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INFLUENCE OF QUERCETIN ON SQUAMOUS CELL CARCINOMA CELL LINE (HEP-2). AN IN-VITRO STUDY

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ABSTRACT

Background: Quercetin is an effective cytotoxic anticancer agent and has been used for the treatment of variety of malignancies. The aim of the current study is to investigate the influence of Quercetin on squamous cell carcinoma cell line (HEP-2) after 24 hours.

Material& methods: Treatment of cell line (HEp-2) by different concentrations of Quercetin was done to assess the viability of the treated cells to determine the percentages of viable cells that was monitored using MTT assay.

Results: the mean viability percentage of Quercetin treated cells (in relation to control cells) increased as Quercetin concentration decreased from 640 mM to 5mM with an IC_{50} of 200 mM. Regarding the cytotoxic effect of Quercetin and related cell cycle profile it was noticed that cell distribution showed a variable percent of arrest at different phase of cell division profile, Where there was non significant difference of arrested cells of Quercetin pre and post treatment compared with its value in non treated G0-G1 phase control cells($P>0.05$) and S phase as well. While there was a significant elevated arrest of treated cells during the G2-M phase ($P<0.05$) and the significant difference of cell arrest at G2-M phase was type of treatment related .

Conclusion: From the results of the current study, we found that Quercetin has cytotoxic effect on squamous cell carcinoma, (HEp-2) cell line, It also induced an effect on the cell cycle distribution, and Quercetin has effect on (HEp-2) cell line resulting in apoptosis and necrosis.

KEY WORDS: Quercetin, oral squamous cell carcinoma cell line (HEp-2), MTT assay.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) representing more than 90% of all oral cancers. It may arise either de novo or from pre existing potentially malignant lesions, within a field of precancerized epithelium [1]. OSCC may affect any oral site, but most frequently the tongue and the floor of the mouth. The main risk factors associated with OSCC include tobacco/betel/areca nut use, alcohol consumption, infection with high-risk human papillomavirus (HPV) genotypes, and a diet low in fruits and vegetables [1].

Quercetin (Qu) (3,3',4',5,7-penta-hydroxyflavone) is one of the most abundant dietary flavonoids. It is found in onions, apples, green tea, grapes, and berries and occurs mainly as glycosides with sugar groups such as glucose, galactose, rhamnose, rutinose, and xylose bound to one of the hydroxyl groups of the flavonol, Quercetin and its glycosylated forms represent 60%–75% of flavonoid intake [2]. Quercetin has been widely used for the prevention and treatment of cardiovascular diseases and cancer. The cancer-preventive effects of quercetin have been attributed primarily to its antioxidant activity; it is able to act as a scavenger of radicals and form complexes with metal ions and DNA [3]. The aim of the current research is to investigate the influence of Quercetin on squamous cell carcinoma cell line (HEP-2) after 24 hours.

MATERIAL AND METHODS

A. Cell line: HNSCC Cell line, larynex cancer cell line (HEP-2)was obtained from cell Culture Department VACSERA- EGYPT.

B. Foetal bovine serum (FBS): FBS was purchased from GIBO COBRAL{R}limited Scoltand as a sterile serum in 500 ml bottle (stored at -20C till used).

C. Phosphate buffer saline (PBS): It was obtained from Sigma Aldrich USA.

D. Trypsin solution:0.25% in PBS, pH 7.5 was supplied from cell culture Media Department VACSERA- EGYPT.

E. Absolute ethanol : (Sigma Aldrich- USA)

F. 70% ethanol in distilled water (BDH-England)

Drug: The drug used was quercetin obtained from MYLAN with molecular formula of $C_{15}H_{10}O_7$ and molecular weight 302.238 g/mol.

Cell line, cell culture and treatment:

Squamous cell carcinoma cell line, larynex cancer cell line (HEp2) was obtained from cell culture line subjected to culture medium for growth and evaluated in the present study. The cell line was maintained in foetal bovine serum (FBS) supplemented medium. Dulbcvco s modified eagle media (DMEM) supplemented with10%(FBS) 2mM, glutamine and sodium bicarbonate (Invitogen) , cell number in the suspension was calculated.

