Journal of Medical & Pharmaceutical Sciences
Volume (6), Issue (4) : 30 Sep 2022
P: 25 - 41

AJSRP
ISSN: 2522-333X

مجلة العلوم الطبية والصيدلانية
المجلد (6)، العدد (4) : 30 سبتمبر 2022 م
ص: 25 - 41

Synergistic Antitumor Effect of Thymoquinone Combined with Resveratrol against Human Lung Cancer Cells Line

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Abstract: Introduction: Thymoquinone (TQ) and resveratrol are natural anticancer and chemo-preventive agents through mechanism(s) that are not fully understood. This study aimed to test the anticancer potential activity of the combination of TQ and Resveratrol, and their underlying mechanisms in the human lung cancer cell line, A549 cells. Materials and methods: The A549 cells were treated with the different concentrations (25, 50, 75, 100, or 200 μM) of TQ, resveratrol, and their combination for 24h. The antiproliferative activity against A549 cells was assessed using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Trypan blue exclusion assay. Apoptosis markers were assessed by using a real-time reverse transcriptase PCR Assay (rRT-PCR). Results : We found that the proliferation of A549 lung cancer cells was inhibited by TQ, and resveratrol and their combination in a concentration-dependent manner, during a defined period. The combination therapy also could inhibit the growth of A549 cells at 25 μM with a 50% inhibitory concentration (IC_{50}) value of 37 ± 3.18μM. These combinations were significantly ($P < 0.001$) elevated caspase-3, caspase-8, caspase-9, p53, p21, Fas, FasL, and Bax mRNA levels in treating A549 cells compared to the untreated cells. The anticancer effect of this combination is mediated by the activation of the extrinsic and the intrinsic pathways. Conclusion: all these results give a new promising combination with the enhanced anticancer effect.

Keywords: Thymoquinone, resveratrol, A549 cells, apoptosis.

التأثير التآزري المضاد للأورام للثيموكينون مع الريسفيراترول ضد خط خلايا سرطان الرئة البشرية

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المستخلص: المقدمة: يعتبر الثيموكينون (TQ) والريسفيراترول من المستخلصات الطبيعية الواقية والمضادة للسرطان من خلال آليات غير مفهومة تمامًا. لذا: هدفت هذه الدراسة إلى اختبار النشاط المحتمل المضاد للسرطان لمزيج من TQ وResveratrol، وآلياتهما الكامنة ضد خلايا سرطان الرئة البشرية، خلايا A549. المواد والطرق: عولجت خلايا A549 بتركيزات مختلفة (25، 50، 75، 100، أو 200 ميكرومتر) من TQ، ريسفيراترول، والعلاج المركب من TQ والريسفيراترول لمدة 24 ساعة. تم تقييم النشاط المضاد للنمو ضد خلايا A549 باستخدام (4،2- 5-dimethylthiazol-2-yl)،5-diphenyltetrazolium bromide (MTT) وطريقة استبعاد التريبان الأزرق. تم تقييم علامات موت الخلايا المبرمج باستخدام فحص PCR للنسخ العكسي في الوقت اللحظي (rRT-PCR). النتائج: وجدنا أن نمو خلايا سرطان الرئة A549 تم تثبيطه بواسطة TQ والريسفيراترول وتركيبهما بطريقة تعتمد على التركيز، خلال فترة محددة. يمكن أن يمنع والعلاج المركب من TQ والريسفيراترول أيضًا نمو خلايا A549 عند 25 ميكرومتر بتركيز مثبط بنسبة 50 ٪ (IC_{50}) بقيمة 37 ± 3.18 ميكرومتر. كانت هذه التوليفات مرتفعة بشكل ملحوظ (P <0.001) في مستويات caspase-3, caspase-8, caspase-9 وp53 وp21 وFas وFasL

وBax وmRNA في علاج خلايا A549 مقارنة بالخلايا غير المعالجة. يُعزى التأثير المضاد للسرطان لهذا المزيج من خلال تنشيط المسارات الموت المبرج للخلايا الخارجية والداخلية. الخلاصة: كل هذه النتائج تعطي توليفة واعدة جديدة لعلاج السرطان.

الكلمات المفتاحية: ثيموكينون، ريسفيراترول، السرطان، الرئة البشرية، موت الخلايا المبرمج.

Introduction.

Cancer is one of the causes of most common causes of death worldwide, accounting for nearly 10 million deaths in 2020 (Sung et al. 2021). Lung cancer is one of the most common and serious types of cancer by far the leading cause of cancer death among both men and women, making up almost 25% of all cancer deaths (Vassallo 2020). Natural product compounds made a major contribution to pharmacotherapy, especially for cancer. Since ancient times, natural products have been used to prevent several chronic diseases, including cancer (Hsieh et al. 2015, Bishayee and Sethi 2016). Notably, several bioactive compounds obtained from dietary or medicinal plant sources have provided an alternative source of bioactive compounds that can be used as preventive or therapeutic agents against a variety of diseases (Shanmugam et al. 2017), actually, up to 200 species are considered as medicinal plants and about 25% of the medicines have plants origins (Gurnani et al. 2014, Shanmugam et al. 2017).

