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

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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 cell 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.

Keywords Quercetin · Sulfamethoxazole · Apoptosis · Antioxidant enzymes · NFkB · caspase3

Abbreviations
Caspase3  Cysteiny1 aspartate-specific proteases 3
EAC  Ehrlich ascites carcinoma
EB/AO  Ethidium bromide and acridine orange
HCT-116  Colon carcinoma
HepG2  Hepatocellular carcinoma
IC50  50% Inhibitory concentration combination
MCF-7  Human breast adenocarcinoma
NFkB  Nuclear factor kappa-B
PC3  Prostate carcinoma
Q  Quercetin
ROS  Reactive oxygen species
S  Sulfamethoxazole
S + Q  Sulfamethoxazole and Quercetin

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 carboxylic anhydrase inhibitory activities (Casini et al. 2002; Scozzafava et al. 2003; Ghorab et al. 2009; Marques et al. 2010). Sulfadiazine (S) is a typical sulfonamide with a –SO₂NH₂ group linked to a phenyl ring. However, S alone produces numerous side effects; therefore, it was used, as medicine, in combination with other compounds (Wormser et al. 1982; Kielhofner 1990; Hamid et al. 2018; J. et al. 2019). Also, recent studies approved the in vivo anticancer effects of S + selenium on hepatocellular carcinoma (Gupta et al. 2013) and its improvement 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). The S and Q combination has also been shown to be an effective antibacterial agent with few side effects (Sahyon et al. 2019). However, it is essential to study compound combinations in order to avoid the toxic effects of chemical mixtures, such as drug–drug mixture as the appearance of carbamazepine toxicity when combined with antiviral drugs (Bates and Herman 2006; Al-Sulami and Alyamani 2019). The toxic effects of a combination may appear even if a natural extract is combined with a synthetic drug, such as the effect of grapefruit juice on the bioavailability of some medications (Schwarz et al. 2005; Glaeser et al. 2007).

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 (Murata 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) or even with a nanomaterial oxide (Elsayed et al. 2021). 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 lyse 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 ((C₁₀H₁₁N₃O₃S) 98.5%, Burroughs-Wellcome Company, Research Triangle Park, NC 27709, USA), quercetin dihydrate ((C₁₅H₁₀O₇·2H₂O) 97% Lot. 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).

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USA; Cat.no. SV30082), l-glutamine (Invitrogen, Grand Island, New York, USA; Cat.no. 25030024), and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenlytetrazolium bromide) were 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, Milwaukie, 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 (no. 33595, cell signaling technology, USA) and monoclonal anti-mouse β-actin (ab6276, Abcam) were obtained. The chemiluminescent western blot ECL substrate (Perkin Elmer, Waltham, MA, USA) reagent was purchased. The kits for serum total bilirubin level (T. Bil.), activities of serum glutathione reductase (GSH), catalase (CAT), and super oxide dismutase (SOD), malondialdehyde (MDA), aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (Alb.), and creatinine were acquired from SPINREACT (SPINREACT, S.A.U., Girona, SPAIN). The kits for measurement of glutathione, uric acid, and creatinine were obtained from American Type Culture Collection, ATCC, NY, Sarthe, were used in exponential growth phases and then washed, trypsinized, and suspended in DMEM. All media were provided with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 2% l-glutamine.

The IC50 value is the half maximal inhibitory concentration. Sigmodial and dose dependent curves were constructed to plot the results of the experiment. The concentration of the compounds inhibiting 50% of cells (IC50) was calculated using the sigmodial curve.

**Combination index analysis**

The combination index (CI) between S and Q was calculated by the isobolographic analysis, a dose-oriented geometric method of assessing drug interactions (Roell et al. 2017). From the IC50 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 IC50 dose of the drug 1 alone; Dx2 is the IC50 dose of the drug 2 alone, D1 is the IC50 dose of drug 1 in combination with drug 2; D2 is the IC50 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’s 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 appear 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 that 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 IC50 of S and/or Q at 37 °C in a humidified 5% CO2 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. We chose the 30% IC₅₀ after testing the cell’s morphology under the microscope at different concentrations of 20, 30, 40, and 50% of the IC₅₀, as well as the real IC₅₀, and noticed that the apoptotic changes appeared at this concentration. 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:

\[
\text{% of apoptotic or necrotic} = \frac{\text{total number of apoptotic or necrotic cells}}{\text{total number of cells counted}} \times 100.
\]