1- Cytotoxicity Assay

a. Principle

Methyl Thiazol Tetrazolium (MTT) assay is a quantitative colormetric method to determine cell proliferation. It utilizes yellow tetrazolium salt (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) which is reduced to an insoluble purple MTT-formazan complex. The MTT cell proliferation assay measures the cell proliferation, when metabolic events lead to apoptosis or necrosis, the reduction for each cell type [4].

b. Procedures

The viability of HEp-2 after treatment with quercetin was determined 24 hours post treatment. For MTT assay, HEp-2 cells were seeded in 96-well culture plates and treated with 5-640 μM of quercetin for 24 hours. Then, 20μL of MTT solution was added to the wells and incubated at 37° C for 5

hours, Thereafter, the medium was gently removed from the wells and 100 pf of DMSO was gently added to each well to dissolve the purple formazan crystals. The data obtained were analyzed using Master Plex Reader Fit program to determine the IC50% (The half maximal inhibitory concentration of the drug). Then Quercetin was added to another 96 well plate for 24 hours and MTT experiment was repeated**.**

2. Microscopic Examination

a. Slides Preparation

The cells were dispended in 25 ml total volume to have a larger examination. HEp-2 cells were treated with either low or high concentration refers to IC50% of Quercetin. The cells were treated with the IC50% of Quercetin for 24 hours. Pelleted cells were re-suspended in PBS and a part (50 µL) was dispended on the clean ethanol washed glass slide, air dried and fixed using methanol while the rest was treated for flow cytometry.

b. Hematoxylin and Eosin Staining

The fixed slides were rehydrated in concentrations of alcohol (100%, 90%, 75% then 50%) and then washed in distilled water for 5 minutes. The slides were immersed in filtered hematoxyline stain for 3 minutes and then washed with distilled water twice. The slides were immersed in filtered eosin stain for 5 seconds and then washed with distilled water. Dried slides were immersed in xylene, and then cover slips were placed and left to dry.

c. Assessment of Hematoxylin and Eosin Stained HEp-2 cells

Ten microscopic fields of each slide were photomicrographed at the power of 400X.This was done using a digital video camera, which was mounted on light microscope. Then, images were transferred to the computer system for analysis. Calculate the circularity and surface area of the cells; from these two parameters we could calculate the nuclear area factor (NAF) which is the product of circularity and surface area.

3. Flow Cytometric Analysis

The remaining cell suspension after taking 50 µL for microscopic examination was fixed by the addition of 700 µL 100% ethanol while vortexing. Fixed cells were centrifuged at 400 g for 5 minutes at room temperature, and ribonuclease was added to the sample to digest RNA-Nuclear staining for DNA content was done using 50 µg /ml propidium iodide (PI) at room temperature for 10 minutes in the dark. Stained cells were filtered through 50 µm pore size nylon mesh; Data were analyzed using Modfit software.

4. ANNEXIN-V staining Kit for detection of apoptosis

Application

Annexin- v is a Ca dependant phospholipid-binding protein with high affinity for ps. This protein can hence be used as a sensitive probe for ps Exposure, necrotic cells expose ps according to the loss membrane integrity. The simulataneous application of DNA stain which is used for dye exclusion tests allows the discrimination of necrotic cells from the annexin V positively Stained cell cluster.

Procedures and required material

Preparation of annexin-v-fluoslabeling solution: predilute 20 µl annexin-v-fluos labeling reagent (vial 1) in 1ml incubation buffer (bottle 3) and add 20 µl PI solution (vial 2) **.**

Staining of cell suspentions

1. 10000 cells with BPS and centrifuge cells were washed at 200 xs for 5 minutes.
2. The cell pellet in 100 µl of annexin-v-fluoslabeling solution was resuspended and incubated 10-15 minutes at 15-25 c.
3. Analysis using flow cytometer was done.

Statistical analysis

The mean values of nuclear area factor (NAF) of different concentrations of the drug and their combinations to the control results were assessed statistically using the statistical package for social science (SPSS 16.0) software for windows.The statistical tests performed included analysis of Variance (ANOVA) for comparison of means of different concentrations of any definite drug and comparison of means of different drugs. The results were considered significant when P value <0,05.

