Understanding the mechanistic aspect of Thymoquinone in breast cancer by employing different nanocomposites

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Vishal Dineshchandra Mistry

30th December 2016
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# Table of contents

List of figures xi  
List of Tables xvii  
List of Abbreviations xviii  
Abstract xix  

## Chapter 1: Introduction

1.1 Cancer ................................................................. 1  
  1.1.1 Current statistics ................................................. 1  
  1.1.2 Types of cancer .................................................. 2  
  1.1.3 Causes of cancer .................................................. 3  
1.2 Methods of cancer therapy ........................................... 3  
  1.2.1 Surgery .......................................................... 3  
  1.2.2 Radiotherapy .................................................... 4  
  1.2.3 Chemotherapy .................................................. 5  
1.3 Drawbacks of current cancer therapy ................................ 6  
1.4 Complementary or alternative therapy ............................ 7  
1.5 Phytochemicals .................................................... 8  
1.6 Thymoquinone (TQ) .................................................. 11  
1.7 Thymoquinone as anticancer agent ................................ 12  
  1.7.1 Anti-proliferative effect of TQ ................................. 12  
  1.7.2 Effect of cell cycle ............................................. 13  
  1.7.3 Apoptotic effect of TQ ........................................ 13  
  1.7.4 Oxidative stress ................................................ 15  
  1.7.5 Synergistic effect of TQ with chemotherapeutic drugs .......... 16  
1.8 Limitations of Thymoquinone ..................................... 17  
  1.8.1 Solubility ....................................................... 17  
  1.8.2 Stability and degradation of TQ ............................ 18  
  1.8.3 Photolytic degradation of TQ ................................ 19  
1.9 Rationale ............................................................ 20  
1.10 Thesis outline ...................................................... 21
Chapter 2: Thymoquinone mediated lysosomal degradation of E-cadherin in breast cancer cells

2.1 Introduction..................................................................................................................38

2.2 Materials and methods.................................................................................................41
  2.2.1 Materials..................................................................................................................41
  2.2.2 Cell Viability............................................................................................................42
  2.2.3 Cell Morphology studies..........................................................................................43
  2.2.4 Immunofluorescence (Double Immunostaining).....................................................43
  2.2.5 Intracellular Ca\textsuperscript{2+} Measurements using fura-2 AM dye......................44
  2.2.6 Flow cytometry......................................................................................................45
  2.2.7 Chromatin Immuno Precipitation Assay (ChIP)....................................................46
  2.2.8 Real-time quantitative Polymerase Chain Reaction (qPCR).................................47

2.3 Results............................................................................................................................48
  2.3.1 TQ shows variable cytotoxicity in cancer cells......................................................48
  2.3.2 Thymoquinone mediated cytoskeleton focal adhesion reorganization in MCF-7 cells.........................................................................................................................53
  2.3.3 TQ promotes loss of E-cadherin from the membrane in breast cancer cells.55
  2.3.4 TQ mediated E-cadherin degradation is associated with calcium influx...........58
  2.3.5 TQ triggers the translocation of β-catenin into the nucleus and transcription of Cyclin dependent Kinase 2 (CdK2)..............................................................................58

2.4 Discussion......................................................................................................................60

2.5 Conclusion......................................................................................................................65

2.5 References.....................................................................................................................66
Chapter 3: Nanosuspension of Thymoquinone: Formulation, characterization and bio-evaluation

3.1 Introduction ........................................................................................................... 72

3.1.1 Nanosuspension .............................................................................................. 72

3.1.2 Precipitation ................................................................................................... 73

3.1.3 Milling .............................................................................................................. 74

3.1.4 Homogenization ............................................................................................ 74

3.2 Materials and methods ...................................................................................... 76

3.2.1 Preparation of nanosuspension .................................................................... 76

3.2.1.1 Nanoprecipitation method ...................................................................... 76

3.2.1.2 High pressure homogenization ............................................................... 76

3.2.1.3 Thymoquinone content determination ................................................. 76

3.2.2 Physico-chemical characterization .................................................................. 76

3.2.2.1 Differential scanning calorimetry (DSC) ............................................... 76

3.2.2.2 Fourier transform infrared spectroscopy (FTIR) ................................... 77

3.2.2.3 Colloidal Stability .................................................................................. 77

3.2.2.4 Storage Stability .................................................................................... 77

3.2.3 Biological evaluation ..................................................................................... 77

3.2.3.1 Cell culture ............................................................................................. 77

3.2.3.2 MTT assay ............................................................................................. 78

3.2.3.3 Intracellular Reactive Oxygen Species (ROS) estimation ..................... 78

3.2.3.4 Mitochondrial membrane potential (MMP) .......................................... 78

3.2.3.5 Apoptosis Assay .................................................................................... 79

3.3 Results and discussion ....................................................................................... 79

3.3.1 Preparation and physicochemical characterization of TQ nanosuspensions... 79
Chapter 3: Thymoquinone (TQ) and its derivatives: A potential therapeutic agent in breast cancer

3.3.2 Dose and time dependent cytotoxicity of TQ-NSP in breast cancer cell line. 83
3.3.3 Intracellular ROS generation. 88
3.3.4 Mitochondrial membrane potential. 90
3.3.5 Apoptosis Assay. 92

3.4 Conclusion. 94
3.5 References. 95

Chapter 4: Thymoquinone encapsulation in dendrimers enhances its efficacy towards breast cancer

4.1 Introduction. 100
4.2 Materials and methods. 102
4.2.1 Chemicals and materials. 102
4.2.2 Preparation of Dend-TQ complexes. 102
4.2.3 Physico-chemical characterization of Dend-TQ complex. 103
4.2.3.1 Determination of hydrodynamic radius and zeta potential. 104
4.2.4 In vitro drug release studies. 104
4.2.5 Stability studies. 104
4.2.6 Biological characterization. 105
4.3 Results and discussion. 105
4.3.1 Preparation of Dend-TQ complexes. 105
4.3.2 Physico-chemical characterization of Dend-TQ complexes. 106
4.3.3 In vitro drug release. 108
4.3.4 Stability studies. 109
4.3.5 Biological evaluation. 110
4.3.5.1 Determination of dose dependent cytotoxicity of Dend-TQ
complex in array of cancer cell lines.................................110

4.3.5.2 Intracellular reactive oxygen species (ROS) generation.........112

4.3.5.3 Mitochondrial Membrane Potential (DΨM)........................115

4.3.5.4 Apoptosis Assay.....................................................117

4.4 Conclusion......................................................................119

4.5 References......................................................................120

Chapter 5: Self-assembled nanostructures of triblock copolymer-
aspartate gold nanoparticles as TQ delivery vehicles with improved stability and therapeutic efficacy

5.1 Introduction......................................................................126

5.2 Materials and methods.....................................................129

5.2.1 Synthesis of P123 amino acid Au micelles.........................129

5.2.2 UV visible spectra .........................................................130

5.2.3 In vitro drug release........................................................130

5.3 Results and discussion......................................................130

5.3.1 Physicochemical characterization.....................................130

5.3.1.1 Dynamic light scattering and Zeta Potential....................130

5.3.1.2 UV-Visible Spectral Results........................................131

5.3.1.3 Transmission Electron Microscopy (TEM).......................132

5.3.2 Biological characterization..............................................133

5.3.2.1 Comparison of dose dependent toxicity of P123 Asp-Au TQ in breast cancer cell lines.........................................................133

5.3.2.2 In vitro drug release.....................................................136

5.3.2.3 Intracellular ROS generation........................................137

5.3.2.4 Mitochondrial Membrane potential (DΨM)......................139

5.3.2.5 Apoptosis Assay.........................................................141

5.4 Conclusion......................................................................144
Chapter 6: Comparative analysis of different drug delivery platforms for TQ

6.1 Introduction (re-visiting the problems of TQ) ................................................. 148

6.2 Materials and Methods ................................................................................. 149
   6.2.1 Calculating IC50 and IC80 for each of the nanoformulations ............... 149

6.3 Result and discussion ..................................................................................... 149
   6.3.1 Comparative analysis on the basis of physico-chemical characterisation..... 149
   6.3.2 Comparative analysis on the basis of cytotoxicity profile ....................... 150
   6.3.3 Flow cytometric analysis for E-cad expression in MCF-7 cells ............ 152

6.4 Conclusion ........................................................................................................ 156

6.5 References ........................................................................................................ 157

Chapter 7: Summary, conclusion and future prospects

7.1 Summary and conclusion ............................................................................... 159

7.2 Future prospects ............................................................................................... 164

7.3 References ........................................................................................................ 166

Appendix

Publications ........................................................................................................... 171
List of Figures:

Figure 1.1  Effect of phytochemicals at different stages of cancer. Phytochemicals have usually more than one target which makes it useful every different stage in cancer development. Diagram adapted with permission from Gonzalez-Vallinas et al, 2013

Figure 1.2  Seed and flowers of Nigella sativa (on the left) and chemical structure of thymoquinone

Figure 1.3  Time-line illustrating 4,000 years of Nigella sativa use in medicinal formulations which includes from the first documented N. sativa formulations made with honey in the Middle Hittite Kingdom to the explosion of published, scientific research over the last decade. Reprint with permission from S. Banerjee et al

Figure 2.1  Illustration of cadherin-catenin complex in association with actin cytoskeleton

Figure 2.2  Dose dependent cytotoxicity of thymoquinone in different cells lines viz. Human Lung cancer cells (A549), Human breast cancer cells (MCF-7 and MDA-MB-231), and prostate cancer cells (PC-3) after 24 h exposure. Cell viability was determined by MTT assay and reported as percentage of viable cells relative to control

Figure 2.3  Cell morphology studies on (a) MCF-7 and (b) MDA-MB-231, treatment with different test concentrations of TQ

Figure 2.4  Dose and time dependent toxicity of TQ on MCF-7 cells. Cell viability was determined by MTT assay and reported as percentage of viable cells relative to control. The data was presented as mean ± standard error (SE) of three independent experiments. Significant differences were determined by ANOVA on Microsoft Excel. The level of statistical significance was *p < 0.05 and **p < 0.001. The statistical significance was calculated between PC-3 and MDA-MB-231 since the viability profile appeared similar in the graph

Figure 2.5  Confocal micrograph depicting the E-cadherin aggregation due to TQ treatment at sub-toxic concentration (10 µM) in MCF-7 cells. The cells are fixed and stained with E-, vinculin (red) and DAPI for DNA (blue) after 2 h incubation with TQ. (Scale bars: 50µm)

Figure 2.6  E-cadherin is expressed as cell surface marker. In the Figure, green
highlighted boundary shows presence of E-cad in MCF-7 cells. While scale bars in left and middle micrograph correspond to 50µm, in right image it is 20µm ................................................................. 53

Figure 2.7 The graph obtained by flow cytometry analysis represents the mean fluorescence intensity of e-cadherin in MCF-7 cells after treatment with TQ along with an untreated control (black), 10 µM treatment (green) and 25 µM TQ (magenta) ................................................................. 54

Figure 2.8 Confocal micrographs of MCF-7 cells along with 10 µM TQ. The cells were stained with Hoechst stain for determination of population viability (Scale bars = 50µm) ........................................................................ 56

Figure 2.9 Localization of E-cad in the untreated and TQ treated (10 µM TQ) cells depicted in the confocal micrograph for MCF-7 cells. After 2 h of TQ treatment the cells were fixed and stained with E-cad antibody (green) and lysotracker (red). (Scale bars: 50µm) ........................................................................ 57

Figure 2.10 Calcium response to TQ treatment in MCF-7 cells. ATP was used as a positive control for calcium influx assay .............................................. 58

Figure 2.11 Fold change expression of CdK2 at 2 h and 8 h with respect to TQ treatment at 10 µM. The RlgG were used as negative control ................................. 59

Figure 2.12 Schematic representation of TQ’s mode of action in MCF-7 cells at sub-lethal concentration. Upon, treatment at sub lethal concentrations of TQ, destruction of cadherin–catenin complex occurs, that leads to lysosomal degradation of e-cadherin and translocation of unrestricted β-catenin into the nucleus. It combines with TCF/LEF in the nucleus to form a functional transcriptional complex for the transcription of genes responsible for cell proliferation and differentiation (EMT). ....................................................... 65

Figure 3.1 Schematic illustrating the bottom up and top down approach for synthesis of nanosuspension ................................................................. 73

Figure 3.2 Flow chart for preparation of drug nanosuspension using precipitation method ................................................................. 74

Figure 3.3 Physicochemical characterization of the TQ Nanaosuspension (a) Size distribution of TQ-NSPA and B (b) FTIR spectra of TQ, TQ-NSPA and TQ-NSP B (c) DSC graph of TQ, TQ-NSPA and B ................................................. 81

Figure 3.4 The graphs represent the stability studies performed with TQ-NSP A and
B. (a) colloidal stability (up to 24 hours) (b) storage stability (up to 60 days)

Figure 3.5 Comparison of cytotoxicity of TQ-NSPs in MCF-7 and MDA-MB-231 at three different time points (24, 48 and 72 h) produced by two different methods

Figure 3.6 Confocal micrographs of fixed cells treated with carboxy-DCFFDA to observe the ROS generation at different treatments in MCF-7 and MDA-MB-231 viz., control, pristine TQ and TQ-NSP B (Scale bar: 50 μm)

Figure 3.7 The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as relative oxygen species generated

Figure 3.8 Confocal micrographs of live cells treated with Rhodamine123 to observe the change in MMP at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and TQ-NSP B (d) (Scale bar: 50 μm)

Figure 3.9 The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as function of change in MMP

Figure 3.10 The flow analysis of Annexin-V FITC Assay for determination of apoptosis in MCF-7 and MDA-MB-231 with TQ and TQ-NSP B treatment for 24 h

Figure 3.11 Graphical representation comparing the ratio of apoptotic cells of TQ and TQ-NSP B in MCF-7 and MDA-MB-231

Figure 4.1 Drug loading in PAMAM dendrimers viz. G4 (4th generation dendrimer) with an amine tail

Figure 4.2 Physicochemical characterization of the TQ loaded dendrimers (A) UV/VIS - spectra of TQ and G4 PAMAM Dend-TQ (B) Size distribution of Dend-TQ complex (C) FTIR spectra of G4 PAMAM dendrimers (G4 Dend), TQ and Dend-TQ (D) NMR spectra of TQ, Dendrimer and Dend-TQ

Figure 4.3 In silico release of TQ from TQ solution and G4 PAMAM dendrimer-TQ complex (Dend-TQ) at 37 °C in PBS up to 24 h

Figure 4.4 In silico stability measured for TQ in solution and Dend-TQ stored at room temperature (25 ± 2 °C)

Figure 4.5 The graph shows cytotoxicity profile of TQ and Dend-TQ at different
concentrations after 24 h exposure, in four different cancer cell lines viz. human breast adenocarcinoma (MCF-7 and MDA-MB-231), lung carcinoma (A549) and human prostate cancer cells (PC-3)................................. 111

Figure 4.6 Confocal micrographs of fixed cells treated with carboxy-DCFFDA to observe the amount of ROS generated at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and Dend-TQ (scale bar: 50 μm) 113

Figure 4.7 The graph represents fluorescence intensities obtained from two samples viz. pristine TQ and Dend-TQ normalized against the untreated cells and plotted as function of change in ROS generation. Live cells were treated with Rhodamine123 to observe the change in MMP in different treatments: Untreated, pristine TQ, Dend-TQ................................. 114

Figure 4.8 Confocal micrographs of live cells treated with Rhodamine123 to observe the change in MMP at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and Dend-TQ (Scale bar: 50 μm) ...................... 116

Figure 4.9 The graph represents fluorescence intensities obtained from two different samples treated with TQ and Dend-TQ normalized against the untreated cells and plotted as function of change in MMP ......................... 116

Figure 4.10 Flow cytometry analysis of Annexin V/ FITC Assay for determination of apoptosis in MCF-7 and MDA-MB-231 with TQ and Dend-TQ treatment for 24 h................................................................. 118

Figure 4.11 Representation of a percentage of apoptotic cells quantitated by Annexin V-FITC/PI analysis of MCF-7 and MDA-MB-231 cells treated with pristine TQ and Dend-TQ for 24 h............................................................... 118

Figure 5.1 Structure of PEO-PPO-PEO tri-block copolymer ......................... 126

Figure 5.2 Synthesis route of Amino acid/Gold nanoparticles functionalized P123 micelles for TQ encapsulation.......................................................... 128

Figure 5.3 UV-Visible spectral data of P123-Amino acid, P123-Amino acid-Au nanoparticles (dotted lines) and after loading of TQ (solid lines)................. 131

Figure 5.4 TEM images of P123-Asp-Au, P123-Lys-Au and P123-His-Au micellar systems .......................................................... 132

Figure 5.5 Cell viability studies of the three different amino acid (aspartate, histidine and lysine) functionalized P123 micelles loaded with TQ for MCF-7(left) and MDA-MB-231(right)................................. 134
Figure 5.6  Dose dependent cytotoxicity of breast cancer cells due to TQ treatment after 24 h……………………………………………………………………………….. 136

Figure 5.7  In silico release of TQ from TQ solution, P123 Asp TQ and P123 Asp-Au TQ at 37 °C in PBS up to 24 h…………………………………………………………………………………….. 137

Figure 5.8  Confocal micrographs of fixed cells treated with carboxy-DCFDDA to observe increase in levels of ROS generation in MCF-7 and MDA MB-231: Control, Pristine TQ and Asp-Au TQ.  (Scale bar: 50 μm)……………………………………… 138

Figure 5.9  The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as function of increase in amount of ROS generated………………………………………………….. 139

Figure 5.10  Confocal micrographs of live cells treated with Rhodamine123 to observe the change in MMP at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and Asp Au-TQ (Scale bar: 50 μm)………………………………… 140

Figure 5.11  The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as function of change in MMP…………………………………………………………………… 141

Figure 5.12  The flow analysis of Annexin V FITC Assay for determination of apoptosis in MCF-7 and MDA-MB-231 with TQ and Asp Au-TQ treatment for 24 h…………………………………………………………………………………… 143

Figure 5.13  Graphical representation of percentage apoptotic cells (Annexin V +ve) with respect to different treatments…………………………………………………………………………………………………… 143

Figure 6.1  Dose dependent cytotoxicity of drug delivery systems viz. dendrimer (Dend-TQ), P123 based amino acid capsules (P123 Asp-Au TQ) and nanosuspension (TQ-NSP B) in (a) MCF-7 and (b) MDA-MB-231 cells after 24 h. Data are mean ± Standard error from three independent experiments……………………………………………………………………………….. 152

Figure 6.2  The bar graph represents the cell viability after treatment with in MCF-7 cells after treatment with TQ along with an untreated control, 10 μM TQ treatment (green) and at titrated TQ concentrations of the three nanoparticles viz. Dend-TQ (3.5 μM), TQ NSP-B (9.72 μM) and P123 Asp-Au TQ (4.25 μM). Data are mean ± Standard error from three independent experiments………………………………………………………………………………. 154