Nigella sativa L. (botanical family, Ranunculaceae) contains more than 30% of fixed oil and 0.4-0.45 % wt/wt of volatile oil. The volatile oil contains 18.4-24% thymoquinone (TQ) and 46% many monoterpenes such as p-cymene and α-piene (El-Agamy and Nader 2012, Kara et al. 2012). Multiple reports have documented the inhibitory role of TQ in a variety of cells lines, including A549 cells (Samarghandian et al. 2019), breast cancer (MCF-7) (Effenberger et al. 2010), Vero, hamster kidney carcinoma (BSR), HeLa (Padhye et al. 2008), and HepG2 cells (Thabrew et al. 2005). However, TQ shows less toxicity toward normal cells (Worthen et al. 1998). These unique properties suggest that TQ may be an effective agent in cancer treatment (Almajali et al. 2021). TQ has been shown to inhibit the proliferation of many types of cancer cells, including breast cancer cells (Kara et al. 2012). TQ also has anti-inflammatory and antioxidant activities (Khan 2018), it scavenges free radicals and prevents the cells damage resulting from oxidative substances. It has been reported that TQ prevented oxidative injury in in-vitro and in-vivo studies in rodents (Rifaioglu et al. 2013, Suguna et al. 2013).

Resveratrol, chemically known as 3,5,4′-trihydroxy-trans-stilbene, is found in over 70 plants including grape skins, peanuts, and red wine (Aluyen et al. 2012). Resveratrol is a natural polyphenol with extensive biological functions such as an antioxidant, promoting nitric oxide production, can also reduce platelet aggregation, and preventing and treating atherosclerosis, and cardiovascular diseases, (Ulrich et al. 2005, Chun-Fu et al. 2013). Furthermore, several studies have shown that resveratrol is a chemo-protective against the development of cancers of the breast, liver, prostate, mammary, colon, and lung cancers (Revel et al. 2003, Stewart et al. 2003, Aggarwal et al. 2004, Le Corre et al. 2005, Fukui et al. 2010, Liao et al. 2010). Therefore, the present study aimed to test the anticancer potential activity of the

combination of TQ and resveratrol and their underlying mechanisms in the human lung cancer cells line, A549 cells.

Materials and Methods.

Cells and Reagents:

The A549 cells line was obtained from (ATCC: CCL-185), resveratrol, TQ, a trypsin-EDTA mixture containing 0.25% trypsin w/v and 0.02% EDTA w/v, dimethyl sulfoxide (DMSO), and MTT assay were purchased from (Sigma-Aldrich LTD, Ayrshire, UK), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin antibiotic were purchased from (Gibco, Invitrogen, Grand Island, NY, USA), RNeasy Micro Kit and RT² SYBR® Green/ROX™ qPCR Master Mix were purchased from (Qiagen, Maryland, USA). High-Capacity cDNA Reverse Transcription Kit was purchased from (Applied Biosystems, Foster City, CA, USA). and RT² SYBR® Green/ROX™ qPCR Master Mix was purchased from (Sigma-Aldrich LTD, Ayrshire, UK). MTT solution was prepared at 0.5mg/mL in PBS and filtered through a 0.2μm filter. Trypan Blue Solution, 0.4% was purchased from (Thermo Scientific, USA). TQ and resveratrol were dissolved in DMSO and then diluted with medium to obtain the working concentration from 25–300 μM.

Cells culture and cytotoxicity assays:

Cells were cultured in DMEM with 4.5 g/L Glucose, L-glutamine, and Pyruvate was supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. The cells were sub-cultured in 25 cm^2 flasks by washing twice with phosphate-buffered saline (PBS) and trypsinization of adherent cells was done using 1.0 mL 25% trypsin with 0.53mM EDT solution, and maintained at 37°C in a 5% CO_2 humidified atmosphere with the media changed every 2–3 days.

The cytotoxicity of TQ, resveratrol, and their combination were tested against A549 cells by MTT assay. This method is based on the transformation of MTT reagent to formazan crystals by the action of mitochondrial dehydrogenases ***(Riss et al. 2016)***. Briefly, exponentially growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96-well plates 1×10^3 cells/ well (100μl) into a 96-well microplate of fresh complete medium for 24 h before treatment. Cells were treated with serial concentrations (25, 50, 75, 100, or 200 μM) of TQ, resveratrol, or predetermined same combinations of both, resulting in a total volume of 200 μL combination at 37°C in a humidified 5% CO_2 atmosphere for 24 h. For combination treatments, the concentration of both agents was combined with the same concentration of each agent. A combination of 25 μM of TQ was prepared and combined with 25 μM of resveratrol. The same procedure was used to prepare combinations for other concentrations. After intended incubation times, 10 μl of a working solution of MTT (5 mg/ml in PBS) was added to the wells,

and incubation continued for 4 h at 37°C. Then, (100 μL/well) DMSO was added to dissolve the produced formazan particles and incubated with gentle shaking for 10 min at 37°C. Finally, the absorbance of each well was read at 590 nm, using an automated microplate reader ELX-808 (Biotek, USA). Cells viability (%) was calculated as [(A-B)/A] x 100, where A and B are the absorbance of untreated control cells, and absorbance of wells with treated cells, respectively ***(Rafieian-Kopaei et al. 2014)***. All experiments were done in triplicate. The data were expressed as the mean value of three independent experiments. The Graph Pad Prism software was applied for determining The half-maximal inhibitory concentration (IC_{50}).

