

ORIGINAL ARTICLE

Inhibition of cancer cell growth by oleanolic acid in multidrug resistant liver carcinoma is mediated via suppression of cancer cell migration and invasion, mitochondrial apoptosis, G2/M cell cycle arrest and deactivation of JNK/p38 signalling pathway

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Summary

Purpose: Liver cancer accounts for considerable mortality across the globe. The sharp upsurge in the incidence of liver cancer, unavailability of standard treatments and the adverse side effects associated with the existing drugs has made it compulsory to explore novel and more effective anticancer molecules. In this study the anticancer effects of a natural compound oleanolic acid were investigated in vitro.

Methods: The human HepG2 liver cancer cells were treated with various concentrations of oleanolic acid for 24 h. The antiproliferative effects of oleanolic acid were measured by CCK8 cell viability assay. DAPI and annexin V/propidium iodide (PI) assays were employed to examine the induction of apoptosis. Transwell assay was performed to examine the cell migration and invasion. Expression analysis was performed by western blot analysis.

Results: The results showed that oleanolic acid decreased the viability of the liver cancer HepG2 cells and exhibited an IC₅₀ of 30 μM. The cytotoxicity of oleanolic acid was also inves-

tigated on the normal liver cells AML12 and it was found that oleanolic acid exerted very low toxic effects on these cells and exhibited an IC₅₀ of 120 μM. Oleanolic acid also caused remarkable changes in the morphology of the HepG2 cells and inhibited their colony formation potential. Flow cytometry indicated oleanolic acid triggered G2/M arrest of the liver HepG2 cancer cells. PI and DAPI staining revealed that oleanolic acid prompted apoptosis of the HepG2 cells. The apoptotic cells increased from 2.2% in control to around 35% at 30 μM concentration. Oleanolic acid also suppressed the migration and invasion of the liver cancer cells via blocking of the JNK/p38 signalling pathway.

Conclusions: The results of the current research revealed that oleanolic acid can be a molecule that may be utilised in the treatment of liver cancer in the future.

Key words: apoptosis, liver cancer, invasion, migration, cell cycle arrest, oleanolic acid

Introduction

In recent years, after facing prolonged impact of industrialization, utilizing extracted edibles and consuming highly active/chemically synthesized/highly specific drugs, there is growing inclination towards basics of life and people started opting natural or close to natural stuffs, edibles and drugs

respectively. Moreover, great amount of research is going on for establishing the pharmacological correlation with ayurvedic/alternative medicines. It is important to note that chemically synthesized drugs are generally improved derivative of prototypes of herbal isolated drugs [1]. Moreover, it has

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been observed that medicinal plants or extracts, used as co-adjuvants or alternative therapies to allopathic ones, are practised possibly due to little or no toxicological effects, low cost and local availability [2]. Oleanolic acid is an important plant secondary metabolite belonging to the class of metabolites known as Triterpenoids [3]. Oleanolic acid is generally isolated from several plant species and has been reported to exhibit strong pharmacological potential including anticancer activities [4,5].

This study was designed to investigate the anticancer effects against human liver cancer cells. Liver cancer is the 5th and 8th prevalent type of cancer in males and females, respectively. The risk of liver cancer has been reported to increase with age [6]. Around 0.56 million new cases of liver cancer are reported annually. Additionally, the frequency of liver cancer is comparatively higher in developing countries [7]. The adverse effects of currently available inefficient chemotherapy remarkably obstruct the treatment of liver cancer [8]. Herein, we examined the anticancer effects of oleanolic acid, an important triterpenoid of plant origin against the human HepG2 liver cancer cells, and attempted to explore the molecular mechanisms responsible for its anticancer effects. The main aim of the current research work was to evaluate the anticancer effects of oleanolic acid in HepG2 human liver cancer cells and normal liver cells along with evaluating its effects on cell apoptosis, cell cycle phase distribution, cell migration and invasion and JNK/p38 signalling pathway.