Figure 6.3  The flow cytometry graphs obtained by flow cytometry analysis
represents the mean fluorescence intensity of in MCF-7 cells after treatment with TQ along with an (a) untreated control, (b) 10 μM TQ treatment and at calculated TQ concentrations of the three nanoparticles viz. (c) Dend-TQ (3.5 μM), (d) TQ NSP-B (9.72 μM) and (e) P123 Asp-Au TQ (4.25 μM)
List of Tables:

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Effect of phytochemicals at different stages of cancer. Phytochemicals have usually more than one target which makes them useful at every different stages in cancer development. Diagram adapted with permission from Gonzalez-Vallinas et al, 2013,</td>
<td>10</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Table 2.1 Sample preparation for real time qPCR for 10µL reactions</td>
<td></td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Comparison of IC50 values (µM) between TQ and TQ-NSP on two different breast cancer cell lines after 24, 48 and 72 h</td>
<td>85</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>DLS and Zeta potential measurements of the P123 amino acid / Au NP functionalized micelles</td>
<td>130</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Comparison of different nanoparticulate systems used for delivering TQ</td>
<td>150</td>
</tr>
</tbody>
</table>
### List of Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aj</td>
<td>Adherens junction</td>
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<tr>
<td>ASP</td>
<td>Aspartate</td>
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<td>Au</td>
<td>Gold</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CCC</td>
<td>Cadherin-catenin complex</td>
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<td>ChIP</td>
<td>Chromatin immuno precipitation</td>
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<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic nucleic acid</td>
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<td>E-cad</td>
<td>E-cadherin</td>
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<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<td>Foetal bovine serum</td>
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<td>FTIR</td>
<td>Fourier transform infrared</td>
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<td>MCF-7</td>
<td>Michigan cancer foundation</td>
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<td>MMP</td>
<td>Mitochondrial membrane potential</td>
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<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>NSP</td>
<td>Nanosuspension</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TQ</td>
<td>Thymoquinone</td>
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</tbody>
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Abstract

Recently, phytochemicals have attracted attention due to their antioxidant, haemo-protective and anti-cancer properties. As compared to most Food and Drug Administration (FDA) approved cancer therapeutic drugs, phytochemicals have shown specificity towards cancer cells and have little or no adverse effects on healthy cells. One such phytochemical is Thymoquinone (TQ), a quinone based constituent in volatile oils of Black cumin. Its antioxidant and anti-inflammatory effect has been reported in various disease models including encephalomyelitis, diabetes, asthma and cancer. While TQ has been shown to affect cancer cell motility and migration, a distinct signalling pathway responsible for this effect has yet to be identified for the effect of TQ on motility and migration of cancer cells. The focus of this research was to elucidate the mechanism by which TQ interacts with the extracellular adhesion proteins of the cells involved in the motility, migration and cancer metastasis. The results revealed that TQ at low concentrations reduces the expression of e-cadherin (E-cad) which is a transmembrane protein responsible for cell-cell adhesion. The reduction of E-cad in cancer is a hallmark of epithelial to mesenchymal transition (EMT). During EMT, epithelial cells lose their adhesion proteins to detach from tumour, exhibiting mesenchymal phenotype, entering the blood, and metastasize to its secondary target site.

This poses a risk under in vivo scenario since TQ exhibits low bioavailability in the body and undergoes rapid elimination. Hence, the effective concentration of phytochemicals in the body are often much lower than when encountering the target cells in vivo. To address the issues of degradation, low bioavailability and low solubility of TQ in aqueous media, nano-based solutions were utilised. Three different types of nanoparticles were used to improve the therapeutic efficacy of TQ viz. dendrimers, nanosuspension and polymer-amino acid gold composite. All three nano-based systems showed significantly higher therapeutic efficacy.
compared to pristine TQ with increased toxicity due to increase in levels of reactive oxygen species and change in mitochondrial membrane potential level.

In order to understand that the pro-cancerous effect of TQ was due to its lower concentration; an experiment was conducted in which the nanoformulations were titrated on the basis of viability of TQ at a sub-lethal concentration. The results from this experiment suggest that low concentration of TQ is responsible for activation of EMT in cancer cells which can be potentially resolved by the use of different nanoparticulate based formulations to increase its therapeutic efficacy against breast cancer.
Chapter 1: Literature review

1.1 Cancer

Cancer is a disease caused by an uncontrolled division of abnormal cells in the body. Cancer is also sometimes referred to as neoplasm, which is defined as a new and abnormal growth of tissues in some parts of the body [1-3].

The word cancer was coined by the Greek physicians, Hippocrates and Galen, and originates from the Greek word Karakinos, which means crab. It then evolved to the Latin term cancer, which also means crab or now refers to a malignant tumour [4, 5].

In Australia, lung, prostate, colorectal, stomach and liver cancers are the most common types of cancer in men; while breast, colorectal, lung, uterine, cervix and stomach cancers are the most common among women [6].

1.1.1 Current statistics

According to the estimation by the GLOBOCAN project, a World Health Organisation (WHO) council initiative, in 2012, there were 14.1 million new cases of cancer, 8.2 million cancer deaths and 32.6 million people were suffering from cancer (within 5 years of diagnosis), worldwide [1].

In Australia, it was estimated that 130,466 new cases of cancer would be diagnosed by 2016. Every 1 in 2 males and 1 in 2 females will be diagnosed with cancer by the age of 85 years. During the census studies in 2013, it was found that there were 44,108 cancer related deaths in 2013, which is estimated to rise to 46,880 by 2016 [7].

According to this estimation, cancer is spreading at a rapid rate and eventually will be the leading cause of death in the world [6].
1.1.2 Types of Cancer

Cancer can be classified according to the organ and tissue type it is found in, and then based on grade, followed by stage. By these methods, there are four main types [1, 7, 8]:

- **Carcinomas**, which arise in epithelial tissue that is found in the internal and external linings of the body. There are 2 types of carcinomas – (i) **Squamous cell carcinomas**, which develop in the squamous epithelium of organs, including the skin, bladder, oesophagus, and lung and (ii) **Adenocarcinomas**, which develop in glandular structure in epithelial tissue.

- **Sarcomas**, account for less than 10% of all cancers, and arise from connective tissues that are found in bones, tendons, cartilage, muscle, and fat.

- **Leukaemia** is cancer of the blood that originates in the bone marrow due to which they are also known as liquid cancers since bone marrow is the site for blood production. During the cancerous stage, the bone marrow produces excessive amounts of immature white blood cells that are not functional, and in turn weakening the immune system.

- **Lymphoma** refers to cancers which develop in the lymph system. Unlike leukaemia, they are solid in state and develop at the lymph nodes in different parts of the body. They can be classified into two types as (i) Hodgkin’s lymphoma or (ii) Non-Hodgkin’s lymphoma depending on the presence of Reed-Stenberg cells.

Once a cancer is classified on the basis of organ or tissue type, it is further graded from 1-4 on the basis of increasing abnormalities of cancer cells [9].

Cancers can be also be individually differentiated on the basis of their stage. The commonly used method is based on tumour size (T) that includes increasing tumour size from T1- T4 , the degree of the spread or the node involved in the cancer (N) N1- N4, and finally on distant
metastasis (M) where M0 signifies no distant spread and M1 signifies the spread to secondary site [7, 10].

1.1.3 Causes of cancer

The causes of cancer are not very well defined as yet, but there are a number of reasons that have been attributed for cancer. Some of the proven risks include genetic instability, alcohol consumptions, smoking tobacco, poor diet, low physical activity and exposure to radiations [10-12].

1.2 Methods of Cancer therapy

The scenario of different approaches for cancer therapy has changed drastically in last few decades. The most common methods of treating cancer include surgery, radiation therapy and chemotherapy. Cancer therapy can be prescribed either before surgery (neoadjuvant chemotherapy) or after surgery (adjuvant therapy). In neoadjuvant therapy, the patient is treated with the chemotherapy/radiation/ hormone therapy before any surgery, so as to reduce the size of tumour, making it operable. Alternatively, adjuvant or additional therapy can be administered after surgical removal of the tumour, to ensure the remaining infected area is cleared of cancer cells. This procedure is performed in order to prevent the spreading or recurrence of the cancer [9]. Since none of the treatments are sufficiently effective to cure cancer by themselves, most treatment regimens involve a combined approach to increase the efficacy of the therapy.

1.2.1 Surgery

Cancer surgery is one of the oldest methods for cancer therapy. One of the main reason for surgery is to remove cancerous cells or tumour at the site within the body in order to prevent the spread of cancer to other parts of the body and restore the bodily functions back to normal [9]. Surgery is used for few different purposes which include[7]:

3
a) **Diagnostic:** Surgery is initially used to perform a biopsy in the patient, which is sent to a pathologist for cancer diagnosis. However, for determining the size or the amount of spread, staging is used, in which the doctor collects the lymph node near to the cancer for analysis.

b) **Primary surgery/ Tumour removal:** This type of surgery is most commonly used for removal of tumour completely to keep the surrounding cells healthy. Some of the side effects include pain, tenderness, scars and swelling.

c) **Debulking:** In this process, the surgeon is unable to remove the complete tumour or cancerous cells from the target site in order to avoid a large impact on the body. The remaining part of the tumour or cancer is treated with techniques such as radiation or chemotherapy.

d) **Palliation:** A tumour or cancerous sites in the body causes a number of side effects such as pain, bowel blockage and excessive bleeding. Hence, palliative surgery is used to relieve these side effects.

### 1.2.2 Radiotherapy

Radiotherapy involves the use of high ionizing electromagnetic radiations in order to shrink the tumours and eradicate cancer cells by preventing their growth and proliferation at the target site.

Radiotherapy can be delivered in two ways [6, 10, 13]:

a) **External radiotherapy:** Radiation beams are generated from a large external machine, also known as a linear accelerator, which is responsible for targeting the radiation towards a particular area of the body.

b) **Internal radiotherapy:** A radiation source is placed within the body, such as the intravenous introduction of a radioactive substance.
1.2.3 Chemotherapy

Chemotherapy is administered in cycles for short periods of time followed by recovery time which can last for up to 6 months. At every treatment the chemo dosage travels through the blood stream to its target site, where rapidly dividing cells either get arrested or turn apoptotic or necrotic in nature and eventually get destroyed. However, there are a number of side effects. The drugs cannot precisely distinguish between cancerous and normal cells thereby killing both populations. Hence, chemotherapy cycles include recovery periods in between treatments lasting for 3-6 months. Some general side effects include hair loss, loss of appetite, mouth sores, nausea, vomiting and low cell blood count [9, 14].

The most commonly used drugs are anthracyclines (doxorubicin/Adriamycin® and epirubicin) and taxanes (such as paclitaxel/Taxol®) in combination with 5’ fluorouracil (5-FU). The different kinds of drugs include [10]:

- Doxorubicin
- Docetaxel
- Paclitaxel
- Platinum based drugs (cisplatin and carboplatin)
- Capecitabine (Xeloda®)
- Vinorelbine (Navelbine®)
- Gemcitabine (Gemzar®)
- Liposomal doxorubicin (Doxil®)
- Albumin-bound paclitaxel (Abraxane®)
- Eribulin (Halaven®)
- Mitoxantrone
- Ixabepilone (Ixempra®)
Some other side effects can vary depending upon the kind of drugs being used and include neuropathy, permanent heart damage (cardiomyopathy), hand-foot syndrome and also increased risk of leukaemia due to bone marrow damage. Even though these drugs show such harmful effects, there are currently no effective alternatives for cancer treatment [7, 13, 15].

1.3 Drawbacks of current cancer therapies
Current anticancer chemotherapy suffers with following major drawbacks:

a) Non-specificity: A number of anticancer drugs available in the market are very effective at destroying cancer cells, but they cannot distinguish a cancerous cell from a normal cell. Hence, non-selectivity of anticancer drugs becomes an issue, leading to systemic toxicity and severe side effects, impacting the livelihood of the patient with increase in after care. Repeated doses of chemotherapy and radiation have the highest impact on the immune system leading to common illnesses, such as common cold and pneumonia [3, 9, 16]. Approximately 67% of deaths in cancer patients are due to infections arising as a result of the compromised immune system [1].

b) Drug resistance: With increase in anticancer drug usage, the cells have started to develop resistance against multiple drugs. This includes altered apoptosis regulation, increased DNA damage repair, alteration in metabolism, under expression of topoisomerase II or topoisomerase II gene mutations, decreased uptake of water-soluble drugs and increased energy-dependent efflux of hydrophobic anticancer drugs. The efflux is due to over expression of glycoproteins involved in the transport of drugs outside the cells. Due to this efflux, patients need to be exposed to high dosages of anticancer drug [17, 18].

c) Inability to cross protective lining of tissues: Anticancer drugs cannot cross the protective lining of some tissues. For example, the brain is well protected by the blood brain barrier (BBB)
and anticancer drugs are unable to cross the BBB to provide therapeutic effects in brain cancer [19, 20].

d) Aggregation or precipitation due to poor aqueous solubility: Most anticancer drugs developed recently such as taxanes and platinum based drugs have shown strong cytotoxicity against cancer cells. However, the major drawback of these anticancer compounds is lipophilicity and poor aqueous solubility which forms an obstacle for further formulation research and development for clinical purposes [4, 9, 21].

e) Short circulation half-life: Most commonly used anticancer drugs have a short half-life in the blood, even though they have strong therapeutic efficacy. Hence the amount of drug to be administered to the patient must be frequent, to improve the efficacy of the dosage. With the increase in dosage of the chemotherapeutic drugs, it would affect the overall well-being of the patient [3, 22].

1.4 Complementary or alternative therapies:
In order to avoid the issues surrounding current conventional therapies, people have started the use of complementary therapies or have opted for alternative therapies. According to literature there has been a rise in the use of complementary and alternative medicine (ECM) in Australia and around the globe [3, 10]. Complementary therapies are used as aides to the conventional therapies, by providing supportive measures to control the side effects, control symptoms and enhance overall well-being of the patient. Some examples of complementary therapies include healing, meditation, aromatherapy, relaxation, reflexology, etc. [23]. Alternative therapies, on the other hand, are used as a replacement to conventional treatments. Alternative therapies include modern techniques (such as homeopathy and ozone therapy) and traditional techniques such as naturopathy, acupuncture, traditional herbal medicines that include traditional oriental medicine, Ayurveda etc. These alternative remedies are known for their therapeutic effects on
a plethora of acute and chronic disorders which range from the common cold to various types of cancer. Since the last few decades, scientists have identified the active constituents in the plant based remedies as phytochemicals [11, 24, 25].

1.5 Phytochemicals

The word phytochemical can be divided into two as “phyto” and “chemical”. The word phyto is derived from a Greek word which means plants, hence they are chemicals derived from plants. These include flavonoids, carotenoids, phenolic compounds and sulphur rich compounds and an estimated five thousand phytochemicals have been detected for treatment of different diseases including cancer (Figure 1.1).

![Diagram of phytochemical effects on cancer stages](image)

**Figure 1.1** Effect of phytochemicals at different stages of cancer. Phytochemicals have usually more than one target which makes them useful at every different stages in cancer development. *Diagram adapted with permission from Gonzalez-Vallinas et al, 2013,[24].*

They have attracted attention due to their roles in the regulation of a variety of physiological functions, such as regulation of enzymes involved in cell metabolism and providing a defence
mechanism against microbial and viral infection. Epidemiological studies suggest that intake of fruits, vegetables and spices on regular basis can act as a source of various phytochemicals to cure diseases such as diabetes, cancer and inflammation [3, 25-30].

The biological activity of medicinal plants is due to the presence of these bioactive phytochemicals as components. They are produced by the plants as secondary metabolites. Most of these secondary metabolites are of little importance to the plants as they are a by-product of metabolic activities occurring within the plants. Sometimes they are required for specific function at certain developmental stages of the plant growth (Table 1.1). At times, they are also produced in response to damage or for protection against the environment conditions. For example resveratrol in red grapes and quercetin in onions can protect against environmental pathogens. Other functions include protection against environmental stress, rapid plant wound healing and as colouration of flowers, seed development and scent emission. Hence they are often produced only in small amounts [11, 16, 25, 28, 30].
Table 1.1 List of some bioactive components in plants and their putative mechanism of action in cancer cells

<table>
<thead>
<tr>
<th>Dietary source</th>
<th>Bioactive components</th>
<th>Putative mechanisms of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red grapes, red wine</td>
<td>Resveratrol</td>
<td>Modulation of cell signalling pathways, inhibition of angiogenesis, induction of apoptosis and type II programmed cell death</td>
<td>[3, 12, 31-33]</td>
</tr>
<tr>
<td>Turmeric</td>
<td>Curcumin</td>
<td>Induction of detoxifying enzymes, inhibition of COX-1, COX-2, iNOS, 5-LOX and PLA₂ enzymes, induction of apoptosis and down-regulation of β-catenin</td>
<td>[34-37]</td>
</tr>
<tr>
<td>Green tea</td>
<td>Catechins (EGCG)</td>
<td>Modulation of cell signalling pathways, inhibition of COX-2 and iNOS enzymes, anti-angiogenic, induction of apoptosis</td>
<td>[38-42]</td>
</tr>
<tr>
<td>Chilli peppers</td>
<td>Capsaicin</td>
<td>Modulation of cell signalling pathways; inhibition of phase I enzymes</td>
<td>[3, 24, 30]</td>
</tr>
<tr>
<td>Onions</td>
<td>Quercetin</td>
<td>Modulation of cell signalling pathways, inhibition of COX-2, 5-LOX, 12-LOX enzymes</td>
<td>[47-49]</td>
</tr>
<tr>
<td>Honey</td>
<td>Caffeic acid phenethyl ester</td>
<td>Induction of phase II enzymes, inhibition of inflammation</td>
<td>[50-52]</td>
</tr>
<tr>
<td>Broccoli</td>
<td>Isothiocyanates</td>
<td>Induction of phase II enzymes, modulation of cell signalling pathways, induction of apoptosis</td>
<td>[25, 53-57]</td>
</tr>
</tbody>
</table>
1.6 Thymoquinone (TQ)
Thymoquinone (TQ) is a phytochemical obtained from a well-known spice with various common names such as Black Seed, Black Cumin, Kalonji, Kalajira, and Roman Coriander [8, 43, 58-79]. The seeds are sourced from an annual herb, *Nigella sativa* which is native to the regions bordering the Mediterranean Sea, Western Asia and India. It belongs to the botanical family Ranunculaceae [58, 59, 64, 80, 81]. These seeds comprise of amino acids, carbohydrates, fixed and volatile oils, alkaloids and saponins to name a few. In 1963, El-Dakhakhny isolated a crystalline substance from the volatile oil, which was identified as Thymoquinone (Figure 1.2). Ghoseh *et al.*, (1998) demonstrated that this component is the major constituent (30-48%) of *Nigella sativa* [82]. A number of studies have tested this compound for its therapeutic effect in many diseases, including cancer, inflammation, atherosclerosis, diabetes and sepsis [82, 83, 89]

![Figure 1.2 Seed and flowers of Nigella sativa (on the left) and chemical structure of thymoquinone](image)

Figure 1.3 represents a timeline showcasing the major milestones in the history of TQ since its discovery. The first formulation of *N. sativa* was documented in 1650 B.C. in Turkey where the seeds were mixed with different bee product such as honey, wax, pollen and jelly. Since then, they have been of religious significance in many civilisations including Egyptian and Greek. The Greek physician Hippocrates showed the applicability of *N. sativa* oils for
treating digestive and liver disorders [58, 65]. Another Persian physician reported its use for stimulatory effects in 980 A.D. During 570-630 A.D. Prophet Muhammad had recognised the black seed as a powerful agent to cure most diseases except death [59, 66].

