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Research Article

Targeting cisplatin resistance in breast cancer using a combination of Thymoquinone and Silymarin: an *in vitro* and *in vivo* study

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Abstract

Background: Breast cancer (BC) is considered the most diagnosed cancer among women globally. This is because of its high possibility of metastasis and high resistance to chemotherapy. Cisplatin is a platinum-based antitumor agent that is used to treat various types of cancer. However, the main obstacle to using this drug is drug resistance. Drug resistance is a cause of most relapses of cancer which eventually lead to death. Nowadays, combining natural products is a trend to overcome drug resistance. Thymoquinone (TQ) is a natural phytochemical that exists mainly in blackseed. It has been used in medicine for decades, especially as an anticancer agent. Silymarin is a milk thistle compound that exhibits anticancer, hepatoprotective, and neuroprotective activity. Hence, the combination of TQ and silymarin could be a probable solution to treat cancer and reduce chemoresistance.

Methods: This study tested this combination on cisplatin-sensitive (EMT6/P) and cisplatin-resistant (EMT6/CPR) mouse mammary cell lines. Apoptotic and antiproliferative activity was assessed for TQ and silymarin *in vitro* using caspase-3 and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) assays, respectively. An *in vivo* study was performed to evaluate the effect of TQ and silymarin combination in mice inoculated with EMT6/P and EMT6/CPR cells. The safety profile was also examined using creatinine and liver enzyme assays.

Results: *In vitro*, the TQ and silymarin combination synergized in both cell lines. Also, this combination caused apoptosis induction at a higher rate than the single treatment in both cell lines. *In vivo*, TQ and silymarin combination resulted in a remarkable reduction in tumor size and enhanced the cure rate in mice implanted with EMT6/P and EMT6/CPR cell lines. According to the safety profile results, TQ and silymarin combination was safe.

Conclusion: In conclusion, the combination of TQ and silymarin provides a promising solution in treating BC resistant to cisplatin by inducing apoptosis. Further studies are needed to define the exact anticancer mechanisms of this combination.

Keywords

breast cancer resistance, thymoquinone, silymarin, cisplatin resistance

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PENSOFT

Introduction

Cancer is the leading cause of mortality and a significant reason for the decrease in life expectancy in all countries around the world (Bray et al. 2021). According to World Health Organization (WHO) estimates, cancer is the first or second major cause of death before the age of 70 in 112 of 183 countries in 2019 and ranks third or fourth in another 23 nations (Sung et al. 2021). In the United States, 1,958,310 new cancer cases and 609,820 cancer losses are expected to occur in 2023, which is equal to 5370 cases daily and 1670 deaths each day (Siegel et al. 2023). Among women, breast, thyroid, lung, colorectal, and cervical cancers are the most frequent types of cancer. In men, prostate, colorectal, lung, stomach, and liver cancer are the most common types of cancer (Bukowski et al. 2020).

Breast cancer (BC) is considered the most diagnosed cancer among women globally. This is because of its high possibility of metastasis and high resistance to chemotherapy (Wang et al. 2021a). In metastatic breast cancer, the five-year survival rate is less than 30%, even with auxiliary chemotherapy (Riggio et al. 2021). Generally, 70–80% of those with early-stage, non-metastatic illnesses can be cured. On the other hand, recent therapies are less effective in curing breast malignancy with distant organ metastases (Harbeck et al. 2019). Despite years of experimental, clinical, and epidemiological research, breast cancer prevalence keeps increasing worldwide (Britt et al. 2020). Recent statistics have shown that breast tumors surpassed lung tumors and became the most detected cancer worldwide in 2020 (Cao et al. 2021). According to Global Cancer Observatory (GLOBOCAN) 2020, breast cancer constitutes 24.5% of cancers in females of all ages worldwide (Ferlay et al. 2021). Consequently, this will generate a serious public health problem, particularly in developing countries (Wang et al. 2021b).

Breast cancer is associated with numerous risk factors. Among these are reproductive factors, which are late marriage, getting first childbirth at delayed age and the age of menopause which correlates strongly to the disease development (Beral et al. 2004; Gold 2011; Marphatia et al. 2017; Kashyap et al. 2022). Exogenic hormonal factors such as using oral contraceptive also lead to a high breast cancer risk (Hunter et al. 2010). In addition, lifestyle factors like unhealthy diet, obesity, lack of physical activity and consuming alcohol (Cancer 2002; Gilsing et al. 2011; Kim et al. 2013; Chang et al. 2017; Picon-Ruiz et al. 2017). There are four major classes of breast tumor based on the expression of the following hormone receptors: human epidermal growth factor receptor 2 (HER2) positive, endocrine receptor (estrogen or progesterone receptor) positive, triple positive (estrogen, progesterone, and HER2 receptor-positive), and triple-negative (absence of estrogen, progesterone, and HER2 receptors) (Schnitt 2010). Needless to say, triple negative breast cancer is the most aggressive type among them (Irvin Jr and Carey 2008; Ensenyat-Mendez et al. 2021). Depending on the stage and the menopausal state, the choice of treatment varies from a lumpectomy, radiation, mastectomy, chemotherapy, and endocrine therapy (Dong et al. 2021; Trayes and Cokenakes 2021). Despite the remarkable progress in cancer treatment during the last years, chemotherapy still has to be the chief method for cancer therapy (Bukowski et al. 2020). Unfortunately, therapeutic resistance has been reported against many antineoplastic agents, resulting in disease relapse and recurrence (Garcia-Martinez et al. 2021). Chemo-resistance is defined as an insufficient response to chemotherapeutic agents which restrict drug efficacy (Trédan et al. 2007). Statistical data displays that more than 90% of mortality in cancer patients is due to drug resistance (Bukowski et al. 2020). This resistance originates from different mechanisms that include: improvement in the drug efflux, growth factors, genetic factors, senescence escape, augmented DNA repair ability, tumor heterogeneity and high xenobiotics metabolism (Luqmani 2005; Wu et al. 2014; Wang et al. 2017; Wang et al. 2019; Dallavalle et al. 2020). Due to the heterogenous nature of BC, drug resistance is a major challenge (Ji et al. 2019).

Cisplatin is a platinum-based antitumor agent that is used to treat various types of cancer (Giacomini et al. 2020). It is a common treatment in breast cancer if combined with paclitaxel drug (Wan et al. 2019). Up to now, it is appraised that about 50% of all cancer patients will receive cisplatin in their anticancer therapy regimen (Ghosh 2019). However, the main obstacle to using this drug is drug resistance. This resistance is acquired through various independent mechanisms including alteration of cell cycle checkpoints, overexpression of breast cancer resistance protein, suppression of apoptosis, and stimulation of multiple signalling pathways (Kartal-Yandim et al. 2016). Accordingly, new aspects of treating BC were developed such as drug carriers, novel agents or combination therapy. Additionally, new therapeutics such as immunotherapy and gene therapy could be promising agents to overcome this resistance (Ji et al. 2019).

In this research, we have focused on using combination therapy to deal with cisplatin resistance. The combination could be between one herbal constituent with another or with a chemotherapeutic agent (Mangla and Kohli 2018). Lee et al. have shown that combination therapy increases the rate of response and avoids drug-induced resistance (Lee and Djamgoz 2018). For decades, natural products and medicinal herbs have been used to cure many different diseases including cancer (Kamble and Gacche 2019). This is due to their bioactive phytochemicals and their antioxidant effect (Najjaa et al. 2020). Add to that their applicability, accessibility, low cost, and low toxicity (Dutta et al. 2019).

Thymoquinone (TQ) is a natural phytochemical, that exists mainly in black seed (*Nigella sativa*; family Ranunculaceae) or black cumin, and it has been widely used in the treatment/prevention of several types of cancer including BC (Adinew et al. 2021). TQ exerts its anticancer effect using several pathways such as: the prevention of oxidative stress and inflammation, induction of apoptosis, suppression of metastasis and angiogenesis, up-regulation of specific tumor suppressor genes as well as down-regulation of tumor promoting genes (Alhmied et al. 2021). Silymarin

is a milk thistle compound (*Silybum marianum*) that exhibits hepatoprotective, neuroprotective, cardioprotective, anti-viral, anti-diabetic, and antineoplastic effects against different types of cancer too (Fallah et al. 2021; Wadhwa et al. 2022). Silymarin anticancer effect is due to several mechanisms including cell cycle arrest at the G1/S-phase, stimulation of cyclin-dependent kinase (CDK) inhibitors, and lowering the synthesis of anti-apoptotic gene production. Moreover, it can modify the expression of gene products related to the proliferation, invasion, metastasis and angiogenesis of different tumor cells (Ramasamy and Agarwal 2008; Hosseinabadi et al. 2019).

To date, no study investigated the effect of combination therapy between thymoquinone and silymarin to overcome cisplatin resistance in BC.

Materials and methods

Chemicals, cell lines, and culture conditions

Pure TQ and silymarin were supplied from Sigma Aldrech. Their CAS-No were 490-91-5 and 65666-07-1 respectively. Sensitive mice mammary cell line (EMT-6/P) and cisplatin resistant cell line (EMT-6/CPR) were attained from the European Collection of Cell Cultures (Salisbury, UK). In our examination, minimum essential medium (MEM) (Caisson, USA) was used to culture both cells. The media which was supplied as 500 ml bottle was supplemented with 5 ml of L-glutamine (Eurobio, France), 0.5 ml of non-essential amino acids (Caisson, USA), 50 ml of 10% fetal bovine serum (Sigma, USA), 5 ml of penicillin-streptomycin solution (Eurobio, France) and 0.5 ml of 0.1% Gentamycin (Sigma, USA). Cells were incubated at 37 °C, with 5% carbon dioxide, and 95% humidity.

