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Pseurotin A Halts Hepatocellular Carcinoma Oncogenic Potential Through Tuning miR-30a and let-7i Tumor Suppressor miRNAs

Mai E. Hussein[a]*, Amira S. El Senousy[a], Hesham I. El-Askary[a], Ahlam M. El Fishawy[a], Ahmed A. Hamed[b] and Rana A. Youness [c]

[a] *Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Kasr el Aini St., 11562 Cairo, Egypt*

[b] *Microbial Chemistry Department, National Research Centre, 33 El-Buhouth Street, Dokki, 12622 Giza, Egypt*

[c] *Biology and Biochemistry Department, School of Life and Medical Sciences, University of Hertfordshire Hosted By Global Academic Foundation, New Administrative Capital, 11865, Cairo, Egypt*

Abstract

Hepatocellular carcinoma (HCC) is a global health challenge with a predicted high mortality rate in the future. Thus, discovering novel natural metabolites trimming the progression of HCC is an urgent necessity. In this study, we aimed to evaluate the effect of cytotoxic alkaloids isolated from endophytic fungus *Aspergillus fumigatus,* extracted from *Albizia lucidior* leaves (Fabaceae), against Huh7 cells and to further reveal their possible mechanisms on TP53 and c-Myc and their downstream tumor suppressor microRNAs (miRNAs). Pseurotin A and chaetominine revealed a concentration and time-dependent inhibition in cellular viability of Huh7 cells as well as significant repression in the proliferation and clonogenic capacities of Huh7 cells, compared to vehicle control. Pseurotin A exhibited more potent effects in halting Huh7 tumorigenic activity than chaetominine. Restoration of TP53, c-Myc, and their downstream tumor suppressor miRNAs; miR-30a, let-7i was achieved by pseurotin A only. Therefore, this study highlights a novel role of alkaloidal compounds from *A. fumigatus* in controlling the progression of HCC.

Keywords: Aspergillus fumigatus; Albizia lucidior; Pseurotin A; Huh7; TP53; miRNAs

1. Introduction

Hepatocellular carcinoma (HCC) is considered one of the most violent malignancies worldwide, being the sixth most common cancer worldwide. The highest incidence and mortality of HCC are noticed in East Asia and Africa [1] with Egypt ranking as the 2nd most populous country worldwide for HCC in 2020 [2]. One of the main obstacles for HCC eradication is the diversity of risk factors and their direct association with HCC development such as hepatitis virus, harmful chemical compounds, alcohol abuse, and genetic factors [1, 3] as well as the lack of molecular targeted therapies.

The molecular pathogenesis of HCC varies according to the distinct genotoxic aetiologies. Genetic alterations in tumor suppressor genes such as TP53, PTEN, RB1 as well as amplification of oncogenic genes such as FGF19, VEGFA, MYC have been highly implicated in HCC, leading to activation of different oncogenic signaling pathways [1]. Tp53, tumor suppressor gene, and c-Myc, oncogenic protein, are vital transcriptional regulators [4, 5] and important influential factors in tuning the malignancy of mutant cells *via* several mechanisms, one of them is modulating microRNAs (miRNAs) expression [6-10]. microRNAs (miRNAs) are small non-coding RNAs (~23 nucleotides) that act as post-transcriptional regulator for respective target mRNAs *via* translational inhibition and mRNA cleavage [11].

*Corresponding author e-mail: mai.husein@pharma.cu.edu.eg; Mai Emad Eldin Hussein
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Xu and his team illustrated the role of miRNAs in the development of HCC and their potential role in several oncogenic hallmarks [12]. In light of this, our research group was interested in discovering natural compounds that could be utilized as cytotoxic drugs against HCC through modulating miRNAs [13].

Based on previous reports, *Aspergillus fumigatus* (*A. fumigatus*) seems to be a valuable source of cytotoxic metabolites [14, 15], consequently we successfully isolated the cytotoxic metabolites; pseurotin A and chaetominine (Fig. 1) from endophytic fungus (*A. fumigatus*), extracted from *Albizia lucidior* (Steud.) I.C.Nielsen leaves (Family Fabaceae) [16]. Pseurotin A exhibited cytotoxic effects against several cancer cell lines such as hepatic carcinoma cells; BEL-7402 [17] and HepG2 cells [18], breast cancer cells; MCF-7 [19] , BT-474 and T47D cells [20] beside other cancer cell lines [21, 22]. In addition, the cytotoxic effect of chaetominine against human leukemia K562 and colon cancer SW1116 cell lines was previously proved [23-26]. However, the mechanistic actions of pseurotin A and chaetominine against miRNAs and transcription factors haven't been explored yet despite the importance of these molecular targets.

