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Biodistribution and Scintigraphic Evaluation of Microemulsion Formulations of Technetium-99m-Radiolabeled-Thymoquinone

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ABSTRACT

The hydrophobic nature of thymoquinone (TQ) compromises its bioavailability and limits its effectiveness as an anticancer compound. The preparation and evaluation of TQ-microemulsion formulations was investigated. Parenteral and oral formulations of TQ-microemulsions were prepared by studying the solubility of TQ in different oils, surfactants and co-surfactants and investigating their stability, globule size, zeta potential, and in vitro and in vivo activity by scintigraphic evaluation of the technetium-99m-radiolabelled TQ-microemulsions. The mean globule size of the TQ-micremulsions was on the nanometer scale. The mean size of the oral formulation remained constant in distilled water and in buffers of various pH for 6 h, while the parenteral formulation was found to be stable for 72 h in distilled water and various aqueous solutions. The in vitro anticancer activity of the TQ formulations against MCF7 and Wehi 164 cell lines was evaluated and determined to be superior to plain TQ. Relative tumor volumes (RTV) of mice transplanted with MCF cells after oral and intravenous administration of TQ-microemulsions were measured to be 6 and 73.2 mm^3 respectively compared to 21.1 nd 231 mm^3 for administration of plain TQ. Similarly, mice transplanted with murine fibrosarcoma cells had RTVs of 68 and 175.3 mm^3 after oral and parenteral administration of TQ-microemulsions compared to 190.1 and 214 mm^3 for analogous administration of plain TQ. Our study is the first to describe the preparation, scintigraphic evaluation and biodistribution of TQ-microemulsion formulations. The superior properties of the microemulsion formulations potentiate their future use in the treatment of solid tumors.

Keywords: ^{99m}Tc-thymoquinone; Biodistribution; Radiolabeling; Scintigraphy; Microemulsions; Formulation

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INTRODUCTION

Thymoquinone (TQ), the active constituent in black seed essential oil, has been reported to exhibit antitumor activities, including anti-proliferative and pro-apoptotic effects on cell lines derived from breast, colon, ovary, larynx, lung, myeloblastic leukemia and osteosarcoma [1-8]. Reports have indicated that the growth inhibitory effects of TQ are specific to cancer cells whereas it is minimally toxic to non-neoplastic cells. This has been observed in colon cancer [3,4], prostate cancer [9], canine osteosarcoma [3], and skin cancer [5]. Many multidrug-resistant variants of human pancreatic adenocarcinoma, uterine sarcoma, and leukemia were found to be sensitive to TQ [10]. Although TQ is potentially a promising anti-cancer drug, its lipophilic nature intrinsically translates into poor aqueous solubility which ultimately affects its bioavailability [11]. In fact, many potential drug candidates, especially hydrophobic molecules, fail in human clinical trials because of poor efficacy which may be in part due to their poor bioavailability [12]. In the case of orally administered formulations, lipophilic drugs show poor gastrointestinal absorption due to low solubility or dissolution rate in water. Consequently, oral bioavailability is low and although the candidate possesses powerful pharmacological activity, the anticipated clinical efficacy is sometimes not realized. Poor aqueous solubility also potentiates problems for parenteral delivery. Parenteral

administration is also of utmost importance in drug development and drug delivery research. The design of parenteral drug delivery systems is a critical and challenging task as the number of excipients approved for parenteral delivery is considerably small. In recent years, much attention has focused on lipid−based formulations to improve the bioavailability of poorly water soluble drug compounds. In fact, an effective approach involves formulation of a drug candidate into a microemulsion. The potential of emulsions in the effective parenteral delivery of several hydrophobic drugs is well documented in the literature [13]. The excellent thermodynamic stability, high solubilization capacity, low viscosity, transparency, ease of manufacture and scale-up, ability to withstand sterilization techniques and their low cost compared to other colloidal carriers make microemulsions an interesting delivery system [14]. Furthermore, microemulsions were found to be less painful on injection as compared to the co-solvent based formulations [15].

In the present study, we synthesized and characterized novel oral and parenteral microemulsion formulations of thymoquinone. In addition, the preparation of ^{99m}Tc-radiolabeled thymoquinone complexes (^{99m}Tc-TQ) enabled us to analyze the biodistribution, stability, anti-tumor activity and pharmacokinetic properites of the aforementioned microemulsion formulations and compared these properties to those of plain TQ.

EXPERIMENTAL SECTION

Preliminary Studies

In the laboratory 10, 45 and 90 mg/kg doses were tried for oral administration and amongst the doses, 90 mg/kg demonstrated optimal efficacy and hence, this dose was used for additional studies. Similarly, for IV administration, 10 mg/kg showed optimal activity and was chosen as the administered dose.

