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Increased anticancer efficacy by the combined administration of quercetin in multidrug resistant breast cancer cells

Krishnamoorthy Kavithaa[1*], Sundaravadivelu Sumathi[1*], Manickam Paulpandi[2], Palakkad Ragunathan Padma[1],

[1] Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher education for Women University, Coimbatore-641 043, TN, India

[2]Proteomics and Molecular Cell Physiology Lab, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore-641 046, TN, India.

Correspondence should be addressed to Krishnamoorthy Kavithaa and Sundaravadivelu Sumathi.

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Abstract

Quercetin is one of the bioactive flavonoid isolated from the leaves of *Euphorbia hirta* (Euphorbiaceae). The isolated quercetin was investigated for its antiproliferative activity in human breast adenocarcinoma MCF-7 cells. Furthermore, the combined efficacy of quercetin with standard chemotherapeutic drug tamoxifen also studied. The results showed greater reduction in the cell viability from the QuerTam group (quercetin with tamoxifen) than tamoxifen and quercetin alone group. The IC_{50} values of test compounds were determined as 2µM, 0.5µM and 1.8µM for quercetin, Quertam and tamoxifen respectively. An elevated induction of apoptosis was found in QuerTam treatment when compared to quercetin and tamoxifen alone. The combined efficacy of QuerTam showed significant inhibition of cell viability by decreasing Bcl-2 protein expression and inducing apoptosis. Flow cytometry revealed a decrease in normal cells and increasing pro and late apoptotic cells indicative of apoptosis induction, while fluorescence microscopy showed evidence of the induction of apoptosis and nuclear fragmentation. To summarize these results showed that quercetin enhances the MCF-7 cell sensitivity to tamoxifen, as demonstrated by the improvement in the anticancer therapy.

Keywords: Quercetin, MCF-7 cells, anti-cancer, *Euphorbia hirta*, Bcl-2, Tamoxifen

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Introduction

The most common group of plant polyphenols are flavonoids which include six subclasses: flavones, flavonols, flavononones, catechins, anthocyanidins and isoflavones (Ross and Kasum 2002). One of the best described flavonol, quercetin, is found in large quantities in onions, apples, broccoli and berries (Erlund, 2004). Quercetin effects are multi-targeted. It has a wide range of biological activities including radical scavenging activity and angiogenesis (Tan et al. 2003). Moreover, quercetin as a pro-oxidant induces apoptosis in cancer cells and as antioxidant and chemopreventive agent protects normal cells against oxidative stress and mutagenesis (Ishisaka *et al.*, 2011).

Of the diverse polyphenols that have been studied, quercetin (Fig 1) has received considerable attention. Dietary quercetin has been shown to increase the resistance of low-density lipoprotein to oxidation, although it did not significantly affect lipoprotein profiles or platelet aggregations, which are also risk factors for heart disease (Serafini *et al.*, 1998). Following oral dosage of quercetin, effects on the predominant antioxidant enzymes were not significant, although inhibition of oxidative damage to lymphocyte DNA was noted (Lean *et al.,* 1999). These results emphasize the importance of further investigating the molecular events responsible for the significant antiproliferative effect afforded by this quercetin.

Breast cancer is a cancer that starts in the tissue of the breast. It could be invasive or noninvasive. The incidence of breast cancer in developing countries is on the increase due to many underlying factors such as birth control measures, sliding to Western culture, lifestyle and genetic mutations. It is the most common cancer diagnosed among women, constituting 23% of all cancer cases in the world. 226,870 new cases of invasive breast cancer are expected to occur among women in 2012; about 2,190 new cases are expected in men .Several differences exist between countries with respect to age, stage at presentation, and biological characteristics of breast cancer cases. In developed countries approximately 50% of all women with newly diagnosed breast cancer are older than 63 years, while in many developing countries women with breast cancer are predominately younger than 50 years of age (Zaemey *et al.,* 2012).

Chemotherapy is one of the important treatment methods, but the side effects are difficult to tolerate, and as a result, people have being paying more attention to searching for new antitumor agents from natural products. Chemotherapy remains the principal mode of treatment for various cancers (Dai *et al.*, 2011). Chemotherapy and radiotherapy, the conventional cancer treatments used nowadays, are expensive and cause many side effects, including such minor ones as vomiting, alopecia, diarrhea, constipation, and major ones such as myelosuppression, neurological, cardiac, pulmonary and renal toxicity. All such side effects reduce the quality of life and discourage patients to observe medication protocols which then lead to the progression of cancer and associated complications (Castro *et al.*, 2011). A major problem of cancer therapy is the side effects of chemotherapy. Another key problem in cancer treatment is the reducing sensitivity of tumor cells to cytotoxic drugs. The majority of chemotherapy medicines have serious adverse effects in addition to their clinical effects. Patients find these side effects hard to tolerate, which often causes chemotherapy failure (Ko *et al.*, 2011).

Minimizing side effects and maximizing efficacy is a major goal in the development of cancer treatment. Now days the usage of combination of naturally derived active compounds with existing anticancer drug has been dramatically increased, because of its selectivity of target cell, reduced level of side effects, enhanced activity in controlling the cancer cell proliferation when compared with standard drug alone. Complementary medicines are a group of medicines and practices that may be used in addition to the standard treatments for enhanced cancer therapy. Furthermore the synergetic effect of complementary medicines is biocompatible,

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satisfactory stability, low toxicity and target-orientation (Kuete and Efferth 2011**)**. However, quercetin has been shown to increase transformed cell sensitivity to cytotoxic drugs and to reduce resistance to anticancer drugs in cancer cells *in vitro* (Woudea *et al.,* 2003). Therefore, the aim of the current study was to determine if quercetin could increase the sensitivity of MCF-7 cells to tamoxifen.

