

## ORIGINAL ARTICLE

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# Naturally occurring triterpene Lupane exerts anticancer effects on colorectal cancer cells via induction of apoptosis and autophagy and suppresses cell migration and invasion by targeting MMP-9

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## Summary

**Purpose:** This study was undertaken with a purpose to examine the anticancer effects of Lupane against human colorectal cancer.

**Methods:** The SW48 colorectal cell line and CDD18Co normal colon cell line were used in this study. The CCK8 assay was used to determine cell proliferation while acridine orange (AO)/ethidium bromide (EB) and DAPI staining assays were used to detect apoptosis. Wound healing and transwell assays were used to detect the cell migration and invasion. Western blotting was used to determine protein expression.

**Results:** Lupane inhibited the proliferation of colorectal cancer cells and the level of inhibition followed dose-dependent pattern. The antiproliferative role of Lupane was exerted via induction of apoptotic cell death. Western blot showed that the expression of Bcl-2 was decreased and that of Bax

was increased. Lupane also prompted the autophagy of the SW48 colorectal cancer cells and enhanced the expression of LC3-II. However, the expression of p62 was depleted. The treatment of Lupane also resulted to inhibition of the migratory potential of cancer cells as revealed by the wound healing assay. The invasion of SW48 cancer cells was also suppressed and was associated with suppression of metalloproteinase-9 (MMP-9) expression.

**Conclusion:** The results indicate the anticancer potential of Lupane against the colorectal cancer growth and propagation. The study envisages the importance of natural compounds for their usage against human cancers.

**Key words:** lupane, anticancer, apoptosis, proliferation, cell cycle arrest, flow cytometry

## Introduction

Plants harbour a fascinating diversity of chemical scaffolds that are involved in a wide array of biological functions [1]. These naturally occurring compounds fall in broad categories of primary and secondary metabolites. The secondary metabolites, although considered the accessory natural compounds, are fairly important for the better survival of host organism for their potential to provide a helping hand to their host in the fluctuating environmental conditions [2,3]. Further, they aid in the chemical defense against the predator attacks [4]. The plant-derived natural products have long been

valued for their health benefitting effects in humans [5,6]. The plant secondary metabolites have been shown to impart positive results on human health and have been used for treating a number of human diseases. The role of plant-derived compounds to be used against human cancers is well recognized [7]. Active research is going on to explore, evaluate and understand the anticancer effects of plant-based natural compounds. Triterpenes comprise a class of plant secondary metabolites synthesized through the mevalonate pathway [8]. These compounds have bioactive properties and

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possess health protective effects. Lupane is an important triterpene found in many plant species [9]. Triterpenes have been reported to exhibit tremendous pharmacological properties against different types of cancers. Additionally, they have been reported to induce apoptosis as well as autophagy in cancer cells via modulation of different signalling pathways. Lupane is an important triterpenoid and has been shown to exhibit cytotoxic effects against different types of cancer cells [10,11]. The purpose of the present work was to study the anticancer effects of Lupane against the human colorectal cancer. Colorectal cancer is ranked 4<sup>th</sup> in terms of cancer mortality. About 0.7 million people died because of colorectal cancer in 2013 alone [12]. Hence, there is urgency in researchers to look for effective measures of preventing and treating colorectal cancer. The aim of the present study was to examine the anticancer effect of Lupane against the human colorectal cancer cells and explore the underlying mechanism.

## Methods

### Growth and proliferation assay

Cell counting kit-8 (CCK-8, MedChemExpress) was used for the estimation of proliferative rates of colorectal cancer cells treated with Lupane (Sigma-Aldrich) and proliferation rates were compared with those of normal colon line CDD18Co. In brief, the cells were placed in 96-well plates at a density of  $1 \times 10^6$  cells/well and cultured for 24 h with 0, 2.5, 5, 10, 20, 40, 80 and 180  $\mu$ M Lupane, after which CCK-8 was employed to estimate the proliferation rates by the addition of 10  $\mu$ l of CCK-8 solution to each well, at the indicated time intervals. Following 2 h incubation at 37°C, absorbance at 450 nm was read for each sample with the help of microplate reader.

