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Biodegradable Thymoquinone Nanoparticles for Higher Therapeutic Efficiency in Murine Colorectal Cancer

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ABSTRACT

Thymoquinone (TQ) is a benzoquinone phytochemical constituent extracted from the black seeds of *Nigella sativa* with a wide range of pharmaceutical and therapeutic applications. However, its therapeutic potential has been hindered by its low aqueous solubility with subsequent low availability, thermal and light instability. One of its promising applications is the use as an anticancer drug against different types of cancer. Herein, we have encapsulated TQ in polymeric nanoparticles (NPs) fabricated from different polymers namely ethyl cellulose (EC), poly (DL-lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) aiming for enhanced anticancer efficiency. Particles were prepared for the aim of colorectal cancer treatment. Prepared NPs ranged in size between 290 and 400 nm with negative surface charge enhancing their stability and cell internalization potential. Particles were spherical in shape with smooth surface texture. In vitro drug release has shown burst release of 50% TQ in the first hour followed by slower release pattern. NPs prepared from PCL were selected for the in vivo experiments on the colorectal cancer xenografts in mice. Both TQ solution and NPs proved to enhance the survival rates of mice via the reduction of tumor growth rate with superior activity observed for the NPs. Encapsulation of TQ into NPs could enhance its accumulation in tumor tissue and enhanced its therapeutic efficiency. Results show that polymeric NPs would be a very promising carrier system for the enhanced anticancer activity of TQ.

1. INTRODUCTION

Thymoquinone (TQ: 2-methyl-5-isopropyl-1,4-benzoquinone) is the main active ingredient in the volatile oil of *Nigella sativa* seed, which is commonly known as the black seed or black cumin. It constitutes around 27% of the seeds volatile oil composition (Abukhader, 2013). It has been known to exhibit various pharmacological effects including anti-neoplastic, anti-oxidant, anti-inflammatory, immunomodulatory as well as anti-diabetic properties. Interestingly, it was observed that black seed is commonly used as a spice in countries of low incidence of colorectal cancer such as Egypt, Pakistan or India (Woo et al., 2012). Such significant prophylactic ability against the development of gastrointestinal tumors has been owed mainly to TQ. Several mechanisms have been proposed as possible pathways of its anticancer activity including inhibition of cell proliferation, apoptosis stimulation, generation of reactive oxygen species and inhibition of angiogenesis and metastasis (Banregiee et al., 2010, Bhattacharya et al., 2015). In addition, TQ was found to be successfully able to inhibit the growth of other different types of cancer cells including glioblastoma, lung cancer and melanoma (Kolli-Bouhafs et al., 2012; Ahmad et al., 2013, Jafri et al., 2010). Moreover, TQ has proved to be able to enhance the antitumor activity of various drugs and help reduce their associated side effects (Badary, 1999). For example, it could ameliorate the nephrotoxicity caused by cisplatin (Badary et al., 1997)and the cardiotoxicity of doxorubicin (Al-Shabanah et al., 1998) making it an excellent candidate for cancer therapy, adjuvant therapy or prophylaxis. The alleviation of toxic side effects of other chemotherapeutic agents has been correlated to its strong antioxidant properties as a phytochemical drug belonging to the antioxidant quinones that contain a p-benzoquinone structure such as coenzyme Q10 (Nehilla et al., 2008).

Although TQ is a potent antioxidant and anticancer drug, its administration has been hindered due to its poor water solubility and bioavailability. Thus might necessitate the administration of very high doses to obtain the desired pharmacological effect leading to aggravation of any associated side effects (Badary et al., 1998).

The encapsulation of TQ into biodegradable polymeric nanoparticles would help to overcome its poor bioavailability due to its low aqueous solubility as well as enhance its thermal and light stability. Protection of TQ with polymeric nanoparticles would also enhance the antioxidant properties, prevent degradation and hence improve the pharmacological efficacy

of the drug. In addition, encapsulation into NPs limits drug diffusion to healthy tissues and prevents its rapid metabolism (Schneider-Stock et al., 2013).