Materials and Methods

Plant material

The leaves of *Euphorbia hirta* (L.) were collected in Western Ghats region in November 2011. Botanical identification was carried out by Prof. G.V.S. Murthy (Botanical Survey of India, Coimbatore). A voucher specimen (No.BSI/SRC/5/23/2011-12/Tech.1077) has been deposited in the laboratory of Botanical Survey of India, Coimbatore.

Cell line and culture conditions

Human breast cancer MCF-7 cells were purchased from National Centre for Cell Science (Pune, India). The cells were maintained in Dulbecco's Modified Eagles medium supplemented with 2mM L-glutamine and Earle's BSS adjusted to contain 1.5g/l Sodium bicarbonate, 0.1mM nonessential amino acids, and 1.0mM of Sodium pyruate in a humidified atmosphere containing 5% CO_2 at 37^{o}C.

Isolation and identification of active compounds

Shadow air-dried leaves of *Euphorbia hirta* were reduced to a fine powder (50g), and soxhlet extracted with absolute methanol (250ml) for 24h. The extract was filtered using Whatman No.1 filter paper and the filtered methanolic extract was concentrated to dryness under vacuum desiccator and the solvent was removed by vacuum evaporation. The solution was partitioned with diethyl ether in a 1:1 ratio using a separating funnel. The diethyl ether part was concentrated to obtain the extract. Quercetin from extract of *Euphorbia hirta* was isolated from fractions separated by distillation and subsequent chromatography.

High performance liquid chromatography (HPLC) analysis

All solvents used were of analytical chromatographic grade (Sigma–Aldrich). HPLC was performed with HPTi series 1050 liquid chromatography, equipped with a photodiode array detector (DAD, HPseries 1050). Solutions of the tested compound (5%, v/v in ethanol) was subjected to normal phase HPLC analysis carried out on a Phenomenex Hypersil 3µm C18 BDS (100 mm ×4.6 mm) column using a mobile phase of ethanol at a flow rate of 1.0 ml/min. The UV detection (DAD) at 210 nm was recorded. Elute fractions obtained from HPLC analyses were further subjected to GC–MS analysis after concentration under vacuum.

Gas Chromatography–Mass Spectrometry (GC–MS) analysis

The chemical composition of the methanolic leaf extract of *Euphorbia hirta* was analyzed using GC–MS. The essential oil (10µl) was dissolved in acetone (100µl) and 1µl of the solution was injected into a GC–MS (QP-2010, Shimadzu Co., Kyoto, Japan). The capillary column was Rtx-5MS (length = 30 m, i.d. = 0.25 mm, thickness = 0.25µm). Helium was used as the carrier gas at a flow rate of 0.94 ml/min. The column inlet pressure was 55.8 kPa. The GC column oven temperature was increased from 60 to 170°C at a rate of 10°C/min, with a final hold time of 10 min. Injector and detector temperatures were maintained at 150°C. EI mode was at 70 eV, while mass spectra were recorded in the 45–450 amu range and ion source-temperature was 200^{o}C. Sample components were identified by matching their mass spectra with those recorded in NIST08s, Wiley-8 and FAME Library.

Cell viability assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, exponentially growing cells (1×10^{4}cells/well) were seeded into a 96 well plate and allowed to attach overnight. Cells were treated with the various concentrations of

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different groups (quercetin alone, QuerTam and tamoxifen alone) in at three replicate wells and left contact for 48h. 500 µg/ml of MTT was added to each well, and the plate was further incubated at 37°C for another 4h. The formazan crystals formed and dissolved in DMSO. Absorbance was determined with a multiwell ELISA reader at 550nm. Absorbance values were expressed as percentage relative to the control.

Cell morphology analysis

MCF-7 cells (5×10^3) grown on 24-well plates were treated with different groups (quercetin, QuerTam and tamoxifen) at different doses of 1, 2, 4and 8µM/ml for 6, 12 and 24h. The morphological changes were observed under an inverted microscope (Nikon Eclipse TS100, Japan).

Fluorescence microscopic analysis of cell death

Approximately 1µl of a dye mixture (100 mg/ml Acridine orange (AO) and 100 mg/ml Ethidium bromide (EtBr) in distilled water) was mixed with 9ml of compound treated cell suspension (1×10^5 cells/ml) on clean microscope cover slips. The MCF-7 cells were collected, washed with phosphate buffered saline (PBS) (pH 7.2) and stained with 1ml of AO/EtBr. After incubation for 2min, the cells were washed twice with PBS (5min) and visualized under a fluorescence microscope (Nikon Eclipse, Inc, Japan) at 400X magnification with an excitation filter at 480nm.

Propidium iodide staining

The cells were seeded ($1x10^6$cells/ml) into wells containing DMEM medium with tested compounds. After 24h incubation at 37°C in a CO_2 incubator, cells were harvested and fixed with methanol and acetic acid fixative. Later cells were smeared over clean dry and non-greasy glass slides. In brief, smeared cells were stained with Ethidium bromide (10mg/ml) solution. The cells were immediately washed with phosphate buffer and observed under (Nikon Eclipse, Inc, Japan) fluorescent microscope to analyze the nuclear fragmentation.

