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Quercetin inhibits hepatocellular carcinoma progression via modulation of HOTAIR/miR-526b-3p/DHX33 axis

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Hepatocellular carcinoma (HCC) remains a highly aggressive malignancy with limited therapeutic options. The long non-coding RNA (lncRNA) HOTAIR contributes to HCC progression through the regulation of the miR-526b-3p/DHX33 axis. This study investigated the antitumor effects of quercetin (Que) on HCC using both Hep3B and HepG2 cell lines, focusing on the modulation of this molecular pathway. In this regard, the study evaluated Que's effects on HOTAIR, miR-526b-3p, and DHX33 expression using qRT-PCR and ELISA. Cell viability was assessed by MTT assay, while apoptosis was measured through Annexin V-FITC/PI staining and analysis of BCL-2/CASP-3 expression. Additional investigations included cell cycle analysis (cyclin A2 and D1), invasion assays (p53 and PTEN), and assessment of oxidative stress markers (SOD, CAT, MDA). Results demonstrated that Que significantly suppressed HOTAIR and DHX33 while upregulating miR-526b-3p in both cell lines (P-value<0.001). Treatment with Que caused dose-dependent apoptosis (54.3% in Hep3B and 48.9% in HepG2 at 100μM) and cell cycle arrest while reducing invasion through TP53 downregulation and PTEN upregulation. Que also ameliorated oxidative stress by enhancing antioxidant enzyme activity (P-value<0.01). These findings suggest Que may exert potent antitumor effects in HCC through modulation of the HOTAIR/miR-526b-3p/DHX33 axis, though further *in vivo* and clinical investigations are warranted.

Keywords: lncRNA, MicroRNA, Cancer, Phytotherapy, Cell cycle arrest

Hepatocellular carcinoma (HCC), stands out as the predominant form of primary liver cancer and holds the position of the world's second most lethal cancer in terms of mortality rates[1]. HCC manifests through various clinical symptoms and recent studies emphasize the role of non-coding RNAs (ncRNA) in disease progression[2,3]. While most ncRNAs do not encode proteins, they regulate protein synthesis indirectly, e.g., via ribosome biogenesis or miRNA sponging. Long non-coding RNAs (lncRNA), characterized by a length exceeding 200 nucleotides[4], play a pivotal role in determining cell fate and act as molecular sponges, sequestering microRNAs (miRNAs)[5]. HOX transcript antisense intergenic RNA (HOTAIR), a gene frequently expressed in various types of cancer, notably HCC, is crucial in advancing the spread and recurrence of the disease[6]. Its elevated levels are consistently tied to rapid disease progression and poor prognosis across various cancers[7], however, the precise function of HOTAIR in the progression of HCC and related mechanisms remains largely unknown. miR-526b has been linked to a worse prognosis for patients suffering from HCC[8]. DEAH-box helicase 33 (DHX33) belongs to the DEAH box family of ATP-dependent RNA helicases, which play a crucial role in RNA metabolism[9]. Indeed it is suggested that DHX33 levels are associated with the prognosis of HCC and its manipulation could contribute to disease management[10].

HCC therapeutic strategies are frequently associated with key challenges such as resistance to chemotherapy and undesired adverse effects that threaten the quality of life[11]. Quercetin (Que), a widely distributed flavonoid, boasts a range of health benefits. Extensive studies have explored its effects through both *in vitro* and *in vivo* experiments, with a particular focus on HCC[12]. Que represents a pivotal contribution to addressing factors that are involved in HCC progression, such as inflammation, migration, apoptosis, fibrosis, and angiogenesis[13]. Currently, genetic factors and intracellular pathways such as the HOTAIR/miR-526b-3p/DHX33 axis are assumed as major contributors to the occurrence and progression of HCC[7]. Despite the challenges in managing this malignancy, phytochemicals such as Que have shown promise in ongoing cell and animal studies. While

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Que′s antiproliferative and pro-apoptotic effects in HCC are well-documented, its regulatory role in the HOTAIR/miR-526b-3p/DHX33 axis remains unexplored. Given that HOTAIR promotes HCC progression via miR-526b-3p sponging and DHX33 upregulation, we hypothesized that Que exerts its antitumor effects by targeting this axis, offering a novel mechanistic insight into its therapeutic potential.

Materials and Methods

Reagents and chemicals

The Que, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Dimethylsulfoxide (DMSO) were procured from Sigma, St. Louis, Missouri. Additionally, essential reagents such as Fetal Bovine Serum (FBS), Dulbecco′s Modified Eagle Medium (DMEM), Penicillin-Streptomycin, and Amphotericin B were provided by Gibco-BRL, Grand Island, New York, USA. Furthermore, ELISA kits designed for the detection of DHX33 (Abbexa, UK,#catalog number: abx510401), BCL-2 (Abcam, #catalog number: ab272102), Cyclin D1 (Abcam, #catalog number: ab214571), Cyclin A2 (Abbexa, #catalog number: abx259510), CASP-3 (Abcam, #catalog number: ab220655), TP53 (MyBioSource, #catalog number: MBS175896), and PTEN (Abcam, #catalog number: ab206979) proteins were provided commercially.

Cell culture and treatment

The human HCC cell lines including Hep3B and HepG2, and the normal liver cell line THLE-2 were obtained from the American Type Culture Collection (ATCC). These cells were subsequently cultivated in 96-well plates at a concentration of 1.0 × 10^4 cells per well. The cultivation medium consisted of Dulbecco's Modified Eagle Medium (DMEM), enriched with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.1% amphotericin B. This setup was maintained under ideal conditions, specifically at 37°C and within a humidified atmosphere containing 5% carbon dioxide[14].

After a 24-h period of incubation, the cells were exposed to varying concentrations of Que prepared in ethanol (0.3%) solutions (0 μM, 25 μM, 50 μM, and 100 μM). This exposure lasted for an additional 24, 48, and 72 h. Following the treatment and removing the supernatant, the cells underwent trypsinization for harvesting. They were then stained with trypan blue, and the suspended cell count was meticulously assessed using a hemocytometer in triplicate to guarantee precision[14].

Cell viability assays

To evaluate cellular viability, the MTT assay was utilized. After 24, 48, and 72 h exposure, 10 μL of the MTT reagent was pipetted into each well of the microtiter plate. This mixture was then incubated at 37°C for a duration of 2 h. Subsequently, 100 μL of DMSO was introduced to the dissolution of the formazan crystals. The optical density was subsequently determined at a wavelength of 570 nm through the use of a microplate spectrophotometer (Biotek ELX800). For comparative analysis, the viability of untreated control cells was arbitrarily set at 100%[15].

