Loading thymoquinone on d -α-tocopherol polyethylene glycol 1000 succinate (TPGS) micelles improves the pro apoptotic properties against breast cancer cells

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Thymoquinone (TQ) has been reported in the literature to inhibit different stages of cancer because of its potency. The great potential was shown by nanoparticles (Micelles) as drug carriers of cytotoxic agents. This work aimed to investigate the ability of Micelles based on to enhance TQ cytotoxicity in MCF-7 cells. TQ was loaded using the antisolvent phase separation technique on TPGS Micelles. The prepared TQ TPGS Micelles were investigated for several factors, including size, shape, *in vitro* release, and cytotoxicity activity in MCF-7 cells. In comparison with either pure TQ or TPGS, TQ-TPGS Micelles revealed spherical shaped Micelles with in vitro TQ sustained release for over 36 h and enhanced cytotoxicity activity in MCF-7 cells. Results of cell cycle analysis showed accumulation of MCF-7 cells in G2 / M, and in MCF-7 cells challenged with TQ TPGS Micelles, pre-G1 phases were observed. A large rise in the percentage of cells for early and late apoptosis, as shown by cells stained with annexin V, in addition to total cell death. TQ formulation in the form of Micelles based on TPGS improved the cellular permeation and apoptotic activity of TQ, contributing to the promise of its cytotoxic activity against MCF-7 cells.

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1. Introduction

Cancer is a global disease that develops by resolving many phases that go from a pre-cancerous state to a malignant state by turning normal human cells into tumour cells[1]. This disease is considered as the second reason for death. Moreover, it accounted for 8.8 million deaths worldwide in 2015. Cancer has caused almost one in six deaths. Furthermore, in low and middle-income countries, about 70% of cancer-related deaths occur. Consequently, the treatment of this disease is a global health priority. Despite the invention of different methods of treatment for cancer, such as surgery, radiotherapy, chemotherapy, immunotherapy, targeted therapy, etc., considerable attention has been paid to the development of new cancer controls using natural plants and their bioactive components[2,3]. Black seeds, besides saponins and alkaloid, are rich in many nutrients, including carbohydrates, proteins, amino acids, essential fatty acids, crude fiber and minerals (zinc, sodium, calcium, iron magnesium, and potassium). Several studies have shown that the biological effects of Nigella sativa seeds are primarily related to TQ, its main bioactive ingredient. It has been intensively studied for its beneficial effects *in vitro* and *in vivo*. TQ has a broad variety of beneficial biological and pharmacological characteristics. Moreover, it has

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excellent anti-inflammatory, antioxidant, anti-cancer, hypoglycemic, neuro, cardio-, nephro- and hepatoprotective activities. Recently, bioactive natural plant components have inspired researchers to study their anti-cancer activity because, in addition to their availability in the ingestive form, they have shown virtually no toxicity. Over the last two decades, about 25% of the medications offered are directly extracted from plants; but, another 25% are natural chemically modified products[4,5]. Black seed, which is also named as Nigella sativa or black cumin, is a flowering plant belongs to the family of Ranunculaceae. In Mediterranean Sea boarding nations, this plant has been widely cultivated and grown. Furthermore, It has a long history and religious tradition of being a miraculous cure used in several cultures to promote health and treat different ailments. In addition, Nigella sativa seeds have an immune-modulating property[6,7]. TQ can also be used to monitor a variety of pathological diseases, including diabetes mellitus, atherosclerosis, heart disease, asthma, arthritis, cancer, and neurodegenerative disorders. The beneficial requirements for Nigella sativa and TQ seeds are mainly linked to the presence of the component of lipophilic quinine in its structure. This lipophilic nature promotes the molecule's effective and simple access to cellular and subcellular structures, as well as addressing tumorigenesis-involved intracellular transcription factors and kinases. A thorough understanding of the structure and the anti-cancer effects of TQ that has been reported in numerous literature papers is given in the current review. It also provides insights into the various mechanisms involved in this impact and offers a window on future prospects for the use of TQ in cancer remedies[8,9].