Cells viability assay:

The viability of the cells was assessed using the Trypan blue exclusion assay, which depended on the interaction of Trypan blue dye with the membrane-damaged cells. Briefly, cells were seeded at 1×10^3 cells/well of A549 in 96-well plates in the presence of DMEM (supplemented with 10% FBS and 1 % penicillin/streptomycin) in 5% CO2 at 37 °C until 70-90% confluence. Cells were treated with 1×10^3 cells/well (100μl) in 96-well plates and incubated at 37°C with 5% CO_2, and 95% air until cells adhered to the plate surface overnight. Cells were treated with TQ, resveratrol, and their combination at concentrations ranging (25, 50, 75, 100, or 200 μM) in experimental cells culture media and were further incubated for 24h at 37°C with 5% CO_2, 95% air. The treated A549 cells were trypsinized and centrifuged at 300 × g for 5 min and re-suspended in 200 μl of PBS. Cells were diluted to 1:1 (100μl cells: 100μl Trypan blue dye) staining solution, after 1 minute at room temperature, the cells were loaded into a hemocytometer, and cells that exhibited dye uptake were counted under an inverted light microscope. Cells viability (%) was counted and calculated [Cells viability (%) = total viable cells (unstained)/total cells (stained and unstained) ×100].

Apoptosis coding genes assay

The expression of apoptosis coding genes and their combinations were determined using rRT-PCR. The number of 2×10^5 A549 cells was harvested into six-well plates containing 3 ml culture medium admixed with different concentrations of TQ, resveratrol, and their combination. After a 24-h incubation at 37°C, the wells were washed with PBS and trypsinized (using 0.02% trypsin prepared in EDTA). After centrifugation at 500*g* for 5 min at 4°C, the upper liquid phase was discarded and, then, the pellet was applied rRT-PCR reaction. Total RNA was extracted from the A549 cells using a RNeasy Micro Kit according to the manufacturer's instructions. cDNA synthesis and genomic DNA elimination were achieved using a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. Archived cDNA libraries were then subjected to rRT-PCR using RT² SYBR® Green/ROX™ qPCR Master Mix. Primer sequences were derived from ***(Honarpisheh et al. 2016, Jiang et al. 2017, Alotaibi et al. 2018, Buskaran et al. 2021)*** as follows: Caspase-3 forward primer 5′- GCTGGATGCCGTCTAGAGTC-3′ and

reverse primer 5′- ATGTGTGGATGATGCTGCCA-3′; Caspase-8 forward 5′-AGAAGAGGGTCATCCTGGGAGA-3′ and reverse primer 5′- TCAGGACTTCCTTCAAGGCTGC-3′; Caspase-9 forward primer 5′- ATTGCACAGCACGTTCACAC-3′ and reverse primer 5′-TATCCCATCCCAGGAAGGCA -3′; Bax forward primer 5′-GAGCTAGGGTCAGAGGGTCA-3′and reverse primer 5′-CCCCGATTCATCTACCCTGC-3′; Bcl-2 forward primer 5′- ACCTACCCAGCCTCCGTTAT-3′ and reverse primer 5′-GAACTGGGGGAGGATTGTGG-3′; Bcl-xL forward primer 5′- CAGAGCTTTGAACAGGTAG-3′ and reverse primer 5′- GCTCTCGGGTGCTGTATTG-3′; p53 forward primer 5′- GCTCTGACTGTACCACCATCC-3′ and reverse primer 5′- CTCTCGGAACATCTCGAAGCG-3′; p21 forward primer 5′-CTCAGAGGAGGCGCCATG-3′ and reverse primer 5′-GGGCGGATTAGGGCTTCC-3′; Fas forward primer 5′-GAAATG AAA TCCAAAGCT-3′ and reverse primer 5′-TAATTTAGAGGCAAAGTGGC-3′; FasL forward primer 5 5′-GGATTGGGCCTGGGGATGTTTCA-3′ and reverse primer 5′-TTGTGGCTC AGG GGC AGG TTG TTG-3′; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control with forward primer 5′- CGGAGTCAACGGATTTGGTC-3′ and reverse primer 5′- AGCCTTCTCCATGGTCGTGA-3′. The real-time PCR reactions were carried out in 7500 Fast real-time PCR (7500 Fast; Applied Biosystems, USA) using the following cycling conditions: an initial denaturation phase at 95°C (3 min) followed by Thirty-five cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 2 min).

Statistical analysis:

Data were analyzed using Statics Package for Social Science (SPSS) version 22 (SPSS Inc. Chicago, IL, USA). Each experiment was done in duplicates. Analysis of results was done using repeated measure analysis. Values are expressed as mean± standard deviation (SD). The student's t-test was used to compare the differences between the two groups. Kaplan–Meier method with a log-rank test was used to estimate the overall survival rate. The results with $p < 0.05$ were considered significant.

Results.

Cytotoxic Efficacy of TQ, resveratrol, and their combination

The effect of TQ, resveratrol, and their combination were studied at various concentrations ranging from 25, 50, 75, 100, or 200 μM for 24 h using MTT assay. As depicted in (Figure 1), the results showed that there was a significant ($P < 0.001$) difference between untreated cells and treated cells at various concentrations. After 25–75 μM of TQ, resveratrol treatment, they could not inhibit the growth of A549 cells, Further concentration of TQ or resveratrol to 75 μM exerted gradient cytotoxic activity with increasing concentration; viability started to drop significantly ($P < 0.001$) at a concentration of 75 μM and

100 μM, with IC_{50} of 66±5.18μM and 63±7.18μM, respectively. However, TQ in combination with resveratrol could inhibit the growth of A549 cells at 25 μM with an IC_{50} of 37 ±3.18μM.