DAPI staining

Cells were plated on glass coverslip in a 24-well plate and treated with compounds for 24h. The fixed cells were permeabilised with 0.2% triton X-100 (50µl) for 10min at room temperature and incubated for 3min with 10µl of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were observed under (Nikon Eclipse, Inc, Japan) fluorescent microscope.

Hoechst staining

Cells were plated on glass coverslips in a 24-well plate and treated with compounds for 24h. After the treatment 10µl of Hoechst stain was added and placed a coverslip over the cells to enable uniform spreading of the stain. The nuclear morphology of treated groups was analyzed under inverted fluorescent microscope.

Apoptotic analysis of MCF-7 cells

The apoptotic effects of tested compounds on MCF-7 cells were determined by the Annexin V-FITC and propidium iodide double staining method of flow cytometry. The MCF-7 cells (1×10^5cells/ml) were treated with various tested compound concentrations and cultured for 6h. The treated cells were harvested, washed with PBS, and then treated with trypsin/EDTA solution. The suspended cells were centrifuged at 200×g for 10min. The cell pellet was added in 100µl of Annexin V-FITC staining-solution (Strong Biotech Co., Taipei, Taiwan) and incubated for 10 –15 min at 25°C. The cells were then analyzed with flowcytometer.

Western blot analysis

Western blotting was performed to detect the proteins of caspase-3 and Bcl-2 family. MCF-7 cells (1.5×10^6) were seeded onto 100mm culture dishes in the presence or absence of treatment compounds, and were treated for 12h. The medium was removed and the cells were washed with PBS (0.01M, pH7.2) for several times. Following removal of the supernantant solution, the cells were lysed with lysis buffer (0.1 ml lysis buffer/each plate) for 20min. The supernatants were collected by centrifugation at 10,000×g for 5min at 4°C, and were used as the cell protein extracts. The

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harvested protein concentration was measured using a protein assay kit (Bio-Rad). The same amounts of proteins from each extract were applied to 12% SDS–polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (Millipore, Bangalore), and then blocked for 1h using 10% skim milk in water. After washing in PBS containing 0.1% Tween 20 for 3 times, primary antibodies against caspase-3 and Bcl-2 or β-actin were added at a v/v ratio of 1:1000. After overnight incubation at 4°C, the primary antibodies were washed away and secondary antibodies were added for 1h incubation at room temperature.

Assessment of DNA fragmentation

Cells were treated with compound individually at 80nM/ml concentration and were lysed in DNA digestion buffer containing 50mM Tris-HCl, pH8.0, 10mM EDTA, 0.1M NaCl and 0.5%SDS. The lysate was incubated with 0.5mg/ml RNase at 37°C for 1h. Phenol extraction of this mixture was carried out and DNA in the aqueous phase was precipitated by 25ml of 7.5M ammonium acetate and 250ml isopropanol. Isolated DNA was subjected to electrophoresis in1.5% agarose gel containing 1mg/ml ethidium bromide at 70 volt and bands were visualized by exposing the gel to UV light and photographed.

Statistical analysis

All the experiments were performed in triplicates for each group. The data were expressed as mean ± standard deviation. Differences with p value <0.05 were considered to be statistically significant.

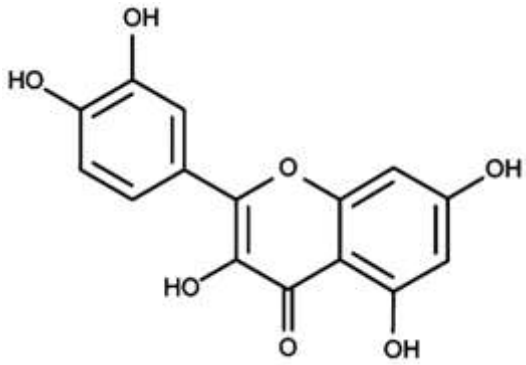

Figure 1. Structure of quercetin

Result

Identification of active compound

The qualitative GC/MS results are listed along with the retention indices of the 10 identified compounds (Fig 2a). The HPLC analyses under normal phase conditions (CN-column and ethanol as a mobile phase) allowed the active components to separate into the different groups of classes of increasing polarity. The separation and purification of quercetin was confirmed with the assistance of HPLC (Fig 2b). The composition of the HPLC fractions was subsequently confirmed again by GC–MS analysis. The GC–MS spectrum revealed the presence of compound quercetin (100%).

Antiproliferative effect of quercetin, QuerTam/ or Tamoxifen

The aim was to investigate the potent combined efficacy of quercetin, QuerTam/or tamoxifen on the viability of MCF 7 cells after treatment with various concentrations (0.1–5 µM/ml) of the tested compounds for 48h. The experimental results indicated that the compounds inhibited cell proliferation in a dose dependent manner (Fig. 3). The IC_{50} value of the tested compounds in MCF-7 cells determined as quercetin, QuerTam and

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tamoxifen (2μM, 0.5 μM and 1.8μM/ml) respectively by MTT assay.

