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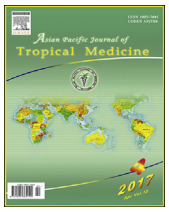

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Antitumor effect of thymoquinone combined with resveratrol on mice transplanted with breast cancer

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ABSTRACT

Objective: To test the anticancer potential activity of the combination of thymoquinone (TQ) and resveratrol (RES) against breast cancer in mice.
Methods: The antiproliferative activity of TQ, RES and their combination was assessed against three breast cancer cell lines and one normal cells using MTT assay. The combination index was calculated using isobolographic method. Balb/C mice were inoculated with EMT6/P cells and *in vivo* antitumor activity was evaluated.
Results: The combination therapy also caused significant decrease in tumor size with a percentage cure of 60%. The combination therapy induced geographic necrosis, enhanced apoptosis, and decreased VEGF expression. Serum levels of IFN-γ were elevated in mice treated with combination therapy with no liver or kidney toxicity.
Conclusions: The combination of TQ and RES against breast cancer in mice can work synergistically. The anticancer effect of this combination is mediated by apoptosis induction, angiogenesis inhibition and immune modulation.

1. Introduction

Cancer is a group of diseases that may appear in any part of the body. Despite the great achievements in the battle against cancer over the past decades, cancer remains to be one of the main causes of death worldwide, occupying the second place after cardiovascular diseases [1].

According to the American Cancer Society, there are 74 different types of cancer. Amongst the different cancer types, breast cancer is the second most common cancer, with approximately 231 840 new cases of invasive breast cancer and 40 290 deaths are expected among US women in 2015. Breast cancer is characterized by high mortality and morbidity, making this malignancy a major health concern and calling on utilizing all of the available possibilities in the field of research and technical development to ensure proper combat once needed [2].

In addition to surgery, the major types of interventions for treatment of cancer include chemotherapy and radiotherapy [3]. These traditional therapies are usually expensive, exhibit low selectivity with extended profile of side effects and concerns; also sometimes they exhibit limited efficiency in treating certain cancer types [3]. Problems and concerns associated with the use of traditional therapies deserve more efforts to discover alternative treatments and/or interventions with optimal efficacy and safety profile.

Due to their low toxicity and multiple mechanisms of action, natural products have significant role in treating different ailments including cancer [4].

Thymoquinone (TQ) is a phytochemical compound and one of the active components of the volatile oil extracted from black seed [*Nigella sativa* (*N. sativa*)]. TQ possesses a wide variety of therapeutic effects including antioxidant, anti-inflammatory, anti-carcinogenic and chemo-sensitizing [5]. Resveratrol (RES), chemically known as 3,5,4′-trihydroxy-trans-stilbene, is a stilbene polyphenolic compound that functionally belongs to phytoalexins [6]. RES has been identified in over 70 plants including grapes, blueberry, cranberry, mulberry and peanuts. This compound is synthesized by plants during environmental stress and pathogenic invasion of bacteria or fungi, hence it possesses strong antioxidant activity [7]. Experimental data show that RES exerts strong anti-inflammatory, anti-oxidant, anti-carcinogenesis and chemo-sensitizing effects [8]. Its effects have been investigated in a variety of diseases and conditions including cardiovascular, diabetes, asthma, kidney, liver and cancer [9].

In the last few decades, trends have been shifted to combination therapy from relying on mono therapies. This trend is

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based on the fact that cancer cells have multiple pathways for survival including enhanced proliferation, angiogenesis stimulation, apoptosis evasion and invasion and metastases [10]. Therefore, the aim of this study was to evaluate the potential anticancer activity of a novel combination of plant derived-natural products consisting of TQ and RES against breast cancer implanted in mice.

2. Material and methods

2.1. TQ and RES working solutions

TQ (99%) and RES (99%) were purchased from Sigma (USA). Ten milligram (mg) of each were dissolved in one milliliter (mL) absolute ethanol to produce a solution of 10 mg/mL, known as 'stock solution one'. Further dilutions was performed to stock solution one with complete tissue culture medium just before used to prepare the desired concentrations of 400 μM down to 10 μM, for both TQ and RES. For *in vivo* study, TQ and RES were dissolved in PBS containing 2% tween 20. Tween 20 was selected for the *in vivo* study as it had lower toxicity and exhibited higher ability to dissolve RES and TQ.

2.2. Cell lines and cell culturing condition

Four cell lines were used in our study. Mouse epithelial breast cancer cells (EMT6/p) was purchased from the European Collection of Cell Cultures (ECACC). EMT6/p cells were maintained in complete MEM medium (Sigma, USA). Two human epithelial breast cancer cell lines MCF-7 and T47D were provided by the University of Jordan. Both cell lines were cultured in complete RPMI 1640 Medium (Sigma, USA). Kidney epithelial cells from African green monkey Vero cell line, used as a normal control, was also obtained from the University of Jordan and cultured in complete DMEM Medium (Sigma, USA). Culture media were supplemented with 10% fetal calf serum and 1% L-glutamine (Gibco, UK). Also 0.1% gentamycin and 1% penicillin-streptomycin (Gibco, UK) solution were added. All cell lines were incubated in a CO_2 incubator at 37 °C in 5% CO_2 and 95% humidity.

2.3. Animals

Standard ethical guidelines were followed in using experimental animals. All experiments were agreed by the Research and Ethical Committee in the Applied Science Private University. Mice were provided by the animal house in the Applied Science University, Amman, Jordan.