RNA extraction and real-time quantitative PCR

Total RNA isolation was performed using the PureLink RNA Mini Kit (Catalog Number: 12183018A, Thermo Fisher Scientific, USA), meticulously following the kit's guidelines. The quality and quantity of the isolated RNA were evaluated using the Biotek Nanodrop system. Subsequently, complementary DNA (cDNA) synthesis was carried out for HOTAIR and DHX33 using the High-Capacity cDNA Reverse Transcription Kit (Catalog Number: 4368814, Thermo Fisher Scientific, USA). For the specific case of miR-526b-3p, cDNA synthesis was achieved with the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Following cDNA synthesis, qRT-PCR was conducted to quantify the cDNA levels. This process utilized the StepOne™ Real-Time PCR System (Applied Biosystems, USA) along with the Maxima SYBR Green qPCR Master Mix (Catalog Number: K0253, Thermo Fisher Scientific, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference gene for normalizing the expression levels of HOTAIR, DHX33, and the apoptosis, invasion, and cell cycle markers. Meanwhile, miR-526b-3p's expression was normalized against U6. The obtained results were expressed as relative quantities, calculated using the $2^{-\Delta\Delta CT}$ method[16]. The primer sequences utilized in this investigation are listed in Table 1. The experimental procedure involved a three-step amplification protocol. It began with a denaturation step at 95°C for 5 min, followed by 45 cycles consisting of denaturation at 95°C for 15 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 15 seconds.

Table 1 — Sequences of primers related to the studied genes

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	GGTGGACCTCATGGCCTACAT	GCCTCTCTCTTGCTCTCAGTATCCT
U6	CTC GCT TCG GCAGCACA	AACGCTTCACGAATTTGCGT
HOTAIR	CAGTGGGGAACTCTGACTCG	GTGCCTGGTGCTCTCTTACC
DHX33	TGCGTGAAGCAATTTCAGAC	AGGTCCACATCCATCGTAGC
miR-526b-3p	GCCGAGGAAAGTGCTTCCTT	CAGTGCGTGTCGTGGAGT
BCL-2	CTGGGAGAACGGGGTACGAT	GACCCCACCGAACTCAAAGA
CASP-3	GGAAGCGAATCAATGGACTCTGG	GCATCGACATCTGTACCAGACC
P53	ATCTGTTCACTTGTGCCCTG	AACCAGCCCTGTCGTCTCTG
PTEN	ACCAGGACCAGAGGAAACCT	GCTAGCCTCTGGATTTGACG
CCNA2	CGCTGGCGGTACTGAAGTC	GAGGAACGGTGACATGCTCAT
CCND1	AGGCCCTGGCTGCTACAAG	ACATCTGAGTGGGTCTGGAG

Preparation of cell lysate

Following the treatment, the cells underwent a thorough washing procedure using phosphate-buffered saline (PBS) at a pH of 7.4, repeated twice to ensure cleanliness. Subsequently, the cells were exposed to a solution of PBS enriched with 0.2% trypsin and 2 mM EDTA for a duration of 10 min. This initial treatment was succeeded by a centrifugation process, executed at a force of 10,000 g for 10 min, to separate the cellular components.

The resulting cell pellet was then processed further by being lysed with a 50 mM phosphate buffer, adjusted to a pH of 7.0, and subsequently subjected to sonication under cold conditions for a span of 2 min. This step aimed to break down the cell structures and release the intracellular contents. To finalize the preparation, the mixture underwent another round of centrifugation, again at 10,000 × g for 10 min. From this process, the supernatant layer, rich in enzymes and proteins, was carefully collected. This supernatant serves as the primary sample for assessing enzyme activities and determining the protein concentration, utilizing Bradford's method for precise quantification.

Enzyme-Linked Immunosorbent Assay

An assessment of apoptosis-related genes including BCL-2 and CASP-3, along with cell invasion indicators TP53 and PTEN, cell cycle regulators Cyclin A2 and D1, and DHX33 was performed through an enzyme-linked immunosorbent assay. This procedure was meticulously followed according to the manufacturer's guidelines.

Annexin V/PI Apoptosis Assay

Apoptosis levels were assessed utilizing an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay. Hep3B and HepG2 cells, at a density of 1 × 10^5 cells, were cultured in a 12-well plate and allowed to incubate for 24 h. Following a 24 h exposure to 6-OHDA, the cells were resuspended in 100 µL of binding buffer composed of 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2. To identify apoptotic and necrotic cells, FITC Annexin V (2 µg/mL) and PI (2.5 µg/mL) were introduced, and the cell suspension was gently mixed and incubated for 15 min at room temperature (25°C) in the absence of light. After this incubation period, 400 µL of binding buffer was added to each tube, and flow cytometric analysis was conducted within one hour. The fluorescence signals of FITC and PI were evaluated using a flow cytometer. Cells that were negative for both PI and Annexin V were deemed viable, those positive for Annexin V but negative for PI were classified as apoptotic, and cells that were positive for both Annexin V and PI were identified as necrotic. Each experimental condition was replicated three times to ensure the reliability of the results.

Measurement of catalase (CAT) and superoxide dismutase (SOD) activity

Following the preparation of the lysate, an assessment was undertaken to quantify the CAT and SOD enzyme activity. The research employed the photometric assay kit, adhering meticulously to the instructions provided by the manufacturer.

Assessment of malondialdehyde (MDA) levels

The level of MDA, indicative of lipid peroxidation, was determined through a colourimetric assay. Specifically, 100 µL of cell extract was combined with 400 µL of a ThioBarbituric Acid (TBA) reagent, composed of 0.375% TBA, 15% trichloroacetic acid, and 0.25 mol/L hydrochloric acid. This mixture was then heated to 95°C in a water bath for 30 min. Following rapid cooling, it underwent centrifugation at 8,000 × g for 15 min at 4°C. The absorbance of the

resulting pink-coloured supernatant was assessed at a wavelength of 532 nm. The MDA concentration was quantified utilizing tetraethoxypropane as the calibration standard and reported in nanomoles per milligram of protein[17].

Statistical analysis

The experiments underwent replication on at least three separate occasions, with the outcomes expressed as the mean ± standard deviation (SD). To conduct statistical analyses and generate visual representations, IBM SPSS Statistics for Windows (version 24) and GraphPad Prism (version 8.0.2.263) were utilized. Statistical significance was determined through the application of one-way and two-way ANOVA analyses. A *P*-value less than 0.05 was deemed statistically significant.