Colorectal cancer is one of the most commonly spread types of cancer and is considered to be a significant leading cause of death especially with its high risk of metastasis to liver and other body parts. Its risk of incidence has been found to be strongly reduced by the intake of certain foods and plants due to their content of various active compounds (Surh, 2003). TQ in non-cytotoxic concentrations has shown to reduce the proliferation and induce apoptosis in human colon cancer cells (Gali-Muhtasib et al., 2004) and has anti-invasive properties in C26 colorectal cancer cells (Gali-Muhtasib et al., 2008). In addition, a therapeutic potential against 1,2- dimethylhydrazine (DMH)-induced cancer in mice has been proven when administered in the early stages of tumor development.

In this work, we investigated the possibility of encapsulating TQ into biodegradable nanoparticles using different polymers and in vivo therapeutic efficiency of a selected formulation was tested on murine colorectal xenografts. For the best of our knowledge, this is the first report about the in vivo evaluation of TQ encapsulated in polymeric nanoparticles using such polymers with in vivo assay of their therapeutic potential.

2. MATERIALS AND METHODS

2.1. Materials:

Polycaprolactone average Mw 48,000-90,000(PCL), Poly(DL-lactide-co-glycolide),PLGA, average Mw 24,000-38,000, Thymoquinone and polyvinyl alcohol 98–99% hydrolyzed, were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Ethyl cellulose (EC) (Ethocel standard 4 premium) was a kind gift from Colorcon. All other chemicals were of analytical grade or equivalent purity.

Cells

Murine colon adenocarcinoma C26 cell lines were obtained from National Cancer Institute (NCI, Frederick, MD, USA). The culture medium RPMI-1640 medium supplemented with 10% FBS, 50 µL/mL streptomycin, 50 U/mL Penicillin G, and 2 mM L-glutamine. Cells were cultivated in a 37°C incubator with 5% CO2 and 95% humidified air.

Animals

Male BALB/c mice (6-week old, n=6 for each group) were purchased from Janvier Labs (Roubaix, France). The animals were kept at room temperature (25±2°C) and relative humidity (40-60%) under a 12 h light/dark cycle. Food and water were provided *ad libitum*. All studies were approved by the Institutional Animal Care and Use Committee of Ain Shams University and were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals in Egypt.

2.2. Preparation of the nanoparticles:

The drug-loaded nanoparticles were prepared according to w/o emulsification solvent evaporation technique. Thymoquinone (20mg) and the polymer (either EC, PCL or PLGA) were dissolved in 3 ml dichloromethane as an organic phase. This organic solution was then poured into 10 ml of aqueous PVA solution where a coarse emulsion was formed. The mixture was then homogenized with an ultrasonic cell disruptor (Bandelin sonopuls, Berlin, Germany) for 4 min in ice bath. Solvent evaporation was then performed in a Buchi Rotavapor RE 120 (Buchi, Flawil, Switzerland) with reducing the pressure stepwise down to 30 mbar with a diaphragm pump.

2.3. Characterization of the nanoparticles

Nanoparticles suspensions were characterized by measuring their particle size and size distribution in terms of the average volume diameters and polydispersity index by photon correlation spectroscopy using particle size analyzer (Brookhaven Instruments Corporation, New York, USA) at fixed angle of 90° at 25 °C. Nanoparticles were diluted with distilled water before analysis.

Entrapment efficiency of thymoquinone in nanoparticles suspensions was determined indirectly by cold centrifugation at 15,000 rpm and 4^0C for 20 minutes. The free unentraped thymoquinone was measured in the supernatant by UV spectrophotometry at λ max of 258 nm. Samples were analyzed in triplicate and average results were obtained.