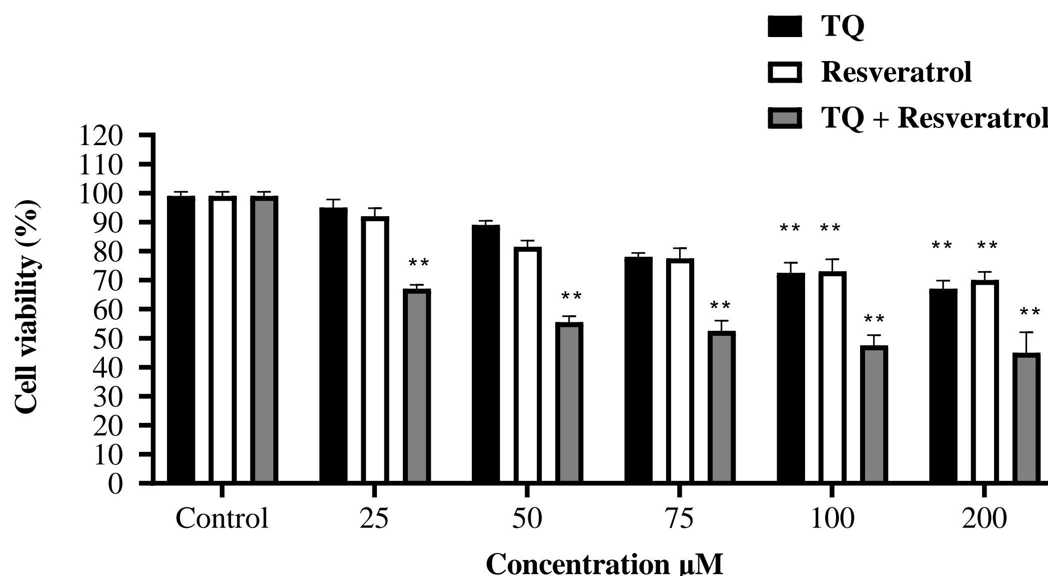

Figure (1) Cytotoxicity effect of TQ, resveratrol, and their combination on A549 cells at various concentrations ranging from 25, 50, 75, 100, or 200 μM for 24 h using MTT assay. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control (* = *P* < 0.05, ** = *P* < 0.01).

Viability Efficiency of TQ, resveratrol, and their combination.

The effect of TQ, resveratrol, and their combination on the growth and viability of A549 cells was evaluated after 24 h using Trypan blue dye exclusion test. As shown in (Figure 2), treatment with TQ or resveratrol resulted in a considerable significant decrease ($P < 0.001$) in the percentage of A549 to about 70 % and 76% respectively after 24 h treatment with 100 μM as compared with untreated cells. However, treatment with a 25 μM combination of TQ and resveratrol for 24 h was found to have 66% viable cells ($P < 0.001$). Further concentration of TQ or resveratrol or their combination to 200 μM caused a significant reduction in viable cells percentage.

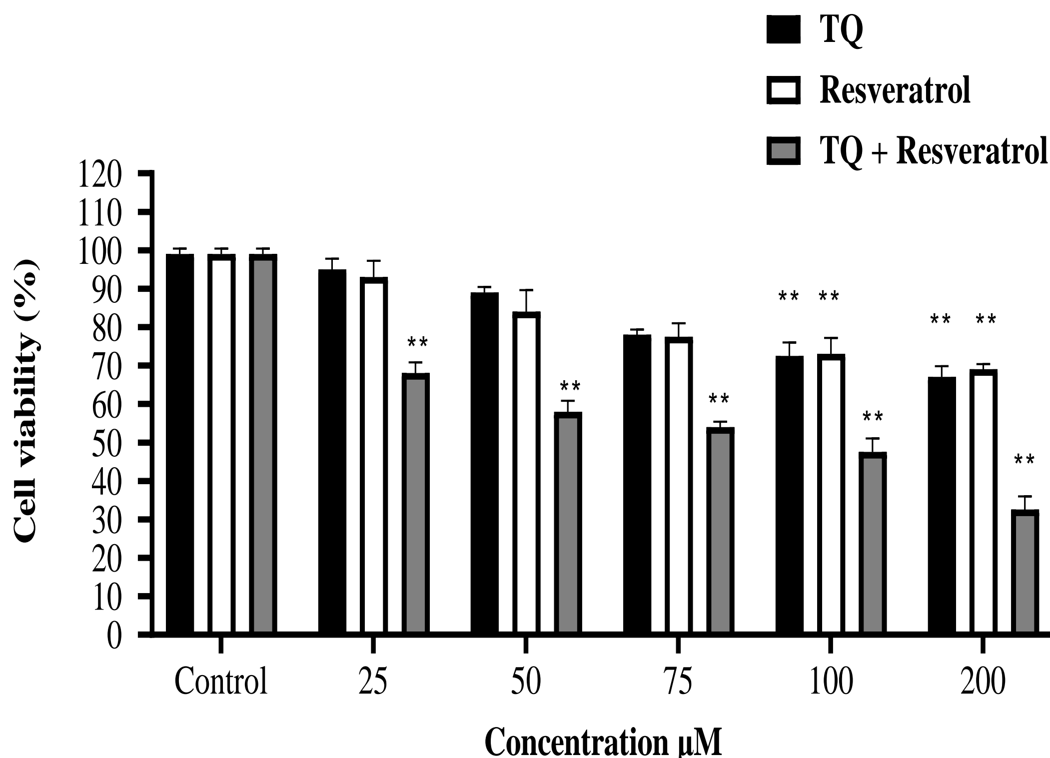

Figure (2) Percentage of cells viability assay with Trypan blue extraction assay on A549 cells tested at different concentrations of various concentrations ranging from 25, 50, 75, 100, or 200 µM of TQ, resveratrol, and their combination for 24 h. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control (* = $P < 0.05$, ** = $P < 0.01$).