Results

Que suppressed the viability of liver cancer cell line

The present findings revealed that Que significantly decreased the survival of Hep3B and HepG2 liver cancer cell lines in a concentration-dependent and time-dependent manner (Fig. 1), whereas the examined doses of Que represented little toxicity on the survival of liver normal THLE-2 cell line. MTT assay also showed that IC50 decreased significantly with increasing treatment duration (Table 2). According to the results of the MTT assay and obtained IC50 values, the current study considered the treatment duration of 48 h for further investigation.

Que regulated *HOTAIR* expression and the downstream mediators

It was previously reported that the *HOTAIR/miR-526b-3p/DHX33* axis is a key contributor to the occurrence and progression of HCC[7]. The findings of qRT-PCR demonstrated that *HOTAIR* expression after treatment with 25 µM of Que was not significantly different from the controls (*P*-value>0.05), whereas a significant decrease in *HOTAIR* expression was achieved after treatment with 50 µM (p-value=0.0003) and 100 µM (*P*-value<0.0001) doses of Que (Fig. 2). This is while only the concentration of 100 µM of Que caused a significant decrease in *DHX33* gene expression (*P*-value=0.0122). On the contrary, the treatment of Hep3B cells with a concentration of 100 µM of Que significantly increased *miR-526b-3p* expression (*P*-value<0.0001). The effects of Que on the *HOTAIR/miR-526b-3p/DHX33* axis were further

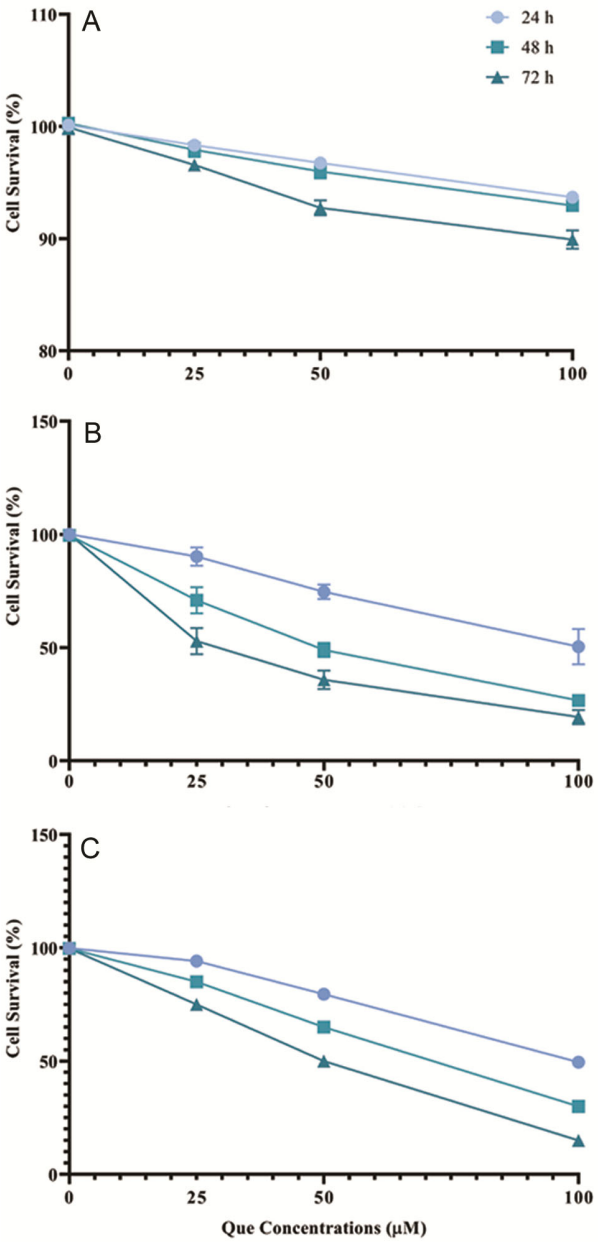

Fig. 1 — MTT assay was performed to determine the cytotoxicity of the studied doses of Que in THEL-2 (A), Hep3B (B), and HepG2 (C) cell lines. The findings showed that the cytotoxicity of Que in Hep3B and HepG2 cells was dependent on dose and time, while IC50 of Que in THEL-2 cells was at a higher concentration than the studied doses.

Table 2 — MTT assay analysis and obtained IC50 values in liver normal and cancer cell lines

Cell lines	IC50 values		
	24 h	48 h	72 h
THLE-2	835.6 µM	236.7 µM	110.1 µM
Hep3B	84.1 µM	51.7 µM	42.8 µM
HepG2	92.1 µM	80.3 µM	56.9 µM

