Anti-proliferative effects of the combination of Sulfamethoxazole and Quercetin via *caspase3* and *NFkB* gene regulation: An *in vitro* and *in vivo* study

Heba Sahyon (heba_sahuon@sci.kfs.edu.eg)
Kafrelsheikh University https://orcid.org/0000-0002-4794-3802
Eman N.M. Ramadan
Damietta University Faculty of Science
Fayez Althobaiti
Taif University College of Science
Mohammad M.A. Mashaly
Damietta University Faculty of Science

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Abstract

Combination therapy comprising natural polyphenols and anticancer drugs has been used to decrease the adverse effects and increase the effectiveness and antioxidant activities of the drugs. The antioxidant and anticancer effects of quercetin (Q), a nutritive polyphenol, have been observed both *in vitro* and *in vivo*. Likewise, the anticancer activity of sulfamethoxazole (S) has been demonstrated *in vitro* and *in vivo*. This study aimed to investigate the *in vitro* and *in vivo* anticancer effects of Q alone and in combination with S. The *in vitro* effects of S, Q, and S + Q on HCT-116, HepG2, MCF-7, and PC3 cell lines were examined. Additionally, the *in vivo* effects of these drugs were evaluated using Ehrlich ascites carcinoma (EAC) tumor-bearing mice. The *in vitro* data revealed the potent anticancer activity of S + Q through the induction of apoptosis and cell cycle arrest. The EAC-inoculated mice treated with S + Q presented with elevated SOD, GSH, CAT, and TAC levels and decreased malondialdehyde levels compared with the untreated EAC group, thus revealing the antioxidant and protective actions of S + Q against EAC cells invasion. Furthermore, the downregulation of *NFkB* and upregulation of the *caspase3* gene in the EAC-inoculated mice treated with the S + Q indicated the induction of the apoptotic pathway and decrease in both cell proliferation and metastasis. In conclusion, the combination of S and Q might exert anticancer effects by inducing apoptosis and exhibiting selective toxicity against the cancer cells and thereby protecting the vital organs.

Introduction

Anticancer, chemotherapeutic, and radio-therapeutic treatments are associated with several drawbacks. The two main drawbacks include the serious side effects and the high expenses involved with these treatments. The search for a solution for these complicated economic and social problems is ongoing. Sulfa-drugs or sulfonamides are a group of biologically active compounds with flexible structures, which allow them to possess a broad range of biological activities, such as anti-inflammatory (Sondhi et al. 2000; El-Araby et al. 2012), antibacterial (Wulf and Matuszewski 2013), antitumor (Ghorab et al. 2009, 2010), protease inhibitor (Stranix et al. 2006), and carbonic anhydrase inhibitory activities (Casini et al. 2002; Scozzafava et al. 2003; Ghorab et al. 2009; Marques et al. 2010). Sulfa-drugs gained a fast approval to be used as anticancer agents in patients with solid tumors (Raymond et al. 2002; Terret et al. 2003) because it has been used as antibacterial agents for centuries (Greenwood 2010). Sulfamethoxazole (S) is a typical sulfonamide with a $-SO_2NH_2$ group linked to a phenyl ring. However, S alone produces numerous side effects; therefore, it is used in combination with other compounds (Wormser et al. 1982; Kielhofner 1990). Recent studies approved the *in vivo* anticancer effects of S + selenium on hepatocellular carcinoma (Gupta et al. 2013) and its improved *in vitro* anticancer activity against colon carcinoma (HCT-116) and human metastatic breast cancer (MDA-MB–231) cells when combined with copper (RAMA and SELVAMEENA 2015).

Quercetin (Q) is a nutritive polyphenol found in fruits, such as apples, red grapes, and berries, and vegetables, such as onion and broccoli (Formica and Regelson 1995). For decades, Q has exhibited numerous biological activities, including antioxidant (Chalet et al. 2018), anti-inflammatory, and

anticancer effects, both *in vitro* and *in vivo* (Shoskes and Nickel 2007; Mohammed et al. 2015; Seo et al. 2015; Granato et al. 2017). Additionally, its role in therapeutic applications has been reported (D'Andrea 2015). The various modes of action of this agent include the prevention of lipid peroxidation (Laughton et al. 1991; Frankel 1999), scavenging of free radicals (Afanas'ev et al. 1989; Ademosun et al. 2016), inhibition of DNA oxidative damage, and thereby protection of the cells from carcinogenic compounds (Murota and Terao 2003). Recent studies confirmed the promising anticancer effect of Q when combined with chemotherapeutic agents such as cisplatin and doxorubicin (Nessa et al. 2011; Wang et al. 2012; Brito et al. 2015). The synergic effects of Q and the chemotherapeutic drugs are exerted by scavenging the reactive oxygen species (ROS) and the antioxidant activities. A recent study demonstrated the synergistic effect of Q and a low dose S in a mice model, which was exerted by an increase in the levels of superoxide dismutase and catalase enzymes (Sahyon et al. 2019).

Apoptosis is the programmed cell death that occurs in normal cells to maintain homeostasis in the body (Elmore 2007). The apoptotic pathway is regulated by various genes that help in removing the dead cells from the body (Fuchs and Steller, 2011). Unlike necrosis, apoptosis does not induce the inflammatory process (Zhang et al. 2018). The anti-inflammatory proteins that protect the healthy cells from inflammation are released during apoptotic cell death (Voll et al. 1997). Inhibition of apoptosis resulting in the rapid proliferation of cells is the main sign of cancer (Hanahan and Weinberg 2000).

Nuclear factor kappa-B (*NFkB*) is a pro-inflammatory gene that regulates the process of inflammation, cytokine generation, and cell survival (Tornatore et al. 2012). The increase in oxidative stress increases the levels of the superoxide radicals and leads to *NFkB* activation (Wang et al. 2004).

Cysteinyl aspartate-specific proteases or caspases are protease proteins that are activated at the end of the apoptosis process to lysis the membrane and degrade the DNA in the damaged cell (Lavrik 2005). *Caspase3* activation is a sensitive assay used to determine cell death via the apoptotic pathway (Yan and Shi 2005). Recent studies have shown that Q treatment stimulated apoptosis in hepatocellular carcinoma (HepG2) and human breast adenocarcinoma (MCF-7) cells by activating *caspase3* (Tan et al. 2009; Chou et al. 2010).

In light of the aforementioned information, this study aimed to investigate the *in vitro* and *in vivo* anticancer effects of S in combination with Q and demonstrate their actions on cell death and the regulation of genes involved in apoptosis.

Materials And Methods

Chemicals and reagents

Sulfamethoxazole[($C_{10}H_{11}N_3O_3S$) 98.5%, Burroughs-WellcomeCompany, Research Triangle Park, NC 27709, USA], quercetin dihydrate [($C_{15}H_{10}O_7 \cdot 2H_2O$) 97% Lot.no. 10181203, Alfa Aesar Company, Germany], Dulbeco's Modified Eagle's Medium (DMEM) (GIBCO, New York, USA; Cat.no.11995073), fetal

bovine serum (GIBCO, Grand Island, New York, USA;Cat.no.10099133), penicillin/streptomycin (Thermo Fisher Scientific; Waltham, MA, USA; Cat.no. SV30082), L-glutamine (Invitrogen, Grand Island, New York, USA; Cat.no. 25030024), MTT reagent **[**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**]** was purchased from Molecular probes, Eugene, Oregon, USA; Cat.no.V-13154, ethidium bromide and acridine orange (EB/AO)(Sigma-Aldrich, Deisenhofen, Germany). Dimethylsulfoxide (DMSO) was purchased from Aldrich, Milwaukee, Wisconsin, USA. Annexin V kit (cat. No. 556547 BD Pharmingen FITC apoptosis kit) was purchased from Becton, Dickinson and Company. Polyclonal anti-rabbit antibodies for caspase3 (ab49822, abcam) and *NFKB* (#33595, cell signaling technology, USA), monoclonal anti-mouse β-actin (ab6276, abcam) were obtained. The chemiluminescent western blot ECL substrate (Perkin Elmer, Waltham, MA, USA) reagent was purchased. Serum total bilirubin level (T.Bil.), activities of alkaline phosphatase (ALP), albumin (Alb.), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and creatinine kits were from SPINREACT diagnostic kits (SPINREACT, S.A.U.,Girona, SPAIN). Super oxide dismutase (SOD), malondialdehyde (MDA), glutathione reductase activity (GSH), catalase (CAT) and total antioxidant capacıty (TAC) kits were from Biodiagnostic, Egypt. All other reagents used in this study were of analytical grade and highest purity.

In vitro **study**

Cell lines

Four human cell lines were used in the *in vitro* experiment; human breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2), colon carcinoma (HCT-116), and prostate carcinoma (PC3) which obtained from American Type Culture Collection, ATCC, NY, Sarthe cells in exponential growth phases were washed, trypsinzed and suspended in DMEM. All media were provided with 10% heat-inactivated fetal bovine serum,1%penicillin/streptomycin and 2% L-glutamine.

Cytotoxicity determination by MTT assay

Anticancer activities of sulfamethoxazole and/or quercetin were evaluated by using MTT (cell viability) assay against MCF-7, HepG2, HCT-116, and PC3 cell lines (Denizot and Lang 1986), as mentioned in supplementary data. The experiment was performed three times independently for each cell line. The percentage of surviving cells was calculated as:

$$\textbf{\% Cell viability} = \frac{\textbf{Absorbance of treated cells}}{\textbf{Absorbance of control cells}} \times \textbf{100}$$

% Cell Inhibition = 100 - % Cell Viability

The IC_{50} value is the half maximal inhibitory concentration. Sigmoidal and dose dependent curves were constructed to plot the results of the experiment. The concentration of the compounds inhibiting 50% of cells (IC_{50}) was calculated using the sigmoidal curve.