Cell morphology

Fig. 4 shows alterations of morphological changes to MCF-7 cells after treatment with the tested compounds treatment for 6–24h. Phase-contrast micrograph reveals that the QuerTam induced more cell shrinkage, membrane blebbing and forming the floating cells rather than the quercetin or tamoxifen alone in a dose-dependent manner.

AO/EtBr staining assay for detection of apoptotic cells

On the basis of the overall cell morphology and the cell membrane integrity, necrotic and apoptotic cells were distinguished from one another using fluorescence microscopy (Nikon Eclipse, Inc., Japan). To examine if the significant effect of QuerTam is due to apoptosis, we examined MCF-7 cells for its nuclear morphology. Fluorescence microscopy of nuclear dye stained MCF-7 cells showed that in the absence of the drug, the nuclei were round and homogeneous, whereas quercetin, QuerTam, and tamoxifen treatment caused a reduction of cell volume, nuclear condensation (a hallmark of apoptotic cells), and increased non-adherence of the cells to the culture surface. Orange apoptotic cells containing apoptotic bodies were observed as orange colored bodies whereas the necrotic cells were observed to be in red color. After treatment with their respective IC_{50} concentrations of tested compounds for a period of 24h, compound induced apoptotic changes in the cells as observed (Fig. 5). It was observed that the total number of apoptotic cells (green to orange and red color) increased when the increased concentrations of tested compounds were added. Exclusively the QuerTam showed more pro and late apoptotic cell at the 0.5μM concentration and it was more significant when comparable to other groups.

Fluorescence analysis of nuclear fragmentation

The fluorescence microscopic studies reveal that the effective concentration 0.5μM significantly induces the apoptosis in MCF-7 cells by causing nuclear fragmentation. Cells that were exposed to quercetin or tamoxifen require increased concentration to cause the same effect by QuerTam. Cells treated with the compound and stained with nuclear dye Propidium Iodide (PI), showed notably elevated levels of nuclear fragmentation has occurred in cells treated with QuerTam rather than quercetin and tamoxifen alone group. These findings concluded that the combined efficiency of isolated quercetin with tamoxifen. Moreover lower to moderate levels of nuclear fragmentation was seen in cells treated with quercetin and tamoxifen alone (Fig 6).

Interestingly, cells were exposed to respective IC_{50} concentrations of quercetin/ QuerTam or tamoxifen and stained with nuclear dye Hoechst 33258, we found that increased fluorescence in the group treated with combined drug whereas, quercetin and tamoxifen alone treated groups showed decreased fluorescence when compared with QuerTam group (Fig 7). Similarly DAPI staining also revealed that significant level of nuclear fragmentation found in QuerTam treated group when compared to rest of quercetin and tamoxifen alone groups (Fig 8).

Flow cytometric analysis of quercetin, QuerTam and/or tamoxifen

An elevated index of apoptosis was observed in cells treated with QuerTam (Fig. 9c) compared to cells that received quercetin and tamoxifen alone. This result was based on data showing that the QuerTam group had more number of apoptotic cells detected by flowcytometer. Consequently, we also observed a significant apoptotic cells in quercetin groups (Fig. 9b, d).

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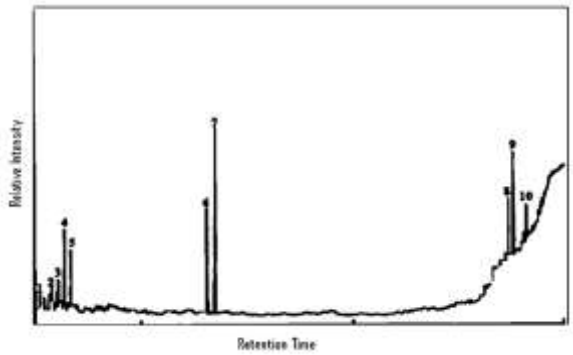

Figure 2a. GC chromotograph of *Euphorbia hirta* fraction.

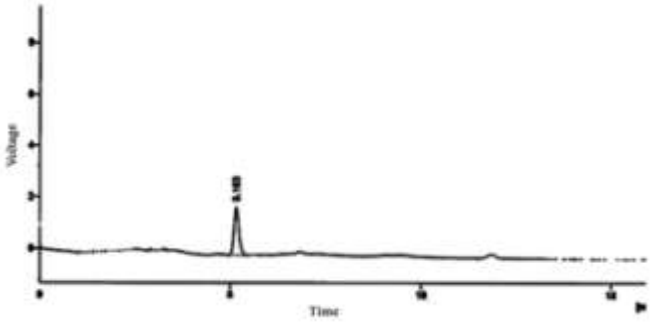

Figure 2b. Purification of quercetin with the assistance of HPLC.

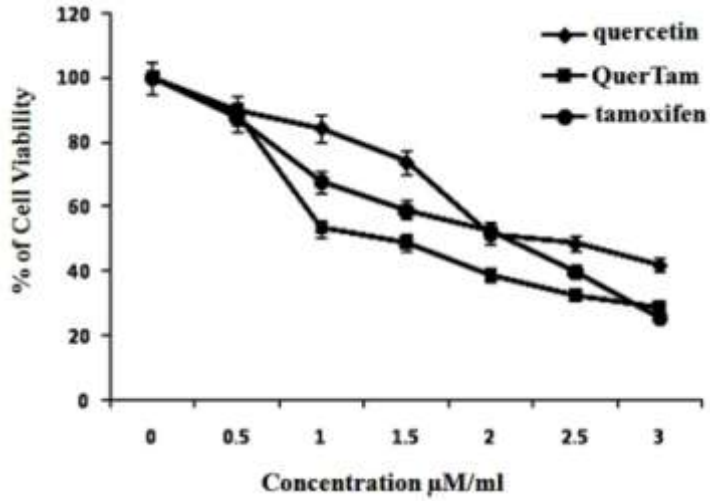

Figure 3. Anti proliferative activity of quercetin, QuerTam and tamoxifen on multidrug resistant MCF-7 cells.

