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ORIGINAL ARTICLE

Pectolinarigenin flavonoid exhibits selective anti-proliferative activity in cisplatin-resistant hepatocellular carcinoma, autophagy activation, inhibiting cell migration and invasion, G2/M phase cell cycle arrest and targeting ERK1/2 MAP kinases

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Summary

Purpose: *The main purpose of the present research article was to investigate the anticancer properties of pectolinarigenin flavonoid in cisplatin-resistant hepatocellular carcinoma cells (SK-HEP-1) and normal liver cells (AML-12), along with examining its effects on autophagy, cell migration and invasion, cell cycle arrest and ERK1/2 MAP signalling pathways.*

Methods: *Antiproliferative effects in cancer and normal cells were assessed by MTT cell viability assay. Cell autophagy effects were studied by electron microscopy as well as western blot. Effects on cell cycle were evaluated by flow cytometry using Annexin V/propidium iodide (PI) staining. Transwell migration assay and in vitro wound healing assay were performed to study the effects on cell migration and invasion, respectively.*

Results: *The results indicated that pectolinarigenin inhibited significantly the growth of the SK-HEP-1 liver cancer cells and exhibited an IC_{50} of 10 μM, while against normal cells the cytotoxic effects were much less pronounced. Further, it was observed that the anticancer effects of pectolinarigenin were due to induction of autophagy which was also associated with upregulation of the expression of Beclin-1, LC3-I and LC3-II. Transmission electron microscopy showed the formation of autophagosomes and vesicles. Pectolinarigenin also caused arrest of the SK-HEP-1 cells at the G2/M-phase of the cell cycle. Wound healing and transwell assays showed pectolinarigenin suppressed the migration and invasive potential of the SK-HEP-1 cells.*

Conclusions: *The present study revealed that pectolinarigenin exhibits antitumor activity in SK-HEP-1 liver cancer cells via multiple mechanisms and may prove promising in the development of systemic therapy for liver cancer.*

Key words: *pectolinarigenin, liver cancer, autophagy, cell migration, cell invasion*

Introduction

Hepatocellular carcinoma (HCC), a leading cause of cancer mortality across the globe, is a common lethal liver malignancy. HCC ranks as 9th major cause of cancer-related mortality in United States [1]. Approximately 21,670 deaths and around 30,640 new hepatocellular and intrahepatic bile duct carcinoma cases were registered alone in 2013 [2]. HCC demonstrated variations based on gender and geographically as well. A ratio of 2.4:1 males to females reveals that males are more vulnerable to HCC than females, with higher risk among the people of Middle and Western Africa, Eastern and

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Southern Asia, Polynesia/Micronesia and Melanesia [3]. Alcohol is one of the main causes of hepatocellular carcinoma; in Europe alone 40-50% of all HCC cases are due to alcohol abuse [4,5]. HCC becomes more lethal by its late diagnosis; if diagnosed early then the most effective treatment is surgical resection. Even after resection of tumor, the recurrence rate of HCC is very high (50% at 2 years) [6]. The best part of the patients are already at advanced stages when diagnosed of HCC and in that cases primary treatment starts with pharmacotherapy. However, due to the increase of drug resistance, the long-term efficacy of chemotherapy is hampered [7]. There are obstacles in the treatment of HCC due to lack of effective chemotherapeutic drugs without side effects [8]. Therefore, to improve HCC outcomes there is an urgent need to develop new drugs. Plants possess a significant potential to produce a vast range of chemical entities for their own needs and are extremely sophisticated natural chemical factories [9].

The current study was undertaken to observe the anticancer effects of Pectolinarigenin flavonoid against human HCC. Pectolinarigenin was extracted from a Chinese herbal plant *Chromolaena odorata*. This molecule shows diverse range of bioactivities like anti-inflammatory, anti-allergic, antitumor, and apoptosis [10-13]. Attention was drawn towards Pectolinarigenin, being a potential molecule to curb different human malignancies. In the current study, we used different assays to evaluate selective anti-proliferative activity in cisplatin-resistant HCC by autophagy activation, inhibiting cell migration and invasion, G2/M phase cell cycle arrest and targeting ERK1/2 MAP pathway. Data from different assays was collected and then the effect of Pectolinarigenin on HCC was evaluated.