Forty female Balb/C mice were used in our study. All mice were at 6–8 weeks old and have body weight of 23–25 g/mouse. Standard cages containing wooden shaving as bedding were used to keep mice.

2.4. Antiproliferative assay

Cells were harvested by employing trypsinization technique using Trypsin–EDTA (Promega, USA) and 1× PBS (Sigma, USA) for 2–3 min, washed, centrifuged at 1500 RPM and re-suspended in 5 mL of fresh tissue culture medium after decant of the supernatant layer and finally counted. Cells were then dispensed (100 μL/well) into 96-well plates (flat bottom) at a concentration of 15 000 cells/well in complete tissue culture medium. After overnight incubation, the media in each well were completely removed and the attached cells were treated in triplicates with different treatments dissolved in 200 μL/well. The following treatments were used: increasing concentrations of TQ (10–400 μM), RES (10–400 μM), and predetermined different combinations of both, resulting in a total volume of 200 μL. Cells then incubated for 48 h. For combination treatments, the concentration of one agent was fixed and combined with increasing concentration of the other agent. Combination of 15 μM RES was prepared and combined with increasing concentrations of TQ (25–300 μM) to treat EMT6/P cells. The same procedure was used to prepare combinations for other cell lines.

Cell viability was then measured by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma, USA). The principle of this assay is to detect the activity of mitochondrial dehydrogenase which reduced MTT to blue formazan crystals, revealing normal mitochondrial function and cell viability. In this assay, 100 μL (of 200 μL/well) of culture media were removed from each well and replaced with 10 μL of Thiazolyl blue tetrazolium solution (final volume 110 μL/well) and placed in CO_2 incubator for 3 h. Then the stop MTT reagent was added (100 μL/well), mixed well and incubated for another 1 h (final volume 210 μL/well). Readings of absorbance was taken at 595 nm by microplate reader (Biotek, USA). Cell viability (% survival) was calculated compared to negative control cells (contain only tissue culture media with 0.05% ethanol). Vincristine sulfate was used as a positive control.

2.5. Calculation of combination index (CI)

CI was calculated for the combinations of TQ and RES against cell lines under study (EMT6/P, MCF-7, T47D and Vero-normal cells) by using an equation published previously [11]: CI = (D) 1/(Dx) 1 + (D) 2/(Dx) 2 + α (D) 1 (D) 2/(Dx) 1 (Dx) 2; where: (Dx) 1 = IC_{50} of thymoquinone alone; (D) 1 = IC_{50} of thymoquinone in combination with resveratrol; (Dx) 2 = IC_{50} of resveratrol alone; (D) 2 = IC_{50} of resveratrol in combination thymoquinone; α = 0 for mutually exclusive or 1 for mutually nonexclusive interaction.

2.6. Antitumor activity assay

Exponentially growing EMT6/P cells were collected by trypsinization and then were washed and re-suspended in complete MEM medium, at a density of 2×10^6/mL. Viable cells were detected using trypan blue exclusion method. A tumorigenic dose of 1×10^5 cells in 0.1 mL 1× PBS was injected subcutaneously in the abdominal area of each female BALB/C mouse (known as day 0 of the *in-vivo* experiment). Tumors were allowed to grow for 14 d, and at day 15 tumor bearing mice started receiving the subjected treatments.

Tumors size was measured using digital calipers and tumors volume was calculated using the following equation [12]. Tumor volume = $A \times B^2 \times 0.5$; where A equaled length of the longest aspect of the tumor; B equaled to the length of the tumor aspect perpendicular to A.

The mice were then divided into four groups (n = 10). Tumors of similar sizes were selected so that the average tumor volume for all groups closely matched. Group I was TQ group (n = 10 mice); mice in this group were injected intraperitoneally

with 50 mg/kg/d of TQ. Group II was the RES group (n = 10 mice); here the mice were injected intraperitoneally with 50 mg/kg/d of RES. Group III was the combination group (n = 10 mice); mice were injected intraperitoneally with 50 mg/kg/d of TQ and 50 mg/kg/d RES. Group IV was the control group (n = 10 mice); mice here were injected intraperitoneally with the vehicle (2% tween 20 in PBS) 0.1 mL daily.

Tumors were measured at the beginning and at the end of the study (after 14 d). At the end of the study, mice were sacrificed; tumors and organs were extracted, weighed and stored in 10% formalin.

2.7. Hematoxylin and eosin (H&E) staining of tumor sections

Further investigation in the effect of TQ, RES and combination of them on breast cancer implanted in mice was mediated through using H&E staining technique. Formalin fixed specimens were gradually dehydrated and embedded into prepared paraffin blocks. Sections of 5 μm thickness were prepared using microtome and were examined under light microscope equipped with digital camera to examine images on the slides looking for the presence of necrotic areas.

2.8. Apoptosis detection in tumor sections

Degree of apoptosis induced by each treatment was detected using DeadEnd Colorimetric TUNEL System (Promega, USA). According to kit instructions, paraffin-embedded sections were deparaffinized using xylene, then rehydrated by immersing through decreasing grades of ethanol (100%–50%). Slides were washed in 0.85% NaCl and fixated in 10% buffered formalin for 15 min. Twenty μg/mL proteinase K solution was added to each slide and incubated for 20 min at room temperature. Sections were re-fixed using 10% buffered formalin then covered with Equilibration buffer for 5–10 min at room temperature. End-labeling reaction occurred through the even distribution of rTdT reaction mixture on the sections while incubating for 60 min at 37 °C in a humidified chamber. Termination of the reaction occurs when slides are immersed in 2X SSC termination solvent. Detailed and step by step procedure was conducted in accordance to DeadEnd Colorimetric TUNEL System G7362 (Promega, USA).