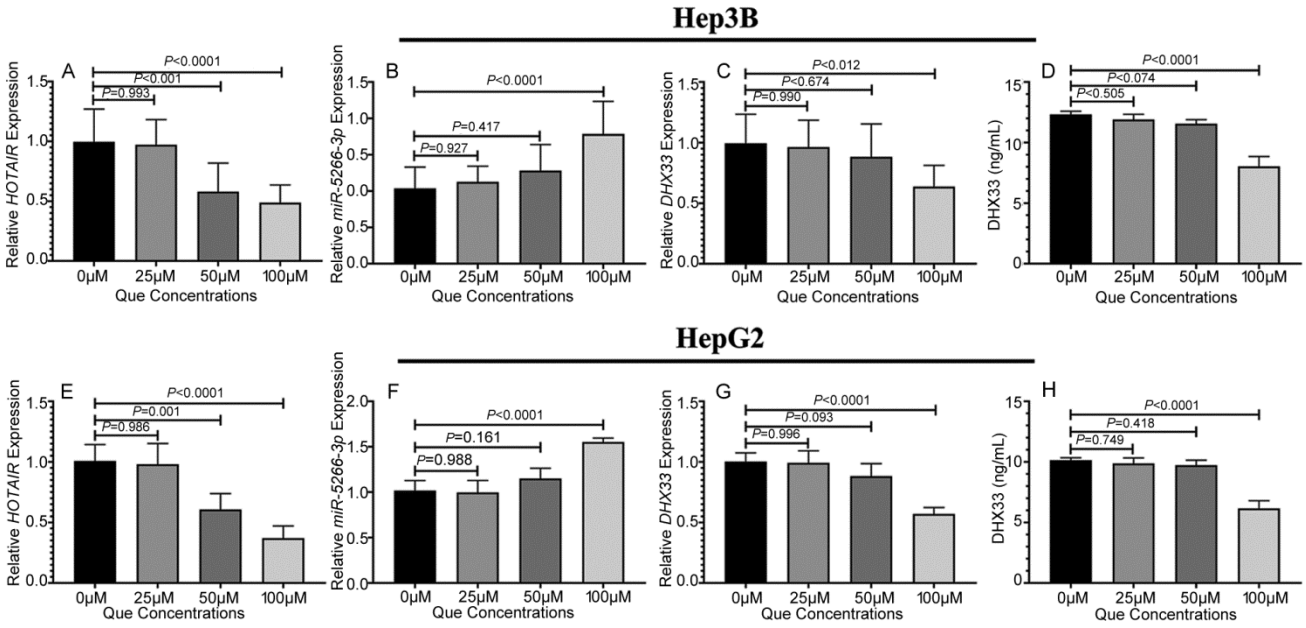

Fig. 2 — Que altered the expression of HOTAIR/miR-526b/3p/DHX33 axis. Que at a dose of 100 μM significantly decreased the expression of HOTAIR (A & E) and DHX33 (C& G), whereas significantly increased miR-526b-3p (B & F) expression in the liver cancer cell lines. Moreover, protein levels of DHX33 were upregulated in Hep3B (D) and HepG2 (H) cell lines upon treatment with Que. [*P*-value<0.05 was considered significant]

evaluated in the HepG2 cell line. Similar to the observations in Hep3B cells, Que treatment significantly modulated the expression of key components in this axis. Que at concentrations of 50 μM (*P*-value=0.001) and 100 μM (*P*-value<0.0001) markedly suppressed *HOTAIR* expression in HepG2 cells, with no significant change observed at 25 μM (*P*-value>0.05). Concurrently, *miR-526b-3p* expression was significantly upregulated at 100 μM (*P*-value<0.0001), mirroring the trend seen in Hep3B cells. For *DHX33*, a significant reduction in expression was noted at 100 μM (*P*-value=0.008), while lower concentrations (25 μM and 50 μM) did not yield statistically significant changes (*P*-value>0.05). These findings align with the results in Hep3B cells, reinforcing the consistency of Que's regulatory effects across different HCC cell lines. Additionally, the protein levels of DHX33 were assessed, revealing a dose-dependent decrease in both Hep3B and HepG2 cell lines, with the most pronounced reduction at 100 μM (*P*-value<0.001). This further corroborates the qRT-PCR results, indicating that Que effectively targets both transcriptional and translational levels of DHX33 in HepG2 cells.

Apoptosis was induced in Hep3B cells after treatment with Que

Apoptosis markers, including BCL-2 and CASP-3, were measured in liver cancer cells using RT- qRT-PCR and ELISA approaches (Fig. 3). Que at a dose of 25 μM did not induce significant changes in the expression of *BCL-2* (*P*=0.997) and *CASP-3* (*P*=0.358) genes, while doses of 50 μM and 100 μM caused a significant decrease and increase in the expression of *BCL-2* (26.62% decrease at 50 μM and 62.62% decrease at 100 μM of Que) and *CASP-3* (2.63 times increase at 50 μM and 5.16 times increase at 100 μM of Que) genes. The findings showed that the concentrations of 50 μM and 100 μM of Que caused a significant decrease (15.61% and 24.81%, respectively) in BCL-2 protein level in the Hep3B cell line (*P*-value<0.05). On the contrary, all doses of 25 μM, 50 μM, and 100 μM of Que caused a significant increase of 1.25 times, 1.27 times, and 1.53 times, respectively, in CASP-3 level (*P*-value<0.01). Additionally, it was also found in HepG2 cells that treatment with Que, especially at doses of 50 and 100 μM, caused a decrease and an increase in gene expression of *BCL-2* and *CASP-3*, respectively (*P*-value<0.05). Subsequently, the levels of BCL-2 and CASP-3 proteins in the HepG2 cell line were downregulated and upregulated, respectively, after treatment with a dose of 100 μM of Que.

The Annexin V assay revealed that Que significantly increased apoptosis in both Hep3B and HepG2 cells in a dose-dependent manner. In Hep3B

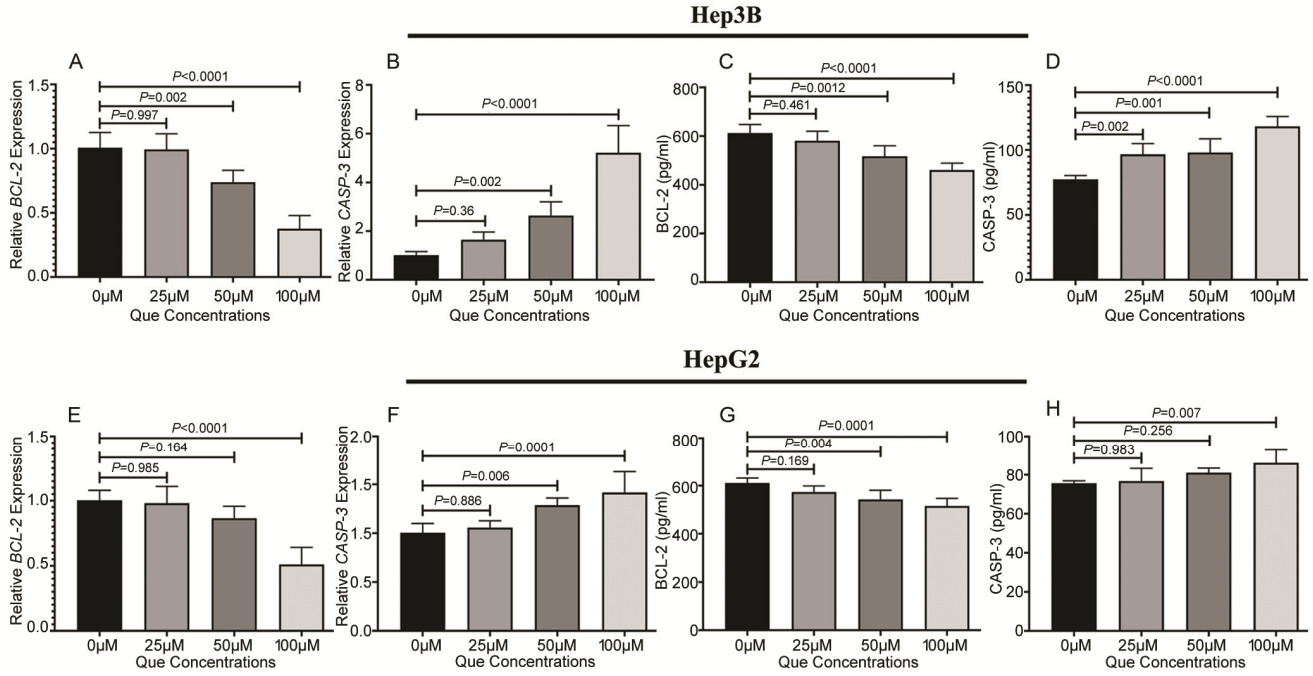

Fig. 3 — The administration of Que induced apoptosis in the liver cancer cell lines. Que modulated the gene expression of *BCL-2* (A & E) and *CASP-3* (B & F). The administration of 100 μM of Que significantly downregulated BCL-2 levels (C & G), while upregulated CASP-3 (D & H) levels. [*P*-value<0.05 was considered significant]