Combination Index (CI) analysis

The CI between S and Q were calculated by the isobolographic analysis, a dose-oriented geometric method of assessing drug interactions (Roell et al. 2017). Form the IC_{50} values, the different CI values for the four cell lines were calculated by the following equation:

$$CI = \frac{D1}{Dx1} + \frac{D2}{Dx2}$$

Where Dx1 is the IC_{50} dose of the drug 1 alone; Dx2 is the IC_{50} dose of the drug 2 alone, D1 is the IC_{50} dose of drug 1 in combination with drug 2; D2 is the IC_{50} dose of drug 2 in combination with drug 1. If CI value > 1 indicates antagonism, CI = 1 additive effect and CI < 1 indicates synergism.

Fluorescence analysis of apoptosis and necrosis in living cells

Higher organisms have a programmed or accidental cell death to reach homeostasis between new growing cells and the old ones. The mode of cell death was determined by investigating apoptosis and necrosis ratios using morphological staining methods such as ethidium bromide and acridine orange (EB/AO) (Smith et al. 2012). EB /AO staining role is to visualize the nuclear changes and apoptotic bodies formation in cells that undergo apoptosis. Fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes (such as EB/AO staining) is a method of choice for its simplicity and accuracy (Taatjes et al. 2008). AO permeates all cells and makes the nuclei appears green. EB is only taken up by cells when cytoplasmic membrane integrity is lost, and stains the nucleus red. Live cells have nuclei staining which present green chromatin with organized structures; early apoptotic cells have bright green to yellow nucleus with condensed or fragmented chromatin; late apoptotic cells display fragmented orange chromatin and cells have died from direct necrosis have similar normal nuclei staining as live cells except the chromatin is dark orange to red instead of green.

MCF-7, HepG2, HCT-116, and PC3 cell lines were cultured and stained in six well plates at a density of 5 x 10^5 cells/well and treated with 30 % of the IC_{50} of S and/or Q at 37°C in a humidified 5% CO_2 atmosphere. Cells were removed by trypsinization and re-suspended in 50 µl cold PBS and 2 µl of a dye mix for the EB/AO staining (100 µg/ml acridine orange and 100 µg/ml ethidium bromide in PBS solution) was added. On a clean microscope slide, 10 µl of the stained cell suspension was placed and covered with a coverslip. Cells were viewed and counted using Fluorescence microscopy BK6000, China. Pictures were taken with a digital camera. Tests were done in triplicate, a minimum of 500 total cells each were counted, and the percentage of apoptotic or necrotic cells was determined as:

% of apoptotic or necrotic = [Total number of apoptotic or necrotic cells/ Total number of cells counted] x 100.

Cell cycle analysis by using propidium iodide [PI] by the flow cytomety

MCF-7 cells were treated with the IC_{50} of S + Q combination and the cell cycle were analyzed per experiment with flow cytometer (Applied Bio-systems, USA), as described in supplementary data.

Western blotting assay

Protein expression of *NFkB* and *caspase3* from the MCF-7 cells treated with 50% IC_{50} of S and/or Q as well as untreated MCF-7 cells were evaluated by western blot analysis as mentioned in the method of Burnette 1981. Briefly, after extraction of protein from the lysed MCF-7 treated/untreated cells, the samples were separated by SDS electrophoresis. Then, the separated protein bands were transferred to a nitrocellulose membrane and blocked with 5% non-fat milk in Tris-buffered saline tween for 2 hr. at 25°C, then washed. Primary antibody for *NFkB*, *caspase3* and *β-actin* were added to the washed membrane and incubated overnight at 4°C. After incubation period, the membrane was rinsed several times with Tris-buffered saline tween and horse radish peroxidase (HRP)- linked secondary antibodies were added then incubated for 60 min. at 25°C. Finally, the membrane was rinsed several times till visualizing the protein bands, then the ECL chemiluminescent western substrate was added to the bands according to the manufacturer's protocol then analyzed by using a CCD camera-based imager (Chemi Doc imager, Biorad, USA), then ImageLab (Biorad) was used to calculate the bands intensities.

In vivo **antitumor activity**

Animal Selection and Care

A total of 80 female Swiss Albino mice were handled according to the experimental practice and standards approved by the guidelines of the animal ethical committee of Kafrelsheikh University, Egypt (4/2018EC). Six-week-old female albino mice (20–22 g) were obtained from the animal holding unit at the Faculty of Science. Female mice were used because female sex hormones are known to aid in the growth of Ehrlich ascites carcinoma (EAC) cells (Ahlström and Jonsson 1960). The animals were housed in polypropylene transparent cages (five mice per cage) at a temperature of ~ 27°C with suitable humidity for equal light/dark cycles. They were fed with regular granulated chow and given drinking water *ad libitum.*

EAC cells Transplantation

EAC cells were obtained from El-Nile Research Centre (El-Mansoura, Egypt), EAC cells were proliferated for 12–14 days in a donor Swiss Albino female mouse by intraperitoneal transplantation of 2.5×10^6 cells per mouse as a model of mammary adenocarcinoma (Jaganathan et al. 2010). The ascetic fluid from the EAC mouse's intraperitoneal cavity was aspirated, washed wit isotonic saline and centrifuged, then the pellets were separated and suspended in sterile PBS. Tumor viability was determined and confirmed by Trypan blue exclusion test to be 98%. A volume of 100 µL of the diluted EAC cells containing 2.5×10^6 cells/ml was injected intraperitoneally (i.p.) into each mice groups.

The mice were randomly distributed into the following groups (10 animals in each group):

i) Control group: healthy normal control mice that were intraperitoneally (i.p.) injected with a vehicle (100 µl of dimethylsulfoxide [DMSO]) for 2 weeks.

ii) Control S group: healthy normal control mice that were i.p. injected with S dissolved in DMSO (30 mg/kg body weight [b.w.]) daily for 2 weeks.
iii) Control Q group: healthy normal control mice that were i.p. injected with Q dissolved in DMSO (200 mg/kg b.w.) daily for 2 weeks.

iv) Control (S + Q200) group: healthy normal control mice that were initially injected with S (30 mg/kg) followed by an injection with Q (200 mg/kg) dissolved in DMSO half an hour later. The animals were injected daily for 2 weeks.
v) EAC group: mice were i.p. injected once with 100 µl of a cell suspension containing viable EAC cells (2.5×10^6).
vi) EAC + S group: mice were i.p. injected once with EAC cells followed by injection with S dissolved in DMSO (30 mg/kg b.w.) after 24 h.
vii) EAC + Q group: mice were i.p. injected once with EAC cells followed by injection with Q dissolved in DMSO (200 mg/kg b.w.) after 24 h.
viii) EAC + (S + Q200) group: mice were first injected with EAC cells; 24 h later, they were injected with S (30 mg/kg) followed by injection with Q dissolved in DMSO (200 mg/kg) after another half an hour.

Then, the abdomens of the animals were monitored for EAC growth for 14 days. The doses of S and Q were administrated, intraperitoneally, once daily for 2 weeks. Fresh S and Q solutions were prepared a few minutes prior to an injection using the DMSO solvent. The dose of S (30 mg/kg/day) was selected based on accessible literature (Gupta et al. 2013) and that of Q (200 mg/kg/day) was selected because it is known to be safe for mice (Ingle et al. 2006).

Sample collection

At the end of the experimental period, all animals were made to fast for 12 h and sacrificed by ether inhalation. Cells from the abdominal regions of the mice in the treated (S, Q, and S + Q200) and untreated EAC-induced groups were aspirated by a sterile syringe to determine the extent of apoptosis via flow cytometry (annexin and propidium iodide [PI] assays). Blood samples were withdrawn by heart puncture and collected in clean tubes with/without EDTA; 250 µl of the blood sample was dispensed into an EDTA tube, and the remaining sample was dispensed in a plain tube to be used for serum biochemical analyses. Serum and plasma samples were then obtained by centrifugation at 4000 rpm for 10 min. The sera were kept in Eppendorfs and stored at − 20°C until further use. The lower erythrocyte layer in the EDTA tubes (packed red blood cells [RBCs)] was washed and centrifuged three times with sterile saline. The supernatant was removed, and the packed RBCs were stored at − 20°C for malondialdehyde (MDA) determination. The liver was carefully excised from some animals in each group and washed with sterile phosphate-buffered saline (PBS; pH 7.4); subsequently, 0.25 g of the liver sample was homogenized in liquid nitrogen and stored at − 80°C until further use. Another sample of the liver was fixed in 10% neutral buffered formalin and sent to the laboratory for histopathological examination.

Apoptosis using annexin V and PI assays

Annexin V and PI assays have been used to reflect the accurate numbers of apoptotic cells (van Heerde, 2000). In the present study, annexin V staining was performed to determine the proportions of early and late apoptotic cells, and the PI staining was used to determine the proportion of necrotic cells. The aspirated cells from the abdominal regions were washed three times with PBS to remove the RBCs and centrifuged at 5000 rpm to separate the pellet. The pellets were aspirated carefully, diluted with sterile PBS (1×10^6 cells), and stained with 5 µl annexin V (fluorescein isothiocyanate label) and 5 µl PI (phycoerythrin label). All tubes were incubated in the dark for 15 min at 27°C. Subsequently, the cells were analyzed using a BD FACS Calibur flow cytometer (Becton Dickinson, Sunnyvale, CA, USA).

Nephrotoxicity biomarkers

Urea was detected in plasma samples by the method of Latt, 1991. Colorimetric creatinine assays are measured by the method of Ogawa et al., 1995.

Hepatotoxicity biomarkers

Serum total bilirubin level was determined by the method by Scott et al., 2012. Activities of serum ALT and AST were assayed according to Wilkinson, Baron, Moss, & Walker, 1972 method, while serum albumin was determined by the method of Dumas, Watson, & Biggs, 1997, and ALP was estimated by the principle of Fowler, 1998.

Oxidative stress biomarkers

The quantitative estimation of TAC in plasma was done according to the method of Koracevic et al., 2001. GSH reduces the glutathione and at the same reaction NADPH is oxidized to $NADPH^+$ and the absorbance value was declined then measured at 340 nm (Anderson 1985). SOD activity in plasma was assessed according to the designation of Nishikimi, Appaji Rao, & Yagi, 1972. CAT activity was detected by the method of Aebi, 1984. Lipid peroxidation was evaluated as the MDA level in packed RBCs which was determined by using the method of Stocks & Dormandy, 1971.