2.9. Degree of VEGF inhibition

Actively growing EMT6/P cells were collected after trypsinization, washed, counted and cultured in four tissue culture flasks at an optimized concentration of 1.5×10^5 cells/10 mL of complete tissue culture medium. After 24 h, the media in each flask was completely removed and the attached cells were treated with TQ, RES, combination of TQ and RES, and blank media (as control). Cells were treated for 48 h and then harvested, washed and tested for VEGF expression using kit instructions (Promega, USA). Briefly, cells were lysed using lysis buffer followed by centrifugation and addition of supernatants to 96 well plate pre-coated with capture antibody for VEGF. The wells were washed four times then biotinylated detection antibody was added to each well followed by washing and adding 100 μL of HRP-conjugated streptavidin. Color development was achieved by adding TMB substrate solution followed by incubation in dark for 30 min. Then the reaction was terminated by adding 50 μL of stop solution. The intensity of the color was measured at 450 nm by microplate reader.

2.10. Determination of TH1 and TH2 serum cytokines levels

Levels of IFN-γ (signature cytokine for TH1 response) and IL-4 (signature cytokine for TH2 response) in the serum were measured using Quantikine ELISA kits (affymetrix eBioscience, California, USA). A monoclonal antibody specific for mouse IFN-γ or mouse IL-4 has been used to pre-coat a 96 well microplate. Standards control and samples were dispensed into the wells where any mouse IFN-γ or mouse IL-4 presented in the serum samples would bound by the fixed antibody. After washing out fee proteins, an enzyme-linked secondary polyclonal antibody specific for mouse IFN-γ or mouse IL-4 was added to the wells. Color development was achieved according to the kit instructions.

2.11. Assessment of liver and kidney toxicity

Liver and kidney toxicity resulted from the use of TQ, RES and their combination was evaluated by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to evaluate liver function, and creatinine serum levels to evaluate the kidney function. The serum levels of AST, ALT, and creatinine were measured using the instructions in the relevant kits (BioSystems, Barcelona, Spain).

2.12. Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS, version 20, Chicago, IL, US). Data were presented using mean ± SEM. Values for IC_{50} (the concentration at which there was 50% cell death compared to negative control) were calculated using non-linear regression in SPSS for each treatment in the corresponding cell line. The one-way analysis of variance (ANOVA) followed by unpaired Student's t-test was used to decide whether there were any statistically significant differences between the means of the four independent study groups with significance defined as P value of ≤ 0.05.

3. Results

3.1. Antiproliferative assay and CI

A dose dependent response was observed after treatment of different cell lines with increasing concentrations of TQ or RES (Tables 1 and 2). The highest activity of TQ was observed against Vero cell line while its lowest activity was observed against MCF-7 cell line (Table 1). On the other hand, RES showed its highest activity against EMT6/P cell line and lowest activity against T47D cell line (Table 2). Combination of 15 μM RES with increasing TQ concentrations caused significant reduction in cell viability of EMT6/P cells compared with TQ single treatment ($P < 0.05$). The highest reduction in cell viability (72% inhibition) was observed for a combination consisting of 250 μM TQ + 15 μM RES (Table 3). Similar significant results were obtained when increasing concentrations of RES combined with 140 μM TQ compared with RES single

treatment ($P < 0.05$) (Table 3). The highest inhibition rate (53%) was observed in combination consisting of 50 μM RES + 140 μM TQ. Based on these *in vitro* results, we decided to use EMT6/P cell line to establish tumors in mice. The effect of the combined treatment on the other cell lines was shown in Table 4. Results for the TQ IC_{50} value showed the highest activity against Vero normal cell [$IC_{50} = (45.00 \pm 2.23)$ μM] and lowest activity against EMT6/P [$IC_{50} = (393.00 \pm 4.33)$ μM]. On the other hand, MCF-7 and T47D cell lines responded almost similarly for TQ (Table 4). In contrast to TQ, RES was most active against EMT6/P [$IC_{50} = (56 \pm 1.07)$ μM] and its IC_{50} values for MCF-7, T47D and Vero normal cell were higher than the values observed with TQ (Table 4).

Combination of TQ and RES led to significant ($P < 0.05$) reduction in the values of IC_{50} compared to the IC_{50} values of single treatments, the most obvious reduction was for TQ against the EMT6/P cell line; here the IC_{50} value for TQ alone was (393.00 ± 4.33) μM, while by combining TQ with RES it decreased to (140.00 ± 2.99) μM. Other cell lines also exhibited significant reduction in the values of IC_{50} for TQ and RES combination (Table 4).

CI along with results interpretations were calculated afterwards [1]. The combination treatment exhibited synergism in all cell lines, with the lowest value of combination index seen in MCF-7 cell line (Table 4).

3.2. Antitumor effects

In order to test the antitumor effect of TQ and RES alone or in combination, BALB/C mice were transplanted with EMT6/P cells and the antitumor effect was evaluated for TQ, RES, and their combination. Treatments started 14 d after tumor inoculation and lasted for 14 d with daily administration of different treatments. Results revealed an increase in tumor size of (435.32 ± 41.30)% in the group treated with 50 mg/kg/d TQ. This change in tumor size was significantly ($P < 0.05$) lower than the change observed in the negative control group (813.62 ± 30.80)% (Table 5). Better results were observed in the group treated using 50 mg/kg/d RES with the increase in tumor size of

Table 3
Antiproliferative activity for combination of RES and TQ with different concentrations against EMT6/P cell line.

