Future Journal of Pharmaceutical Sciences 4 (2018) 41–46

Contents lists available at ScienceDirect

Future Journal of Pharmaceutical Sciences

journal homepage: http://www.journals.elsevier.com/future-journal-of-pharmaceutical-sciences/

Novel combination of thymoquinone and resveratrol enhances anticancer effect on hepatocellular carcinoma cell line

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ARTICLE INFO

Article history:
Received 26 April 2017
Received in revised form
19 July 2017
Accepted 2 August 2017
Available online 15 March 2018

Keywords:
HepG2 cell line
Neutraceuticals
Thymoquinone
Resveratrol

ABSTRACT

Hepatocellular carcinoma remains one of the most dominant malignancies worldwide. Neutraceuticals have become under focus in anticancer treatment. Resveratrol is one of the major components of *Polygonum Cuspidatum* and is known as chemo-preventive agent. Thymoquinone is one of the most potent constituents in *Nigella Sativa* and has many medicinal effects. The aim of the present study is to investigate the combined effect of thymoquinone and resveratrol on treatment of hepatocellular carcinoma cells (HepG2). We evaluated the effect of thymoquinone and resveratrol separately and in combination on HepG2. Cell viability, caspase-3 activity, glutathione and malondialdehyde content were determined. The IC50 values of thymoquinone and resveratrol were (46 μM and 64.5 μM) respectively, where each showed potent anti-tumor activity on HepG2. The cell viability was 47.2% and 49.9% respectively. Comparing to the control group, treatment with thymoquinone and resveratrol increased caspase-3 enzyme by 77% and 98.5% respectively, while content of glutathione decreased by 22.8% and 35.6% while malondialdehyde content decreased by 18% and 29.6% correspondingly. The combination (thymoquinone + resveratrol) affected the cell viability leading to further decrease by 9.9% and 12.6%. The content of caspase-3 increased by 89% and 67.5% while the glutathione content had further decrease by 25.6% and 12.8%. Malondialdehyde content decreased by 32.3% and 20.7% all are comparing to thymoquinone and resveratrol separate treatment.

Thymoquinone and resveratrol combination showed significant cell inhibition and increase in caspase-3 indicating cell apoptosis. Both drugs raised reactive oxygen species leading to decrease of glutathione and minor effect on lipid peroxidation, all these results give a new promising combination with enhanced anticancer effect.

1. Introduction

Hepatocellular carcinoma (HCC) the primary malignancy of the liver is considered as the second most common cause of cancer-related death [1]. Many therapeutic agents have been involved in HCC treatment such as sorafenib, brivanib, oxaliplatin and many other chemotherapeutic agents [2]. Chemotherapy remains unsatisfactory. As many side effects decrease the patient compliance and quality of life. To improve the outcome of the therapy and increase the patient's quality of life, many natural compounds are under the spotlight. (see Table 1)

In addition to chemotherapeutic drugs, natural remedies have been used to support the treatment of cancer. *Nigella Sativa*, that is also called Habbah Al-Baraka in Egypt dates back to the Egyptian Pharaoh Tutankhamun and is commonly used in the Middle East countries as a folklore medicine for the treatment of various diseases [3]. Thymoquinone (TQ) which is one of the main constituent in *Nigella Sativa* has been shown to exert anticancer effect [4]. The molecular pathways of TQ as anti-cancer agent include anti-proliferation, apoptosis induction cell cycle arrest and anti-angiogenesis [5]. Moreover, TQ exhibits anticancer activity through the activation of caspases and generation of reactive oxygen species [4,6]. Previous studies showed that TQ induced

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Peer review under responsibility of Future University.

https://doi.org/10.1016/j.fjps.2017.08.001

Table 1
Summary for the results of the four experimental groups: control (0.1%DMSO), TQ (46.03 μM) Resveratrol (64.54 μM) and as combination after determining the cell viability, caspase-3, GSH and MDA contents. The table is pointing to a synergistic action for the combination between TQ + resveratrol.