Methods

Cell viability determination

In brief, the HepG2 liver cancer and normal AML12 cells were seeded in 96-well plates and subjected to treatment with varied concentrations of oleanolic acid at 37°C for 24 h. Thereafter, 10 µL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C in a humidifier (5% CO₂, 95% O₂). OD₄₅₀ was taken with the help of a microplate reader to determine the cell viability.

Detection of apoptosis

The HepG2 cells (0.6×10⁶) were seeded in 6-well plates and subjected to 24-h incubation with varied concentration of oleanolic acid at 37°C. As the cells disposed off, 10 µl of cell culture were put onto glass slides and stained with DAPI. The slides were cover-slipped and examined with a fluorescent microscope. Annexin V/PI staining was performed as described previously [9].

Cell cycle analysis

The HepG2 cells were treated with varied concentrations of oleanolic acid and incubated for 24 h at 37 °C.

The cells were washed with phosphate buffered saline (PBS). Afterwards, the oleanolic acid-treated HepG2 cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Transwell assay

The migration and invasion abilities of the HepG2 cells were examined by transwell chamber assay. In brief, 1×10⁴ HepG2 cells were seeded in the upper chamber of the transwell (8 µm pore size polycarbonate filters). This was followed by placement of the chambers into 24-well plates and subjected to incubation at 37 °C for 24 h. The inserts were coated with extracellular matrix gel (50 µl) (ECM, Sigma, USA). Swabbing was performed to remove the non-invaded cells from the upper surface. However, the invaded cells on the lower surface were subjected to fixation with methanol for about 35 min, followed by staining with crystal violet (0.5%) for about 50 min, subjected to washing with PBS and finally counted under light microscope (5 fields).

Western blot analysis

Protein expression estimation was carried out by western blotting. The oleanolic acid-treated HepG2 cells were harvested with centrifugation. The HepG2 cells were then lysed in lysis buffer containing protease inhibitor. Around 45 µg of proteins from each sample were subjected to separation 10%, followed by transferring it to polyvinylidene difluoride (PVDF) membrane. Next, skimmed milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

The experiments were performed in triplicate and data are shown as mean ± SD. Differences between groups were examined using Student's *t*-test and *p*<0.05 was considered to indicate statistically significant difference.

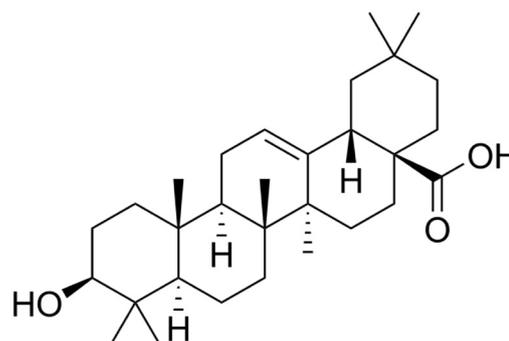


Figure 1. Chemical structure of oleanolic acid.

Results

Suppression of liver cancer cell growth by oleanolic acid

To assess the growth inhibitory effects of oleanolic acid (Figure 1), the HepG2 cells were treated with 0-200 μM concentrations of oleanolic acid and then subjected to CCK8 assay. The results of the CCK8 cell viability assay revealed that Oleanolic acid caused concentration-dependent decrease in the viability of the HepG2 cells (Figure 2A). It was further found that at 24 h of incubation, oleanolic acid showed an IC_{50} of 30 μM against the HepG2 liver cancer cells. However, oleanolic acid did not exhibit significant toxic effects on the normal human liver AML12 cells as evidenced from

the IC_{50} of more than 100 μM (Figure 2B). Microscopic analysis also revealed that oleanolic acid caused significant changes in the morphology of HepG2 cells (Figure 3). These changes were characteristic of apoptotic cell death.