2. Materials and methods

The TQ, TPGS, and ethanol were all obtained from Sigma-Aldrich (St. Louis, MI, USA). Streptomycin, trypsin-EDTA (0.05%), Dulbecco's Modified Eagle Medium (DMEM), penicillin, phosphate buffer Fetal and Calf Serum (FCS) (PBS pH 7.4) were bought from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.1. TQ-TPGS Micelles formulation

As previously stated[10], TQ-TPGS Micelles were prepared with slight modification. Briefly, TQ was dissolved in absolute ethanol, and then the TPGS was dissolved in 90% ethanol. After that, both alcoholic solutions were mixed and then added to an aqueous solution of PVA (1% w/v). After that, the formed solution was stirred for 4 h. Then, it was subjected to evaporation of ethanol. The TQ-TPGS Micelles dispersion was separated by centrifugation at 20,000 rpm for 30 mins at 8°C. Afterwards, it was washed with deionized water (two cycles), and subsequently lyophilized and stored until fully characterized.

2.2. TQ-TPGS Micelles size and zeta potential evaluation

A sample of prepared TQ-TPGS Micelles was dispersed in deionized water using Zetasizer Nano ZSP (Malvern Panalytical, Malvern, UK) to be analyzed for size and zeta potential. The average of 5 runs was used.

2.3. TQ-TPGS Micelles encapsulation efficiency

The prepared sample of the TQ-TPGS Micelles formula was dissolved in ethanol and then injected into a high-performance liquid chromatography (HPLC) and, as previously stated[11], the sample was analysed for TQ material. Equation (1) was utilized to determine the encapsulation efficiency (EE) percentage of TQ:

$$EE\% = \left(\frac{Amount\ of\ TQ\ in\ the\ NP}{Amount\ of\ TQ\ initially\ added}\right) \times 100$$

2.4. TQ-TPGS Micelles in vitro diffusion study

For the diffusion sample, an automated vertical Franz diffusion cell (MicroettePlus; Hanson Research, Chatsworth, CA, USA) as previously mentioned [12,13] was used. A diffusion

buffer (pH 7.0) with Tween 20 (0.5% w/v) stirred at 400 rpm was used, and a diffusion membrane (0.1 µm) was used. Samples were collected at specified time intervals. HPLC examined the TQ content in the withdrawn aliquots as previously stated[14]

2.5. Transmission electron microscopy

Several samples were prepared, and then they analysis after being secured to metal stubs using double-sided sticky tape. The tape had already been connected to the aluminium stubs, and a vacuum was used to add a gold coating. In order to ensure that the surface morphology of the chosen TQ-TPGS formula could be seen well enough for the study, a scanning electron microscope (JEM 100-CX; JEOL, Tokyo, Japan) was then applied.

2.6. Cells and cell culture

Dulbecco's modified Eagle medium (DMEM) was utilized in order to maintain the MCF-7 cells that were bought from Vacsera (Giza, Egypt). The culture medium was supplemented with different components, including penicillin (100 units/mL), streptomycin (100 µg/mL), and heat-inactivated fetal bovine serum (10%). Subsequently, in order to keep the cells in a sub-confluent state, a 5% CO2 humidified (v/v) atmosphere was utilized at 37^{o}C.

2.7. Assessment of anti-proliferative activity

The anti-proliferative activity of prepared TQ-TPGS micelles and TQ and TPGS (plain formula) against MCF-7 liver cancer cells was assessed using sulforhodamine B (SRB). In addition, the anti-proliferative activities against normal human oesophagal epithelial cells (HEEpiC) of the investigated preparations were evaluated. A 96-well plates (1000–2000 cells/well), and 0.25% Trypsin-EDTA was utilized in order to seed the cells for the trypsinization. In order to treat the cells for 72 h, the serial concentrations of the isolated compounds were utilized. Then, the cells were fixed at 4°C for 1 h utilizing 10% of trichloroacetic acid (TCA). After that, the cells were washed multiple times by water. Then, a 0.4% SRB solution was utilized to stain the cells. The stained cells were maintained for 10 mins at room temperature in the dark, and then the cells were washed using 1% glacial acetic acid. The plates were left overnight in order to dry. Moreover, Tris-HCl was utilised to dissolve the SRB-stained cells. The colour intensity (OD) was determined using a monochromator SpectraMax® M3 plate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. The OD measurements taken at this point were used to calculate the percentage of growth inhibition (IC50). The concentrations were determined in triplicate. Also, the whole experiment was repeated three times.