**Cell cycle analysis by using propidium iodide by the flow cytometry**

MCF-7 cells were treated with the IC₅₀ of S + Q combination and the cell cycle was 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% IC₅₀ of S and/or Q as well as untreated MCF-7 cells was 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 h at 25 °C, then washed. Primary antibody for *NFkB*, *caspase3*, and *β-actin* was 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 until 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 cell 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 × 10⁶ 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 with 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%. After measuring the abdominal circumferences of all animals, a volume of 100 μl of the diluted EAC cells containing 2.5 × 10⁶ 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 × 10⁶).
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 administered, 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 euthanized by ether inhalation as approved by the Ethics Committee of Kafrelsheikh University. 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 while the rat was euthanized, 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 pellets were aspirated carefully, diluted with sterile PBS (1×10⁶ 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 the Wilkinson et al. (1972) method, while serum albumin was determined by the method of Dumas et al. (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 et al. (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 and 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’s protocol (Thermo Scientific, Fermentas, #K0731, USA). The extracted RNA were evaluated by Nanodrop. Nanodrop revealed presence of pure RNA with large concentrations (1240–1890 ng/µl). Five micrograms 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 2 × Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer’s 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 NFκB 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 housekeeping gene encoding liver after treatment with S and/or Q compounds. The changes in transcription levels of these genes in mice NFkB and the pro-inflammatory gene that reflects the caspase3 (USA). The amplified cDNA was used to conclude 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.

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−∆∆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. Tukey analysis was done for the calculation of significances and probabilities and for the comparison between groups, a probability of p < 0.05 is considered significant. The R² data were calculated using the Regression tool in Excel 2010.

### Table 1

| Gene    | Forward primer (‘5 — ‘3) | Reverse primer (‘5 — ‘3) |
|---------|--------------------------|--------------------------|
| Caspase3 | GGAGGCTGACTTCTCTGTATGCTT | CCTGTTACGCAGTGAGAATG     |
| NFκB    | GAAATCCCTGTACAGACAAAAAC | ATCACTTTCAATGGCCCTGTGTAG |
| β-actin | ACTATTGGCAACGAGCGGTT    | CAGGATTCCATAACCAGAAGGA   |

### Table 2

| 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 |
| W138       | 828.1 ± 12.9 | 969.6 ± 13.8 | 1146.3 ± 45.4 | 6.52 ± 1.53 |

### 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, in addition to W138 as normal cell line. The anti-proliferative effect was assessed using the MTT reagent to calculate the IC₅₀. The determination coefficient (R²) for every cell line was explored from the viability data and confirmed to meet the value > 0.90 (Table 1S). 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 IC₅₀ of 55.97 ± 3.49, 135.9 ± 8.5, and 162.2 ± 10.1 µg/ml, respectively, when compared with the IC₅₀ 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 (IC₅₀) 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 (IC₅₀ 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. The safety of the S + Q combination on a normal cell line (W138) was detected. As represented in Table 2, S + Q showed a high IC₅₀ value (1146.3 ± 45.4 µg/ml) on the W138 cell line compared with both S and Q (p = 0.001 and p = 0.011, respectively); however, both S and Q also have higher IC₅₀ values compared to doxorubicin. These data
indicates the safety of the S + Q combination on normal tissues, which can be used safely in the in vivo experiment.

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 IC₅₀ values of S, Q, and S + Q) for 24 h compared with untreated human cancer cells. The percent apoptosis to necrosis was calculated from the dot-plot that was divided into four quarters. The apoptotic rate was calculated from both the late and early apoptosis percentages. The percent of cells in the upper and lower right quarters (Q-UR and Q-LR) represented early and late apoptosis, respectively. While the percent of cells in the upper left quarter (Q-UL) represented the necrotic cells, the cells in the lower quarter represented the necrotic cells (Q-LL). 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.

**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 IC₅₀ 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.

**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% IC₅₀ 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 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 treating 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 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.

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

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 24 h 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 ± 4.78        | 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            | 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             |
|            | 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≤0.05 indicates significant differences compared to the untreated control cells

**P>0.05 indicates non-significant differences compared to the untreated control cells

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

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.
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 \( (P < 0.0001, P < 0.0001, P = 0.009, P = 0.0004, \text{ and } P < 0.0001, \text{ respectively}) \) 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 \( (P = 0.031, P < 0.0001, P = 0.0003, P = 0.013, \text{ and } P < 0.0001, \text{ respectively}) \) compared with the untreated EAC group; the GSH, CAT, and TAC

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 ± 1.7  | 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)
values were similar to those in the control group. These data confirmed that treatment with S + Q 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.

