

Baicalein 5, 6, 7-trimethyl ether from *Physalis pubescens* L. leaves ameliorate the effect of epirubicin in HepG2 cancer cells by inducing apoptosis and autophagy

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Abstract

Context:

flavonoids have a pivotal cytotoxic effect against hepatocellular carcinoma (HCC).

Aims

To reveal which flavonoid isolated from *Physalis pubescens* L. leaves exerts the most cytotoxic effect against HepG2 liver cancer cells and if it could ameliorate epirubicin efficacy and safety through regulating apoptosis and autophagy.

Methods

Baicalein trimethyl ether (BTE), rutin, quercitrin and myricitrin were isolated from *Physalis Pubescens* L. leaves. HepG2 cells were treated with the isolated flavonoids as well as a combination of BTE and epirubicin. Cell viability was assessed by MTT assay after 48hr treatment. In addition, the chromosomal DNA fragmentation in HepG2 cells, a biochemical hallmark of apoptosis, was assessed. Autophagy and apoptosis markers, ATG7 and TGF- β 1, were determined by RT-qPCR.

Results

BTE showed the best cytotoxic effect against HepG2 cells ($IC_{50} = 95.6 \pm 5.849 \mu\text{g/mL}$). Combination of epirubicin with (200 $\mu\text{g/mL}$) BTE significantly decreased the IC_{50} of epirubicin from $2.79 \pm 0.626 \mu\text{g/mL}$ to $0.76 \pm 0.258 \mu\text{g/mL}$ ($p = 0.04$). Moreover, the same combination significantly increased the IC_{50} of BTE against WI-38 normal cells ($p < 0.0001$). DNA fragmentation was increased in HepG2 cells treated with BTE and BTE + epirubicin compared to untreated cells. BTE and BTE + epirubicin significantly downregulated gene expression of TGF- β 1 while upregulated ATG7 gene expression.

Conclusions

BTE (200 $\mu\text{g/mL}$) significantly increased the cytotoxicity of epirubicin against HepG2 cells and increased its safety profile. It could exert anti-hepatocarcinoma effect via increasing apoptosis and autophagy. It could be a promising adjuvant therapy to ameliorate epirubicin cytotoxicity against HCC with less adverse effects.

Introduction

Hepatocellular carcinoma (HCC) is a universal problem and it is considered the fifth most common malignancy and the second leading cause of cancer-related death worldwide [1]. Chemotherapy remains the main treatment of malignancy but it is linked with harmful side-effects and acquired cancer resistance. Consequently, substantial efforts have been conducted to develop new alternative therapeutic options for malignant diseases [2] [3].

Epirubicin is a chemotherapeutic drug belongs to anthracyclines topoisomerase II inhibitor. It is an effective anticancer drugs, but the cardiotoxicity and the development of malignant cell resistance limit its use [4]. The need to solve the resistance problem and to reduce the effective dose of epirubicin to overcome its cardiotoxicity is necessary [5].

Natural products from plants are interesting attractive candidates for development of anticancer agents due to their strong anticancerous efficiency, low toxicity, and high availability [6]. Moreover, phytochemicals like resveratrol, gingerol, curcumin, ginsenoside, genistein, and quercetin can ameliorate the adverse effects of chemotherapy due to their antioxidation, anti-inflammation, antimutagenesis, immunomodulation, and myeloprotection [7].

Flavonoids are polyphenolic compounds which are found in plants and are consumed as part of the human diet. They attracted high attention due to their broad biological applications [8]. Flavonoids compounds among the different natural products were very useful in preventing and treating different human cancer with less adverse effects, through their antioxidant, anti-inflammatory, and antiapoptotic effects as well as modulating autophagy (Yan et al. 2018) [9] [10].

Autophagy is a homeostatic process involved the degradation of aged and damaged organelles and proteins by the lysosome. It is consider as a survival mechanism which provides energy for cell [11]. Autophagy plays a critical role in cancer and there is a strong link between autophagy and malignancy. The autophagy process can be a tumor suppressor or a pro-oncogenic mechanism and many studies have revealed the dual role of autophagy, promoting either cell death or cell survival in cancer progression [12]. Consequently, regulation of autophagy using both natural product or synthetic drugs can be used as reliable interventional strategies for cancer treatment [13].

This study aimed to reveal which one of the flavonoids isolated from *Physalis pubescens* L. leaves exerts the most cytotoxic effect against HepG2 liver cancer cells and if it could ameliorate epirubicin efficacy and safety and the underlying molecular mechanism.

Materials And Methods

Plant material:

This study was approved by the Research Ethical Committee, Faculty of Pharmacy, Tanta University, Egypt (BC0009) and all methods were performed in accordance with the relevant guidelines and regulations. Leaves of *P. pubescens* L. were collected from Shebin el-kanater of Qalyubia governorate.

They were collected in May and identified by Prof. Dr. Mohammed Ibrahim Fotoh, professor of ornamental horticulture and landscape design, Faculty of Agriculture, Tanta University. Voucher specimens (PG00413) was deposited at Pharmacognosy Department Herbarium in Tanta University. Leaves were dried to afford two kg powdered leaves of *P. pubescens* L [14].

Extraction and fractionation of *Physalis Pubescens* L. leaves:

Concentrated methanol extract (95%) was obtained of leaves of *P. pubescens*. The yield of dry residues of crude extract was 11.3%. The air-dried powdered leaves of *P. pubescens* (2 kg) were extracted three times with 7 L of MeOH (95%) at each time. The total extract was concentrated under vacuum to obtain a gummy residue which was redissolved in 50% MeOH and partitioned successively with petroleum ether, methylene chloride, ethyl acetate and *n*-butanol.