Apoptosis induction by oleanolic acid in HepG2 cells

Apoptosis in the oleanolic acid-treated HepG2 cells was assessed by DAPI staining which revealed that oleanolic acid triggered apoptosis as evidenced from nuclear fragmentation of the oleanolic acid-treated HepG2 cells (Figure 4). Moreover, the results of DAPI positive cells increased with increase in the concentration of oleanolic acid, indicative of apoptotic cell death. Annexin V/PI staining showed that the apoptotic HepG2 cell percent increased to

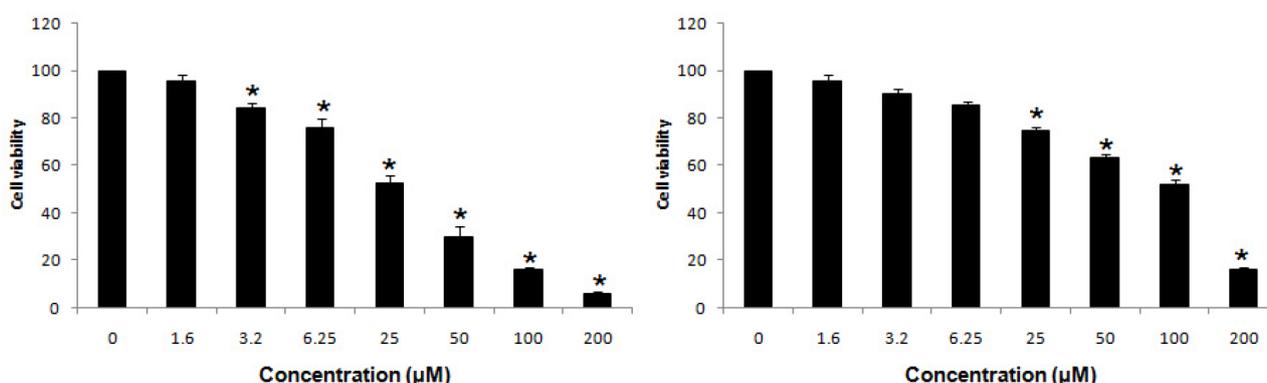


Figure 2. Effect of oleanolic acid on the viability of (A) HepG2 and (B) AML12 cells as determined by CCK8 assay. The values are mean of three replicates \pm SD (* $p < 0.05$).