Quantitative RT-PCR

To conduct real time PCR, RNA from mice liver was extracted using total Gene JET™ RNA Purification Kit following the manufacturer protocol (Thermo Scientific, Fermentas, #K0731, USA). The extracted RNA were evaluated by Nanodrop. The Nanodrop revealed presence of pure RNA with large concentrations (1240–1890 ng/µl). A 5µg of the isolated RNA was reverse transcribed using Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, Fermentas, #EP0451, USA) into complementary DNA (cDNA). The cDNA templates was then amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol (Thermo scientific, # K0221, USA). The amplified cDNA was used to conclude the relative expression of the pro-apoptotic gene (*caspase3*) and the pro-inflammatory gene *NFkB* that reflects the changes in transcription levels of these genes in mice liver after treatment with S and/or Q compounds. The housekeeping gene encoding *B actin* was used, during the RT-qPCR experiment, as a

reference to normalize the results and to calculate the relative gene expression or fold change in target gene. The data was expressed as mean ± SEM and every experiment has triplicated measurements. The quantitative measurements of the target gene expression level in the control group was considered the baseline. The forward and reverse primers used in the qPCR are listed in Table 1.

Table 1
Forward and reverse primers sequence used in qPCR.

Gene	Forward primer(′5 —— ′3)	Reverse primer (′5 —— ′3)
Caspase3	GGAGGCTGACTTCCTGTATGCTT	CCTGTTAACGCGAGTGAGAATG
NFkB	GAAATTCCTGATCCAGACAAAAAC	ATCACTTCAATGGCCTCTGTGTAG
β-actin	ACTATTGGCAACGAGCGGTT	CAGGATTCCATACCCAAGAAGGA

The final reaction mixture was placed in a Step One Plus real time thermal cycler (Applied Biosystems, Life technology, USA) and the PCR program was carried out with the PCR conditions. After a few steps, the cycle threshold (Ct) values were calculated for target genes and the housekeeping gene, and relative gene expression was determined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

All data were expressed as means ± standard error of Mean (SEM). The statistical significance was evaluated by one-way ANOVA using SPSS 18.0 software, a probability of $p < 0.05$ is considered significant.

Results

In vitro **assay**

Analysis of the cytotoxicity of S + Q and the combination index values

The *in vitro* anti-proliferative effects of S and/or Q were examined in the MCF-7, HepG2, HCT-116, and PC3 cell lines. The anti-proliferative effect was assessed using the MTT reagent to calculate the IC_{50}. The combination of S and Q (50% S + 50% Q) induced cytotoxicity in most of the tested cell lines in a dose-dependent manner, whereas S and Q alone exhibited lower cytotoxicity. As represented in Table 2, S + Q exerted cytotoxic effects on the MCF-7, HepG2, and PC3 cells with a mean IC_{50} of 55.97 ± 3.49, 135.9 ± 8.5, and 162.2 ± 10.1 µg/ml, respectively, when compared with the IC_{50} values obtained with S and Q alone. These results demonstrate the synergistic effect of Q and S as anti-proliferative and anticancer agents. The cytotoxic effect (IC_{50}) of S + Q on HCT-116 cells was 48.10 ± 3.00 µg/ml, which was similar to that obtained with Q alone (47.39 ± 2.95 µg/ml); however, a limited cytotoxicity was observed using S

alone (IC_{50}, 292.9 ± 18.2 µg/ml). Thus, our results reveal that the S + Q combination can be promising chemotherapy for the clinical outcome of cancer treatment.

Table 2
IC_{50}s (µg/ml) of sulfamethoxazole, quercetin and 50% S + 50% Q on different human cancer cell lines.

IC_{50} Cell Lines	S µg/ml	Q µg/ml	50 % S + 50 % Q µg/ml	Doxorubicin µg/ml
MCF-7	182.5 ± 11.4	91.59 ± 5.7	55.97 ± 3.49	4.97 ± 0.3
HepG2	204.5 ± 12.7	374.2 ± 23.3	135.9 ± 8.5	24.36 ± 2.9
HCT-116	292.9 ± 18.2	47.39 ± 2.95	48.10 ± 3.00	6.81 ± 0.5
PC3	227.6 ± 14.2	203.5 ± 12.67	162.2 ± 10.1	23.61 ± 2.73

The combination index (CI) of S and Q on MCF-7, HepG2, HCT-116, and PC3 cells was calculated using the isobolographic analysis method. The CI values of the concentrations of Q and S exhibited synergistic interactions in the MCF-7 (CI, 0.42–0.57), HepG2 (CI, 0.5–0.66), HCT-116 (CI, 0.63–0.94), and PC3 (CI, 0.75–0.86) cells.

Evaluation of cell death mode and apoptosis

Apoptotic and necrotic cell death in the four cell lines (MCF-7, HepG2, HCT-116, and PC3) were estimated using DNA dyes (acridine orange/ethidium bromide [AO/EB]). Programmed cell death or apoptosis is a normal occurrence in all cells but is turned off in the cancer cells. During apoptosis, the nucleus of the cell is disintegrated resulting in its shrinkage; the plasma membrane of the cell is then altered and is finally removed from circulation via phagocytosis (Lemasters et al. 1999). Alternatively, in necrosis, the cells swell up and explode, causing inflammation in the tissues around the cells (Proskuryakov and Gabai 2010). In the present study, the cells were counted in each color population, and the percentage of vital, as well as apoptotic and necrotic, cells was calculated. The mode of cell death in the four cell lines was analyzed after treatment (30% of the IC_{50} values of S, Q, and S + Q) for 24 h compared with untreated human cancer cells. As seen in Fig. 1, a higher apoptotic rate was observed in the MCF-7, HCT-116, HepG2, and PC3 cell lines after treatment with S + Q, whereas the cells treated with S or Q alone exhibited lower levels of apoptosis. On the contrary, decreased percentages of necrotic cells were observed in the MCF-7, HCT-116, HepG2, and PC3 cell lines after treatment with S + Q; the percentage of necrosis in the MCF-7 cells was similar to that in the control group. These results indicated that the combination of S and Q might induce the natural cell death pathway and inhibit cancer cell proliferation (Fig. 1; Table 3). On the basis of these findings, we chose the MCF7 cell line to perform the cell cycle analysis using S + Q to evaluate the phase when cell cycle arrest is induced.

Table 3
The percentage of vital, apoptosis and necrosis in the four cell lines, after being treated with 30% of the IC_{50} of S, Q and S + Q for 24h compared to control untreated cells.

Cell lines	Treatment	Vital % Mean ± SEM	Necrotic % Mean ± SEM	Apoptotic % Mean ± SEM
MCF-7	control	93.6 ± 5.83	4.2 ± 0.24	2.2 ± 0.14
	S	75.4 ± 4.70*	9.7 ± 0.61*	14.9 ± 0.93*
	Q	67.6 ± 4.21*	11.2 ± 0.69*	21.2 ± 1.32*
	S + Q	60.3 ± 3.75*	4.5 ± 0.28^N	35.2 ± 2.19*
HepG2	control	89.0 ± 5.50	6.2 ± 0.39	4.8 ± 0.29
	S	71.0 ± 4.42*	22.6 ± 1.41*	6.4 ± 0.39*
	Q	79.8 ± 4.96*	9.8 ± 0.41*	10.4 ± 0.64*
	S + Q	60.3 ± 3.75*	17.7 ± 1.1*	22.0 ± 1.37*
HCT-116	control	91.9 ± 5.72	5.6 ± 0.35	2.5 ± 0.16
	S	76.0 ± 4.73*	11.6 ± 0.72*	12.4 ± 0.77*
	Q	67.5 ± 4.21*	9.4 ± 0.58*	23.1 ± 1.44*
	S + Q	65.0 ± 4.05*	8.8 ± 0.54*	26.2 ± 1.63*
PC3	control	92.6 ± 5.77	4.1 ± 0.26	3.3 ± 0.25
	S	75.3 ± 4.69*	19.9 ± 1.23*	4.8 ± 0.29 ^{N}
	Q	73.7 ± 4.58*	12.4 ± 0.77*	13.9 ± 0.86*
	S + Q	62.4 ± 3.88*	11.2 ± 0.69*	26.4 ± 1.64*

* $P \leq 0.05$ indicates significant different of means compared to the untreated control cells.

$^N P > 0.05$ indicates non-significant different of means compared to the untreated control cells.

Cell cycle distribution in MCF-7 cells

The cell cycle (or cell division) is a cascade of steps that occur inside a cell to promote its replication and consist of four phases: G1, synthesis, G2 (interphase), and M (mitosis). In the present study, we examined the association between apoptosis, induced by S, Q, or S + Q (50% S + 50% Q), and cell cycle arrest in

MCF7 cells. The treatment effect of the IC_{50} values of the S, Q, and S + Q on the cell cycle in MCF-7 cells was estimated by flow cytometry using the PI assay. The distribution of cells in different phases was examined, and cells containing DNA were scheduled in the G0/G1, synthesis, or G2/M phase. The number of cells in each phase was divided by the whole number of examined cells, and the percentage was calculated. As shown in Fig. 2, a significant increase in the G0/G1 phase was observed in the cells treated with S, whereas no significant change in this phase was observed in cells treated with Q when compared with that in the untreated cells. Conversely, a significant decrease in the G0/G1 phase was observed in cells treated with S + Q (26 ± 2.90%) compared with that in the untreated cells (50 ± 3.2%), indicating a cell cycle shift from the G0/G1 phase to the G2/M phase following the S + Q treatment. However, treatment with S or Q alone resulted in significant increases in the G2/M phase with no change in the synthesis phase in the MCF-7 cells when compared with that in the untreated cells (Table 4). By contrast, a significant increase in the G2/M phase was observed following S + Q treatment (52 ± 2.03%) when compared with that in the untreated cells (38 ± 2.48%), with no significant change in the synthesis phase (Table 4). This accumulation in the G2/M phase indicates that S + Q treatment inhibits the proliferation of the cancer cells and prevents their entry into a new cell cycle.