### Analysis of cellular apoptosis

DAPI and AO/EB staining procedures followed to examine the effect of Lupane on the viability of colorec-

tal cancer cells and induction of cell apoptosis. The cells were placed into 12-well plates at a density of  $0.6 \times 10^6$  cells/well. Lupane at the concentrations of 0, 7.5, 15 and 30  $\mu$ M was added to each well and cells were incubated at 37°C for 24 h. Afterwards, the cells were harvested and washed twice with phosphate buffered saline (PBS), followed by fixing with 4% paraformaldehyde. The DAPI or AO/EB solutions were then used separately to stain the cells. Afterwards, the cells were examined for the fluorescence measurements using fluorescent microscope.

### Transmission electron microscopy (TEM)

For electron microscopy, the SW48 cells were fixed with 4% glutaraldehyde, 0.05 M sodium cacodylate solution, post-fixed in 1.5% OsO<sub>4</sub>, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and then observed using Zeiss CEM 902 electron microscope.

### Cell migration assay

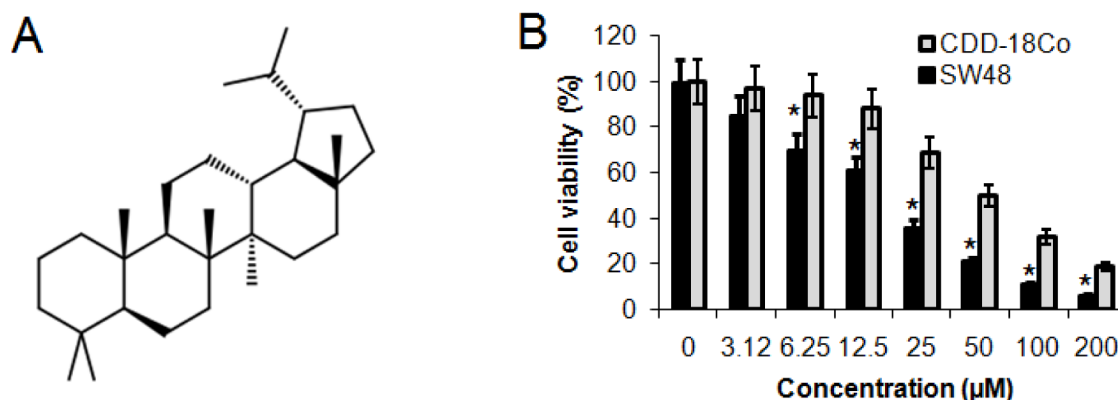
The migration of SW48 cancer cells treated with 0 and 7.5  $\mu$ M Lupane was assessed by wound healing assay. In brief, culturing of cancer cells was done in 6-well plates. A scratch line was drawn on the cell surface with the tip of a sterile pipette. After 24 h of incubation at 37°C, the scratch mark was examined for assessment of cell migration.

### Cell invasion assay

The transwell assay was used to determine the invasion of SW48 cancer cells treated with 0, 7.5, 15 and 30  $\mu$ M Lupane. Briefly, about 250  $\mu$ l of cancer cells at a density of  $4 \times 10^5$ /ml were put into the upper chamber and only Dulbecco's modified Eagle's medium (DMEM) was kept in the lower chamber. Then, incubation was followed at 37°C for 24 h. The cells that invaded the lower chamber were fixed using ethanol, stained with crystal violet and visualized using high magnification (200 $\times$ ) microscopy.

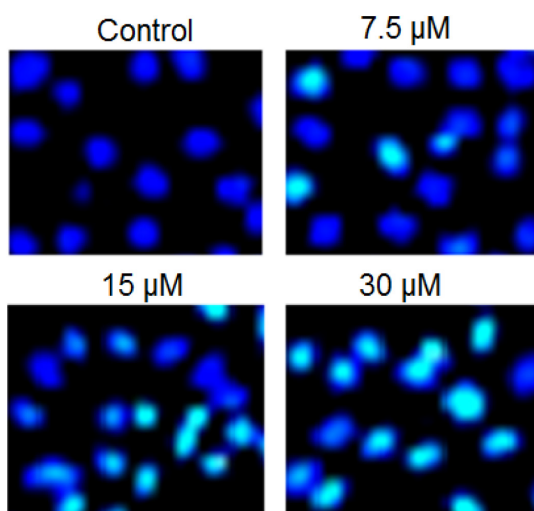
### Western blotting

Using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, Mass, USA), total proteins