RESULTS

To show the effect of quercetin on viability of HEp-2 cells, MTT Assay was employed. Data obtained reveled that the mean viability percentage of quercetin treated cells (in relation to control cells) increased as quercetin concentration decreased from 640 to 5mM with an IC_{50} of 220 mM. **Table (1)**. Cytological evaluation revealed that Control cells showed relatively regular,hyperchromatic nuclei and regular cellulare outline with minimum folding in the cellular or nuclear membrane as they are malignant cells. Only a few number of malignant cells showed nuclear and cellular pleomorphism. A small number of control cells showed the morphological criteria of apoptosis as nuclear fragmentation **Fig. (1**a). On the contrary, drug-treated cells showed nuclear morphological modifications which corresponds to the morphological parameters of apoptosis in its various stages. **Fig. (1a &2 a,b). Morphometric Analysis, Nuclear Area Factor (NAF):** The mean value of NAF of HEp-2 cells treated cells treated with Quercetin are shown in **table (2).** The data recorded revealed a decrease in the mean values of NAF of HEp-2 cells treated with quercetin when compared to that of control cells the mean value decreased from 0.056 to 0.042 in pre to post treatment of quercetin. **Flow Cytometric Analysis:** cell distribution showed a variable percentage of arrest at different phases of cell division profile. The concentration of quercetin produced pre G1 apoptosis with cell cycle arrest at G1/S phase. With post quercetin concentration, the percentage of cells in S G2/M phases has significant change **Fig. (3).**

Effect of quercetin on cell apoptosis: The highest fraction of control cells are living cell (in lower left quadrant) with only few necrotic and apoptotic cells, necrotic cells (apoptotic cells in the lower right quadrant and necrotic cells in upper right quadrant)with increasing the dose of quercetin the fraction of apoptotic cells increased **Fig.(4)**.

TABLE (1): The mean viability percentage of HEp-2 cells treated with different concentrations of quercetin for 24h.

Quercetin concentration	640mM	320mM	160mM	80Mm	40Mm	20mM	10mM	5mM
%Viability	24.21	51.07	53.36	88.21	96.93	97.50	100.29	100.0

TABLE (2): The mean values of nuclear area factor of HEp-2 cells post treatment with Quercetin.

	Control	Quer Pre	Quer IC50%	Quer Post
NAF	0.056	0.055	0.047	0.042

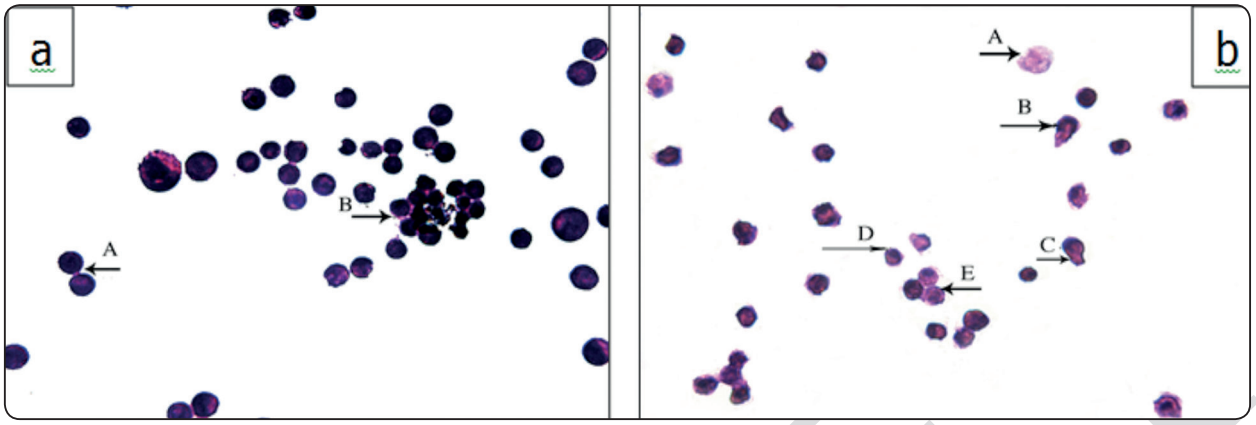

Fig. (1) (a) A photomicrograph of HEp-2 cells (control cells) 24hours showing Hyperchromatic and condenced nuclei (A) and abnormal mitotic figures (B). (b) A photomicrograph of Hep-2 cell 24 hours post treatment with pre IC50 concentration of Quercetin showing necrotic cell (A), irrigular nuclear out line (B), membrane blebbing (C), nuclear shrinkage (D), adhesion of cells(E),) (H&E ,x 400).