cells, treatment with 25 μM Que resulted in a modest but detectable increase in apoptotic cells. At 50 μM, apoptosis rose sharply to 32.6% (*P*-value<0.01), and the highest dose (100 μM) induced apoptosis in 54.3% of cells (*P*-value<0.001). Similarly, in HepG2 cells, 25 μM Que triggered 12.7% apoptosis, while 50 μM and 100 μM treatments led to 28.4% (*P*-value<0.01) and 48.9% (*P*-value<0.001) apoptotic cells, respectively. These findings confirm that Que effectively promotes programmed cell death in HCC cells, with Hep3B exhibiting slightly greater sensitivity. The data support Que's role as a pro-apoptotic agent in HCC (Fig. 4).

Liver cancer cell invasion was suppressed after Que treatment

The present findings showed that *p53* gene expression was significantly reduced by 36.60% (*P*-value=0.003) and 48.94% (*P*-value<0.0001) at doses of 50 μM and 100 μM of Que, respectively, while doses of 50 μM and 100 μM caused a significant increase in *PTEN* gene expression by 1.60-fold (*P*-value=0.033) and 2.26-fold (*P*-value<0.0001), respectively. The measurement of TP53 protein level in the Hep3B cell line revealed that the doses of 50 μM and 100 μM of Que had caused a significant decrease of 5.36% and 21.40% (*P*-value<0.05), respectively, while the dose of 25 μM of Que did not induce a significant alteration in TP53 levels (*P*-value>0.05). In addition, the PTEN protein level was measured by the ELISA technique (Fig. 5). The obtained data showed that the doses of 25 μM and 50 μM of Que did not cause a significant change in the level of PTEN (*P*-value>0.05), while after treating the cells with a dose of 100 μM of Que, the level of PTEN was significantly increased by 1.10 times (*P*=0.0047). However, in HepG2 cells, only a dose of 100 μM of Que was able to significantly decrease and increase the expression of *p53* and *PTEN* genes, respectively (*P*-value<0.05). Subsequently, the levels of TP53 and PTEN proteins were also significantly changed only at a dose of 100 μM of Que.

Administration of Que arrested the cell cycle in the Hep3B cell line

Administration of Que caused a significant dose-dependent decrease in the expression of genes encoding cyclins A2 (*CCNA2*) and cyclins D1 (*CCND1*). The protein levels of cyclins A2 and D1 were measured using available ELISA kits (Fig. 6). Obtained results showed that 100 μM of Que significantly downregulated cyclin A2 levels (*P*-value<0.001), whereas no significant difference was achieved after treatment with 25 μM and 50 μM doses of Que (*P*-value>0.05). Moreover, a remarkable

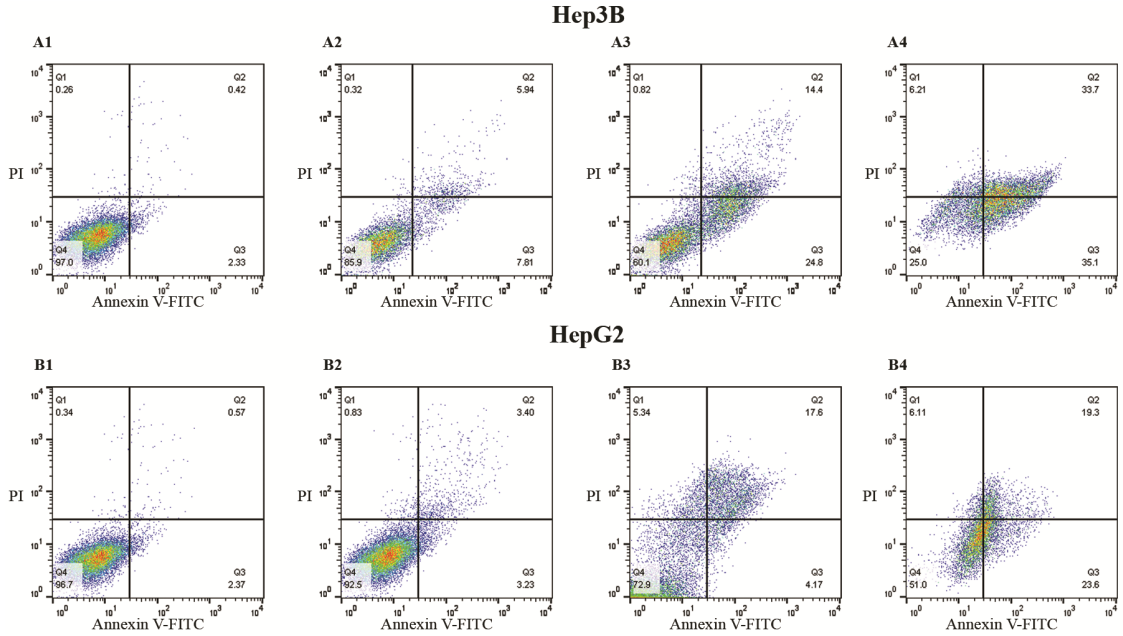

Fig. 4 — Dose-dependent pro-apoptotic effects of Que in HCC cells. Annexin V/PI flow cytometry of Hep3B (A) and HepG2 (B) cells treated with 0-100 µM Que for 48h. Quadrants show viable (Q3), early apoptotic (Q4), late apoptotic (Q2), and necrotic (Q1) populations. Increasing Que concentrations elevate combined early+late apoptotic fractions in both cell lines.

