemical structure of acetoxyroleanone

### *Effect of acetoxyroleanone treatment on NF-κB and caspase-3 expression in Colo-205 cell line*

In next experiment, the effect of acetoxyroleanone on NF-κB and caspase-3 protein expression levels by Western blotting was observed. Following acetoxyroleanone (10 and 30 μM) treatment for 48 hours, Western blot analysis revealed that acetoxyroleanone decreased expression level of NF-κB (p65) and increased caspase-3 expression level (Figure 3). However, significant effect on both proteins was observed at 30 μM acetoxyroleanone concentration as compared to untreated and BEZ-235 (10 nM) (positive control) (Figure 4), suggesting NF-κB down-regulation and caspase-3 up-regulation by acetoxyroleanone.

### *Treatment of acetoxyroleanone inhibits cell migration of Colo-205 cell monolayers*

Wound closure experiments were performed to establish the inhibitory effect of acetoxyroleanone in Colo-205 cell migration. As shown in Figure 5, the wound got almost healed in untreated and slightly so at the lower concentrations (10 μM) of acetoxyroleanone. However, at higher concentration (30 μM) invasiveness of Colo-205 cells showed potent cell migration inhibition. Acetoxyroleanone treatment results in significant inhibition of Colo-205 cells invasion at a concentration of 30 μM compared to negative control (0 μM) and positive control (BEZ-235, 10 nM) (Figure 6).

## Discussion

In the present study, an interesting correlation was discovered for the first time between various regulatory and phenotypic events of acetoxyroleanone with apoptosis. First, the growth inhibitory and cytotoxicity effect of acetoxyroleanone against a panel of human cancer cell lines which include leukemia (THP-1), prostate (PC-3), breast (MCF-7, T47D), pancreas (MIAPaca2), colon (Colo-205, Caco-2) including normal

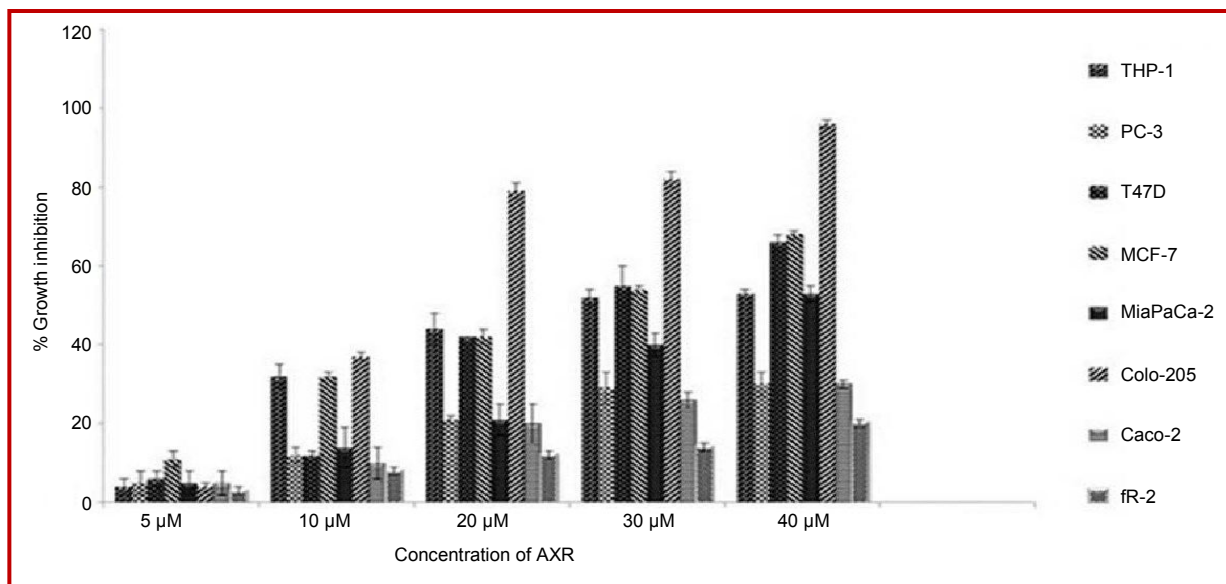


Figure 2: Growth inhibitory effect of acetoxyroyleanone against panel of cancer cell lines including normal epithelial cell line. Cells grown in 96 well plate was treated with different concentration of acetoxyroyleanone (5, 10, 20, 30 and 50  $\mu\text{M}$ ). Data represent mean  $\pm$  SD (n=3) of three repeats