Herein we paid more attention to miR-30a and let-7i due to their importance in controlling HCC. miR-30a inhibits the metastasis of HCC *via* various pathways such as modulating other important target genes as CREB1, PAWR, NEDD4, etc [27, 28], controlling the autophagy process by inhibition of pro-autophagic protein Beclin 1 and gene Atg5, consequently mediated anoikis inhibition of HCC cells [29, 30], inducing caspase-3/7 activity and reducing AEG-1 protein level [31], blocking K-Ras/c-Raf/MEK/ERK [32], regulating the expression of REEP3 [33] and targeting MTDH/PTEN/AKT pathways [34].

Besides, the role of miR-30a in inhibition of epithelial–mesenchymal transition by targeting SNAI1 [35] and regulation vimentin, MMP3 expression and E-cadherin expression [36] was illustrated. On the other hand, let-7i controls the progression of HCC *via* abrogating the interplay between IGF2BP1, 2 and 3 and IGF1R [37] in addition to reducing RAF1 [38] and anti-apoptotic protein, Bcl-xL, levels [39]. Therefore, targeting these molecular targets may have therapeutic revenues for the treatment of HCC. Consequently, the aim of this study was to depict the cytotoxic activity of ethyl acetate (EtOAc) extract of *A. fumigatus* and its isolated compounds against Huh7 cells as well as unveil the impact of the isolated compounds on TP53 and c-Myc and their downstream tumor suppressor miRNAs; miR-30a and let-7i.

2. Experimental

2.1. Isolation of natural compounds

Pseurotin A and chaetominine were isolated from ethyl acetate extract of endophytic fungus, *Aspergillus fumigatus*, extracted from healthy leaves of *Albizia lucidior* (Fabaceae) by our research group [16]. The structures were confirmed by ^{1}H-NMR, ^{13}C-NMR and electrospray ionization mass spectrometry (ESI-MS).

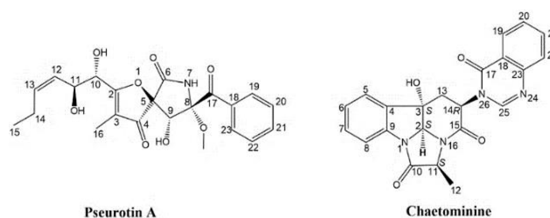

Fig. 1. Isolated alkaloidal metabolites from endophytic fungus *A. fumigatus.*

2.2. Cell culture and treatment

The current study employed human hepatic cancer cells (Huh7 and HepG2) and human breast cancer cells (MDA-MB-231 and MCF-7) that were purchased from American Type Culture Collection (Manassas, VA, USA) and Vacsera, Egypt. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 4.5 g/L glucose, 4 mmol/L L-glutamine, 10% fetal bovine serum (FBS) and MycoZap (1:500; Lonza) at 37°C in 5% CO_2 atmosphere, The cells were passaged upon reaching 70–80% confluency as previously described in [40]. Stock solutions of the respective tested samples were prepared in 0.2% DMSO in culture media. Serial concentrations of EtOAc extract (from 10 μg/ml to 100 μg/ml) and respective compounds (from 20 μM to 100 μM) were prepared and used to treat different cell lines, each cell line was seeded in either 96-well or 24-well plates based on the experimental setup. Cells treated with 10 μM of 5-fluorouracil (equivalent to 1.3 μg/ml) were used as positive control, while the cells that were exposed to 0.2% DMSO were used as vehicle control as previously described in [13].

2.3. MTT assay

Cellular viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described in [41],

all experiments were done in triplicates and repeated at least 3 times. IC_{50} values of the extract and its respective compounds were calculated from linear regression analysis.

