Journal of Environmental Sciences

JOESE 5

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Kholoud Abdelhamed[$*], Doaa sakr[$], Ibrahim Elsherbini[#], Mohamed E. Abdraboh [$]

[$] Zoology dept., faculty of Science, Mansoura University, Egypt.

[#]University of science and Technology (UST), Zewail city, Egypt

Reprint

Journal of Environmental Sciences

JOESE 5

ISSN 2090-9233

Journal homepage http://Joese.mans.edu.eg

Original Article

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Kholoud Abdelhamed*, Doaa sakr$, Ibrahim Elsherbini#, Mohamed E. Abdraboh$

$ **Zoology dept., faculty of Science, Mansoura University, Egypt.**
#**University of science and Technology (UST), Zewail city, Egypt**

Article Info	Abstract
	Quercetin (Qu.) is considered as one of the flavonoids that were known by their anti-inflammatory and anti-carcinogenic effects**.** Even though, its implication in clinical trials of cancers is still limited. This study aims to determine the effect of Qu. against progression of Liver cancer using an *in vitro* model of hepatocellular carcinoma. Hepatocellular carcinoma cell line HepG2 was treated with serial concentrations of Qu. and the MTT assay data indicated a significant dose dependent effect of Qu. in lessen the cell proliferation rate in a dose dependent manner. This effect on cell proliferation was confirmed by the significant ceasing of cell cycle at G1/S phase upon Qu. treatment. The wound healing assay illustrated the inhibitory effect of Qu. on the capability of HepG2 to migrate to the induced wound. Finally, Qu. significantly induced HepG2 cell apoptosis via the significant upregulation of proapoptotic Bax expression and downregulation of antiapoptotic Bcl2. In conclusion, Qu. showed potent anti-cancer characteristics that been indicated by its prominent effect on HepG2 cells proliferation, motility, and survival.

1. Introduction

According to their origin, liver cancer can be classified as angiosarcoma, hepatoblastoma, intrahepatic cholangiocarcinoma, and hepatocellular carcinoma. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer that used to seen in patients of chronic liver cirrhosis, which were mostly developed at patients virally infected with either hepatitis C or B **(de Oliveria Andrade *et al.*, 2009)**. Recently, in United states an estimated 42,030 adult patients were diagnosed with primary liver cancer. This number of diagnosed patients was increased by approximately 3% annually in between 2006 and 2015. The incidence of HCC diagnosed cases were found to be three times

higher in men than women. Drastically, more than 44% of people who were diagnosed with an early stage of HCC, their 5-year survival rate was 31%. This ratio was dramatically lowered to 11% when liver cancer was spread to If liver to other organs and/or the regional lymph nodes **(Siegel *et al.*, 2019).**
Early treatment of Hepatitis B and C, could improve the chances of HCC treatment or even lowering the number of liver cancer cases by half **(Harrod *et al.*, 2019).** Surgery and other techniques for cancer ablation is the first choice for cancer treatment, even though; one of surgeon's biggest concern is the rate of tumor recurrence. Clinical trials and research experiments proved the high ability of chemotherapy to stop the progression of liver cancer with severe side

effects on normal cells and healthy organs (**Yu *et al.*, 2017).** For long time, traditional medicine had used natural sources of medicinal plants to treat several diseases. The high content of antioxidants and anti-inflammatory compounds in such plants suggested the study of their possible anticancer effects (**Shilpha *et al.*, 2017, Cragg and Pezzuto, 2016).**

Several bioactive natural compounds were recorded to be implicated in the prevention and treatment of cancers via targeting several signaling pathways that regulated cancer cell proliferation, migration and survival (**Bishayee and Sethi, 2016, Varoni *et al.*, 2016).**

The high flavonoid content of Quercetin (Qu.) (3,3',4',5,7-pentahydroxy flavone) that is naturally found and extensively present in several dietary plants, with an estimated human dietary consumption of 16-1000mg/day, suggested its potent antioxidant activity (**Tieppo *et al.*, 2007**). Moreover, Qu. has proved its effect on targeting vital signaling pathways for cancer cell proliferation and survival such as PI3K and MAPK pathways (Kedhari Sundaram *et al.*, 2019). Qu. significantly induced a dose dependent cytotoxicity in three leukemic cell lines (CEM, K562 and Nalm6) with an incidence of cell cycle arrest at G0/G1 phase (**Srivastava *et al.*, 2016**). Interestingly, Qu. can stimulates both intrinsic and extrinsic pathways of cell apoptosis in a tissue specific effect which can be shown in breast cancer cell line (MDA-MB-231) and liver cancer (HepG2), respectively (**Rather and Bhagat, 2020, Rao *et al.*, 2018**).