Preparation of Microemulsion Formulations

Solubility studies of TQ: Solubility studies in pharmaceutically acceptable solvents and oils were carried out to identify appropriate excipients for formulation of microemulsions. An excess of TQ (approximately 50 mg) was placed in 1 mL of the vehicle in a screw-capped glass vial and the mixture was vortexed to facilitate solubilization. The above mixture was equilibrated at 25°C for 24 h and then centrifuged at 30 000 xg for 10 minutes to separate undissolved TQ. Aliquots of supernatant were diluted with ethanol and quantified by a validated spectrophotometric method at a wavelength of 254 nm. Formulation development: The compositions for oral and parenteral microemulsions are as described by Date et al. [16] and are presented in Table 1. Microemulsions for oral formulation (F1) were prepared by thorough mixing of 200 mg of PC1 with 45 mg TQ whereas the parenteral formulation (F2) involved mixing 100 mg of PC2 with 5 mg of TQ. Both microemulsion formulations were diluted with distilled water to 1 mL.

Table 1: Composition of formulations (F1 and F2)

Composition	Quantity	Purpose
For oral formulation (F1)		
Cremaphor EL	195 mg	Surfactant
Akoline –MCM	65 mg	Cosurfactant
Capryol 90	260 mg	Oil
Total PC1	520 mg	
For parenteral formulation (F2)		
Ethyl oleate	123 mg	Oil
Solutol	660 mg	Surfactant
Ethanol	217 mg	Cosurfactant
Total PC2	**1000 mg**	

Characterization of Microemulsion Formulations

Globule size analysis: Mean globule size and polydispersity index (PI) of F2 were determined by photon correlation spectroscopy (PCS) at 25°C, using Beckman Coulter N5 plus Submicron Particle Size Analyzer.

Measurement of Zeta Potential

ZetaPALS instrument was used for measurement of surface charge (zeta potential) and electrophoretic mobility of the blank and TQ-loaded microemulsions at 25°C. The measurements were carried out using diluted microemulsion formulations.

Morphology of microemulsions:
The oral (F1) and parenteral (F2) formulations were diluted with distilled water (1:200) and mixed by slightly shaking. One drop of diluted sample was deposited on a film-coated copper grid and stained with one drop of 2% aqueous solution of phosphotungstic acid (PTA). The mixture was allowed to dry prior to making observations under the transmission electron microscope (TEM).

Effect of various buffers for oral formulation:
25 µL of oral formulation (F1) was diluted with various media namely, double distilled water, buffer pH 1.2, buffer pH 3.0 and buffer pH 6.8, to 25 mL in a volumetric flask and the solution was gently mixed by inverting the flask. Visual observations were made immediately after dilution for assessment of appearance (transparency), phase separation, and precipitation of the drug. The mean globule size and polydispersity index (P.I.) of the resultant emulsions was determined by PCS wherein measurements were obtained at an angle of 90º. The emulsions were diluted with the respective vehicles to ensure that the light scattering intensity was within the instrument's sensitivity range. The resultant emulsions were also allowed to stand for 6 h at room temperature to assess stability to dilution.

Effect of various vehicles on globule size for parenteral microemulsions:
Various diluents, namely, double-distilled water, 0.9% w/v sodium chloride, 5% dextrose solution and 2.25% glycerol, were used for dilution. Microemulsions were diluted to ensure that light scattering intensity was within the instrument's sensitivity range. Samples were placed in transparent polystyrene cuvettes and loaded in a thermostated chamber. Light scattering was monitored at an angle of 90° to the incident beam.

Robustness to dilution:
Robustness of TQ-microemulsions to dilution was studied by diluting 50, 100 and 1000 times with various media, namely, water, buffer pH 1.2, buffer pH 3.0 and buffer pH 6.8 and the effect of dilution on particle size was observed. The resulting emulsions were kept at 25 ± 2°C for 24 h and observed for any signs of phase separation and drug precipitation.

TQ content:
The TQ content in both formulations (F1 and F2) was determined using a validated HPTLC method as described in the literature [17].

Biodistribution of Radiolabelled Formulation
Radiolabeling procedure: The oral (F1) and IV (F2) formulations were radiolabeled with ^{99m}Tc using a modified protocol to that described by Reddy H et al. [18]. The optimized method involved radiolabeling TQ-microemulsions with $Na^{99m}TcO4$ by reduction with stannous chloride dihydrate. Briefly, in a sterile vial the pertechnetate (4 mCi) was reduced with 250 µg of stannous chloride dihydrate in 25 µL of freshly prepared 0.1 N HCl and pH was adjusted to 5.5 - 6 with 0.1 M sodium bicarbonate solution. To this, 125 µL of TQ stock solution was added and the solution was allowed to mix in a sonicator at room temperature for 30 minutes. The contents were passed through 0.22 µm filter into an evacuated sterile sealed vial and volume made up with 0.1 M sterile tris buffer to 1 mL (final pH=7). A radiolabeling efficiency of 95% was measured.

Anticancer Activity Testing using *In vitro* and *In vivo* Models
The efficacy of formulations (F1 and F2) was evaluated *in vitro* and *in vivo* and compared to plain TQ.