Isolation of baicalein 5, 6, 7-trimethyl ether from *Physalis Pubescens* L. leaves:

The total methanol extract 95% of *P. pubescens* leaves was fractionated successively with petroleum ether, methylene chloride, ethyl acetate and *n*-butanol. Column chromatography of methylene chloride residue (3 g) was performed using silica gel column (120 g, ϕ 3.5 x 30 cm). The elution was done using methylene chloride- methanol mixtures, polarity increased with increasing percentage of methanol in CH₂Cl₂: methanol mixtures. At methylene chloride: methanol (94: 6) fractions (31–39) were combined based on their analytical TLC profile and evaporated under vacuum to obtain 233 mg of dry residue. This residue was subjected to a silica gel column chromatography (20 g, ϕ 2 x 15 cm). The elution was done with mixtures of CH₂Cl₂: methanol (using methanol to increase polarity). Sub-fractions 5–11 eluted by methylene chloride: methanol (95: 5) were combined to give 105 mg of dried matter, they were re-chromatographed with silica gel column (10 g, ϕ 1.2 x 10 cm) to have 6 mg of dry white powder of baicalein 5, 6, 7-trimethyl ether or 5, 6, 7-trimethoxy-2-phenyl-4H-chromen-4-one.

Baicalein trimethyl ether (BTE), rutin, quercitrin [15] and myricitrin [14] were separated from *Physalis pubescens* L. leaves. The cytotoxic effect against HepG2 cancer cells of the separated flavonoids were evaluated by MTT cell viability/toxicity assay. The flavonoid which exerted the best cytotoxic effect was combined with epirubicin (Epirubicin® vials, Mylan S.A.S., France) to examine the ability of natural flavonoid to reduce the cytotoxic dose of epirubicin against HepG2 cancer cells. The molecular mechanism of action of the flavonoid exerted the most cytotoxic effect against HepG2 cells or its combination with epirubicin could be determined by measuring its effects on DNA fragmentation (apoptosis). Moreover, the effect of the most cytotoxic flavonoid or its combination with epirubicin on the gene expression of transforming growth factor beta 1 (TGF- β) and autophagy-related 7 (ATG7) in HepG2 cells was observed.

Cell lines:

HepG2 liver carcinoma and WI-38 normal human cells were purchased from the Cell Culture Department of the Holding Company for Biological Products & Vaccines (Vacsera®), Dokki-Giza, Egypt. Cells were

cultured in Dulbecco's modified Eagle medium (DMEM), with glucose and L-glutamine (Lonza Verviers Sprl®, Belgium) supplemented with 10% fetal bovine serum (Sigma-Aldrich®, USA), 1% of penicillin/streptomycin/amphotericin B (Gibco®, USA) and incubated at 37°C in a 5% CO₂ prior to use. The medium was then replaced with fresh DEMEM-10% FBS. The cells were maintained by sub-culturing them after arriving 90% confluence.

MTT cell viability assay against HepG2 cancer cells

HepG2 and WI-38 cancer cells were seeded on a 96-well plate at a concentration of 10⁴ cells/well in a volume of 100 µl in fresh medium and left for 24 hours, at 37°C under 5% CO₂ atmosphere, to attach to the wall of the plate and reach exponential growth. After 24 hours, cells were incubated with different concentrations of drugs. Determination of IC₅₀ was done by treating different groups of HepG2 cells as following: I treated with Dimethyl Sulfoxide (DMSO) (BDH®, England) (control group), II treated with 0.21, 0.42, 0.85, 1.7, 3.4 µg/mL (epirubicin), III, IV, V and VI treated with 31.25, 62.5, 125, 250, 500 µg/mL of baicalein trimethyl ether, rutin, quercitrin and myricitrin. Triplicate wells were prepared for each individual concentration. After 48 hours, the medium was aspirated off 20 µl yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT solution 10X, Sigma- Aldrich®, USA) were added to each well, and the plate was incubated for an additional 4 hours at 37°C. One hundred microliter of acidified isopropanol was added to each well to solubilize the formed purple formazan crystal, and absorbance was then measured at 630 nm using ELISA microplate reader (Bio-RAD micro plate reader, Japan). The flavonoid with the most cytotoxic effect against HepG2 was added to epirubicin and IC₅₀ of epirubicin in the combined form was determined on HepG2 cells. Moreover, the cytotoxic effect on WI-38 normal cells of this flavonoid and the combination were carried out to assess the IC₅₀ and safety profile.

% cell survival = (absorbance of test cells / absorbance of control untreated cells) X 100

The IC₅₀ values were determined, which are the concentrations of drugs required to produce 50% inhibition of cell growth [16].

DNA fragmentation

To determine drug-induced apoptosis in HepG2 cells, chromosomal DNA fragmentation, a biochemical hallmark of apoptosis, was performed. The HepG2 cells (1 × 10⁶ cells) of different groups were incubated with media containing different drugs for 24 h. After cells were harvested and washed with PBS and DNA was isolated using Blood and Cell Culture DNA Mini Kit (Qiagen®, Germany) as per manufacturer's protocol. Total genomic DNA isolated from each sample was subjected to RNase digestion (10 µg/ml) followed by separation on 2% agarose gels. The gels were stained with 0.5 µg/ml ethidium bromide; and images of the stained gels were depicted [17].