This study aimed to investigate the anticancer effects of Qu. on the proliferation, survival and motility of HepG2 cells as an *in vitro* model of hepatocellular carcinoma.

2. Materials and Methods

Cell line

The Hepatocellular carcinoma (Hepg-2) cell lines were obtained from VACSERA company, Cairo, Egypt. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100µg/ml streptomycin, then cells were incubated at 37 $^{\circ}$C in 5% CO_2 incubator.

Chemical reagents

RPMI-1640 medium, Methylthialazole Tetrazolium (MTT), and DMSO were purchased from Sigma Aldrich (sigma co., St. Louis, USA), Fetal Bovine serum was obtained from (GIBCO, UK). Doxorubicin (Sigma-Aldrich, Germany) was used as a standard anticancer drug to be compared with Qu anticancer activities.

MTT assay

MTT assay was conducted to assess the inhibitory effects of Qu. on cell growth. This colorimetric assessment of cell proliferation depends on the ability of the viable cells to reduce the tetrazolium salt (yellow) to form formazan (purple). Hepg-2 cells were seeded in a 96-well plate ($3x10^3$ cells/well) and incubated for 24h at 37 $^{\circ}$C and 5% CO_2. Cells were then treated with different concentrations of Qu. and incubated for extra 48 hr. After incubation, 10µl of (5mg/ml) MTT solution was added to each well and incubated for 4h. 100 µl of DMSO was added into each well to dissolve the purple formazan. The cell viability dependent change in color was measured at 540 nm using a plate reader (EXL 800, USA). The percentage of relative cell viability was calculated as (A540 of treated samples/A540 of the untreated sample) X 100.

Cell migration assay

cells were sub-cultured in a six-well plate and let to grows to confluence. The monolayer cells were scratched vertically with a 200 µl pipette tip to develop a wound, then the plate was washed two times with PBS to remove cell debris. Afterwards, a new medium with two different conc. of Qu. (7.22 and 0.722µg/ml) was added. The migrating cells were photographed at 0hr and 24hr which indicated by the time required by the control cells to migrate to heal the induced wounds.

Quantitative real-time polymerase chain reaction (RT-PCR)

The cells treated with Qu. for 6 hr and 24hr were collected to extract cellular RNA

(Qiagen, USA), then total RNA was transferred to cDNA (Qiagen, USA). The synthesized cDNA was then amplified with the β-actin, Bcl2 and Bax genes specific primers and SYBR green PCR master mix (Bioline, UK) at the following conditions: the 40 cycles were conducted at 94°C for 1 min, then 60°C for 1 min and 72°C for 1 min; with 10 min final extension at 72°C. The house keeping gene β-Actin, was used as a loading control, and each experiment was repeated three times; the following primers sequences were used: β-actin 5′TTC CTT CCT GGG CAT GGA GTC C-3′ (forward), 5′-GGA GGA GCA ATG ATC TTG ATC TTC ATT G-3′ (reverse); bcl2, 5'GCC GGT TCA GGT ACT CAG TCA3'(forward)5'CAT GTG TGT GGA GAG CGT CAA 3'(reverse); Bax, 5′-' GGA CGA ACT GGA CAG TAA CAT GG-3′ (forward), and 5′-GCA AAG TAG AAA AGG GCG ACA AC-3′ (reverse). Data analysis was conducted and the change at fold change was calculated according to the formula $2^{-\Delta\Delta Ct}$ method for relative quantitation.

Flow cytometry

Hepg2 cells were treated with Qu. at EC50 (7.22µg/ml) and (0.722 µg/ml) doses for one day. The treated and control cells were collected by trypsinization, centrifuged at 200gx, and fixed in cold 70% ethanol for 2 hrs then centrifuged again. Cells were incubated with propidium iodide (PI) in dark for 1 hr at room temperature (Sigma, CA, USA). To eliminate cell aggregates, cells were filtered through a 30mm nylon mesh filter of flow cytometry tubes (BD Bioscience, US). The cell cycle profile analysis was conducted using Cell Quest analysis program (FACS can, Becton Dickinson, Germany) **(Clemente-Soto *et al.*, 2019)**.