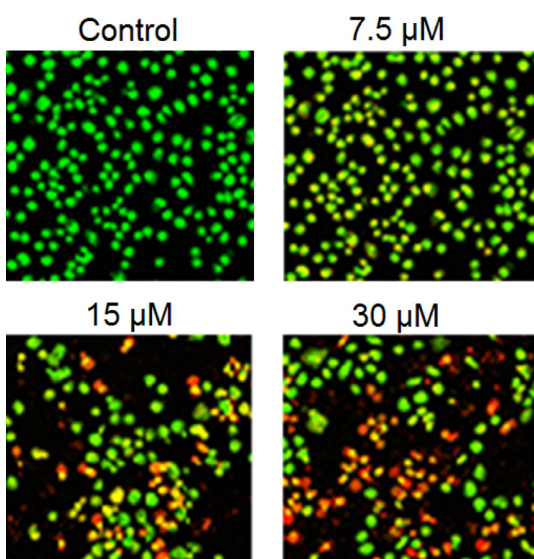


**Figure 1. A:** Chemical structure of Lupane. **B:** Effect of Lupane on the viability of the SW48 colorectal cancer cells. The experiments were performed in triplicate and expressed as mean  $\pm$  SD (\* $p < 0.05$ ).

were isolated from untreated colorectal cancer cells and cancer cells treated with 10, 20 and 40  $\mu\text{M}$  Lupane for 24 h. Bradford method was used to quantify the protein concentrations. About 45  $\mu\text{g}$  of total proteins from each sample were separated electrophoretically on 10% SDS-PAGE. The gel was blotted to nitrocellulose membrane which was exposed to primary antibodies, followed by exposure to secondary antibodies. Enhanced chemiluminescence reagent (ECL) was used for detection of bands corresponding to proteins of interest. The protein expression procedures were normalized with human GAPDH protein.



**Figure 2.** DAPI staining showing the effect of Lupane on the nuclear morphology of the SW48 colorectal cancer cells. Lupane induced apoptosis in SW48 cancer cells in a dose-dependent manner. The experiments were performed in triplicate.



**Figure 3.** AO/EB staining showing Lupane induces apoptosis in a concentration-dependent manner in the SW48 colorectal cancer cells. Green color depicts normal, yellow depicts early apoptotic and red depicts necrotic cells. The experiments were performed in triplicate.

### Statistics

The experiments were performed in triplicate and expressed as mean  $\pm$  SD. Graphpad Prism 7 software was used to perform t-test. P value  $< 0.05$  was taken as an indicator of statistically significant difference.

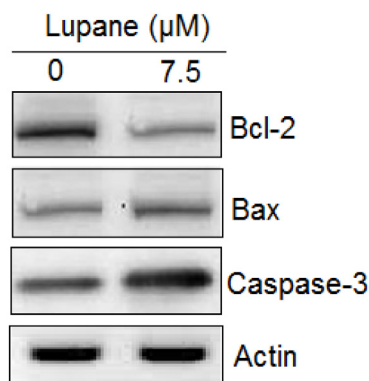
## Results

### Colorectal cancer growth is inhibited by Lupane via apoptotic cell death

To ascertain the effects of Lupane (Figure 1A) on the proliferation of colorectal cancer, the normal (CDD-18Co) and SW48 colorectal cancer cells were treated with 0 to 200  $\mu\text{M}$  concentrations of Lupane for 24 h. Using CCK-8 kit, the proliferation rates of cells were determined and it was found that the viability of SW48 cancer cells decreased proportionally with increasing doses of Lupane with an  $\text{IC}_{50}$  of 15  $\mu\text{M}$  (Figure 1B). Interestingly, the effects of Lupane on the normal cells were less severe with  $\text{IC}_{50}$  47  $\mu\text{M}$ . Next, several apoptosis assays were performed to investigate if the Lupane induced growth inhibitory effects were due to induction of apoptosis. Interestingly, the DAPI as well as the AO/EB stained cancer cells were seen with clear nuclear deformation which is indicative of apoptotic cell death (Figure 2 and Figure 3). Moreover, the effects were more prominent at higher doses of Lupane. Further support was obtained from western blotting results where it was found that the positively regulating apoptotic proteins, Bax and active Caspase-3 were upregulated under Lupane treatment (Figure 4). Taken together, the results showed that Lupane induces apoptosis in colorectal cancer cells and thus reduces their proliferation rates.