***In vitro* Activity**
In vitro studies were performed on two different cell lines: Wehi 164 (Mouse fibrosarcoma) and MCF7 (human breast adenocarcinoma). The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. Briefly, 1×10^3 /well cells were inoculated into 96 well microtiter plates in 100 µL at plating densities. After cell innoculation, the microtiter plates were incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of TQ/vehicle and formulation (F1 or F2). After 24 h, one 96 well plate

containing 5×10^3 cells/well was fixed in situ with TCA, to represent a measurement of the cell population at the time of TQ addition. TQ was dissolved in ethanol and the volume made up with water to 1 mL. The formulation was also prepared according to this method. Plain TQ (TQ1 and TQ2) and the formulations (F1 and F2) were then initially solubilized in dimethyl sulfoxide (DMSO) at 100 mg/mL and diluted to 1 mg/mL with water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1 mg/mL) was thawed and diluted to 100 µg/mL, 200 µg/mL, 400 µg/mL and 800 µg/mL with complete medium containing test article. Aliquots of 10 µL of these different drug and formulation dilutions were added to the appropriate microtiter wells already containing 90 µL of medium, resulting in the required final drug concentrations of 10 µg/mL, 20 µg/mL, 40 µg/mL and 80 µg/mL. After compound addition, plates were incubated at standard conditions for 48 hours and the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µL of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 µL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:

$[(Ti-Tz)/(C-Tz)] \times 100$ for concentrations for which $Ti \geq Tz$ (Ti-Tz) positive or zero and $[(Ti-Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$. (Ti-Tz) negative.

The dose response parameters were calculated for each test article. Growth inhibition of 50% (GI 50) was calculated from $[(Ti-Tz)/(C-Tz)] \times 100=50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti=Tz. The LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from $[(Ti-Tz)/Tz] \times 100= -50$. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

***In vivo* Activity**

Animal studies: Swiss female albino mice weighing 25-30 g were obtained from the animal house facility of the Biomedical Group, Bhabha Atomic Research Centre (BARC), Mumbai, India, and were maintained under standard housing conditions of temperature i.e., (12 h light and 12 h dark and 22 ± 4ºC) and standard mouse feed and water ad libitum. Mice were housed in groups of four in each steril polypropylene cage containing sterile paddy as bedding. All animal experiments were conducted adhering to the ethical guidelines of the Institutional Animal Ethics Committee. To develop tumor in mice, 2×10^6/0.1 mL/mouse murine fibrosarcoma cells were injected by subcutaneous injection on the right dorsal side of the hind limb of the experimental mice. 10- 12 days later after the tumor was developed, these mice were termed 'donor mice' and used for tumor transplantation in experimental animals. Thus, murine fibrosarcoma was serially maintained in mice under standard laboratory conditions at the institution. For tumor transplantation, aseptic conditions were observed and all procedures conducted under laminar hood. All instruments like scissors, forceps, and syringe needles used were previously sterilized and separate instruments were used for excision of tumor. The donor mice were sacrificed by cervical dislocation and immersed in 70% alcohol for approximately three minutes. Then the mouse was removed and placed in a sterile towel to remove excess alcohol. The tumor was excised, washed in phosphate buffered saline (PBS), cut into small pieces and minced to obtain a single-cell suspension. About 100 µL of this murine fibrosarcoma single-cell suspension (1×10^6 cells) in PBS was transplanted by subcutaneous injection on the right dorsal side of the hind limb of the experimental mouse. After the 7^{th} day of tumor transplantation, overnight fasted animals were either orally or parenterally administered (10 mg/kg) the radiolabeled formulation as follows: Prior to start of the experiment, the mice were anaesthesized with a combination of ketamine and xylazine administered intraperitoneally wherein xylazine (10 mg/kg body weight) was used as preanaesthetic agent and ketamine (100 mg/kg body weight) was employed as anesthetic agent. For *in vivo* studies ^{99m}Tc-TQ complex was administered intravenously at 10 mg/kg

body weight as a single dose. The mice were placed in a ventral (sternal) recumbent position with head extended forward under the Millenium MPS single head Gamma camera (GE) fitted with LEGP pin hole collimator and images were acquired by using GENIE acquisition station. The static images were acquired in 256 × 256 matrix at 140 keV and 20% window for 1 minute at 4, 6, 12 and 24 h post injection. The images acquired were transferred to eNTEGRA workstation for further processing. The biodistribution of radiolabeled TQ formulations were studied by analyzing the scintigrams obtained from the gamma camera by drawing constant regions of interest (ROI) on the RES organs (liver, lung spleen). In order to validate the results obtained from the static images, the mice were sacrificed at various time points and the organs were dissected out and the percentage uptake of the radiolabeled complex was calculated as % ID per gram of tissue/organ. Blood was obtained by cardiac puncture at predetermined time points. Subsequently, tissues (heart, lung, liver, spleen, kidney, stomach, intestine and tumor in case of tumor bearing mice), were dissected, washed with normal saline, made free from adhering tissues and dried in the paper folds. The organs were then weighed on an analytical balance and the radioactivity in each organ was measured using a dose calibrator and results expressed as percent injected dose per gram/tissue after decay correction i.e., the activity in each organ was divided by the total activity administered to determine the percentage of radioactivity (% ID) in each organ. The % ID was divided by the mass of each tissue to determine the percentage of radioactivity per gram (% ID/g) after applying the decay correction. All data are reported as mean ± SD. Results were calculated as % ID after subtracting the individual organs background.