2.4. Scanning electron microscopy (SEM):

The shape and surface morphology of the TQ NPs were examined using scanning electron microscopy (Hitachi S 2460N, Tokyo, Japan). Nanoparticles suspensions were mounted

on metal (aluminum) stubs, using double-sided adhesive carbon tape and allowed to completely dry overnight. The samples were then sputter-coated with gold using gold sputter module and examined using SEM at an acceleration voltage of 15 kV.

2.5. *In vitro* drug release experiments

Drug release from the different nanoparticles was tested using dialysis technique.Simply 1 ml of the nanoparticles suspension was placed in a dialysis bag sealed from both ends by plastic clips (regenerated cellulose dialysis tubing of MWCO 12-14 KDa soaked in release medium overnight). Dialysis bags were then immersed in 50 mL of phosphate buffer pH 7.4 at 37°C in a shaking water bath moving at a speed of 100 rpm. Aliquots of 1 ml were withdrawn at predetermined time intervals and the TQ content was measured spectrophotometrically at 258 nm. All experiments were performed six times and average results were obtained.

2.6. In vivo therapeutic efficiency

For the evaluation of therapeutic efficiency and the anticancer activity against colorectal tumors of the prepared TQ NPs, tumor-bearing BALB/c mice were employed. Tumors were induced by subcutaneous injection of 3X105 C26 cells into the lower right flank of 6-week-old male BALB/c mice. Animals were surveyed for tumor growth daily and treatment started once the tumor volume reached average volume of 25 mm3. Treatments given to mice were either in the form of TQ-NPs or TQ solution (0.5mg/ml in PBS) with corresponding doses. Injections were given twice weekly and treatment continued until the average volume of the tumor exceeded 2000 mm3 where animals had to be sacrificed. With such volume, the tumor weight never exceeded 2 g which is considered to be much less than 10% of the average body weight of the bearing mice (around 25 g) so that total tumor burden of the animals were within the permissible ranges (Bhattacharya et al., 2015). Tumor size was measured periodically using digital caliper and volume was calculated according to the formula (volume= (width)2 X length / 2). At the end of the experiment, samples from the tumors and animals livers were taken, fixed in 10% formalin, embedded in Paraffin and stained for histological examination with typical hematoxylin and eosin staining procedure.

2.7. Statistical analysis

All results were expressed as mean values ± SD. ANOVA on ranks was used to investigate differences statistically. GraphPad Prism (version 7.0a) was used to construct the survival rate curve and the statistical analysis. Differences were considered significant at $p < 0.05$.

3. RESULTS

The preparation of thymoquinone-loaded nanoparticles was successfully performed using the o/w emulsification solvent evaporation technique using the three different polymers namely EC, PCL and PLGA. Particle size analysis of the prepared NPs revealed that all of them have been in the nano range with the smallest size achieved using EC (around 235 nm) while the largest particles were the ones prepared from PCL with an average diameter of 406 ± 29.6 nm (Table 1).

Table 1: Particle size analysis and zeta potential of the different nanoparticles.

Formula	Particle diameter (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)
EC-NPs	235 ± 3.6	0.106 ± 0.012	-37 ± 1.4	91 ± 3.6
PCL-NPs	406 ± 29.6	0.005 ± 0.001	-13 ± 2.0	88 ± 2.5
PLGA-NPs	287± 6.1	0.055 ± 0.027	-19 ± 2.2	76 ± 1.5

Polydispersity indices for the different NPs indicated high degree of particle size homogeneity and very narrow size distribution, especially for the PCL-NPs. These results were confirmed by the images obtained from the scanning electron microscopical examination of the different nanoparticles. However, certain degree of aggregation and larger particle size for the PLGA NPs was observed using SEM compared to the photon correlation spectroscopy size measurements (Figure 1). Zeta potential measurements indicated that the nanoparticles carried a negative potential indicating potential high stability of the nanosuspensions. Measurement of the entrapment efficiency of TQ into the different NPs showed high encapsulation rates ranging from 76 to 91% with the lowest encapsulation rates observed for the PLGA NPs.