### Lupane promotes autophagy in colorectal cancer cells

Next, TEM analysis of the SW48 cells after treatment with 7.5  $\mu\text{M}$  Lupane showed that this



**Figure 4.** Western blot showing that Lupane inhibits the expression of Bcl-2 and increases the expression of Bax and Caspase-3 in SW48 cells. The experiments were performed in triplicate.



molecule led to development of autophagosomes in the SW48 cells, suggestive of autophagy (Figure 5). The autophagy induction was further authenticated by determining the expression of LC3-II, LC3-I and p62 expression. The results showed that the expression of LC3-II was enhanced and the expression of p62 was decreased (Figure 6).

#### *Lupane inhibits the migration of colorectal cancer cells*

The wound healing assay was employed to assess the effects of Lupane on the migration of the SW48 cells. The results showed that Lupane inhibited the migration of the SW48 cells at 7.5  $\mu$ M concentration (Figure 7).

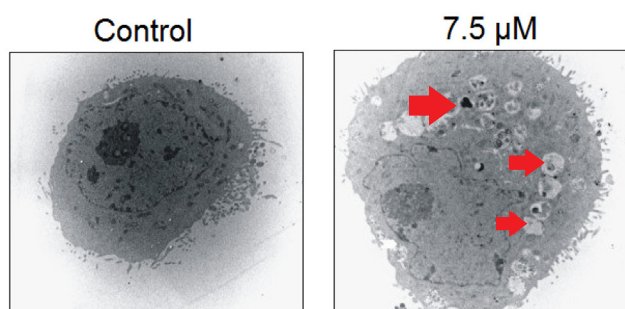
#### *Lupane inhibits the invasion of colorectal cancer cells*

The transwell assay based assessment of colorectal cancer cell invasion revealed that invasion of cancer cells was remarkably inhibited by Lupane treatment. The extent of restriction of cell invasion

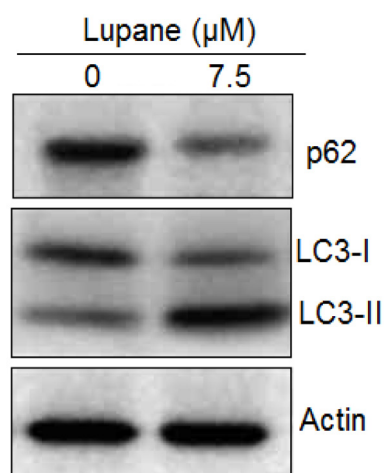
by Lupane increased with increasing doses (Figure 8A). The western blotting of matrix metalloproteinase-9 (MMP-9) showed that the molecule exerted its anti-invasive effects on colorectal cancer cells by decreasing the protein concentrations of MMP-9 (Figure 8B).

## Discussion

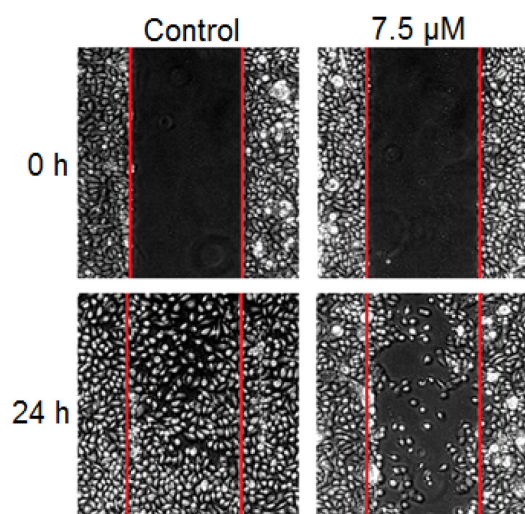
Synthesized via the mevalonate pathway, the triterpenes are isoprenoids with diverse biological functions [13]. They are spread across the plant kingdom and generally perform defence-related functions. Pharmacologically, they exhibit a wide range of functions such as antioxidant, antimicrobial and anticancer to name a few [14]. This study was undertaken to evaluate the anticancer effects of Lupane, a naturally occurring triterpenoid [15] of plant origin against the human colorectal cancer cells. The cell proliferation assay showed significant inhibition of the colorectal cancer cells growth upon Lupane treatment. Previous researches have also shown that Lupane and its derivatives halt the proliferation of different types of cancer cells [16]. The investigation of the underlying mechanism revealed induction of apoptosis by Lupane in the SW48 cells. The Bax/Bcl-2 ratio was also increased, which is an important indicator of apoptosis [17]. Apoptosis plays key role in eliminating defective cells and thus drugs that promote apoptosis are currently being studied extensively [17]. TEM analysis showed that Lupane also induces autophagy in the SW48 colorectal cancer cells which was accompanied by alteration of the LC3