RT-qPCR

The gene expression of TGF-β1 and ATG7 in harvested cells were determined by real time polymerase chain reaction (RT-qPCR). Total RNA was extracted from HepG2 cells using RNeasy® Mini kit (Qiagen®,

Germany). The extracted RNA concentration was measured using Namodrops (Denovix®, USA) and the extracted RNA was used for the synthesis of Complementary DNA (cDNA) by QuantiTect® reverse transcription (Qiagen®, Germany). The synthesized cDNA was used for determination of ATG7 and TGF- β 1 genes using QuantiTect® SYBR Green PCR Kit (Qiagen®, Germany). The used primers were TGF- β 1 (Cat. No. QT00000728), ATG7 (Cat. No. QT00008974), and β -actin or ACTB (Cat. No. QT00095431). They were all purchased from (Qiagen®, Germany).

The threshold cycle (Ct) of target genes were compared to the Ct of the housekeeping gene (β -actin) and expressed as fold change. The real-time PCR program started with initial heat activation step at 95°C for 15 min, followed by 50 cycles (94°C for 15 s, 55°C for 30 s, and at 72°C for 30 s) using Rotor-Gene Q 5plex (Qiagen®, USA). The fold change gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method [18].

Statistical analysis

The data are represented as mean $\pm$ standard error (SE) and standard deviation (SD). The statistical analysis of data was carried out by using unpaired t-test and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, *P*-value less than 0.05 were considered as statistically significant.

Results

Baicalein 5, 6, 7-trimethyl ether (BTE) was efficiently isolated from *Physalis Pubescens* L. leaves:

In our study we isolated BTE from *Physalis Pubescens* L. leaves using column chromatography technique (Fig. 1). Cytotoxicity of BTE alone or its combination with epirubicin was evaluated against HepG2 hepatocellular carcinoma cells to detect if the use of BTE as adjuvant therapy could help in augmenting the cytotoxic effect of epirubicin and reducing its side effects.

Baicalein trimethyl ether isolated from *Physalis pubescens* L. was the flavonoid with the most cytotoxic effect against HepG2 liver cancer cells:

The percent of viability of HepG2 cancer cells after incubation with different concentration of our treatments for 48 hr were determined using MTT assay (Fig. 2). Figure (3C) demonstrated the 50% inhibitory concentration (IC₅₀) of different treatments on HepG2 cancer cells. It was figured out that IC₅₀ values on HepG2 cancer cells were 2.79 $\pm$ 0.626 μ g/mL (epirubicin), 95.6 $\pm$ 5.849 μ g/mL (BTE), 219.36 $\pm$ 9.667 μ g/mL (rutin), 111.73 $\pm$ 7.445 μ g/mL (quercitrin), 241 $\pm$ 10.908 μ g/mL (myricitrin). The flavonoid with the most cytotoxic effect against HepG2 hepatocarcinoma was BTE, so combinations between BTE and epirubicin were carried out to assess if BTE could increase cytotoxicity of epirubicin against HepG2 and reduce its adverse effects. The first combination was BTE (95.6 μ g/mL) + 0.4, 0.8, 1.2, 1.6, 2, 2.4 and 2.8 μ g/mL epirubicin (EP). IC₅₀ was more than 2.8 μ g/mL (more than the IC₅₀ of epirubicin alone) (Fig. 2C), so the second combination was prepared as BTE (200 μ g/mL) + 0.4, 0.8, 1.2, 1.6, 2, 2.4 and 2.8 μ g/mL epirubicin. IC₅₀ was 0.76 $\pm$ 0.258 μ g/mL of epirubicin combined with (200 μ g/mL) BTE (Fig. 2D).

Baicalein trimethyl ether (200µg/mL) augmented the cytotoxic effect of epirubicin against HebG2 liver cancer cells and increased its safety profile:

Our data from MTT viability assay showed that, the combination of epirubicin with 200µg/mL BTE significantly decreased the IC_{50} of epirubicin against HebG2 cells from $2.79 \pm 0.626 \mu\text{g/mL}$ (Fig. 2B) to $0.76 \pm 0.258 \mu\text{g/mL}$ (Fig. 2D) ($p = 0.040$).

On the other hand, IC_{50} of BTE on WI-38 normal cells was $243.4 \pm 11.667 \mu\text{g/mL}$ (Fig. 3A). Moreover, IC_{50} of BTE in the combination of BTE 100, 200, 300, 400, 500 µg/mL and 600 + epirubicin 0.76 µg/mL on WI-38 normal cells was $570.8 \pm 13.72 \mu\text{g/mL}$ (Fig. 3B). More interestingly, combination of epirubicin (0.76 µg/mL) with BTE significantly increased the 50% inhibitory concentration (IC_{50}) of BTE against WI-38 normal cells from $243.4 \pm 11.667 \mu\text{g/mL}$ to $570.8 \pm 13.72 \mu\text{g/mL}$ ($p < 0.0001$) increasing the safety profile of the combination (Fig. 3D).

Baicalein trimethyl ether and its combination with epirubicin increased DNA fragmentation and induced apoptosis in HebG2 liver cancer cells:

To determine the effect of BTE or its combination with epirubicin on DNA fragmentation of liver cancer cells, DNA fragmentation of HebG2 cells treated with epirubicin (0.76µg/mL) or BTE (95.6µg/mL) or epirubicin (0.76µg/mL) + BTE (95.6µg/mL) or epirubicin (0.76µg/mL) + BTE (200µg/mL) was analyzed by electrophoresis. Interestingly, DNA fragmentation was observed in all the treated groups compared to untreated HebG2 control cells. The present data strongly suggest the induction of apoptosis in liver cancer HebG2 cells as the possible antiproliferative mechanism of our different treatments (Fig. 4).