Thymoquinone is one of the chief constituent extracted from *N. sativa* along with the other components that include dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY). Its molecular weight is 164.2 g/mol and the chemical formula is C$_{12}$H$_{10}$O$_{2}$. The seeds also contain essential components such as carbohydrates, fat, vitamins, minerals, proteins and essential amino acids.

Figure 1.3 Time-line illustrating 4,000 years of *Nigella sativa* use in medicinal formulations which includes from the first documented *N. sativa* formulations made with honey in the Middle Hittite Kingdom to the explosion of published, scientific research over the last decade. Adapted from S. Banerjee et al. with permission [83]

1.7 Thymoquinone as an anticancer agent

1.7.1 Anti-proliferative effect of TQ
A promising strategy for cancer treatment is to attack the hyper-proliferating cells that give rise to the tumour. With respect to TQ, proliferation of mouse neoplastic keratinocytes was inhibited by as about 50% at non-cytotoxic concentrations. Later on it was found to inhibit various kinds of cancers which include glioblastoma (U87 MG and T98G, M059K and M059J), breast adenocarcinoma (multi-drug-resistant MCF-7/TOPO, MCF-7, MDA-MB-231 and BT-474), leukaemia (HL-60 and Jurkat), lung cancer (NCI-H460 and A549), colorectal carcinoma (HT-29, HCT-116, DLD-1, Lovo and Caco-2), pancreatic cancer (MIA PaCa-2, HPAC and BxPC-3), osteosarcoma (MG63 and MNNG/HOS) and prostate cancer (LNCaP, C4-2B, DU145 and PC-3) [14, 28, 40, 43, 58, 60, 63, 65, 66, 71, 77, 78, 84-108]. However, TQ showed little effect on the non-cancerous cells such as mouse fibroblasts, prostate epithelial cells, normal human intestinal cells and normal human lung fibroblast [58, 60, 95, 96]. The results from the above studies indicated that TQ only affects an array of cancerous cells while having insignificant effect on the normal cells.

1.7.2 Effect of TQ on cell cycle

Recent reports have demonstrated the impact of TQ on the cell cycle progression by arresting the cancer cells at different stages of cell cycle. Thymoquinone has demonstrated G0/G1 phase cell cycle arrest in papilloma carcinoma cells and HCT116 human colorectal carcinoma cell line and G1 to S phase arrest in LNCaP prostate cancer cells and in acute lymphoblastic leukaemia [109, 110]. A number of studies have also shown G2/M phase arrest in mouse spindle carcinoma cell, MNNG/HOS human osteosarcoma cells and in doxorubicin resistant breast cancer cells [62, 111, 112]. A sum total of all the above results suggest that TQ’s mode of action involves the cell cycle arrest as an important event in the anticancer mechanism.

1.7.3 Apoptotic effect of TQ
Apoptosis is the process of programmed cell death in multicellular organism. A multicellular organism is made up of highly organized community of different types of cells. The numbers of cells in the community are tightly regulated by two processes viz. controlled rate of cell division and controlled death rate [28, 113]. The controlled process is mediated by a cascade of intracellular proteins. When disruption of the programmed cell death occurs, it gives rise to uncontrolled proliferation of cells, which gives rise to cancer cells. Hence, apoptosis is a major event that is targeted by the chemotherapeutic drugs to cure cancer. Thymoquinone is known for its pro-apoptotic effect in cancer cells [52, 57, 60, 61, 63, 65, 66, 84, 96, 99, 114]. The induction of apoptosis in the cells occurs in numerous ways through various molecular targets and pathways. Thymoquinone induces apoptosis via both pathways viz. p53-dependent and independent pathway [76, 115]. The involvement of p53 was noted in some recent studies which showed resistance to TQ in case of apoptosis in HCT116 human colorectal cancer cells and p53-null myeloblastic leukaemia HL-60 cells [84, 116]. However, El-Mahdy et.al found TQ to induce apoptosis in p53-null HCT116 cells, simultaneously activating caspase 8, 9 and 3 as well as Bax/Bcl ratio. This finding suggested a p53 independent pathway for induction of apoptosis by using a caspase inhibitor z-VADFMK [117].

Further studies have observed the effect of TQ not only by p53 but also of other pathway such as signal transducer and activator of transcription 3 (STAT3), Phosphatase and Tensin homolog (PTEN) [77, 92]. Thymoquinone suppressed the proliferation of MCF-7/DOX, doxorubicin resistant breast adenocarcinoma cells, but also upregulated the PTEN expression levels in the cells [14]. Thymoquinone has also been able to show apoptosis in multiple myeloma cells, activating the caspases and PARP cleavage [118-120]. Inhibition of both constitutive and IL-6 inducible STAT3 phosphorylation was also observed in U266 cells.

Thymoquinone has accounted for apoptosis by supressing Tumour necrosis factor (TNF-alpha via NF-κb regulation) which includes IAP1, IAP2, Bcl-2, Bcl-xL, Survivin, and COX-2 cyclin
D1, c-myc, DΨM and vascular endothelial growth factor in KBM-5 in human myeloid cells [121]. The human umbilical vein endothelial cells and glioblastoma cells showed inhibition of VEGF-dependent ERK and Akt activation [20, 43, 73]. Thymoquinone simultaneously upregulated Bax and down regulate Bcl-2 and Bcl-xL, Mucin-4 in human pancreatic cells while suppressing the proteins survivin, XIAP and Mcl-1 via a proteosomal pathway [61, 98, 122]. Thymoquinone mediated apoptosis seen in SiHa (human cervical squamous carcinoma) cells was more potent compared to cisplatin [66, 123]. The highlights mentioned from the above recent literature suggest that TQ has an ability to induce apoptosis in the cancer cells through not only different targets but also through activation of different pathways.

### 1.7.4 Oxidative Stress

Almost all cancer cells are known to have elevated levels of reactive oxygen species [124]. The increased reactive oxygen species (ROS) are one of the causes that increase, to some extent, tumour development and cancer progression. A delicate balance of ROS levels is maintained intracellularly for cancer cell function [125, 126]. It appears to be a challenge for any novel therapeutic drug for targeting the intracellular levels of ROS. The strategy involves interference with the ROS levels so that the ROS-induced tumour progression is halted along with initiation of apoptosis.

Recent studies have revealed initiation of apoptosis in the cancer cells via the ROS production, rather than anti-oxidant/anti-inflammatory effects [4, 8, 46, 58, 85, 87, 101, 102, 106, 114, 122, 127-133]. Thymoquinone has exhibited increased levels of ROS leading to induction of apoptosis in primary effusion lymphoma cells [106]. It was further confirmed by addition of a strong antioxidant which reverses the effect of TQ, and promotes Akt activation, thereby preventing apoptosis. Another study conducted in DLD-1 human colon cancer cells showed the same TQ mediated apoptosis due to change in ROS levels and also supported that it could
be reversed upon use of N-acetyl cysteine [134]. In fact, one study showed that the application (pre-treatment) of N-acetyl cysteine could save prostate cancer cells (C4-2B) from TQ mediated apoptosis. In addition to this, Alhosin et al. established that TQ has an ability to generate ROS metabolites, which in turn activates mitochondrial membrane potential loss in Jurkat cells [84]. An interesting observation by Zubair et al. (2013), suggested that TQ like most phytochemicals can act as either an antioxidant or a pro-oxidant when used at low concentrations in in vitro studies. The author suggest that it acts as a pro-oxidant in presence of transition metal ions such as copper. Hence, TQ leads to increase or decrease in oxidative stress due to their dual mode of action [101]. The reason behind TQ’s ability to act as an antioxidant or pro-oxidant was suggested to be dependent upon the variations of TQ into hydroquinone or semiquinone in any given cell type [80, 82, 135-138]. Altogether the results from different studies indicate that ROS levels play a critical role in the TQ mediated apoptosis which leads to either anti-inflammatory/ anticancer activity.

1.7.5 Synergistic effect of TQ with chemotherapeutic drugs

Chemo-treatment for cancer is mostly based on single drug administration. Localized treatment involves surgery and/or radiation to remove the bulk of the tumour, followed by treatment with cytotoxic chemicals that kill both fast dividing tumoral and normal cells [7]. Many years of clinical research involving in vitro and in vivo models have shown that cytotoxic drugs when combined are more effective since they exhibit synergistic effects (such as combinatorial or additive effects) [132]. The rationale behind the use of two or more cytotoxic drugs as chemotherapy is to administer drugs such that they use different mechanism to kill the tumour cells thereby reducing the likelihood of drug resistance in the cells and improve its cytotoxic effect. A synthetic, natural or a biological agent that has an ability to reduce or delay the occurrence of malignancy at different stages in cancer is called chemopreventive agent.
However combining chemotherapy drugs can greatly increase adverse effects to normal cells [14].

This synergistic effect was also observed in the case of TQ, when combined with certain chemotherapeutic agents. Thymoquinone was found to increase the cytotoxicity when combined with cisplatin on non-small cell lung cancer cells (NCI-H460) [90]. Combination with TNF, paclitaxel or doxorubicin showed higher toxicity towards KBM-5 cells [97]. Earlier exposure of TQ to the pancreatic cells (HPAC and BxPC-3) could increase the cytotoxic effect of gemcitabine and oxaliplatin. It also leads to down regulation of Bcl-xL, survivin and XIAP proteins with increase in the amount of caspase-3 activity. However, it had no effect on human pancreatic ductal epithelial cells [61, 133]. Thymoquinone was able to increase the induction of apoptosis in U266 when combined with thalidomide and bortezomib [120, 139]. Thymoquinone also demonstrated sensitization across MCF-7 cells and T47D and increased cytotoxicity against radiation [86]. The above findings suggest that TQ could complement the chemotherapeutic drugs to achieve greater therapeutic effect in a clinical setting. When TQ was combined with 5-fluoro uracil for the treatment of the gastric cancer cells, it showed increase in apoptotic cells and also a regression pattern of growth was seen in gastric cell xenograft nude mice [105]. Thymoquinone was found to delay the growth of tumour cells in HCT116 cell xenograft mice and increased the induction of apoptosis shown by the TUNEL staining of tumour cells. In colon cancer induced by 1, 2 dimethyl hydrazine, it was found that TQ was able to reduce the number and size of aberrant crypt foci [140].

1.8 Limitations of TQ in cancer therapy

1.8.1 Solubility

The solubility of a substance may be defined as the maximum quantity of a substance that may be dissolved in a solvent. It is a key factor that is necessary for a successful formulation of most
anticancer drugs required for delivery to the target in the body. Thus, it is a challenge to increase the solubility of the phytochemical /drug due to its structural and chemical properties. The TQ solubility in different solvents was extensively studied by Salmani et al [135]. According to them, TQ solubility ranged from 549-669 µg/mL in all aqueous solutions. They concluded that observed profile of aqueous solubility was good enough to maintain effective therapeutics level of in vivo studies. Even though the solubility was observed to be increasing up to 72 hours in water, it was not possible for them to determine a value in extreme conditions of acid and base [135]. One of the possible reason suggested, was due to chemical/physical instability of the TQ in these solvents as, it may interfere with soluble fraction of TQ. Hence to further understand this, degradation studies were necessary to check the production of various degradation products in each of the solvents.

1.8.2 Stability and degradation of TQ

The stability of TQ in aqueous solutions was evaluated in 0.1 N HCl solution (acidic) and at three different pH viz. 5, 7 and 4 of phosphate buffer solution. In order to determine the stability of TQ, high performance liquid chromatography (HPLC) was performed to investigate the degraded products after 96 hours. The residual concentrations in all the different solutions showed time dependent decrease in stability of TQ and the HPLC chromatograms showed decrease in the area under the peak, with simultaneous rise in new degradation peaks post 96 hours. At acidic pH, TQ showed minimal amounts of degradation towards by-products [135]. Studies on rate of degradation predicted that the TQ showed similar patterns of degradation in water and saline solutions but slightly higher in phosphate buffer solutions and the highest was seen in the alkaline phosphate buffer. This was also accompanied by increase in the number and the intensity of the degradation peaks. The R² values suggested that, in acidic and basic conditions, TQ degradation tends to follow first order kinetics and that in pH 5, water, saline
and pH 7.4 followed second order kinetics. This means, the degradation of TQ in highly acidic and basic condition (0.1 N HCl and pH 9 respectively) depends upon the concentration of TQ, whereas in case of solutions of pH 5, water, saline, and pH 7.4 solution it depends upon concentration of TQ, pH or phosphate salts [135]. These factors play an important role in preparation of a successful drug formulation.

Finally, it was concluded that degradation in aqueous media poses a major problem as it is not possible to exactly quantify the amount of TQ in a drug release study. Therefore, there is a need of a suitable media which offers least degradation in aqueous conditions for improvement of formulation.

1.8.3 Photolytic degradation of TQ

Light plays a crucial role in the degradation of TQ. The study from Salmani et al. suggests that TQ is highly sensitive to light even for short period of exposure [135]. The results of their study suggest that almost all solvents showed 80% degradation of drug within 24 hours and 90% after 48 h. Shorter time points showed that rapid degradation < 70% occurs within 10 hours of incubation while following the second order kinetics which means that it is dependent on both the concentration and the type of solvent being used as well.

Furthermore, the study claimed that to test the purity of TQ solution, UV-visible spectroscopy was the best method. The results indicated that the initial spectral analysis revealed a peak at 257 nm which after 48 h incubation disappeared with rise of few more peaks at different wavelength other than 257 nm [135].

Under dark conditions, it was noticed that there was no significant degradation of TQ when monitored in either presence of ethanol or dimethyl sulphate with water or buffer in ratio 1:1 [135, 137-140]. This suggests the combination of either of the above mentioned would be a
good strategy for *in vitro* drug release studies for the formulation. However, all studies need to be performed in absence of light to avoid any photolytic degradation.

### 1.9 Rationale:

Recently, phytochemicals have attracted a lot of attention as being a natural source of antioxidants, chemo-protection and anti-cancer model drugs [141]. Thymoquinone is known to initiate apoptosis in several cancers such as prostate, breast, pancreatic, lung, etc. via different signalling pathways including PI3K/Akt and JAK-STAT [60, 102, 117, 120, 142-146]. The two effects that are well explored with respect to TQ in cancer are apoptosis and its antioxidant activity [8, 87, 104, 106-108, 120, 146-150].

The current study is based on two major objectives that are unexplored with regards to TQ. Firstly, TQ has been reported for decreased cell motility and migration in cancers cells. However, the exact mechanism by which TQ affects cell motility and migration is not well understood. Hence, this study was undertaken to unravel the pathway by which TQ affects motility and migration at cellular level.

Secondly, even though the therapeutic activity of TQ has been promising, there are some major drawbacks which hinder its biological activity and its bioavailability, such as poor solubility and rapid degradation in aqueous environment. This hampers the formation of successful therapeutic formulation of TQ. In order to obtain an efficacious formulation of TQ, it should remain protected until it reaches its target site and promote its uptake into the tumour cells. This problem may be solved with the help of a nanoformulation, allowing increased solubility in an aqueous environment.

A nanoformulation would prevent the degradation of TQ, which in turn would improve the pharmacokinetic profile of the compound. This would also protect the compound from the harsh aqueous environment, thereby, preventing degradation before reaching the target site.
These nanoformulations can also help to improve strategies for higher distribution and targeting for anti-tumour treatment. Nanocarrier can be designed for easy penetration into the cells by targeting them in a way that they selectively enter the tumour or into the stromal compartments of the cells.

Another advantage is the small size of the nanocarriers, which can increase the payload of the compound, thereby playing a pivotal role in improving the efficacy of the treatment. They can also be designed to release the compound only under some kind of stimulus. For example the most popular property of the tumour environment that has been exploited involves pH change. Nanocarriers can be designed such that they release their payload only under certain pH conditions.

Hence, the focus of this study is to use different nanoformulation with TQ, to test the increase in therapeutic efficacy by increasing its solubility and stability.

1.10 Thesis outline

Chapter 1 comprises of introduction and review of the literature related to the aim of this thesis. An in-depth discussion with regards to need for phytochemicals such as TQ in cancer therapy is provided. The chapter also includes the recent advances and efforts done to understand the anticancer effects of TQ.

Chapter 2 sheds light on a new biochemical pathway in order to understand the anticancer properties of TQ and in particular, to study the effect of low concentration of TQ in *in vitro* conditions. In order to addresses the problems due to low bioavailability and stability of TQ, use of 3 nanoformulations (3 different types) were proposed.
Chapter 3 includes the use of a 1st type of nanoformulation also known as nanosuspension. The nanosuspensions of TQ were characterised and evaluated for the increase in stability, thereby, increasing efficacy against human breast cancer cells.

In Chapter 4, a dendrimer based nanoformulation was introduced to encapsulate TQ to improve its aqueous solubility and stability. The phytochemical loading and release studies were also performed to investigate the improvement of efficacy in the cells.

Chapter 5 aims at exploring the self-assembled pluronic based polymeric micelles functionalized by a gold and amino acid nanoparticles as a drug delivery vehicle. The chapter investigates the effect of TQ encapsulated within the nanoformulation and study the possibility of increase in efficacy of the phytochemical.

Chapter 6 comprises of a comparative analysis of the all three nanoformulations, in order to choose the nanoformulation with best TQ efficacy. At the same time, it also discusses the effect of nanoformulations on pro-cancerous effects of TQ

Chapter 7 summarises and concludes the findings in this thesis along with some future prospects for continuation of this work.
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Chapter 2: Thymoquinone mediated lysosomal degradation of E-cadherin in breast cancer cells

2.1 Introduction

Breast cancer is the most common malignant disease in the western world. Although there has been a decrease in the rates of breast cancer due to early diagnosis by mammographic scanning and adjuvant therapy, chemotherapy itself has a wide range of acute and long term side effects which substantially affect the quality of patient’s life [1, 2]. Hence the focus is now shifting towards alternative therapies such as using metabolites from natural products. This alternative way of chemotherapy involves the use of natural (herbal) plant metabolites with lesser or no side effects. Thymoquinone (TQ, 2-isopropyl-5-methyl-1, 4-benzoquinone) is a bioactive component obtained from volatile oil of black cumin (Nigella sativa) and is a commonly used spice in traditional Middle Eastern medicinal formulations.