Table 4
Average % of cells in each cell cycle phase in untreated MCF-7 and S, Q and S + Q treated cells.

Groups	% of cells in each cell cycle phase (Mean ± SEM)		
	G0/G1 phase	S phase	G2/M phase
Untreated MCF-7 cells	50 ± 3.2	12 ± 3.76	38 ± 2.48
MCF-7 treated with S	67.8 ± 3.15*	13.6 ± 1	18.6 ± 1.6*
MCF-7 treated with Q	57.8 ± 4.2	13.8 ± 1.7	28.4 ± 5.9*
MCF-7 treated with S + Q	26 ± 2.90*	22 ± 4.65	52 ± 2.03*

* $P \leq 0.05$ indicates significant different of means compared to the untreated cells based on Duncan's multiple range test (DMRT).

Protein expression of NFKB and Caspase3 determination by western blot analysis

The protein expression levels of both *NFkB* and *caspase3* in MCF-7 cells treated with 50% IC_{50} of S, Q or S + Q (50% S + 50% Q) as well as untreated MCF-7 were determined by the western blot method. *NFkB* was considered as pro-inflammatory gene and *caspase3* is a pro-apoptotic gene. As shown in Fig. 3, a significant increase was observed in the *caspase3* protein expression levels in MCF-7 cells treated with S, Q or S + Q compared with untreated cells ($P = 0.002$, $P = 0.033$, and $P = 0.001$, respectively) with a maximum increase in the S + Q treated cells. On the other hand, a significant decrease in the *NFkB* protein

expression levels in MCF-7 cells treated with S, Q or S + Q compared with untreated cells ($P = 0.003$, $P = 0.021$, and $P = 0.0001$, respectively) with a maximum reduction in the S + Q treated cells.

In vivo **antitumor activity**

Effect of treatment with S, Q, or S + Q200 on the abdominal circumference

EAC is a liquid tumor that spreads in the abdominal region and affects all vital organs. The proliferated EAC cells increase the size of the abdomen in the affected mice. The mice in the control groups that were injected with S, Q, or S + Q200 showed non-significant changes in the abdominal circumference (7.42 ± 0.3, 7.28 ± 0.29, and 7.94 ± 0.16 cm, respectively) compared with those in the healthy control group ($P = 0.94$, $P = 0.69$, and $P = 0.71$, respectively) indicating that the increase in abdominal circumference was due to EAC proliferation and not inflammation, which could occur following injection with S and/or Q. The EAC-inoculated mice treated with S presented with a nonsignificant reduction ($P = 0.06$) in mean abdominal circumference compared with the EAC group (10.97 ± 0.39 vs. 11.43 ± 4.2 cm, respectively). However, the EAC-inoculated mice treated with Q and S + Q200 demonstrated a significant reduction ($P < 0.0001$) in mean abdominal circumference (8.93 ± 0.08 and 8.2 ± 0.1 cm, respectively) compared with that in the EAC group. Furthermore, a small but statistically significant increase in mean abdominal circumference was observed in the EAC mice treated with S + Q200 ($P = 0.03$) when compared with that in the healthy control group (7.58 ± 0.21 cm).

Induction of apoptosis

Figure 4 shows the effect of S, Q, and S + Q200 treatment on the induction of apoptosis in the EAC-inoculated mice. A significant increase in the percentage of apoptotic cells was observed in all the treated groups with a highly significant increase in the S + Q200 treated group ($P < 0.0001$) when compared with that in the untreated EAC group. However, after treatment the EAC-inoculated mice with S alone, the number of viable cells was only decreased by 9.8%, and the number of dead cells was not significantly altered ($P = 0.57$) when compared with that in the untreated EAC mice. After treatment with Q alone, the number of viable cells was decreased by 30.01% and the number of dead cells was significantly increased ($P < 0.0001$) by 149.7% when compared with that in the untreated EAC mice. In EAC-inoculated mice treated with S + Q200, the number of viable cells was decreased by 46.69% and the number of dead cells was significantly increased ($P < 0.0001$) by 268.86% when compared with that in the untreated EAC mice.

Effect of treatment with S, Q, or S + Q200 on liver and kidney functions tests

Table 5 shows that the GOT, GPT, ALP, and total bilirubin levels were significantly increased in the sera of the EAC-inoculated group along with a significant reduction in albumin concentration when compared with those in the control group, indicating that the cancer burden can cause damage to the hepatocytes. Similarly, the creatinine and urea levels were significantly increased ($P < 0.0001$) in the EAC-inoculated mice compared with those in the control mice, indicating the effect of EAC cells on the kidney tissues.

The EAC-inoculated mice treated with S did not show any significant changes ($P > 0.05$) in both the liver and kidney function tests compared with those in the untreated EAC group, thus indicating the permanence of cancer burden in these mice. However, the EAC-inoculated mice treated with Q demonstrated significant decreases in GOT, GPT, total bilirubin, and ALP levels ($P = 0.014$, $P = 0.029$, $P = 0.036$, and $P = 0.04$, respectively) compared with those in the untreated EAC group; however, the levels were significantly higher ($P = 0.042$, $P = 0.031$, $P = 0.035$, and $P = 0.022$, respectively) than those in the control group. The creatinine and urea levels were significantly decreased ($P = 0.027$ and $P = 0.019$, respectively) in the EAC-inoculated mice treated with Q compared with those in the untreated EAC group, but the levels were higher than the normal levels. Additionally, EAC-inoculated mice treated with S + Q200 demonstrated significant reductions in GOT, GPT, ALP, and bilirubin levels and a significant elevation in albumin compared with those in the untreated EAC group. Furthermore, the levels of creatinine and urea in the EAC-inoculated mice treated with S + Q200 were significantly lower than those in the untreated EAC group and were similar to those in the control group. These findings indicate the removal of the cancer burden and the protection of the vital organs from oxidative damage following treatment with S + Q200.

Table 5
Mean serum levels of liver and kidney function tests in controls, untreated EAC, and the four treated groups

Group	GOT U/L	GPT U/L	Albumin g/dL	T. Bil. mg/dL	ALP U/L	Creatinine mg/dL	Urea mg/dL
Control DMSO	145.6 ± 5.2	49.2 ± 3.56	2.87 ± 0.03	0.2 ± 0.024	51.3 ± 2.7	0.63 ± 0.05	22.3 ± 1.04
Control S	142 ± 6.5	82.9 ± 6.65*	2.78 ± 0.03	0.23 ± 0.26	57.8 ± 1.85	0.72 ± 0.045	27.7 ± 1.81
Control Q	137.3 ± 12	57.1 ± 5.63	2.85 ± 0.05	0.225 ± 0.26	56.1 ± 3.47	0.66 ± 0.068	23 ± 1.10
Control S + Q200	149.4 ± 9.83	72.1 ± 2.72*	2.86 ± 0.049	0.2 ± 0.26	41.22 ± 2.45	0.73 ± 0.03	28.1 ± 1.38
EAC	231 ± 19.7*	120.8 ± 4.9*	1.7 ± 0.06*	0.27 ± 0.032*	100.3 ± 5.78*	0.97 ± 0.049*	34.9 ± 1.37*
EAC + S	195 ± 14.9^{N}	109.8 ± 4.3^{N}	1.83 ± 0.04 ^{N}	0.19 ± 0.029^{N}	92.3 ± 4.2 ^{N}	0.95 ± 0.053 ^{N}	32.8 ± 1.4^{N}
EAC + Q	176.1 ± 9.2$^{\#}$	94.7 ± 6.7$^{\#}$	1.84 ± 0.036^{N}	0.17 ± 0.028$^{\#}$	81.9 ± 6.87$^{\#}$	0.79 ± 0.043$^{\#}$	30.4 ± 0.78$^{\#}$
EAC + (S + Q200)	166.8 ± 6.5$^{\#}$	90.2 ± 2.7$^{\#}$	2.6 ± 0.04$^{\#}$	0.21 ± 0.026$^{\#}$	73.2 ± 4.48$^{\#}$	0.68 ± 0.036$^{\#}$	25.6 ± 1.39$^{\#}$

Data are expressed as mean ± Standard error of the mean (SEM), ($n = 10$), * $P < 0.05$ significant compared to control group, $^{\#}$indicates significant difference at < 0.05 compared to EAC group, ^{N} indicates non-significant difference at $P \geq 0.05$ compared to EAC group.

Effect of treatment with S, Q, or S + Q200 on oxidative stress

ROS are frequently produced as byproducts of metabolism and can be used by the cell during phagocytosis and signal transduction (Kohchi et al. 2009). Excess ROS generation can trigger lipid, protein, organ, and DNA oxidative damage (Valko et al. 2007). Free radicals, such as the superoxide radical, do not cause direct cell damage but are involved in the Fenton reaction to produce a highly active hydroxyl radical (Benov 2001). In normal cells, antioxidant enzymes were produced to neutralize excessive ROS (Poljšak and Fink 2014). Superoxide dismutase can deactivate the superoxide radical and produce hydrogen peroxide, which is converted to water by catalase (CAT) (Urso and Clarkson 2003). Another way to remove the excessive ROS is by converting oxidized glutathione, in the presence of glutathione reductase, to reduced glutathione. The total amount of antioxidant enzymes inside the cell can be determined by estimating the TAC level (Scandalios 2005). Increased ROS along with an insufficient antioxidant system can result in the production of large amounts of MDA (Marnett 1999).

EAC cells have a high proliferation rate and are associated with elevated levels of metabolic byproducts, which lead to increased levels of ROS. That ROS elevation leads to MDA elevation therefore, the antioxidant enzymes must be depleted (Halliwell 2007).