The same procedure was followed for oral administration with a modification in the anesthesia procedure wherein only ketamine was administered intraperitoneally (100 mg/kg body weight) just before oral feeding of the radiolabeled complex using a feeding needle. The animal models selected for *in vivo* antitumor activity were mouse spontaneous mammary tumor and murine fibrosarcoma as they represented the *in vivo* models of the *in vitro* cell lines which were used previously, namely, MCF7 and Wehi 164. Animal experiments were performed adhering to the guidelines of Institutional Animal Ethics Committee of Bombay College of Pharmacy.

Mouse Spontaneous Mammary Tumor

ICRC female mice (20 - 25 g) were received from animal breeding centre of Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Tata Memorial Centre at the beginning of the experiments. Mice were maintained under artificial light for 12 hours light-dark cycle (lights on from 08:00 to 20:00 h) and at constant temperature of 23 ± 20°C and 65% humidity. Standard rodent pellet diet (Amrut, Pranav Agro Ind. Ltd., Sangli, India) and tap water was provided ad libitum. The animals were acclimatized for > 7 days before use. Animal care and handling throughout the experimental procedure were performed in accordance to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The experimental protocol was approved by the animal ethical committee of ACTREC. All mice were injected subcutaneously on the dorsal right flank with 106 MCF cells in sterile PBS. The tumor mice were divided into five groups of 6 mice each as follows: Group I: Control (No treatment), group II: TQ1 and group III: F1 (each treated with 90 mg/kg); group IV: TQ2 and group V: F2 (each treated with 10 mg/kg). Tumor mice in group II were administered TQ1 orally (90 mg/kg) while group III were administered F1 on day 5 after tumor transplantation which was considered as day 1 of treatment. Similarly, tumor mice in group IV were administered parenterally i.e., IV TQ2 (10 mg/kg) while group V were administered F2 on day 5 after tumor transplantation which was considered as day 1 of treatment. The dosing schedule observed was 1, 7, 10, 13, 16, 19, 22, 25, 28, 31 and 34 days for both oral as well as parenteral treatment. The size of tumors in the study was measured in two dimensions (area) with vernier calipers on alternate days.

Murine Fibrosarcoma

The grouping of the animals and the dosing schedule was the same as described under mouse spontaneous mammary tumor.

RESULTS AND DISCUSSION

Solubility of TQ in Different Oils and Surfactants

The solubility of TQ was studied in different oils, surfactants and co-surfactants as a part of a preliminary screening. The solubility of TQ in these vehicles is presented in Figure 1. TQ was soluble in oils such as capryol 90, ethyl oleate, and lauroglycol and in surfactants such as labrafil M2125, solutol HS 40 and co-surfactants such as akoline-MCM. This study formed the basis of selecting the formulae shown in Table 1. The described compositions are

known to give microemulsions [16], thus these systems were characterized for their stability upon incorporation of TQ. TQ was soluble in the components of the formulation inidcating that the systems possess good stability.

Furthermore, ethyl oleate was chosen as an oily phase in case of parenteral formulation due to its safety. It is included in the FDA Inactive Ingredients Guide [19], parenteral medicines licensed in the UK, and Canadian List of Acceptable Nonmedicinal Ingredients [20] and is used as a vehicle in parenteral preparations and considered of low toxicity with minimal tissue irritation potential. Ethyl oleate is also reported as a suitable solvent for steroids and other lipophilic drugs [21]. Because of these properties, in addition to its solubilizing potential for TQ, ethyl oleate was selected as an oily phase. Further, solutol HS 15 was chosen as the surfactant because it has been generally regarded as a relatively nontoxic and nonirritant excipient and has been used in preclinical injectable formulations of water-insoluble molecules [20].