Baicalein trimethyl ether and its combination with epirubicin decreased the gene expression of TGF-β1 in HebG2 liver cancer cells:

RT-qPCR was used to evaluate the effect of epirubicin (0.76 µg/mL), BTE (200 µg/mL), and their combination on the gene expression of TGF-β1 (as marker of apoptosis) in HebG2 cells. Epirubicin, BTE and their combination significantly downregulated the gene expression of TGF-β1 (0.523, 0.421, and 0.457 fold respectively) compared to the control untreated HebG2 cells (1 fold) ($p = 0.000$) (Fig. 5A).

Baicalein trimethyl ether and its combination with epirubicin increased the gene expression of ATG7 in HebG2 liver cancer cells

In the current study RT-qPCR was used to evaluate the effect of epirubicin (0.76 µg/mL), BTE (200 µg/mL), and their combination on the gene expression of ATG7 (as marker of autophagy in HebG2 cells). Epirubicin, BTE and their combination significantly upregulated the gene expression of ATG7 (8.281, 10.773 and 13.653 fold respectively) compared to the control untreated HebG2 cells (1 fold) ($p = 0.000$). More interestingly, the gene expression of ATG7 was significantly higher in HebG2 cells treated with the combination of BTE and epirubicin than HebG2 cells treated with BTE or epirubicin alone ($p = 0.001$ and 0.03 respectively) (Fig. 5B).

Discussion

Hepatocellular carcinoma (HCC) is one of the most common malignancies with high fatality rate worldwide [1]. Unfortunately, treatment protocols of HCC include traditional resection and chemotherapy, all of which can lead to high mortality rate [19] [3].

Epirubicin is a potential chemotherapeutic agent but its cardiotoxicity and the development of drug resistance limit its use [4]. Many studies have showed that flavonoids have a potential anti-cancer effects against wide range of malignancy [20] and it can ameliorate efficacy of chemotherapeutic agents, increase its safety profile and decrease cancer resistance [21] [22] [14].

Our study aimed to reveal which flavonoids isolated from *Physalis pubescens* L. leaves extract could exert the most cytotoxic effect against HepG2 liver cancer cells and if it could ameliorate epirubicin efficacy and safety.

Flavonoids include compounds of phenylalanine derivatives, and flavonol glycosides which are represented in this study by baicalein, quercitrin, rutin and myricitrin. In the present study we isolated BTE for the first time from *Physalis Pubescens* L. leaves using column chromatography technique. This plant belongs to family Solanaceae A. L. de Jussieu, Nightshade or Potato family. Genus *Physalis* includes 95 species [23].

In the present study, HepG2 cells were treated with extracted flavonoids for 48hr then cell viability was determined by MTT assay and 50% inhibitory concentration (IC_{50}) was calculated. The flavonoid with the most cytotoxic effect against HepG2 liver cancer cells was BTE ($IC_{50} = 95.6 \pm 5.849 \mu\text{g/mL}$). Our findings were supported by those demonstrated by [24], who found that baicalein could suppress human liver cancer cell SMMC-7721 proliferation and induce apoptosis.

Herein, the flavonoid with the most cytotoxic effect against HepG2 cells was BTE, so combinations between BTE and epirubicin were carried out to assess if BTE could increase the cytotoxicity of epirubicin against HepG2 and reduce its adverse effects. The first combination was by adding $95.6 \mu\text{g/mL}$ BTE to epirubicin. The IC_{50} of epirubicin in combination with $95.6 \mu\text{g/mL}$ BTE was more than the IC_{50} of epirubicin alone. Consequently, there was no increase in epirubicin cytotoxicity by using this concentration of BTE. So we prepared a second combination by using higher concentration of BTE ($200 \mu\text{g/mL}$). The IC_{50} of epirubicin against HepG2 cells was decreased significantly from $2.79 \pm 0.626 \mu\text{g/mL}$ to $0.76 \pm 0.258 \mu\text{g/mL}$ by using this higher concentration of BTE ($p = 0.04$).

In support of our results, [25] found that baicalein could enhance the sensitivity of the resistant HCC (Bel7402/5-FU) to 5-FU and epirubicin by increasing the intra-cellular concentrations of epirubicin and decreasing drug efflux.

In this study human lung fibroblasts (WI-38) cells were used to determine the safety profile of BTE and its combination with epirubicin. Our MTT viability assay results revealed that, combination of epirubicin

(0.76 µg/mL) with BTE significantly increased the 50% inhibitory concentration (IC₅₀) of BTE against WI-38 normal cells from 243.4 ± 11.667 µg/ mL to 570.8 ± 13.72 µg/ mL ($p < 0.0001$) increasing the safety profile of the combination. Our results were supported by [26] who revealed that oral baicalein was generally safe and well-tolerated in healthy Chinese subjects.

DNA fragmentation and chromatin condensation are the two hallmarks of apoptosis [27]. In this study, we determined the effect of epirubicin, BTE, and their combinations on DNA fragmentation of HepG2 liver cancer cells. Epirubicin, BTE, and their combinations significantly increased the DNA fragmentation of HepG2 cells. The present data strongly suggest that our treatments induced apoptosis in liver cancer HepG2 cells as the possible antiproliferative mechanism.

Transforming growth factor (TGF)-β1 is a cytokine which regulates a variety of cell functions, including growth, differentiation, and apoptosis [28]. In the early-stage adenoma, TGF-β1 plays a role as a tumor inhibitor in non-cancerous epithelial cells. In the carcinogenic stage, TGF-β1 changes from a tumor suppressor to a carcinogen and promotes the development of cancer [29].