As shown in Table 6, the untreated EAC mice presented with significant reductions in SOD, GSH, CAT, and TAC levels and a significant elevation in MDA concentration ($P < 0.0001$, $P < 0.0001$, $P = 0.009$, $P = 0.0004$, and $P < 0.0001$, respectively) compared with the healthy control mice. There were slight but nonsignificant increases in the levels of SOD, GSH, CAT, and TAC and a decrease in MDA values in the EAC-inoculated mice treated with either S or Q compared with those in the untreated EAC group (Table 6). These results indicate that treatment with S or Q can inhibit the growth of EAC cells but not kill them. However, the EAC-inoculated mice treated with S + Q200 demonstrated significantly elevated levels of SOD, GSH, CAT, and TAC and a significant reduction in MDA values ($P = 0.031$, $P < 0.0001$, $P = 0.0003$, $P = 0.013$, and $P < 0.0001$, respectively) compared with the untreated EAC group; the GSH, CAT, and TAC values were similar to those in the control group. These data confirmed that treatment with S + Q200 combination can inhibit the growth of EAC cells and improve the levels of antioxidant enzymes to prevent oxidative damage (Davies 2000). Overall, the findings of the *in vivo* experiment in this study confirm that treatment with S + Q can stimulate the antioxidant system by increasing the levels of SOD, GSH, TAC, and CAT and decreasing lipid peroxidation to eliminate excess ROS produced from dead EAC cells.

Table 6
Mean serum SOD, CAT and TAC activities, GSH levels and MDA concentration in red blood cells in controls, untreated EAC and the four treated groups

Group	SOD U/ml	GSH U/L	Catalase U/L	T A C mM/L	MDA/RBCs (mol/mL packed cells $\times 10^{-5}$)
Control DMSO	203.2 ± 3.2	179 ± 3.37	90.8 ± 2.87	1.54 ± 0.027	1.04 ± 0.02
Control S	178.1 ± 3.38	176.6 ± 4.17	89.13 ± 3.53	1.29 ± 0.03	1.49 ± 0.143
Control Q	176.91 ± 3.28	181.38 ± 6.49	97.75 ± 1.69	1.43 ± 0.03	1.09 ± 0.085
Control S + Q200	200.46 ± 4.68	196.2 ± 3.78	103.89 ± 2.04	1.43 ± 0.07	0.92 ± 0.067
EAC	157.5 ± 3.65*	108.7 ± 3.8*	74.2 ± 2.57*	0.85 ± 0.03*	3.85 ± 0.11*
EAC + S	165.5 ± 5.1^{N}	111.2 ± 4.65 ^{N}	79.0 ± 2.1 ^{N}	1.05 ± 0.04 ^{N}	3.70 ± 0.08 ^{N}
EAC + Q	169.4 ± 3.0 ^{N}	120.2 ± 3.46 ^{N}	81.8 ± 2.4 ^{N}	1.11 ± 0.043 ^{N}	3.57 ± 0.08 ^{N}
EAC + (S + Q200)	186.3 ± 5.6$^{\#}$	181.6 ± 3.48$^{\#}$	95.9 ± 1.76$^{\#}$	1.45 ± 0.036$^{\#}$	1.82 ± 0.04$^{\#}$

Data are expressed as mean ± Standard error of the mean (SEM), ($n = 10$)„ *P < 0.05 significant compared to control group, $^{\#}$ indicates significant difference at P < 0.05 compared to EAC group, ^{N} indicates no significant difference at $P \geq 0.05$ compared to EAC group.

Effect of treatment with S, Q, or S + Q200 on the relative expression levels of caspase3 and NFkB genes

The expression levels of the pro-apoptotic gene, *caspase3*, and the pro-inflammatory gene, *NFkB*, were measured by real-time reverse transcriptase-polymerase chain reaction, which expresses the variations in the transcription levels of these genes after treatment with S and/or Q (Fig. 1S&2S **and** Table 1S&2S, **supporting data**). A significant (P = 0.001) upregulation in the expression level of *caspase3* was observed in the liver tissues of the mice in the untreated EAC group (**Fig. 5 and** Table 7) compared with those in the control (normal) mice. This elevated expression was significantly (P < 0.05) upregulated after treatment with S or Q or S + Q, with the highest expression in S + Q200 group.

Table 7
Effect of S and/or Q treatment on the relative expression of *caspase3* and *NFkB* gene in mice liver of control, untreated EAC and the four treated groups.

Groups	*Caspase3*	*NFkB*
Control DMSO	1.00 ± 0.09	1.00 ± 0.03
EAC	2.66 ± 0.08*	5.90 ± 0.25*
EAC + S	4.38 ± 0.12$^{\#}$	4.26 ± 0.2$^{\#}$
EAC + Q	6.36 ± 0.12$^{\#}$	3.14 ± 0.17$^{\#}$
EAC + (S + Q200)	8.22 ± 0.21$^{\#}$*	1.88 ± 0.08$^{\#}$*

Results are expressed by mean ± SEM, (n = 10).*P< 0.05 significant compared to control group, $^{\#}$ indicates significant difference at P< 0.05 compared to EAC group.

A significant (P= 0.002) upregulation in the expression level of *NFkB* was observed in the liver tissues of the mice in the untreated EAC group (**Fig. 5 and** Table 7) compared with those in the control group. This elevated expression was significantly (P< 0.05) downregulated after treatment with S, Q, or S + Q with the lowest expression observed in the S + Q200 group; however, these reduced levels were higher than those in the control group.

Effect of treatment with S, Q, or S + Q200 on the liver histopathology

Histopathological examination of the untreated EAC mice showed the presence of hepatic nodules surrounded by mononuclear cells followed by a halo zone of suspected EACs, and most of the hepatocytes were markedly vacuolated (Fig. 6A). The livers of the EAC-inoculated mice treated with S presented with small foci of suspected EACs associated with moderate hepatic vacuolation (Fig. 6B). Similarly, liver examination of the EAC-inoculated mice treated with Q showed small foci of suspected EACs associated with mild hepatic vacuolation (Fig. 6C). However, the liver tissues of EAC-inoculated mice treated with S + Q200 demonstrated the presence of normal structures without any hepatic vacuolation (Fig. 6D). These results established the effectiveness of S + Q in inhibiting EAC proliferation by improving the activities of the antioxidant enzymes and thereby protecting the liver from ROS produced by the EAC cells.

Discussion

Q is an edible polyphenol widely distributed in fruits and vegetables (Kumar and Pandey 2013). It has several therapeutic effects, such as anti-proliferative and antioxidant activities, both *in vitro* and *in vivo* (Choi et al. 2001; Shoskes and Nickel 2007; D'Andrea 2015; Nguyen et al. 2017; Granato et al. 2017; Chalet et al. 2018). The use of Q in combination with anticancer drugs such as doxorubicin and cisplatin

has been established and proven to result in superior anticancer with antioxidant activities (Nessa et al. 2011; Wang et al. 2012; Brito et al. 2015). The structure of sulfonamide makes it conducive for use as an anticancer agent (Ghorab et al. 2009). Sulfonamide derivatives have been recently used as anti-proliferative agents against different human cancer cell lines (Lu et al. 2015; Ghorab et al. 2016; Gul et al. 2018; Koyuncu et al. 2019; Apaydın and Török 2019). They are reported to act as an anticancer agent (by arresting the G1 phase cell cycle and inhibiting carbonic anhydrase) and exert anti-angiogenic effects (Owa et al. 1999; casini et al. 2002; van Kesteren et al. 2002; Huang et al. 2019). S is the most commonly used sulfonamide, and when successfully combined with selenium, it acts as an anticancer agent against hepatocellular carcinoma *in vivo* (Gupta et al. 2013). S was combined with copper to produce a potent anticancer copper complex, which demonstrated a higher *in vitro* activity than cisplatin (RAMA and SELVAMEENA 2015). In the present study, to increase the anticancer activity of S and the antioxidant activity of the combined drug and to reduce the toxic effect of sulfamethoxazole, a combination of a natural edible polyphenol (Q) and low dose S was used *in vitro* and *in vivo*.

Programmed cell death is a normal process that maintains homeostasis in the body (Elmore 2007). The anti-inflammatory proteins that protect the healthy cells from inflammation are released during apoptotic cell death (Voll et al. 1997). Hence, it was known that the better anticancer drug is that can induce apoptotic over necrotic cell death. Apoptosis has many signaling pathways, all of which end with caspases activation (Li and Yuan 2008). Caspases are pro-apoptotic proteins that cause DNA fragmentation and change the biochemical and morphological features of the cells (Degterev et al. 2003).

In the present study, decreased IC_{50} was observed with S + Q rather than with S or Q alone in the four human cancer cell lines, thus exhibiting the efficiency of the combined drug as an anticancer agent. Also, the CI values established that S + Q combination exhibited synergistic interactions. The anticancer activities of S, Q, and S + Q were detected by the induction of apoptosis in the human cancer cell lines. The *in vitro* results confirmed the elevations in the percentages of apoptotic cells and the decrease in the percentage of necrotic cells in the MCF-7, PC3, and HCT-116 cell lines after treatment with S + Q200 when compared with those in cells treated with S or Q alone.

Several *in vitro* studies have reported that sulfonamide and its derivatives act as anticancer agents by inducing cell cycle arrest in the G0 or G1 phase with accumulation in the G2 or M phase (Yokoi et al. 2002; casini et al. 2002; Scozzafava et al. 2003; Koyuncu et al. 2019; Azevedo-Barbosa et al. 2019). Similarly, recent *in vitro* studies had revealed the anticancer effect of Q via the elevation in the percentage of apoptotic cancer cells; additionally, the proportions of cells in the G0/G1 phases were reduced, whereas those in the G2/M phases were increased (Ong et al. 2004; Chien et al. 2009; Kumar and Pandey 2013; Massi et al. 2017; Nguyen et al. 2017). These results were in agreement with those in the present study, wherein the percentage of MCF-7 cells was elevated in the G2/M phase after treatment with S + Q200 and decreased in the G0/G1 phase, thus indicating the efficacy of S + Q as an anti-proliferative agent by preventing the cancer cells from entering into another cell cycle.