Test	Control (0.1%DMSO)	TQ (46.03 μM)	Resveratrol (64.54 μM)	TQ + resveratrol
MTT (% of Cell Viability)	97.69 ± 0.5467%	52.83 ± 1.635%[a]	49.91 ± 2.552 %[a]	37.27 ± 0.9473 %[a,b,c]
Caspase-3(n mol/10^6)	14.73 ± 0.36	26.06 ± 1.72[a]	29.23 ± 2.466[a]	39.17 ± 2.436[a,b,c]
GSH (n mol/10^6)	136.52 ± 3.90	105.29 ± 2.30[a]	95.09 ± 3.91[a]	76.33 ± 2.77[a,b,c]
MDA (n mol/10^6)	10.88 ± 0.08	8.9 ± 0.17[a]	9.88 ± 0.26[a]	6.977 ± 0.25[a,b,c]

Data is reported as mean ± SEM for four independent experiments for each parameter.
Date at $p < 0.05$ showed.
a: Significant difference from Control (0.1% DMSO).
b: Significant difference from TQ (46.03 μM) group.
c: Significant difference from Resveratrol (64.54 μM) group.

apoptosis and inhibited proliferation in pancreatic ductal adenocarcinoma cells [7]. Many other studies reported that TQ exhibited inhibitory effects on cell proliferation of many cancer cell lines, including colon, ovarian, lung, and myeloblastic leukemias [8].

Many other neutraceuticals have beneficial effect in treatment of several types of cancer, such as resveratrol which is a polyphenol compound belonging to the class of the stilbenes present in many vegetables and fruits including grapes [9]. Resveratrol possesses an apoptosis-dependent anticancer activity and minimal toxicity to normal cells at certain doses [10]. Since many tumors show sensitivity to resveratrol including lung carcinoma, acute myeloid leukemia, promyelocytic leukemia, multiple myeloma, prostate cancer, oral epidermoid carcinoma, and pancreatic cancer great attention has been given to resveratrol [11].

Resveratrol has various anti-cancer cellular mechanisms including inhibition of angiogenesis and metastasis [12,13]. Additionally resveratrol modulates many cell cycle signaling factors resulting in cell cycle arrest or apoptosis and it also increases caspases which are apoptosis associated enzymes [14]. Caspase-3 is one of the executioner caspases that are believed to be responsible for the actual damage of the cell [15]. Many agents, which induce apoptosis, are either oxidants or stimulators for cellular oxidative metabolism [16]. TQ and resveratrol have oxidative stress effect within cancer cells that makes them potential candidates for treatment of cancer [4,17]. ROS production is one of apoptosis predisposing factor within the cell leading to glutathione (GSH) reduction and the loss of cellular redox balance [18].

A need for a safer and more effective therapy is required either by using single or combined drugs, by offering a great potential in treating HCC and counteracting the side effects of chemotherapeutic agents. Here we investigate the effect of TQ and resveratrol either separately or in combination as anticancer agents on HCC cell line (HepG2).

2. Material and methods

2.1. Chemicals

TQ (2-methyl-5-propan-2-ylcyclohexa-2, 5-diene-1, 4-dione) drug, Resveratrol (3,4,5-trihydroxy-trans-stilbene) drug and reduced glutathione (GSH) kit all were purchased from Sigma-Aldrich St.louis, Mo, USA. Caspase-3 immune assay kit was purchased from Quantikine ELISA, USA. Fetal bovine serum (FBS), RPMI-1640 medium (culture media) and streptomycin/penicillin were purchased from Lonza Co., Almaadi, Egypt.

2.2. Cell line and culture condition

The HCC cell line (**HepG2**) was purchased from VACSERA **(Dokki, Giza, Egypt)**. Cells were cultured in PRMI media with 10% fetal bovine serum FBS and 100 U/mL Penicillin in 5% CO_2 at 37 °C.

2.3. Design of the work

Cells were classified as follow; control cell lines with 0.1%DMSO, TQ treated cells with 46 μM concentration, Resveratrol treated cells with 64.5 μM concentration and (TQ + resveratrol) in combination treated cells.

2.4. Cytotoxicity assay

Cells were plated on 96-well plates at a density of 5000 cells/well. They were plated in triplicate for each concentration. Different concentrations of TQ and resveratrol were prepared by serial dilution. All serial dilutions were transferred to the cells in the 96-well plate, each plate included untreated cells as control with 0.1% DMSO and was incubated for 24 h. After incubation the viability of the cells was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) 20 μl were added to cells and incubated for 2–3 h at 37 °C. After incubation, all of the media were removed and 100 μl of DMSO were added to the cells for solubilization. The absorbance was measured at a wavelength of 570 nm using a micro plate reader. The percentage of cell viability was calculated as survival fraction = T/C × 100 (T mean absorbance of test /C mean absorbance of control).

Data analysis was carried out using prism software program (Graph pad Software incorporated, version 5).