2.8. Cell cycle progression analysis

A six-well cell culture plates was utilized, and about 3× 105 cells/well were seeded in the plate. The cells were challenged with 5.3 µg/mL TQ-TPGS Micelles in addition to the control incubations (cells treated with drug-free media), as well as equal concentrations of TQ and TPGS were utilized for 24 h. In order to perform the cell cycle analysis, the CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was utilized. Cells with a known DNA content (PBMCs) were used as a control in order to achieve the DI (DNA Index) of the test samples. According to the kit manufacturer's instructions, the Propidium iodide was used for the staining. Then, In order to determine the distribution of the cell cycle, a DNA cytometer was used, and CELLQUEST software (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) was used.

2.9. Caspase 3 enzyme assay

The cells were treated with the same preparation for the same time (24 h), as defined in the cell cycle analysis experiment. Then, using a commercial kit according to the direction of the manufacturer (USCN Life Science Inc., Wuhan, Hubei, China), the cells were lysed, extracted, and exposed to the caspase 3 content determination assay.

2.10. Statistical analysis

The data is shown as the mean ± SD. Moreover, the Statistical tests were performed using IBM SPSS® statistics software, version 25 (SPSS Inc., Chicago, IL, USA). In order to compare the

means, analysis of variance (ANOVA) followed by Tukey's post hoc test were utilized. Statistical significance was indicated by $p < 0.05$.

3. Results

As determined by Zetasizer Nano ZSP, particle size results of the prepared TQ-TPGS formulation showed an average size of (75± 9.2 nm). The TEM micrograph of the prepared TQ-TPGS (Fig. 1) showed spherical nanospheres with comparative results with those of the zetasizer.

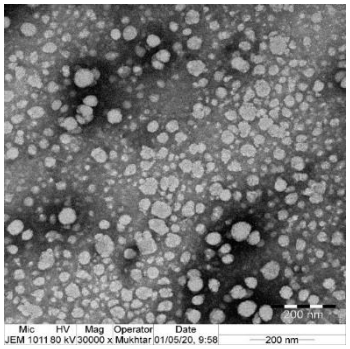

Fig. 2. Transmission electron microscope image of TQ-TPGS Micelles (×30,000).

Fig. 2 demonstrates the *in vitro* release profile of an optimized TQ-TPGS. In both profiles, the optimized Micelles showed a burst release of TQ, which was far higher than the raw TQ. After 2 h, Micelles released about 70% of TQ, while the release of the TQ-raw was about 20%. Consequently, at 2 h, as compared to the TQ-raw, the drug release was more than twice that of the Micelles. TQ release from Micelles was almost complete at the end of 12 h, compared with TQ-raw release of just 65%.

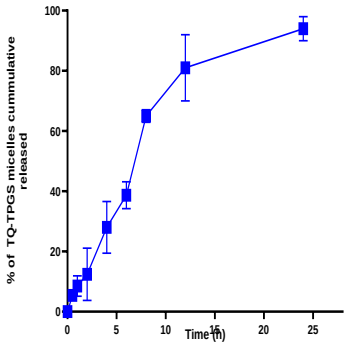

Fig. 3. In vitro release profile of TQ-M in phosphate-buffered saline (PBS) buffer pH 7.4 at 37 ± 0.5°C. (Results are presented as mean ± SD, n = 3).

In Fig. 3, the IC50 values obtained for the samples are shown. TQ-TPGS Micelles had the lowest IC_{50} value of11.9 µM, while TQ-raw had the lowest IC_{50} value of17.48 µM. Consequently, there was a reduction to about one-third of the IC_{50} value of TQ when loaded in the Micelles.