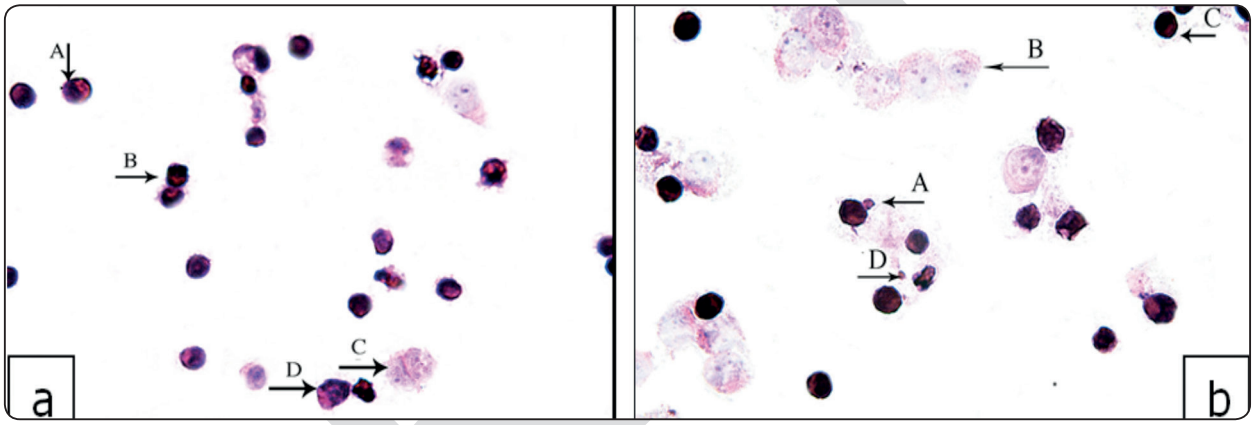

Fig. (2) (a) A photomicrograph of Hep-2 cell 24 hours post treatment with IC50 concentration of Quercetin showing peripheral chromatin condensation (A), apoptotic body (B),swollen necrotic cells with cellular swelling(C).patchy chromatin (D). (b):A photomicrograph of HEp-2 cells 24 hours post treatment with post IC50 concentration of Quercetin showing budding of apoptotic body(A) and necrotic cells (B) and peripheral condensation of chromatin (C) apoptotic body (D)(H&E,x 400).

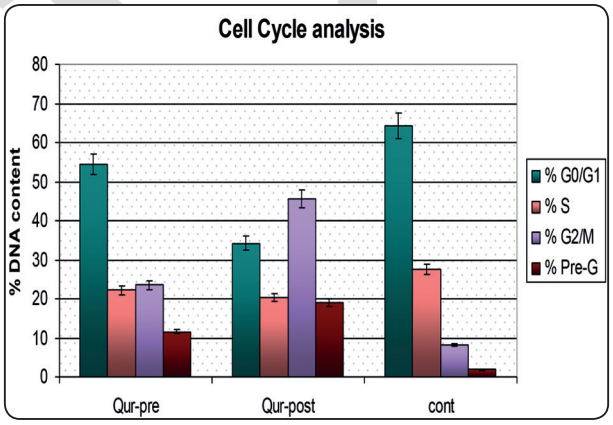

Fig. (3) The effect of different quercetin concentrations on cell cycle distribution

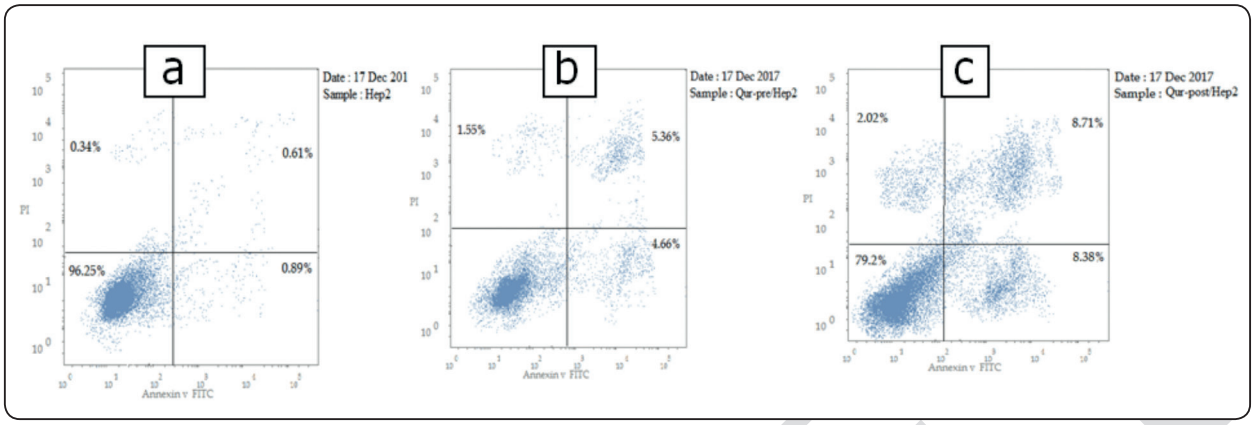

Fig. (4): Effect of different concentration of quercetin on cell apoptosis showing. (a)The highest fraction of control cells are living cell (in lower left quadrant) with only few necrotic and apoptotic cells. (b) in pre IC50 apoptotic cells in the lower right quadrant and necrotic cells in upper right quadrant increased, (c) with increasing the dose of quercetin (post IC50) the fraction of apoptosis cells increased.