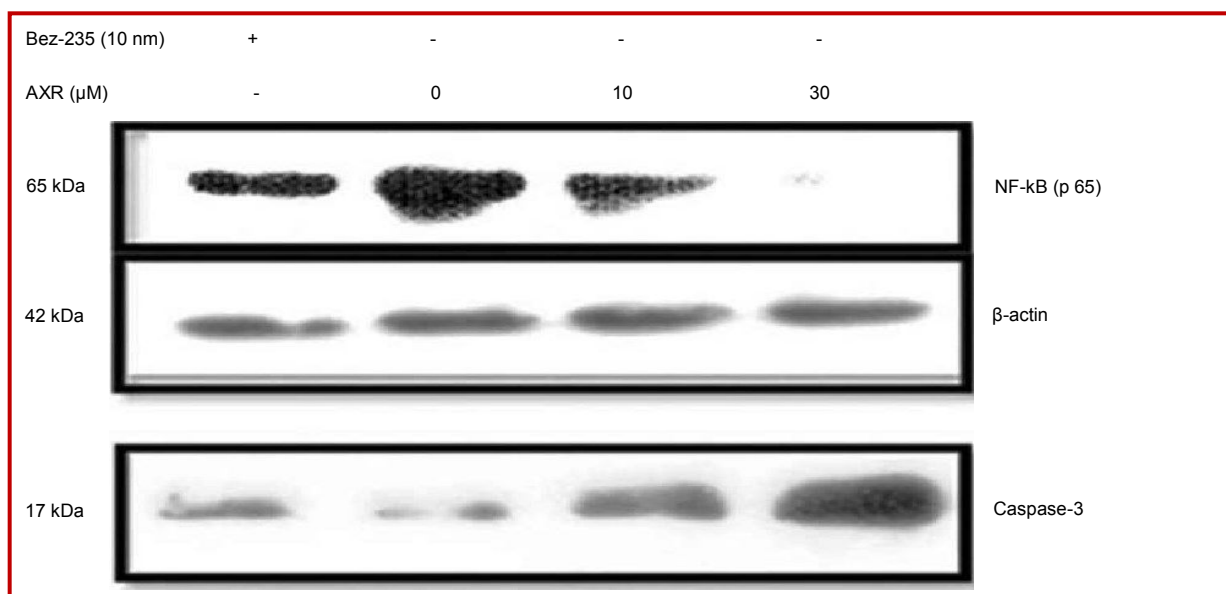


Figure 3: Western blot analysis reveals decrease in NF- $\kappa$ B (p65) and increase in caspase-3 expression at 10 and 30  $\mu\text{M}$  of acetoxyroyleanone for 48 hours incubation

epithelial cell (fR-2) was evaluated. Particularly, it was found that acetoxyroyleanone inhibits cell growth and more significantly showed concentration-dependent inhibition against a panel of cancer cell lines tested. Interestingly, maximum and potent growth inhibition following acetoxyroyleanone were observed at 50, 30 and 20  $\mu\text{M}$  in human colon cancer cell line i.e. Colo-205. Keeping this in view, the  $\text{IC}_{50}$  value of acetoxyroyleanone against all shown human cancer cell lines by cell viability assay was evaluated. The calculated  $\text{IC}_{50}$  values of acetoxyroyleanone were of the order of 27, 24, 28, 25 and 12  $\mu\text{M}$  for 48 hours incubation in the case of THP-1,

MCF-7, T47D, MiaPaca-2 and Colo-205 respectively. While as no significant cytotoxic effects were found in the normal epithelial cell line (fR-2). Overall, these results depicted that acetoxyroyleanone showed significant effect against Colo-205 colon cancer cell proliferation as reflected by relative  $\text{IC}_{50}$  value.