Effects of TQ, resveratrol, and their combination on A549 induced apoptosis signaling

The effect of TQ, resveratrol, and their combination on A549 induced apoptosis signaling was measured after 24 h using rRT-PCR. As shown in (Figure 3), control cells showed the weakest caspase-3, caspase-8, and caspase-9 mRNA levels. There was a significant increase in caspase-3, caspase-8, and caspase-9 mRNA levels in TQ or resveratrol treated A549 cells than in the control untreated cells, whereas, TQ in combination with resveratrol treated cells showed the significantly stronger caspase-3, caspase-8, and caspase-9 expressions than control untreated cells ($P < 0.001$). As shown in (Figure 4), TQ, resveratrol, and TQ in combination with resveratrol for 24 h significantly elevated Bax mRNA and significantly reduced Bcl-2 and Bcl-xL anti-apoptotic gene in treated A549 cells compared to the control untreated cells ($P < 0.001$). As depicted in (Figure 5), TQ, resveratrol, or TQ in combination with resveratrol treated A549 cells were showing the higher mRNA of p53 and p21 levels than the control untreated cells ($P < 0.001$). There were a significantly higher p53, p21, Fas, and FasL mRNA levels in TQ, resveratrol, or TQ in combination with resveratrol treated cells compared to the control untreated cells.

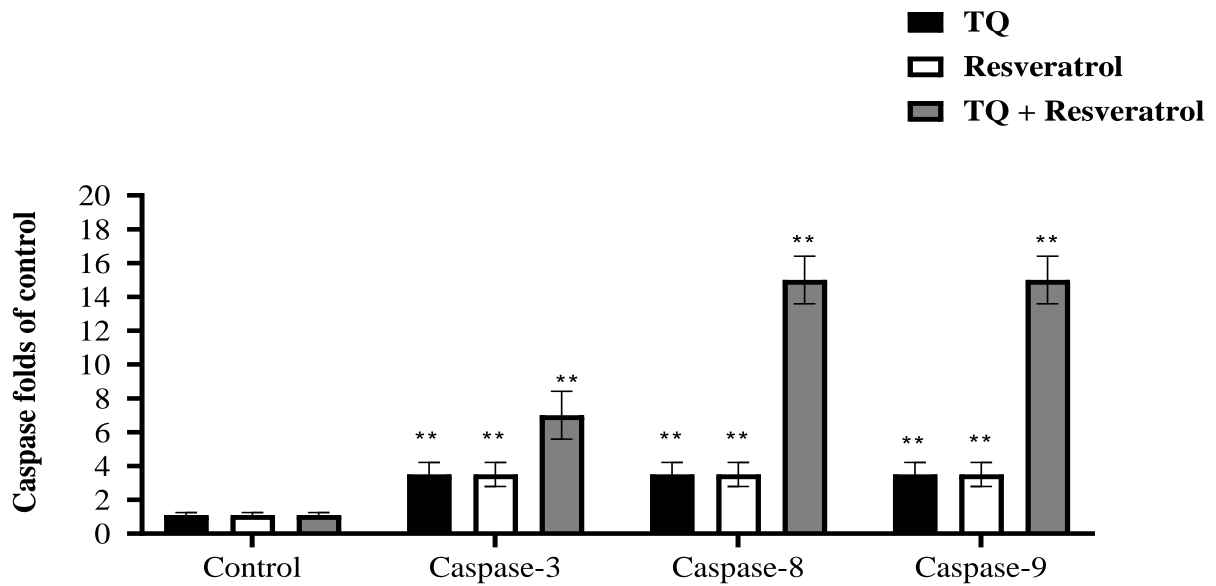

Figure (3) The mRNA of caspase-3 caspase-8 and caspase-9 on A549 cells. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control (* = P< 0.05, ** = P< 0.001).