Methods

Cell proliferation assay

MTT assay was used to determine the proliferation rate of human HCC SK-HEP-1 and normal liver cells AML-12. Briefly, 96-well microtiter plates were used to seed SK-HEP1 (HCC cells) and AML12 (normal liver cells) at 1×110 [???] cells/mL with fetal bovine serum (FBS) in Minimum Essential Medium. After incubation overnight for attachment, Pectolinarigenin was added in serial three-fold dilutions in triplicates. Further, cells were incubated for 48h at 37°C and 5% CO^2. Later, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT) was added to the culture (5 mg/mL) and left for 4h. Thereafter, all of the medium including MTT solution was discarded. Dimethylsulfoxide (DMSO) was used to dissolve the remaining formazan crystals and hence absorbance was measured with a microplate reader (SynergyTM HT, Bio-Tek Instruments, Inc.) at 570nm to determine proliferation.

Electron microscopy

Electron microscopy was used to assess that Pectolinarigenin-induced autophagy in human HCC cells. Briefly, SK-HEP1 cells were treated for 24h with 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM of Pectolinarigenin. Collection of the cells were done with trypsinization and later they were washed with PBS. Thereafter, fixation was done in glutaraldehyde (2%) in phosphate buffer (0.1 M). Post fixation was done with 1.5% osmium tetroxide. After post fixation the cells were treated with ethanol embedded in resin. Then, ultramicrotome was used to cut thin sections and then the cells were subjected to electron microscopy.

Cell cycle analysis

The SK-HEP1 HCC cells at a density of 1×10^5 cells/ml were cultured in DMEM medium. SK-HEP1 cells were treated with different concentrations (0, 5, 10, and 20 μM) of Pectolinarigenin, and then harvested using trypsinization. Prior to the treatment with 15 μg/ml of RNase A, 70% ethanol was used for fixation. Later, cells were washed and stained. Phosphate buffered saline (PBS) was used for washing, and after washing, Annexin V/PI (20 μg/mL) staining was performed. FACS Calibur flow cytometry (FACS Calibur; BD Biosciences) was employed in the determination of different phases of cell cycle of SK-HEP1 HCC cells.

Cell invasion assay

Cell invasion ability of SK-HEP1 HCC cells after treatment with Pectolinarigenin was evaluated by transwell chambers with Matrigel. Onto the bottom wells only medium was placed and upper chambers were filled with cell cultures (around 200 ml). Prior to the fixation with methyl alcohol, cells were incubated for 24h and then stained with crystal violet. Using inverted microscope the invaded cells were counted at 200x magnification.

Wound-healing assay

SK-HEP1 HCC cells were treated with Pectolinarigenin (10 μM). After that, cells were removed from the medium and washed with PBS. A wound in each well was made by scratching with a sterile pipette tip and cells were washed again. Before cells were subjected to culturing for 24 h a picture was captured and after culturing, again a picture was captured with an inverted microscope (Leica, Germany).

Western blotting

Following the lysis of the SK-HEP1 HCC cells in RIPA lysis buffer, the protein content of the each lysate was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 h. Then, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

Statistics

Data are shown as mean ± SD. Statistical analyses were done using Students t-test with GraphPad prism 7 software. P<0.05 was taken as indicative of significant difference.

Results

Selective inhibition of liver cancer cells by Pectolinarigenin

The MTT assay was used for determining the cytotoxic effects of Pectolinarigenin (Figure 1) against the proliferation of the SK-HEP1 liver cancer and AML12 normal liver cells. The molecule resulted in significant reduction in the rate of cell proliferation of SK-HEP1 cells selectively. The inhibitory effect of Pectolinarigenin on SK-HEP1 cells was dose-dependent and the IC_{50} was 10 µM against the SK-HEP1 cells (Figure 1). Interestingly, lower cytotoxic effect was observed in normal AML12 cells, and the IC_{50} was 75 µM against normal liver cells.

Pectolinarigenin induced autophagy in SK-HEP1 HCC cells

Subsequently, Pectolinarigenin-treated SK-HEP1 HCC cells were analysed under an electron microscope. Formation of autophagosomes or autophagic vesicles was observed in the Pectolinarigenin-treated SK-HEP1 cells which marked the confirmation of autophagy (Figure 2). An increase in the protein expression levels of Beclin-1, LC3-II and LC3-I (Figure 3) was observed after Pectolinarigenin treatment which also confirmed autophagy. The expression levels of p62 were shown to decrease with increase in the dose of Pectolinarigenin.