In the current study RT-qPCR was used to evaluate the effect of epirubicin (0.76 µg/mL), BTE (200 µg/mL), and their combination on the gene expression of TGF-β1 (as marker of apoptosis) in HepG2 cells. Epirubicin, BTE and their combination significantly downregulated the gene expression of TGF-β1 (0.523, 0.421, and 0.457 fold respectively) compared to the control untreated HepG2 cells (1 fold) ($p = 0.000$). In support of our findings [30] illustrated that baicalein could inhibit cell proliferation and activate apoptosis in breast cancer cells in vitro.

Autophagy is a homeostatic process which plays a critical role in cancer. The autophagy process can be a tumor suppressor or a pro-oncogenic mechanism [12]. In our study, the gene expression of autophagy associated gene (ATG7) was determined using RT-qPCR as marker of autophagy. Epirubicin, BTE and their combination significantly upregulated the gene expression of ATG7 (8.281, 10.773 and 13.653 fold respectively) compared to the control untreated HepG2 cells (1 fold) ($p = 0.000$). More interestingly, the gene expression of ATG7 was significantly higher in HepG2 cells treated with the combination of BTE and epirubicin than HepG2 cells treated with BTE or epirubicin alone ($p = 0.001$ and 0.03 respectively). Our findings were in line with [30] who found that baicalein could increase autophagy of breast cancer cells in vitro.

Conclusions

BTE (200µg/mL) significantly increased the cytotoxicity of epirubicin against HepG2 cells and increased its safety profile. It exerted anti-hepatocarcinoma effect via increasing apoptosis and autophagy. It could be a promising adjuvant therapy to ameliorate epirubicin cytotoxicity against HCC with less adverse effects on normal cells. Further in vivo studies on the use of BTE in combination with epirubicin to confirm the safety and efficacy of the combination are recommended.

Declarations

Conflict of Interest:

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards:

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability:

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

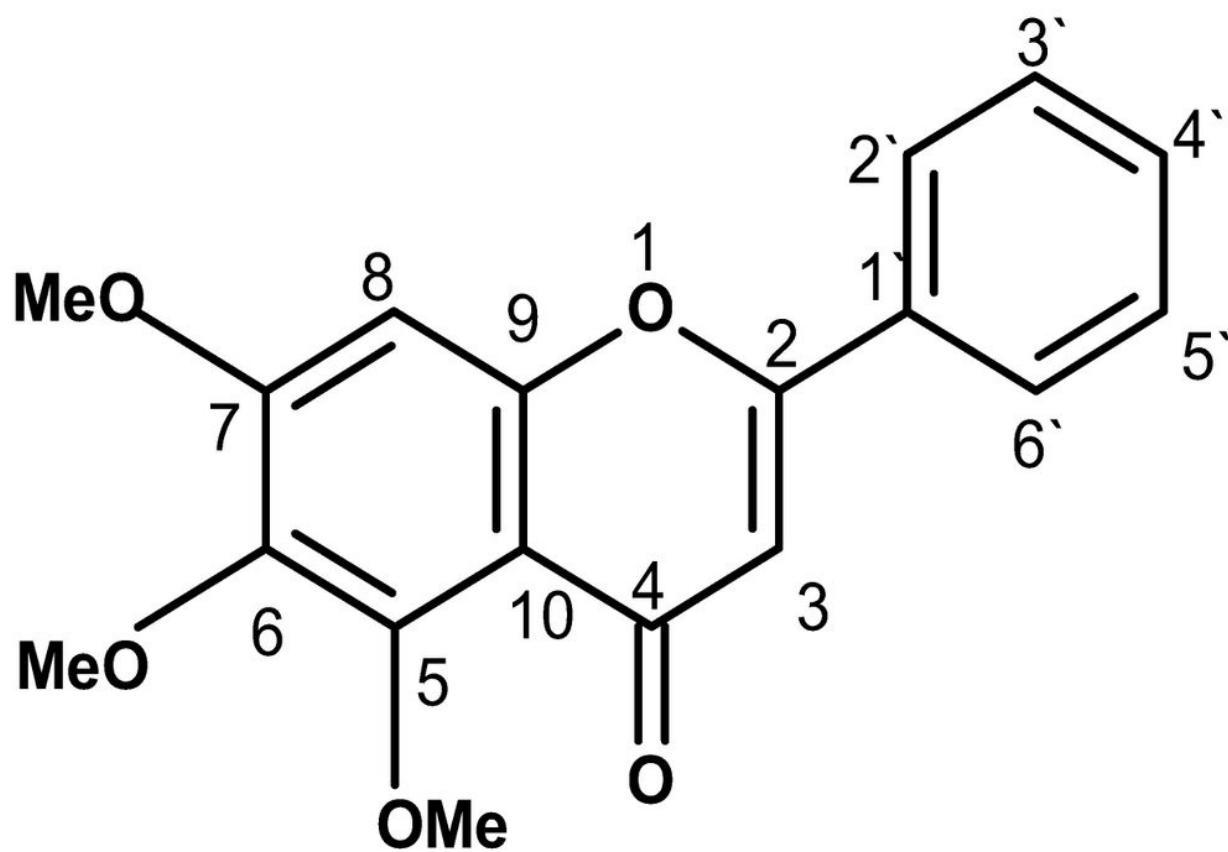


Figure 1

Baicalein 5,6,7-trimethyl ether.