In vitro TQ release experiments have shown comparable release patterns for the three polymers under study. There was a burst release of around 50% of the thymoquinone content in the first hour followed by slower gradual release of the remaining drug content over the experiment duration. Release profiles from both PCL and PLGA were nearly identical while EC NPs have shown slightly slower release with lower released quantitates at the same time points compared to the other two polymers (Figure 2).

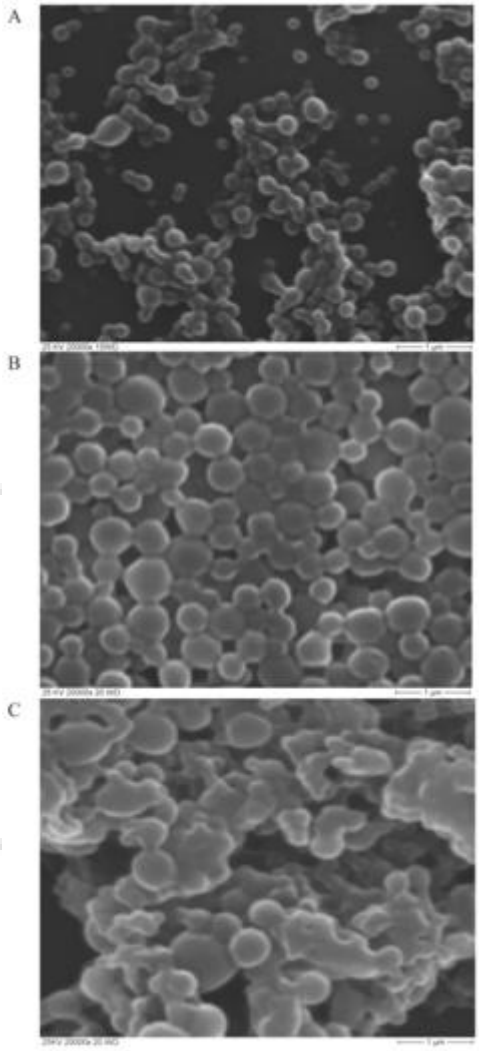

Figure 1: Scanning electron microscopy images of the different thymoquinone loaded polymeric nanoparticles A: EC-NPs, B: PCL-NPs, C: PLGA-NPs.

Based on the above results, PCL nanoparticles were chosen as the best candidate for the further in vivo investigations and testing their therapeutic efficiency in the inhibition of colorectal tumor cells growth. Colon cancer xenografts were induced by the subcutaneous injection of C26 cells in BALB/c mice. All animals varied in their weight from 20 to 22 g in the beginning of the treatments and at the end, they were all around 24-25 g without any significant

differences between the different groups. Animals were then treated by the injection of either TQ solution in PBS or TQ loaded PCL NPs until the tumor volume reached the volume of 2000 mm3. It was found that the treatment with both TQ solution or NPs suspension significantly reduced the tumor volume and tumor growth rate compared to the untreated animals with a superior growth inhibiting ability observed for the NPs, $p<0.05$ (Figure 3).