In order to separate the adherent cells from the walls of the cell culture flask, the trypsinization method was applied. This is accomplished by adding trypsin ethylene diamine tetra acetic acid (trypsin EDTA) (Eurobio, France) with phosphate buffer saline (PBS) (Eurobio, France). For MTT assay, cell counting was performed by using trypan blue (0.4%) (Sigma, USA). Dimethyl sulfoxide (DMSO) (Alpha Chemika, India). To conserve tumors after vivisection, buffered formalin (10%) (S.D. Fine-Chem ltd, India) was employed.

Commercial kits

Besides, to measure the percentage of viable cells, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) assay kit (Sigma, USA) was utilized. A Colorimetric Caspase-3 Assay Kit (Invitrogen ThermoFisher, USA) with a catalog number: BMS2012INST was applied to detect apoptosis. For ALT serum level assessment, Alanine aminotransferase ALAT (GPT) FS kit (BioMajesty, Germany) was used. For AST serum level assessment, aspartate aminotransferase ASAT(GOT) FS kit (BioMajesty, Germany) was utilized. Furthermore, a Creatinine FS assay kit (BioMajesty, Germany) was performed to measure Creatinine serum levels.

Preparation of TQ, silymarin and cisplatin working solutions

Regarding the MTT assay procedures, TQ and silymarin were dissolved directly in the medium with DMSO (less than 1%) to produce a concentration of 2000 μM as a working solution. These concentrations were selected depending on previous testing conducted in our laboratory (Talib 2017). After that, serial dilution was applied in order to obtain 50% reduction in concentration. As a result, the highest concentration was 1000 μM, then it was diluted to 500, 250, 125, 62.5, 31.25, 15.62, 7.81 μM for single treatments in EMT-6/P and EMT-6/CPR cells.

The positive control, cisplatin (Ebewe Pharma, Austria), was given as a stock solution of 50 mg per 100 mL (0.5 mg/mL) as it is already prepared as a drug in the market. In order to achieve the requisite concentrations of 100 μM down to 0.8 μM in single treatment experiments for both cell lines, further dilutions of the stock solution manipulation MEM were prepared before use (Talib 2020).

In combination treatment, 66 μM stock solution of TQ was prepared to produce a serial dilution of 50% to 0.51 μM with fixed dose of silymarin (106.63 μM) in EMT-6/CPR cells. While in EMT-6/P cells, 29.58 μM of TQ stock solution was prepared to generate a serial dilution of 50% to reach 0.23 μM with fixed dose of silymarin (142.40 μM). For silymarin, a stock solution of 106.63 μM was prepared to produce a serial dilution down to 0.83 μM with fixed dose of TQ (66 μM) in EMT-6/CPR cells. While in EMT-6/P cells, 142.40μM of silymarin stock solution was ready to dilute it down to 1.11 μM with fixed dose of TQ (29.58 μM).

Antiproliferative assay

MTT (the tetrazolium salt, 3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) is a colorimetric reduction test (Sigma, USA) (Kumar et al. 2018). It was used to identify the antiproliferative activity. The test recognizes the normal operation of mitochondria and cell viability by detecting the reduction of MTT by mitochondrial dehydrogenase to blue formazan. The mouse mammary cell lines (EMT6/P and EMT6/CPR) were cultured overnight. Next, cells were collected using the trypsinization technique by applying 0.5 mL of trypsin-EDTA and 1 mL of 1X PBS for 2–3 min, washed using 5 mL media, centrifuged at 1000 rpm for 10 min at 4 °C, and resuspended in 5 mL of fresh tissue culture medium after removing the supernatant layer. The trypan blue exclusion technique was then used to determine the viability of the exponentially developing cells. The cells were then counted and planted onto 96-well tissue culture flat-bottom microplates with 10,000 cells per well. Then a 200 μL of growth media was added and incubated for a 24-hours. After seeding and monolayer formation, the medium was withdrawn, and cells were then treated with TQ and silymarin. Eight concentrations of TQ and silymarin were

applied to both cell lines (1000, 500, 250, 125, 62.50, 31.25, 15.62, 7.81 μM) for 48 h. Additionally, they subjected both cell lines to 48 hours of exposure to cisplatin at the following doses (0.80–100 μM). In combined treatment, TQ concentrations ranging from (29.58, 14.79, 7.39, 3.69, 1.84, 0.92, 0.46, to 0.23 μM and a fixed dosage of silymarin (142.40 μM) were applied to EMT-6/P cells. Also, these cells were exposed to concentrations of silymarin ranged from (142.40, 71.20, 35.60, 17.80, 8.90, 4.45, 2.22 to 1.11 μM) with fixed dose of TQ (29.58 μM). In resistant cell line, cells were treated with TQ solution with concentrations ranging from (66, 33, 16.50, 8.25, 4.12, 2.06, 1.03 to 0.51 μM) with fixed concentration of silymarin (106.63 μM). Then, EMT6/CPR cells were treated with silymarin in different concentrations (106.63, 53.25, 26.62, 13.31, 6.65, 3.32, 1.66 to 0.830 μM) with fixed dose of TQ (66 μM). After incubation, the 200 μL was removed and 100 μL of medium was then added with 10 μL of the MTT solution in every well. After that, the microplate was incubated in CO2 incubator for another 3 h so the blue crystals could form. After adding 100 μL of DMSO (stop solution) and an additional hour of incubation, the reduced MTT was then measured at 550 nm using a microplate reader (Biotek, Winooski, VT, USA). Direct solvation of the chemical stock solutions in the medium was followed by sterilization using 0.22 μm syringe filters. The average of three replicates was used to derive the IC_{50} values. Cell viability (% survival) for each treatment was determined and compared to the untreated (negative control) cells, which solely contain tissue culture medium.

Calculation of inhibitory concentration (IC_{50})

The term "IC_5" refers to the drug concentration needed to kill or inhibit cells by 50% as compared to untreated cells, which suggests that at that concentration, the inhibitory substrate only exerts 50% of its maximum inhibitory action. The statistical software for the social sciences (SPSS) version 26 (Chicago, IL, US) was used in our experiment to compute and evaluate IC_{50} values. To determine the IC_{50} values for both single and combination treatments, a nonlinear regression test was performed on the data.

Calculation of combination index (CI)

To calculate the combination index (CI) of the combinations of TQ and silymarin against the two cell lines (EMT6/P and EMT6/CPR), we applied an equation published previously (Ichite et al. 2009):

$$CI = (D)\,1/(Dx)\,1 + (D)\,2/(Dx)\,2 + a\,(D)\,1\,(D)\,2/(Dx)\,1\,(Dx)\,2$$

Where:

(Dx) 1 = IC_{50} of TQ alone
(D) 1 = IC_{50} of TQ in combination with silymarin
(Dx) 2 = IC_{50} of silymarin alone
(D) 2 = IC_{50} of silymarin in combination with TQ

a = 0 for mutually exclusive or 1 for mutually nonexclusive interaction.

According to the literature study, silymarin and TQ each have a unique mode of action for fighting cancer. Therefore, in CI calculations, we used the mutually nonexclusive model, where α = 1. CI results are explained as follows:

If CI > 1.3 that shows antagonism, CI = 1.1–1.3 represents moderate antagonism, CI = 0.9–1.1 indicates additive effect, CI = 0.8–0.9 shows slight synergism, CI = 0.6–0.8 reveals moderate synergism, CI = 0.4–0.6 means synergism, CI = 0.2–0.4 reveals strong synergism, and CI < 0.1 reveals a very strong synergism (Chou 2006).

Calculation of resistance fold

To compare the resistant cell line to the sensitive cell line, we used the term "resistance fold". It described the times of change in the concentration required to achieve 50% death in the resistant cell line against the sensitive cell line. The resistance fold is determined in this study by comparing the IC_{50} values between the resistant EMT-6/CPR and the sensitive EMT-6/P cell lines using the formula:

$$\text{Resistance fold} = IC_{50}\ \text{of Resistant Cell Line} / IC_{50}\ \text{of Parental Cell Line.}$$

By subjecting EMT-6/P and EMT-6/CPR cell lines to a range of cisplatin concentrations (208.33, 104.16, 52.08, 26.04,13.02,6.51, 3.25, 1.62 μM) for 48 hours, the anti-proliferative assay (MTT) was carried out to assess the cisplatin-mediated anti-proliferative effect on these cell lines.

Colorimetric assay of caspase-3 activity in EMT-6/P and EMT-6/CPR cells (Niles et al. 2008)

The tested cell line (EMT-6/CPR) was taken out of the liquid nitrogen tank, thawed at 37 °C, and grown in flasks measuring 75 cm2 and containing 15 ml of MEM. The cultivated cells were then incubated overnight at 37 °C, 5% CO2, and 95% humidity. Using 0.5 mL trypsin-EDTA and 1 mL 1X PBS, cells were separated from the flask walls and allowed to incubate for 2–3 minutes. Cells were then transferred to 15-mL sterile centrifuge tubes, rinsed with 5 mL MEM, and centrifuged at 1000 rpm for 10 minutes at 4 °C. Cells (pellets) were resuspended in 5 mL MEM and counted.

The cells were seeded at a density of 100,000 cells/mL in a 75 cm2 area that was pre-labelled with the cell type, passage number, and date. Five flasks were made, and they underwent a 24-hour incubation period to ensure optimal adhesion and growth. At that point, the treatments were dissolved in fresh media, and the old media was discarded. The negative control flask contained only MEM. A single dose of TQ (10 μM) and a single dose of silymarin (10 μM) were added to flasks two and three. A combined dose of (10 μM) TQ and (10 μM) silymarin was in flask four. Eventually, a dose of cisplatin (0.50 mg/ml) was added to flask five.