The EAC-inoculated model was used in this study to indicate the *in vivo* anticancer effect of the combination of S and Q. EAC cells are undifferentiated cancer cells that quickly spread in the abdominal region, resulting in an increase in the abdominal circumference and damage to vital organs such as the liver and kidney. Our results demonstrated a significant decrease in abdominal circumference in EAC-inoculated mice treated with S + Q200 compared with those treated with S or Q alone, thus indicating the potent anti-proliferative effect of the combined drug

To demonstrate the effect of S + Q treatment on apoptosis induction, the annexin V assay was conducted using EAC cells aspirated from the abdominal regions of the mice in each group. EAC proliferation can elevate ROS levels and induce oxidative damage in the organs (Davies 2000). An effective chemotherapeutic agent can eliminate ROS and increase cancer cell death via apoptosis (Conklin 2004). In the present study, the apoptosis percentage was elevated in all the treated groups, particularly the group treated with S + Q200, and decreased in the untreated EAC group. The number of viable cells in the EAC-inoculated mice treated with S + Q200 was significantly decreased, and the number of dead cells was increased via the apoptotic pathway, thus indicating the anti-proliferative effect of the combined drug. These results coincided with those of the *in vitro* experiments in this study, thus confirming that the combination of S and Q can act as a promising chemotherapeutic drug that selectively favors the apoptotic pathway rather than necrosis.

The apoptotic pathway is regulated by various genes that help in removing dead cells safely from the body (Fuchs and Steller 2011; Elmore 2007).). It ends with the activation of caspase3 (Kataoka et al. 1998) and is terminated by the cleavage of this protease resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors, and finally uptake by phagocytic cells (Kerr 2002). Apoptosis impairment can lead to prolonged survival of the cancer cell and lead to metastasis. The *NFkB* gene is an important anti-apoptotic gene that can activate the transcription of growth-promoting genes in cancer cells. When activated, it can enhance anti-apoptosis and increase the propagation, survival, and metastasis of cancer cells (Coornaert et al. 2009; Pepper et al. 2009). Deactivation of apoptosis has been correlated with increased cell proliferation and cancer development (Plati et al. 2008; Fulda 2009). Effective therapeutic approaches involve the enabling of certain apoptotic genes to force cancer cells into apoptotic cell death (Gimenez-Bonafe et al. 2009; Fulda 2015; Rathore et al. 2017). *NFkB* is reported to inhibit apoptosis by deactivating the pro-apoptotic protein, *Bcl-2*, in various cancer cell lines (Sohur et al. 1999; Chendil et al. 2002; Martinez-Castillo et al. 2016; Rawat and Nayak 2021). Furthermore, *NFkB* stimulation, often found in cancer cells, regulates a cascade of genes and causes a significant increase in cell growth (cell cycle), proliferation, and metastasis (Subramaniam et al. 2018). Moreover, it deactivates the caspase family leading to the downregulation of *caspase3* and apoptosis resistance (Bai and Wang 2015; Yu et al. 2020). In our *in vitro* study, the *NFkB* protein expression level was increased in untreated MCF-7 cells and decreased with S + Q treatment which indicated the efficiency of that combination in decreasing cell proliferation and triggering apoptotic pathway ends with *caspase3* activation. *Caspase3* activation was confirmed, in our study, as its level was increased in MCF-7 cells treated with S + Q more than its level in untreated cells. In the *in vivo* study, the EAC group had elevated

levels of *NFkB*, which suggested increased cancer cell propagation and the blockage of the apoptosis signaling pathway via downregulation of the *caspase3* gene. EAC-inoculated mice treated with S or Q had reduced expression levels of *NFkB* and increased expression levels of *caspase3*, thus indicating their apoptotic action on EAC cell proliferation. However, a highly significant downregulation in the expression level of *NFkB* and upregulation in the expression level of the *caspase3* gene was detected in the EAC-inoculated mice treated with S + Q200 when compared with those in the mice treated with S or Q alone. These significant changes in the S + Q200 treated group confirmed the induction of the apoptotic pathway, which ended with the lysis of the cells after the cleavage of *caspase3*; thus, the proliferation and metastasis of the EAC cells were prevented.

Superoxide anion is one of the ROS normally produced inside cells (Ray et al. 2012). SODs are a group of enzymes that can balance the metabolic production of ROS by neutralizing the superoxide anion (Davies 2000; O'Neil 2013). Removal of the superoxide anion from the system prevents damage to the proteins, lipids, and DNA and thereby the development of various diseases complications (Fattman et al. 2003). SODs were associated with other enzymes that degrade H_2O_2, such as CAT and glutathione peroxidases. SOD mainly neutralizes superoxide anion ($O_2^{-\bullet}$) to hydrogen peroxide, which is then degraded by CAT into water and oxygen.

Increased cancer cell proliferation leads to elevated levels of ROS in the form of waste metabolites, which can consume antioxidant enzymes and produce oxidative stress leading to tissue damage (Birben et al. 2012). Furthermore, the superoxide radical can directly activate *NFkB* (Wang et al. 2004). *NFkB* gene has been shown to markedly increase cancer proliferation, enhance cell cycle propagation, and block apoptosis (Tornatore et al. 2012). The untreated EAC mice presented with decreased proportions of apoptotic cells, increased the levels of *NFkB*, and decreased the levels of SOD, GSH, CAT, and TAC in the present study. However, the EAC-inoculated mice treated with S + Q200 presented with elevated SOD, GSH, CAT, and TAC levels and decreased lipid peroxidation (MDA) levels compared with those in the EAC group, indicating the antioxidant and protective actions of the combination of S and Q against EAC cell invasion. Superoxide anion levels were decreased, and *NFkB* was inhibited because of the increase in antioxidant activity following treatment with S + Q200, which resulted in the inhibition of EAC cell proliferation. On the contrary, *caspase3* was observed to be increased in apoptotic cell death of cancer cells treated with a chemotherapeutic drug. (Pistritto et al. 2016).

A successful anticancer agent must be selectively toxic to the cancer cells and safe for the vital organs. The results of this study revealed that treatment with S alone did not improve the liver and kidney function tests and the action of the antioxidant enzymes. Conversely, the EAC-inoculated mice treated with Q alone demonstrated slight improvements in the liver and kidney function tests and the antioxidant enzyme activities; however, they did not reach the levels observed in the control group. The EAC-inoculated mice treated with S + Q200 demonstrated improved liver and kidney function tests, thus indicating that they were safe to use as anticancer agents. The liver sections of the untreated EAC-inoculated mice consisted of numerous hepatic nodules and EAC cells, whereas marked improvement

was observed in the liver sections of the EAC-inoculated mice treated with S + Q200 (normal hepatocytes).

In conclusion, this study revealed the potential anticancer activities of the combination of S and Q on MCF-7, HepG2, HCT-116, and PC3 cell lines via the induction of the apoptotic pathway. The anti-proliferative effect of S + Q200 was established by the induction of apoptosis during sustained cell cycle arrest in MCF-7 cells. The *in vivo* experiments using the EAC mouse model demonstrated the anticancer and antioxidant potentials of S + Q200. The combination of S and Q appeared to act as a chemotherapeutic agent by increasing apoptosis via *caspase3* activation and *NFkB* inhibition in the EAC-inoculated mice. Furthermore, selective cancer cell toxicity and protection of the vital organs were observed following treatment with the combination of S and Q *in vivo*. Thus, the combination of S and Q might be considered as a promising anticancer agent with limited side effects and could be tested on cancer patients.

Declarations

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Ethical approval The animal experiment (mice) was approved in accordance with the Ethics Committee of Kafrelsheikh University, Egypt (4/2018EC). No human participants were used in this study.

Consent to Participate All authors have approved the participation section of the manuscript.

Consent to publish All authors have read and approved the final version of the manuscript.

Author contributions ER did the experimental section, analyzed the data, and wrote the article. HS designed and helped in experimental section, contributed part of kits, statistical analysis, writing and revise the article. F. A. participated in the *in vitro* study, revise the manuscript. MM contributed reagents, and revising the article. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Competing Interests No conflict of interest was stated by the authors.

Availability of data and materials All data and materials as well as software (SPSS 18.0 software) support the published claims in this manuscript and comply with field standards. Manuscript data will be available on demand.