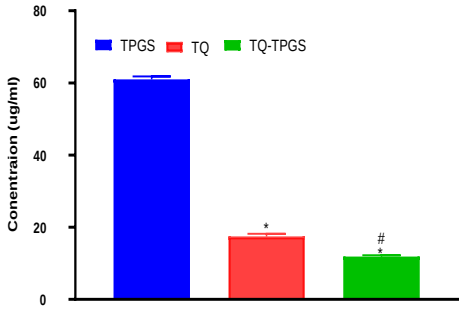

Fig. 4. IC50 of the TPGS, TQ-raw, or TQ-TPGS micelles in the MCF-7 cells.
** Significantly different from control at p < 0.05, # significantly different from plain-M at p < 0.05, $ significantly different from TQ-raw at p < 0.05.*

Fig. 4 demonstrates the outcomes of cell cycle analysis. There was a significant difference observed in the cell cycle. In all phases, the TQ-TPGS was found to work as predicted from the observations. There was no significant effect of plain-M on the G0-G1 phase. The % of cells in the G2-M phase showed marked enhancement on incubation with TQ-TPGS. The percentage of cells in the G2-M process showed marked improvement in TQ-TPGS incubation

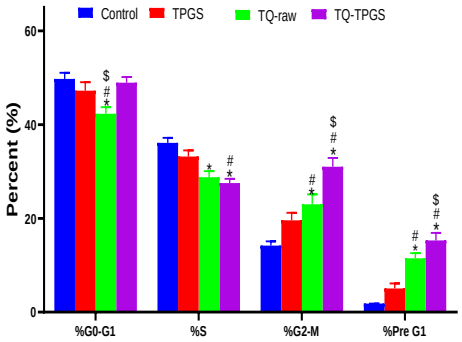

Fig. 5. Assessment of MCF-7 cell death using flow cytometric analysis after annexin V staining.
** Significantly different from control at p < 0.05, # significantly different from plain-M at p < 0.05, $ significantly different from TQ-raw at p < 0.05.*

Annexin V staining: In order to study the apoptosis determination by flow cytometry, Annexin V-FITC apoptosis detection kit was used. Figure 5 shows that when compared to other therapies, TQ-TPGS displayed a distinct and much higher early, late, and complete cell apoptosis as well as necrotic cell death.

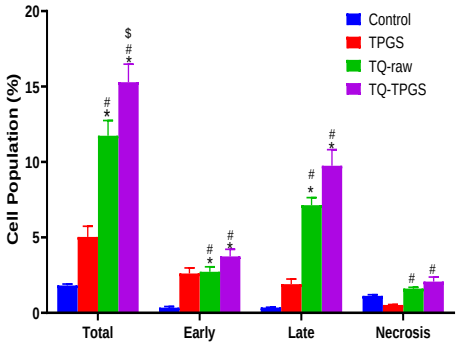

Fig. 6. Assessment of MCF-7 cell death using flow cytometric analysis after annexin V staining.
** Significantly different from control at p < 0.05, # significantly different from plain-M at p < 0.05, $ significantly different from TQ-raw at p < 0.05.*

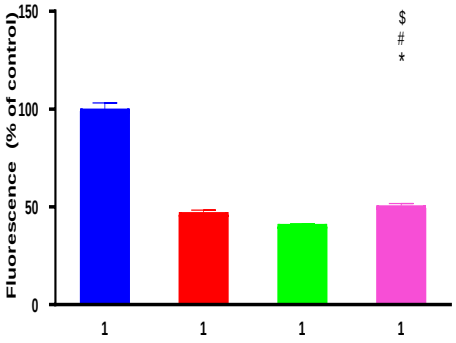

Fig. 7. Effect of TQ-TPGS on caspase-3 content in MCF-7 cells.
** Significantly different from control at p < 0.05, # significantly different from plain-M at p < 0.05, $ significantly different from TQ-raw at p < 0.05.*

Caspase-3 assay: The data represented in Figure 7 suggest that TQ-TPGS caused a significant enhancement in caspase-3 content amounting to an about two-fold increase from that of the TQ-raw-treated cells. Also, Plain-M induces caspase-3 content as compared to control value.

4. Discussion

In this study, TPGS Micelles were loaded with the formulation of TQ to evaluate the role of TPGS as a drug carrier and to maintain TQ release and enhance TQ cytotoxic activity against breast cancer cells. In comparison with either pure TQ or TPGS, TQ-Zein Micelles revealed spherical shaped Micelles with *in vitro* TQ sustained release for over 36 h and enhanced cytotoxicity activity in MCF-7 cells.