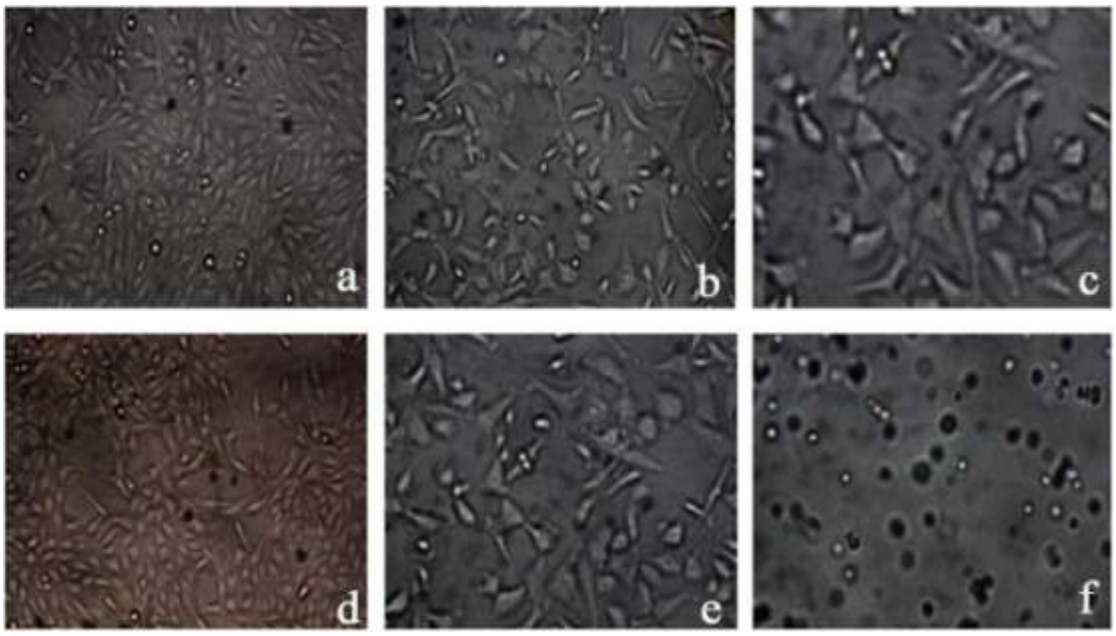

Figure 4. Morphological characterization of MCF-7 cells after 48h exposure to quercetin, QuerTam and tamoxifen.

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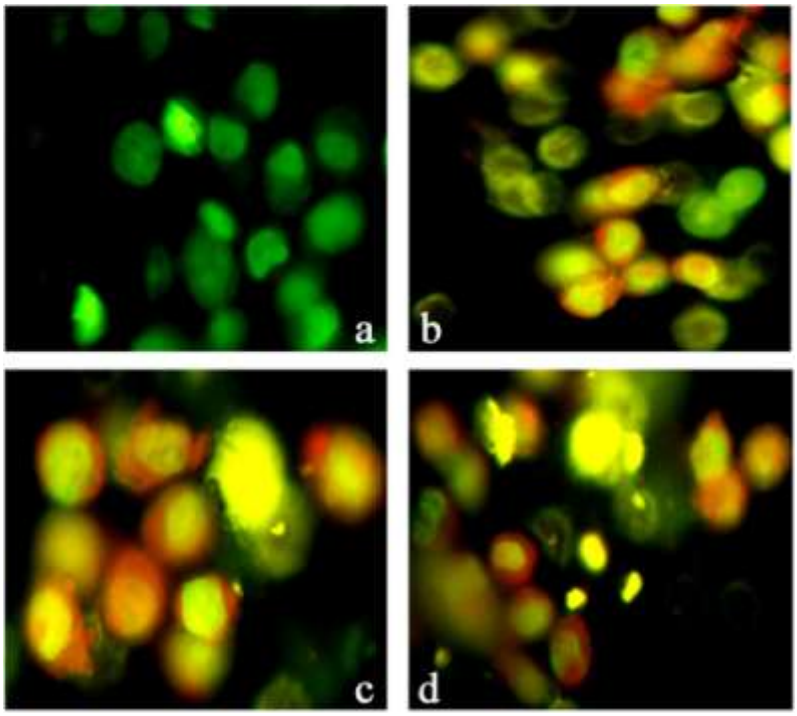

Figure 5. MCF-7 cells were stained with AO/EtBr. (a) Control, (b) 2μM quercetin treated, (c) 0.5μM QuerTam treated and (d) 1.8 μM tamoxifen treated.