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Figures

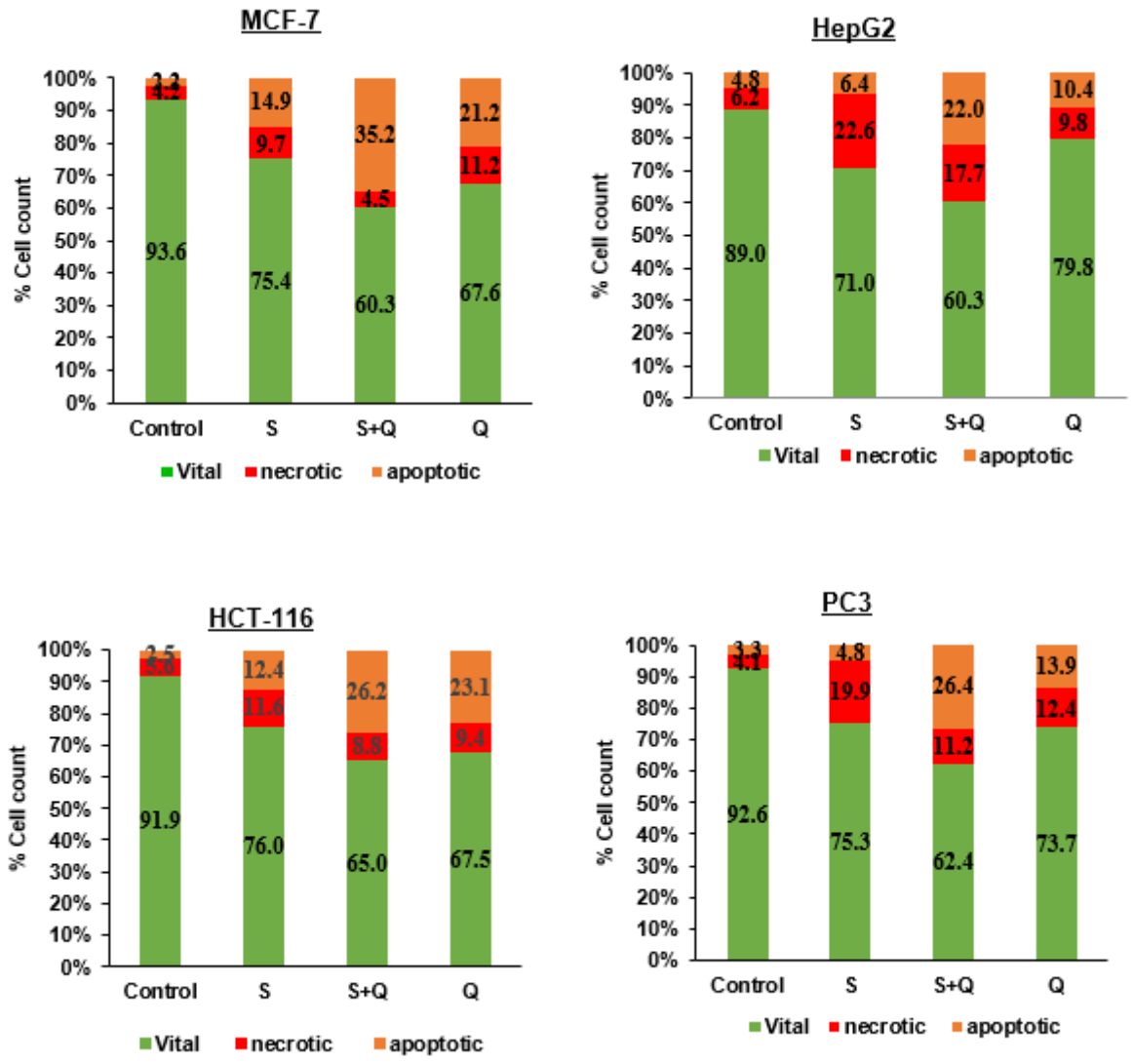


Figure 1

The percent of apoptosis and necrosis in MCF-7, HepG2, HCT-116 and PC3 cell lines, after being treated with 30% of the IC50 of S, Q and S + Q for 24 h compared to control untreated cells. All the experiments were performed three times independently for each cell line.

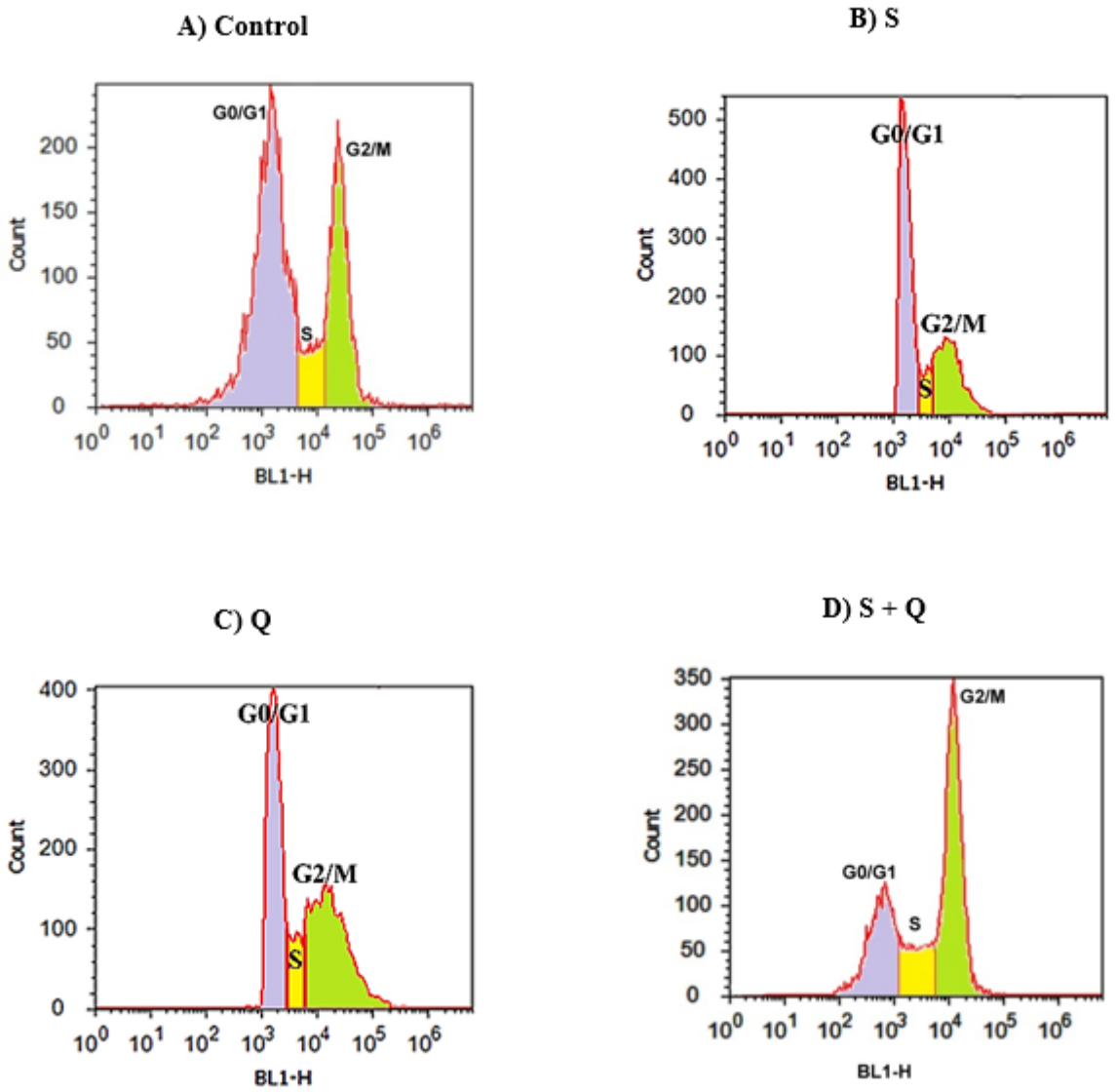


Figure 2

The cell cycle distribution in MCF-7 cells before and after treatment with S, Q and S+Q (IC50) measured by the flow cytometry using PI.

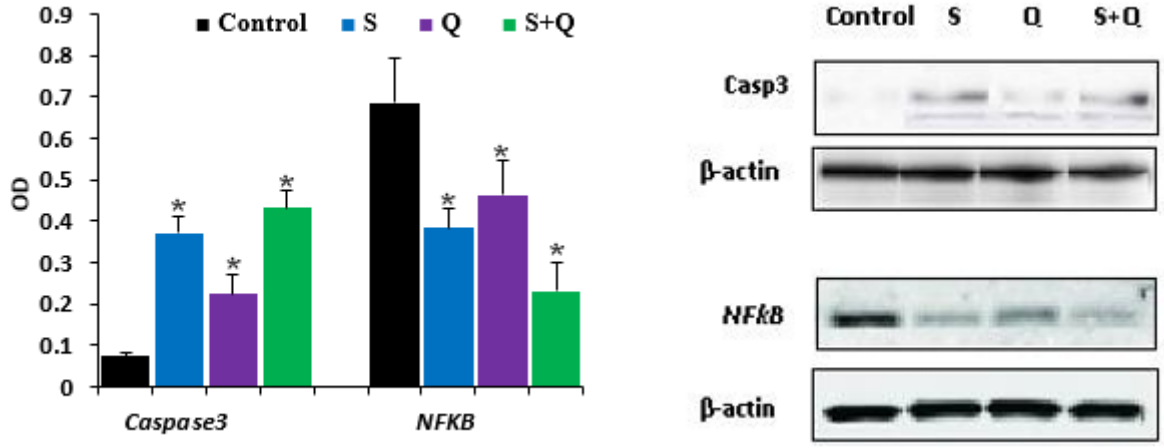


Figure 3

Protein expression of both caspase3 and NFKB in MCF-7 cells before and after treatment with S, Q and S+Q (IC50s), (A) bar plots and (B) representative western blot image of caspase3 and NFKB. * indicates significant difference at $P < 0.05$ compared to untreated MCF-7 (control).