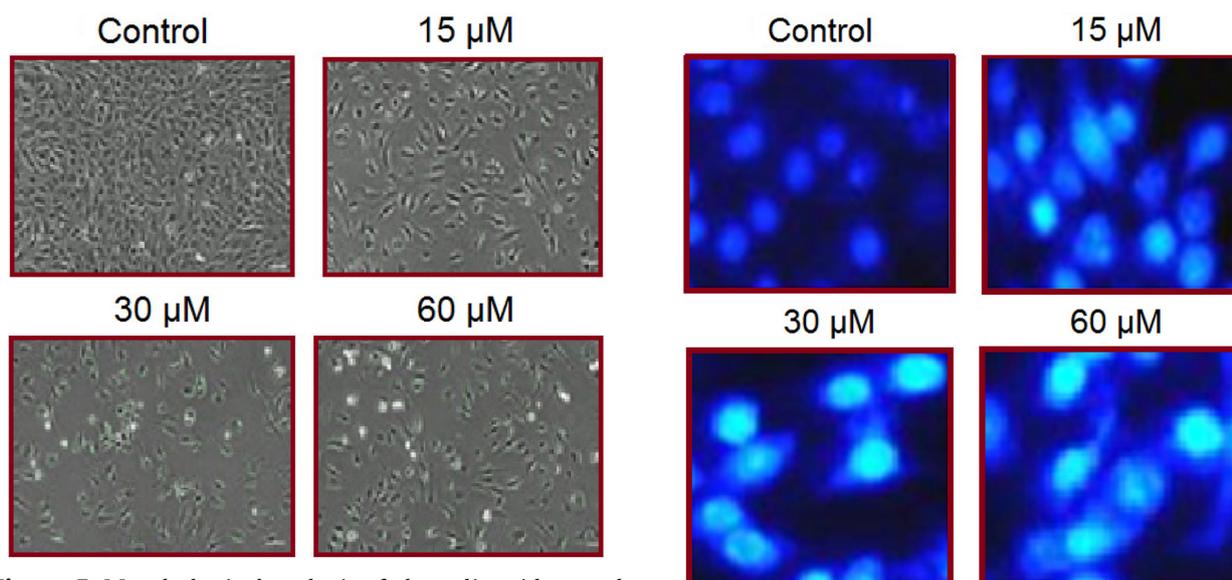


Figure 3. Morphological analysis of oleanolic acid treated HepG2 cells as determined by microscopy. Microscopic analysis revealed that oleanolic acid caused significant changes in the morphology of HepG2 cells characteristics of apoptotic cell death. The experiments were performed in triplicate.

Figure 4. Oleanolic acid induces apoptosis in the HepG2 cells as indicated by DAPI staining. Oleanolic acid triggered apoptosis as evidenced from nuclear fragmentation of the oleanolic acid-treated HepG2 cells. The experiments were performed in triplicate.

about 25.19% at 60 μM concentration of oleanolic acid as compared to approximately 4.3% in the untreated HepG2 cells (Figure 5).

Oleanolic acid caused G2/M arrest of HepG2 cells

The effects of oleanolic acid were also investigated on the cell cycle distribution of the HepG2 cells by flow cytometry. It was found that oleanolic acid caused significant increase in the percentage

of the G2/M phase HepG2 cells. The percentage of the G2/M phase cells increased to 40.05% at 60 μM as compared to 9.12% in the control (Figure 6).

Oleanolic acid inhibited the migration and invasion of the HepG2 cells

The impact of oleanolic acid was examined on the migration and invasion of the HepG2 cells by transwell assay. The results showed that migration (Figure 7) and invasion (Figure 8) was considerably decreased upon treatment of HepG2 cells with oleanolic acid. These effects of oleanolic acid on the HepG2 cells were found to be concentration-dependent.

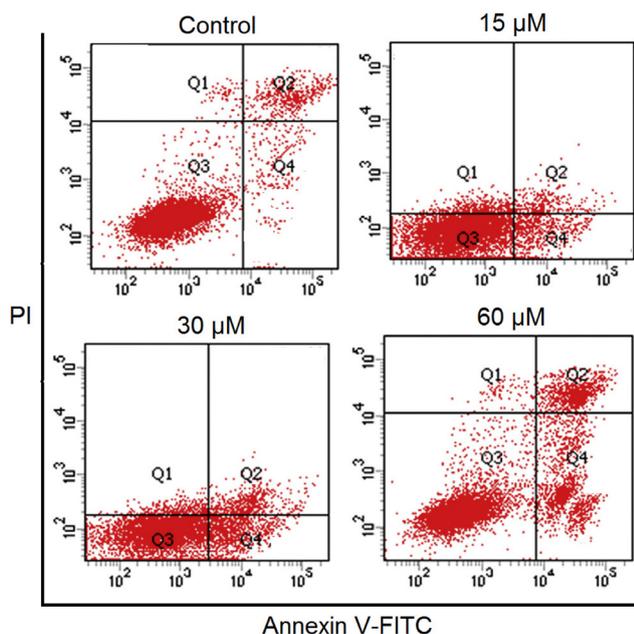


Figure 5. Determination of the percentage of the apoptotic HepG2 cell populations as determined by Annexin V/PI staining. Apoptotic HepG2 cell percent increased to about 25.19% at 60 μM concentration of oleanolic acid as compared to approximately 4.3% in the untreated HepG2 cells. The experiments were performed in triplicate.