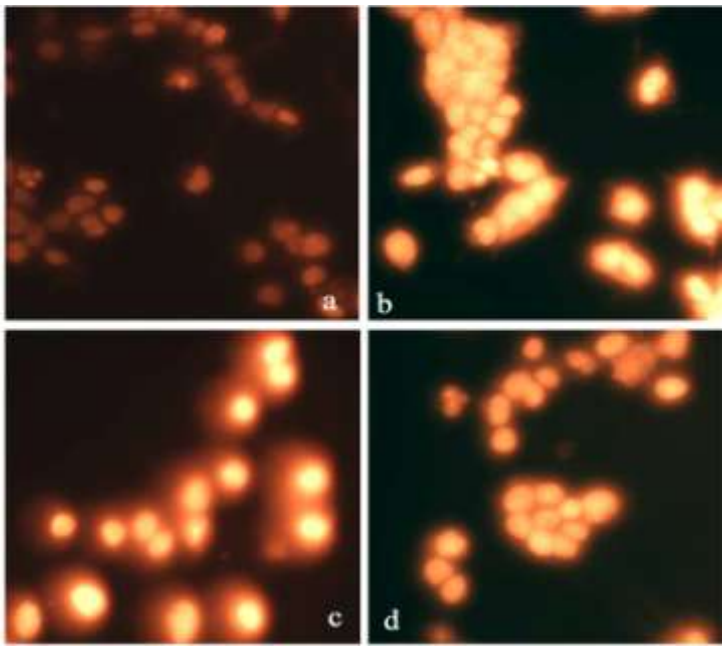

Figure 6. Propidium iodide staining. (a) Control, (b) 2μM quercetin treated, (c) 0.5μM QuerTam treated and (d) 1.8 μM tamoxifen treated.

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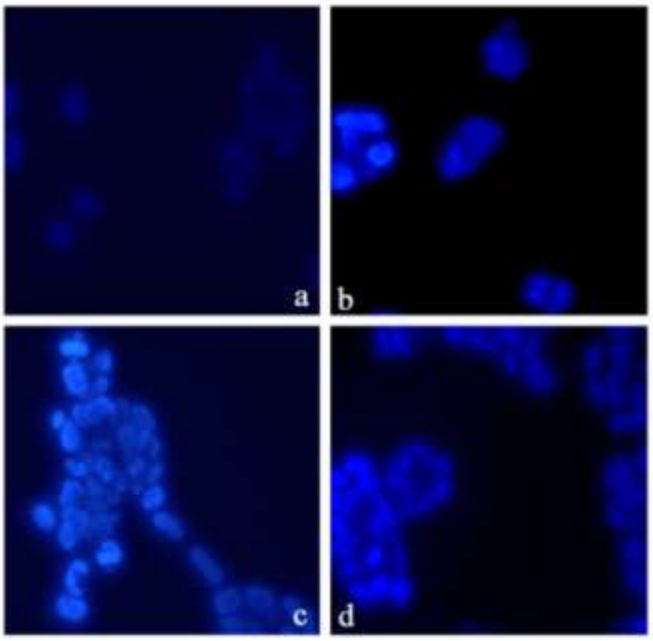

Figure 7. Hoechst 33258 staining. (a) Control, (b) 2μM quercetin treated, (c) 0.5μM QuerTam treated and (d) 1.8 μM tamoxifen treated.

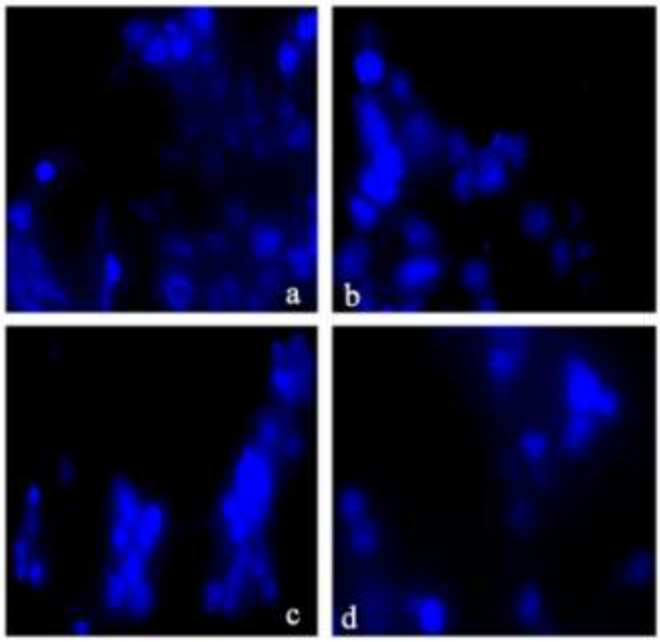

Figure 8. Nuclear dye DAPI staining. (a) Control, (b) 2μM quercetin treated, (c) 0.5μM QuerTam treated and (d) 1.8 μM tamoxifen treated.