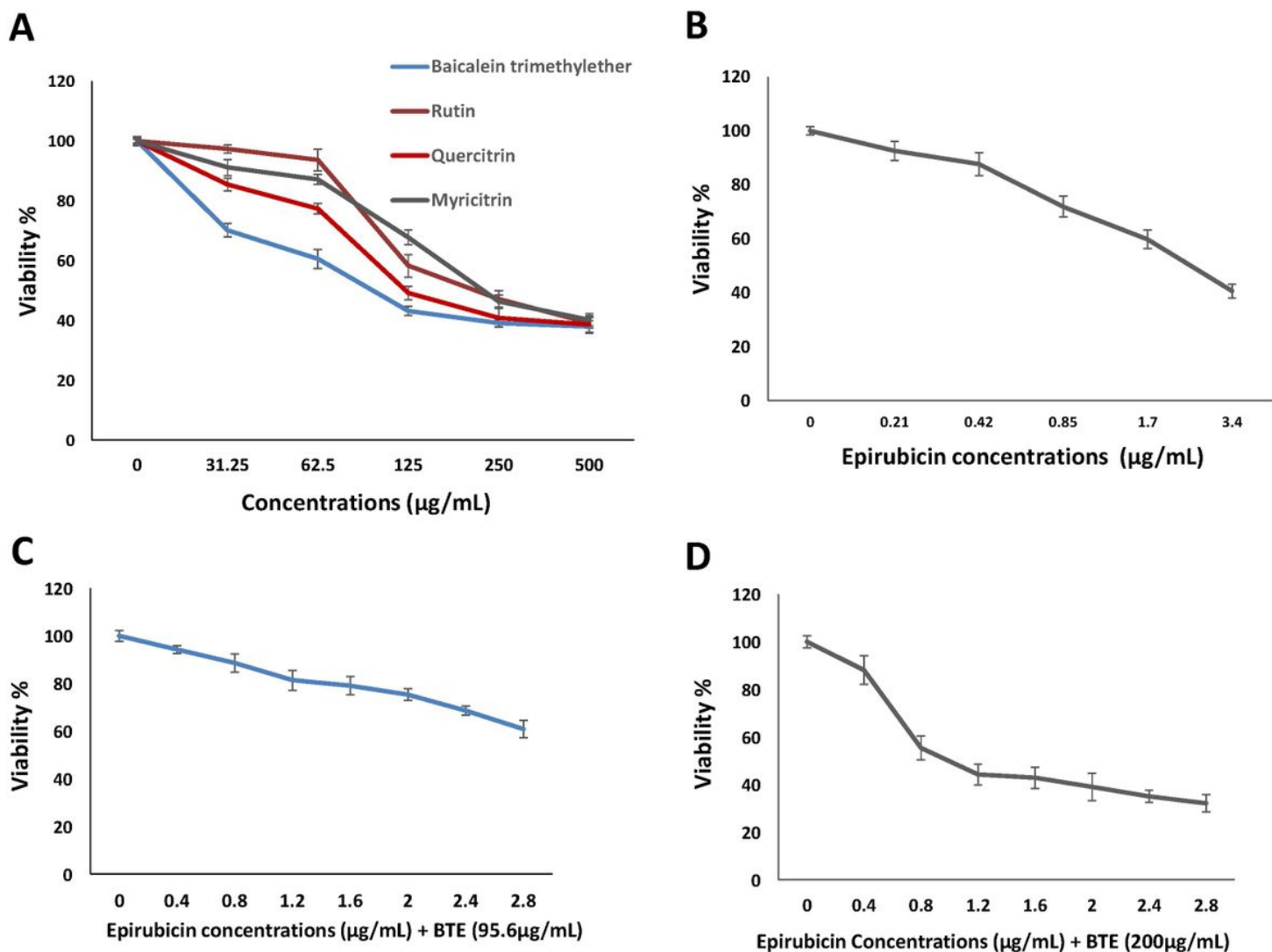


Figure 2

MTT cell viability assay of HepG2 cancer cells after incubation for 48 hr with different concentration of (A): flavonoids extract from *Physalis pubescens* L. showed that BTE is the flavonoid with the most cytotoxic effect on HepG2 liver cancer cells ($IC_{50} = 95.6 \pm 5.849 \mu\text{g/mL}$), (B): epirubicin showed that the 50% inhibitory concentration (IC_{50}) = $2.79 \pm 0.626 \mu\text{g/mL}$ for epirubicin alone, (C): epirubicin in combination with (95.6 µg/mL) BTE showed that IC_{50} is more than 2.8 µg/mL for all the used concentrations of epirubicin (D): epirubicin in combination with (200 µg/mL) BTE showed that combination of epirubicin with 200 µg/mL BTE significantly decreased the IC_{50} of epirubicin from $2.79 \pm 0.626 \mu\text{g/mL}$ to $0.76 \pm 0.258 \mu\text{g/mL}$ ($p=0.04$). Data are presented as mean $\pm$ SE, $n=3$, BTE: baicalein trimethyl ether.