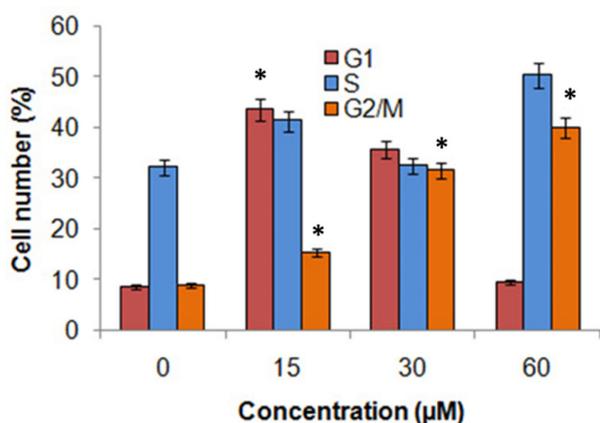


Figure 6. Effect of oleanolic acid on the distribution of the HepG2 cells in different cell cycle phases at indicated concentrations. Oleanolic acid caused significant increase in the percentage of the G2/M phase HepG2 cells and the values are the mean \pm SD of 3 independent experiments (* $p < 0.05$).

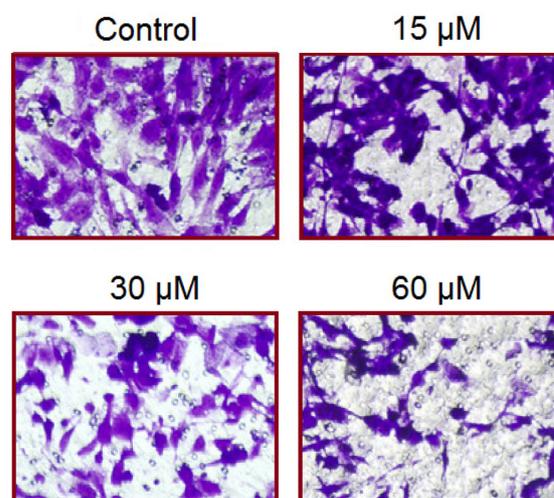


Figure 7. Effect of oleanolic acid on the migration of the HepG2 cells at indicated concentrations as determined by transwell assay. Oleanolic acid led to dose-dependent inhibition of cancer cell invasion. The experiments were performed in triplicate.

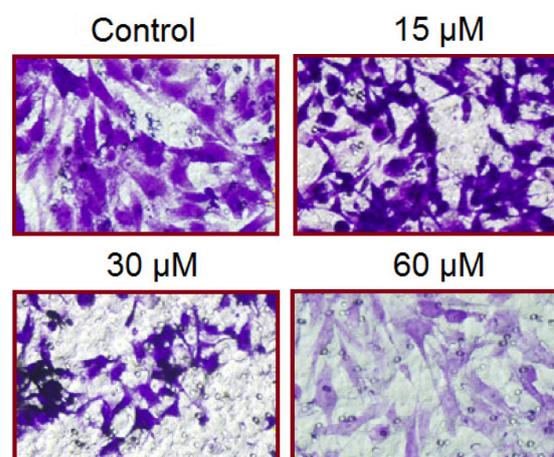


Figure 8. Effect of oleanolic acid on the invasion of the HepG2 cells at indicated concentrations as determined by transwell assay. Oleanolic acid led to dose-dependent inhibition of cancer cell invasion. The experiments were performed in triplicate.

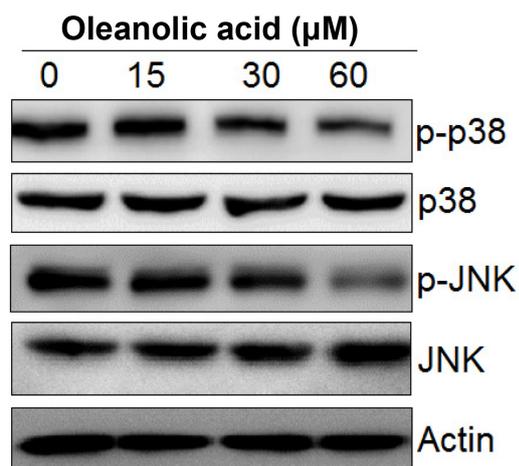


Figure 9. Inhibition of the phosphorylation of JNK and p38 by oleanolic acid indicated concentrations as depicted by western blot analysis. The results showed that oleanolic acid caused decrease in the expression of p-p-38 and p-JNK in a concentration-dependent manner. However, no visible effects were observed on the total JNK and p38. The experiments were performed in triplicate.