Recent studies in relation to the effect of TQ in different cancers have exhibited TQ as having antioxidant, anti-inflammatory, anti-neoplastic, anti-metastasis and anti-angiogenesis properties in vitro as well as in vivo [1-4]. Thymoquinone has generated great attention due to its pleiotropic nature i.e. it is specific to only the tumour cells without harming the non-cancerous (epithelial) cells [5]. Modern research claims TQ as a pro-apoptotic agent against several types of cancers such as prostate, breast, pancreatic, lung, etc. activating signalling pathways including PI3K/Akt and JAK-STAT [1, 6-12]. The two effects that are well explored with respect to TQ in cancer are its antioxidant activity and apoptosis [13]. Thymoquinone has been also reported for decreased cell motility and migration in cancer cells. However, the exact mechanism involved is not well understood.

Epithelial to Mesenchymal transition (EMT) is an integral process of development, wound
healing, stem cell behaviour, and a pathological contributor to cancer progression [14]. It is essential for changing the nature of cancer cells from non-metastatic to metastatic and is the phenomenon in which epithelial cells undergo biochemical changes and differentiate to give a motile mesenchymal phenotype. The process of EMT involves reorganization of the cytoskeletal architecture comprising of actin cytoskeleton and focal adhesion points which in turn is responsible for the stability of cadherin-catenin complex (CCC). The rearrangement of the CCC causes reduction in epithelial properties and an enhancement in mesenchymal properties such as increased migratory capacity, resistance to apoptosis and increased invasiveness [15]. EMT is achieved by the degradation of underlying basement membrane and the formation of a mesenchymal layer, causing the cells to drift away from the epithelial layer in which it originated and settle down at other favourable locations within the body [16]. From recent literature, it is known that adherens junctions (AJs), which are essential for preserving the integrity of the basal lamina of epithelial tissues, play a critical role in tissue formation and developmental stages of organisms as well as maintaining cell polarity and integrity [14]. During diseases such as cancer, the adherens junctions play a major role in deciding its nature i.e. non-metastatic to metastatic.

A major component of the adherens junctions are the cadherin proteins. Classical cadherins such as E-cadherin (E-cad) are transmembrane glycoproteins, which are necessary for Ca\textsuperscript{2+} dependent cell-cell adhesion and are among the traditional markers of an epithelial phenotype. Being a transmembrane glycoprotein, E-cads have an extracellular as well as intracellular part. The extracellular region of E-cad spans the cell surface and is usually involved in homophilic recognition by E-cad present on adjacent cells [17]. The intracellular cytoplasmic tail of E-cad binds to different cytosolic proteins like the \( \alpha \) and \( \beta \) catenins to form the CCC (Figure 2.1), which provides an anchorage to form stable cell to cell contact and are linked to fundamental intracellular processes like the actin cytoskeleton, cell signalling and vesicular trafficking [18].
Loss of E-cad has been implied in disruption of stability at adherens junctions at any given time in the cell. Destruction of AJs due to degradation of E-cad has not only been associated with loss of cell polarity leading to cell death, but this also activates the migratory machinery leading to EMT [16, 19]. Loss of E-cad releases the \( \beta \)-catenin, and this loss can be due to different reasons in the cell, like increase in the calcium influx leading to proteasomal degradation of the glycoprotein. Previous studies suggest that intracellular fate of internalized E-cad leads to its sorting for either degradation or recycling, which is a crucial step, as it eliminates existing AJs or re-deploys E-cad in formation of the new junctions.

\( \beta \)-catenin, when separated from E-cad, translocates to the cytoplasm and is eventually transported to the nucleus where it acts as a transcription factor in the canonical Wnt-signalling pathway [18]. Here it combines with T-cell factor/lymphoid enhancer factor (TCF/LEF) to form a transcriptional complex which up regulates the transcription of its target genes. Most of the targets of the \( \beta \)-catenin transcriptional complex are anti-apoptotic and lead to cell differentiation and proliferation, which in the case of tumours, make them more aggressive in nature [18].

The current study has been undertaken to understand the role of TQ and its potential as an anti-
cancer agent. Although there is no shortage of studies conducted that establish the toxicity and pro-apoptotic effects of TQ towards cancer cells at high concentrations [2-4, 6-8, 10, 12, 20-31], the exact mode of action of TQ remains elusive at sub-toxic concentrations. In real time, TQ concentration reduces as it has to overcome several barriers in the body in order to reach its target site. In an attempt to shed light on the biochemical pathways of TQ activity, this chapter will outline in clear and concise steps, the difference in the effects of low concentration of TQ against high concentrations of TQ on cell toxicity.

A coherent rationale has been postulated that sequentially links TQ with E-cad degradation and resultant β-catenin up regulation. The objectives underling this chapter are:

1. Determining the effect of TQ in an array of cancer cells.
2. Assessing the effect of sub-lethal concentrations of TQ on cancer cells.

2.2 Materials and Methods

2.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin 10,000 U/mL, TrypLE Express, Phosphate Buffered Saline (PBS), 5-(and-6)-carboxy-2’,7’-difluorodihydrofluorescein diacetate (carboxy-H₂DFFDA), Rhodamine 123, Ultrapure DNase and RNase free water, MTT purchased from Life Technologies, Mulgrave, Victoria, Australia. A stock solution of 1 mM carboxy-H₂DFFDA was prepared in ethanol and stored under nitrogen, at -20°C and in the dark. A stock solution of 1 mg/mL Rhodamine123 was prepared in methanol and stored at 4°C and in the dark. Foetal Bovine Serum (FBS) was purchased from Interpath Services, Heidelberg West, Victoria, Australia. Paraformaldehyde was purchased from Electron Microscopy Sciences, The Patch, Victoria, Australia. Working solution of 4% was prepared in PBS and
chilled to 4°C. Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), cell culture ethanol, propidium iodide (PI), Triton-X 100, phosphate buffer saline (PBS) tablets, cell culture grade chloroform and isopropanol were purchased from Sigma-Aldrich, Castle Hill, New South Wales, Australia. Cell culture wares such as 25 cm², 75 cm², and centrifuge tubes were purchased from Corning, Castle Hill, New South Wales, Australia. Tissue culture grade 6 well plates and 24 well plates were obtained from Nunc, Scoresby, Victoria, Australia. Pipette tips and eppendorf tubes were purchased from Eppendorf, North Ryde, New South Wales, Australia. The Primary antibody were purchased from Santa Cruz Biotech Pty Ltd which include: Mouse monoclonal E-cadherin (1:150 dilution); Rabbit polyclonal β-catenin (1:200 dilution); all the secondary antibodies conjugated with Alexa 488 (1:500) and Alexa 594 (1:700) and Lysotracker (Red-DND) were purchased from Thermofisher Pty Ltd., Australia.

2.2.2 Cell viability assay

The basic principle of the assay involves the conversion of the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), tetrazolium salts into purple formazan crystal by viable cells in culture. The formazan crystals are solubilized by the addition of acidified isopropanol or acidified sodium dodecyl sulphate and the resultant colour is measured in a spectrophotometer between 570-600 nm. The absorbance correlates directly with cell number. Briefly, the 10,000 cells were seeded in a 96 well plate, incubated overnight and treated with 5, 10, 25, 50, 100 µM TQ for predetermined time period of 24, 48 and 72 h for each cell line. A 10 µL aliquot of MTT (5 mg/mL) was added to each well and further incubated at 37°C for 4 hours. The medium was replaced with acidified isopropanol and plate absorbance was measured at 595 nm in Perkin-Elmer UV-vis spectrophotometer. The selected cell lines were treated with MTT, further to study at three different time points viz. 24, 48 and 72 hours.
2.2.3 Cell Morphology studies

The qualitative technique to confirm the cell viability assay is obtaining pictures at different time point after the introduction of the drug. MCF-7 and MDA-MB-231 cells (50,000 cells) were seeded in each well of six well plate. After overnight incubation, cells were treated with different test concentration and snapshot using phase contrast microscope fitted with Nikon CCD camera were taken at different time points.

2.2.4 Immunofluorescence

Immunofluorescence studies were used to determine protein levels in the MCF-7 cells. The cells were seeded in six well plate containing coverslips. They were treated with different concentrations of TQ and fixed after the stipulated time using 4% paraformaldehyde. The coverslips were washed twice with ice-cold PBS (1X) and subjected to either permeabilisation or blocked (without permeabilisation) with 2% bovine serum albumin (BSA) with 250 µL foetal bovine serum FBS. After the blocking step, the coverslips were treated with appropriate dilutions of primary antibody in 2% BSA/FBS for 1 h in humidified chamber. The coverslips were washed with chilled 1X PBS (containing 2% BSA/BSA) at least 3 times, followed by incubation with fluorophore-tagged secondary antibody for 30 min to 1 h in a dark humidified chamber. The coverslips were washed 2-3 times with PBS and then treated with Alexa Fluor 488® phalloidin (1:40), a high-affinity filamentous actin (F-actin) probe, for 20 min in dark humidified chamber at room temperature. The coverslips were thoroughly washed with PBS and mounted onto a glass slide using Prolong gold antifade mounting media containing Hoechst 333628 (nuclear stain). Finally the coverslips are sealed and stored at 4°C.

The confocal micrographs were imaged using Nikon confocal laser scanning microscope and analysed further using NIS Elements 4.0 (Advanced research) and a 60 X oil objective lens.
(Nikon) with an NA of 1.4 was used. Images were obtained using the Nikon CFLSM. Data shown are representative of three independent experiments.

For the Lysotracker Red-DND 1 mM probe stock solution was diluted to the final working concentration (50 nM) in the growth medium (DMEM). For the LysoTracker® probes, the recommended procedure involves the cells grown on the coverslip were added the pre-warmed (37°C) probe-containing medium. Cells were incubated for 30 minutes at 37°C. The loading solution was then replaced with fresh medium and cells were fixed and then regular immunostaining protocol was followed as above.

The primary antibodies purchased from Santa Cruz Biotech Pty Ltd include: Mouse monoclonal E-cadherin (1:150 dilution); Rabbit polyclonal β-catenin (1:200 dilution); secondary antibodies conjugated with Alexa 488 (1:500) and Alexa 594 (1:700) Lysotracker Red DND (1 mM Stock) were purchased from Thermofisher Pty Ltd., Australia.

2.2.5 Intracellular Ca$^{2+}$ Measurements using fura-2 AM dye

Activation of MCF-7 cells was assessed by monitoring variations in calcium levels using a Flexstation. Briefly, MCF-7 cells were seeded at 1 x10$^5$ cells/mL (0.2 mL/ well) in a 96-well plate for 24 h. Ratiometric measurement of calcium was achieved after loading the cells for 30 min with the membrane-permeant fluorescent indicator FURA-2AM (Molecular probes). Dye loading medium constituted with 0.25% Pluronic F-127 (Molecular Probes) and 10 μM FURA-2AM in flex assay buffer (137 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl$_2$ 2H$_2$O; 1 mM MgSO$_4$.7H$_2$O; 0.3 mM NaH$_2$PO$_4$. H2O; 0.3 mM KH$_2$PO$_4$; 10 mM HEPES) pH 7.4. Effectors to be tested were prepared in parallel in flex buffer and automatically added onto the cells during the assay. Measurements were typically carried out for 300 secs with a measurement every second. To obtain a baseline, fluorescence signals (excitation 340/380 nm - emission 510 nm) were measured for 30 sec in flex assay buffer prior to a washing step and addition of
compounds on the cells. Intracellular free calcium concentration (nM) was estimated using the equation of Grynkiewicz,

\[
[Ca^{2+}]_i = \frac{K_d \beta (R - R_{\text{min}})}{(R_{\text{max}} - R)}
\]

where \(K_d\) (apparent dissociation constant) was determined at 224 nM for FURA-2AM, \(\beta\) being the fluorescent ratio (EGTA 380 nM), \(R\) is the fluorescent ratio measured upon effector stimulation, \(R_{\text{max}}\) provided by adding 0.1% Triton X-100 on the cells after a washing step and \(R_{\text{min}}\) by the subsequent addition of 10 mM EGTA. Adenosine triphosphate (5 μM) was used as a positive control in the experiment. Each effector was tested in triplicates.

A separate drug plate was prepared and loaded in the Flex station (Molecular devices Pty Ltd) for further steps. The results obtained were analysed using the Soft Max Pro 5.4 (Molecular devices Pty Ltd).

2.2.6 Flow cytometry

The extracellular protein level of E-cad after TQ treatment to MCF-7 cells was determined using flow cytometry analysis. Briefly, MCF-7 cells were washed and dislodged using EDTA-sodium buffer from the 6-well plate and pelleted in eppendorf tubes. A detergent free medium was used for the dislodgement of cells in order to prevent damage on extracellular protein on the cells. Dislodged cells were washed and resuspended in FACS (Fluorescence activated cell sorting) buffer, followed by blocking with 10% FBS. The cells were treated with primary antibody mouse monoclonal E-cad (1:150 dilution) dissolved in the 2% BSA and incubated in a humid chamber for 1 h with respect to the E-cad antibody (1 μL in 200 μL suspension). After the designated time for incubation, the cells were washed at least 3 times. The cells were then resuspended in 200 μL of FACS buffer and treated with secondary antibody (Alexa Fluor 488: goat anti-mouse FITC (0.5 μL in 200 μL suspension). Finally the cells were washed and resuspended in FACS buffer.
Data was collected using BD FACS Canto II flow cytometer (Beckton Dickinson, USA). The data obtained for flow cytometer was analysed with help of FlowJo Software (Version 10.0.8r.1, LLC, USA). The number of events (cells) used for analysis were 20,000 cells. The computational analysis was performed using FlowJo Software (Version 10.0.8r.1, LLC, USA). A flow cytometry dot-plot was plotted using forward v/s side-scattered. The MCF-7 cells were then gated to include 10,000 events for each of the treatments (untreated, 10 μM and 25 μM ) to further plot the histogram

2.2.7 Chromatin Immuno Precipitation Assay (ChIP) Assay

Cells were fixed with 1% formaldehyde for 10 min. Cross-linking was arrested by adding glycine (0.125 M) for 5 min at room temperature. The cells were subsequently harvested in SDS lysis buffer (0.5% SDS, 100 mM NaCl, 50 mM Tris-Cl pH 8.1, 5 mM EDTA pH 8.0, protease inhibitor mixture Complete (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were pelleted and resuspended in IP buffer (2 volumes SDS lysis buffer:1 volume Triton-X buffer (100 mM Tris-Cl, pH 8.6, 100 mM NaCl, 5 mM EDTA pH 8.0, 5% Triton X-100)). The lysates were sonicated using BIORUPTOR sonicator for 12 cycles of 30 s and centrifuged at maximum speed. The sheared chromatin was diluted to 1 mL with IP buffer and pre-cleared with salmon sperm DNA/recombinant protein A-agarose (Thermo Scientific) for 2 h. One per cent of the sample was used as the input control and the remaining precleared chromatin was incubated overnight with 10 μg by incubation with salmon sperm DNA/protein-A agarose (50% slurry) and centrifugation. The samples were diluted with IP buffer to obtain the desired concentration (between 10 -50 μg per IP) with 1% of lysate was used as total control. The diluted chromatin lysate (1mL) was added to each safe lock tube containing the primary antibodies: β-catenin (dilution 1:20) and rat immunoglobulin (1: 1000). These safe locktubes were then incubated overnight on a shaking rotor at 4°C. The bead pellets were washed in low- or high-salt conditions (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0,
20 mM Tris-HCl pH 8.0, and 150 mM (low)/500 mM (high) NaCl). The beads were then washed once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA and 10 mM Tris-HCl pH 8.0) followed by two washes with Tris-EDTA. Elution buffer (0.1% SDS, 0.1 M NaHCO3) was added to the samples and the cross-linking was reverted by incubation at 65 °C overnight. Samples were incubated 1 h at 37 °C with RNAse A (Sigma) and 45 min at 50 °C with proteinase K (Ambion, Life Technologies). The DNA was purified using Minelute PCR Purification Kit (Qiagen) and then amplified by qPCR.

2.2.8 Real-time quantitative Polymerase Chain Reaction (qPCR)

Real-time quantitative PCR (qPCR) was performed using Fast SYBR® Green Fast Fast Gene Expression Assay on a 7500 Fast Applied Biosystems instrument. The experiment was set up as per manufacturer’s instructions. Samples were prepared as shown in Table 2.1. A total of 10 μL reaction mix was added to each well of a 96 well reaction plate, sealed and centrifuged briefly before loading into the instrument. Samples were run with two holding stages at 50°C for 2 minutes, 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were in triplicates and three independent experiments were performed. Gene expression of CdK2 is expressed as fold change or delta delta threshold cycle (ΔΔCT), the housekeeping genes GAPDH were used for normalization of qRT–PCR data, unless otherwise stated.

Table 2.1: Sample preparation for real time qPCR for 10μL reactions

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast SYBR® Green Master Mix (2X)</td>
<td>5.0</td>
</tr>
<tr>
<td>Forward and Reverse Primers‡</td>
<td>1.0</td>
</tr>
<tr>
<td>DNA (25ng)</td>
<td>2.0</td>
</tr>
<tr>
<td>Ultrapure RNase, DNase free water</td>
<td>2.0</td>
</tr>
</tbody>
</table>
2.3 Results

2.3.1 Thymoquinone shows variable cytotoxicity in cancer cells:

Initial investigation was based on dose dependent cytotoxicity of TQ on different cell lines which include Human lung cancer cells (A549), human breast cancer cells (MCF-7 and MDA-MB-231) and Prostate cancer cells (PC-3). To determine the dose response, the cells were pre-treated with different test concentrations of TQ in each cell line for 24 hours. MTT assay was used to determine the cell viability which was an endpoint assay. The assay depicted that the lung carcinoma cells (A549) showed marginal toxicity as they were highly resistant to the TQ concentration up to 100 µM. (Figure 2.2). The cell viability percentage at the highest concentration showed only about 20 % decline in cell population. The prostate cancer cells at lower concentration show proliferation of cells but with steady increase in dosage, it eventually lead to cell death. Amongst the four cell lines tested MCF-7 and MDA-MBA-231 cells (Human breast adenocarcinoma) showed the most sensitivity to TQ. The graph (Figure 2.2) suggests that TQ has been most effective in MDA-MB-231 followed by MCF-7 cells. Both the cell lines did not show much decline at the sub-toxic concentrations (5 and 10 µM) but there was a drastic decrease in the cell viability at higher concentrations causing almost complete clearance of cancer cells at 100 µM. Hence, breast cancer was chosen for further to study the effect of TQ.