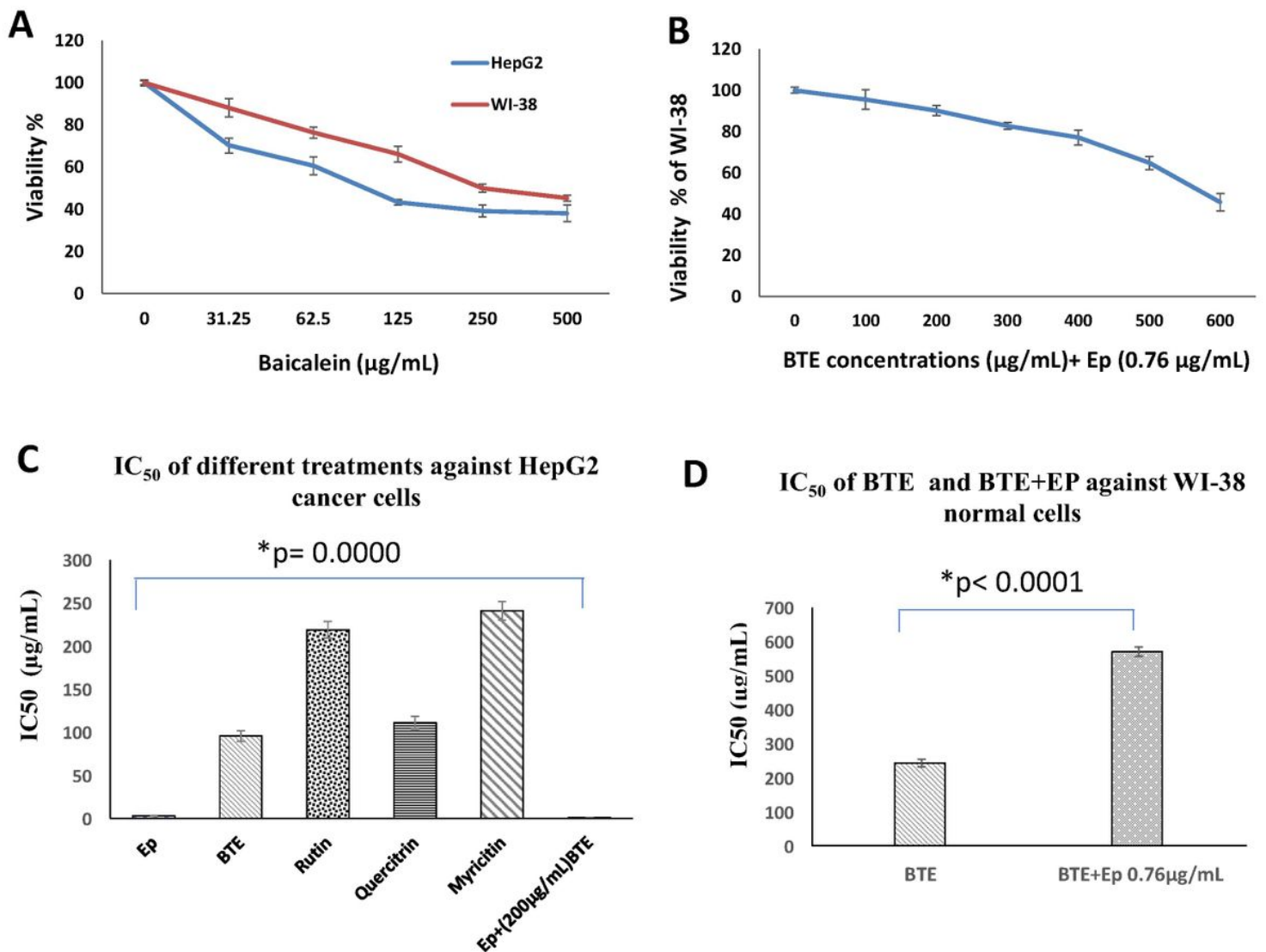


Figure 3

(A) MTT cell viability assay of baicalin trimethyl ether (BTE) against HepG2 (IC₅₀ = 95.6 ± 5.849 μg/mL) and WI-38 cells (243.4 ± 11.667 μg/mL). (B) MTT cell viability assay of BTE + epirubicin (0.76 μg/mL) against WI-38 normal cells (IC₅₀ = 570.8 ± 13.72). (C) 50% inhibitory concentration (IC₅₀) of different treatments against HepG2 cancer cells. (D) 50% inhibitory concentration of BTE alone or combined with epirubicin (0.76μg/mL) against WI-38 normal cells. Data are presented as mean ± SE, n=3, BTE: baicalin trimethyl ether, Ep: epirubicin.