Combination of RES and TQ (μM)		% survival
RES 15	TQ 25	52.80 ± 10.31
	TQ 50	51.20 ± 10.27
	TQ 75	48.80 ± 2.70
	TQ 100	46.60 ± 14.60
	TQ 125	33.20 ± 1.90
	TQ 150	30.10 ± 15.90
	TQ 175	34.70 ± 2.60
	TQ 200	33.30 ± 0.99
	TQ 250	28.10 ± 2.31
	TQ 300	29.20 ± 8.60
TQ 140	RES 10	109.70 ± 4.70
	RES 20	64.80 ± 30.10
	RES 30	50.10 ± 20.90
	RES 40	48.30 ± 8.70
	RES 50	45.10 ± 4.70
	RES 60	54.20 ± 2.31
	RES 70	62.90 ± 20.10

Data were expressed as the mean ± SEM.

Table 1
Antiproliferative activity of increasing concentrations of TQ (μM) against three different breast cancer cell lines and Vero cell line.

TQ concentration	% viability			
	EMT/6	T47D	Vero	MCF-7
0	90.01 ± 7.09	73.20 ± 4.50	87.50 ± 3.90	78.50 ± 3.40
25	89.40 ± 24.30	82.20 ± 3.20	79.72 ± 1.91	63.70 ± 30.91
50	92.31 ± 2.41	70.00 ± 3.30	39.80 ± 0.90	47.30 ± 1.20
100	103.20 ± 1.10	36.60 ± 1.10	8.61 ± 0.72	11.30 ± 1.60
150	108.20 ± 5.20	38.30 ± 1.31	8.70 ± 0.81	10.10 ± 0.09
200	81.30 ± 0.09	24.40 ± 1.20	7.82 ± 1.87	11.01 ± 0.09
250	90.10 ± 20.10	22.30 ± 1.20	7.82 ± 0.90	11.00 ± 0.99
300	82.40 ± 10.30	24.80 ± 0.99	7.91 ± 0.89	9.99 ± 2.93
350	69.80 ± 14.50	25.20 ± 0.80	8.21 ± 0.90	12.30 ± 3.21
400	44.30 ± 7.10	24.70 ± 2.30	8.12 ± 1.60	15.30 ± 6.10

Data were expressed as the mean ± SEM.

Table 2
Antiproliferative activity of increasing concentrations of RES (μM) against three different breast cancer cell lines and Vero cell line.

RES concentration	% viability			
	EMT/6	T47D	Vero	MCF-7
0	39.70 ± 24.50	110.40 ± 10.90	98.81 ± 2.30	79.90 ± 1.10
25	30.90 ± 1.90	82.30 ± 2.60	99.61 ± 11.10	72.50 ± 9.51
50	27.60 ± 7.30	88.20 ± 1.10	93.70 ± 3.50	68.20 ± 3.45
150	29.90 ± 2.30	78.70 ± 3.10	79.30 ± 1.10	57.60 ± 1.40
200	29.20 ± 2.40	60.10 ± 5.20	50.20 ± 2.40	41.40 ± 1.30
250	22.51 ± 2.20	41.60 ± 10.31	19.90 ± 4.20	34.50 ± 11.60
300	24.80 ± 3.30	37.90 ± 1.90	23.80 ± 2.20	27.70 ± 1.81
350	27.30 ± 3.10	33.10 ± 2.20	24.20 ± 4.10	27.70 ± 2.80
400	24.20 ± 5.30	38.70 ± 0.07	27.10 ± 7.64	28.20 ± 0.09
450	22.80 ± 5.71	44.20 ± 10.41	26.30 ± 1.90	30.10 ± 0.07

Data were expressed as the mean ± SEM.

Table 4

IC_{50} (μM) values along with CI and interpretation for the different treatment groups against study cell lines.

Cell line	IC_{50}					CI	Interpretation
	Vincristine sulfate	TQ alone	RES alone	TQ in combination	RES in combination		
EMT6/P	45.00 ± 2.23	393.00 ± 4.33	56.00 ± 1.07	140.00 ± 2.99*	15.00 ± 0.17*	0.719 4 ± 0.0900	Moderate synergism
MCF-7	14.00 ± 0.87	55.00 ± 2.32	117.00 ± 4.09	16.00 ± 0.33*	33.00 ± 1.09*	0.654 9 ± 0.0500	Moderate synergism
T47D	16.00 ± 1.01	85.00 ± 2.90	185.00 ± 3.88	29.00 ± 0.65*	58.50 ± 3.15*	0.765 1 ± 0.1200	Moderate synergism
Vero-normal cells	47.00 ± 1.97	45.00 ± 5.96	151.00 ± 3.21	14.50 ± 2.11*	49.00 ± 1.04*	0.751 2 ± 0.0600	Moderate synergism

Interpretation of results: CI > 1.3 antagonism; CI 1.1–1.3 moderate antagonism; CI 0.9–1.1 additive effect; CI 0.8–0.9 slight synergism; CI 0.6–0.8 moderate synergism; CI 0.4–0.6 synergism; CI 0.2–0.4 strong synergism. Data were expressed as the mean ± SEM. Compared with single treatment values, $^{*}P < 0.05$. For statistical difference measurement values of TQ in combination treatment were compared with values of TQ alone and values of RES in combination treatment were compared with values of RES alone.