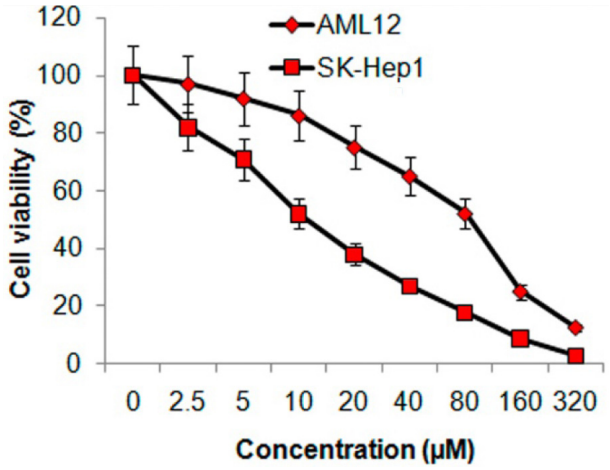

Figure 1. Effect of Pectolinarigenin flavonoid on the viability of SK-HEP1 HCC cells and normal AML-12 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Pectolinarigenin induced G2/M phase cell cycle arrest

The SK-HEP1 HCC cells were assessed with different concentrations of Pectolinarigenin and their distribution at each phase of the cell cycle was evaluated using flow cytometry. Remarkably, on treatment with Pectolinarigenin there was an increase in G2/M phase cells. The percentage of G2/M HCC cells were 5.11, 8.41, 18.02 and 27.53%

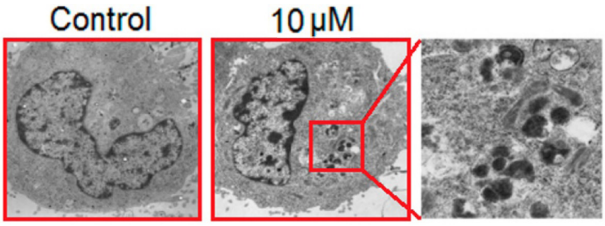

Figure 2. TEM analysis showing induction of autophagy in SK-HEP1 liver cancer cells at indicated concentrations of Pectolinarigenin. The experiments were performed in triplicate.

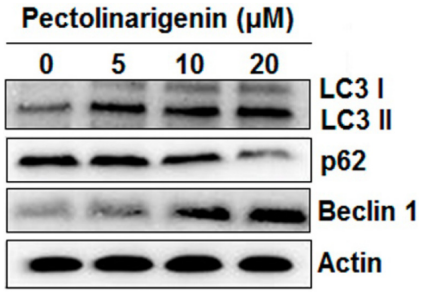

Figure 3. Effect of Pectolinarigenin on the autophagy-related proteins as depicted by western blot analysis. The Figure shows that the expression of Beclin1 and LC3II increases and that of p62 decreases upon Pectolinarigenin treatment. The experiments were performed in triplicate.

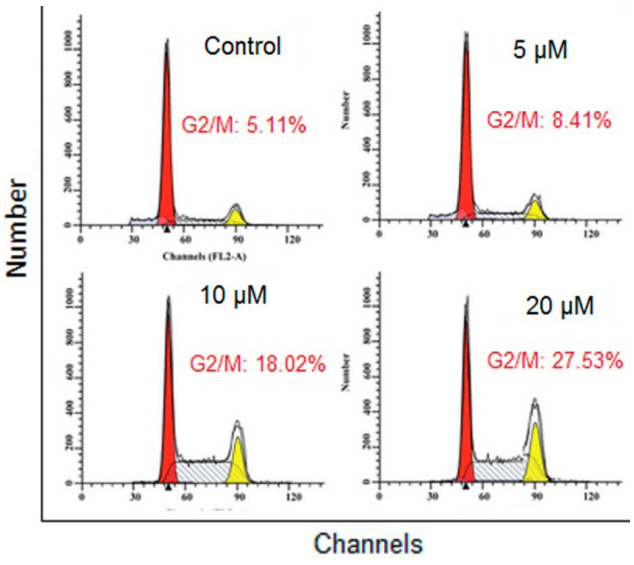

Figure 4. Flow cytometry showing that Pectolinarigenin induces dose-dependent G2/M cell cycle arrest in SK-HEP1 liver cancer cells. The experiments were performed in triplicate.

at 0, 5, 10 and 20 μM concentrations of Pectolinarigenin, respectively. Therefore, the above results are indicative of G2/M arrest of the SK-HEP1 cells (Figure 4). To evaluate the effects on the cell cycle-related proteins by Pectolinarigenin, western blot analysis was performed which showed that the molecule was responsible for downregulation of cyclin B1 (Figure 5).