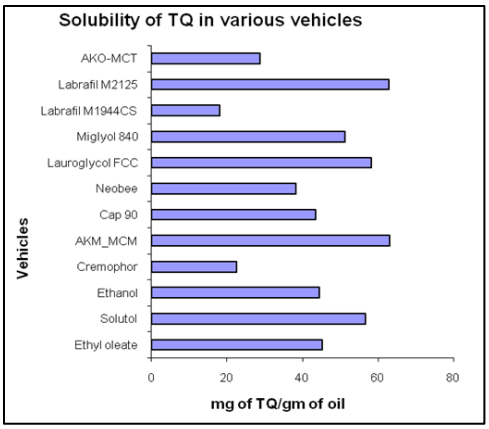


Figure 1: Solubility of TQ in different oily vehicles

Characterization of TQ-Containing Microemulsions

Globule size analysis: The droplet size of the emulsion is a crucial factor in self-emulsification performance because it determines the rate and extent of drug release as well as absorption [22–24]. The mean globule size and polydispersity index (PI) of microemulsions were determined by photon correlation spectroscopy (PCS). The mean globule size and PI of formulations F1, F2 and their respective blank formulations are presented in Table 2. The microemulsions were diluted with double distilled water and mean globule size and PI was noted for 24 hours in order to evaluate the stability of the formulations when stored at an ambient temperature for a short period of time. The data in Table 2 indicates that the formulations were stable with respect to mean globule size for a period of 24 hours.

Table 2: Mean globule size and PI (in parentheses) of blank and formulations

Time (h)	Blank F1	Formulation F1	Blank F2	Formulation F2
0	156.3 ± 0.72	127.1 ± 0.31	17.0 ± 0.11	20 ± 0.3
	(0.101 ± 0.047)	(0.297 ± 0.029)	(0.008 ± 0.053)	(0.111 ± 0.091)
24	160.5 ± 0.47	143 ± 0.07	20.1 ± 0.12	22.6 ± 0.87
	(0.284 ± 0.008)	(0.665 ± 0.058)	(0.018 ± 0.03)	(0.388 ± 0.184)

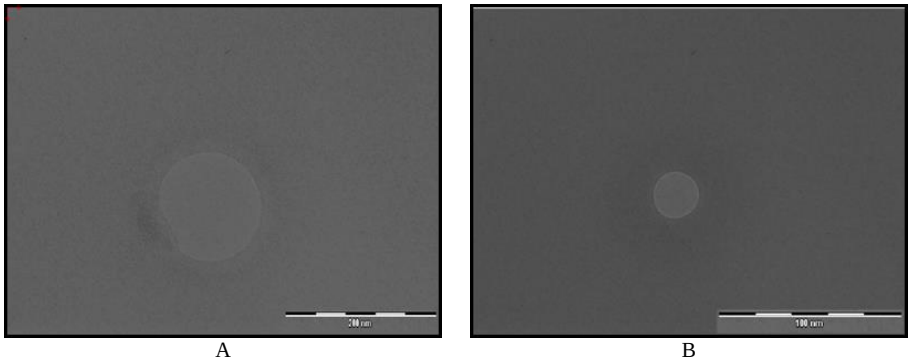
Measurement of Zeta potential: The zeta potential of the formulations are listed in Table 3. All formulations showed negative zeta potential, indicating that the surfactant boundary surrounding the oil globules is negatively charged. This suggests that the systems should be stable on storage [23,25,26].

Table 3: Zeta potential of formulations

Sample	Zeta Potential
Blank F1	-5.88
Formulation F1	-4.98
Blank F2	-15.46
Formulation F2	-17.13

Morphology of Microemulsions

The morphology of microemulsions was determined by transmission electron microscopy (TEM). The TEM picture indicated that the oral formulation has a spherical nature and the particle size obtained was in accordance with that obtained by PCS. Also, no drug crystals were noted in the field suggesting the absence of drug crystallization. The TEM images for F1 and F2 are shown in Figure 2.



A B

Figure 2: TEM images of formulations of (a) F1 and (b) F2

Effect of various buffers for oral formulation: The mean globule size of formulation F1 after dilution with various dissolution media is presented in Table 4. The formation showed mean globule size within a range of 118 -138 nm when diluted with various dissolution media differing in pH. The time required for dispersion of microemulsions after dilution with various dissolution media was just 2 minutes. The resulting microemulsions were translucent in appearance and did not show any signs of phase separation and drug precipitation even after 6 h indicating that the formulation would remain stable during its transit into gastrointestinal tract.

Table 4: Mean Globule size and PI of formulation F1 in different buffers

Dissolution medium	Water	Buffer pH 1.2	Buffer pH 3.0	Buffer pH 6.8
Globule size (nm)	127.1 ± 0.31	118.8 ± 0.65	121.8 ± 0.72	138 ± 0.65
P.I.	0.297 ± 0.029	0.418 ± 0.021	0.402 ± 0.011	0.445 ± 0.71

Effect of various vehicles on globule size for parenteral ME: The mean globule size of formulation F2 and its blank in different vehicles is shown in Table 5. The mean globule size remained constant when diluted with various diluents indicating that the formulations were stable to dilution in these vehicles for 24 h. Interestingly, an increase in PI was observed for formulations diluted in 2.25% glycerol.