2.5. Caspase-3 immuno-assay

In-vitro determination of the proteolytic activity of the enzymes in lysates of HepG2 cells were performed. TQ and resveratrol as single therapy and then as combination (TQ + resveratrol) induced apoptosis in cells at different time intervals (12, 24 and 48 h) using the Quantikine ELISA Kit. Briefly, cells were collected, washed with cold PBS (1800 rpm in 10 min) and subsequently re-suspended in protein lysis buffer and incubated for 10 min on ice. Then, centrifugation was done at 1500 rpm for 1 min, and the supernatant (protein) was collected. Protein was transferred to 96-well plates in triplicates, then 50 μl of reaction buffer and 5 μl of caspase were added to each well, then incubated for 1–2 h at 37 °C. The plates were read using the micro plate reader at a wavelength of 405 nm.

2.6. Glutathione content determination

Determination of non-protein sulfhydryl compounds (NPSH) in HepG2 cell lysate (indication to reduced glutathione) was performed to each micro centrifuge tube. The tubes were gently shaken intermittently for 10–15 min. This was followed by centrifugation at 2000 rpm for 5 min at room temperature. Then

200 μl of the resulting clear supernatant was taken and mixed with 1.7 ml phosphate buffer in separate test tubes. Ellman's reagent (0.1 ml) was added to each tube. After 5 min, the optical density was measured at 412 nm against a reagent blank.

2.7. Malondialdehyde content determination

The content of lipid peroxidation was determined by Malondialdehyde (MDA) assay. An aliquot of cell lysate (200 μl) was pipetted into a 10-ml test tube followed by the addition of 3 ml of 1% orthophosphoric acid and 1 ml of 0.6% thiobarbituric acid. The tubes were incubated in water bath at 95 °C for 45 min. After cooling, 4 ml of n-butanol was added to each tube and mixed vigorously. The n-butanol phase (upper layer) was separated by centrifugation at 2000 rpm for 10 min. The color was measured at 450 nm using a spectrophotometer.

2.8. Statistical analysis

Data are presented as mean ± SEM (N = 6) and subjected to non-linear regression statistical analysis. For each concentration, percent inhibition values were calculated and IC50 values were determined by Graph-pad prism version [5]. Data were analyzed using one-way ANOVA followed by Tukey-Kramer as a post-ANOVA test at $p < 0.05$.

3. Results

3.1. Effect of TQ and resveratrol on cell viability

The drugs inhibited the proliferation of HepG2 cells in a dose-dependent manner with an IC_{50} of TQ = 46 μM (Fig. 1) and resveratrol = 64.5 μM (Fig. 2).

3.2. Cytotoxicity results of the combined drugs

The cell viability was determined after treatment with TQ and resveratrol separately and it became (47.2 and 49.91%) respectively and after combination of TQ + resveratrol (37.27%) comparing with the control group 97.69% (Fig. 3).

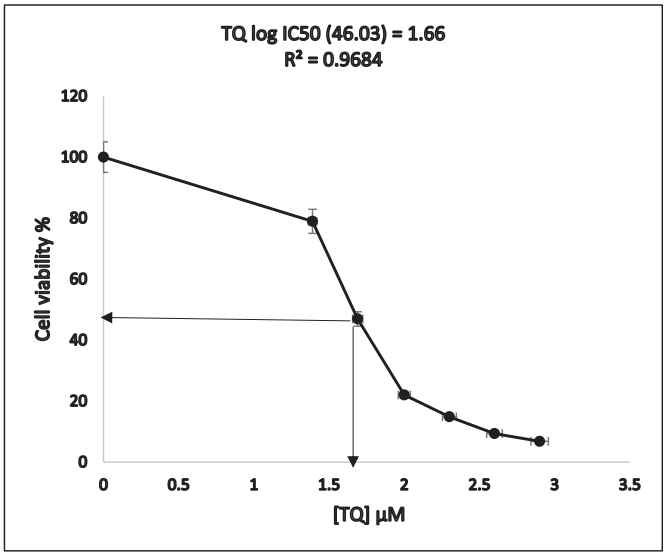

Fig. 1. Cell viability determined after treatment with serial concentrations of TQ (800, 400, 200, 100, 50 and 25 μM) using MTT assay and data measured after 72 h obtaining the IC50 of TQ = 46.03 μM. Data are presented as mean ± SEM (N = 6) and subjected to computerized non-linear regression for determination of IC50 value using Graph Pad Prism (version-5).