In order to understand the effect of TQ phenotypically in both breast cancer cell lines, cell morphology assay was performed. Both cell lines viz. MCF-7 and MDA-MB-231 were subjected to TQ treatment with three different concentrations viz. after 12 h. Figure 2.3 below depicts MCF-7 cells untreated as well as spread with sharp edges. This is an indication of healthy cells. The cells treated with TQ showed change in morphology with increase in TQ concentration.
Figure 2.2 Dose dependent cytotoxicity of TQ in different cells lines viz. Human Lung cancer cells (A549), Human breast cancer cells (MCF-7 and MDA-MB-231), and prostate cancer cells (PC-3) after 24 h exposure. Cell viability was determined by MTT assay and reported as percentage of viable cells relative to control. The data was presented as mean ± standard error (SE) of three independent experiments. Significant differences were determined by ANOVA on Microsoft Excel. The level of statistical significance was *p < 0.05 and **p < 0.001. The statistical significance was calculated between PC-3 and MDA-MB-231 since the viability profile appears to be similar in the graph.

At 25 µM, the sharpness at the edges of the cells disappears making them appear with smooth edges. However, the cells are still well spread and adhered to the surface of a 6 well plate. This suggests the effect of TQ in extracellular region of the MCF-7 cells. Along with change in morphology it was also noticed that as soon as the media containing TQ was administered to the MCF-7 cells, they would immediately dislodge from bottom of the plate without any change in morphology of the cells. This suggests an indication that TQ has direct effect on the extracellular proteins of the cells. This effect was not visible in MDA-MB-231 cells. Upon
treatment of TQ in media the cells rounded off completely, thereby detaching from the surface of well.

![Figure 2.3 Cell morphology studies on (a)MCF-7 and (b)MDA-MB-231, treatment with different test concentrations of TQ](image)

Hence, there was a clear indication that TQ affects the adhesion properties of MCF-7 cells. It was chosen for further studies, as MCF-7 cells are slow migrating cells with higher expression of adhesion proteins. However, before initiating further studies, the right concentration needed to be chosen for investigating the effect of TQ at sub-toxic concentration.

In order to further understand the effect of TQ, MCF-7 cells were subjected to TQ for longer exposure periods (24, 48 and 72 h) presented in Figure 2.4. At 24 h time point the cells showed a steady decline in the viability. Higher concentrations such as 50 and 100 µM were the most effective concentration where the cell viability was negligible. However, this was not the case in the longer exposure periods. In case of lower concentration there were not any changes in the cell viability at 48 h. Most recovery of cells was noticed at the 72 h time point only. The result from the graph below reveals the recovery of cells at lower concentration of TQ after 72 h incubation. There is slight increase in viability at 5 µM, that further lead to a significant rise
at 10 µM by 22.3%. There was rise in viability at each of the concentrations of TQ, suggesting an overall recovery of cells in case of 72 h time point.

The aim of this project was to explore the mechanistic aspect of TQ in MCF-7 cells. Hence, the cytotoxic assay was performed to determine the sub-toxic concentration required to study the mechanistic aspects of TQ without killing the MCF-7 cells. The 10 µM concentration was chosen as the ideal concentration for further studies, because at 10 µM, the cell showed low toxicity at 24 and 48 hr along with significant recovery 72 hours. At this concentration, it would also help to understand the effect of low bioavailability of TQ in *in vitro* conditions.

Figure 2.4 Dose and time dependent toxicity of TQ on MCF-7 cells. Cell viability was determined by MTT assay and reported as percentage of viable cells relative to control. The data was presented as mean ± standard error (SE) of three independent experiments. Significant differences were determined by ANOVA on Microsoft Excel. The level of statistical significance was *p < 0.05 and **p < 0.001

In order to improve the understanding about the changes in the morphology of the MCF-7, the next task involved the investigation of cytoskeletal framework of the cells. Therefore, TQ treated cell were fixed and probed for actin and vinculin, the main cytoskeletal proteins
responsible for cellular shape, adhesions and migration. In order to stabilise the cell shape as well for migration, actin forms a circumferential network (CAF) around the inner periphery of the cells. These CAF are in turn linked to the cadherin catenin complex (CCC) stabilising them at the periphery. Upon TQ treatment, disruption of circumferential actin network is noticed in the MCF-7 cells (Figure 2.5) leading to reorganization of actin filaments.

![Untreated](image1.png) ![10 µM TQ](image2.png)

*Figure 2.5 Confocal micrograph depicting the E-cad aggregation due to TQ treatment at sub-toxic concentration (10 µM) in MCF-7 cells. The cells are fixed and stained with E-, vinculin (red) and DAPI for DNA (blue) after 2 h incubation with TQ. (Scale bars: 50µm)*

When closely observed the focal adhesion points also seem affected with TQ treatment. The focal adhesion points were highlighted using the vinculin antibody. The red spots towards the outer perimeter indicate the presence of Vinculin in the untreated sample which tends to reduce at the edges in MCF-7 cells when treated with TQ. This reduction and reorganization of the focal adhesion points is an indication of cells in migratory state [18, 32].

Probing the cytoskeleton shed some light on the morphological changes occurring in the cells at sub-toxic concentration in terms of cytoskeleton reorganization. Furthermore, it is essential to decipher the effect of TQ in the MCF-7 cells with respect to the adhesion protein especially the CCCs as the CAF is found to be disrupted. Further exploration on TQ’s mode of action on cell-cell adhesion complex (such as CCC) will help to develop an understanding behind detachment of cells from the substrate immediately after the TQ treatment.
2.3.2 Thymoquinone affects the cytoskeleton and focal adhesion in MCF-7 cells

Observations from the previous experiment revealed changes in the cytoskeletal architecture along with diminishing circumferential actin filaments (CAF) upon treatment of TQ. The presence of CAF requires the CCC to be located at the periphery. Hence it was necessary to investigate the involvement of CCC during TQ treatment. During treatment, the first exposure to the TQ containing media is encountered by extracellular adhesion proteins in the cells. As mentioned in the introduction, the CCC’s consists of the intracellular cytoplasmic tail of E-cad bound to the actin cytoskeleton via α and β-catenin.

As part of the initial investigation the levels of E-cad were checked qualitatively by subjecting MCF-7 cells to sub toxic concentration of TQ for 2 hours after which they were immunostained with E-cad antibody. The confocal images captured revealed a substantial decline in the E-cad levels (in green) at 10 µM (Figure 2.6).

Figure 2.6 E-cad is expressed as cell surface marker. In the figure, green highlighted boundary shows presence of E-cad in MCF-7 cells. While scale bars in left and middle micrograph correspond to 50µm, in right image it is 20µm

However, the cells at 25 µM, did not show much decrease in the level of E-cad. The results obtained clearly suggest that at sub-toxic concentration TQ might have a different mode of action.
In order to further validate the above result, qualitative studies were performed at both concentrations using flow cytometry. The cytometric graph (Figure 2.7) below represents the mean fluorescence intensity which consistently depicted two distinct populations of MCF-7 cells that includes E-cad expressing population (positive) and E-cad absent /low level population (negative). A significant decline in the mean fluorescence intensity of E-cad following the TQ treatment was visible at 10 μM, but this was not the case at higher TQ concentration (25 μM).

![Cytometric graph](image)

**Figure 2.7.** The graph obtained by flow cytometry analysis represents the mean fluorescence intensity of E-cad in MCF-7 cells after treatment with TQ along with an untreated control (black), 10 μM treatment (green) and 25 μM TQ (magenta)

The quantitative and qualitative results together validate that decline in E-cad is due to TQ especially at low or sub-toxic concentration. However, it is important to check if the cells were still viable with decrease in E-cad. Hence the next essential experiment was conducted to check cell viability. Before the cells were fixed, they were stained with Hoechst live cell stain. The dye selectively stained only the live cells. The results from the confocal micrographs (Figure 2.8) below suggest that at 10 μM, the viability was not affected; however there was distinctive decline in the E-cad levels in the treated cells.
2.3.3 Thymoquinone mediated lysosomal degradation of E-cad

Internalization of E-cad is followed by either recycling back to the lateral membrane or shuttled to the lysosome [33]. In order to investigate the fate of E-cad, MCF-7 cells were treated with 10 μM TQ and then stained with E-cad antibody and LysoTracker for 15 minutes. Figure 2.9 shows the three micrographs, one for each individual channel/ filter (FITC or Texas red) and an overlay image. The E-cad was highlighted in green and the lysosomes were highlighted in red. The overlay image obtained by merging the two individual images depicted an orange/yellow signal (co-localization). This is an indication of E-cad being localized within the lysosomes. Hence, suggesting the fate of E-cad to be degraded in lysosomes.

*Figure 2.8 Confocal micrographs of MCF-7 cells along with 10 μM TQ. The cells were stained with Hoechst stain for determination of population viability (Scale bars: 50μm)*
Figure 2.9 Localization of E-cad in the untreated and TQ treated (10 μM TQ) cells depicted in the confocal micrograph for MCF-7 cells. After 2 h of TQ treatment the cells were fixed and stained with E-cad antibody (green) and lysotracker (red). (Scale bars: 50µm)

From the above results, it can be established that TQ treatment at low/ sub-toxic concentration might lead to degradation of E-cad in the cells. Degradation of E-cad in cancer cells is a hallmark of epithelial to mesenchymal transition (EMT). E-cad degradation leads to the destruction of cadherin-catenin complex consisting of detachment from actin filaments and separation of components such as β-catenin and α-catenin that are stabilised by E-cad. Upon degradation of E-cad, β-catenin is known to relocate into the cytoplasm and either degrade or by localising in the nucleus, act as a transcription factor [18, 34-36]. The latter process helps in facilitating the survival or EMT in cells.
2.3.4 TQ mediated E-cad degradation is associated with calcium influx

Loss of E-cad is triggered by the calcium induced calcium influx resulting in translocation of β-catenin from the cell-cell contacts to nucleus.

Fura-2 AM assay was performed in order to investigate whether TQ could interact with calcium in the cellular context as E-cad expression has been reported to be linked with changes in intracellular calcium levels [36]. Fura-2 AM assay was performed in order to investigate whether TQ could interact with calcium in the cellular context as E-cad expression has been reported to be linked with changes in intracellular calcium levels [36, 37]. Fura-2 AM is a dye which enters the cell and measures changes in cytoplasmic calcium levels. Cells were grown in a calcium containing buffer, and then treated with different concentrations of TQ (1, 10, 25 µM) [38] and adenosine triphosphate (ATP). The mean fluorescence intensity of Fura-2 AM was measured at every second up to 50 seconds immediately after TQ treatment.

The graph below (Figure 2.10) depicts a delayed increase in intracellular calcium levels at 10 µM and 25 µM TQ (starting from 10 sec onwards). In contrast, ATP showed an immediate rapid increase in calcium signals. 1 µM TQ concentration was comparable to the control sample which showed no change in intracellular calcium levels. Calcium influx, which is a response to an increase in the calcium levels outside the cell (as caused by the calcium buffer), can be due to two possible reasons. First, an instantaneous influx can occur from the cell's own stores as this can be visualized by the addition of ATP which activates the calcium channels or it can be a delayed calcium influx which results from the entry of calcium ions across the plasma membrane [38]. As can be seen in Figure 2.10, presence of TQ showed a gradual increase instead of the instantaneous influx whereas control and 1 µM TQ did not show any significant influx in calcium levels. Hence, presence of 10 µM TQ has undoubtedly increased in
extracellular calcium influx, which is consistent with reports that stated the increase in extracellular influx, associated with destruction of cadherin-catenin complex [36, 39].

Figure 2.10 Calcium responses to TQ treatment in MCF-7 cells. ATP was used as a positive control for calcium influx assay.

2.3.5 TQ triggers the translocation of β-catenin into the nucleus and transcription of Cyclin dependent Kinase 2 (CdK2)

In order to investigate the translocation of β-catenin, Chromatin Immuno Precipitation (ChIP) assay was performed on MCF-7 cells. After the treatment of TQ (10 μM) the cells were fixed at different time point viz. 2 h and 8 h. In order to take into account a delay in the response to TQ, an 8 h time point was selected. The R IgG (human immunoglobulin generated in rabbit), was used as a negative control meaning it shows little or no response to the treatment in the cells. A small amount of response is observed in the graph (Figure 2.11) below which is considered as noise because most IgG antibodies are not obtained from true pre-immune serum from the same animal in which the specific antibody was raised.
CdK2 is a well-known target of β-catenin and the Figure 2.11 represents the fold change in expression of CdK2 in presence and absence of TQ. The increase in expression corresponds to increase in CdK2 gene transcription level; indirectly suggesting an increase in β-catenin levels in the nucleus. The results suggested, highest nuclear localization of β-catenin was observed at 2 h time point. According to literature, β-catenin is a transcription factor with multiple target genes that is stabilised by CCCs [15, 18]. The ChIP assay involves the probing of β-catenin bound chromatin complex. The chromatin from this complex is separated and is quantitated using target (CdK2) for qPCR. The function of CdK2 is well established as a cell proliferator in normal cells [38]. The higher expression of CdK2 is known as trigger to uncontrolled proliferation of cancer cells. The β-catenin being a transcription factor has CdK2 as its target during its localization in the nucleus [36]. This provided an insight into the mode of action for TQ. At low concentration of TQ, the E-cad complex that stabilizes β-catenin is dismantled and it eventually relocated into nucleus increasing the transcription of CdK2. This aberrant
expression of CdK2 has been labelled as one of the main causes for initiation of phenomenon known as epithelial to mesenchymal transition (EMT) [15, 18].

2.4 Discussion

Thymoquinone is a well-known quinone based natural phytochemical which forms an essential part of the traditional medicinal systems such as Unani and Ayurveda [2, 40]. Being pleiotropic in nature and finding its use in variety of ways for cancer and other therapies, it has attracted a lot of attention recently [4, 9]. Current literature claims TQ as a potential anticancer agent. However, there is very little understanding on the actual mode of action against cancer cells.

The aim of this study was to elucidate the mode of action of TQ in breast cancer cells at low or sub-toxic concentrations. Present literature has always showed TQ against cancer cells at higher concentrations that were very effective under in vitro conditions. However, this does not represent a true scenario as many phytochemicals do not graduate as anticancer agents, due to low bioavailability in the cells. In the in vitro environment, TQ comes in direct contact with the target cells, however, in the in vivo environment, it has to cross several barriers such as cell membrane permeability, low pH in stomach and insufficient time for absorption, thereby losing its activity due to degradation, before reaching the target site and this in turn leads to low bioavailability of TQ at the target site.

In this study, cytotoxicity assay on the arrays of cancer cells was performed in order to find the most sensitive cell line to TQ in a dose dependent manner. The aforementioned results showed that breast cancer cells viz. MCF-7 and MDA-MB-231 were most susceptible to TQ treatment, of which MDA-MB-231 was most sensitive. Both the cell lines originate from malignant adenocarcinoma and are obtained by pleural effusion [41, 42]. However, MCF-7 cells retain their characteristics of differentiated mammary epithelium and have the capability to grow in colonies in the form of domes [43]. The latter property makes it more resistant compared to
MDA-MB-231 cells that grow individually and do not form colonies or grow in domes, as well as having low amount of cell-cell adhesion proteins for attachment to the substrate. When TQ was administered in dose and time dependent manner, there was significant amount of recovery (by ~25%) in the MCF-7 cells at low concentrations of TQ after 72 hours of incubation. With higher concentrations the MCF-7 cell viability declined faster after 48 and 72 h, compared to 24 h. The reason behind recovery at lower concentrations and long exposure period could be due to the poor solubility, lower stability and photo-degradability of TQ that has been discussed in the detail in Chapter 1, section 1.8.

As shown in the Figure 2.2, the most significant rise in MCF-7 cell viability was observed at 10 µM concentration of TQ following 72 h incubation. Cell morphology assay at 10 µM concentration also showed significant changes in morphology of the cells. Change in cell morphology results from the reorganization of cytoskeletal proteins that determine its shape, size and adherence to the substratum (Figure 2.3).

According to Adam et al, cell-cell adhesion depends on clustering of cadherins and is associated with the organization of actin cytoskeleton [28]. The actin cytoskeleton was visualised by staining of fixed cells with a nuclear stain (DAPI) and F-actin fibres were labelled with conjugated phalloidin toxin. This highlighted the F-actin fibres supporting the cytoskeleton in confocal micrographs and revealed that at low TQ concentrations, disruption of circumferential actin network can be observed (i.e. conversion from F-actin fibre into its monomeric G-actin form) towards cell boundary (Figure 2.5). These results are in agreement with Bracke et al. who demonstrated that constitutive loss of the apical cortical actin ring results in components of the CCCs to get localized inside the intracellular vesicles [18]. The mechanism involves the conversion of cortin F-actin filaments (polymeric units) to G-actin (monomers). In this case, the cadherin components if intact move into Golgi vesicles where it gets recycled back to the surface of the cell or is degraded in the lysosomes of the cells [44].
The cells were also stained for cell adhesion protein known as vinculin. These results indicate that after treatment of TQ (sub-toxic) there was reduction and rearrangement of vinculin from the edge of the cells (Figure 2.5). The reduction of vinculin is usually associated with the poor adhesive properties of the cell as vinculin has long been identified as a protein involved in cell adhesion to substratum. Furthermore, studies by Hazan et al. (1997) discovered the association between vinculin and CCCs [45]. They suggested that vinculin plays a vital role in regulation of cadherin mediated cell adhesion by direct interaction with β-catenin. Another report regarding vinculin found that depletion of vinculin decreased the level of E-cad at cell surface via binding to β-catenin [45]. Hence these studies underline the dual role of vinculin in cell adhesion to substratum and cell–cell adhesion. From the above results, it is clearly observed that the actin cytoskeleton and vinculin, the two most important cytoskeletal proteins less expressed, which might be directly associated to CCCs (Figure 2.5). Hence, the effect of TQ needed to be examined on the CCC’s as they are in first line for exposure.

The results also reflected on the fact that E-cad’s adhesiveness is modulated by signalling events that dynamically influence vinculin binding to catenin. A well-established link between the loss of vinculin from adhesion sites and poor survival rates in patients suffering from cancers has been reported as well [17, 18, 28, 45, 46].

Upon treatment with TQ, E-cad was found to be degraded at later time points, activating a signalling cascade. A similar kind of pattern was visible at protein level when tracked using immunofluorescence. Results obtained from measurement of E-cad quantitatively (confocal) and qualitatively (flow cytometry) helped to establish this fact (Figure 2.6 and 2.7). This effect was particularly seen at 10 µM TQ (sub-toxic) concentration.