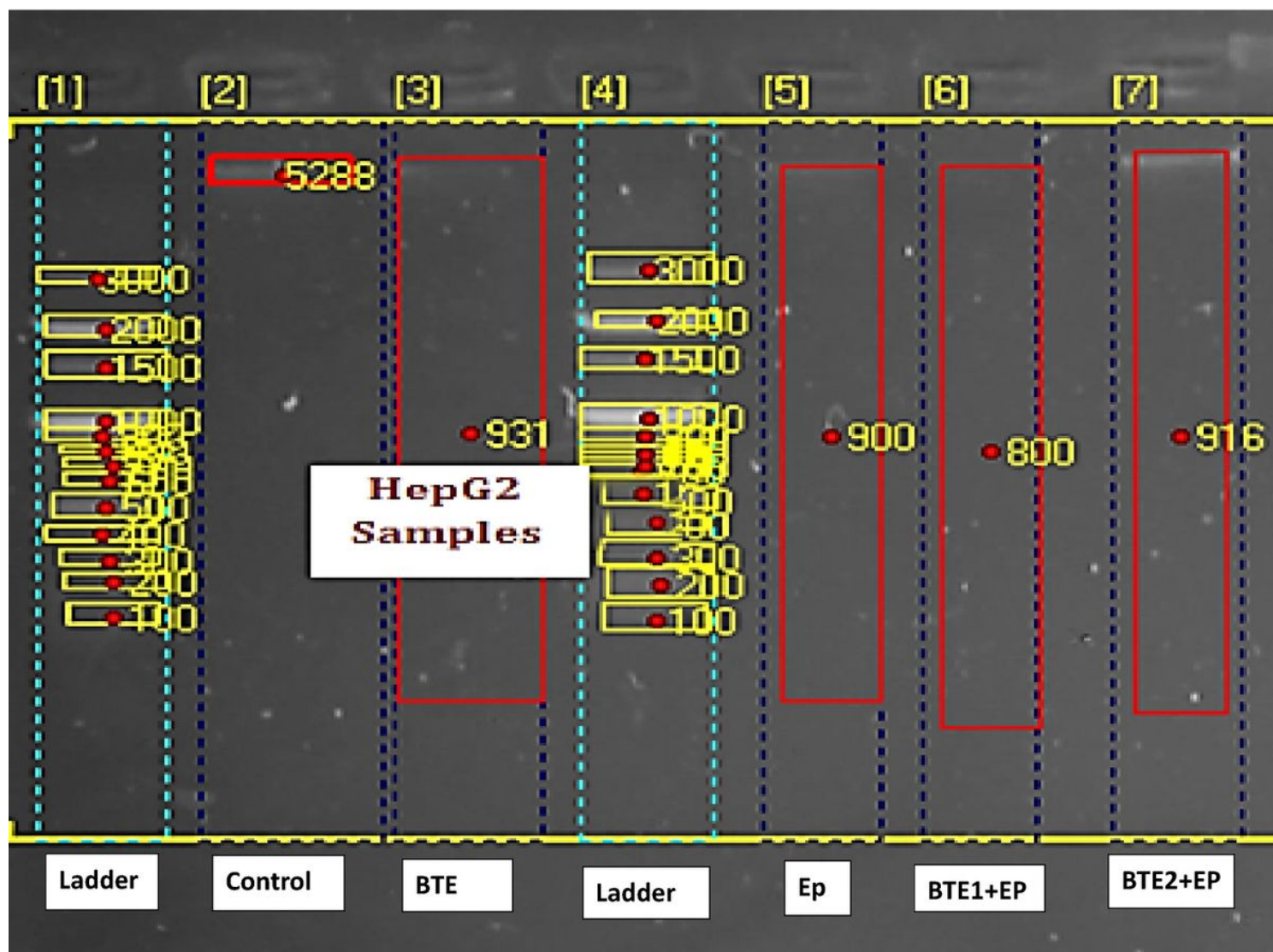


Figure 4

Drug-induced genomic DNA fragmentation in HepG2 liver cancer cell. BTE: baicalein trimethyl ether (95.6 μ g/mL), Ep: epirubicin (0.76 μ g/mL), BTE 1 and 2, : different concentrations of BTE (95.6 and 200 μ g/mL) respectively, DNA ladder (0.1 μ g/ μ l) 100bp (GeneRuler™ DNA ladders, Co,SM0243, Thermo Fisher Scientific®, America): DNA ladder 10 mMTris-HCl (pH 7.6).

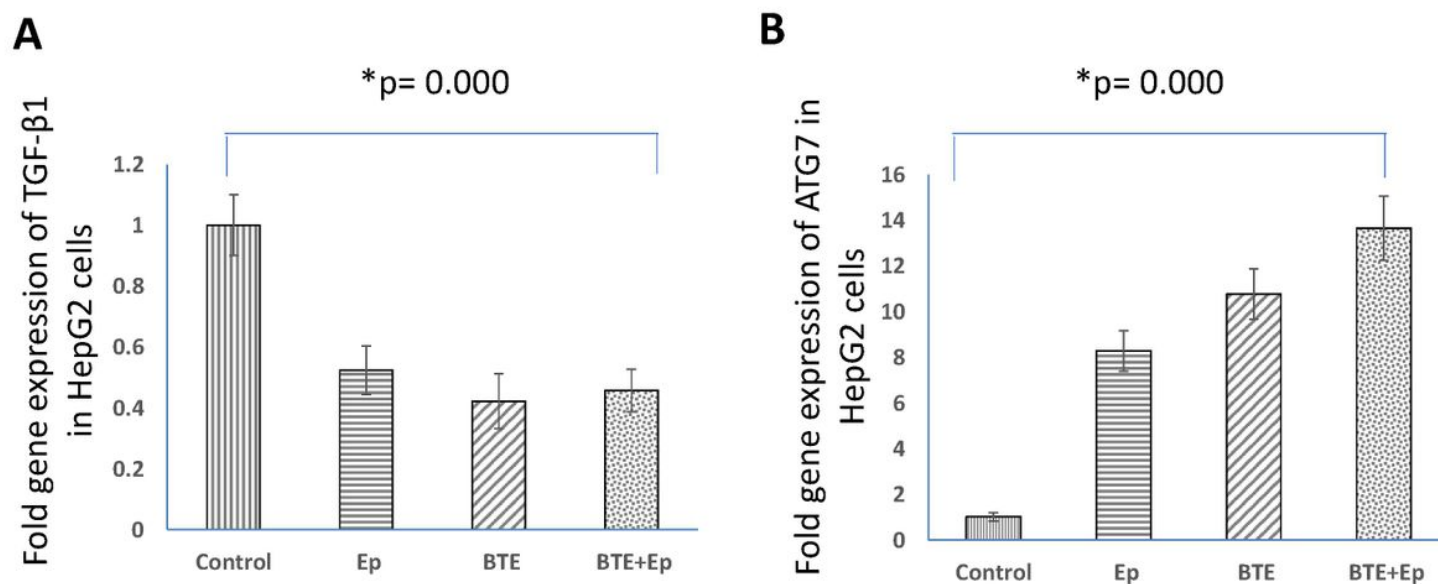


Figure 5

Effect of different treatments on the gene expression of ATG7 (A) and TGF- β 1 (B) in HepG2 liver cancer cell. Ep: epirubicin (0.76 μ g/mL). BTE: baicalein trimethyl ether (95.6 μ g/mL), BTE+Ep: combination of BTE (200 μ g/mL) and epirubicin (0.76 μ g/mL). Data are presented as mean $\pm$ SD, n=3