2.4. Cellular proliferation

For the cellular proliferation assessment, bromodeoxyuridine (BrdU) incorporation assay was employed [13]. Different cancer cells were seeded into black 96-well plates with an initial cell count of 5×10^4 cells/well. Post-treatment, cells were labeled with BrdU labeling reagent for 4 hr using the Cell Proliferation ELISA Kit (Roche Applied Science, Penzberg, Germany). The cells were then exposed to Fix-Denate for 30 min for fixation and then incubated with Anti-BrdU POD for 90 min. All experiments were performed in triplicates and repeated at least three times.

2.5. Colony-forming assay

The assay was performed as illustrated in [41]. Post-treatment with the tested extract and its respective compounds, cells were trypsinized, suspended, counted, and seeded in six-well plate with an initial count of 2,000 cells/well. Cells were kept to colonize in full DMEM under normal conditions (37°C and 5% CO_2) for 14 days. Colonies were fixed using 6% glutaraldehyde and stained using 0.3% crystal violet and then manual counting was performed. All experiments were done in triplicates and repeated at least three times.

2.6. Total RNA and miRNA extraction

Total RNA and miRNAs were extracted from Huh7 cell line using Biozol Reagent after treatment with compounds. Extracted RNA was then quantified spectrophotometrically. RNA purity was examined by 18s rRNA bands detection on 1% agarose gel electrophoresis. RNA samples were assessed at two wavelengths (260/280 nm), optical density more than two were excluded [13].

2.7. Quantitative real-time PCR analysis

c-Myc and TP53 were reverse-transcribed into complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription Kit (ABI, Foster City, CA) according to the manufacturer's instruction. However, the extracted miRNAs were reverse transcribed into single stranded cDNA using specific primers for hsa-miR-30a-5p and let-7i and TaqMan_MicroRNA Reverse Transcription Kit (ABI). Relative expression of c-Myc and TP53 as well as miR-30a-5p and let-7i was quantified using TaqMan Real-Time q-PCR (measured on StepOne™ Systems (ABI). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All PCR reactions were run in triplicates and repeated at least three times [42].

2.8. Lactate dehydrogenase (LDH) assay

Peripheral blood mononuclear cells (PBMCs) were extracted from peripheral blood of healthy donors upon their written informed consent using Ficoll–Hypaque centrifugation (Axis-Shield PoC AS, Norway) as previously described [43]. Lactate dehydrogenase (LDH) assay was performed using lactate dehydrogenase (LDH) activity assay kit (MAK066-1K1-Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instruction and according to [44] . The experiment was done in triplicate and repeated at least three times.

2.9. Statistical analysis

Non-parametric unpaired student-t-test was performed to compare between every two independent groups, p-value less than 0.05 was considered statistically significant. Data were presented as mean ± S.E.. All the data were statistically analyzed using GraphPad Prism 9.00 software.

3. Results and Discussion

3.1. Cytotoxic effects of *A. fumigatus* EtOAc extract against different cell lines

The fungus, *Aspergillus fumigatus*, is an endophyte associated with the healthy leaves of *Albizia lucidior*. The crude EtOAc extract of the fungus was subjected to repeated chromatographic isolation, yielding the cytotoxic compounds; pseurotin A and chaetominine (44 mg and 38mg, respectively) [16].

The EtOAc extract were screened for its cytotoxic activity in four cancer cell lines viz. HepG2, Huh7, MDA-MB-231 and MCF-7. Upon treating different cell lines with increasing concentrations of the EtOAc extract (10-100 µg/ml), treated cells revealed a significant reduction in cellular viability compared to vehicle control with IC_{50} values ranging from 49.92 to 93.50 µg/ml. The extract revealed the most potency against Huh7 based on experimental results (Table 1 and Fig. 2).

Table 1. IC_{50} of *A. fumigatus* EtOAc extract against four cell lines

Cell line	IC_{50} of EtOAc extract (µg/ml)
HepG2	93.41 ± 1.54
Huh7	49.92 ± 2.66
MDA-MB-231	93.50 ± 1.66
MCF-7	54.16 ± 3.65