The initial TQ release is associated with the surface-bound release of TQ. The Micelles showed sustain release of TQ as a consequence of the diffusion of TQ from Micelles core through the hydrophobic TPGS matrix [15,16]

The raw TQ MCF-7 cells outcomes are in accordance with the finding reported by Acharya et al. [34]. On the other hand, IC50 data of blank (free of TQ) TPGS Micelles agree with the results reported with Alhakamy et al. [17–19]. The decrease in the IC50 value of TPGS Micelles loaded by TQ, indicates that the nanocarrier system has improved TQ activity. This finding is comparable with the recorded human lung cancer line (MCF-7 cells) TQ results[20–22]. Our results showed that the efficacy of TQ as a cytotoxic agent was augmented by loading in TPGS Micelles. This may be attributed to improved TQ's cellular permeability by the nanocarrier, modification of TQ cellular uptake mechanism. RawTQ is transported by passive transport to cells,

while the transport mechanism of TQ-TPGS Micelles is endocytosis. Also, TPGS encapsulation of drugs as hydrophobic nanospheres may enhance cellular absorption across the cell membrane, leading to increased concentration of intracellular TQ[23].

Apoptosis is a mechanism which, at the genetic level, regulates cell death, leading to the removal of damaged cells. The effect of TQ-TQ-TPGS Micelles on the pre-G1 and G2-M phases of the MCF-7 cell line showed that the TQ loaded formula of TPGS Micelles improved the effectiveness of TQ. The increased percentage of MCF-7 cells is an indicator of apoptotic potential in the pre-G1 cell cycle process[24]. In addition, the induction of apoptosis with increased efficacy of TQ in arresting at different cell cycle stages may be associated with its ability to modulate cellular component expressions during apoptosis and cell cycle arrest cell signalling pathways. The literature has identified similar findings on cancer cells [25,26]

It could be concluded, according to our findings and the previous results on cell-cycle analysis, that the increased apoptotic potential of the TQ could be due to the arrest of various phases of cell cycles. These findings are confirmed by a previous TQ study that highlighted TQ's ability to trigger colorectal cancer cell arrest (HCT 116) in the G2-M phase; TQ caused *in vitro* G2 / M phase cell cycle arrest in both TFK1 and HuCCT-1 cells, resulting in decreased G2 / M control point protein cyclin B1 expression.

In the pre-G step, TQ also significantly increased the percentage of apoptotic cells[27–29]. However, published studies have also indicated that in the G0-G1 process, TQ blocks cells[30,31]. This is in agreement with previous research suggesting apoptosis induction as a mechanism of anti-proliferative properties of TQ [40]. With regard to annexin V, these findings are backed by previous studies showing improved staining of melanoma cells with annexin V following a TQ challenge. Additionally, TQ-TQ-TPGS Micelles showed a significant rise in the proportion of MCF-7 cells with positive annexin staining. This coincides with studies demonstrating the ability of TQ-TPGS Micelles to increase the annexin V-staining of icariin-loaded ovarian cells [32–34]. The lipophilic aspect of the formulation that enhanced the delivery of anti-proliferative agents may be responsible for these observations[35]. TQ-TPGS Micelles significantly improved the content of caspase-3 in the present analysis. This is consistent with previous studies demonstrating TQ's ability to increase cellular caspase-3 [36]. Also, the improved effects of TQ-TPGS Micelles on the cleaved caspase-3 material of A543 cells have been demonstrated previously[37]. Elevation of cleaved caspase-3 content is the last cytosolic event preceding apoptosis. Therefore, It can be concluded that formulating TQ significantly improves caspase-3 content in a nanostructured system. In Fact, the increase in the activity of cleaved caspase-3 by nanostructured anti-tumour agent systems has been well reported [38,39]. The observed enhancement of caspase-3 content confirms these findings.

5. Conclusion

The TQ-mixed formulated micelles were almost spherical. The release of TQ from the mixed micelles *(in vitro)* was markedly delayed- TQ- release, suggesting improved tumour cell presentation. The mixed micelles loaded by TQ greatly improve their cytotoxic activities against mcf-7 breast cancer cells. This is mediated, at least in part, by improved Apoptosis, as shown by cell cycle study, staining of annexin V, and caspase 3 determination, enhancing the cytotoxicity of TQ-mixed micelles.

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