The flasks were then incubated for 48 hours. After incubation, the old medium was taken out of each flask and discarded. The flasks were then cleaned with 2 ml of PBS before each was given 1 ml of trypsin to separate the cells. The entire 5 mL was then transferred to a centrifugation type (15 mL) and centrifuged at 1000 rpm at 4 °C for 10 min. After adding 4 ml of the new medium, we added trypsin. Pellets of cells are now prepared for testing for caspase-3 activity.

According to the kit's instructions, the lysis buffer was made, and then 1 ml of it was added to every $5*10^6$ cells. The tubes were then gently shaken while being incubated for one hour at room temperature. The tubes were then centrifuged for 15 minutes at 1000×. Then, 100 µL, 100 µL, and 140 µL, of distilled water were added to the blank, standard, and sample wells respectively. Then, 10 ml of each sample in each well was added in two separate additions. The plate was then covered and shaken for three hours at room temperature. Using the washing buffer previously produced per the kit instructions, the microwell strips were washed six times after three hours, each time adding 140 µL to each well containing the blank, standard, and samples. After the final wash, the microwell strip was tapped on a paper towel to remove the extra buffer. The substrate solution (tetramethylbenzidine) was then added to each well at a volume of 100 µL. The microplate was then kept at room temperature for almost ten minutes, or until the highest standard turned dark blue. The stopping solution (1M phosphoric acid) was then added to each well at a volume of 100 µL. After that, a spectrophotometer employing a 450nm wavelength immediately scanned the plate.

Mice

This investigation utilized 35 female *Balb/C* strain mice, aged 4–6 weeks, weighing between 21 and 25 g each. The animal house of the Applied Science Private University in Amman, Jordan, provided mice. The Research and Ethical Committee of Applied Science University approved all animal experimentation methods in accordance with accepted ethical standards. The approval No. was 2023-PHA-11. Separate cages with bedding made of wood shavings were used to house the animals. The animal housing was set up with a constant temperature of 25 C, a 50–60% humidity range, continuous air ventilation, and 12-hour cycles of light and dark.

Establishing regimens for the in vivo experiment

On female Balb/C mice with EMT-6/P and EMT-6/CPR tumors, we carried out the *in vivo* part. Based on research that was published in the literature, the doses of TQ, silymarin, and cisplatin were selected. For TQ, an intraperitoneal dose of 25 mg/kg (0.625 mg/mouse) was chosen. It was given every day for 10 days based on the dosage used by Fatfat et al. (Fatfat et al. 2019). In contrast, Kim et al. study (Kim et al. 2021) stated that silymarin dosage was a daily intraperitoneal injection of 50 mg/kg (1.25 mg/mouse) for 10 days. Cisplatin dose was based on the literature of Talib et al. (Talib and Jawarneh 2022) and it was 5 mg/kg/week (0.125 mg/mouse).

Tumor inoculation and antitumor activity in vivo

EMT-6/CPR and EMT-6/P cells were defrosted, cultivated, separated, calculated and seeded using MEM. After that, they were kept in the incubator to grow for 24 h. Using trypsinization method, exponentially growing EMT-6/P and EMT-6/CPR cells were collected, washed, and resuspended in MEM, at a density of 1.5×10^6 cells / ml. Then, viable cells were assessed using trypan blue exclusion technique. Each female BALB/C mouse received a subcutaneous injection of 1.5×10^5 tumor-inducing cells in a 0.1 mL medium. Following the injection of cancer cells, the tumor-bearing mice got the therapies on day 15, after the tumors had grown for 14 days. Tumor dimensions were measured with a digital caliper and tumor volumes were calculated using the method below:

$$\text{Tumor volume} = A \times B2 \times 0.5$$

Where:

A = length of the longest aspect of the tumor.

B = the length of the tumor aspect perpendicular to A.

It was decided to choose tumors of comparable sizes, and the average tumor volume for each group was approximately matched. EMT-6/P, a parent BC cell line, was injected into the right side of each mouse, and EMT-6/CPR, a cisplatin-resistant BC cell line, was injected into the left side of each mouse. After 14 days of the tumor injection, the treatment began. In this study, 35 tumor-bearing mice were employed, and they were split into five groups (n = 7 for each group), Fig. 1. As a negative control, Group 1 received 0.1 mL of vehicle (olive oil) intraperitoneally every day. Group 2 was exposed to TQ (25 mg/kg/ day) by intraperitoneal injections. Group 3 was treated with daily dose of silymarin (50 mg/kg/day) administered intraperitoneally. Group 4 was treated with a combination of TQ and silymarin (25 and 50 mg/kg/ day respectively). Group 5 was administered with cisplatin (5 mg/kg/ week). Blood samples were taken three times throughout the course of the 10-day therapy, on days 1, 5, and 10.

Three-time periods during the treatment period days (1, 5, and 10) were used to quantify the tumor volumes. The following calculation was used to compute the percent change in tumor volumes when comparing beginning and final volumes:

$$\%\ \text{Tumour change} = ((F–I)/\ I) * 100\%$$

Where F is the final tumor volumes, and I represent the initial tumour volumes. After 10 days of treatment, mice were sacrificed by cervical dislocation, and blood samples

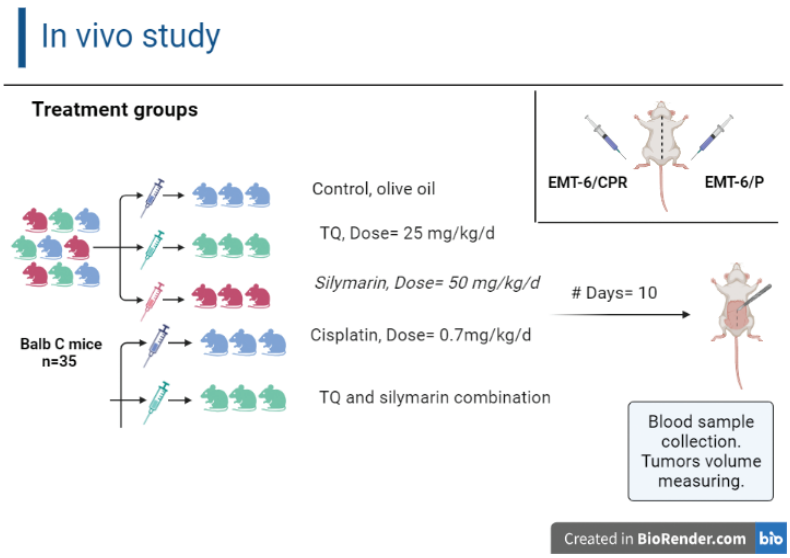

Figure 1. Shows the treatment groups for the *in vivo* study with the subjected doses.

were taken retroorbitally for each group. To maintain the morphology of the tumors, they were excised, weighed, and kept in 10% formalin. For 10 minutes, blood samples were centrifuged at 5000 rpm. The produced serum was transferred to a fresh, pre-labelled Eppendorf tube for each sample. For the next studies, serum samples were kept at -20 C.

Evaluation of liver function in treated mice

The blood levels of the liver enzymes ALT and AST were measured for TQ, silymarin, cisplatin, and their combinations that were previously described. In addition, the negative control group was assessed to determine the levels of liver damage caused by the utilized therapies. Alanine aminotransferase ALAT (GPT) FS kits and aspartate aminotransferase ASAT (GOT) FS kits were used to evaluate ALT and AST after serum samples were collected. To create functioning reagents, reagents were combined according to the protocol's instructions. In ALAT (GPT) FS kit, the first reagent was a combination of Tris (hydroxymethyl) aminomethane buffer, L-Alanine, and Lactate dehydrogenase (LDH). While the second reagent was a mixture of 2-Oxoglutarate along with Nicotinamide adenine dinucleotide (NADH). In ASAT (GOT) FS kit, Tris (hydroxymethyl) aminomethane buffer, L-Aspartate, Malate dehydrogenase (MDH) and LDH were in the first reagent. The second reagent was a combination of 2-Oxoglutarate and NADH. Then, to achieve the reaction's ideal temperature, working reagents were incubated at 37 °C. Next, 100 µL of each sample were combined with 1 mL of the working reagent in a single-use cuvette. After one minute of incubation, the initial absorbance at time zero was noted. Readings of absorbance were taken after 0, 1, 2, and 3 minutes. The spectrophotometer was calibrated to zero absorbance using distilled water and synced to read absorbance at 340 nm. Working reagents were used as blanks as well.

Evaluation of kidney function in treated mice

We also assessed the levels of kidney injury via the several treatments; creatinine level was measured for TQ, silymarin, cisplatin and their combinations beside the negative control group. The level of creatinine in the obtained serum samples was then determined using a creatinine kit. The standard (S) was ready to use. In accordance with the guidelines, reagents were combined to create functioning reagents. In order to accurately determine the reaction temperature, the working reagent was then incubated at 37 °C. After mixing 100 µL of each sample with 1 mL of the working solution in a disposable cuvette, absorbance measurements were taken after 30 and 90 seconds. Utilizing distilled water, the spectrophotometer was adjusted to zero absorbance and calibrated to read absorbance at 505 nm. Additionally, working reagents were used as blank samples.

Data analysis

SEM (Standard Error of Mean) was used to represent the results as a mean ±SEM. The IC_{50} values for TQ, silymarin, cisplatin, and their combinations against EMT-6/P and EMT-6/CPR cell lines were really obtained. Additionally, ANOVA nonlinear regression was used in SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA) version 26 to statistically examine the IC_{50} results. To determine the statistical significance between groups, GraphPad prism 8 software was used. It was considered a significant difference between the groups when the probability level (P-value) was less than 0.05 (P-value < 0.05). At least two separate animal experimentation studies were carried out, with a minimum of six animals per group.