Since the NF- $\kappa$ B pathway is important for cell survival, proliferation, cell cycle progression and migration. Activation of NF- $\kappa$ B pathway affects regulation of proliferative, anti-apoptotic, pro-apoptotic and cell cycle regulatory molecules and therefore results in cell prolifera-

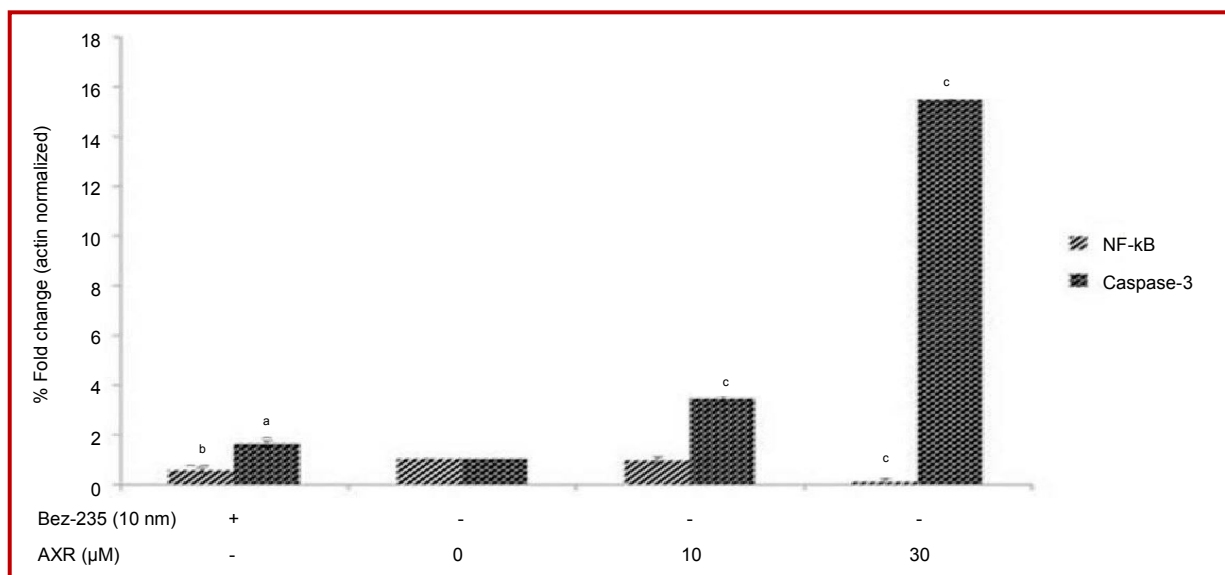


Figure 4: Acetoxyroleanone treatment led significant decrease in NF- $\kappa$ B (p65) and increase in caspase-3 expression at 30  $\mu$ M with respect to untreated and BEZ-235 (10 nm); <sup>a</sup> $p \leq 0.05$ ; <sup>b</sup> $p \leq 0.01$ ; <sup>c</sup> $p \leq 0.001$