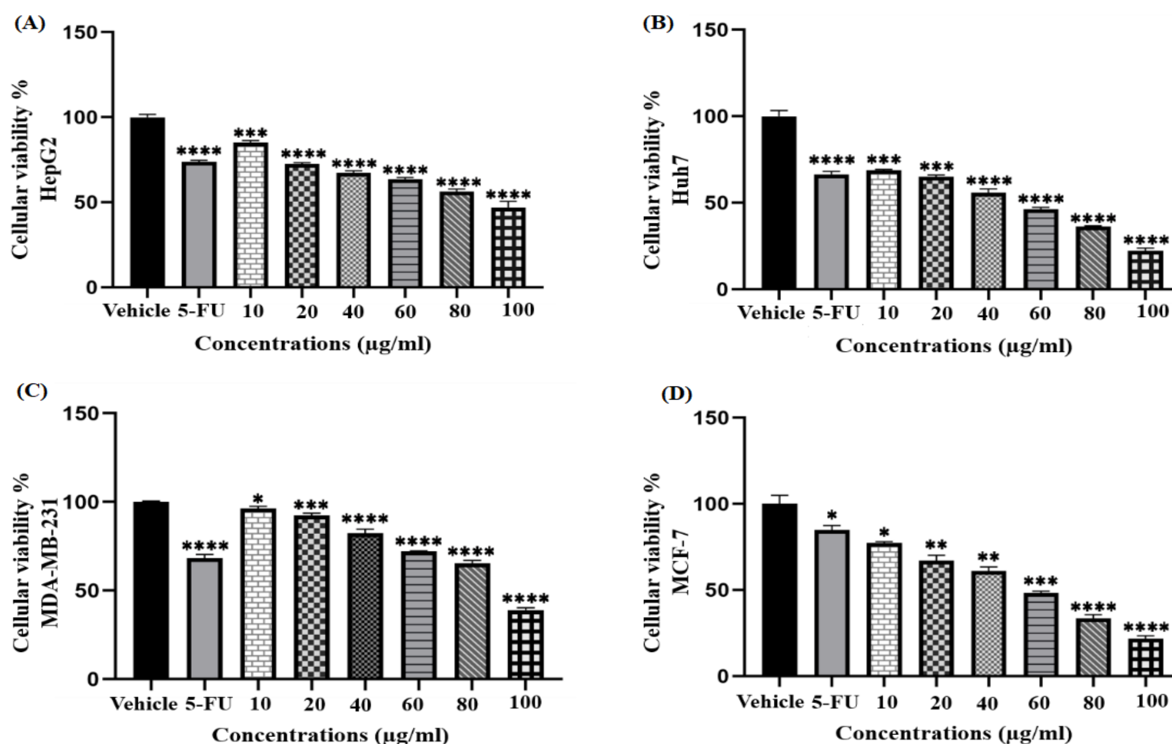

Fig. 2. Concentration dependent effect of *A. fumigatus* EtOAc extract on cellular viability of cell lines.
The % viability of different adherent cells (A) HepG2, (B) Huh7, (C) MDA-MB-231 and (D) MCF-7 were calculated relative to cells treated vehicle control. Data are expressed as M ± S.E., **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, compared to the vehicle-treated control group. The positive control cells were treated with 10 µM of 5-flurouracil (equivalent to 1.3 µg/ml). Student's unpaired T-test was performed to compare each two groups.

3.2. Cytotoxic effect of pseurotin A and chaetominine on Huh7 cells

Although there are several approved drugs for treatment of advanced stages of HCC patients, the interest in the use of bioactive metabolites from fungi as possible chemotherapeutic agents against HCC has become a promising strategy [18, 45]. In such context, pseurotin A and chaetominine were evaluated against the most sensitive cells to their mother extract; Huh7 cells. Significant inhibition of cellular viability of Huh7 cells after treatment with the isolated compounds was observed, showing a time and concentration-dependent effect (Fig. 3). Based on IC_{50} results, pseurotin A was more potent than chaetominine (Table 2). These findings go in line with previous reports highlighting the proliferation inhibitory effects of pseurotin A against human hepatic carcinoma cells; BEL-7402 [17] and HepG2 cells [18] in addition to its anti-hepatocarcinogenic effect in diethylnitrosamine and carbon tetrachloride-induced hepatocellular carcinoma in rats [18]. On the other hand, our study represents the first to unravel the cytotoxic activity of chaetominine against HCC cell lines.