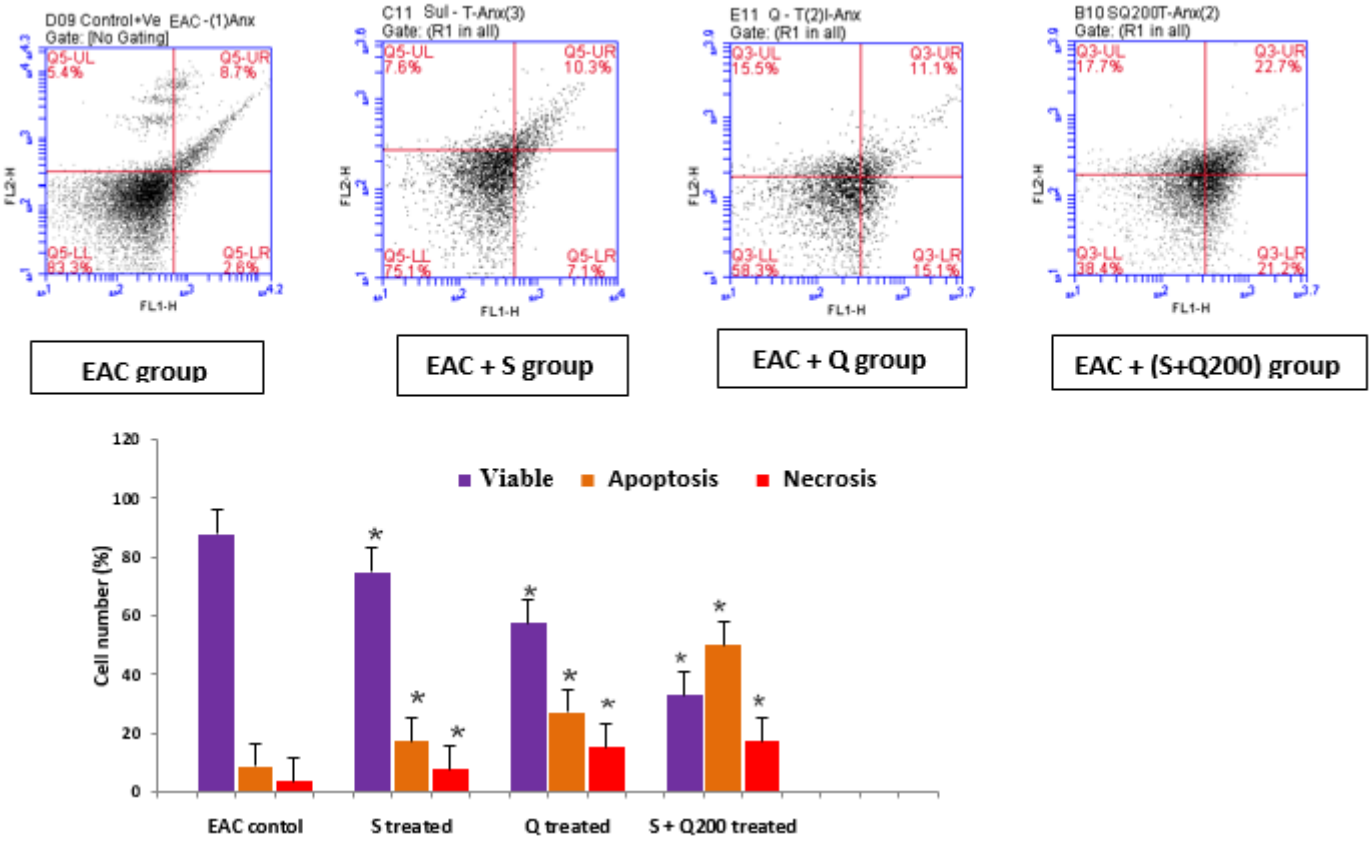


Figure 4

Live, dead and apoptotic rate in EAC cells aspirated from the abdominal region of untreated EAC and the three treated groups compared to the untreated EAC measured by the flow cytometry using annexin V kit.The upper left quarter (QUL) represents necrotic cells, the upper and lower right quarters (QUR and QLR) represent late and early apoptotic cells, the low left quarter (QLL) represents viable cells. n = 10, * $P \leq 0.05$ indicates significant different of means compared to the untreated EAC inoculated mice.

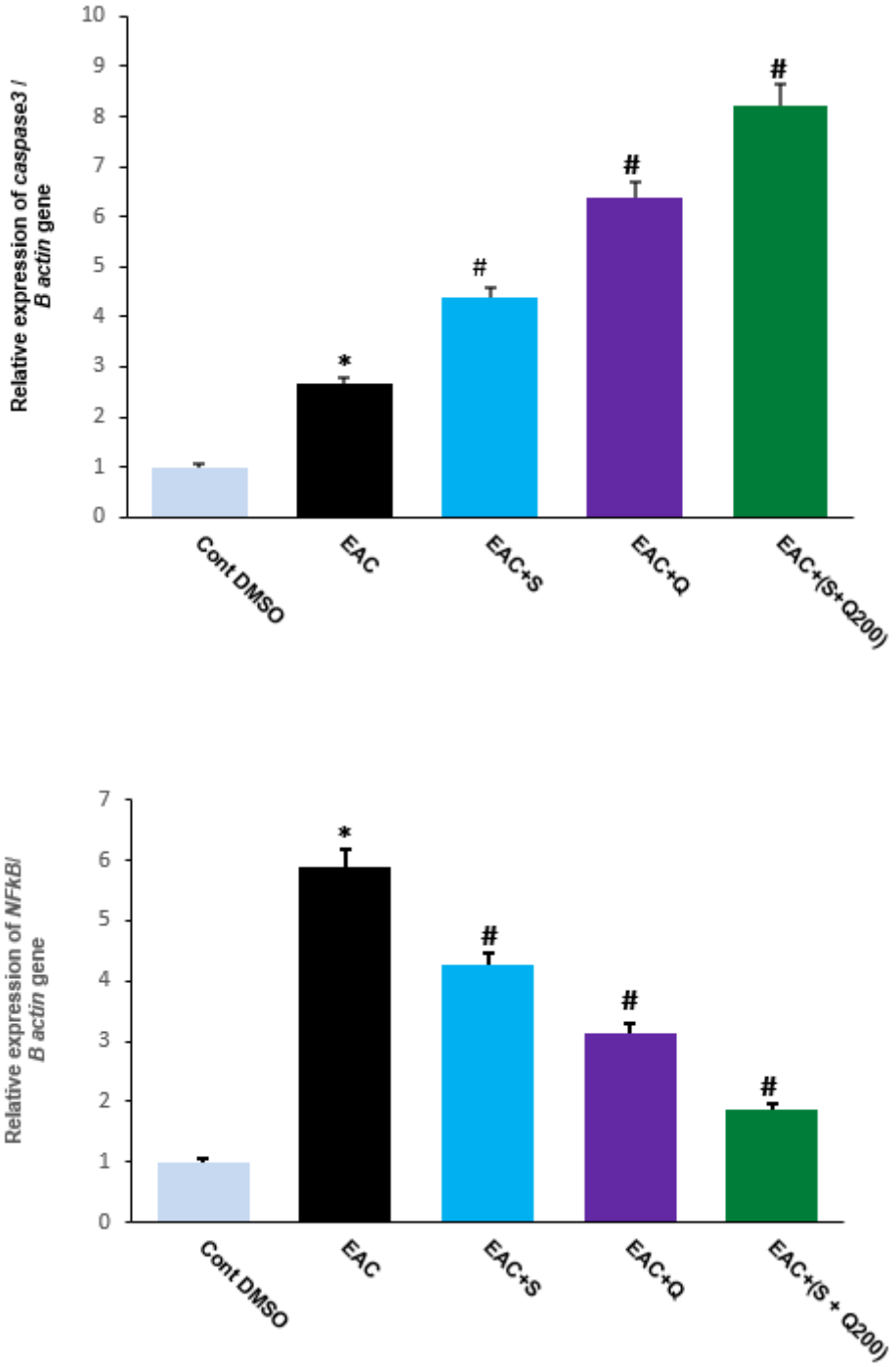
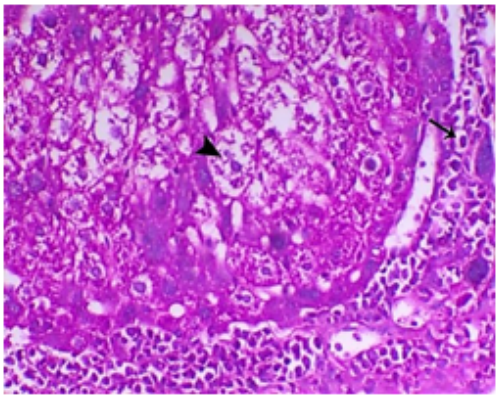
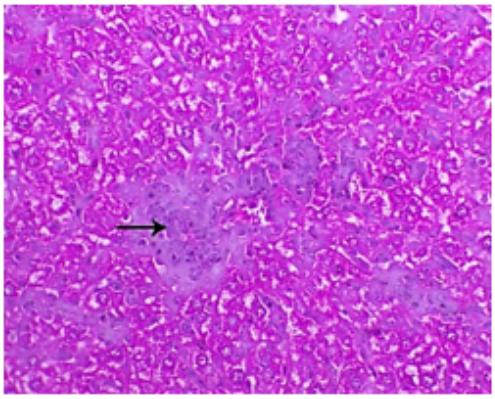


Figure 5

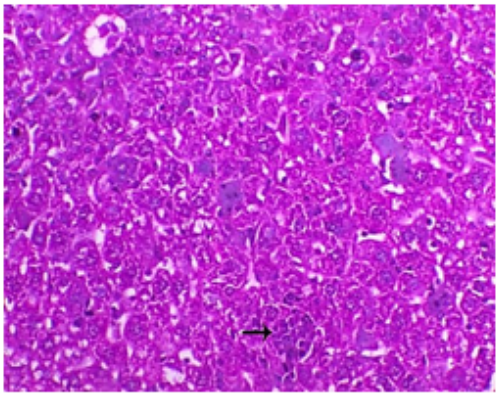
Graphical presentation of real-time quantitative PCR analysis of the expression of both caspase3 gene and NFkB gene in mice liver after administration of S and/or Q. *P < 0.05 significant compared to control group, # indicates significant difference at P < 0.05 compared to EAC group. All the experiments were performed three times independently.



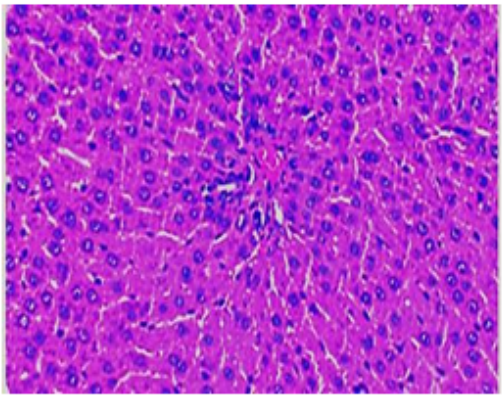
A. Liver of untreated EAC mice



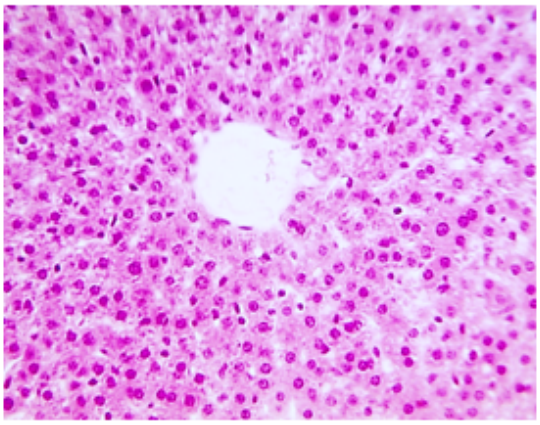
B. Liver of E+S treated mice



C. Liver of EAC + Q treated mice



D. Liver of E+ S + Q200 treated mice



E. Liver of control mice

Figure 6

Histopathological examination of mice liver of untreated EAC and the S, Q and S + Q200 treated groups, H&E, X200

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AnticancerSQresults.xlsx
- Graphicalabstract.pdf
- Supportingdata.docx