In vitro results

Anti-proliferative activity of TQ and silymarin as single treatments on both EMT-6/P and EMT-6/CPR cell lines

The MTT test was carried out on EMT-6 cisplatin-sensitive parent BC cells (EMT-6/P) and EMT-6 cisplatin-resistant BC cell lines (EMT-6/CPR) to assess TQ's efficacy as a single treatment. As shown in Fig. 2, TQ reduced cell proliferation in a concentration-dependent manner when compared to the vehicle control. Our findings also showed that, when exposed to the same TQ doses, EMT-6/CPR had greater survival rates than EMT-6/P. For EMT-6/P and EMT-6/CPR, the IC_{50} values for TQ were 59.16 ± 1.8 μM and 132 ± 4 μM, respectively.

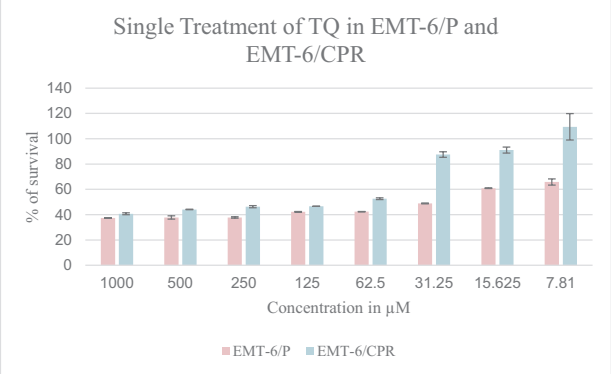

Figure 2. Anti-proliferation effect of TQ in single treatment against EMT-6/P and EMT-6/CPR cell lines.

To evaluate the effectiveness of silymarin as a single treatment, the MTT test was performed on EMT-6 cisplatin-sensitive parent BC cells (EMT-6/P) and EMT-6 cisplatin-resistant BC cell lines (EMT-6/CPR) compared to the vehicle control, Fig. 3. The test demonstrated that silymarin reduced cell proliferation in a concentration-dependent way. Additionally, our research revealed that both cell lines exhibited lower survival rates when exposed to a high dose of silymarin. The IC_{50} values for silymarin in EMT-6/P and EMT-6/CPR were 284.8 ± 38 μM and 213.27 ± 6 μM, respectively.

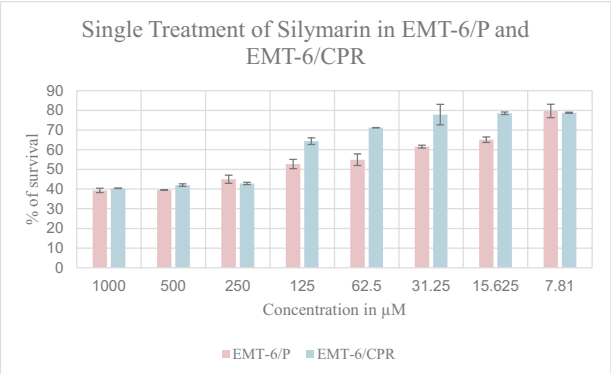

Figure 3. Anti-proliferation effect of silymarin in single treatment against EMT-6/P and EMT-6/CPR cell lines.

Anti-proliferative effect of cisplatin treatment on EMT-6/P, EMT-6/CPR cell lines

The anti-proliferative assay (MTT) was applied to evaluate the cisplatin-mediated anti-proliferative effect on both EMT-6/P and EMT-6/CPR cell lines. That was achieved by exposing EMT-6/P and EMT-6/CPR cell lines to a range of cisplatin concentrations (208.33, 104.16, 52.08, 26.04,13.02,6.51, 3.25, 1.62 μM) for 48 hours. Then, it was determined that the cisplatin concentration needed to inhibit cell growth by 50% (IC_{50}) was 26.50 ± 0.01 μM for the EMT-6/P cell line and 70 ± 0.006 μM for the EMT-6/CPR cell line. According to the aforementioned equation, IC_{50} values of cisplatin in both cell lines were used to determine the resistance fold. The findings revealed that EMT-6/CPR cells were 2.64 times more resistant to cisplatin than EMT-6/P cells, as shown in Fig. 4 and Table 1.

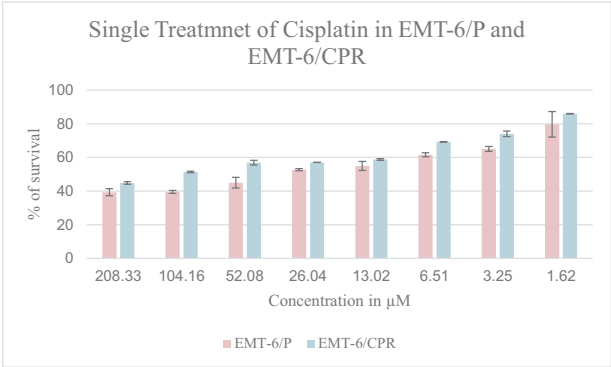

Figure 4. The anti-proliferative assay (MTT) was used to examine the EMT-6/P and EMT-6/CPR cell lines' sensitivity to cisplatin at varying doses.

Anti-proliferative effect of TQ and silymarin combination treatment on EMT-6/P, EMT-6/CPR cell lines

To assess the inhibitory impact of TQ and silymarin combination treatment, EMT-6/P, and EMT-6/CPR cell lines were treated with different doses of TQ and a constant concentration of silymarin. Also, these cells were treated with different doses of silymarin and a fixed dose of TQ; both treatments lasted for 48 hours.

According to the MTT assay findings, Figs 5–8 demonstrate that the combination of TQ and silymarin greatly decreased cell viability in a concentration-dependent manner. Furthermore, combined therapy required much lower dosages to achieve 50% killing. When EMT-6/P cells were placed next to EMT-6/CPR cells, EMT-6/CPR cells were more susceptible to combination at lower doses, but EMT-6/CPR cells were more vulnerable when silymarin dosages were raised.

The 50% inhibitory concentrations (IC_{50}) of the combination treatment were 25.23 ± 16.5 μM of silymarin and 29.95 ± 3.5 μM of TQ in EMT-6/P and 0.939 ± 0.308 μM of silymarin and 1.83 ± 1.01 μM of TQ in EMT-6/CPR.

Table 1. IC_{50} values of TQ, Silymarin and Cisplatin on EMT-6/P, EMT-6/CPR cell lines, along with the combination index (CI), interpretation and resistance folds.

Cell line	IC_{50} of Single TQ (μM)	IC_{50} of Single Silymarin (μM)	IC_{50} of Cisplatin (μM)	TQ IC_{50} in combination with silymarin (μM)	Silymarin IC_{50} in combination with TQ (μM)	CI	Interpretation
EMT6/P	59.16	284.8	26.5	29.95	25.23	0.632	Synergism
EMT6/CPR	132	213.27	70	1.83	0.939	0.018	Very strong synergism
Resistant folds	2.23	0.75	2.64	0.061	0.037		

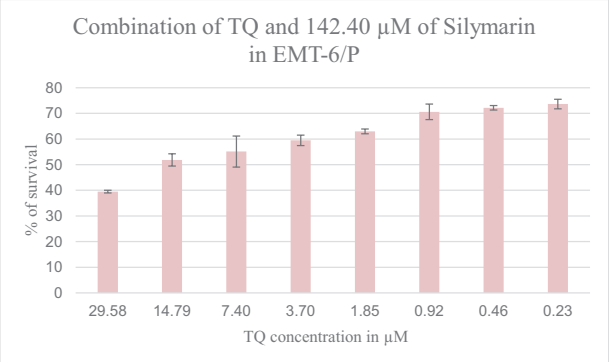

Figure 5. Anti-proliferation activity of various concentrations of TQ with 142.40 μM silymarin against EMT-6/P cell lines.

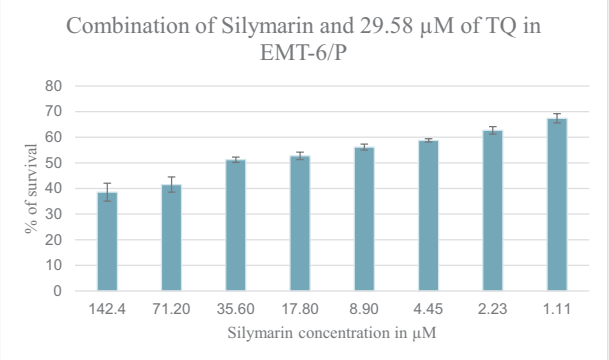

Figure 6. Anti-proliferation activity of various concentrations of silymarin with 29.58 μM TQ against EMT-6/P cell lines.

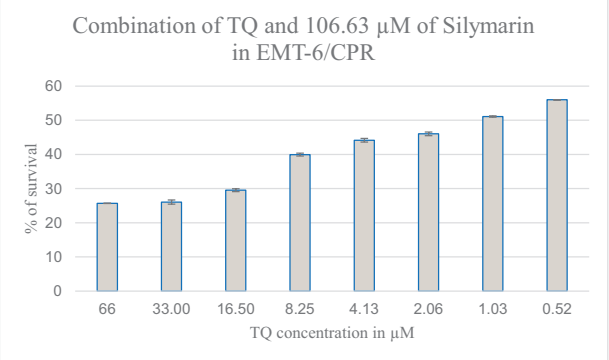

Figure 7. Anti-proliferation activity of several concentrations of TQ with 106.63 μM silymarin against EMT-6/CPR cell lines.