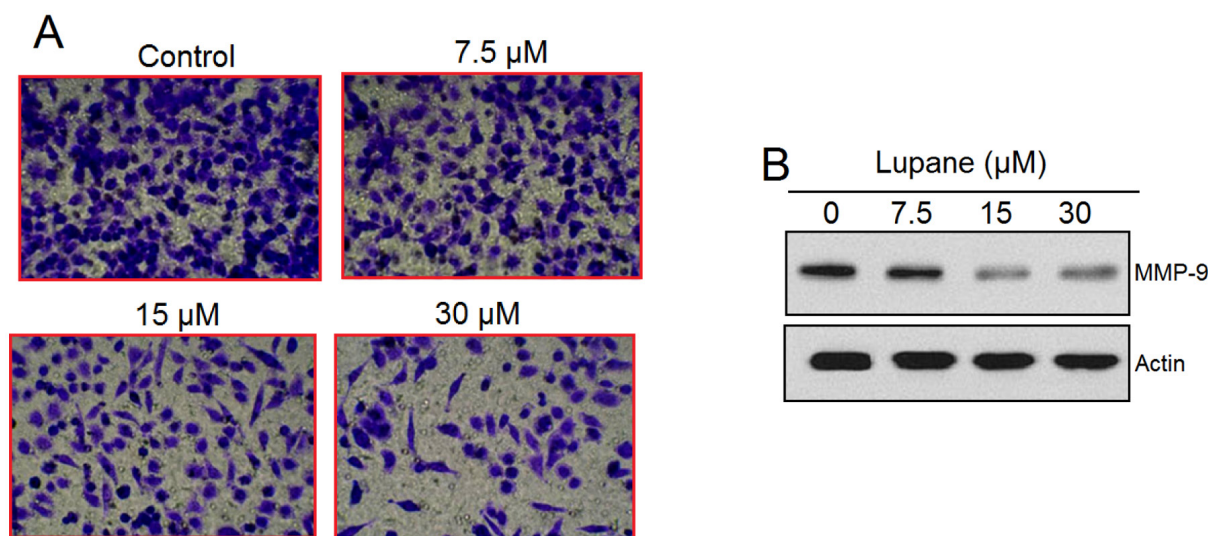
**Figure 5.** TEM analysis showing Lupane induction of autophagy in the SW48 colorectal cancer cells. Arrows show autophagosomes. The experiments were performed in triplicate.



**Figure 6.** Western blot showing the effect of Lupane on the expression of autophagy-related proteins in SW48 cells. The Figure shows that Lupane decreased p62 and increased LC3-II expression in SW48 cells. The experiments were performed in triplicate.



**Figure 7.** Wound healing assay showing that Lupane inhibits the migration of SW48 cells dose-dependently on the inhibition of migration in SW48 cells. The experiments were performed in triplicate.



**Figure 8. A:** Transwell assay showing that Lupane inhibits the migration of SW48 cells dose-dependently. **B:** Western blot showing that Lupane suppresses the expression of MMP-9 dose-dependently. The experiments were performed in triplicate.

and p62 expression. Several studies have shown that LC3-II expression is increased and that of p62 is significantly decreased during the induction of autophagy [18]. We also observed similar results in our study. This study also examined the effects of Lupane on the migration and invasion of the SW48 cells. The wound healing assay used for cell migration analysis showed inhibition of cell migration upon Lupane treatment. The transwell assay showed inhibition of SW48 cell invasion together with suppression of MMP-9 expression. The migration and invasion of cancer cells facilitates the metastasis of cancer cells [19]. Hence, the ability of this molecule to inhibit the migration and invasion of cancer cells indicates that it may exhibit antimetastatic potential *in vivo*. More studies are required to establish Lupane as a lead molecule for the development of systemic therapy for colorectal cancer.

## Conclusion

The results of the current study shed light on the apoptosis and autophagy inducing potential of Lupane against human colorectal cancer. The efficacy of its anticancer properties may be augmented via semisynthetic chemistry approaches and it may act as a crucial lead molecule for discovery of more efficient drugs against colorectal cancer.

## Acknowledgements

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## Conflict of interests

The authors declare no conflict of interests.

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