Oleanolic acid inhibited the JNK/p38 signalling pathway in HepG2 cells

We also wanted to know the effects of oleanolic acid on the JNK/MAPK signalling pathway by western blot analysis at 0, 15, 30 and 60 μM concentrations. The results showed that oleanolic acid caused decrease in the expression of p-p-38 and p-JNK in a concentration-dependent manner (Figure 9). However, no visible effects were observed on the total JNK and p38.

Discussion

Liver cancer is devastating type of malignancy and considered as the second major cause of cancer related mortality around the globe [10]. The incidence of liver cancer has significantly increased over the last few decades and varies geographically [11]. Plant-derived compounds have shown amazing potential to curb the growth and development of cancers [12]. Molecules of plant origin suppress the proliferation of cancer cells via multiple mechanisms which include but are not limited to the induction of apoptosis, autophagy and arrest of the cancer cells at different cell cycle phases [13]. Some anticancer molecules deactivate the signalling pathway that is generally activated in cancer cells, while others activate the signalling pathways that are generally deactivated in cancer cells [14]. Moreover, plant-derived molecules are believed to be safer for human consumption owing to their minimal adverse effects [15]. As such it is believed that anticancer drugs that are of plant origin may

exhibit lower or even no side effects on the overall health of cancer patients [15]. Herein, the anticancer effects of a plant-derived triterpenoid, oleanolic acid, were examined against the human HepG2 cells as well as the normal human liver cells. The results showed that oleanolic acid dose-dependently inhibited the growth of cancer cells at IC_{50} 30 μM . Nevertheless, it was interesting to see that oleanolic acid exhibits minimal growth inhibitory effects on normal human AML12 cells, while exhibiting an IC_{50} of more than 100, i.e. almost 3 times higher than that against the HepG2 cells. These observations suggest that oleanolic acid selectively targets the liver cancer cells. Several of the plant-derived molecules have been shown to halt the growth of cancer cells, for example Daidzein triggers cell cycle arrest [16] and apoptosis of breast cancer cells [17]. To investigate the underlying mechanisms for the anticancer effects of oleanolic acid, DAPI and Annexin V/PI staining assays were performed and both of these assays showed that oleanolic acid induces apoptosis and the percentage of the apoptotic cells increases with increase in the concentration of oleanolic acid. Cell cycle arrest is another mechanism by which plant-derived anticancer agents have been reported to exert their anticancer effects [18]. Herein, we found that oleanolic acid caused arrest of the HePG2 cells in the G2/M checkpoint of the cell cycle. The migration and invasion of cancer cells is considered as an essential step in the metastasis of cancer cells. Herein, it was found that oleanolic acid inhibited the migration and invasion of the HepG2 cells, indicative of the antimetastatic potential of oleanolic acid. JNK/p38 signalling cascade has been shown to be activated in cancer cells and believed to be responsible for the development and progression of different mechanisms [19,20], and herein we found that oleanolic acid blocks this pathways, suggestive of the potent anticancer effects of this compound.

Conclusion

The results of the present study indicate that oleanolic acid exerts significant anticancer effects on the drug-resistant human liver cancer cells. The anticancer effects of oleanolic acid are mainly due to apoptosis induction and cell cycle arrest. Taken together, oleanolic acid may be utilised in the development of systemic therapy for liver cancer and deserves further studies.

Conflict of interests

The authors declare no conflict of interests.

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