Combination index (CI) calculations

Several doses of TQ are administered to both cell lines with a fixed dosage of silymarin using the MTT test to acquire the IC_{50} values, which allow the CI to be calculated

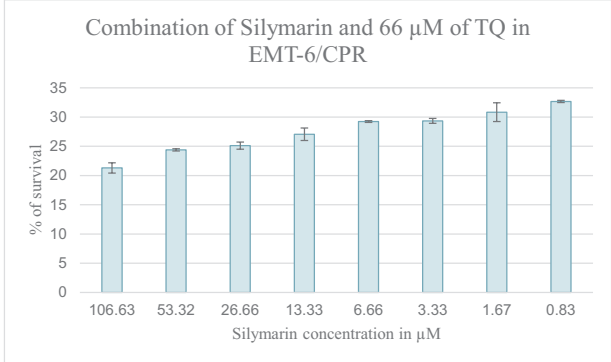

Figure 8. Anti-proliferation activity of several concentrations of silymarin with 66 μM TQ against EMT-6/CPR cell lines.

using the previously described equation and further explanation. The same method was applied in the combination of several doses of silymarin with a fixed dose of TQ in both cell lines.

The combination test analysis revealed that the combination therapy had a synergistic impact on EMT-6/P cells and a strong synergism on EMT-6/CPR cells, with CIs of (0.632) and (0.018), respectively (Table 1).

Resistance fold

The MTT test was used to compare the IC_{50} values of TQ, silymarin, and cisplatin combination therapy for both cell lines in order to assess EMT-6/CPR resistance to these therapies. Table 1 shows the mean IC_{50} values, which are defined as the concentration of medication required for 50% suppression of viable cell number in vitro. The concentration of TQ that was required to achieve 50% of inhibition was 59.16 and 132 μM for EMT-6/P and EMT-6/CPR, respectively, with resistance fold of 2.23, which indicates that EMT-6/CPR cells were 2.23 times more resistant to the treatment than EMT-6/P. For silymarin, the IC_{50} values were 284.80 μM in EMT-6/P and 213.27 μM in EMT-6/CPR with a resistance fold of 0.75. On the other hand, the IC_{50} values of cisplatin were 26.50 μM and 70 μM for, EMT-6/P and EMT-6/CPR, respectively. This means that EMT-6/CPR cells were 2.64 times more resistant to cisplatin compared to EMT6/P. For the combination treatment, the resistance fold for TQ reduced to 0.061 and for silymarin was decreased to 0.037. The resistance fold was obtained by dividing the IC_{50} value for each treatment in the resistant EMT-6/CPR cell line by the treatment's IC_{50} value for the sensitive EMT-6/P cell line.

Effect of TQ, silymarin, cisplatin and their combination on caspase-3 levels in EMT-6/P and EMT-6/CPR cells

A colorimetric caspase-3 test kit was used to assess the apoptotic effect of TQ, silymarin, cisplatin, and their combinations on caspase-3 levels in EMT-6/CPR cell line. As clarified in Fig. 9, the combination treatment showed the highest concentration of Caspase-3 in ng/ml. Also, Fig. 10 demonstrated that the combination treatment exhibited the highest Caspase-3 activity.

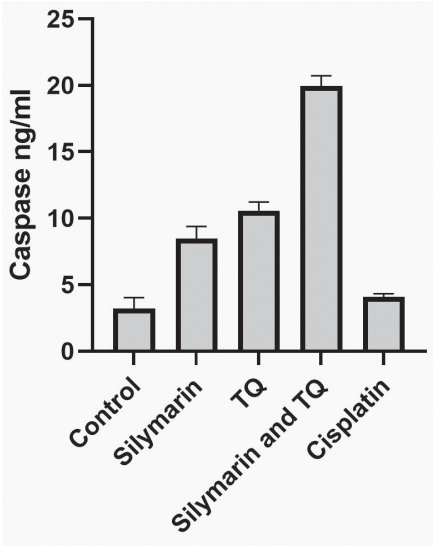

Figure 9. Demonstrates concentration of Caspase-3 in ng/ml in each treatment group along with the control group.

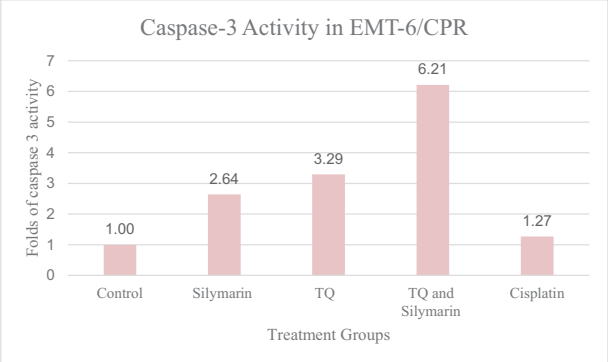

Figure 10. Folds increase in caspase-3 activity and apoptosis induction in concentrations of TQ (10 µM), silymarin (10 µM), and their combination in EMT-6/CPR cell line.

In vivo Results

Antitumor effects of TQ, silymarin, their combination and, cisplatin on EMT-6/P cells implanted in mice

In contrast to the negative control group, which had an increase in tumor size of 47.62%, all the treated groups displayed a significant reduction (P-value < 0.05) in tumor size Fig. 11. Notably, as shown in Table 2, the TQ

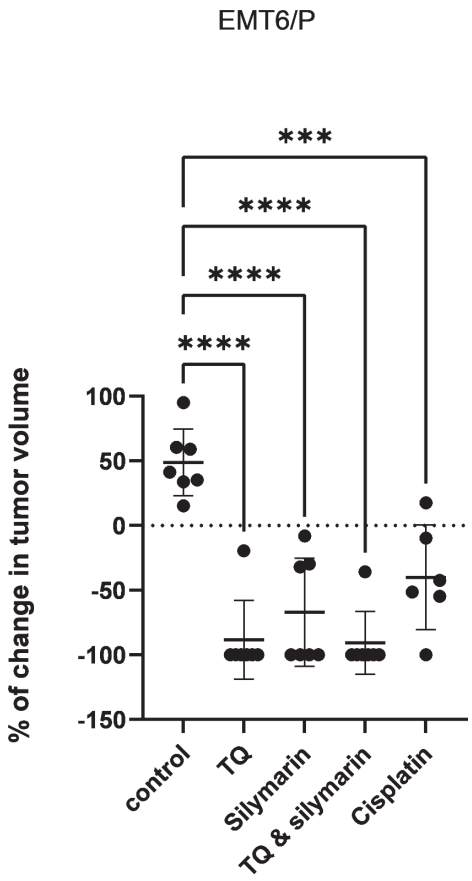

Figure 11. Shows that the percentage of change in tumor volume in EMT-6/P cell line was significant (P-value < 0.05) in all treatment groups. This graph was obtained using GraphPad prism

and silymarin combination had the highest percentages of size reduction (92.60%) and curability (100%). On the other hand, TQ single treatment showed 86.86% reduction in tumor size with also a highest curable rate (100%). Silymarin revealed 68.65% decreasing in tumor size and a curable rate of 57.14%. Cisplatin group displayed the lowest reduction in tumor size (43.12%) among the other groups with the lowest curable rate of (16.66%). To have a better understanding, Fig. 12 demonstrates how the combination therapies offered higher tumor size reduction at the end of the therapy when graded according to initial tumor size.

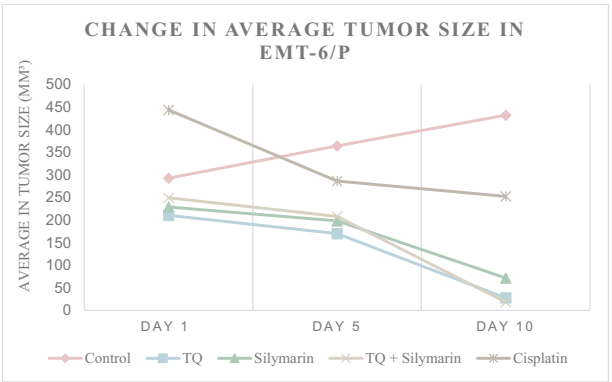

Figure 12. A plot of change in average tumor size (mm^3) vs. time in (days) of treatment in EMT-6/P cell line.

Table 2. Results of TQ, silymarin, their combinations, and cisplatin regarding tumor size changes, percentages of change in tumor size and average tumor weight in EMT-6/P cell line (n = 7).

Treatment group EMT-6/P	Av. initial tumor size (mm^3)	Av. final tumor size (mm^3)	%Change in tumor size (%)	Mice with no detectable tumor (%)	Av. tumor weight (g)
Control	292.66	432.06	47.62	0	0.261
TQ	210.46	27.65	-86.86	100	0.00
Silymarin	228.90	71.75	-68.65	57.14	0.156
Cisplatin	443.85	252.41	-43.12	16.66	0.239
TQ + Silymarin	249.33	18.42	-92.60	100	0.029

Abbreviations: Av. = Average, mm^3 = cubic millimeter.

Antitumor effects of TQ, silymarin, their combination and, cisplatin on EMT-6/CPR cells implanted in mice

As a result, the same treatment procedures were used on EMT-6/CPR. Table 3 demonstrates the reduction in tumor size in different treatment groups along with the negative control group which registered an increase in the tumor size of 59.78%. This reduction was significant (P-value < 0.05) in all groups except the silymarin group, Fig. 13. Again, TQ and silymarin combination showed the highest percentage in tumor size reduction of 56.40% with a curable rate of 42.85%. Remarkably, TQ in EMT-6/CPR was less active than EMT-6/P cell lines. TQ results showed a reduction in tumor size of 45.07% along with 42.85% curable rate. Also, silymarin was less active and insignificant in EMT-6/CPR cell lines with 20% reduction in tumor size and 14.28% curable rate. For cisplatin, the dropping in the tumor size was 28.55% and its curable rate was equal to 16.66%. Fig. 14 shows the change in average tumor size during the therapy at three points. Fig. 15 illustrates the sizes of tumor after dissection at day 10 in all groups compared to each other in EMT-6/CPR.