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. 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) 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.

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) 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.

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 N | 94.7 ± 6.7 N | 1.84 ± 0.036 N | 0.17 ± 0.028 N | 81.9 ± 6.87 N  | 0.79 ± 0.043 N   | 30.4 ± 0.78 N  |
| 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, *significant difference at < 0.05 compared to the EAC group, Nnon-significant difference at P ≥ 0.05 compared to the EAC group.

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 | TAC mM/L | MDA/RBCs (mol/mL packed cells × 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 | 89.1 ± 3.53 | 1.29 ± 0.03 | 1.49 ± 0.14                          |
| 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, *significant difference at < 0.05 compared to the EAC group, Nnon significant difference at P ≥ 0.05 compared to the EAC group.
**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. 2022).
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 one that can induce apoptotic over necrotic cell death. Apoptosis has many signaling pathways, all of which end with caspase 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. To some extent, the IC_{50}s of both S and Q were similar in both HepG2 and PC3 cell lines, which concluded the similarity in their anti-proliferative effects. Agreeing with our results, Q was proven to have anticancer activity against several cancer cell lines (Hassanzadeh et al. 2019; Kumar et al. 2020). Also, sulfonamide derivatives have been shown to inhibit the proliferation of HCT-116 colorectal, A2780 ovarian (Lu et al. 2015), HepG2, HeLa, CNE1, and A549 cell lines (Guo et al. 2019). However, our data revealed the in vitro anticancer effect of Q was mainly through apoptosis and the anticancer effect of S was through necrosis.

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 efficacy of S + Q 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, leading to nucleic acid double strand breakdown, extracellular matrix and cytoplasmic protein denaturation, protein cross-linking, appearance of apoptotic bodies, production of ligands for phagocytic cell receptors, and eventually ingestion 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 H2O2, such as CAT and glutathione peroxidases. SOD mainly neutralizes superoxide anion (O2^-) 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).

EAC is a type of carcinoma that can spread in the abdominal region affecting both liver and kidney (Mutar et al. 2019, 2020). A successful anticancer agent must be selectively toxic to the cancer cells and safe for the vital organs. Our data revealed significant increase in both liver and kidney function tests in EAC inoculated mice indicating sever tissues injury. The results of this study revealed that treatment with S alone did not improve neither liver and kidney function tests nor the action of the antioxidant enzymes. Conversely, the EAC-inoculated mice treated with Q alone demonstrated slight improvements in both 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 remarkable improvement in both liver and kidney function tests, and the antioxidant enzyme activities, 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).

From our data, we can hypothesize that during treatment with both S + Q combinations, a small dose of S has no toxic effect on healthy cells but can induce both apoptosis and necrosis in cancer cells. The high antioxidant activity of Q can induce the antioxidant system that scavengers the ROS produced by the cancer cells, preventing DNA damage to the healthy cells while at the same time preventing cancer metastasis. However, S + Q can force cancer cells to apoptotic cell death via cell cycle arrest at the G2/M phase, which prevents the cancer cells from entering a new cell cycle. From the molecular point of view, treatment with the S + Q combination can reduce the NFkB gene in cancer cells, preventing its metastasis along with enhancement of caspase3 protein that stimulates the apoptotic death of cancer cells.

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 by its action.
on the normal cell line and following treatment with the combination of S and Q in vivo. Thus, the combination of S and Q might be considered a promising anticancer agent with limited side effects and could be tested on cancer patients.

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Author contribution E. R. did the experimental section, analyzed the data, and wrote the article. H. S. designed and helped in experimental section; contributed parts of the kits, statistical analysis, and writing; and revised the article. F. A. participated in the in vitro study, and revised the manuscript. M. M. contributed reagents, and revised 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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Data availability 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.

Declarations

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.

Competing interests The authors declare no competing interests.