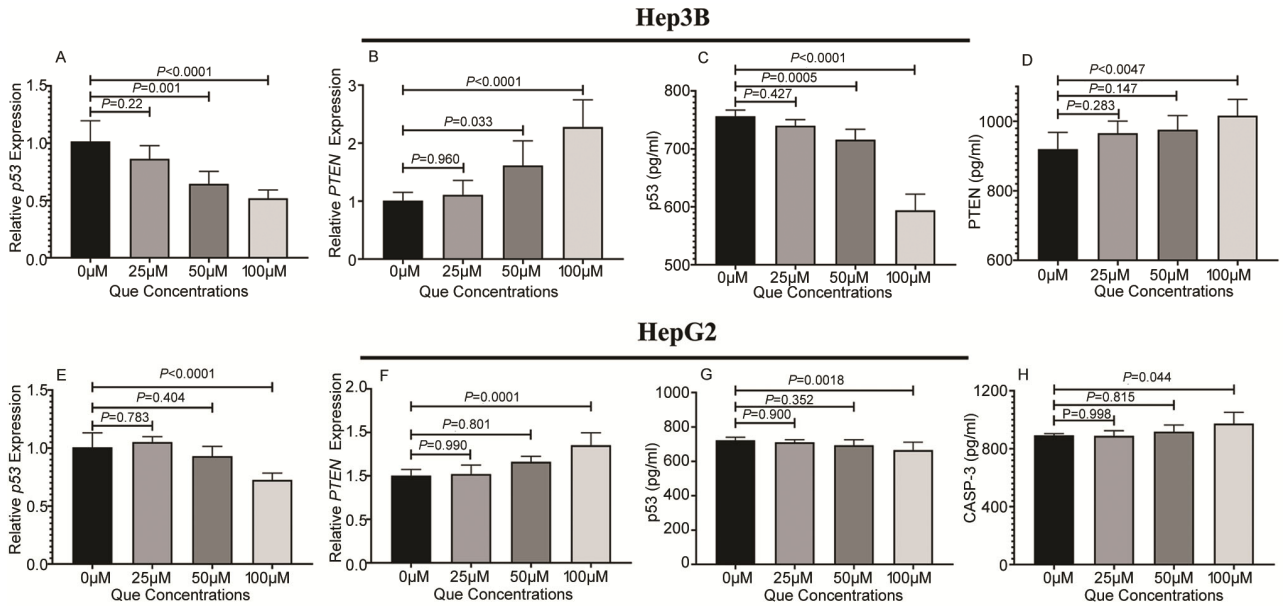

Fig. 5 — Tumor invasion was suppressed in liver cancer cells after treatment with Que. The expression of *p53* (A) and *PTEN* (B*)* was modulated by Que. 100 µM of Que significantly reduced the levels of TP53 (C) and increased PTEN (D) levels in Hep3B cells. Moreover, the expression of *p53* (E) and *PTEN* (F) along with protein levels of TP53 (G) and PTEN (H) in the HepG2 cell line were altered after treatment with Que. [*P*-value<0.05 was considered significant]

decrement was observed in the levels of cyclin D1 after treatment with 50 µM and 100 µM doses of Que (*P*-value<0.001), however, treatment with 25 µM of Que did not alter cyclin D2 levels significantly (*P*>0.05). However, changes in the expression of *CCNA2* and *CCND1* genes, as well as the levels of cyclins A2 and D1 proteins in HepG2 cells, were mainly achieved at high doses of Que, especially after treating the cells with a dose of 100 µM of Que (*P*-value<0.05).

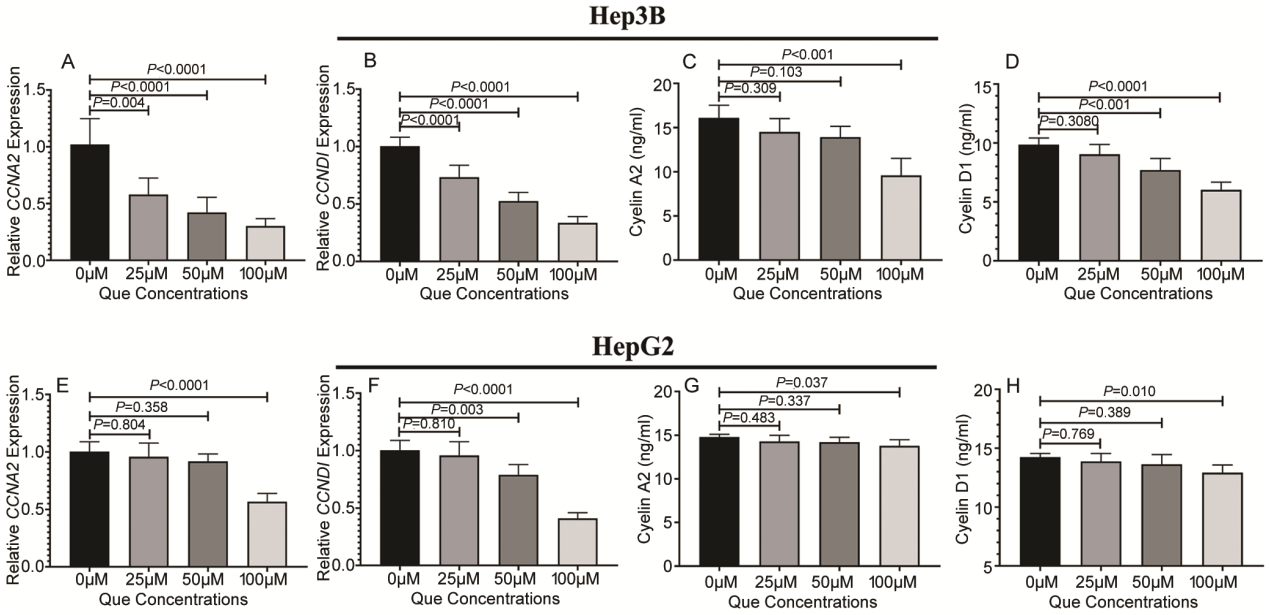

Fig. 6 — Que administration caused cell cycle arrest in hepatic tumor cells. The levels of *CCNA2* (A) and *CCND1* (B) along with cyclin A2 (C) and cyclin D1 (D) were downregulated after treatment of Hep3B cells with Que at a dose of 100 μM for 48h. Moreover, the expression of *CCNA2* (E) and *CCND1* (F) along with protein levels of cyclin A2 (G) and cyclin D1 (H) in the HepG2 cell line were altered after treatment with Que. [*P*-value<0.05 was considered significant]

Effects of Que on oxidative stress and lipid peroxidation in Hep3B cell line

Measurement of SOD enzyme activity showed that all three administrated doses of Que were able to induce a significant increase in enzyme activity (*P*-value<0.05). In addition, doses of 50 μM and 100 μM of Que have significantly increased the activity of the CAT enzyme in the Hep3B cell line (*P*-value<0.001). This is while only the dose of 100 μM of Que caused a significant decrease in the MDA levels in the studied human HCC cell line (*P*-value=0.002, Fig. 7). In the HepG2 cell line, treatment of cells with 50 and 100 μM concentrations of Que significantly increased SOD and CAT activity, while only 100 μM doses of Que significantly decreased MDA levels (*P*-value<0.01).