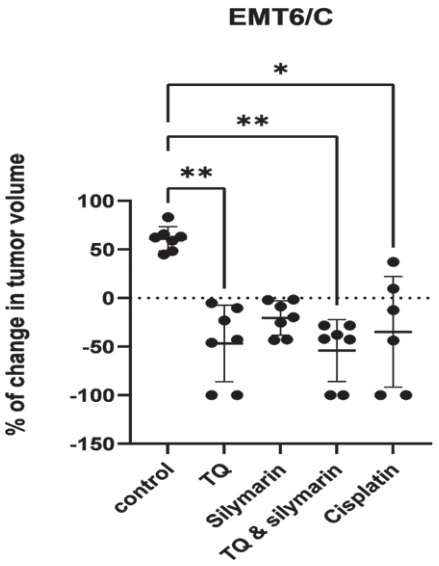

Figure 13. Shows that the percentage of change in tumor volume in EMT-6/CPR cell line was significant (P-value < 0.05) in all treatment groups except the silymarin group. This graph was obtained using GraphPad prism.

Table 3. Results of TQ, Silymarin, their combinations and, cisplatin regarding tumor size changes, percentages of change in tumor size and average tumor weight in EMT-6/CPR cell line (n = 7).

Treatment group for EMT-6/CPR	Av. Initial tumor size (mm^3)	Av. Final tumor size (mm^3)	change in tumor size (%)	Mice with no detectable tumor (%)	Av. tumor weight (g)
Control	327.25	522.91	59.78	0	0.757
TQ	259.73	142.64	-45.07	42.85	0.193
Silymarin	436.27	348.21	-20.18	14.28	0.373
Cisplatin	285.31	203.83	-28.55	16.66	0.296
TQ + Silymarin	361.05	157.39	-56.40	42.85	0.250

Abbreviations: Av. = Average, mm^3 = cubic millimetre.

Table 4. Serum ALT, AST and Cr levels for groups with different treatments, negative control treated with only vehicle.

Treatment groups	ALT (IU/L)	AST (IU/L)	Cr (mg/dl)
Control	47.9 ± 2.1	212.4 ± 15.3	0.25 ± 0.01
TQ	41.1 ± 6.4	245.4 ± 7.0	0.24 ± 0.02
Silymarin	43.8 ± 1.7	222.8 ± 3.5	0.25 ± 0.01
Cisplatin	38.1 ± 1.2	187 ± 18	0.26 ± 0.01
TQ + silymarin	40.8 ± 5.5	230.8 ± 11.7	0.23 ± 0.01

Liver toxicity evaluation in mice

Since ALT and AST assays are regarded as indicators for liver damage, blood levels of liver enzymes were evaluated for all treated groups with TQ, silymarin, their combinations, and cisplatin, in addition to the negative (untreated) control, Table 4. Then, as a reference for liver function, we compared their results to a healthy group that didn't bear any tumors. Our investigation discovered that serum ALT and AST levels for all treatment groups were within the normal range compared to those observed for the control and healthy groups with insignificant difference (P-value > 0.05) (Figs 16–19).

Kidney toxicity evaluation in mice

In the case of creatinine, normal levels of serum creatinine were observed between the healthy mice and the other mouse groups that received the previously mentioned therapies, except the cisplatin group which showed a significant increase in creatinine level to 0.27 mg/dl (Figs 20, 21).

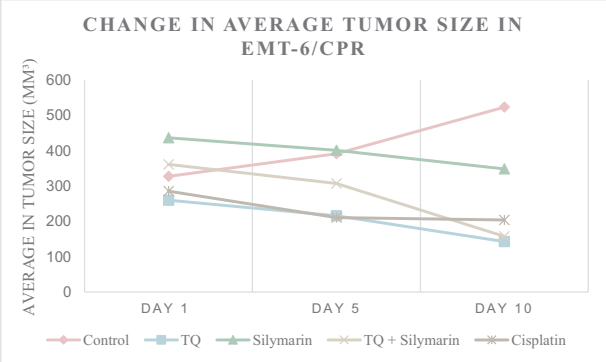

Figure 14. A plot of change in average tumor size (mm^3) vs. time in (days) of treatment in EMT-6/CPR cell line.

Figure 15. Changes of tumor sizes in EMT-6/CPR after dissection at day 10 in all groups compared to each other, n = 7.

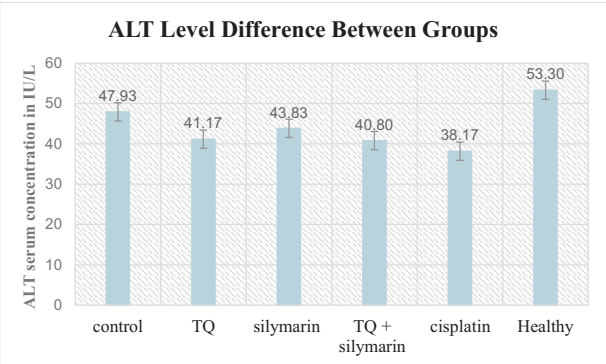

Figure 16. Effect of TQ (25 mg/kg), silymarin (50 mg/kg), their combinations, cisplatin (0.7 mg/kg), and control group on serum ALT level measured by (IU/L).

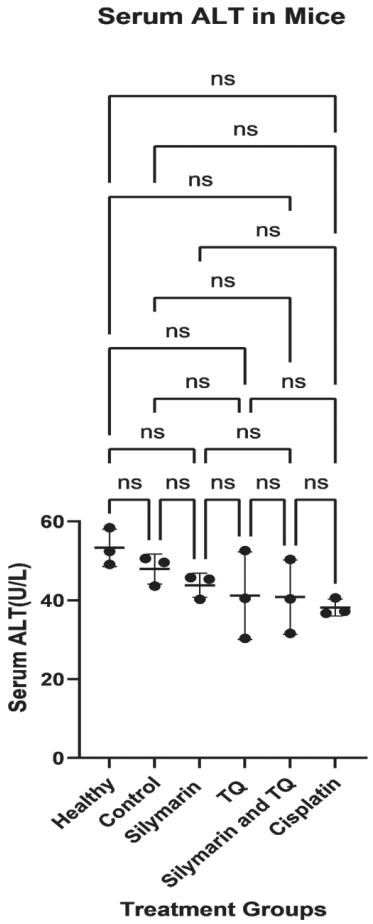

Figure 17. Showed that there is no significant (ns) difference in serum ALT between the healthy group and other treatment groups. This graph was obtained by GraphPad Prism.

Discussion

BC incidence continues to rise despite decades of epidemiological, laboratory, and clinical studies. BC is still the major cancer-related cause of disease burden for women, impacting one in every 20 people worldwide and up to one in every eight in high-income nations (Britt et al. 2020). Although it is commonly accepted that chemotherapy is the major therapeutic choice for BC traditional chemotherapy still has numerous substantial obstacles. First of all, a lack of selectivity which leads to inevitable side effects (Omidi et al. 2022) such as nausea, vomiting, alopecia, and tiredness (Łukasiewicz et al. 2021). Add to that, the specific side effects of the chemotherapy like ototoxicity and nephrotoxicity of cisplatin (Dilruba and Kalayda 2016). Additionally, poor chemical stability, short half-life and limitations in crossing blood brain barrier (Mills et al. 2020; Malik et al. 2022). Another obstacle is drug resistance which resulted in reducing the effectiveness of the chemotherapeutic agents toward cancer cells. The last obstacle is the main one because it is accountable for most relapses of cancer, which eventually lead to death (Wang et al. 2019). Over the last decades, scientist developed various solutions to overcome drug resistance such as combination therapy (Housman et al. 2014). Combination

therapy is now frequently utilized to reduce pharmacological adverse effects, prevent the development of drug resistance, and improve positive benefits with lower dosages. Additionally, it can provide targeted synergy against a certain illness, like cancer (García-Fuente et al. 2018). Natural products have always been seen as attractive anticancer agents (Varghese and Dalvi 2021). This is due to their variety in chemical structure and relatively low toxicity (Talib et al. 2022).