Table 5: Mean globule size and PI (in parentheses) of blank and F2 in various vehicles

Vehicles	Blank		F2	
	0 hr	24 hr	0 hr	24 hr
Saline (-0.9%)	17.6 ± 0.36 (0.189)	17.5 ± 0.26 (0.130)	18.1 ± 2.28 (0.722)	20.7 ± 0.06 (0.058)
Water	16.8 ± 0.04 (0.221)	18.8 ± 0.32 (0.114)	18.3 ± 2.37 (0.401)	21.0 ± 1.37 (0.34)
Dextrose (-5%)	17.7 ± 0.26 (0.204)	17.0 ± 0.32 (0.562)	18.3 ± 0.75 (0.542)	22.3 ± 0.78 (0.261)
Glycerol (-2.25%)	18.6 ± 0.61 (0.030)	31.6 ± 0.93 (0.787)	20.8 ± 0.23 (0.876)	30.2 ± 8.77 (0.188)

Robustness to Dilution

The stability of microemulsion formulations after dilution was checked. The results are as listed in Table 6. From the results obtained it is apparent that both formulations remained stable for 6 hours after dilution with hardly no change in the mean globule size for the F1 formulation and only a slight increase in globule size of formulation F2 from 43.3 to 47.2 nm upon diluting 1000-fold. Also the formulations did not show any signs of phase separation and drug precipitation. This is indicative of the stability of microemulsions after incorporation of TQ.

Table 6: Mean globule size and PI of formulation F1 after dilution

Dilution Factor	F1		F2	
	Size (nm)	PI	Size (nm)	PI
50	39.8 ± 0.41	0.258 ± 0.027	43.3 ± 0.02	0.252 ± 0.030
100	39.6 ± 2.2	0.292 ± 0.028	38.2 ± 0.47	0.202 ± 0.118
1000	36.9 ± 2.27	0.877 ± 0.021	47.2 ± 5.70	0.817 ± 0.272

Biodistribution Studies in Fibrosarcoma Bearing Tumor Mice

IV administration: The biodistribution studies were performed in fibrosarcoma mice for plain TQ and its formulation (F2). In tumor bearing animals after IV administration of plain TQ, the % ID at 4, 6, 12 and 24 h was 66.46, 71.80, 63.24 and 26.69 respectively. This indicates a high uptake of the radiolabeled complex by the organs of the RES. While the % ID observed at 4, 6, 12 and 24 h for tumor mice injected with formulation of TQ (F2) was 70.89, 58.76, 60.78 and 22.83 respectively. At 4 and 6 h, the % ID in the tumor of F2 injected mice was 0.013% and 2.723%, respectively whereas it was not observed for mice administered with plain TQ indicating accumulation of F2 in the tumor tissue as can also be evident from Figure 3. The accumulation of F2 in the tumor could be attributed to the permeability of tumor vasculature, which is generally increased as compared with normal tissues [27-30], although this varies among tumors.

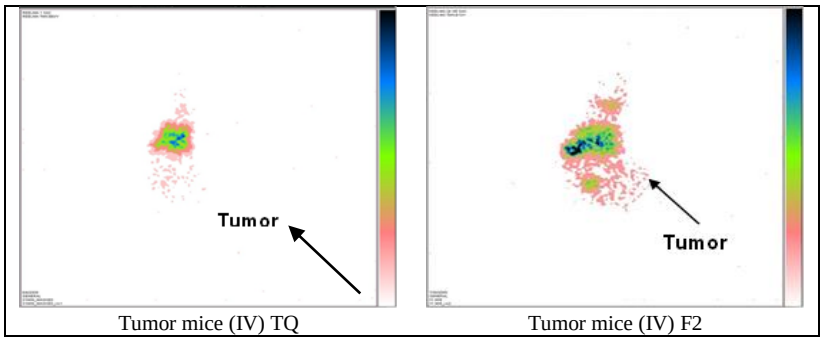


Figure 3: Scintigrams obtained after 4 h of IV administration of radiolabeled formulation

Anticancer Activity Testing using *In vitro* and *In vivo* Models

In vitro activity of MCF-7: The results for *in vitro* activity of plain TQ and its formulations in MCF-7 cell line are displayed in Figure 4. Results showed that the growth inhibitory concentration (GI 50) calculated for plain TQ (TQ1) was 29.3 µg/mL, while for the formulation (F1) GI 50 value was found to be 12.4 µg/mL. Thus, the formulation meant for oral administration exhibited enhanced activity compared to plain TQ in MCF-7 cell line as evidenced by the 50% reduction of the GI 50. Furthermore, parenteral administration of F2, the GI 50 calculated was less than 10 µg/mL. This demonstrated that formulation F2 also exhibited superior activity compared to plain TQ as evidenced by reduction of the growth inhibitory concentration to nearly half the GI 50 concentration, when compared to plain TQ2. Thus, both the formulations F1 and F2 displayed better inhibitory effect on MCF-7 as compared to plain TQ.