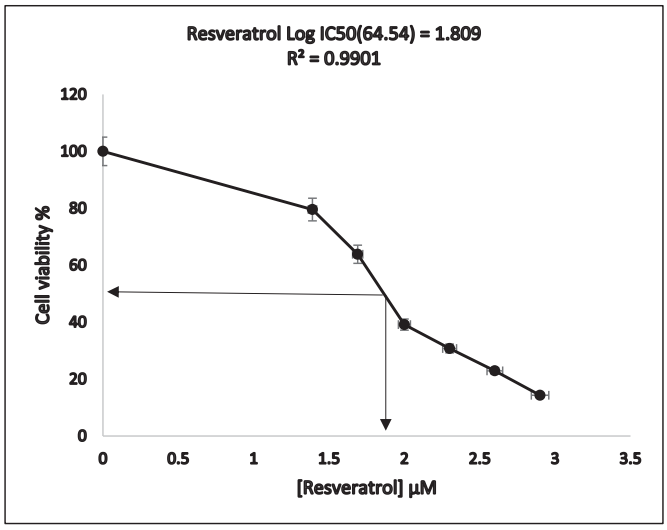

Fig. 2. Cell viability determined after treatment with serial concentrations of resveratrol (800, 400, 200, 100, 50 and 25 μM) using MTT assay and data measured after 72 h obtaining the IC50 of Resveratrol = 64.54 μM. Data are presented as mean ± SEM (N = 6) and subjected to computerized non-linear regression for determination of IC50 value.

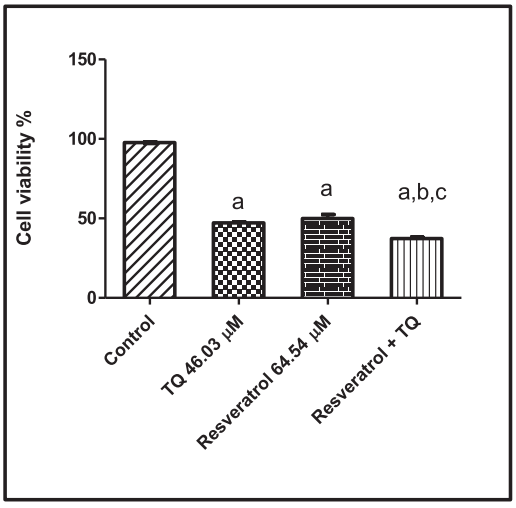

Fig. 3. The effect of TQ and resveratrol and their combination on cell viability of HepG2 cells using MTT assay compared to control group (0.1%DMSO) Data are presented as means ± SEM (n = 6) and were analyzed using one-way ANOVA followed by Tukey-Kramer at $P < 0.05$.

3.3. Caspase 3 analysis

The apoptotic effect of TQ, resveratrol and their combination were also examined by measuring the activities of caspases 3. The enzyme activities were determined in relation to the different concentrations of protein content (50–250 μg/sample) for cells treated with IC_{50}. The results obtained showed an increase of the enzyme activities with the two drugs separately by 77% and 98.5% for TQ and resveratrol respectively and increase of the enzyme activity upon the TQ + resveratrol) increase by 166% comparing to control group. (Fig. 4).

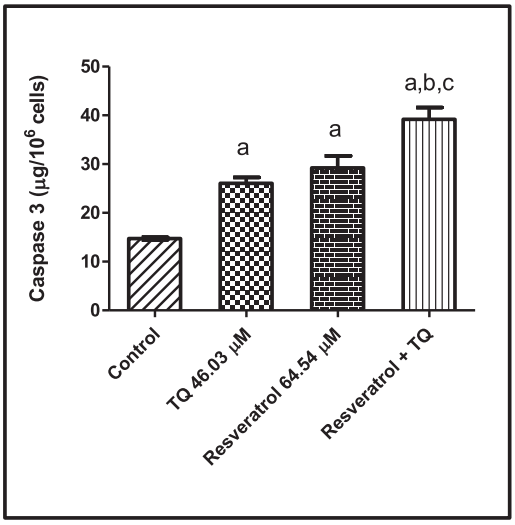

Fig. 4. Effects of TQ and resveratrol either individually or combined with each other on Caspase-3 content in the cell lysates of HepG2 treated cells. Data are presented as means ± SEM (N = 6) and were analyzed using one-way ANOVA followed by Tukey-Kramer at P value < 0.05.