Pectolinarigenin induced inhibition of cell migration and invasion in SK-HEP1 HCC cells

Next, to examine the effects of Pectolinarigenin on cell migration and invasion, we performed wound healing and transwell assay, respectively. The results showed that the molecule led to significant reduction of migration of SK-HEP1 liver carcinoma cells at a concentration of 10 μM (Figure 6). Pectolinarigenin treatment also resulted in dose-dependent inhibition of cell invasion in these cells (Figure 7). These two assays provide a hint whether the tested molecule could be utilised in inhibiting cancer metastases to other tissues.

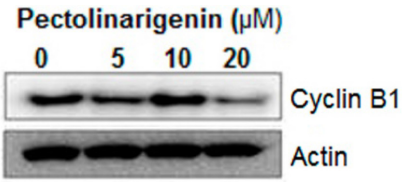

Figure 5. Effect of Pectolinarigenin on cyclin B1 as depicted by western blot analysis. The Figure shows that Pectolinarigenin inhibits the expression of cyclin B1 dose-dependently. The experiments were performed in triplicate.

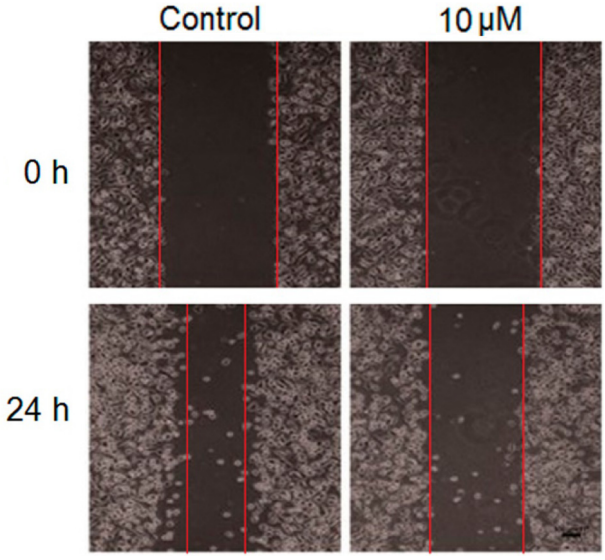

Figure 6. Effect of Pectolinarigenin on the migration of the SK-HEP1 liver cancer cells as depicted by wound healing assay. The Figure shows that Pectolinarigenin suppresses the migration of SK-HEP1 cells dose-dependently. The experiments were performed in triplicate.

Blocking of ERK1/2/MAPK pathway by Pectolinarigenin

Pectolinarigenin-treated human SK-HEP1 carcinoma cells were investigated to reveal the effects on ERK1/2 /MAPK signalling pathway at 0, 5, 10 and 20 μM doses. The results showed that Pectolinarigenin caused inhibition in ERK1/2 phosphorylation dose-dependently. However, the overall ERK1/2 expression remained almost constant (Figure 8). The results also revealed that the phosphorylation of MAPK p38 decreased while the expression of MAPK p38 remained almost constant.

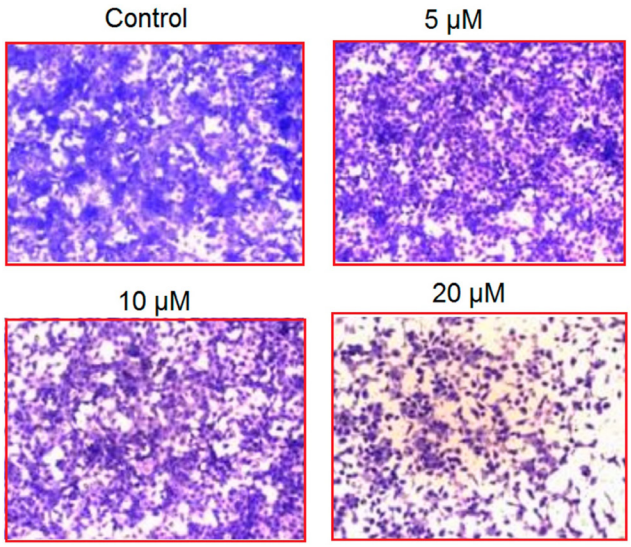

Figure 7. Effect of Pectolinarigenin on the invasion of the SK-HEP1 liver cancer cells as shown by transwell assay. The Figure shows that Pectolinarigenin led to dose-dependent inhibition of cell migration. The experiments were performed in triplicate.