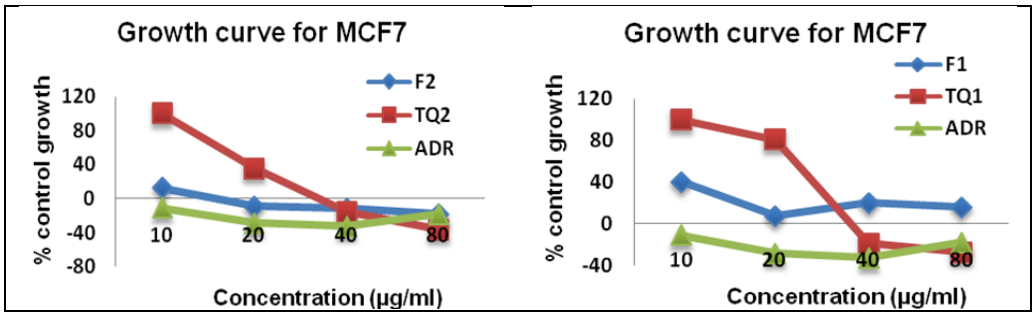


Figure 4: *In vitro* % cell growth inhibition of MCF7 by (a) F2, TQ2, ADR (b) F1, TQ1, ADR

In vitro Activity of Wehi 164

The *in vitro* activity of plain TQ and its formulations in Wehi 164 are displayed in Figure 5. Results showed that the growth inhibitory concentration (GI 50) for plain TQ (TQ1) was 61.8 µg/mL, while the formulation (F1) exhibited GI 50 less than 10 µg/mL. Thus, the formulation meant for oral administration exhibited a very good activity in Wehi 164 cell line as evidenced by the significant reduction of the GI 50 as compared to plain TQ. Further, in case of F2, the GI 50 was less than 10 µg/mL. This showed that the formulation F2 also exhibited enhanced activity as compared to plain TQ as evidenced by reduction of GI 50 when compared to plain TQ. Thus, both the formulations F1 and F2 displayed better inhibitory effect on Wehi 164 as compared to plain TQ.

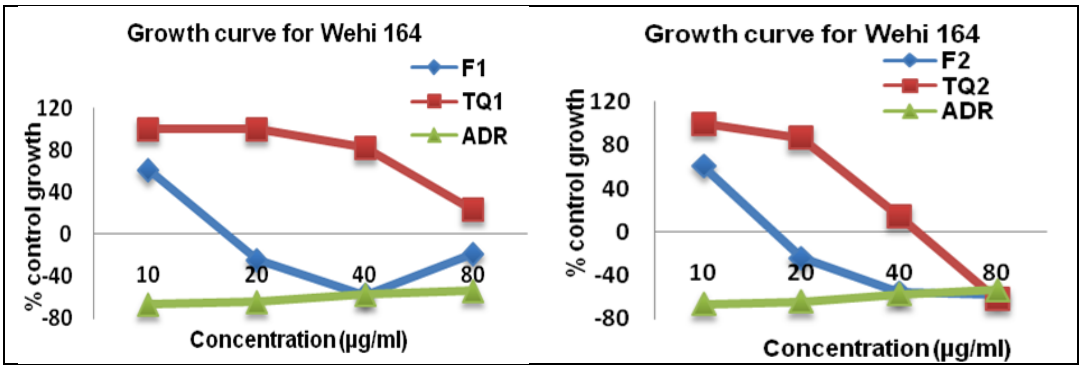


Figure 5 *In vitro* % cell growth inhibition of Wehi 164 (Mouse fibrosarcoma cells) by TQ1 plain and its formulation F1 and TQ2 plain and its formulation F2

In vivo Antitumor Activity

In order to evaluate *in vivo* antitumor activity, MCF7 cells were injected subcutaneously into ICRC mice. Evidently, tumors developed with average relative tumor volumes (RTV) of 244.3 mm^3 on day 35 in the control group (Figure 6). Treatment of mice with TQ1 and TQ2 and their respective formulations F1 and F2, five days after tumor cell inoculation significantly retarded the growth of these tumors. The RTV values for plain TQ1 and its corresponding formulation F1 were 21.1 mm^3 and 6 mm^3 respectively, whereas for plain TQ2 and its corresponding formulation F2 the RTV values were 231 mm^3 and 73.2 mm^3 respectively.