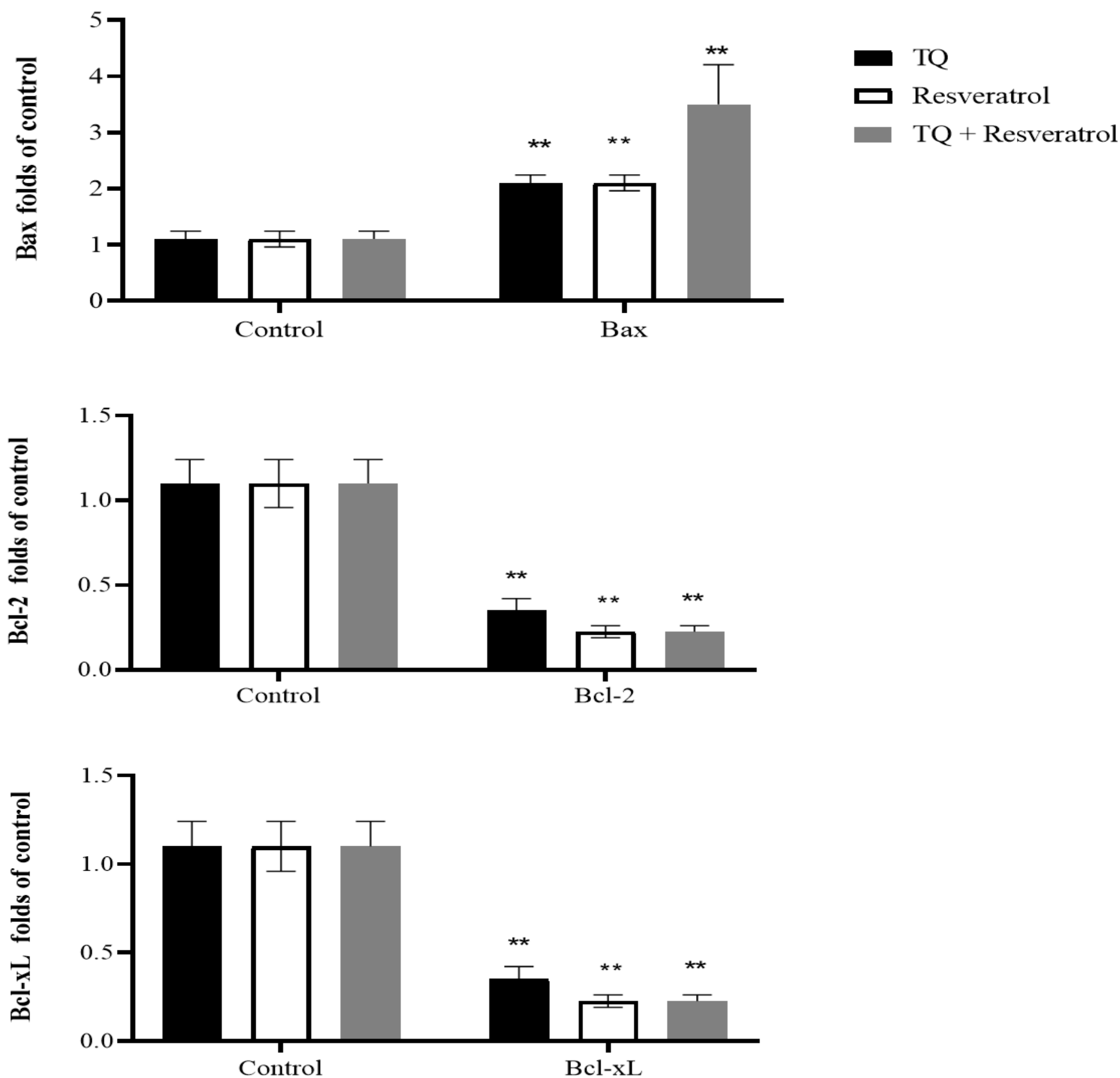

Figure (4) The mRNA of of Bax, Bcl-2 and Bcl-xL on A549 cells. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control (* = P< 0.05, ** = P< 0.001).