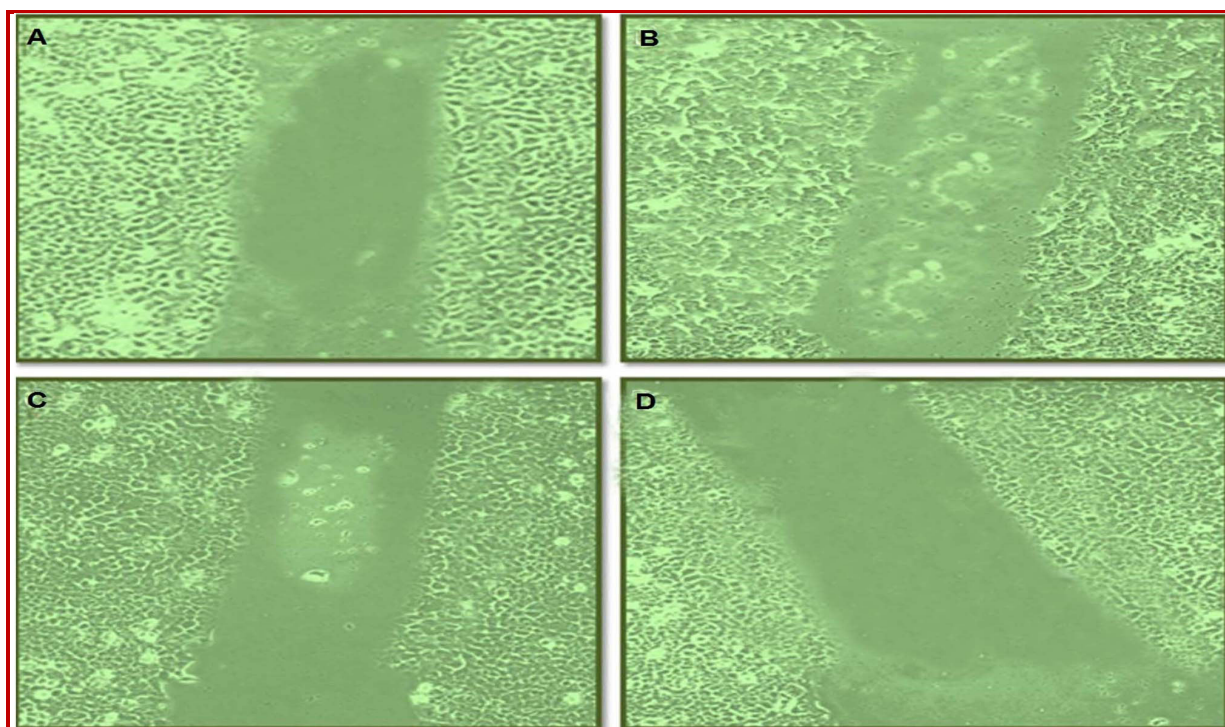


Figure 5: Effect of acetoxyroleanone on cell migration of Colo-205 monolayers. Cells were exposed to different concentrations of acetoxyroleanone (10 and 30  $\mu$ M) for 48 hours compared to untreated. A, represents BEZ-235 treatment which was used as positive control, B, C and D represent 0, 10 and 30  $\mu$ M concentration of acetoxyroleanone

tion, progression and migration of numerous cancers (Bhart et al., 2003; Garg and Aggarwal, 2002). Notably, NF- $\kappa$ B promotes cell survival via the induction of proteins that inhibit components of the apoptotic machinery in normal and cancerous cells (Shen and Tergaonkar, 2009). To evaluate the mechanism by which the effect of the acetoxyroleanone occurred,

further experiments of the effect of acetoxyroleanone on NF- $\kappa$ B protein expression was determined. The most abundant form of NF- $\kappa$ B consists of a p50 subunit and a p65 subunit. In its inactive form, NF- $\kappa$ B is located in the cytoplasm, however, upon activation by various stimuli, it translocates to the nucleus, where it may activate genes leading to cell survival or proliferation