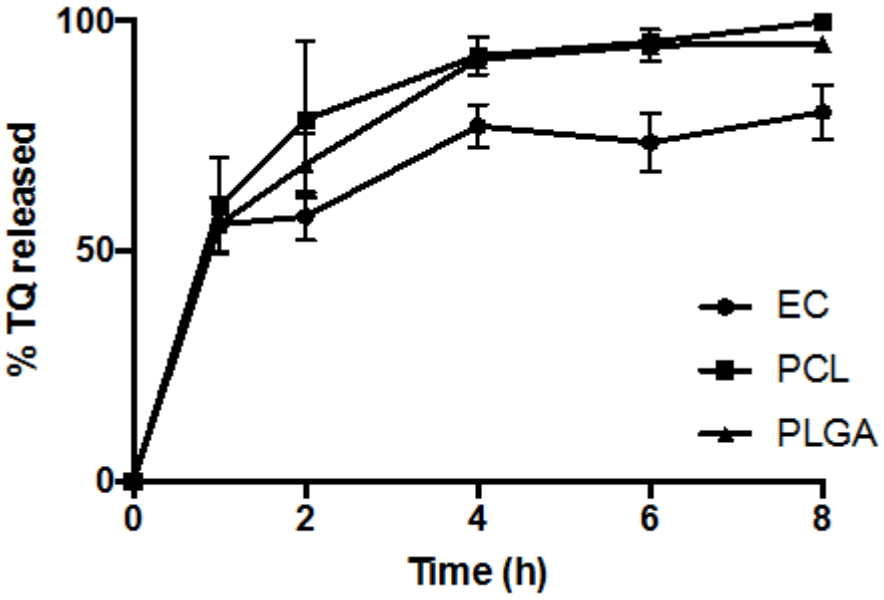

Figure 2: In vitro thymoquinone release profiles from different polymeric nanoparticles.

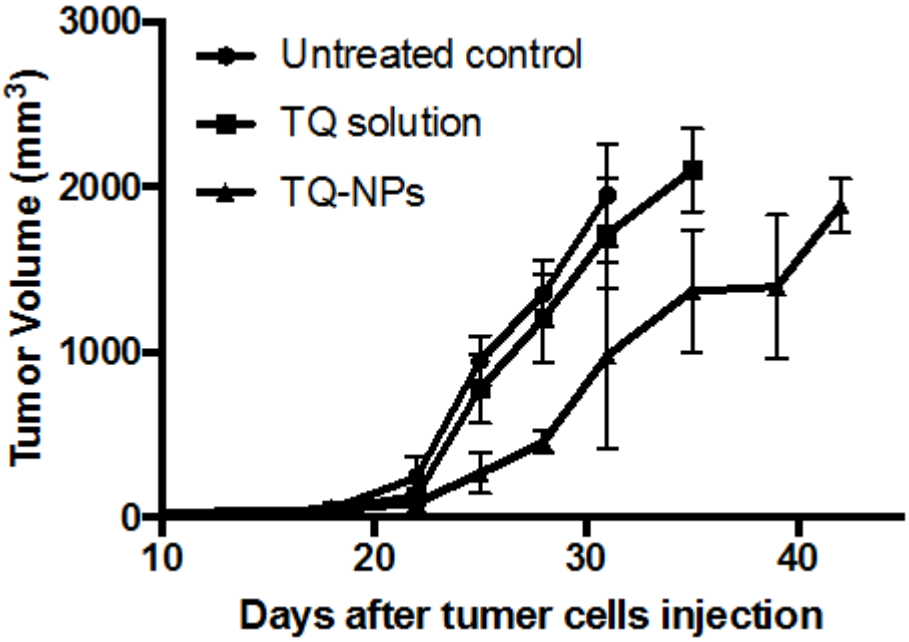

Figure 3: Tumor volume of the subcutaneous C26 colorectal cancer tumors in mice of different groups.

This has been significantly reflected on the survival rates of the animals where it was found that the best survival rates were obtained for animals treated with TQNPs (up to 50days) compared to 35 and 31days in case of TQ solution and untreated control groups respectively (Figure 4). Histological examination of the untreated tumors has shown an intensive growth pattern with dense accumulation of the highly proliferating cells with enlarged and multiplied nuclei. In contrast, treatment with TQ solution had shown slight detachment of the cells and less density of cell multiplication. NP striated sections showed significant loss of cells adhesion yielding sponge-like shape with significantly increased apoptosis features. Examination of the liver sections from different groups revealed the absence of any signs of toxicity neither from TQ solution nor the used NPs (Figure 5).