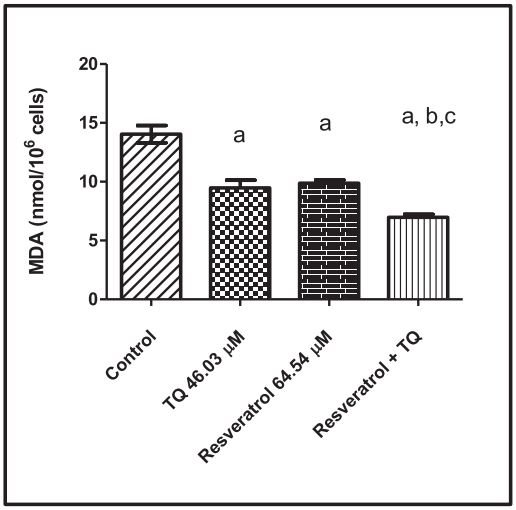

Fig. 6. Effects of TQ and Resveratrol either individually or combined with each other on MDA content of HepG2 cells. Data are presented as means ± SEM (N = 6) and were analyzed using one-way ANOVA followed by Tukey-Kramer as a post-ANOVA test at P < 0.05. a: Significant difference from Control (0.1% DMSO). b: Significant difference from TQ (46.03 µM) group. c: Significant difference from Resveratrol (64.54 µM) group.

3.4. Glutathione

Exposure of HepG2 cells to TQ and resveratrol for 72 h significantly decreased the content of GSH by 22.8% and 35.6% correspondingly. While TQ + resveratrol decreased 48.4% comparing to the control group. (Fig. 5).

3.5. Malondialdehyde

Exposure of HepG2 cells to TQ and resveratrol for 72 h showed significant decrease in MDA content by 18% and 29.6% respectively. While MDA content decreased to 50.3% after treatment with TQ + resveratrol combination comparing to the control. (Fig. 6).

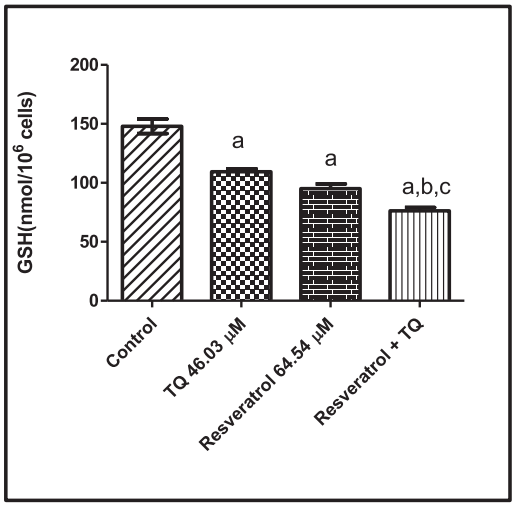

Fig. 5. Effects of TQ and resveratrol either individually or combined with each other on GSH content of HepG2 cells. Data are presented as means ± SEM (N = 6) and were analyzed using one-way ANOVA followed by Tukey-Kramer as a post-ANOVA test at P < 0.05.

3.6. Coefficient of drug interaction (CDI)

The coefficient of drug interaction was used to analyze the synergistically inhibitory effect of the drug combination. CDI was calculated as follows: **CDI=AB/(A×B)**.

AB is the ratio of the two drugs combination group to the control group and A or B is the ratio of the single drug group to the control group.

CDI <1 indicates synergism, especially CDI <0.7 indicates a significantly synergistic effect, CDI = 1 indicates additive.

MTT assay for cell viability CDI = 32.72/52.8*49.9 = 0.012.

Caspase-3: CDI = 39.17/26.06*29.23 = 0.05.

GSH: CDI = 76.33/105.29*95.09 = 0.0075.

MDA: CDI = 6.97/9.88*8.9 = 0.079.

4. Discussion

Neutraceuticals have become the focus of many studies aiming at improving current cancer therapy and decreasing its detrimental side effects.

In this regard TQ and resveratrol have been assessed on HepG2 cells separately and in combination. We demonstrated that TQ and resveratrol have significant cell viability inhibitory effect on HepG2 cell lines. This is in agreements with previous studies of Salim et al. who found decrease in viability of lymphocyte leukemia cells when treated with TQ [19]. In another study resveratrol showed decrease in the cell proliferation of HCC hepa1-6 cell line [20].