E-cad is a transmembrane protein existing as a complex which harbours the neighbouring cells. The loss of E-cad in cells is a hallmark of epithelial to mesenchymal transition (EMT) [47, 48].
The process of EMT serves many different purposes in growth and maintenance of individual organism [14, 16, 19]. However, its effect in the cancer progression is vital for metastasis. Recent literature suggests that EMT can also contribute to resistance in chemotherapy [49-51], which further indicates that use of TQ as chemo-sensitizing agent is not preferable. Thymoquinone being quinone, are known to be highly reactive in nature. The mutagenicity of certain quinones has already been shown by formation of reactive oxygen species and others via their transformation to DNA-binding semiquinone free radicals. This not only makes quinones mutagenic but potentially carcinogenic, but paradoxically they are also well known anticancer agents. In a study on quinone based phytochemical, Shikonin was reported as anticancer and anti-inflammatory agent, showing initiation of EMT activities along with suppression of micro-RNA required for wound healing [51]. The Chapter 1 section 1.7 discussed the dual nature of quinones especially TQ where it acts as pro-oxidant or antioxidant depending on the type of cell lines used i.e. it either generates reactive oxygen species or chelates the free radicals, respectively.

The extracellular span of E-cad consists of two heavy chains associated with calcium ions [18, 52]. However, this affects the CCC’s intracellular regions of E-cad such as β-catenin. The intracellular part of the transmembrane protein consist of the heavy chains connected to the p120, β-catenin and α-actinin attached to actin fibres providing the cell support and adherence to each other [18, 53, 54].

The intracellular part of E-cad is known to stabilize β-catenin while simultaneously providing the cell a backbone to remain attached to the neighbouring cell. β-catenin is a known transcription factor facilitating the transcription of multiple gene targets. Unrestricted β-catenin localization in the nucleus eventually forms a complex with LEF/TCF family and initiates transcription [15, 44, 47]. Increase in β-catenin localization into the nucleus is directly related to increase in transcription of target genes. Chromatin immuno-precipitation assay (ChIP) was
employed to determine the levels of β-catenin in the nucleus and the cytoplasm, which revealed the presence of β-catenin in nucleus after the treatment with TQ (Figure 2.12).

**Figure 2.12 Schematic representation of TQ’s mode of action in MCF-7 cells at sub-lethal concentration (10 µM).** Upon treatment at sub lethal concentrations of TQ, destruction of cadherin–catenin complex occurs, that leads to lysosomal degradation of E-cad and translocation of unrestricted β-catenin into the nucleus. It combines with TCF/LEF in the nucleus to form a functional transcriptional complex for the transcription of genes responsible for cell proliferation and differentiation (EMT).

β-catenin affects transcription of various cell cycle proteins, a prominent member of which is cyclin dependent kinase (CdK2). Among several targets of β-catenin, CdK2 showed promising results. The CdK2 plays an important role in the apoptosis (programmed cell death) and DNA damage repair especially during cell division [35, 36, 38]. Elevation in CdK2 levels leads to the activation from G1 to S checkpoint signalling. The G1 checkpoint plays an important role in preserving the genomic integrity of the cells along with prevention of S-phase transition for
cells with damaged DNA. CdK2 prevents the destruction of DNA leading to prevention of apoptosis [55]. The increase in the transcriptional level of CdK2 promotes proliferation, of cancer cells, thereby preventing cytotoxicity and in the case of cancer, promoting its aggressiveness (Figure. 2.12) [36, 39].

The extracellular span of E-cad consists of two heavy chains associated with calcium ions. Quinones are well known chelators [56, 57] and as mentioned in the results above, TQ showed association with the increase in the extracellular calcium influx (Figure 2.10). Hence this study proposes that TQ at sub-lethal concentration (10 µM) decreases E-cad levels via interaction with calcium ions on plasma membrane.

2.5 Conclusion

From the aforementioned results, it is reasonable to say that TQ may play a significant role in inhibiting cancer progression and metastasis in breast cancer cells at higher concentration but not at sub-lethal concentrations. At this point, TQ at sub-lethal concentrations depicts a pro-cancerous profile in the cells, where the MCF-7 cells upon TQ treatment initiate proliferation instead of cell death. Furthermore, the decrease in E-cad expression can activate EMT, which would enable cells to migrate to secondary sites in the body; conceding cancerous cells the power to metastasize.

In order to mitigate this issue, a suitable nanoparticle based approach for drug delivery to increase TQ’s stability may be chosen, which in turn would also enhance its therapeutic efficacy. In conclusion, our findings here not only help to contribute to the elementary understanding of TQ functions but also reveal novel strategic target to treat breast cancer.
2.6 References


Chapter 3: Nanosuspension of thymoquinone: formulation, characterization and biological evaluation

3.1 Introduction

From Chapter 2 of the thesis, it could be inferred that even though TQ showed promising cytotoxicity at higher concentrations, sub-toxic concentrations depicted a pro-cancerous profile. This effect makes the basis for hypothesis that the observed effect was due to lower stability of TQ in the medium. In order to address this, a nano-particulate system was suggested to be employed. This chapter deals with conversion of phytochemicals into novel nanoformulation in order to increase its stability and therapeutic efficacy.

3.1.1 Nanosuspensions

Nanosuspensions (NSPs) are sub-micron size colloidal form of dispersions of insoluble compounds that are stabilised using surfactants. Nanosuspensions have shown characteristics that include increase in stability and solubility which in-turn increases bioavailability [1, 2]. In addition, nanosuspensions can be used to disperse phytochemicals and/or drugs that are difficult to dissolve in both oil and water. The small particulate characteristics of nanosuspension have potential to change the pharmacokinetic profiles. Therefore, nanosuspensions offer exciting avenues to transform highly insoluble compounds which are often referred as “brick dust” during various drug discovery programs [3].

The top down technology (Figure 3.1) encompasses disintegration of larger particles into the nanosuspension with the help of high pressure homogenization and milling methods whereas the bottom up technology involves assembling method to form the nanoparticles like precipitation, melt emulsification and micro emulsion [3]. The two basic methods of nanosuspension formulation techniques used in the following study are: precipitation and homogenization.
3.1.2 Precipitation

This method is more commonly used method for preparation of nanosuspension from water insoluble compounds such as anticancer drugs and phytochemicals. It is a part of bottom-up technology that uses a drug already dissolved in an organic solvent [3, 4]. Firstly the phytochemical/drug is dissolved in the appropriate solvent. This mixture is then mixed with the hydrophilic medium (such as water) in which drug is insoluble in presence of a surfactant. This rapid process of addition of the mixture into the solvent and continuous stirring leads to quick supersaturating of the drug solution and it precipitates out of the solution to form ultra-fine amorphous or crystalline drug particles (Figure 3.2). One important factor in this method is maintenance of low temperatures to prevent phytochemicals/drugs from degradation [4]. The nuclei formation and crystal growth for a stable nanosuspension formulation is also dependent on the temperature. For a stable formulation, high nucleation rates and low crystal growth are required for small particle size [5].
3.1.3 Milling
During the process of wet milling, powdered phytochemicals/drugs are subjected to milling media for nanoparticle production. This process involves a milling chamber that is charged with the milling media or glass or zirconium beads/balls enclosed in highly cross-linked polystyrene resin or ceramic-sintered aluminium oxide filled with drug, stabilizer and either water or suitable buffer. The chamber is rotated at a high shear rate to generate suspensions. The high shear forces are generated as an outcome of impaction of milling media with drug or phytochemical to convert into nano-sized particles [3, 6].

3.1.4 Homogenization
This technique involves three major steps: First, the phytochemical is dispersed in a stabilizer solution to form pre-suspension; after that, pre-suspension is homogenized by high pressure homogenizer at a low pressure sometimes for pre-milling, this is done in order to reduce the particle down to micron size. At the final step, the suspension, which contains drug and stabilisers is passed through a valve with a ultra-small orifice under high pressure (10-25 cycles) to obtain desired particle size [3, 4].
In order to overcome the hurdle of low stability and bioavailability, nanosuspensions of TQ were prepared using two different methods viz. precipitation and homogenization were employed for this study. The production of thymoquinone based nanosuspension requires stabilisers that play an important role in maintaining the stability of the formulation. The most important characteristic of nanosuspension is its small particle size, which also is responsible for its physical instability. They consist of small hydrophobic particles (drugs or phytochemical) dispersed in a hydrophilic medium. Due to the large surface area of these nanoparticles, it results in high surface tension. This in turn results in increase in the free energy of the system which leads to aggregation or flocculation disrupting its stability [7]. Hence, in order to reduce this free energy on the nanosuspension, stabilisers are added. The different types of stabilisers include surfactants, polymers or a mixture of both. For example, Vitamin E/TPGS (D-α- Tocopherol polyethylene glycol succinate), sodium lauryl sulphate, Tween 80, hydroxypropyl methyl cellulose (HPMC), hydroxypropyl cellulose (HPC), and polyvinyl alcohol (PVA).

This study employs water soluble Vitamin E based stabiliser called D-α-tocopherol polyethylene glycol 1000 succinate that is obtained by the esterification of Vitamin E succinate with polyethylene glycol (PEG) 1000. It comprises of an amphiphilic structure consisting a hydrophilic polar head and a lipophilic alkyl tail. As mentioned above, it water soluble and has a low critical micellar concentration of 0.02% [7]. TPGS is well known non-ionic surfactant which has found its application in wetting, emulsification, detergency spreading and solubilisation [2, 3, 8].

This chapter details the synthesis of two nanosuspension formulation of TQ. The thymoquinone based nanosuspensions (TQ-NSPs) were characterized for the particle size, colloidal stability, \textit{in vitro} release and its biological activity against breast cancer cells.
3.2 Materials and Methods

3.2.1 Preparations of nanosuspension (Precipitation and homogenization method)

3.2.1.1 Nanoprecipitation method

A total of 25 mg of TQ was dissolved in 1 mL of ethanol and then dispersed in 0.25% w/v α-Tocopherol polyethylene glycol 1000 succinate (TPGS) aqueous solution. The mixture was sonicated at 45% amplitude for 1 min followed by stirring at 1000 rpm for 3 h. The solution was filtered through 0.22 µm syringe filter.

3.2.1.2 High pressure homogenization

Thymoquinone (TQ) (25 mg) was initially dissolved in chloroform (1 mL) and added to 0.25% w/v TPGS aqueous solution. The dispersion was homogenised using high pressure homogenizer for 20 min and then the organic phase was removed by rotary evaporation at 100 rpm and room temperature.

3.2.1.3 Thymoquinone estimation in nanosuspension

The drug content was determined by freeze drying both TQ-NSPs. Nanosuspensions were quickly pre-freezed using liquid nitrogen and then freeze-dried overnight at -50°C and pressure < 1 mbar. An amount (5 mg) of lyophilized powder was dissolved in 1 mL of methanol and then absorbance was measured at 260 nm using a UV-VIS spectrophotometer after appropriate dilution.

3.2.2 Physico-chemical characterization

3.2.2.1 Differential scanning calorimetry (DSC)

DSC is a thermo-analytical technique that measures the thermal transitions of a sample as a function of temperature against time [4]. DSC curves were recorded for TQ and TQ-NSPs using DSC (TA Instruments, USA). An accurately weighed (3.5 mg) amount of TQ and TQ-
NSPs were placed in aluminium pan and then scanned in a temperature range from 25° to 100°C with a heating rate of 5 °C/min.

### 3.2.2.2 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectral measurements of thymoquinone and nanosuspension formulations were performed using a PerkinElmer-FTIR spectrometer, with a resolution of 4 cm⁻¹, and 64 scans. Pure TQ and TQ nanosuspensions were mixed with the KBr to result in pellet and then examined for FTIR spectra with the scanning range of 400 to 4000 cm⁻¹ (Frontier Optica, PERKIN ELMER).

### 3.2.2.3 Colloidal Stability

For the determination of short-term colloidal stability, TQ-NSPs were stored at 4°C for 24 h and change in particle size, and drug content was determined up to 24 h.

### 3.2.2.4 Storage stability

For long term stability, lyophilized TQ-NSP were stored at 4°C for a period of 2 months and change in particle size, and drug content was determined using UV-vis spectrophotometer

### 3.2.3 Biological evaluation

#### 3.2.3.1 Cell culture

MCF-7 cells were maintained in DMEM media, while PC-3, MDA-MB 231 and A549 cells were maintained in RPMI 1640 media. All cell lines were cultured in 10% FBS with 100 units/mL penicillin and 100 µg/mL streptomycin. Cell lines were maintained at 37 °C with 5% CO₂.
3.2.3.2 MTT assay

Actively growing MCF-7, PC-3, MDA-MB 231 and A549 cells in their log phase of cell cycle were seeded into a 96 well plate at a concentration of 10,000 cells per well. Cells were treated with increasing concentrations of TQ or TQ NSPs ranging from 5, 10, 25, 50 and 100 µM for a period of 24, 48 or 72 h. The toxicity was assessed using the standard MTT assay, which analyses cellular viability by measuring the mitochondrial dehydrogenase activity due to reduction of yellow tetrazolium (MTT) to purple needle like formazan crystals. These crystals are solubilised in acidified isopropanol and measured by a UV-Vis multiwell plate reader at 570 and 630 nm.

3.2.3.3 Intracellular Reactive Oxygen Species (ROS) estimation

MCF-7 cells were seeded at a density of $1 \times 10^5$ cells per mL and allowed to grow for a period of 24 h. The cells were then treated with 10 µM TQ or Dend-TQ (as per the IC50 values for 48 h) for 24 h. ROS production was assessed using carboxy-H$_2$DFFDA. This compound gets oxidized to a fluorescent product in the presences of ROS within the cells. After treatment, cells were fixed in 4% paraformaldehyde for 10 minutes, washed with PBS (at least twice) and 5 µM of carboxy-H$_2$DFFDA was added to the cells and incubated for 30 minutes in the dark at room temperature. The cells were washed thoroughly and the levels of ROS generation were observed using a Nikon fluorescent microscope (Nikon Eclipse TS100, Japan) with a 475-490 nm excitation wavelength.

3.2.3.4 Mitochondrial membrane potential ($\Delta$ΨM)

$\Delta$ΨM was assessed using Rhodamine 123 after MCF-7 cells were treated with increasing concentrations of TQ and Dend-TQ for 24 h. About $1 \times 10^5$ cells were seeded in a 12 well plate and incubated at 37 °C overnight, in presence of 5% CO$_2$. The cells were washed with warm PBS and treated for 30 minutes with 5 µg/mL of Rh123 after which the plate was observed
using a Nikon fluorescent microscope (Nikon Eclipse TS100, Japan) with a 475-490nm excitation wavelength. For quantitative analysis the cells were seeded at same concentration as above but after treatment, the cells were washed with PBS and trypsinized and collected in 1 mL eppendorf tubes. The cells were then checked for fluorescence at excitation of 480 nm and emission at 490–700 nm. The graphs were plotted as a function of relative mean fluorescence intensities.

3.2.3.5 Annexin V-Propidium iodide analysis

The cells were analyzed for apoptosis by using the Annexin-V FITC and evaluation was done using flow cytometry. Briefly, MCF-7 cells were seeded in 6-well plate at the density of 1x 10⁶ cells per well and incubated overnight at 37 °C at 5% CO₂. The cells were dislodged and washed twice with ice cold PBS. Finally the cells were resuspended in 1X Annexin binding buffer and incubated with 5 μL of Annexin-V FITC and 1 μL of propidium iodide (5 mg/mL) for 15 minutes in dark at room temperature. The cells were then analysed on the BDS Canto II flow cytometer by measuring fluorescence at 575 nm emission and 488 nm excitation. The data was analyzed with help of FlowJo software (Tree Star Inc., Ashland, US).

3.3 Results and discussion

3.3.1 Preparation and physicochemical characterization of TQ nanosuspensions

Nanosuspension has emerged as an important technology in the development of the formulation of water insoluble drugs. It reduces the particle size of the solid drug below sub-micron, typically 5-50 nm. This enhances the solubility of the drug/phytochemical [9]. The small size is responsible for reducing the rate of sedimentation of the particles and hence increases the physical stability. In this study, TQ-NSPs were synthesized and stabilized using TPGS by both bottom-up and top-down approaches.
In the precipitation method, TQ was dissolved in ethanol and dispersed in TPGS surfactant solution. The dispersion was required to control the process of nucleation and accelerate molecular diffusion. The organic solvent (ethanol) was then evaporated by magnetic stirring to precipitate the phytochemical in its nanoparticle form. The presence of TPGS in the aqueous phase inhibited the agglomeration of nanoparticles (TQ-NSP A).

In homogenization approach, TQ was dissolved in chloroform and then emulsified in TPGS solution. The formed coarse emulsion was homogenized to form nano-emulsions. In this approach, the chloroform was evaporated by rotary evaporation to give nanosuspension of TQ in TPGS solution (TQ-NSP B).

Both TQ-NSP A and TQ NSP-B, were evaluated for particle size, polydispersity index PDI and surface charge using Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK). The observed particles size for TQ-NSP A and TQ NSP-B was found to be 12.7±2.12 nm and 11.2±2.2 nm, respectively (Figure 3.3). Zeta potential represents the surface charge of a nanoparticle which depends upon the chemical properties of the coating or dispersing agent. There was no significant difference in the zeta potential of both nanosuspension formulations. The observed zeta potential values for TQ-NSP A and TQ NSP-B were -15.8 and -16.3 mV, respectively. Since as dispersing agent, TPGS (0.25 % w/v) was used for the preparation of both nanosuspensions, a similar zeta potential is observed for both the suspensions. The polydispersity index (PDI) of TQ-NSP A and TQ NSP-B was 0.271 and 0.195 respectively. The results revealed that TQ NSP-B had smaller particlesize along with lower PDI in comparison to TQ-NSP A.
Figure 3.3 Physicochemical characterization of the TQ Nanosuspensions (a) Size distribution of TQ-NSPA and B (b) FTIR spectra of TQ, TQ-NSPA and TQ-NSP B (c) DSC graph of TQ, TQ-NSPA and B

In the Figure 3.3 (b) FTIR spectral studies of TQ and nanosuspension formulation of TQ were carried out to investigate the interaction between the functional groups of TQ with the nanosuspension. FTIR spectral analysis of TQ vibrational modes in its native form and its nanosuspension form provide insight into its structural integrity and retention of functional group in the nanosuspension form. Figure 3.3 (b) showed the FTIR spectra of TQ and its nanosuspension forms. Pure TQ showed a strong vibrational peak at 1637 cm$^{-1}$, which
corresponds to the –C=O group vibration. However, this carbonyl group was shifted to 1747 cm\(^{-1}\), which may be probably due to confinement of nanoparticulate TQ within the suspension that restricts the keto-enol tautomerism. Moreover, the intense band at 2967 cm\(^{-1}\) corresponds to C-H stretching of aliphatic group, but this peak was shifted to 2925 cm\(^{-1}\) and a broad O-H stretching centered around 3400 cm\(^{-1}\). This was probably due to the interaction of aliphatic chains interaction with the hydrophobic interior of nanosuspension. Appearance of new O-H broad band in the nanosuspension was attributed to the hydrogen bonded interaction between the keto oxygen with the solvent hydrogens. From the FTIR analysis, it is clearly shown that TQ entrapped within the nanosuspension without any modification of TQ itself. The nature of interaction between TQ and nanosuspension are mainly the hydrophobic and hydrogen bonded interaction, which was concluded from the shift in the vibrational frequencies correspond to the TQ functional groups. Therefore, TQ encapsulated within the nanosuspension is likely to retain its functionality, which is essential for the TQ nanosuspension to render the desired therapeutic action.