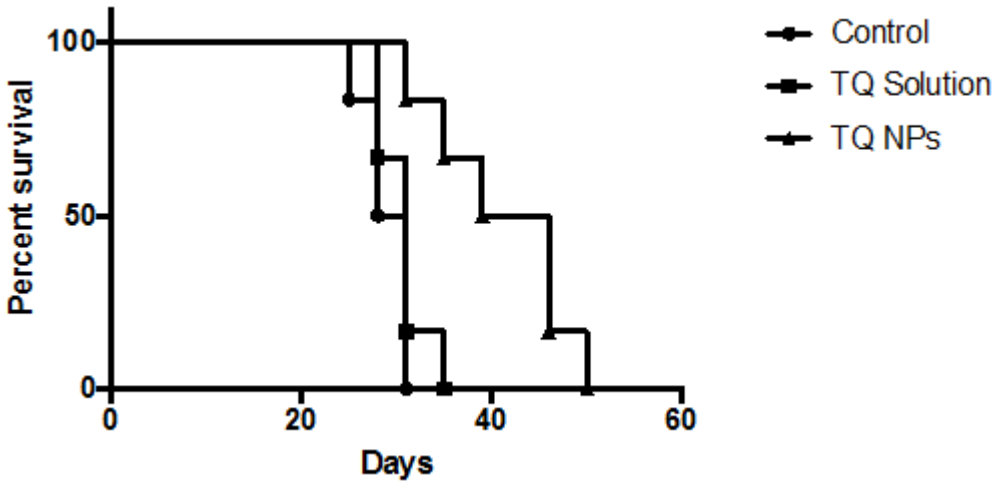

Figure 4: Survival rates of Balb/c mice carrying C26 colorectal cancer tumors treated with either TQ solution of PCL-TQ loaded NPs.

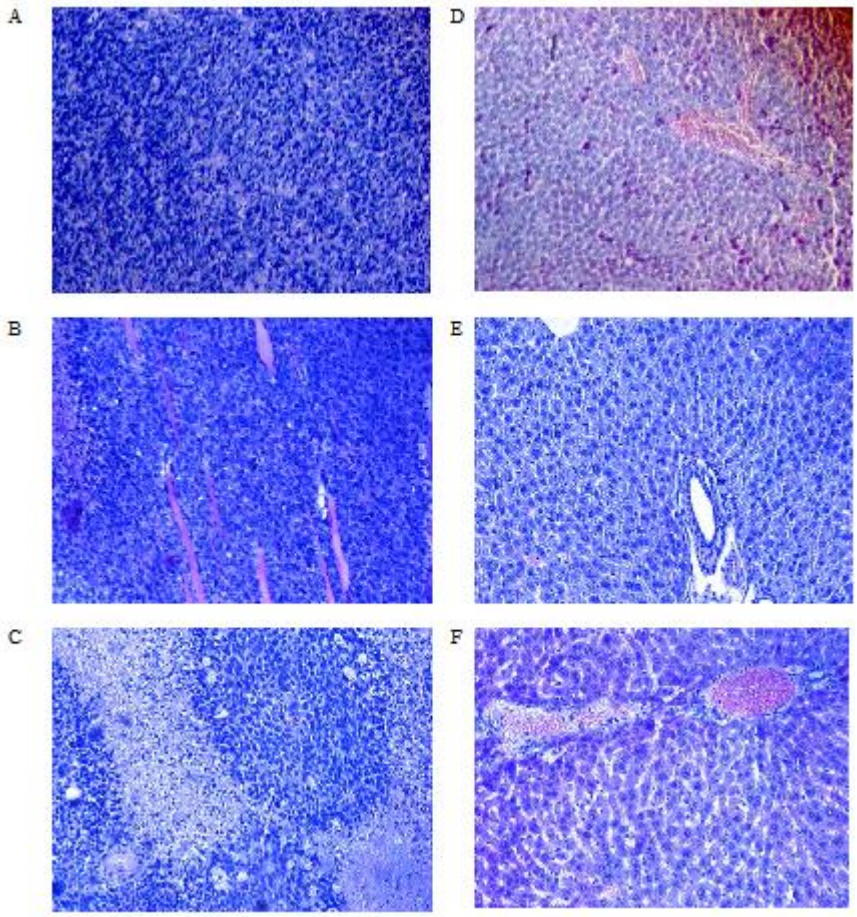

Figure 5: Histological sections stained with typical H&E staining of tumor (A,B and C) or Liver (D, E and F) samples from nontreated, TQ solution or TQ PCL-NPs respectively.