DISCUSSION

Squamous cell carcinoma (SCC) is the second most common form of skin cancer comprises 90-95 % of all oral malignancies. Oral squamous cell carcinoma (OSCC) which is the most common form of head and neck cancer, is accounting for ~3% of malignancies worldwide and 500,000 newly diagnosed cases annually [5] ; [6]. Quercetin is widespread in nature and can be found in various food products and plants, including fruits, seeds, vegetables, tea, coffee, bracken fern, and natural dyes. Qu is one of the natural antioxidant; its anticancer properties have been proved by in vivo and in vitro experiments [7]. MTT assay revealed the inhibitory effect of each concentration of Qu on the viability of all cancer HEp-2 cell lines after 24 h incubation; reveled that the mean viability percentage of Qu treated cells (in relation to control cells) increased as Qu concentration decreased from 640 mM (24.21%) to 5 mM (100%) with an IC_{50} (half maximal inhibitory concentration) of 220 mM. IC_{50} value is a measure of the effectiveness of a compound in inhibiting biochemical processes and biological functions. It represents the concentration of the particular drug that is required for 50% inhibition of the cell viability which is safe for normal cells and it was calculated as previously described by [8].This approach revealed that the administration of Qu significantly reduced the tumor volume at all utilized doses. Therefore, the inhibitory effect of Qu on the growth of utilized cancer cell lines was dose dependent, as demonstrated by the obtained IC_{50} values of Qu. Treatment with quercetin revealed obviously increased the percentage of apoptotic cells. The percentage of cells in early apoptosis increased from 4.66 with pre IC_{50} to 8.38 with post IC_{50} compared to control cells 0.89. At the late apoptosis, The percentage of cells were in pre IC_{50} 5.36 increased to 8.71 in post compared to 0.61 in control cells. At last, there was increase in the total cell death (apoptosis and necrosis) as quercetin concentration increased from pre IC_{50} 11.57 to post IC_{50} 19.11 when compared with untreated control cells 1.89. In the current study we found increasing of apoptotic cells with increase concentrations of quercetin and this agreed with [7], [9] who found that microscopic observations after the 24-h incubation of Hep-2 cells demonstrated an increased number of apoptotic cells especially after the treatment with Qu and imperatorin administered at the same time. The concentration of quercetin produced pre G1 apoptosis with cell cycle arrest at G1/S phase. With post quercetin concentrations, the percentage of

cells in S G2/M phases has significant change. Ren et al., 2015[10] discussed in his study the effect of Qu on the proliferation of the human ovarian cancer cell line that cell and found that apoptosis are playing a key role in the regulation of the proliferation of tumor cells. Also, Hashemzaei et al., 2017[11] who assessed the anticancer activity of Qu at 10, 20, 40, 80 and 120 μM in vitro by MMT assay tumor cell lines and found that there was induction of apoptosis in LNCaP, MOLT-4 and Raji cell lines treated with Qu as assessed by flow cytometer Annexin V/PI and found the results revealed that Qu significantly induced apoptosis in comparison to the control group (P<0.001) and this was in accordance with our results. In this study we need to confirm whether Qu caused apoptosis or necrosis, so, another confirmatory assay was needed to clarify that annexin flow cytometry staining assay which showed a dose dependent increase in the number of apoptotic cells in relation to control cells with a minor increase in the fraction of necrotic cells as necrosis can peak into Sub-G1 area but close to G1 peak where as apoptotic peak will be far off from G1 peak.

CONCLUSION

Cytotoxicity assay, microscopic examination, and flow cytometry analysis revealed that Quercetin able to suppress cancer cell by inducing tumor cell apoptosis or cell cycle arrest. In the present study, the total effects of Qu on apoptosis assay of Hep-2 cancer cells were investigated in order to provide an experimental basis for the clinical application of Qu in the treatment of OSCC cancer.

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