Table 5

Effect of different treatment groups (n = 10) on tumor size and mortality.

Group	Initial tumor size (mm^3)	Final tumor size (mm^3)	% increase in tumor size	% of cured mice	% death
TQ	291.60 ± 93.90	1561.00 ± 63.50	435.32 ± 41.30*	40	0
RES	240.86 ± 53.00	583.86 ± 18.70	142.40 ± 25.60*	60	0
Combination of TQ and RES	187.70 ± 20.50	188.40 ± 28.20	0.37 ± 0.05*#€	60	0
Negative control	214.86 ± 44.90	1963.00 ± 37.20	813.62 ± 30.80	0	10

Data were expressed as the mean ± SEM. Compared with the negative control group, $^{*}P < 0.05$; compared with single TQ treatment group, $^{\#}P < 0.05$; compared with single RES treatment group $^{€}P < 0.05$. Mice were considered cured if they had undetectable tumors in the inoculation site after 14 d treatment. Death percentage was calculated by counting the number of dead animals in each group during 14 d treatment time.

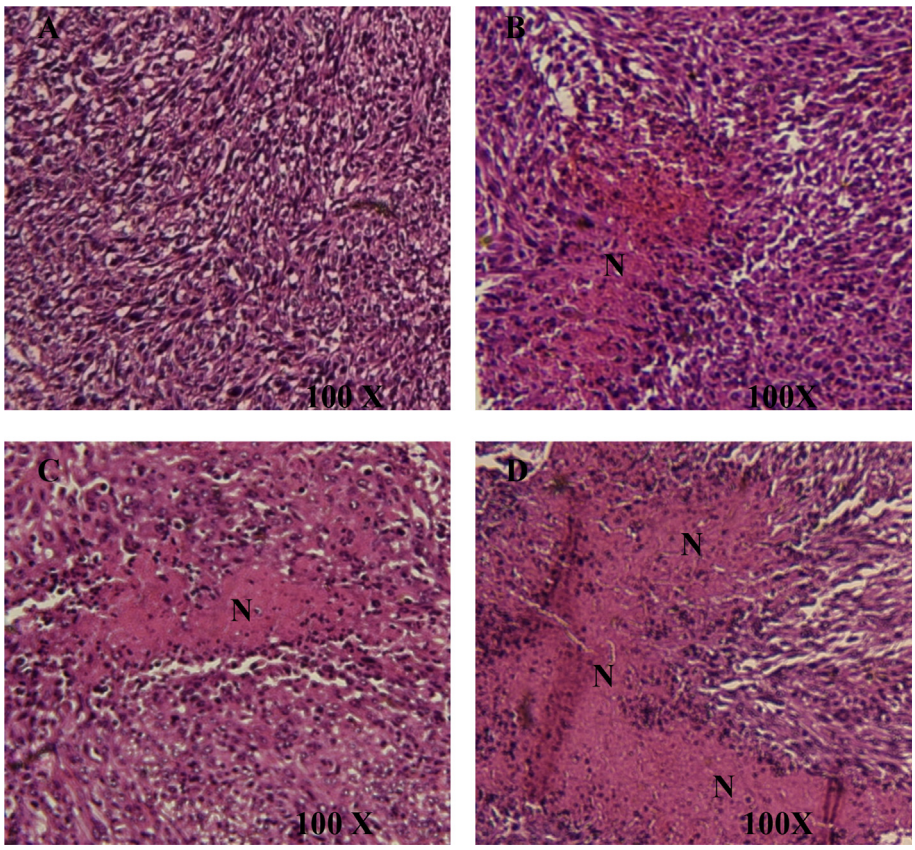

Figure 1. H&E of tumors treated with vehicle (A), 50 mg/kg/d of TQ (B), 50 mg/kg/d of RES (C), and a combination of 50 mg/kg/d of each TQ and RES (D).
N: Necrotic area. Four mice were used in each treatment.

(142.40 ± 25.60)%. However, the strongest antitumor response was reported for the combination therapy group which caused a increase in tumor size of (0.37 ± 0.05)%. This change was significantly lower than the change observed in the negative control group (Table 5). TQ treatment caused 40% cure in the treated mice and 60% cure was reported for RES and combination therapy. All mice exhibited normal behavior throughout the study, and death occurred only in the control group (10%).

3.3. Effects of treatments on tumors histology

It was observed that tumors treated with either TQ or RES had more necrotic areas compared to control group where no necrotic areas were detected. It was difficult to clearly judge which treatment elicited more necrosis, but the most dramatic and obvious effects were seen in the combination of TQ and RES where extensive and more frequent necrosis was seen compared to the other treatments (Figure 1).

3.4. Apoptosis induction ability

Both TQ and RES induced apoptosis and DNA fragmentation in treated mice compared to untreated tumor bearing mice, where no apoptotic cells were recognized. A higher percentage of apoptotic cells were observed in the mice group treated with TQ. The highest degree of apoptosis was detected in tumor sections treated with the combination of TQ and RES (Figure 2).

3.5. Effect on VEGF expression

VEGF was highly expressed in the negative control group that received only tissue culture media. Significant reduction in VEGF expression was observed after treatment with TQ and RES single treatments compared with the negative control (untreated tumor bearing mice) ($P < 0.05$) (Table 6).