References

Ademosun AO, Oboh G, Bello F, Ayeni PO (2016) Antioxidative properties and effect of quercetin and its glycosylated form (Rutin) on acetylcholinesterase and butyrylcholinesterase activities. J Evid Based Complement Altern Med 21:NP11–NP17. https://doi.org/10.1080/2156587215610032

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126. https://doi.org/10.1016/0076-6879(84)05016-3

Afanas’ev IB, Dcrozhko AI, Brodskii AV et al (1989) Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochem Pharmacol 38:1763–1769. https://doi.org/10.1016/0006-2952(89)90410-3

Al-Sulami A, Alyamani S (2019) Carbamazepine toxicity during combination therapy with levetiracetam in two pediatric patients. J Neurol Sci. https://doi.org/10.1016/j.jns.2019.10.099

Anderson ME (1985) [70] Determination of glutathione and glutathione disulfide in biological samples. In: Methods in Enzymology 548–555. https://doi.org/10.1016/S0076-6879(85)3073-9

Ayapin S, Török M (2019) Sulfonamide derivatives as multi-target agents for complex diseases. Bioorg Med Chem Lett 29:2042–2050. https://doi.org/10.1016/j.bmcl.2019.06.041

Azedo-Do-Barbio H, Ferreira-Silva GA, Silva CF et al (2019) Phenylpropanoid-based sulfonamide promotes cyclin D1 and cyclin E down-regulation and induces cell cycle arrest at G1/S transition in estrogen positive MCF-7 cell line. Toxicol In Vitro 59:150–160. https://doi.org/10.1016/j.tiv.2019.04.023

Bai CY, Wang HL (2015) Paeoniflorin induces HepG2 cell apoptosis by regulating Caspase3 activation and nuclear factor kappa B signaling pathway. World Chin J Dig. https://doi.org/10.11569/wcjdz.v3.i2.j.3582

Bates DE, Heran J (2006) Carbamazepine toxicity induced by lopinavir/ritonavir and nelfinavir. Ann Pharmacother. https://doi.org/10.1345/aph.1G630

Benov L (2001) How superoxide radical damages the cell. Proto- plasma 217:33–36. https://doi.org/10.1074/PP01289410

Birben E, Sahiner UM, Sackesen C, et al (2012) Oxidative stress and antioxidant defense. World Allergy Organ J. https://doi.org/10.1016/j.waoj.2012.03.008

Brito AF, Ribeiro M, Abrantes AM et al (2015) Quercetin in cancer treatment, alone or in combination with conventional therapeutics? Curr Med Chem 22:3025–3039. https://doi.org/10.2174/092986732266150812145435

Burnette WN (1981) “Western Blotting”: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiiodinated protein A. Anal Biochem. https://doi.org/10.1016/0003-2697(81)90281-5

Casini A, Scozzafava A, Mastrolorenzo A, Supuran C (2002) Sulfonamides and sulfonlated derivatives as anticancer agents. Curr Cancer Drug Targets 2(55):75. https://doi.org/10.2174/156800902334060

Chen X-Y, Wu Y-C, Chung J-G et al (2009) Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. Hum Exp Toxicol 28:493–503. https://doi.org/10.1177/096032710907002

Choi JA, Kim YJ, Lee JY et al (2001) Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. Int J Oncol. https://doi.org/10.3892/ijo.19.4.837

Chou CC, Yang JS, Lu HF et al (2010) Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. Arch Pharm Res. https://doi.org/10.1007/s12272-010-0808-y

Conklin KA (2004) Chemotherapy-Associated oxidative stress: impact on chemotherapeutic effectiveness. Integr Cancer Ther 3:294–300. https://doi.org/10.1177/1534735404270335
from across disciplines. Front Pharmacol https://doi.org/10.3389/fphar.2017.00158

Sahyon HA, Ramadan ENM, Mashaly MMA (2019) Synergistic effect of quercetin in combination with sulfamethoxazole as new antibacterial agent: in vitro and in vivo study. Pharm Chem J 53:803–813. https://doi.org/10.1007/s11994-019-02083-z

Scandalias JG (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res 38:995–1014. https://doi.org/10.1590/S0100-879X2005000700003

Schwarz UJ, Seemann D, Oertel R et al (2005) Grapefruit juice ingestion significantly reduces talinolol bioavailability. Clin Pharmacol Ther. https://doi.org/10.1016/j.clpt.2004.11.111

Scott MG, LeGrys VA, Hood JL (2012) Electrolytes and blood gases. In: Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. Elsevier, pp 807–835. https://doi.org/10.1016/p978-1-4160-6164-9.00028-7

Scozzafava A, Owa T, Mastrolorenzo A, Supuran C (2003) Anticancer and antiviral sulfonamides. Curr Med Chem 10:925–953. https://doi.org/10.2174/0929867033457647

Seo M-J, Lee Y-J, Hwang J-H et al (2015) The inhibitory effects of dexamethasone on nuclear transcription factors transformations in pro-B lymphocytes. Gene Expr pp. 219-229