Thus, in our study, TQ and silymarin natural products were compared to cisplatin as single treatments or in combination. In particular, they were examined *in vitro* on EMT-6/P and EMT-6/CPR BC cell lines and *in vivo* on female *Balb/C* mice injected with both EMT-6/P and EMT-6/CPR BC cell lines. According to our findings, TQ inhibited the viability of EMT-6/P and EMT-6/CPR cell lines in a concentration-dependent manner. These findings are consistent with Talib et al. study, where they examined TQ on EMT-6/P BC cell line and the investigated results displayed a dose-dependent anti-cancer activity (Odeh et al. 2018). Another study demonstrated that TQ acted as antiproliferative agent in three different types of BC cell lines (EMT-6/P, MCF-7, and T47D) in a concentration dependent manner (Alobaedi et al. 2017). Also, in MCF-7/DOX cells, which are doxorubicin-resistant human breast adenocarcinoma MCF-7 cell line, TQ exerted an antiproliferative activity (Arafa et al. 2011). In colorectal cancer, TQ had stimulate apoptosis and DNA damage in 5-fluorouracil-resistant HCT116 colorectal cancer cells, resulting in reduction in cell viability (Ballout et al. 2020). In order to understand the mechanism of action of TQ as an anticancer agent, it is important to examine its various properties. Initially, experimental investigations revealed that TQ's antioxidant activity was responsible for its chemo preventive actions; however, other research reported that TQ induces death in cancer cells by oxidative damage (Richards et al. 2006), (Dergarabetian et al. 2013). TQ's antioxidant, antiproliferative, and proapoptotic properties were further clarified by Cecarini et al. (Cecarini et al. 2010), who showed that it causes selective proteasome inhibition, which may be related to the induction of apoptosis in cancer cells. More research by Torres et al. found that TQ causes apoptosis in pancreatic cancer cells via activating the c-Jun NH (2)-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways and down-regulating glycoprotein mucin 4 (MUC4) expression through the proteasomal pathway (Torres et al. 2010). TQ also affects DNA structure by interfering with it. It attacks cellular copper, which is found in chromatin and is tightly linked to the DNA base guanine. This results in oxidative DNA damage, which kills cancer cells (Zubair et al. 2013). Silymarin also showed antiproliferative activity in EMT-6/P and EMT-6/CPR cell lines. In fact, its activity against EMT-6/CPR was more potent than its activity against EMT-6/P cell line. This can be explained by silymarin's ability to interact with P- glycoprotein and inhibiting P-glycoprotein ATPase activity (Zhang and Morris 2003). It is well known that elevated P- glycoprotein expression is closely associated with cisplatin resistance (He et al. 2019). Silymarin's antiproliferative activity is due to its various properties. Actually, silymarin can regulate the imbalance between cell survival and apoptosis, by interfering with the expression of cell cycle regulators and proteins involved in apoptosis. Silymarin efficiently modifies G0-G1 and G2-M cell cycle regulators and checkpoints to limit proliferation and cause growth arrest (Singh and Agarwal 2002). Additionally, silymarin possess both anti-inflammatory and anti-metastatic properties (Ramasamy and Agarwal 2008). In fact, our findings are similar to Kalla et al. study. In the previous study, silymarin acted as a strong anticancer agent against NCI-H23 (Lung cancer) and MCF-7 (BC) cells by encouraging the apoptotic gene expression (Kalla et al. 2014). Another study on human prostate cancer LNCaP and 22Rv1 cells revealed that silymarin diastereoisomers cause the cleavage of poly (ADP- ribose) polymerase PARP, caspase 9, and caspase 3, which lowers the levels of survivin (Deep et al. 2007). Moreover, silymarin inhibits various angiogenic responses of vascular endothelial cells, including growth and survival, matrix metalloproteinase-2 (MMP-2) production, and *in vitro* angiogenesis. As well, silymarin prevents the release of a main angiogenic cytokine, vascular endothelial growth factor (VEGF), by cancer epithelial cells (Jiang et al. 2000). Also, in multidrug-resistant colon cancer, silymarin and doxorubicin combination was found to be effective at high but therapeutically acceptable dosages (Colombo et al. 2011). Interestingly, TQ and silymarin combination exerted a synergistic activity in EMT-6/P cell line and a very strong synergistic activity in EMT-6/CPR cell line with a CI = 0.632 and 0.018 respectively. The suggested mechanism of their synergism activity is due to their various mechanisms of action and multi-target effects as anticancer agents (Ma et al. 2009; Yang et al. 2014). In a previous study, TQ with melatonin revealed a synergistic effect against EMT-6/P BC cell line. This combination's anticancer impact is achieved via angiogenesis suppression, and apoptosis induction (Odeh et al. 2018). Also, TQ and emodin combination synergistically induce apoptosis, slow cell migration, and diminish stemness in MCF-7 BC cells (Bhattacharjee et al. 2020). Again, TQ combination with gefitinib showed a synergistic activity toward gefitinib-resistant A549 cells in non-small cell lung carcinoma (Upadhyay et al. 2021). For silymarin, a previous study tested a combination of silymarin and curcumin demonstrated a synergistic activity on different colon cancer cell lines (DLD-1, LoVo, HCT116) (Montgomery et al. 2016). Both curcumin and silymarin have been shown to reduce protein phosphorylation, hence inhibiting NF-kB activation (Gao et al. 2015) (Miroliaee et al. 2011). Chen et al. showed that baicalein and silymarin combination exerted synergistic anti-cancer activity on HepG2 human hepatoma cells. It appears that this combination significantly changed the expression of the genes, particularly those related to the

G1/S transition. (Chen et al. 2009). Silymarin and quercetin together showed synergistic antiproliferative activity in HepG2 liver cancer cell lines (Raut et al. 2023).

Apoptosis has been extensively documented as a key process of controlled death that takes place not just in response to external stress or cell damage but also during normal development and morphogenesis (Nikoletopoulou et al. 2013). Extrinsic and intrinsic pathways are the two primary processes that contribute to the induction of apoptosis. The intrinsic route is a mitochondrial-mediated pathway, whereas the extrinsic pathway is a death receptors proteins (DR) mediated one (Elmore 2007). To understand how apoptosis happens, let's explain its mechanism. When extracellular ligands like TNF (tumor necrosis factor), Fas-L (Fas ligand), and TRAIL (TNF-related apoptosis-inducing ligand) are bound to the extracellular domain of the DR (transmembrane receptors), apoptotic signalling through the extrinsic pathway is activated. The FasL/FasR (Fas receptor) and TNF-α/TNFR1 models accurately describe the sequence of events involved in the extrinsic phase of apoptosis (Jin and El-Deiry 2005; Elmore 2007; Guicciardi and Gores 2009). When DRs are activated by certain death ligands (DLs), a death-inducing signalling complex (DISC) forms (Bredesen et al. 2006). Caspases are key apoptosis initiators and executioners, and their function is intimately tied to their structure, which has diverse substrate preferences. Some caspases feature lengthy pro-domains with specific motifs, such as the death effector domain (DED) and caspase recruitment domains (CARD), that allow them to interact with other proteins and link to signalling networks. Caspase-8 and Caspase-10 are involved in DED, whereas Caspase-1, Caspase-2, Caspase-4, Caspase-5, Caspase-9, Caspase-11, and Caspase-12 are involved in CARD (Ghavami et al. 2009). As mentioned before, the "intrinsic pathway" mostly refers to the apoptotic process mediated by mitochondria. The intrinsic route, which is activated by a variety of extracellular and intracellular stressors, includes oxidative damage, radiation, and cytotoxic drug therapy (Ghavami et al. 2004), (Hashemi et al. 2004). The intrinsic route is mediated by the insertion of Bax/Bak into the mitochondrial membrane, followed by the release of cytochrome c from the mitochondrial intermembranous region into the cytosol (Kim 2005). Anti-apoptotic proteins Bcl-2 and Bcl-xL (a member of the Bcl-2 family) prevent the release of cytochrome c (Ghobrial et al. 2005). Apoptosomes are formed when cytochrome c interacts with Apaf-1 and procaspase-9. Apoptosome is a multi-protein complex composed of a seven-spoke ring-shaped complex that activates caspase 9, followed by activation of caspase-3 signalling cascade, resulting in cell deconstruction and apoptosis (Los et al. 1995; Jin and El-Deiry 2005; Yuan and Akey 2013). Together, extrinsic and intrinsic paths meet at the same point (execution phase). The term "execution phase" describes the last stage of apoptosis (Sankari et al. 2012). While caspase-3, caspase-6, caspase-7, caspase-10, PARP (Poly (ADP-ribose) polymerase), and CAD (Caspase-activated DNAse) are categorized as effector or executioner caspases, caspase-8 and caspase-9 are initiator caspases (Hengartner 2000), (Hu et al. 2013)

It is generally known that caspase-3 is regarded as a crucial enzyme in the execution of apoptosis, and is therefore often targeted to detect apoptosis (Lossi et al. 2018). In light of this, we evaluated the role of TQ and silymarin in single and combination treatment in the activation of the caspase-3. Our outcomes displayed that both TQ and silymarin increased the level of caspase-3 activity. But their combination showed the strongest elevation in the level of caspase-3 in EMT-6/CPR cell lines (Figs 9, 10). These results were consistent with other research published previously where TQ and silymarin usage on various malignant tissues increased caspase-3 activation and concurrently increased apoptosis. For instance, TQ inhibited JAK2/STAT3 in human renal carcinoma Caki-1 cells via pro-oxidant effect. This then resulted in apoptosis induction (Chae et al. 2020). Besides, TQ in single or in combination with temozolomide (TMZ) enhanced the apoptosis of TMZ-resistant glioblastoma cells. That was achieved through the activation of p38 mitogen-activated protein kinase signalling pathway (Mai et al. 2023). In addition, silymarin induced apoptosis in human acute promyelocytic NB4 cells when combined with all-trans retinoic acid (ATRA) (Parsa et al. 2023). Similarly, silymarin significantly augmented the apoptosis process and cell death through caspases pathways on K562 leukemia cells (Zhong et al. 2006). In another study, silymarin suppresses the Nuclear Factor-Kappa B Pathway and induces apoptosis in Epstein-Barr Virus-Positive Lymphoma Cells (Lu et al. 2020).

In accordance with the *in vitro* findings, this study discovered that TQ reduced tumor size in both cell lines *in vivo* to 86.86% and 45.07% in EMT-6/P and EMT-6/CPR, respectively. The treatment was intraperitoneal (i.p.) injection of 25 mg/kg of TQ for 10 days in a daily basis treatment, Tables 2, 3. Our findings are consistent with a previous study showing that treatment with TQ (10 mg/kg/i.p.) for 18 days, decreased the tumor growth of LNM35 lung cells by 39% significantly in athymic mice (Attoub et al. 2013). Others observed that in gastric cancer, TQ resulted in tumor growth inhibition in a gastric mouse xenograft model (Zhu et al. 2016).

Silymarin exhibited a reduction in the tumor size in EMT-6/P significantly to 68.65% after daily i.p. injection of 50 mg/kg for 10 days, Table 2. It has been reported that silymarin reduced tumor volume and growth in oral cancer by Won et.al. (Won et al. 2018). Also, oral administration of 500 mg/kg/week of silymarin significantly decreased tumor volume in melanoma cancer to 60% (Vaid et al. 2015). On the other hand, its activity against EMT-6/CPR cell line showed a 20% reduction in tumor size but this reduction was insignificant, Fig. 13. This result is similar to a previous study which presented that silymarin exhibited a non-significant reduction in tumor size by 20% in a mouse skin model (Katiyar et al. 1997). This was possibly due to low tumor incidence and tumor multiplicity in the control group.