The physical state of TQ in its native form and nanosuspension was determined by DSC [Figure 3.3 (c)] TQ, TQ-NSP A and TQ-NSP B. The powder of pristine TQ showed a sharp melting endothermic peak at 46.5 °C, while no such melting was observed for TQ-NSP-A and TQ-NSP-B. The results suggested that the crystalline form of native TQ was transformed to amorphous form during nanosuspension preparation.

It is evident from the DLS data (p>0.05) how the size of the nanosuspension of TQ after storage as lyophilized powder (Figure 3.4). After storage as colloidal suspension, the size of the formulation was slightly increased after 24 h. The particle size changed from 12.7 ± 3.1 nm and 11.2 ± 2.2 nm at 0 day to 19.29 ± 1.8 nm and 16.4±2.6 nm within 24 h, respectively. After 60 days the size of TQ-NSP A increased from 19.29±1.8 nm to 34.52 ± 5.7 and that of TQ-NSP B increased from 26.8 ± 1.4 The observed drug content for TQ-NSP A and TQ-NSP B
was 98.7% and 99.4% after storage as colloidal suspension after 24 h when stored as lyophilized powder respectively. The results indicated that TQ-NSP A and TQ-NSP B are equally unstable during long term storage.

![Graphs](image)

*Figure 3.4 The graphs represent the stability studies performed with TQ-NSP A and B. (a) colloidal stability (up to 24 hours) (b) storage stability (up to 60 days)*

### 3.3.2 Dose and time dependent cytotoxicity of TQ-NSP in breast cancer cell lines

Two different types of human breast adenocarcinoma viz. MCF-7 and MDA-MB-231 were exposed to TQ, TQ-NSP A (homogenization) and TQ-NSP B (precipitation). The cells were treated with increasing concentrations (5, 10, 25, 50 and 100 µM) of the TQ, TQ-NSP A and TQ-NSP B in a dose dependent manner for 3 different time points viz. 24, 48 and 72 hours. (Figure 3.5)
Figure 3.5 Comparison of cytotoxicity of TQ-NSPs in MCF-7 and MDA-MB-231 at three different time points (24, 48 and 72 h) produced by two different methods

From Figure 3.5, TQ appears to be effective in both breast cancer cell lines. In MCF-7 cells there is a steady decline in the viability with increasing concentrations of pristine TQ at 24 h. The 48 h time point is similar at sub-toxic concentrations of TQ but slightly more effective at higher concentrations viz. 25, 50 and 100 µM. Another important observation included that the cells at 72 h showed recovery in MCF-7 (table 3.1). MDA-MB-231 cells also showed a similar trend to MCF-7 cells, where the viability decreases with increase in concentration of pristine TQ. However, MDA-MB-231 cells show higher sensitivity to TQ over MCF-7 cells.
Table 3.1: Comparison of IC50 values (µM) between TQ and TQ-NSP on two different breast cancer cell lines after 24, 48 and 72 h

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cell Lines</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TQ</td>
<td>MCF-7</td>
<td>25.40</td>
<td>15.43</td>
<td>24.63</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>16.18</td>
<td>15.46</td>
<td>22.08</td>
</tr>
<tr>
<td>TQ-NSP A</td>
<td>MCF-7</td>
<td>21.59</td>
<td>16.56</td>
<td>12.28</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>14.24</td>
<td>7.14</td>
<td>8.01</td>
</tr>
<tr>
<td>TQ-NSP B</td>
<td>MCF-7</td>
<td>15.57</td>
<td>13.72</td>
<td>7.81</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>8.33</td>
<td>7.23</td>
<td>1.23</td>
</tr>
</tbody>
</table>

When MCF-7 cells were treated with TQ-NSP A, it did not show considerable effect at lower concentrations of TQ and the toxicity was similar to that of pristine TQ at 24 and 48 h. However at 72 h, TQ-NSP A showed a decline in the viability in contrast to the results of pristine TQ. This decline was similarly noticed in the case of TQ-NSP B, though MCF-7 showed more sensitivity towards TQ-NSP A at 72 h. The results from the graph suggest that recovery of MCF-7 cells was subsided by both TQ-NSP formulations at 72 hours in comparison to pristine TQ.

This improvement in the toxicity profile would possibly address the problems associated with TQ that are previously discussed in (Chapter 1 section 1.6). The stability of the compound plays a vital role in the effectiveness of the phytochemical [10-14].

MDA-MB-231 showed higher sensitivity towards both TQ-NSP formulations. In case of TQ-NSP A, the cells at lower TQ concentrations did not show much change in the viability. However, there was a sharp decrease in viability (below 20 %) observed at concentrations-25 µM and above at each time point (Figure 3.5) with less effect seen due to higher cell death at 72 h time point.
In case of TQ-NSP B, results from graph showed similar effects at 72 h in MDA-MB-231 cells. Overall TQ-NSP B showed better improvement in the efficacy, in comparison to TQ-NSP A. The viability decreased to 20% at 10 µM upon treatment with TQ-NSP B in contrast to 20% viability at 50 µM in case of pristine TQ.

In order, to increase the understanding on the cytotoxicity profiles of each TQ-NSP formulation, IC50 was calculated (Table 3.1) at each time point and cell line with various treatments viz. TQ, TQ-NSP A and TQ-NSP B. IC50 is a quantitative measurement that indicates the concentration of phytochemical showing 50% inhibition of cancer cells. This measurement helps to determine the efficacy of TQ or TQ-NSP in each cell line.

With pristine TQ, it showed an initial decrease in the IC50 value when moving from 24 h to 48 h in case of both lines. However, after 72 h treatment, IC50 values of both cell lines increase which a clear sign of recovery. It also indicates that MDA-MB-231 is more sensitive to TQ compared to MCF-7 cells which is similar to some earlier studies by other investigators [15]. The reason behind better efficacy of TQ in MDA-MB-231 cells was suggested due to the morphology of cells. MCF-7 cells grow in colonies/clusters attached to each other unlike the MDA-MB-231 cells that grow and migrate individually presenting more surface area exposed to TQ [16]. The IC50 values from the Table 3.1 also indicated the decrease in IC50 value by 2 times in MCF-7 cells and ~3 times in MDA-MB-231 cells during TQ-NSP A treatment (72 h) in comparison to pristine TQ. In case of TQ-NSP-B treatment, the IC50 values showed decline by 3.15 times in MCF-7 cells and ~18 times in MDA-MB-231 over 72 h. These values suggest that MCF-7 to be a slightly more resistant to both nanosuspensions in comparison to MDA-MB-231. TQ-NSP B’s toxicity is much better compared to TQ-NSP A.

A possible reason to explain the effectiveness of nanosuspension is the size of nanosuspension of TQ compared to pristine TQ in aqueous form. Smaller size increases the ease of crossing
the phospholipid bilayer membrane of the cells. This provides higher biosorption and bioavailability at the target site [17, 18]. In 2015, Han et al. showed that the IC50 value in HepG2 cells (liver carcinoma) of Glaucocalyxin A (GLA) nanosuspension was lowered in contrast to the pristine GLA solution after 24 h incubation. The increase in the effectiveness was as result of either increase in the number of particles internalized into the cells via endocytosis or phagocytosis due to smaller size or by non-specific adsorption by pinocytosis after accumulation on the cells [19]. Another report on silybin nanosuspension prepared by high pressure homogenization method also showed changes in morphology of PC-3 cells along with increase in formation of apoptotic bodies and increased cell death due to increase in efficacy in contrast to the pristine silybin solution [20]. One of the reports suggested that honokiol, a phytochemical when converted to nanosuspension form, it was found to improve the oral bioavailability along with alteration in the bioavailability during intraperitoneal administration with increase in levels in blood, heart and brain [21].

The overall results suggest that reduction in the size of TQ to nano-particulate form increases the cytotoxicity at 10 µM TQ concentration improving its therapeutic efficacy compared to pristine TQ.

From the table 3.1 above, a distinct difference is seen in the IC50 values with respect to MDA-MB-231 cells when treated with TQ-NSP A & B. However, TQ-NSP B shows higher efficacy (by 7 times) and higher sensitivity towards MDA-MB-231 compared to TQ-NSP A (Table 3.1). This is evident from reduction in IC50. Hence, TQ-NSP B was chosen for further studies in MCF-7 and MDA-MB-231 cells.
3.3.3 Intracellular ROS generation

The MTT assay results helped to assess the change in toxicity profile upon decreasing the size of TQ. Out of both NSP formulations, TQ-NSP B showed better improvement in toxicity profile on both cell lines and showed higher anti-proliferative effect on MDA-MB-231. Therefore, MDA-MB-231 was also included for all further studies along with MCF-7 cells. As mentioned earlier, aim of this chapter was to improve the stability and therapeutic efficacy of TQ. Hence, a 10 µM dose was chosen as the optimal concentration of TQ for performing all *in vitro* assays. The cells were treated with pristine TQ and TQ-NSP B. The cells were stained with carboxy-H$_2$DFFDA; a dye that is cell membrane permeable. It is deacetylated with help of cellular esterases within the cells which leads to formation of non-fluorescent compound, which upon reacting with ROS get oxidised giving a fluorescent signal. The amount of ROS generation increased in the cells in the following order:

Untreated cells < TQ < TQ-NSP B

![Confocal micrographs of fixed cells treated with carboxy-DCFFDA to observe the ROS generation at different treatments in MCF-7 and MDA-MB-231 viz. control, pristine TQ and TQ-NSP B (Scale bar: 50 µm)](image)

*Figure 3.6 Confocal micrographs of fixed cells treated with carboxy-DCFFDA to observe the ROS generation at different treatments in MCF-7 and MDA-MB-231 viz. control, pristine TQ and TQ-NSP B (Scale bar: 50 µm)*
The results shown in the Figure 3.6 suggest an increase in ROS generation by TQ and TQ-NSP B. However, there was an elevation in the levels of ROS generated TQ-NSP B compared to pristine TQ. These results were further proven with the help of quantitative analysis using a fluorescence spectrophotometer presented below in Figure 3.7. The fluorescence intensities obtained from all three samples were normalized against the untreated cells and plotted as function of change in ROS generation. The quantitative results from the fluorescence measurement study are in agreement with the Figure 3.6. According to the graph in the Figure 3.7, over untreated there is a 0.75 and 1.25 fold increase in ROS activity in MCF-7 cells and MDA-MB-231, respectively while using TQ-NSPB. These results indicate that MDA-MB-231 cells are more sensitive to TQ-NSP B when compared to MCF-7 cells, which was also observed in terms of toxicity.

![Graph showing ROS generation](image)

*Figure 3.7 The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as relative reactive oxygen species generate. The data was presented as mean ± standard error (SE) of three independent experiments.*

According to Zubair *et al*, TQ can switch between pro and anti-oxidant activity in presence of low concentration of metal ions [22]. They showed TQ could possibly interact with cellular
copper in cancer cells and lead to cytotoxicity by pro-oxidant mechanism. Another study in 2013, reported increase in DNA damage due to increase in ROS activity in chondrocytes (primary cell line) in a dose dependent manner in support of this finding [23].

As mentioned in published reports, TQ is well known as pro and anti-oxidant in cancer cells [22-26]. As a pro-oxidant, it was observed that TQ increases ROS levels, which is involved in a variety of biological functions that include mutation, inflammation, aging, development, carcinogenesis and many other diseases. These superoxide radicals, hydrogen peroxide, singlet oxygen and hydrogen radicals as a result of various metabolic processes are known to be fatal to healthy as well as cancerous cells. Chronic increase in the ROS production can lead to accumulation of ROS associated cellular damages affecting protein lipids and DNA [27, 28]. In general, cancer cells are resistant to higher levels of ROS compared to healthy cells, but there is fine balance, which if disturbed leads to cell death via apoptosis. Excess ROS generated is harmful to cells and usually connected with changes or disruption of mitochondrial membrane too. Hence, it was necessary to probe the effect of ROS in relation to the change in the mitochondrial membrane potential.

3.3.4 Mitochondrial Membrane potential (DΨM)

The change in the DΨM was assessed in MCF-7 cells and MDA-MB-231 in presence of TQ and TQ-NSP B. After the 24 hour incubation the cells were washed and treated with Rh123. Since Rh123 is a cell-permeant, cationic, fluorescent dye; it is readily sequestered by active and intact mitochondria without inducing cytotoxic effects. Intact mitochondria have relatively high negative electric potential across the mitochondrial membrane which in turn allows accumulation of cationic Rh123 molecules within it [29-31]. However, change in membrane potential leads to membrane depolarization which leads to low uptake of Rh123; hence less
fluorescence intensity is observed. The fluorescence micrographs in the Figure 3.8 were used for qualitative analysis.

![Confocal micrographs of live cells treated with Rhodamine123 to observe the change in $D\Psi M$ at different treatments in MCF-7 and MDA MB-231](image)

Figure 3.8 Confocal micrographs of live cells treated with Rhodamine123 to observe the change in $D\Psi M$ at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and TQ-NSP B (d) (Scale bar: 50 μm)

The qualitative results suggested a steady decline in the intensities in confocal micrographs in Figure 3.8, which was in agreement with quantitative measurement of mean fluorescent intensities. The Figure 3.9 depicts MCF-7 cells, upon pristine TQ treatment which showed the decrease of ~ 0.3 fold in $D\Psi M$ with regards to untreated. This effect is retained in the case of TQ-NSP B which is slightly higher than 0.6 fold when compared to untreated cells.

$D\Psi M$ is the charge on the mitochondrial membrane which allows it to remain intact in the cytoplasm. The decrease in the charge intensities suggests that there is a change in $D\Psi M$ which could be responsible for a leaky organelle. Mitochondria are considered as the main source of reactive oxygen species (ROS). When disruption of the mitochondrial membrane occurs due to change in the membrane potential, it allows the release of catabolic activators that exert pressure on both vital and lethal cellular functions, simultaneously in both viz. physiological and pathological pathways. The termination of mitochondria’s biological redox function occurs with release of catabolic enzymes that lead to cellular destruction [23, 31, 32]. Catabolic
enzymes play a pivotal role in initiating apoptosis by causing DNA damage along with destruction of mitochondrial stability (which is a powerhouse of the cell) [32-34]. Hence, it is also prescribed as “a point of no return” since the programmed cell death is initiated at this stage. The above results suggest that since TQ-NSP B caused a significant shift in the membrane potential, the likelihood of apoptosis would also increase. Hence, it was essential to determine the occurrence of apoptosis in both cell lines due to TQ-NSP B in comparison to pristine TQ.

![Graph](image_url)

*Figure.3.9 The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as function of change in $\Delta \Psi_M$. The data was presented as mean ± standard error (SE) of three independent experiments.*

### 3.3.5 Apoptosis

With the increase in mitochondrial membrane permeabilisation, a critical threshold in cancer cells is surpassed, which leads to rapid cell death via a variety of independent and redundant mechanism [30, 34].

The process of maintenance and renewal of cells to main homeostasis in normal functioning of cells is vastly governed by the phenomena known to us as apoptosis. The disruption caused
within genetically defined programmed cell death would be a major cause for endless proliferation of cancerous cells. In order to understand the effect of change in levels of ROS and membrane potential of mitochondria, cells were checked for apoptosis using Annexin V/FITC-PI staining assay [35-40]. When apoptosis is initiated, phosphatidyl serine residues appear on extracellular part of cell membrane which is a hallmark of apoptosis. FITC tagged Annexin V is known to bind these phosphatidyl serine residues and its fluorescence is measured using flow cytometry. PI is used in conjunction with Annexin V to determine the damaged and dead cells in the population.

Figure 3.10 The flow analysis of Annexin-V FITC Assay for determination of apoptosis in MCF-7 and MDA-MB-231 with TQ and TQ-NSP B treatment for 24 h

The flow cytometry results from (Figure 3.10) indicated that TQ-NSP B at 10 µM TQ concentration induced apoptosis at higher levels compared to TQ. After 24 hour treatment, Annexin positive cells increased 24.9 % in TQ to 48.2% in MCF-7 cells and 24.9 % to 55 % rise in MDA-MB-231 cells due to TQ-NSP B treatment suggesting a rise in early apoptotic
phase. The graphical representation (Figure 3.11) for the apoptotic cells below suggests that there is a distinct rise in the apoptotic cells with respect to TQ-NSP B.

A similar formulation study involving oridonin (extracted from *Rabdosia rubescens*) suggested that the nanosuspension formulation not only enhanced the toxicity in a time dependent manner but also showed improvement in the ratio of early apoptotic cells [41]. Zheng *et al.* in 2011 also showed similar results which included increase in the apoptotic bodies with rise in the ratio of apoptotic cells upon silybin nanosuspension [20].

![Figure 3.11](image_url)

*Figure 3.11 Graphical representation comparing the ratio of apoptotic cells of TQ and TQ-NSP B in MCF-7 and MDA-MB-231*

### 3.4 Conclusion

In summary, nanosuspensions of TQ with help of a surfactant TPGS were prepared using two different methods viz. precipitation (TQ-NSP A) and homogenization (TQ-NSP B). Nanosuspension method was used to increase the solubility and stability of TQ in the solution. Upon characterization, both the methods resulted with DLS sizes and zeta potential within similar range. However, the polydispersity index of TQ-NSP-B was lower than TQ-NSP A.
This effect might have potentially translated to the cytotoxicity profile of both breast cancer cells lines. The MTT assay conducted on the breast cancer lines showed a decline in the viability for both TQ-NSPs. However, TQ-NSP-B showed improvement in efficacy in comparison to the TQ-NSP A. Upon further investigation results revealed that TQ-NSP B had increased range of apoptotic cells through increased generation of ROS and damage to the mitochondrial membrane leading to increased cell death.

Overall results from the above study suggest that nano-formulation of TQ can be an alternative for the improvement in stability and therapeutic efficacy of TQ.

3.5 References


Chapter 4: Thymoquinone encapsulation in dendrimers enhances its efficacy towards breast cancer

4.1 Introduction

Thymoquinone (TQ) has shown promising anti-cancer activity against various cancers such as breast, lung, prostate, liver and colon cancers, [1-5]. This effect of TQ is shown to be due to its antioxidant, anti-inflammatory, anti-neoplastic, anti-metastasis and anti-angiogenesis properties [2, 3, 6-9]. Thymoquinone has the ability to induce apoptosis and inhibit tumour cell growth \textit{in vitro and in vivo} model such as mice [4, 5, 10-13]. Due to its broad spectrum of antioxidant potential, it is associated with the ability to alter “redox state” and scavenge free radicals, generating reactive oxygen species (ROS) through variation of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase.