3. Results
Effect of Quercetin on Cell viability

To determine half maximum effective inhibitory concentration value (EC50), cells were seeded in 96 well plate (3×10^3 cells/well) then exposed to different concentrations (100,50.25,12.5,6.75,3.75,1.69,0 µg\ml) of Qu for 48hr. A significant decrease in MTT absorbance was recognized Vs Qu. conc. increases indicating a significant inhibitory effect of Qu. on cell growth. The percentage of survived cells in comparison to control untreated cells were plotted (fig. 1).
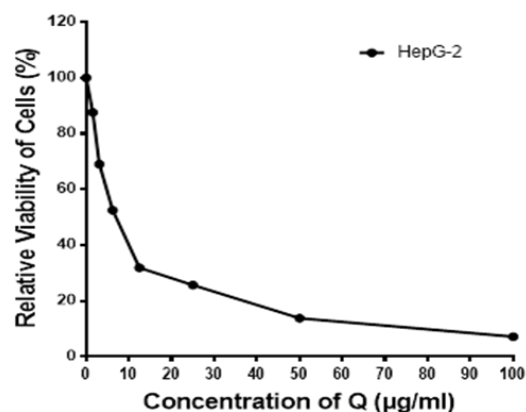

Fig. 1: Effect of Quercetin on Cell proliferation after 48 hr of treatment. The data showed a conc. dependent effect of Qu. In inhibiting cell proliferation.

Induction of cell cycle arrest of Hepg2 cell line by Quercetin

In order to confirm the data of MTT assay regarding the inhibitory effect of Qu. on cell proliferation, cell cycle analysis of cells treated with EC50 (7.22 µg\ml) and EC5 (0.722µg\ml) of Qu. for 24 hr revealed a significant cell cycle arrest at G1/S phase compared to control cells (fig.2).
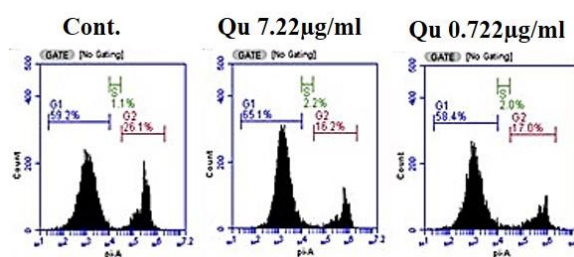

Fig. 2: Effect of Quercetin on HepG2 cell cycle. Cells treated with 7.22 and 0.722 µg/ml of Qu. showed a marked induction of cell cycle arrest at G1/S phase, in a dose dependent manner.

Effect of Quercetin on cell motility

A marked dose dependent effect of Qu. on the size of the artificially induced wound after 24 hr treatment with (7.22 and 0.722 µg\ml) of Qu. (fig.3). These data indicated a marked effect of Qu. in lessen the cells motility and their ability to migrate to close

the induced wound in comparison with control untreated cells which successfully migrate into and close the wound after 24 hr of starting the experiment.
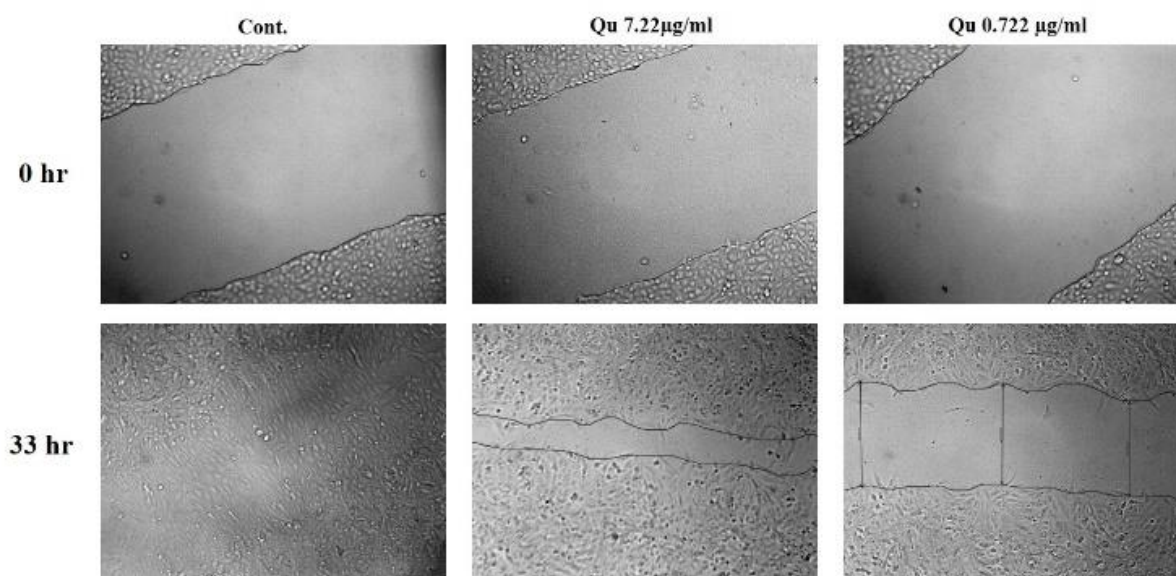

Fig. 3: Effect of Quercetin on cell motility. The ability of cells to migrate was monitored and recorded at 0 hr and 24 hr till closure of the induced wound of the control group. The dose of 7.22 µg/ml showed a marked inhibition at cell migration compared to the 0.722 µg/ml dose of Qu. which indicated by the size of the wound after 24 hr of treatment.