Table 2. IC_{50} of pseurotin A and chaetominine against Huh7 cells

Compounds	IC_{50} (µM)	
	24 hr	**72hr**
Pseurotin A	146.98 ± 1.68	< 20
Chaetominine	183.55 ± 2.14	< 20

3.3. Impact of pseurotin A and chaetominine on proliferation rate and colony forming ability of Huh7 cells

Tumor invasion and metastasis are considered as major causes of clinical treatment failure and mortality. Thus, the evaluation of the efficacy of isolated compounds towards proliferation and clonogenic capacities of Huh7 cells is an important step in treatment of liver cancer. Significant reduction of the cellular proliferation rate (Fig. 4) and clonogenic capacity (Fig. 5) were shown after treating Huh7 cells with the isolated compounds in a concentration dependent manner, compared to vehicle control. It was observed that pseurotin A possessed more inhibitory effect than chaetominine.

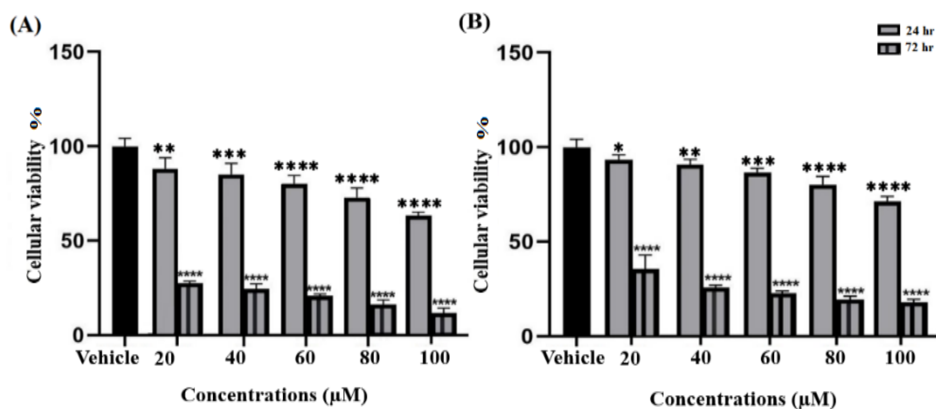

Fig. 3. Time dependent and concentration dependent effect of (A) pseurotin A and (B) chaetominine on cell viability of Huh7 cells.

Compared to the vehicle control, Huh7 treated with (A) pseurotin A showed a dose dependent decrease in cellular viability after 24 hr of treatment at concentrations of 20 μM ($p < 0.01$), 40 μM ($p < 0.001$) and (60-100 μM) ($p < 0.0001$), while (B) chaetominine showed a less significant decrease in Huh7 cellular viability after 24 hr of treatment at concentrations of 20 μM ($p < 0.05$), 40 μM with ($p < 0.01$), 60 μM with ($p < 0.001$) and 80-100 μM ($p < 0.0001$). Both compounds revealed significant effect $p < 0.0001$ after 72 hr of treatment. Student's unpaired T-test was performed to compare each two groups

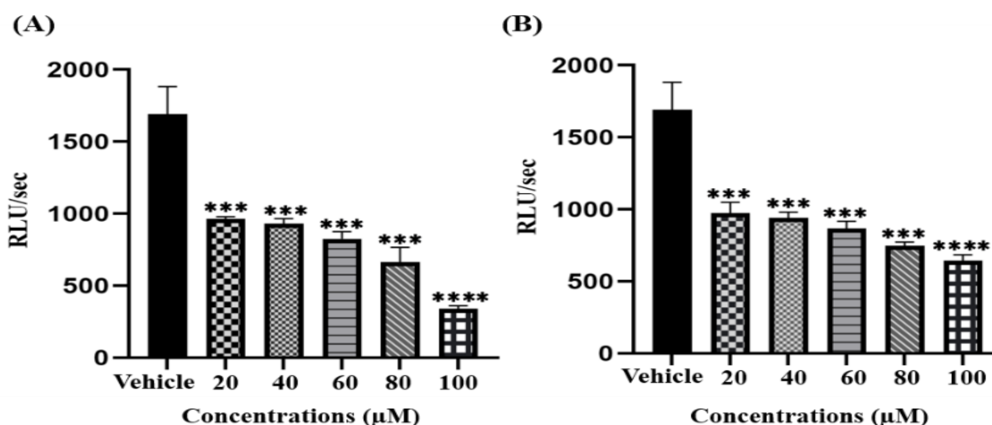

Fig. 4. Impact of isolated compounds on cellular proliferation rate of Huh7 cells

Treatment with (A) pseurotin A and (B) chaetominine resulted in a significant reduction of Huh7 cellular proliferation rate, compared to vehicle control in a dose dependent manner, (20-80 μM, $p < 0.001$), and (100 μM, $p < 0.0001$), Student's unpaired T-test was performed to compare each two groups.