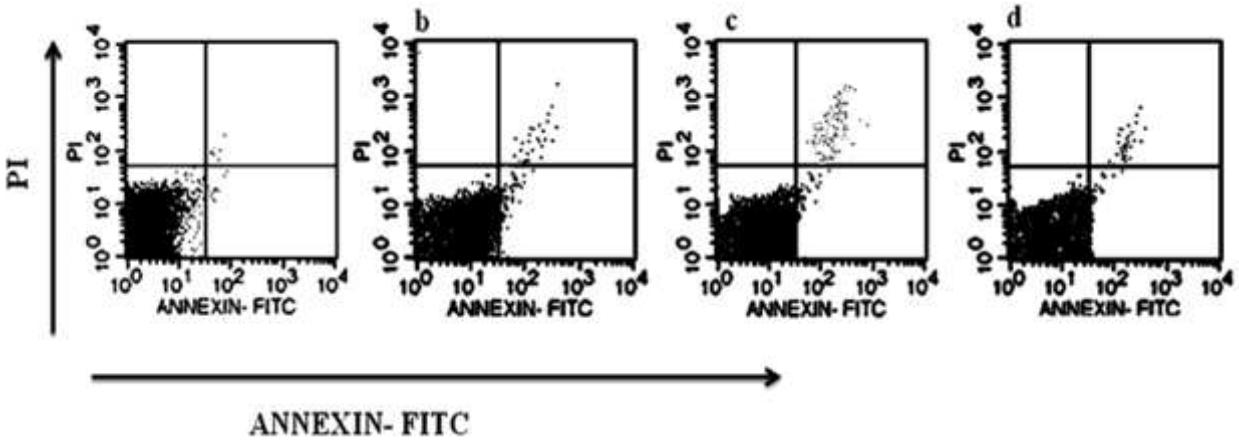

Figure 9. Flowcytometric analysis of apoptotic detection. (a) Control, (b) 2μM quercetin treated, (c) 0.5μM QuerTam treated and (d) 1.8 μM tamoxifen treated.

Effect on Bcl-2/ Bax/ or caspase expression

Recent investigations of the Bcl-2 gene family have shown a complex network regulating apoptosis. Bcl-2 is an integral membrane protein that prevents apoptosis in multiple biological systems. The expression of Bcl-2 correlates inversely with the susceptibility of cells to apoptosis. We examined the cellular levels of Bcl-2 expression after treatment of MCF-7 cells with 0.5μM, 2μM and 1.8μM test compounds respectively. In the mean time we also assessed the pro apoptotic protein Bax. We found that the significant decrease in Bcl-2 (fig. 10b) and elevated level of caspase-3 were observed after treatment and the expression of Bcl-2 was not comparable to groups treated with quercetin and tamoxifen alone (fig. 10a, c). The apoptotic protein Bax expression was gradually increased with the increased concentration of tested compounds in all groups.

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Effect of quercetin/ QuerTam/ or tamoxifen on DNA fragmentation

Initial results of Propidium Iodide, Acridine Orange, DAPI and Hoechst orange staining had shown that the compounds induce apoptosis and it was intended to compare the DNA fragmentation pattern. Therefore, nuclear DNA was isolated from treated MCF-7 cells and subjected to agarose gel electrophoresis. As illustrated in (Fig. 11), electrophoresis of DNA isolated from 0.5µM QuerTam treated MCF-7 cells for 8h exhibited a typical ladder formation in contrary to, quercetin and tamoxifen where clear cleavage pattern was not observed.