4. DISCUSSION

Due to the general toxicity of most chemotherapeutic agents and lack of selectivity yielding severe life complicating side effects, drugs of natural origins are sometimes favored due to their relative tolerability and reduced toxicity risks (Odeh et al., 2012). Therefore, use of drugs of herbal origin has been always an attractive approach for the treatment of various diseases. TQ extracted from the black seed (Nigella sativa) has been known for years with its antitumor activity against various types of tumors. It was recently found to possess antiproliferative properties and enhance apoptosis of human colon cancer cells (Gali-Muhtasib et al., 2004). Its inhibitory activity against the growth of C26 cells spheroids has been proven but the exact mechanism is yet not clear. Its anticancer properties could be owed to its potent anti-inflammatory and antioxidant effects and in particular to its strong potential to induce apoptosis (Gali- Muhtasib et al., 2008).

In the current work, encapsulation of TQ into polymeric nanoparticles was performed with the aim of enhancing its bioavailability, therapeutic efficiency as well as its light and thermal stability. The particle sizes of all particles were in the nano range but we observed the best size homogeneity and stability of the particles with the PCL nanoparticles. Due to the high hydrophobicity of the polymer, high encapsulation rate was also achieved compared to the PLGA. Compatibility of TQ was found to be higher for high molecular weight polymers than lower molecular weights. This could explain the higher entrapment efficiency in PCL compared to the PLGA. Also, drug precipitation was observed if drug loading was higher than 25 mg for each 100 mg polymer (Ganea et al., 2010). Therefore, with the concentration used in our study, stable formulations could be prepared with no precipitation of the drug over the experimentation period.

The *in vitro* release profiles of the different formulation has shown a fast release of around 50% of TQ in the first hour followed by slower release over the remaining 7 hours. This might be explained by the non-uniform distribution of TQ inside the polymer matrix where higher proportion of the drug was located near the particles' surface. Similar release profiles from PLGA nanoparticles and solid lipid nanoparticles (Ganea et al., 2010, Singh et al., 2013) have been previously reported. The antioxidant properties of TQ role in the prevention and treatment of cancer should not be also neglected. It is known that the excessive production of free radicals may contribute significantly to the alteration of cellular functions leading to different diseases mainly cancer (Ganea et al., 2010).

TQ although having a promising anti-cancer activity, its use has been hindered due to its low aqueous solubility and subsequently reduced bioavailability. Its encapsulation into polymeric nanoparticles would enhance its uptake by the cancer cells especially with the leaky vasculature along with poor lymphatic drainage into tumors, which is known as the enhanced permeation and retention effect (EPR). In addition, protection from external factors will lead to higher stability of TQ with its known thermal and light sensitivity. Controlled release of TQ from the nanoparticles accumulating into tumor tissue will also reduce normal tissue exposure and delay the rapid metabolism of the administered drug (Schneider-Stock et al., 2014). Higher cytotoxicity was also observed from the TQ-loaded PLGA nanoparticles compared to TQ solution where cell viability was reduced from 69% to 16% for the solution and nanoparticles respectively (Ganea et al., 2010). All these factors would contribute to the higher therapeutic efficiency obtained from the TQ loaded NPs compared to the free drug