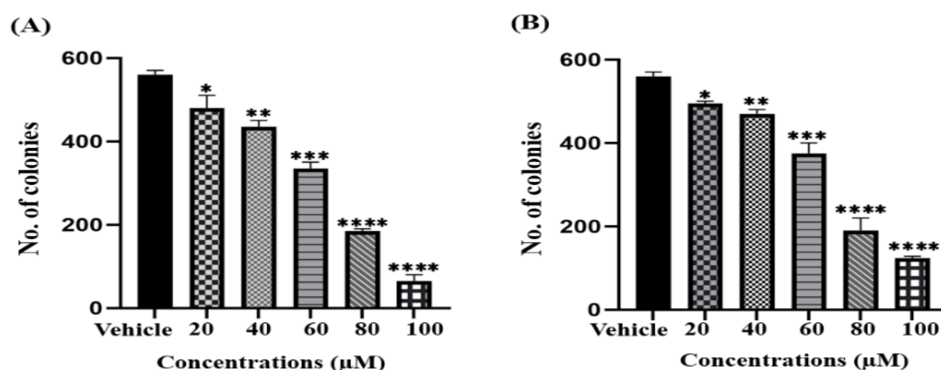

Fig. 5. Impact of isolated compounds on clonogenic properties of Huh7 cells.

Huh7 colony forming ability was assessed after treatment with serial concentrations of (A) pseurotin A and (B) chaetominine. Compared to the vehicle control, Huh7 treated with (A) pseurotin A and (B) chaetominine noticeably decreased colony forming ability of Huh7 cells (20 μM, $p < 0.05$), (40 μM, $p < 0.01$), (60 μM, $p < 0.001$) and (80-100 μM, $p < 0.0001$). Student's unpaired T-test was performed to compare each two groups.

3.4. Impact of pseurotin A and chaetominine on transcription factors, TP53 and c-Myc, and their downstream miRNAs

TP53 and c-Myc are vital players in HCC development and progression. Upon screening literature, it was revealed that the impact of isolated compounds on TP53 and c-Myc transcription factors was not tested before. Thus, we were inspired to dig deeper to probe the molecular mechanism of these compounds in halting Huh7 hallmarks. Accordingly, pseurotin A successfully resulted in a marked induction of TP53 expression level (20 µM concentration showed > 5 folds increase, compared to vehicle control) and a significant inhibition in c-Myc expression levels in Huh7 cells. While, chaetominine showed non-significant effect on TP53 levels and less significant inhibitory effect on c-Myc than pseurotin A (Fig. 6).

Based on the recent crosstalk between transcription factors, TP53 and c-Myc, and miRNAs, we further plotted the impact of the isolated compounds on tumor suppressor miRNAs that are drawn downstream TP53 and c-Myc. Surprisingly, only pseurotin A revealed significant increase in the levels of let-7i and miR-30a (20 µM concentration displayed ~ 8 and~ 4 folds increase, respectively, compared to vehicle control) while chaetominine showed non-significant effect. The induction in TP53 levels upon treatment with pseurotin A together with the subsequent increase in expression levels of miR-30a and let-7i strongly alludes to the dominant role of TP53 in modulating miRNAs in Huh7 cells (Fig. 6). It is worthy to note that both compounds had nearly similar cytotoxic activities against Huh7 cells, illustrated *via* their near inhibitory effects on cellular viability, cellular proliferation rate and colony forming ability. Based on our detailed molecular study, pseurotin A exerted its anticancer activity against Huh7 cells *via* modulating TP53 and c-Myc expression levels and subsequently increasing the levels of miR-30a and let-7i axis. While, chaetominine might exhibit its cytotoxic effect by other molecular mechanism not illustrated yet. Chaetominine had different molecular mechanisms in halting of human leukemia and colon cancer cells [23-26], chaetominine affected the human leukemia cells *via* regulation the Bax/Bcl-2 ratio and activation of caspase-9 and caspase-3 as well as modulation of the expression of ATR/Chk1/cdc25A. On the other hand, chaetominine inhibited PI3K/Akt/Nrf2 signaling pathway in treatment of K562/Adr human leukemia cells. Furthermore, chaetominine involved in the regulation of p53/p21 and ATM and Rad3-related (ATM)/Chk2 signaling in colon cancer cells. Thus, it might be one of these mechanisms involved in halting of Huh7 cells. Yet, further investigations are in need to evaluate the exact mechanism of action.