In this study, several combination strategies were examined. Firstly, our TQ and silymarin combination which reduced tumor size in the studied cell lines in vivo significantly with the highest percentage of reduction. The shrinking in tumor sizes were 92.6% and 56.4% in EMT-6/P and EMT-6/CPR, respectively. This was attained after i.p. daily injection of 25 mg/kg TQ and 50 mg/kg of silymarin for 10 days Tables 2, 3. However, this combination has never been tested before. Talib et al. demonstrated that TQ and piperine combination resulted in significant dropping in tumor size in breast carcinoma syngraft (Talib 2017). Again, TQ and resveratrol together inhibited the growth of tumor in mice implanted with BC (Alobaedi et al. 2017). In addition, silymarin, curcumin and boswellic acid combination avoid congenital intestinal cancer in mice (Girardi et al. 2020).

Cisplatin is one of the most effective and popular medications for the treatment of different solid malignancies. Cisplatin's antineoplastic properties are primarily a result of its capacity to cross-link with DNA, preventing transcription and replication (Qi et al. 2019). However, it has two intrinsic problems that restrict its use and efficacy: side effects and drug resistance (Ghosh 2019). Generally, it has about 40 distinct toxicities, the most frequent of which is nephrotoxicity. Ototoxicity, gastrointestinal toxicity, neurotoxicity, hematological toxicity, hepatotoxicity, and cardiotoxicity are other frequent side effects (Qi et al. 2019). Major factors contributing to cisplatin resistance inside the tumor cell include reduced drug import, enhanced drug inactivation by detoxification enzymes, increased drug export, improved DNA damage repair, and inhibited cell death signalling (Chen and Chang 2019). In order to reduce the adverse effects and resistance of cisplatin, combination therapy have been applied and shown to be more successful in treating malignancies (Ghosh 2019). In our investigation, cisplatin attended as a positive control both in vitro and in vivo. Our results showed an increase in IC_{50} of cisplatin in EMT-6/CPR cell line in comparison with EMT-6/P cell line. These findings are similar to Jawarneh et.al study, where the IC50 of cisplatin in EMT-6/CPR was more than the IC50 in EMT-6/P cell line (Jawarneh and Talib 2022).

In terms of safety, anticancer drugs' safety profiles are frequently evaluated to determine their toxicity (Levêque and Becker 2019). This is because it is well known that many anticancer medications have negative effects on the kidney, liver, and spleen. Hepatotoxicity is ruled out by the biochemical criteria like ALT and AST values and nephrotoxicity is ruled out by the serum creatinine levels (Pal et al. 2008). As a result, we employed the liver enzymes ALT and AST in our investigation to determine liver functions, whereas creatinine indicates renal function. In our experiment, the outcomes of the treated groups were contrasted with those of the normal, untreated group, which had any tumor implants. The ALT and AST results showed that all treated groups had normal levels of ALT and AST with no significant change when compared to the normal-untreated group,

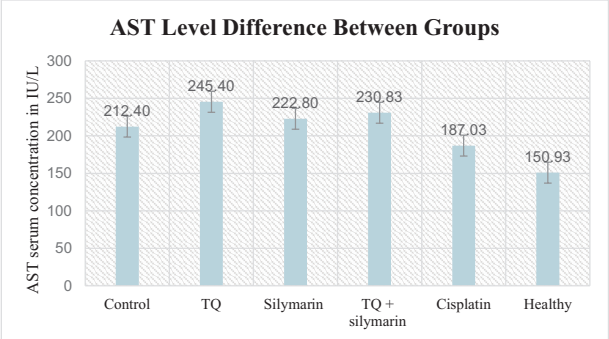

Figure 18. Effect of TQ (25 mg/kg), silymarin (50 mg/kg), their combinations, cisplatin (0.7 mg/kg), and control group on serum AST level measured by (IU/L).

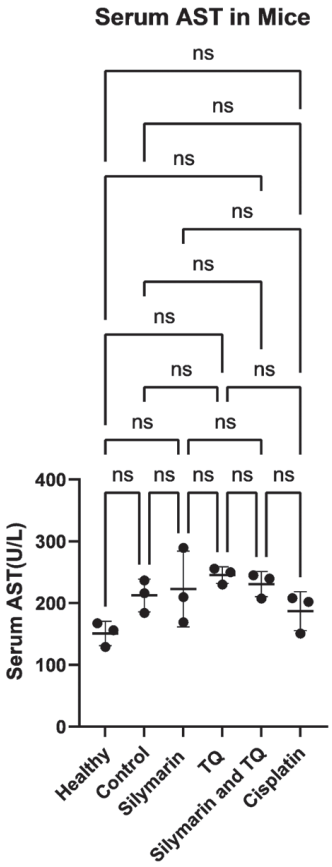

Figure 19. Showed that there is no significant difference in serum AST between the healthy group and other treatment groups. This graph was obtained by GraphPad Prism.

Figs 17, 19. In fact, many literatures proved that TQ exhibited hepatoprotective activity. This is due to its ability to prevent the accumulation of fatty acids in the hepatocytes (Noorbakhsh et al. 2018). Also, silymarin is a well-known hepatoprotective agent by inhibiting the apoptosis and fibrogenesis in the liver (Mukhtar et al. 2021). That is, the combination of TQ and silymarin reduced cisplatin's hepatotoxicity. On the other hand, all treatment groups showed normal levels of creatine except the cisplatin group, Fig. 21. The reason for this is cisplatin-induced nephrotoxicity, one of the most significant barriers to cisplatin usage and it results from the accumulation of cisplatin in kidney cells (Miller et al. 2010).

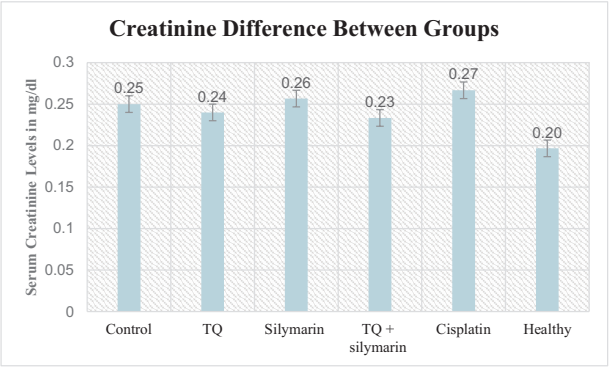

Figure 20. Effect of TQ (25 mg/kg), silymarin (50 mg/kg), their combinations, cisplatin (0.7 mg/kg), and control group on serum creatinine level measured by (mg/dl).

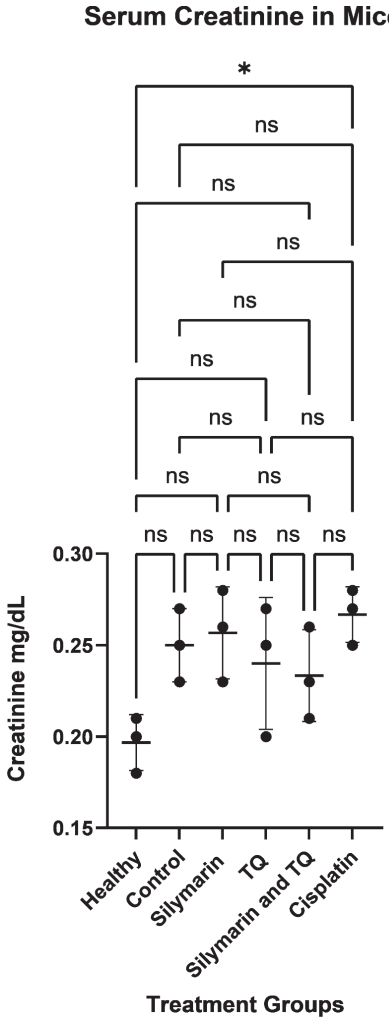

Figure 21. Showed that there is no significant difference in serum creatinine between the healthy group and other treatment groups except cisplatin group. This graph was obtained by GraphPad Prism.

Conclusion

Based on the information provided, we draw the conclusion that the combination of TQ and silymarin demonstrated a synergistic anticancer effect against both the parent (EMT-6/P) and resistant (EMT-6/CPR) cell lines *in vitro* and *in vivo* through apoptosis induction and caspas-3 activation better than each treatment alone. But when TQ and silymarin were combined in novel ways, the tumor size reduction was superior to that of a single therapy in both cell lines. These combinations are less harmful to the liver and kidneys than traditional cisplatin treatment. Such unusual combinations make it worthwhile broadening the scope of study to ascertain additional information in order to benefit from it further in breast cancer therapy.

Recommendations

Research and development are a never-ending process, with constant scrutiny for useful, futuristic, and significant results that add to a greater understanding of cancer prevention and therapy. As a result, the following recommendations for further work on this subject are provided:

- To investigate the triple therapy of thymoquinone, silymarin and cisplatin to overcome cisplatin resistance in breast cancer implanted in mice.
- To further explore the mechanisms of the combination's synergistic activity by testing VEGF assay.
- To examine this combination along with different chemotherapies on various cancer types.

Author contributions

RAH contributed to design the study, the writing and editing of the report, and the analysis and interpretation of the data. WT contributed to conceptualize, plan, and revise the experiments.

Ethical approval

The Research and Ethical Committee of Applied Science University approved all animal experimentation techniques in accordance with standard ethical principles. The Institutional Review Board (IRB) panels at the IRB authorized the methods used to collect tumor tissue samples (Approval number: 2023-PHA-11). This was all conducted in conformity with the moral guidelines established by the 1964 Helsinki Declaration and its ensuing revisions.

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