The group treated with the combination therapy showed the significantly higher reduction in VEGF expression compared with TQ and RES single treatments group but still showed higher VEGF expression than the normal control group (Table 6).

3.6. Effects on serum levels of TH1 and TH2 cytokines

To have better understanding of the effect of each treatment on the immune system, serum levels of IFN-γ and IL-4 were measured. TQ, RES and the combination therapy caused an

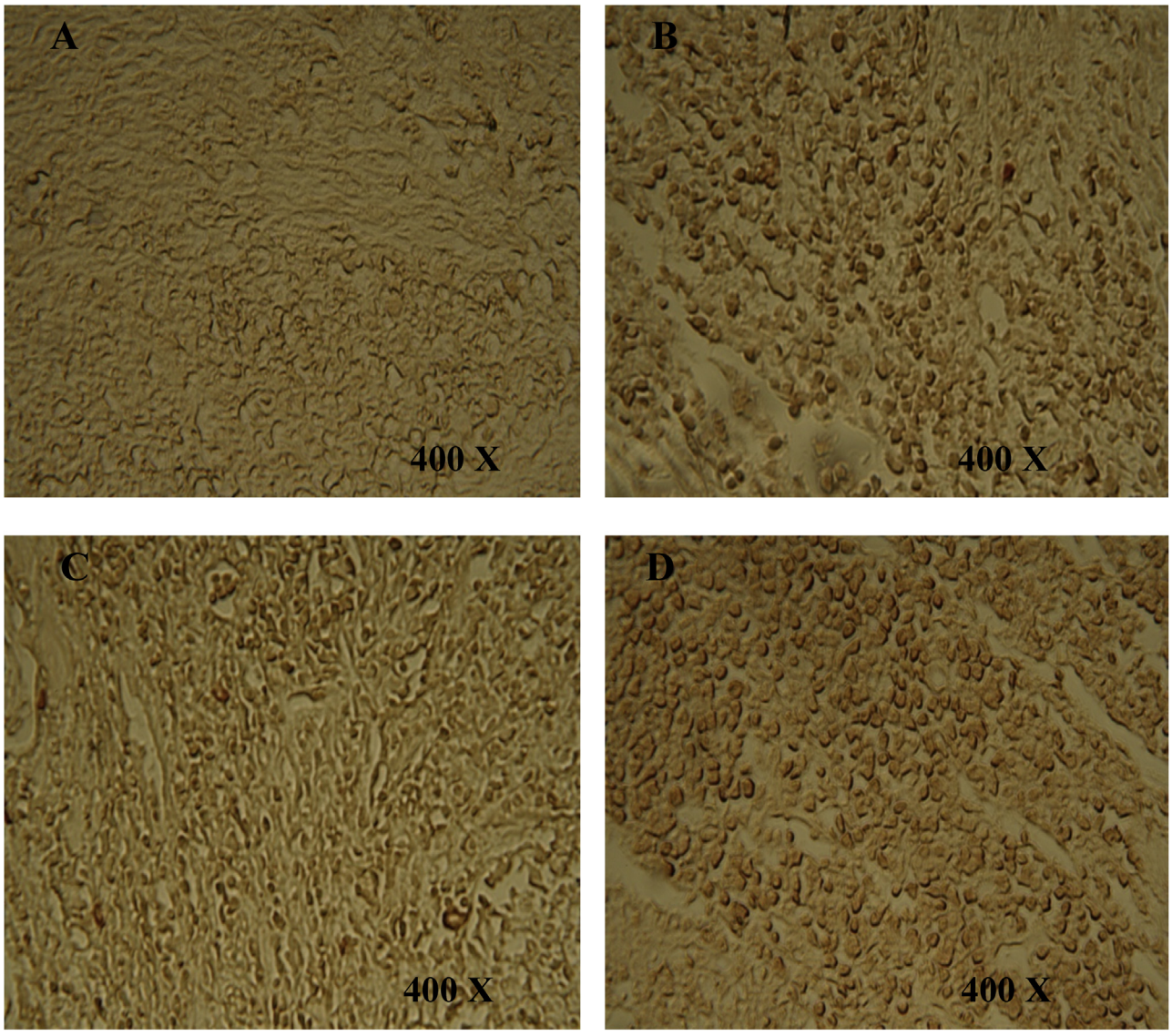

Figure 2. Cell apoptosis of tumor sections assayed by DeadEnd colorimetric TUNEL system.
Tumors were treated with vehicle (A), tumors treated with 50 mg/kg/d TQ (B), tumors treated with 50 mg/kg/d RES (C), tumors treated with a combination of TQ and RES 50 mg/kg/d of each (D). Brown stained nuclei indicate DNA fragmentation and nuclear condensation. Four mice were used in each treatment.

Table 6
Serum levels of IFN-γ, IL-4, and VEGF (pg/mL), creatinine (μmol/L), ALT and AST (IU/L) for the different treatment groups.