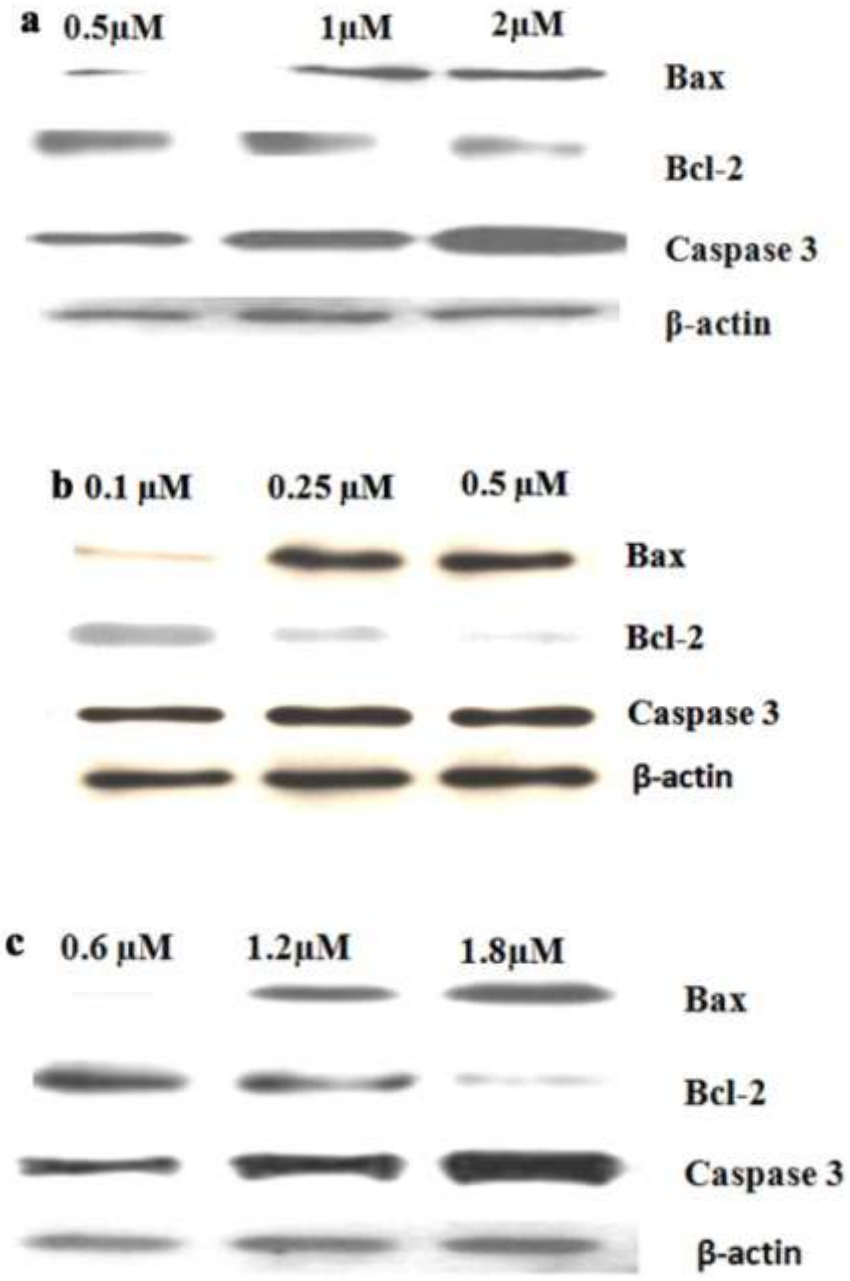

Figure 10. Effect of tested compounds on the expression of apoptosis-related proteins in MCF-7 cells. (a) Expression pattern of, casepase-3, Bcl-2 and Bax in cells treated with quercetin; (b) Cells treated with QuerTam; (c) Cells treated with tamoxifen. β-Actin was used as internal control.

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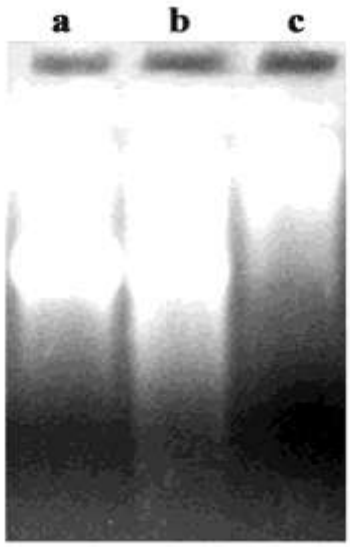

Figure 11. DNA fragmentation analysis of MCF-7 cells. Lane a: 2μM quercetin treated, Lane b: 0.5μM QuerTam treated and Lane c: 1.8 μM tamoxifen treated.

Discussion

Tamoxifen is considered to be one of the most potent chemotherapeutic agents discovered to date; however, severe side effects in normal tissues and acquired resistance to tamoxifen in tumor cells has limited its use (Dorssers et al. 2001). Therefore, combination therapies that enhance the anticancer effectiveness of tamoxifen without increasing the toxicity are highly desirable. This study demonstrates, for the first time, that quercetin enhances the sensitivity of MCF-7 cells to tamoxifen and suggests that quercetin could be useful in therapies that utilize tamoxifen in multidrug resistant cancer cells.

In this study, at a lower concentrations of QuerTam showed significant inhibitory effect on cell proliferation. So far, a combined effect of QuerTam on cell proliferation has been reported for the multidrug resistant human breast carcinoma cell line MCF-7, which showed growth inhibition after a 24h exposure to 0.5μM (Fig. 3). There are so much mechanism have been reported to explore on MCF-7 inhibition through activation apoptosis but, the mitochondrial pathway both intrinsic and extrinsic related efficacy of quercetin with tamoxifen yet not been reported. Herein we reported that the combined administration of quercetin with tamoxifen causes significant reduction of viability in multidrug resistant human breast carcinoma cells through activation of apoptotic related pathways (Fig. 5).

Generally, apoptosis can occur via two fundamental pathways, the mitochondrial or intrinsic pathway and the death receptor or extrinsic pathway (Yoon and Gores 2002). The intrinsic pathway is triggered by release of mitochondrial proteins, such as cytochrome C, which bind with Apaf-1 and procaspase-9 in a dATP-dependent manner to form the apoptosome (Mignotte and Vayssiere 1998). The apoptosome can induce activation of caspase-9, thereby initiating apoptotic caspase cascades (Hengartner 2000). The QuerTam induces mitochondrial enzymes leading to caspase-3 activation and cause apoptotic related cell death (Fig. 9). The intrinsic pathway of apoptosis is regulated by the Bcl-2 family of proteins. Anti-apoptotic (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic (e.g. Bad, Bax and Bak) are two of the major members in Bcl-2 family (Zhong et al. 1993). Anti-apoptotic Bcl-2 and Bcl-xL inhibit apoptosis by sequestering proforms of capsases or by preventing the release of mitochondrial apoptogenic factors (Adams and Cory 2007). Bad, Bax and Bak inhibit Bcl-2 activity and promote apoptosis (Reed 1995). In this study, QuerTam treatments altered the

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expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins, resulting in apoptosis of MCF-7 cells apoptosis (Fig. 10). Furthermore, the elevated intracellular ratios of Bax/Bcl-2 occur during increased apoptotic cell death (Fig. 10) therefore, the increased Bax expression promotes apoptosis. These experimental findings suggest that combined administration QuerTam induced multidrug resistant MCF-7 cells to apoptosis via the mitochondrial pathway. QuerTam exhibited more significant anti proliferative activity on multidrug resistant MCF-7 human breast carcinoma rather than the quercetin and tamoxifen alone groups. In summary this combinatorial effect had a strong anti-proliferative effect on MCF-7 cells and also quercetin enhances the multidrug resistant MCF-7 cell sensitivity to tamoxifen.

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