Caspases are proteases enzymes that play a key role in the initiation and execution of apoptosis, necrosis and inflammation [21]. Generally, caspases are expressed in the cells as an inactive proenzyme (procaspase). Almost all apoptotic stimuli induce the activation of procaspases and once activated can trigger other caspases leading to amplification of apoptotic signaling pathway followed by cell death due to their proteolytic activity [22]. Caspase-3 is an executioner caspases that activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease, which then degrades chromosomal DNA within the nuclei and causes chromatin condensation [23].

We demonstrated a significant effect of TQ and resveratrol inducing the cell apoptosis by significant induction of caspase-3 activity in the HepG2 cells. The increase in caspase-3 activity was higher in resveratrol treated cells than TQ group. Resveratrol have been proven to induce caspase-3 activity within prostate cancer LNCaP cells [24]. Along with TQ, it was reported that it also increases caspase-3 activity in HCC after treatment of cells in a dose dependent manner showing potential anti-cancer effect [25].

Recent studies showed that generation of reactive oxygen species (ROS) by oxidative damage play an important role in cells undergoing apoptosis [26]. In the current study we investigated the generation of ROS through measurement of the content of GSH within the cells after treatment with TQ and Resveratrol. GSH level was significantly decreased with both drugs indicating liberation of ROS within the cells. This was harmonious with previous work showing that TQ increased the ROS and decreased GSH level on prostate cancer cells possess an anticancer effect by this oxidation process [27]. On the other hand, an in vivo study demonstrated that GSH content increased after treating potassium bromate oxidative stress induced rats with TQ [28]. This data supports TQ to have multiple effect in redox process as it possess either anti-oxidant effect and in some researches it liberates ROS causing oxidation. The illustration of this phenomenon may be related to the dose of TQ used during the experiments, as presented in previous study treatment with TQ clearly resulted in a dose-dependent decrease in GSH level within Jurkatard Hut-102 cells [29]. Depletion of GSH is due to formation of ROS in large amount thus involved in transduction pathway of apoptosis producing anti-cancer effect.

Resveratrol generates reactive oxygen species when used as anticancer treatment in HCC cells leading to lowering the GSH level within cells [24]. Resveratrol also has a dual property towards redox process within the cells [17], it is known as antioxidant drug and protect from many diseases in small regular doses, on the other hand when used in cancer cells with high doses it liberates hydroxyl radicals causing oxidative stress lead to cell death [30].

Lipid peroxidation or reaction of oxygen with unsaturated lipids yields a wide variety of oxidation products. The main products of lipid peroxidation are lipid hydroperoxides. Among the many different aldehydes which can be formed as secondary products during lipid peroxidation, is MDA [31]. MDA has been widely used for many years as biomarker for lipid peroxidation [32].

In the current study we investigated the level of MDA content in HepG2 cells after drug treatment with TQ and resveratrol, they showed decrease in MDA content thus decreasing the lipid peroxidation process. These findings were supported by a previous study at which TQ decreased MDA content in diabetic experimental rats [33]. In parallel with resveratrol findings it was proven that the level of MDA decreased in the serum of strenuous exercise rats after resveratrol treatment [34]. In our study although TQ and resveratrol have decreased the GSH content and produced an oxidative stress, lipid peroxidation didn't increase and this may be due to that the amount of ROS produced wasn't enough to affect the MDA content. Previous studies showed similar results which need further investigation for underlying the possible mechanism that makes TQ and resveratrol affect GSH level while not increasing the lipid peroxidation [35].

In this study, a new combination of TQ with resveratrol was assessed for treatment of HCC HepG2 cell line. By calculating the CDI ratio, the ratio was less than 0.7 showing a significant synergistic effect when combining TQ and resveratrol [36].

Other neutraceuticals combination as resveratrol and curcumin were good combination when added together and enhanced apoptosis and anticancer effect [20]. Combinations of cytotoxic anti-tumor agents and inhibitors from phytochemicals are believed to act together producing inhibitory mechanisms on cancer growth. This combination strategy shows promise in cancer therapy and need further investigation to develop a better treatment strategy.

5. Conclusion

The combination of TQ and resveratrol showed a promising outcome. TQ and resveratrol enhanced each other's effects when added together and further cell viability inhibition occurred with increment of caspase-3 activity and a significant decrease in GSH and MDA content compared to TQ and resveratrol individually. This study showed that though TQ and resveratrol are promising cytotoxic agents and their combination on HCC showed potent anticancer efficacy.

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