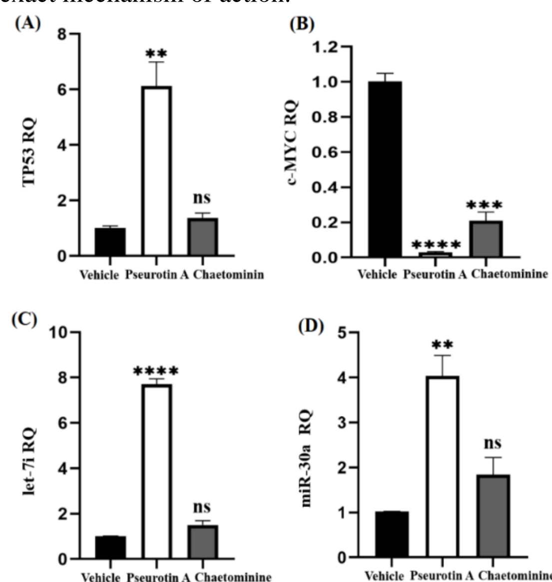

Fig. 6. Impact of isolated compounds on transcription factors, TP53 and c-Myc, and their downstream miRNAs.

Compared to vehicle control cells, Huh7 cells treated with 20 µM of pseurotin A resulted in a significant increase in (A) TP53 ($p < 0.01$) (C) let-7i ($p < 0.0001$) and (D) miR-30a ($p < 0.01$) expression levels while a significant decrease in (B) c-Myc ($p < 0.0001$) level. On the other hand, chaetominine showed no significant effect on TP53, miR-30a, let-7i levels and less effect on c-Myc ($p < 0.001$) than pseurotin A.

3.5. Cytotoxic effect of pseurotin A and chaetominine on primary peripheral blood mononuclear cells

The emergence of discovering selective cytotoxic drugs become an urgent require. Therefore, the selectivity of both compounds was tested, by LDH assay, against healthy primary peripheral blood mononuclear cells (PBMC), revealing the safety of both against normal cells (Fig. 7). This result goes in parallel with studies revealing that pseurotin A was non cytotoxic against Vero cells [46] and PBMCs were not affected at the highest concentration of chaetominine [24]. Several trials were devoted to develop selective cytotoxic agents to overcome the harmful revenues of chemotherapy on normal cells. Nevertheless, few approval drugs showed selectivity to tumor cells. Thus, finding cytotoxic drugs with high selectivity is considered an important therapeutic tool.

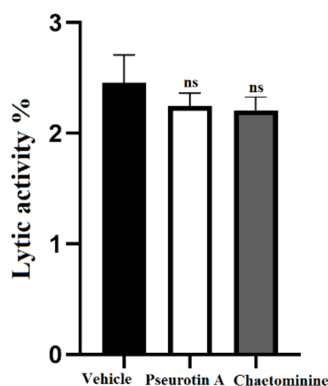

Fig. 7. Impact of isolated compounds on primary peripheral blood mononuclear cells (PBMCs). Pseurotin A and chaetominine showed no toxicity on normal PBMCs that was evaluated by LDH assay.

4. Conclusion

This study represents the first evidence on the promising role of alkaloidal metabolites, isolated from *A. fumigatus*, as therapeutic agents for halting the progression of Huh7 cells. In this study, a novel molecular mechanism behind pseurotin A was drawn. Pseurotin A was found to modulate the tumor suppressor TP53 and the oncogene c-Myc in Huh7 cells and accordingly their downstream miRNAs: miR-30a and let-7i were found to be involved. On the other hand, the molecular mechanism of chaetominine remained unclear. It is worthy to note that the structural difference between the metabolites might be contributed mainly in the variation of their mechanisms. In addition, this study highlights on the selectivity of both metabolites against PBMC cells, while their selectivity against primary liver cells was not evaluated yet, thus further study is warranted.

5. Conflicts of interest

The authors declare that they have no conflict of interest.

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Graphical abstract