Group	VEGF	IFN-γ	IL-4	Creatinine	ALT	AST
TQ	600.00 ± 2.31*	69.600 ± 0.065*	98.100 0 ± 0.014 0*	14.82 ± 0.24*	21.10 ± 1.82*	124.65 ± 3.77*
RES	755.00 ± 3.53*	73.300 ± 0.069*	107.400 0 ± 0.002 0*	36.23 ± 0.45*	23.33 ± 2.83*	120.21 ± 9.49*
Combination of TQ and RES	295.00 ± 2.29*# €	82.800 ± 0.075*# €	51.300 0 ± 0.000 2*# €	26.52 ± 0.13*# €	18.33 ± 0.55*	91.65 ± 2.05*# €
Negative control	112 6.00 ± 5.86	46.900 ± 0.011	42.600 0 ± 0.008 0	24.27 ± 0.56	15.99 ± 2.21	99.10 ± 2.79
Normal control	255.00 ± 1.56	59.300 ± 0.026*	33.900 0 ± 0.003 0*	25.33 ± 0.41	17.36 ± 0.58	62.77 ± 1.67*

Compared with the negative control group, $^{*}P < 0.05$; compared with single TQ treatment group, $^{\#}P < 0.05$; compared with single RES treatment group, $^{€}P < 0.05$.

significant increase in IFN-γ production compared with the negative control (untreated tumor bearing mice) and normal control groups (untreated tumor free mice) ($P < 0.05$, Table 6). On the other hand, serum levels of IL-4 associated with the use of each treatment was highest in the group of mice treated with RES (Table 6).

3.7. Effects on serum levels of AST, ALT, creatinine

An increase in the levels of AST and ALT for all tumor bearing mice groups compared to normal mice was noticed. However, the elevation in ALT levels was very limited in all groups. Single treatment of either TQ or RES resulted in slight increase in the levels of both liver enzymes compared to normal mice as well as untreated tumor bearing mice. The combination therapy caused significant reduction in the levels of both liver enzymes compared to single treatment groups and the untreated tumor bearing mice group (Table 6).

The highest level of creatinine was observed in the group of mice treated with RES, while the lowest level was detected in the group of mice treated with TQ. Combination therapy resulted in creatinine levels below the level associated with RES (Table 6).

4. Discussion

Our study might be the first to investigate the anticancer activity of plant derived-natural product combination consisting of TQ and RES. The combination was evaluated both *in-vitro* against different breast cancer cell lines (EMT6/P, MCF-7, and T47D) and *in-vivo* against EMT6/P induced breast cancer in female Balb/C mice. The combination of TQ and RES showed high ability to inhibit cell growth *in-vitro* as well as to control the progression and growth of breast cancer *in-vivo*. Hence, in this attempt to augment the anticancer activity of TQ and RES, promising results indicating synergistic anticancer activity without noteworthy toxicity have been shown.

The effectiveness of TQ and RES as single agents in breast cancer prevention and treatment have been shown previously [13]. Looking at TQ, it was able to inhibit cell growth of the subjected breast cancer cell lines (EMT6/P, MCF-7, and T47D) in a dose dependent response; which is consistent with the previous studies that showed similar behavior of human breast adeno carcinoma upon exposure to TQ [14]. However, EMT6/P was more resistant to TQ treatment compared with other cell lines. Such resistance can be explained by the ability of this cell line to produce large quantities of factors that promote cell progression, transformation, and tumorigenesis. These factors include type I insulin-like growth factor receptor (IGF-IR), tissue-type plasminogen activator (tPA), and urokinase-typeplasminogen activator (uPA) [15]. Additionally, Transforming growth factor-beta (TGF-beta) was also detected as one EMT6/P products [16]. Mice treated with TQ showed a delay of tumor growth approximately of 47% compared to untreated tumor bearing mice, which is consistent with previous work highlighting the ability of TQ to stop tumor growth of breast, liver, prostate and colorectal cancer [17]. The rest of the results complemented the effectiveness of TQ, shown in the relatively low change in tumor size, zero death and high percentage of cured mice. TQ showed good ability to induce necrosis in tumors implanted in mice; this result is in agreement with other reports that documented the potential of TQ to induce necrosis in hepatocellular carcinoma (HepG2) cell line [18]. TQ was able to inhibit VEGF expression in comparison with untreated mice, which agrees with the previous findings revealing the ability of TQ to inhibit VEGF expression in a rat model of DMH-induced colon carcinogenesis [19]. TQ was also able to induce apoptosis in tumor bearing mice, being consistent with previous reports that documented the ability of TQ to induce apoptosis in breast cancer tumors [17]. Changes in the immune system due to the exposure for TQ were also evident, showing increased production of IFN-γ which is a signature cytokine in TH1 immune response that has an antitumor effect. Keyhanmanesh *et al* previously unveiled the ability of TQ to elicit stimulation in the production of IFN-γ in ovalbumin-sensitized guinea pigs [20]. As for the levels of IL-4, they were higher than the levels observed in untreated tumor bearing mice as well as normal mice. These results are in contrast with the previous findings where the treatment of guinea pigs model of asthma with TQ caused reduction in the levels of IL-4 compared to negative control [21].

N. sativa is the main source of dietary TQ. The volatile oil of this plant represent 0.40%–0.45%. This volatile oil fraction is consisted of nigellone, TQ, thymohydroquinone, dithymoquinone, thymol, carvacrol, α and β-pinene, D-limonene, d-citronellol, and *p*-cymen in addition to other components [22]. In our study, an effective anticancer effect was achieved by using 50 mg/kg TQ. Such high concentration cannot be obtained by consuming *N. sativa* seeds. However, our results support using pure TQ to treat cancer which is proved by previous studies [5].