solution. The safety and protective activity of TQ would be also improved by the use of NPs. Singh et al., 2012 has shown promising results by the encapsulation of TQ into solid lipid nanoparticles with five-fold increase in TQ bioavailability in rats plasma with significant hepatoprotective properties against cirrhosis (Singh et al., 2013). However, in our study, no major differences were found between the histological evaluation of liver samples of animals treated with Solution or nanoparticles. This can be explained by the safety of the doses used in both cases. Mechanism of anticancer activity of TQ is not yet quite clear although several studies have been performed in such area. It was found to be a potent inhibitor of the NF- κB pathway and reduce tumor angiogenesis (Sethi et al., 2008, Yi et al., 2008). It was reported its ability to enhance the sensitivity of pancreatic cancer cells to antineoplastic agents (Banerjee et al., 2009). It could also reduce the growth of androgen sensitive and insensitive prostate cancer cells through the suppression of expression of androgen receptor and E2F-1 (Kaseb et al., 2007). TQ was found to induce p38 phosphorylation and ROS production in breast cancer cells thus suppressing xenograft tumors growth via the subsequent anti-proliferative and pro- apoptotic effects. TQ also increased the levels of catalase, superoxide dismutase and glutathione in mouse liver tissues (Woo et al., 2013). There was an evidence that the oral administration of thymoquinone has resulted in the inhibition of benzo(a)pyrene- induced forestomach carcinogenesis (Badary et al., 1999) and 20-methylcholanthrene- induced fibrosarcoma tumorigenesis in mice (Badary et al., 2001). A successful study encapsulated TQ into polyethyleneglycol (PEG) and Polyvinylpyrrolidone (PVP) based nanoparticles have been performed (Bhattacharya et al., 2015). The prepared NPs were used for inhibition of breast cancer cells migration and successfully reduced the tumor growth rate, volume and increased the life span of the treated animals compared to the untreated animals. It was found that PEG4000- TQ-NPs could up-regulate the miR-34a in a p53- dependent manner thus retarding cancer cell migration significantly.A major advantage of TQ as an antineoplastic agent is its ability to interfere with cancer cells growth with minimal or no effect on the healthy tissues (Soni et al., 2015). TQ was found to interfere with polyp progression in ApcMin mice (only in a dose of 375mg/kg and not 37.5 mg/kg) via the induction of tumor cells apoptosis only in the neoplastic tissue and not in the normal tissue. In addition, β-catenin was retained at the membrane and c-myc decreased in the nucleus, which was associated with a reduced cell proliferation in the villi. These observations proposed the use of Thymoquinone as a nutritional supplement for the prevention and protection from colorectal cancer (Lang et al., 2013). This could have a great application especially that clinical trials had shown that human

body could tolerate doses of TQ up to 2600 mg/day (Al-Amri and Bamosa, 2009). TQ was also found to protect against testicular injury in male mice treated with methotrexate. This might suggest a complementary role of TQ in improving the quality of life of cancer patients (Woo et al., 2012).Encapsulation of TQ and paclitaxel into PLGA nanoparticles has been achieved before and the particles proved to be significantly able to enhance the anticancer activity of paclitaxel in MCF-7 breast cancer cells and were proposed to alleviate the associated side effects of paclitaxel by lowering the dose used (Soni et al., 2015). However, only in vitro evaluation was performed. Our data confirmed the superiority of TQ-NPs therapeutic effects compared to the solution in terms of tumor growth retardation and animals survival. Therefore the PCL-TQ-NPs prepared here could be used for several applications in cancer therapy either as a sole, adjuvant or prophylactic therapy.

CONCLUSION

Encapsulation of TQ in PCL based polymeric nanoparticles for the enhancement of its bioavailability; thermal, light and chemical stability has been successfully performed. Particles were tested in vivo for the treatment of colorectal tumors in murine model. Significantly higher therapeutic activity was observed compared to TQ solution as seen from the significantly higher survival rates and reduced tumor volumes. Therefore, PCL-TQ loaded NPs are considered a promising carrier system for the utilization of TQ for various therapeutic purposes in colorectal cancer.

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