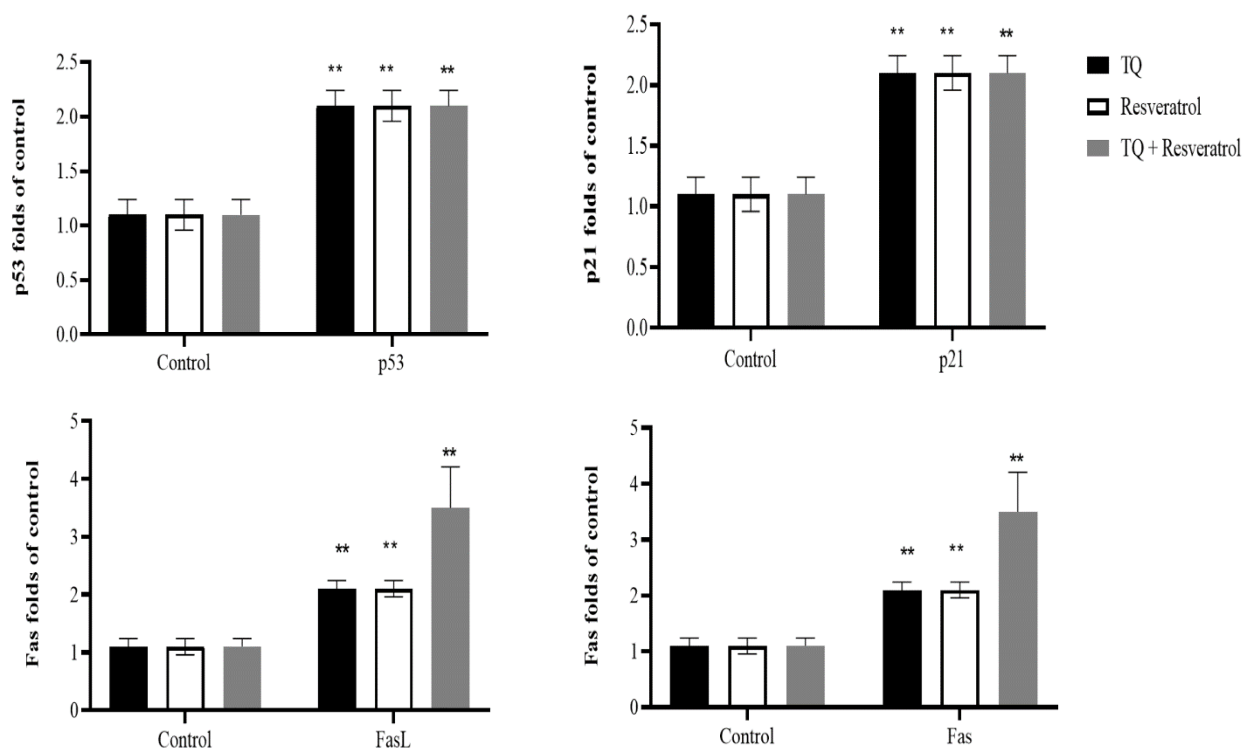

Figure (5) The mRNA of p53, p21, Fas, and FasL on A54 cells. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control (* = *P*< 0.05, ** = *P*< 0.001).

Discussion.

The limitations of conventional cancer therapies have pushed the field of anti-cancer drugs towards exploring other novel strategies to improve cancer therapy. Since the last decade, scientists exploring the anticancer effects of the natural compound as they are considered to have less toxic or no side effects have been recognized compared to current treatments such as chemotherapy ***(Roepke et al. 2007, Greenwell and Rahman 2015)***. TQ is the main biological ingredient of *Nigella sativa* (black seed oil); a traditional medicinal plant, several TQ compounds have been introduced as potential useful targeted therapeutics and cancer-preventive agents (i.e., influencing only cancerous cells rather than normal cells ***(Malik et al. 2016, Samarghandian et al. 2019)***. It has been confirmed to possess anti-cancer potentials and anti-inflammatory and antioxidant activities ***(Yi et al. 2008, Woo et al. 2012***). resveratrol, extracted from grape or polygonum is such a natural compound and was found to possess anti-cancer potentials, reduce blood viscosity, antioxidant, and inhibit platelet aggregation ***(Kennedy et al. 2010, Wightman et al. 2014)***. However, the mechanisms of how TQ and RES sensitized tumor cells to the drugs have not been fully understood.

In the present study, we investigated the inhibitory effect of TQ, resveratrol, and their combination on cells growth in A549 lung cancer cells. Our results indicated that TQ, resveratrol, and their combination dramatically reduced the viability rate of carcinomic lung cells in dose-dependent manners using MTT assay and Trypan blue dye exclusion test. The MTT has been widely used to assess cells survival, proliferation, and testing for cytotoxic effects of anticancer compounds on cells lines ***(Riss et al. 2016)***. TQ or resveratrol alone was found to be stressful enough to induce cells death observed at 100 µM concentration. Inte resveratrol ting, we observed that TQ in combination with resveratrol significantly reduced the viability of the A549 cells at 25 µM with IC_{50} of 37 ±3.18 µM. The effect of TQ and resveratrol both as a chemopreventive as well as a chemotherapeutic agent for lung cancer cells have previously been reported ***(Lee et al. 2004, Whyte et al. 2007, Samarghandian et al. 2019).*** These growth-suppressing properties are confirmed for A549 cells in this report. Whyte et al. reported that resveratrol significantly inhibited the growth of A549 cells at 25 µmol/L after 48-h incubation (IC_{50}, 50 µmol/L). Furthermore, they found a linear growth inhibition up to 100 µmol/L resveratrol, and thereafter, no significant difference in growth inhibition was shown ***(Whyte et al. 2007).*** Another previous study suggested that resveratrol significantly induced A549 cells death at (25.5 µmol/L) and the maximum cytotoxic activity reached 80% with resveratrol ***(Wu et al. 2010)***. A recent study reported that the IC_{50} value of TQ was 47 ± 0.09 µM after 72 h of incubation ***(Samarghandian et al. 2019)***.

Apoptosis is the process of programmed cells death ***(Wong 2011)***. Apoptosis of cancer cells plays lays an important role in the treatment of cancer as it is a popular target of many treatment strategies, a lot of receptor-mediated cells signal transduction, and many different genes are involved in the activation of cancer cells apoptosis, and regulation of cancer cells apoptosis respectively ***(Elmore 2007, Wong 2011)***. In mammalian systems, various apoptotic pathways exist that can be distinguished by the adapters and initiator caspases involved. Most apoptotic programs fall into either the extrinsic (death receptor pathway) and the intrinsic or mitochondrial pathway ***(Yaacoub et al. 2016)***. There is an additional pathway that involves T-cells mediated cytotoxicity and perforin-granzyme-dependent killing of the cells ***(Elmore 2007).*** The extrinsic apoptotic pathway is activated upon the binding of cytokine ligands (i.e., FasL, TNF, and TRAIL) to members of the TNFα receptor super-family, which are usually called the death receptors (i.e., Fas, also called TRAIL receptors ***(Khosravi-Far 2004)***. Apoptosis involves the activation of a group of cysteine proteases called caspases and a complex cascade of events that are involved in apoptosis. As an upstream protein involved in exogenous apoptosis, caspase-8 becomes activated and then activates downstream apoptosis-inducing proteins such as caspase-3, caspase-6, and caspase-7, to induce apoptosis ***(Chen et al. 2015)***. Caspase-9 is the initiator caspase of the intrinsic apoptosis pathway which is involved in various stimuli, including chemotherapies, stress agents, and radiation ***(Li et al. 2017)***. Caspase-3 involves both exogenous and endogenous apoptosis, and many apoptotic factors work on downstream effector caspase-3 ultimately to induce cells apoptosis ***(Agostini-Dreyer et al. 2015)***. (Apaf-