Single treatment of RES also showed significant inhibition of cell growth both *in-vitro* and *in-vivo*. This result is in agreement with the previous findings that indicated the ability of RES to stop breast cancer cell proliferation *in-vitro* [23] as well as inhibit tumor growth of breast, gastric, prostate, liver, lung and cervical cancer [24,25]. Additionally, %change in tumor size, %cured of mice and %death were also improved in comparison to untreated tumor bearing mice and mice treated with single TQ therapy. Tumors sections treated with RES showed necrotic

areas almost similar in size and frequency to that found in the tumors treated with TQ. These results confirm the previous findings regarding the ability of RES to induce necrosis as one of the mechanisms responsible for cell death upon exposure to RES [26]. Induction of apoptosis and inhibition of VEGF expression were also observed in tumors treated with single RES therapy.

Previous studies showed that the diverse health benefits of RES are dose dependent. This natural product has a biphasic anticancer effect. An increase in cell proliferation was observed in treatments using low doses of RES and apoptosis induction was reported after treatment of different cancer cell lines with high doses of RES [27]. On the other hand, a very high dose (3 000 mg/kg) of RES was toxic and caused nephrotoxicity [28]. In our study a high concentration of RES (50 mg/kg) was selected to avoid cancer promoting effects of low doses and to secure efficient anticancer effect without toxicity. In another study the therapeutic concentration of RES reached 625 mg/kg to treat prostate cancer in mice [29]. Such therapeutic concentrations cannot be achieved by using grape seeds as a source of RES and results of our study are applicable only on pure RES that can be used to reach high therapeutic doses to treat established tumors.

Significant breast anticancer activity results have been shown for the first time in this study due to the combination of TQ and RES as a plant derived natural products based combination. This combination worked synergistically both *in-vitro* and *in-vivo*. These results were expected considering previous reports on the synergistic activity of both combination of TQ and diosgenin against squamous cell carcinoma, and combination of RES and curcumin on colorectal cancer [30,31]. Percentage change in tumor size, percentage cure of mice and percentage death were highly improved in the mice group that received the combination of TQ and RES compared to all other mice groups. Despite the same percentage of cured mice in both combination group and single RES group, the lowest percentage change in tumor size was seen in the combination group. Degree and frequency of necrosis were highest in the combination group compared to the single treatment groups. In part, this could suggest a strong ability of this novel combination to induce necrosis in tumors implanted in mice. Also, inhibition of VEGF expression was most obvious in the combination group. Cancer proliferation depends on high levels of VEGF to stimulate angiogenesis and secure continuous blood supply. The geographical necrosis observed in tumors treated with combination therapy is mainly due to high degree of VEGF inhibition. Such inhibition reduces blood vessel formation and cause starvation of cancer cells leading to more necrosis.

Induction of apoptosis was more pronounce in the combination group compared to the single treatment groups, suggesting the existence of real synergism between TQ and RES. Previous studies showed the ability of TQ to induce apoptosis by up-regulation of pro-apoptotic genes (*Bax*, *caspase 9*, and *p53*) and down-regulation of antiapoptotic genes (*Bcl-2* and *Bcl-xL*) [32]. Apoptosis induction by RES is mediated by activation and nuclear translocation of mitogen-activated protein kinase (ERK1/2) followed by p53 phosphorylation and apoptosis [33]. In our study, it seems that the high apoptosis degree observed in combination therapy is a result of integrative apoptosis induction mechanisms of TQ and RES. Changes in immune system toward the combination therapy were also explored. The highest level of IFN-γ among groups was seen in the combination group; this strengthens the possibility of existence of synergism between TQ and RES in stimulating the immune system. IFN-γ is the signature cytokines of Th-1 anticancer immune response while IL-4 is dominating Th-2 immune response. Healthy individuals have balance Th-1/Th-2 cytokines ratio. On the other hand, cancer patients are characterized by low Th-1/Th-2 cytokines ratio. Our results clearly indicate the potential of our combination to shift the immune response toward Th-1 response [34].Such results are consistent with previous findings reporting the immunomodulatory effect of TQ by inhibiting Th-2 response and enhancing Th-1 anticancer immune response [35]. Also RES enhance anticancer immune response by stimulating lymphocyte proliferation, NK cell cytotoxic activity and inhibition of immune suppressive Treg [36]. Combination of TQ and RES may activate various anticancer immune mechanisms to cause significant antitumor immune response.

In our study TQ and RES were injected intraperitoneally and caused significant reduction in tumor size. Such results cannot be obtained if both agents were given orally. Therapeutic effects of orally consumed natural products are dependent on the ability of gastrointestinal tract to absorb such agents [37]. Most widely consumed polyphenols have little bioavailability which reduce or eliminate their therapeutic effects [38]. TQ and RES have low toxicity and can be consumed in good quantities. However, further testing using TQ and RES given orally to tumor bearing mice is essential to evaluate their anticancer effects if consumed with food.

In conclusion, a combination of TQ and RES has the potential to work synergistically to inhibit cell growth in breast cancer cell lines, as well as to stop the progression of breast cancer inoculated in mice. Antitumor activity of TQ was shown to be mediated through different modes of actions, including induction of apoptosis, inhibition of VEGF expression and stimulation of immune system. RES showed ability to augment the anticancer activity of TQ through overlapping with the same pathways triggered by TQ. This effective plant based natural combination is widely available and relatively uncostly, hence deserves further investigations to be considered as a possible therapeutic option for breast cancer.

Author contributions

Wamidh H. Talib and Iman A. Basheti conceived and designed the experiments; Omar H. Alobaedi conducted the experiments, Iman A. Basheti and Omar H. Alobaedi performed the statistical analysis, Wamidh H. Talib wrote the paper.

Conflicts of interest statement

The authors declare no conflict of interest.

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