Discussion

While this study provides compelling evidence As a highly malignant disease, HCC is recognized with poor prognosis, difficult early diagnosis, and effectiveness of radio- and chemo-therapeutical approaches[18]. Although conventional surgical resection is considered the major management strategy for patients with HCC, the overall survival rate after hepatic resection is reported to be low[19]. Therefore, there has been an increasing demand to propose an efficient treatment approach with

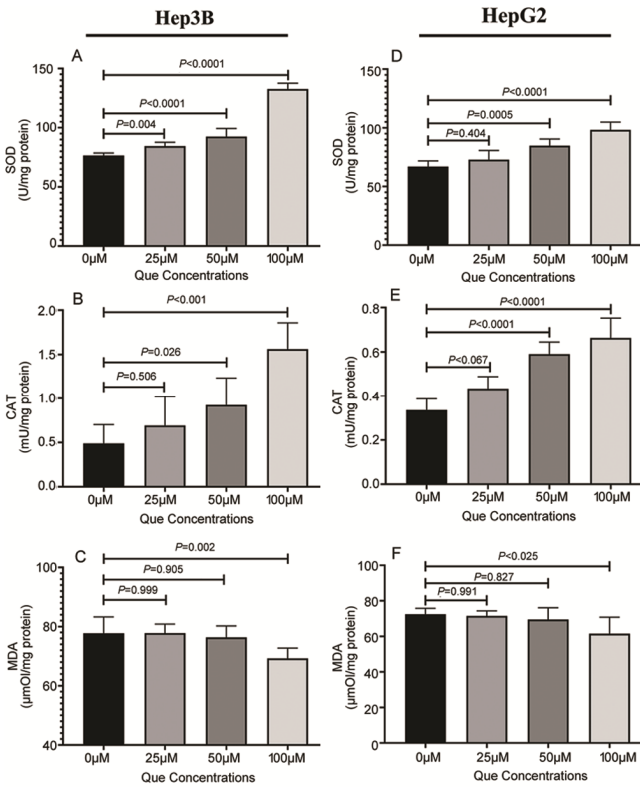

Fig. 7 — Oxidative stress was suppressed in the Hep3B cell line upon treatment with Que. The findings revealed that Que significantly increased the levels of SOD (A & D) and CAT (B & E) whereas decreased MDA (C & F) levels in Hep3B and HepG2 cells. [*P*-value<0.05 was considered significant]

acceptable adverse effects and several studies have been directed toward this content. Along with that, the uncertainty of the underlying mechanism of HCC occurrence and progression is assumed to be one of the key challenges of disease management[20]. Recently, it has been emphasized that lncRNAs and downstream mediators could modulate HCC[21]. Considering the properties of phytochemicals, including anti-tumor, anti-stress, and antiproliferative activities, the present study aimed to investigate the therapeutic properties of Que on Hep3B and HepG2 cell lines by examining the expression of genes encoding *HOTAIR*, *miR-526b-3p*, and *DHX33* along with analysis of cytotoxicity, apoptosis, cell cycle, and oxidative stress.

Analysis of the data from the MTT assay showed that Que decreased the survival rate of the Hep3B cell line in a dose- and time-dependent manner, whereas the studied doses of Que did not cause a significant decrease in the survival rate of normal liver cells. In fact, a plethora of *in vitro* and *in vivo* studies have emphasized the non-toxicity of phytochemicals (e.g. Que[17], morin[22], etc.) in untargeted cells and tissues. The high safety of phytochemicals along with their ability to limit the survival of cancer cells has caused these chemicals to be proposed as promising approaches for the management of chronic diseases such as cancer[23]. Although several compounds have been proposed for HCC therapy[24], investigating their mechanism of action is of critical importance to elucidate the antitumor activity. The findings of the current investigation showed that higher doses of Que significantly decreased the expression of *HOTAIR* and *DHX33*, whereas the expression of *miR-526b-3p* increased as the dose of Que administrated to the Hep3B cells increased. LncRNAs often alter the expression of genes by targeting miRNAs, such as miR-331-3p[25], miR-145-5p[26], etc. are considered downstream targets of *HOTAIR* affecting different types of cancer. According to the data obtained from the present research, Liu *et al.* have shown that the overexpression of *HOTAIR* has promoted HCC through regulation of the miR-331-3p/DHX33 axis[7]. DHX33 is an RNA helicase assumed as a potent oncogenic agent in patients with HCC, in which its overexpression may function as a poor prognostic biomarker due to the contribution of DHX33 in tumor proliferation and invasion[7]. The current study expanded its investigation to include the HepG2 cell line, reinforcing the consistency of Que antitumor effects across different HCC models. Similar to Hep3B cells, Que treatment in HepG2 cells significantly suppressed *HOTAIR* and *DHX33* expression while upregulating *miR-526b-3p*, corroborating the pivotal role of the *HOTAIR/miR-526b-3p/DHX33* axis in HCC progression. These findings align with recent studies highlighting the universality of this axis in HCC pathogenesis, as demonstrated by Liu *et al*.[7] and further supported by Wu *et al*.[27], who identified miR-526b-3p as a tumor suppressor in HCC. The dose-dependent reduction in DHX33 protein levels in both cell lines underscores Que's dual transcriptional and translational regulatory capabilities, a phenomenon also observed in other cancers treated with flavonoid compounds[28]. The apparent lag between *HOTAIR* suppression and miR-526b-3p/DHX33 modulation at intermediate doses (50 μM) may reflect incomplete *HOTAIR-miR-526b-3p* decoy activity below a critical threshold, or time-dependent accumulation of miR-526b-3p required to suppress DHX33 translation. In other words, the delayed miR-526b-3p/DHX33 response at 50 μM Que may suggests a threshold effect, where *HOTAIR* suppression must reach a critical level to derepress miR-526b-3p and downstream DHX33 inhibition.Similar kinetic delays are reported in other lncRNA-regulated pathways[27]. Therefore, even though it was found that Que reduced the survival of Hep3B and HepG2 liver cancer cells through the manipulation of the *HOTAIR/miR-526b-3p/DHX33* axis, the present study aimed to further evaluate the effect of Que on apoptosis, cell cycle, proliferation, and oxidative stress.