Smith SM, Ribble D, Goldstein NB, et al (2012) A simple technique for quantifying apoptosis in 96-well plates. In: Methods in Cell Biology. pp 361–368. https://doi.org/10.1016/B978-0-12-405914-6.00020-2

Soehn US, Dixit MN, Chen CL, et al (1999) Rel/NF-kB represses bcl-2 transcription in pro-B lymphocytes. Gene Expr pp. 219-229

Sondhi SM, Johar M, Singhal N et al (2000) Synthesis and anticancer, antiinflammatory, and analgesic activity evaluation of some sulfonamides. Bioorg Med Chem Lett 16:3459–3462. https://doi.org/10.1016/s0960-894x(03)00158-6

Stocks J, Dormandy TL (1971) The autoxidation of human red cell lipids induced by hydrogen peroxide. Br J Haematol 20:95–116. https://doi.org/10.1111/j.1365-2141.1971.tb00790.x

Stranix BR, Lavallée J-F, Sévigny G et al (2006) Lysine sulfonamides as novel HIV-protease inhibitors: Ne-Acyl aromatic α-amino acids. Bioorg Med Chem Lett 16:3459–3462. https://doi.org/10.1016/j.bmcl.2006.04.011

Subramaniam M, Liew SK, In LLA et al (2018) Inactivation of nuclear factor kB by MIP-based drug combinations augments cell death of breast cancer cells. Drug Des Devel Ther. https://doi.org/10.2147/DDDT.S141925

Taatjes DJ, Sobel BE, Budd RC (2008) Morphological and cytotoxicological determination of cell death by apoptosis. Histochem Cell Biol 129:33–43. https://doi.org/10.1007/s00418-007-0356-9

Tan J, Wang B, Zhu L (2009) Regulation of survivin and Bcl-2 in HepG2 cell apoptosis induced by quercetin. Chem Biodivers. https://doi.org/10.1002/cbdv.200800141

Terret C, Zanetta S, Roché H et al (2003) Phase I clinical and pharmacokinetic study of E7070, a novel sulfonamide given as a 5-day continuous infusion repeated every 3 weeks in patients with solid tumours. A study by the EORTC Early Clinical Study Group (ECSG). Eur J Cancer 39:1097–1104. https://doi.org/10.1016/S0959-8049(03)00128-X

Tornatore L, Thotakura AK, Bennett J et al (2012) The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. Trends Cell Biol 22:557–566. https://doi.org/10.1016/j.tcb.2012.08.001

Urso ML, Clarkson PM (2003) Oxidative stress, exercise, and antioxidant supplementation. Toxicology 189:41–54. https://doi.org/10.1016/j.tox.2003.04.038.xx(03)00151-3

Valko M, Leibfritz D, Moncol J et al (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84. https://doi.org/10.1016/j.biocel.2006.07.001

van Heerde W (2000) Markers of apoptosis in cardiovascular tissues focus on Annexin V. Cardiovasc Res 45:549–559. https://doi.org/10.1016/S0002-0464(99)00396-X

van Kesteren C, Beijnen JH, Schellens JHM (2002) E7070: a novel synthetic sulfonamide targeting the cell cycle progression for the treatment of cancer. Anticancer Drugs 13:989–997. https://doi.org/10.101700018181-2002110000-00002

Voll RE, Herrmann M, Roth EA et al (1997) Immunosuppressive effects of apoptotic cells. Nature 390:350–351. https://doi.org/10.1038/37022

Wang G, Zhang J, Liu L et al (2012) Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/Bcl-xl. PLoS ONE 7:e51764. https://doi.org/10.1371/journal.pone.0051764

Wang Z-Q, Porreca F, Cuzzocrea S et al (2004) A newly identified antiinflammatory, and analgesic drug and acridine derivatives. Monatshefte Für Chemie/chemical. pp 219-229

Wulf NR, Matuszewski KA (2013) Sulfonamide cross-reactivity: is there evidence to support broad cross-allergenicity? Am J Health Pharm 70:1483–1494

Yan N, Shi Y (2005) Mechanisms of apoptosis through structural biology. Annu Rev Cell Dev Biol 21:35–56. https://doi.org/10.1146/annurev.cellbio.21.012704.131040

Yokoi A, Kuromitsu J, Kawai T et al (2002) Profiling novel sulfonamide derivatives. Curr Med Chem 10:925–953. https://doi.org/10.2174/0929867033457647

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