1) and procaspase-9, called the apoptosome. This results in the auto-activation of caspase-9, which in turn activates the executioner caspases-3, -6 and -7, and ultimately inducing endogenous apoptosis of cells ***(Guerrero et al. 2008)***. The tumor suppressor p53 and the proto-oncogene Bcl-2 were two of the earliest identified cancer genes. p53 is the primary regulator of the Bcl-2 family, can up-regulate Bax and down-regulate Bcl-2 or Bcl-xL, affecting the apoptosis of cancer cells, and changing the permeability of mitochondria, thus affecting the function of downstream pro-apoptotic genes ***(Zhang et al. 2016)***. The anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL) and apoptosis-promoting factors (Bax), their ratio determines whether the cells can accept the apoptotic signal or not ***(Ghobrial et al. 2005, Nakazawa et al. 2016)***. As the main members of the Bcl-2 family, Bcl-2, Bax, and Bcl XL mainly regulate and mediate the process by which mitochondria contribute to cells death. When cells get death signals, the Bax which is bonded to Bcl-2 or Bcl-xL will be displaced, resulting in an increase in the permeability of the mitochondrial membrane and leading to the release of a series of substances, thus eventually causing the death of cells ***(O'Neill et al. 2016)***. Apoptosis mediated by Fas–FasL is an important protein mediating the apoptosis of cells. The induction of apoptosis is triggered by the interaction of Fas with its ligand (FasL) ***(Fesik 2000)*** allowing recruitment of the adaptor protein Fas-associated death domain (FADD) and ultimately inducing effector caspase-3 by active Caspase-8 ***(Volpe et al. 2016)***.

In the present study, we showed TQ, or resveratrol treated A549 cells was significant significantly increased the expression of caspase-3, caspase-8 caspase-9, p53, p21, Fas, FasL, and Bax mRNA levels. Interestingly, the TQ combination with resveratrol treated A549 cells showed that the significantly stronger of these mRNA levels and further induction of apoptosis, this combination had a good anticancer effect in A549 cancer cells. Consistent with these results reported for lung cancer A549 cells, a previous study reported that treatment of A549 cells with resveratrol (25 µmol/L) for 48 h was significantly increased p53, p21, and p27 and the induction of apoptosis by the activation of the caspases and the disruption of the mitochondrial membrane complex ***(Whyte et al. 2007)***. Another study reported that TQ promoted apoptosis in A546 cells by p53 and caspase cascade-dependent pathways. They found that TQ treatment of A549 cells with TQ for 24 h significantly evaluated caspase-3, -9, Bax, Bcl-2, and upregulated p53 expression ***(Samarghandian et al. 2019)***.

For the safety of TQ, resveratrol, and their combination, we have already tested peripheral blood mononuclear cells (PBMCs) from healthy donors and used them as our control in the experiments. We found no effect of TQ, resveratrol, and their combination on the growth, and lysis of normal cells. Moreover, it is already known from existing literature reported that less toxic or less toxic toward normal cells ***(Worthen et al. 1998, Roepke et al. 2007, Greenwell and Rahman 2015)***. Resveratrol appears to have some toxic effects at high concentrations treatment, reducing their therapeutic concentrations could be conducive to cancer treatment ***(GENG et al. 2012, Xiong et al. 2017)***. A combination of TQ and resveratrol may enhance the effects of the treatment at a lower concentration than the alone treatment

with TQ or resveratrol, this combination could reduce the usage amount of the drug, this is the most important mechanism of action.

We acknowledge the shortcomings and limitations in terms of the methodology we used in our present study. The mRNA expression levels are commonly used as a proxy for estimating functional differences that occur at the protein level, mRNA levels are easier to measure genome-wide and hence are typically used to infer the corresponding protein abundances ***(Kuchta et al. 2018)***. Since *in vitro* study is not sufficient for proving the anticancer effects, *in vivo* model is required to determine the exact predictive value of these findings.

Conclusion.

In conclusion, our results proposed that the proliferation of A549 lung cancer cells was significantly inhibited by the combination of TQ with resveratrol treatment at 25 μM with a 50% IC_{50} value of 37 ±3.18μM, this may be a potential anticancer effect for the management of lung cancer. The combination of TQ with resveratrol treatment promoted apoptosis in A549 lung cancer cells by the activation of caspase-3, caspase-8 caspase-9, p53, p21, Fas, FasL, and Bax mRNA levels and reduced Bcl-2 and Bcl-xL anti-apoptotic gene in treated A549 cells. The effective concentration of TQ and resveratrol combination was lower than the alone treatment, this combination could reduce the usage amount of the drug. Our findings suggested that this combination might be a promising anti-cancer therapeutic agent for lung cancer. Future studies are needed to demonstrate if this mechanism is typical of A549 cells in other cells lines with a long period and *in vivo* models to determine the exact predictive value of these findings.

Conflicts of Interest:

The author declares that they have no competing interests.

Acknowledgments:

The author gratefully acknowledges the support of the Deanship of Scientific Research at Prince Sattam bin Abdulaziz University, kharj, Kingdom of Saudi Arabia.

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