The present findings showed that Que downregulated the level of BCL-2 whereas upregulated CASP-3 in cancer cell lines. BCL-2 is an anti-apoptotic protein and a member of the larger BCL-2 family, whose overexpression is associated with a decrease in programmed apoptotic cell death, hence assumed as a target for cancer therapy[29]. It has been reported abundantly that cancer cells halt the process of apoptosis and increase cell survival by regulating BCL-2 expression[30]. Meanwhile, CASP-3 is a major serine protease in the apoptotic pathway, whose hyperactivity ends with apoptotic death[30]. Therefore, one may conclude that the administration of Que was associated with the induction of apoptosis in Hep3B cells. Que's pro-apoptotic effects were replicated in HepG2 cells, with significant downregulation of BCL-2 and upregulation of CASP-3 at higher doses (50–100 μM). The Annexin V assay further confirmed Que's ability to induce apoptosis in Hep3B and HepG2 cells, albeit with slightly lower sensitivity in HepG2 compared to Hep3B. This differential response may reflect intrinsic

variations in apoptotic pathway activation between cell lines, as suggested by recent work on HCC heterogeneity[18]. Similarly, it has been shown that the change in the apoptosis pathway is one of the most important characteristics of a variety of phytochemicals, which causes cytotoxicity in cancer cells and decreases the survival rate[31]. The present findings indicated the downregulation of cyclins A2 and D1 after treating Hep3B cells with the Que. Additionally, Que-induced cell cycle arrest in HepG2 cells, evidenced by reduced *CCNA2* and *CCND1* expression, mirrors findings in Hep3B cells and aligns with studies linking cyclin dysregulation to HCC proliferation[32,33]. The consistency of these results across cell lines strengthens the potential of Que as a broad-spectrum HCC therapeutic. Cyclin A2 is considered an activating partner of cyclin-dependent kinase 1 and its upregulation causes high proliferation of hepatic stellate cells leading to liver fibrosis while targeting cyclin A2 results in apoptosis induction and cell cycle arrest[34]. Moreover, the reduction of cyclin A2 expression is followed by cell cycle arrest and apoptosis induction mediated by BCL-2 downregulation in rats with HCC[35]. Concordantly, the overexpression of cyclin D1 is assumed to be a marker of HCC progression, and its targeting is associated with arresting the cell cycle and inhibiting liver cancer cell survival[33]. Unlike previous studies focusing on Que's broad anticancer properties, our work is the first to demonstrate that Que suppresses HCC progression by specifically modulating the *HOTAIR/miR-526b-3p/DHX33* axis. This axis has recently been implicated in HCC aggressiveness, and our data reveal Que as a potent regulator of this pathway, providing a new molecular basis for its therapeutic application.

Both TP53 and PTEN are known tumor suppressors, while mutations in TP53[36] and downregulation of PTEN[37] have been associated with poor prognosis and malignant liver cell invasion. The findings of the present study determined that Que administration was followed by TP53 downregulation and PTEN upregulation in the Hep3B cell line. In HepG2 cells, Que's anti-invasive effects were pronounced at 100 μM, with TP53 downregulation and PTEN upregulation, echoing trends observed in Hep3B. The restoration of PTEN activity is particularly noteworthy, as PTEN loss is a hallmark of HCC metastasis[37]. Similarly, it has been found that Que decreases the expression of TP53 in various types of cancers, which ultimately ends in the death of tumor cells, although TP53 activation is reported to be involved in the suppression of cancer cell invasion[38]. The association of TP53 overexpression with cancer is generally explained by mutations that alter the activity of this tumor suppressor[39]. In addition, it has been reported that treatment with Que suppressed the progression and invasion of cancer cells through the activation of PTEN and related signaling pathways such as PI3K/Akt/PTEN[40,41], and Akt/mTOR/PTEN[38]. Proliferation and invasion of cancer cells are often associated with the overproduction of free radicals and the induction of oxidative stress, therefore Que with inherent antioxidant properties has been proposed as a strategy to inhibit the progression of cancer[39]. The increase in the activity of SOD and CAT enzymes along with the reduction of lipid peroxidation is a sign of the Que's ability to disrupt oxidative stress in cancer cells. In fact, Que's ability to modulate oxidative stress in HepG2, elevating SOD/CAT activity and reducing MDA,further supports its role in mitigating HCC-associated redox imbalance. Recent studies emphasize the interplay between oxidative stress and lncRNA-mediated pathways in HCC[42], suggesting Que's dual antioxidant and epigenetic actions could synergistically suppress tumor progression. SOD and CAT enzymes, by converting reactive oxygen species into less reactive molecules, establish redox homeostasis in tumor cells and can confront the processes necessary for the survival and invasion of cancer cells, such as angiogenesis.

for Que antitumor effects via the *HOTAIR/miR-526b-3p/DHX33* axis in both Hep3B and HepG2 cell lines, certain limitations should be acknowledged. First, the reliance on qRT-PCR and ELISA, without Western blot validation, leaves some uncertainty regarding protein-level changes in key markers such as DHX33, TP53, and PTEN. Future studies should incorporate Western blotting to confirm translational regulation. Second, expanding the investigation to additional HCC cell lines (e.g., Huh7, SNU-398) and non-cancerous liver models would strengthen the generalizability of these findings. Third, mechanistic studies, including luciferase reporter assays and RNA immunoprecipitation, are needed to definitively establish the interaction between *HOTAIR*, *miR-526b-3p*, and *DHX33*. Additionally, *in vivo* experiments using HCC xenograft or chemically induced liver cancer models would validate Que′s efficacy in a more physiologically relevant setting. Finally,

clinical trials are essential to assess Que′s safety, bioavailability, and therapeutic potential in HCC patients, particularly in combination with existing treatments like sorafenib or immunotherapy.

Conclusion

The findings of this study demonstrate that Quercetin exerts potent antitumor effects in HCC by modulating the *HOTAIR/miR-526b-3p/DHX33* axis, leading to apoptosis induction, cell cycle arrest, invasion suppression, and oxidative stress reduction in both Hep3B and HepG2 cell lines. These results highlight Que′s potential as a promising therapeutic agent for HCC, particularly given its ability to target multiple oncogenic pathways while sparing normal liver cells. However, further mechanistic investigations, *in vivo* validation, and clinical trials are necessary to fully elucidate Que′s molecular actions and translate these findings into clinical applications. Future research should prioritize comprehensive preclinical studies and human trials to establish Que as a viable adjunct or alternative therapy for HCC.

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Conflict of interest

The authors declare that they have no competing interests.

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