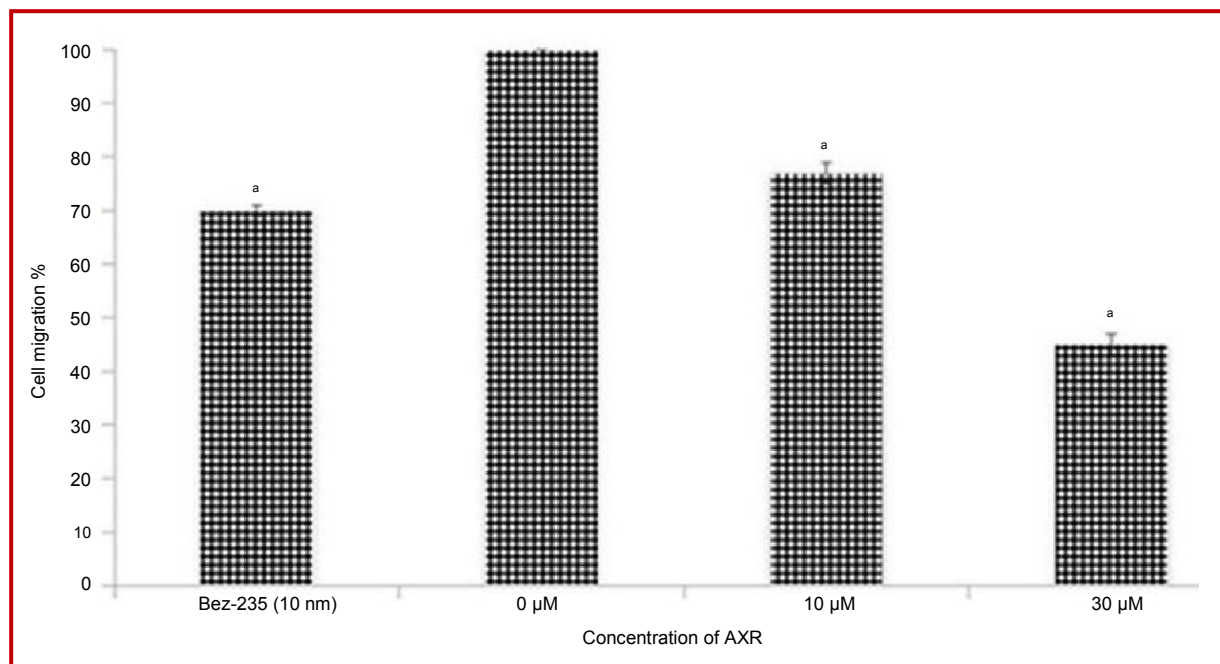


Figure 6: Significant decrease in cell migration at 30  $\mu\text{M}$  as compared to untreated (0  $\mu\text{M}$ )  $^*p \leq 0.01$

(Schmid et al., 2000; Birbach et al., 2002; Swinney et al., 2002). Notably, our study demonstrates that exposure to acetoxyroleanone resulted in remarkable down-regulation in the expression of NF- $\kappa\text{B}$  (p65). Furthermore, caspases play a central role in apoptosis and are central to the mechanism of apoptosis as they are both the initiators and executioners. Among caspases, caspase-3 is a frequently activated death protease, which catalyzes the specific cleavage of many key cellular proteins resulting apoptosis (Porter and Jänicke, 1999). Importantly, caspase-3 is crucial for apoptotic chromatin condensation and DNA fragmentation in all cell types. Here, it is reported that acetoxyroleanone mediates caspase-3 up-regulation in colo-205 cells. Taken together, these data indicate that NF- $\kappa\text{B}$  and caspase-3 play a pivotal role in mediating acetoxyroleanone-induced apoptosis in Colo-205 cells.

Cell migration plays a critical role in tumor cell invasion and metastasis (Yamaguchi et al., 2005). Importantly, cell migration and invasion represents an important property for chemotherapeutic agent other than having the potential to cause specific cancer cell death. Molecules involved in cancer cell migration could be the potential target for anti-metastasis therapy.

This study describes how colon cancer cell migrate using acetoxyroleanone tested by measuring the gap between control and treatment groups. Acetoxyroleanone was found actively inhibit colon cancer cell migration. Notably, apoptosis is morphologically characterized by chromatin condensation, internucleosomal fragments, cell shrinkage, membrane blebbing and formation of apoptotic bodies

without disruption of the plasma membrane (Wyllie et al., 1984).

## Conclusion

Acetoxyroleanone has a target based antiproliferative activity. A successful anticancer drug must have the ability to induce tumor cell apoptosis and these results showed that acetoxyroleanone does show this characteristic feature of inducing apoptosis in Colo-205 cancer cells.

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