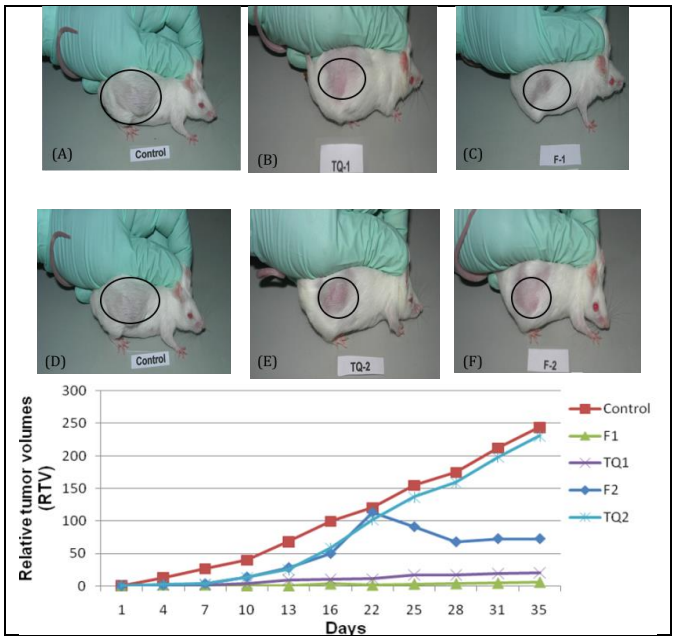


Figure 6: Inhibition of tumor growth by TQ1 and TQ2 and its formulations (F1 and F2) in mouse spontaneous mammary tumor model. (A) Control (B) Plain TQ1 (90 mg/kg) (C) F1 (D) Control (E) Plain TQ2 (10 mg/kg) (F) F2

Similarly, murine fibrosarcoma cells were injected subcutaneously into swiss mice and tumors developed with average relative tumor volumes (RTV) of 260.3 mm^3 on day 35 in the control group. Treatment of mice with TQ1 and TQ2 and their respective formulations F1 and F2, five days after tumor cell inoculation significantly retarded the growth of these tumors (Figure 7). The RTV values for plain TQ1 and its corresponding formulation F1 were 190.1 mm^3 and 68 mm^3 respectively, whereas for plain TQ2 and its corresponding formulation F2 the RTV values were 214 mm^3 and 175.3 mm^3 respectively.

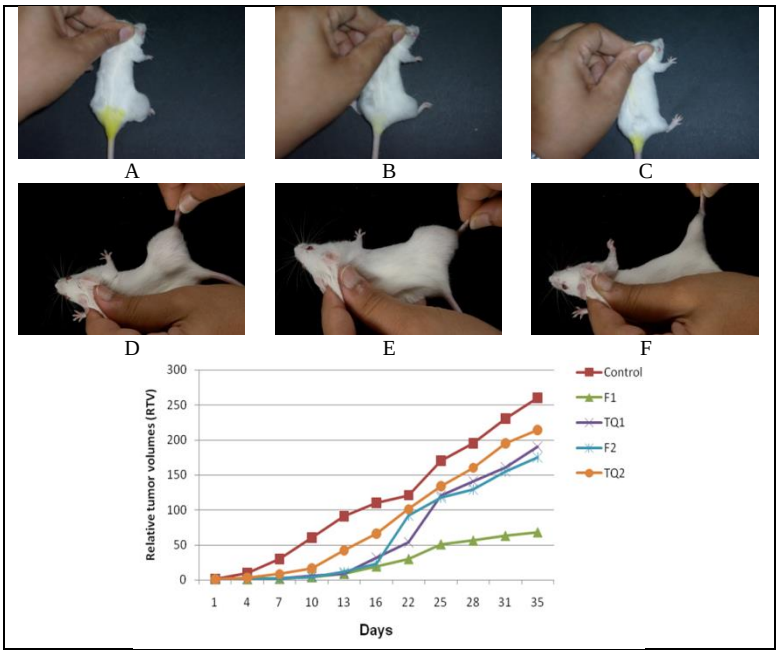


Figure 7: Inhibition of tumor growth by TQ (TQ1 and TQ2) and its formulations (F1 and F2) in murine fibrosarcoma. (A)Control (B) Plain TQ (90 mg/kg) (C) F1 (D) Control (E) Plain TQ2 (10 mg/kg) (F) F2

Thus, the formulations F1 and F2 were effective in reducing the tumor volumes in mouse spontaneous mammary tumor and murine fibrosarcoma tumor models as displayed by the low values of RTV as compared to their respective plain TQ controls.

CONCLUSION

Formulations of TQ-microemulsions for oral and parenteral administration were prepared and characterized based on their solubility, shape, size, zeta potential and stability to dilution and various buffer systems. The mean globule sizes of the oral and parenteral TQ-microemulsions were measured on a nanometer scale and measurement of the polydispersity indexes are indicative of unimodal distributions in both formulations. Oral and parenteral formulations showed negative zeta potentials which is representative of their relative stabilities. The mean globule size of the oral formulation in various buffers remained constant for 6 h whereas the parenteral formulation was found to be stable for 72 h in in various isotonic solutions. Both formulations were determined to be stable upon 1000-fold dilution for 6 h. The *in vitro* anticancer activity of TQ and its microemulsion formulations was determined by measuring percent growth inhibition (GI 50) in MCF7 and Wehi 164cell lines. The reduction in GI 50 values observed for formulations is indicative of superior cellular uptake of TQ in formulations as compared to plain TQ. Oral and intravenous administration of TQ-microemulsions into mice transplanted with MCF cells and mice transplanted with murine fibrosarcoma cells showed significant reduction in relative tumor volumes (RTV) compared to plain TQ, highlighting the potential utility of microemulsion formulations in the delivery of TQ for the treatment of solid tumors.

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