Effect of Quercetin on HepG2 cell survival

A dose and time dependent effect of Qu. on the expression of proapoptotic marker protein Bax and antiapoptotic Bcl2 was assessed using RT-PCR approach in presence and absence of EC50 (7.22µg /ml) and EC5 (0.722µg/ml) of Qu. after 6 and 24 hr of treatment. The data revealed a significant dose and time depnednt effects of Qu. on upraising Bax exression and lessen Bcl2 expression after 6 and 24 hrs (fig.4).
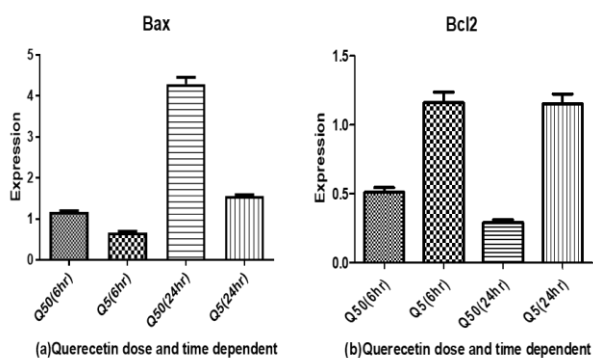

Fig. 4: Quantitative assessment of Quercetin proapoptotic effect on HepG2 cells. RT-PCR data illustrated the significant effect of Qu. on inhibiting Bcl2 expression and upregulating Bax expression.

4. Discussion

The main target in searching for novel drugs, is to develop molecules that are more effective and selective for cancer treatment to lessen the cytotoxicity and impact of serious side effects on normal cells, that been recorded with current chemotherapeutic drugs. Phytochemicals have an essential role in regulating various cell signaling pathways that prevent many physiological disorders such as cancer progression, cardiovascular diseases, and neurodegeneration **(Sak *et al.*, 2016).**

Previous studies showed that Qu. induced cytotoxicity in a diverse of cancer cell lines with various sensitivity for different doses **(Raja *et al.*, 2017).** This *in vitro* study was devoted to elucidate the anticancer activity of Qu. on HepG2 cell line as an *in vitro* model of hepatocellular carcinoma.

The inhibitory effect of Qu. on HepG2 cell proliferation was first illustrated using MTT assay which indicated a dose dependent effect of Qu. in diminishing the ability of cells to proliferate. Furthermore, these data

were confirmed by the ability of the Qu. calculated EC_{50} (7.22µg/ml) and not (0.722 µg/ml) to induce cell cycle arrest at G1/S phase. The resemblance between the structure of Qu. and the PI3K selective inhibitor LY 294002 illustrated its selective abrogation of PT3K/Akt/PKB pathway to inhibit the proliferation of glioblasroma (**Gulati** *et al.***, 2006).** Qu. significantly inhibited the proliferation of LM3 hepatocellular carcinoma cell line via targeting JAK2/STAT3 signaling pathway (**Wu** *et al.***, 2019).**

On the other hand, the data revealed a marked reduction at the ability of HepG2 cells for migration when treated with Qu. in a dose dependent manner. Qu. significantly inhibited the ability of gastric cancer cell lines BGC823 and AGS to metastasis via downregulating the urokinase plasminogen activator (uPA) which in turn decreased the activity of matrix metalloproteinases -2 and -9 (**Li and Chen, 2018**).

A xenograft model of non-small cell lung cancer (NSCLC) cell lines illustrated the role of Qu in diminishing the ability of cells for bone metastasis by suppressing the Snail/Akt mediated epithelial mesenchymal transition (**Chang** *et al.***, 2017).**

Furthermore, Qu. significantly upregulated the expression of the pro-apoptotic protein Bax and downregulating the anti-apoptotic Bcl2 expression pointing out the proapoptotic effect Qu. on HepG2 treated cells.

The higher incidence of cell apoptosis after Qu. supplementation was claimed to its implication in increasing the enzymatic antioxidant activity or inhibition of calcium ions flow and increased pro-apoptotic protein expression that antagonize Bcl-2 expression (**Duo** *et al.***, 2012**).

Qu. activated apoptosis in HepG2 cells by upregulating the Bax translocation to the mitochondrial membrane which led to a subsequent activation of caspases, along with associated inhibition of the PI3K/Akt and ERK signaling pathways (**Rather and Bhagat, 2020**).

In conclusion, our data illustrated the Qu. anticancer effects on the HepG2 cell line as it inhibited cell proliferation, migration, and induction of cell apoptosis via up-regulating pro-apoptotic Bax and down-regulating anti-apoptotic Bcl-2 transcription.

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