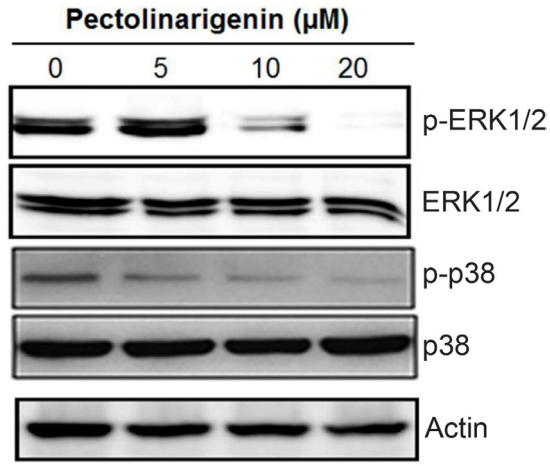

Figure 8. Western blot analysis showing the effect of Pectolinarigenin on the ERK1/2/MAPK signalling pathway in SK-HEP1 liver cancer cells. The Figure shows that Pectolinarigenin inhibits the phosphorylation of p-ERK 1/2 and p-38 dose-dependently. The experiments were performed in triplicate.

Discussion

Despite recent advancements made concerning HCC survival, this disease remains a major cause of mortality across the globe. In the past 20 years there is an increase of 80% cases of HCC in United States was registered [14]. Liver cancer still remains prevalent and at highest rates in Asia and Africa [15]. HCC accounts for 90% of primary liver cancers in comparison to other liver cancer types [16,17]. There is an improvement in the prognosis and 5-year survival of HCC, yet, due to molecular and phenotypic heterogeneity, the systemic treatment is limited [18,19]. Herein, the investigation regarding the anticancer effects of Pectolinarigenin on HCC revealed that the proliferation rate of human HCC cells was supressed by this molecule in a dose-dependent manner, while, on the contrary, the effect of the drug against normal liver cells was significantly lower. This indicated that Pectolinarigenin specifically targeted the human SK-HEP1 HCC cells. A study has reported that Pectolinarigenin inhibited colony formation and cell proliferation of human C666-1 nasopharyngeal carcinoma cells [20]. Due to the promising results revealed by the cell proliferation assay, we tried to disclose the molecular mechanisms accountable for the anticancer effects of Pectolinarigenin. It was shown by electron microscopic analysis that Pectolinarigenin administration resulted in inhibition of SK-HEP1 cells through autophagy induction and also upregulation of Beclin-1 and LC3B II and LC3B I expressions. Previous investigations [10-13,20] regarding plant molecules resulting in initiating autophagy in cancer cells were confirmed by this observation. Next, it was revealed by cell cycle analysis that Pectolinarigenin resulted in cell cycle arrest in human liver carcinoma cells at G2/M phase which was evaluated by decrease in cyclin B1. This molecule also revealed its antimetastatic potential on human SK-HEP1 carcinoma cells by showing inhibition of cell invasion and migration of HCC cells, further revealing its potential as an anticancer drug. ERK1/2 MAPK signal transduction pathway has shown therapeutic implications in curbing liver cancer [21] and herein Pectolinarigenin showed to block this pathway as well.

Conclusion

In conclusion, the results of the current study indicated that Pectolinarigenin flavonoid exhibits selective antiproliferative activity by targeting different caspases in cisplatin-resistant hepatocellular carcinoma cells. Pectolinarigenin resulted in autophagy activation, inhibition of cell migration and invasion, G2/M phase cell cycle arrest and targeting ERK1/2 MAP pathway which revealed its potential against cancer cells. All these characteristics create a place of Pectolinarigenin to become a lead molecule in liver cancer treatment.

Conflict of interests

The authors declare no conflict of interests.

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