However, from the previous chapter, it was observed that TQ at sub-toxic concentration can lead to triggering of Epithelial to mesenchymal transition (EMT) making cancer more aggressive or immune to TQ. Clinical and therapeutic applications of TQ are limited due to its poor aqueous solubility and high physico-chemical instability. TQ is highly sensitive towards light, pH and temperature changes [9, 12, 14].

The therapeutic efficacy of any drug/ phytochemical is most often reduced due to its incapability to gain access to the site of action due to its insolubility in aqueous environment. Advances in nanotechnology provide an insight into various drug delivery vehicles to increase the longevity of the drug as well as reduces the dosage to achieve effective therapy. Dendrimer is one such nanocarrier that is branched, synthetic polymers with layered architecture that show promise in several biomedical applications. Dendrimers are monodisperse, macromolecular,
globular polymeric architecture having several unique advantages over conventional drug delivery system [15].

The dendrimers possess some unique properties, such as uniform size, water solubility, high degree of branching, high surface area for adsorption, multi-valency, well defined molecular weight and available internal cavities, which make it a suitable candidate for drug delivery applications. This provides an enormous surface area in relation to volume. Its globular shape and internal cavities provide possibility to encapsulate guest molecules within the macromolecular interior.

With well-defined nano-size structural design and the internal cavities dendrimers have the ability to carry the water insoluble drugs in its internal cavities that help to cross biological barriers, to increase the blood circulation time of drugs needed to exert a clinical effect, and to enhance the stability of drug molecules [15, 16]. Another important attribute of dendrimers is its biodegradability, which prevents its bioaccumulation of possible toxic effects in the body. Polyamidoamine (PAMAM) dendrimers are the most studied that hydrolytically degrade due to presence of amide backbone at physiological temperatures [17, 18]. Dendrimers also contain thiol-reactive disulfides within their branches that can be cleaved under the reducing conditions encountered within the cells. Moreover, the dendrimers functions itself as an enzyme substrate, which leads to possibility for its degradation by enzymes in the cell [15, 18, 19].

The first dendrimer family to be completely synthesized and characterized (in 1984) was poly (amido amine) dendrimers, also known as PAMAM dendrimers. Divergent method is used to synthesize dendrimers. This involves a two-step iterative reaction sequence that generates concentric shells of branches around the initiator core. The core shell architecture grows linearly in diameter, while the surface groups grow exponentially. Due to this growth, causing
congestion between core and branch cell diversities to produce geometrically closed nano structures that exhibit hydrophobic host-guest cavities [20].

The rationale behind using dendrimers:

- The process of biodistribution and its pharmacokinetic properties can be controlled by fine tuning the size and conformation of the dendrimer.
- Dendrimers maintain high structural and chemical homogeneity due to which there is less batch to batch variations in production as well as its facilitates the reproducibility of pharmacokinetic data within different batches.
- They have the ability to be functionalised or conjugate with various drugs, chromophores ligands or phytochemicals either on their surface or within their hydrophobic cavities. They help to increase the drug payload by attaching to other carriers such as biocompatible polymers or antibodies.
- Dendrimers are biocompatible and biodegradable in an in vivo system. These characteristics can be attained by careful choice of the available dendrimer chemistry.

In this study, a PAMAM dendrimer formulation is demonstrated to encapsulate TQ in order to improve its solubility in aqueous media and improve its stability and cytotoxicity by protecting within the internal cavities of the dendrimer. The TQ loaded dendrimers were characterised for particle size, encapsulation efficiency, in vitro release and improvement of anticancer activity against different cancer cells was tested.

4.2 Materials and methods

4.2.1 Chemicals and Materials

G4 amine-terminated PAMAM dendrimers with diaminobutane core were purchased from NanoSynthons LLC (Mt Pleasant, US). TQ was purchased from Sigma Aldrich (St. Louis, US).
MCF-7 human breast cancer, PC3 human prostate cancer cells, MDA-MB-231 human breast cancer cells and A549 human lung cancer cells were purchased from American Type Cell Culture (Rockville, USA). Roswell Park Memorial Institute (RPMI) 1640 media, Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin 10,000U/mL, trypsin-EDTA (0.25%), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), phosphate buffered saline pH 7.4 (PBS), carboxy-H2DCFDA, rhodamine 123 (Rh123), Dead cell apoptosis kit with Annexin V FITC was purchased from Life Technologies Pty Ltd. Foetal Bovine Serum (FBS) was purchased from Interpath Services Pty Ltd. Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, US) and brought to 4% in PBS and chilled to 4°C right before use.

4.2.2 Preparation of dendrimers-TQ complexes

Dendrimer-TQ (Dend-TQ) complexes were prepared by solvent casting method. Dendrimers (0.2 and 0.4% w/v) and TQ were dissolved in methanol and then Dend-TQ complexes were extracted in PBS for 24 h. Dend-TQ complexes were kept overnight for equilibrium between dendrimers and TQ molecules. The aqueous solution were filtered through 0.2 µm syringe filter and stored at 4 °C. TQ concentration in each dendrimer formulation was determined at 260 nm absorbance wavelength using UV-vis spectrophotometer (Agilent Technologies, Melbourne, Australia).

4.2.3 Physico-chemical characterization of Dendrimer-TQ complexes

TQ and Dend-TQ were dissolved in PBS and scanned in the wavelength-range of 200-800 nm using UV/vis spectrophotometer. For Fourier Transmission Infra-Red (FTIR) spectrum analysis, dendrimers, TQ and lyophilized Dend-TQ complexes were scanned for transmittance against wave number 650-4000 cm⁻¹ using a FTIR system (Perkin Elmer, US). For ¹H NMR analysis, dendrimers, TQ and Dend-TQ were dissolved in either D₂O or D6-dimethyl sulfoxide
and analysed using Nuclear Magnetic Resonance - Bruker Avance 300 MHz from Bruker Pty Ltd (Melbourne, Australia).

4.2.3.1 Determination of hydrodynamic radius and zeta potential

The mean particle size and zeta potential of different formulations were determined by dynamic light scattering (DLS) technique using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

4.2.4 In vitro drug release studies

*In vitro* drug release from Dend-TQ complex and TQ solution formulation was evaluated from the diffusion of the drug from the formulation kept within the dialysis bags [16, 17]. Dend-TQ complex or TQ solution, containing TQ equivalent to 2.5 mg, were placed in a dialysis bag (Cellophane membrane, molecular cut off 2000 Da). After that, the dialysis bag was immersed in 100 mL of PBS in a beaker. The system was maintained at 37 ± 0.5 °C under 100 rpm speed. At predetermined time intervals, 5 mL of aliquot was collected and the same amount of fresh medium was replaced to maintain the sink condition. The collected aliquots were filtered, diluted appropriately and analysed for the absorbance at 260 nm using a UV-vis spectrophotometer.

4.2.5 Stability studies

Thymoquinone was shown to degrade in the solution, therefore the stability of TQ in Dend-TQ complex and in solution was estimated. Dend-TQ complexes and TQ solution were stored at room temperature in dark and evaluated for drug content. For the accelerated stability, samples were stored at room temperature (25 ± 2 °C) in light and measured the absorbance at 260 nm at different time intervals up to three days using a UV-vis spectrophotometer.
4.2.6 Biological characterisation

The methods used for biological characterisation are described in Chapter 3, section 3.2.3.

4.3 Results and discussion

4.3.1 Preparation of Dend-TQ complexes

Dend-TQ complexes can be prepared by either dispersing an excess of solid drug/phytochemical in aqueous solution of dendrimers or by solvent casting method [21]. In solvent casting method, drug and dendrimers are dissolved in a common, evaporating solvent and then dendrimer-drug complexes in aqueous phase. Deep et al. observed that hydrophobic drugs such as docetaxel and paclitaxel were encapsulated more by solvent casting method than dispersion method [21]. Therefore, in this study Dend-TQ complexes using solvent casting method were prepared. The absorbance-concentration curve was rectilinear in the concentration range 0.05-5 µg/mL with regression equation \( y=0.1895x-0.0002 \) and regression coefficient \( r^2 = 0.9997 \).

The results of TQ encapsulation in different dendrimer formulations are shown in Figure 4.1. The TQ concentration was found to be 741.9 and 1332 µg/mL with 0.2% and 0.4% w/v G4-NH₂ PAMAM dendrimers, respectively. The results clearly indicated that the solubility of TQ was increased with increasing dendrimer concentration. PAMAM dendrimers have been well reported as solubility enhancer for hydrophobic drugs [17]. The enhanced solubility of TQ could be explained by host-guest interaction between dendrimers and TQ. The interior cavities of dendrimers have hydrophobic environment which encapsulate water-insoluble drugs. The encapsulation of TQ in these cavities could be a reason for the enhanced solubility of TQ in Dend-TQ complexes.
Figure 4.1 Drug loading in PAMAM dendrimers viz. G4 (4th generation dendrimer) with an amine tail

Apart from concentration of dendrimers, dendrimer-mediated solubility enhancement also depends on change of pH and surface groups present on dendrimers [16, 17]. Salmani et al reported that TQ is unstable at both acidic and basic pH. Therefore, Dend-TQ complexes were prepared at pH 7.4 [22]. Since 0.4% w/v G4-NH$_2$ PAMAM dendrimers showed the highest amount of drug loading, it was chosen for further in vitro studies to determine the improvement in stability and therapeutic efficacy.

### 4.3.2 Physico-chemical characterization of Dend-TQ complexes

In order to investigate the nature of interaction between TQ and Dend, Dend-TQ complexes were characterized by UV-vis spectroscopy, FTIR and NMR analysis. Figure 4.2 (A) shows the UV/VIS spectra of TQ and Dend-TQ. Both plain TQ and Dend-TQ showed characteristic absorption band of TQ at 260 nm. There was no shift in the absorption band of TQ in Dend-TQ indicating that TQ was encapsulated by simple host-guest interaction [15].
DLS was used to calculate the hydrodynamic radius of Dend-TQ complex. The average particle diameter estimated from DLS analysis Dend-TQ was 15.9±1.7 nm [Figure 4.2 (B)] and zeta potential 14.1±0.9 mV.

Figure 4.2 Physicochemical characterization of the TQ loaded dendrimers (A) UV/VIS - spectra of TQ and G4 PAMAM Dend-TQ (B) Size distribution of Dend-TQ complex (C) FTIR spectra of G4 PAMAM dendrimers (G4 Dend), TQ and Dend-TQ (D) NMR spectra of TQ.

To confirm the interaction between dendrimers and TQ, an FTIR study was performed (Figure 4.2 C). The FTIR spectra of TQ showed bands at 2967 cm⁻¹ (C-H stretching of aliphatic group), 1642 cm⁻¹ (C=O vibration) and (C=C vibration). Two methyl groups of the isopropyl group showed doublet peaks at 1355 cm⁻¹ and 1376 cm⁻¹. Dendrimer’s FTIR spectra showed bands at 3276 (N-H stretching), amide (1632 cm⁻¹ and 1543 cm⁻¹) and 1033 cm⁻¹ (C-N stretching) [17].
With an insignificant shift, Dend-TQ complex exhibited all the characteristic peaks of TQ at 2964 cm\(^{-1}\), 1376 cm\(^{-1}\) and 1355 cm\(^{-1}\), indicating an electrostatic interaction between dendrimers and TQ.

Further, NMR analysis also suggested successful complex formation between dendrimers and TQ (Figure. 4.2 D). The peak assignments in the \(^1\)H NMR spectra of TQ were as follows: \(\delta 1.12\) (6H of two methyl groups of isoproyl side chain), \(\delta 2.08\) (3H of methyl side chain), \(\delta 3.1\) (1H of CH group of isoproryl side chain) and \(\delta 6.4\) and \(\delta 6.6\) for aromatic protons. The NMR spectra of lyophilized Dend-TQ exhibited peaks of TQ without significant shift, suggesting the lack of chemical interaction between TQ and dendrimers. Similar reports with regards to host-guest interaction have been reported in case of other phytochemicals/drugs [15, 16, 18, 19, 21, 23].

4.3.3 In vitro drug release

The release of TQ from Dend-TQ complex was investigated in PBS and compared with the release from TQ solution in DMSO (Figure 4.3). The TQ dissolved in DMSO exhibited a complete release and degradation in PBS within 4 h. However the release kinetics of Dend-TQ displayed about 81% release of TQ in 12 h. The slow release of TQ from Dend-TQ formulation could be attributed to encapsulation of TQ in interior cavities of dendrimers which act as hydrophobic pockets for storage. Hydrophobic interactions between interior cavities of PAMAM-NH\(_2\) dendrimers and hydrophobic drugs are mainly responsible for slow and sustained release of TQ molecules from PAMAM dendrimers (as mentioned above) [17].
4.3.4 Stability studies

Stability of TQ in the solution is a major concern in realizing its therapeutic efficacy. Figure 4.4 shows the degradation behaviour of pure TQ and Dend-TQ. TQ concentration was rapidly declined in TQ solution in comparison to Dend-TQ complex. After 24 h, the stability rate constant was observed to be $3.9 \times 10^{-3}$ and $12.6 \times 10^{-3}$ for TQ in solution form and for TQ encapsulated in dendrimers. The linear regression line helps to determine the stability constant for each of the formulations. Higher the degradation constant indicates higher degradation rate of TQ in the formulation. The results demonstrate that TQ by itself in DMSO degrades faster than being encapsulated in the dendrimer based formulation.
4.3.5 Biological evaluation

4.3.5.1 Determination of dose dependent cytotoxicity of Dend-TQ complex in array of cancer cell lines

Four different cancer cell lines namely, A549 human lung cancer, PC-3 human prostate cancer and human breast adenocarcinoma (MCF-7 and MDA-MB-231) were exposed to pristine TQ and Dend-TQ complex to observe the effect of TQ. The cells were treated with increasing concentrations of pristine TQ or Dend-TQ complex for 24 hours.

The ability of TQ and Dend-TQ to inhibit the proliferation of different cancer cell lines was determined by MTT Assay (Figure 4.5). In case of pristine TQ treatment, the breast cancer cells showed much higher sensitivity compared to lung carcinoma and prostate cancer cells after 24 h. MDA-MB-231 was most sensitive to TQ amongst all the cell lines and lung carcinoma cells almost remained unaffected even at high concentrations. Higher concentrations of TQ such as 25-50 μM showed most effect in all cell lines except lung carcinoma. At sub-toxic concentrations of TQ in PC-3 the cells exhibited proliferation. This is an indication of
hormesis within 24 h. Hormesis is a phenomenon of dose-response relationship in which a toxic substance at low or sub-toxic concentrations depicts beneficial effects towards cancer cells and allows its proliferation [24].

Figure 4.5 The graph shows cytotoxicity profile of TQ and Dend-TQ at different concentrations after 24 h exposure, in four different cancer cell lines viz. human breast adenocarcinoma (MCF-7 and MDA-MB-231), lung carcinoma (A549) and human prostate cancer cells (PC-3). Data are mean ± Standard error from three independent experiments.

Furthermore, this trend was also reported by Koka et al. (2010), in case of prostate cancer cells where there was increase in the cell viability at lower concentrations of TQ treatments [6]. The IC50 after 24 h was recorded to be 25.4 μM and 16.18 μM for pristine TQ in MCF-7 and MDA-MB-231 cells respectively. However, in case of dendrimer formulation the IC50 was reduced to 7.61 μM and 3.68 μM in MCF-7 and MDA-MB-231 after 24 h treatment. The hormesis effect seemed to disappear from the PC-3 cells and even lung carcinoma cells showed a steady decline in cell viability at low or sub-toxic concentration. According to Darakshan et al. (2011), TQ was able to suppress the growth of three independent breast cancer cell lines viz. MCF-7, MDA-MB-231 and BT-474 [40]. The results were similar to various studies performed in vitro and in vivo that indicated thymoquinone was cytotoxic and prevent carcinogenesis, and inhibit tumorigenesis through different molecular mechanisms [11, 25-35] The reason behind increase in the therapeutic efficacy of the TQ could be due to increase in the stability of TQ within the
dendrimer pockets and slow sustained release up to 24 h. Another factor involved would be efficient delivery of dendrimers into the cells. Dendrimers are small in size. Kannan and coworkers studied the dynamics of cellular uptake of PAMAM dendrimers (G4-NH$_2$, G3-NH$_2$, G4-OH, PEGylated G3 (G3-PEG)) into the A549 human lung epithelial carcinoma cells which showed that G4-NH$_2$ and G4-OH entered into the cells more rapidly than did G3-NH$_2$ or G3-PEG [36]. The rapid entry of G4-NH$_2$ dendrimer was a results of the cationic nature of the amine surface groups, which may interact electrostatically with negatively charged epithelial cells and enter via fluid phase pinocytosis. Usually the nanoparticles used as drug delivery carriers find the most difficult to cross the Blood brain barrier (BBB). However, Lee et al., were able to demonstrate the cellular internalization of G4- PAMAM dendrimers in to the primary neuronal cultures and central nervous system in vivo [20].

### 4.3.5.2 Intracellular reactive oxygen species (ROS) generation

The cells were treated with TQ and Dend-TQ at concentration of 10 µM so as to compare the effect of TQ encapsulated within the dendrimer. The carboxy-H$_2$DFFDA dye was used after fixing the cell in paraformaldehyde to preserve the activity within them. After diffusion into the cell, carboxy-H$_2$DFFDA is deacetylated by cellular esterases within the cells to a non-fluorescent compound, which is later oxidized by ROS [37]. This oxidation is responsible for the fluorescence and helps us to determine the increase or decrease in ROS generated within the cell.

The results from the confocal micrographs in Figure 4.6 show decrease in fluorescence with both TQ and Dend-TQ. The amount of ROS generated was found to be higher in case of Dend-TQ treatment in comparison to TQ treated cells. This result was further confirmed by quantitative analysis using a fluorescence spectrophotometer in Figure 4.6. The fluorescence intensities obtained from all three samples were normalized against the untreated cells and
plotted as function of change in ROS generation. The results were in agreement with those obtained from the fluorescent images. Similar results have been reported in case of the other cell lines viz. ovarian and colon cancer cell lines. Taha et al., (2016) reported the increase the cellular oxidative stress in the Caov-3 cells. Another study associated with the increase in ROS activity with increasing amounts of TQ was reported in the malignant T- cells. The amount of ROS generation increased in the cells in the following order:

Untreated cells< TQ < Dend-TQ

Figure 4.6 Confocal micrographs of fixed cells treated with carboxy-DCFFFDA to observe the amount of ROS generated at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and Dend-TQ (scale bar: 50 μm)

Figure 4.7 suggested that there was increase in the ROS generation with respect to TQ and Dend-TQ in both breast cancer cells lines. However, in case of MCF-7 cells there is relatively less ROS generation in comparison to TQ. A much better response was observed in MDA-MB-231 cells, with respect to both cell lines in both treatments viz. untreated and pristine TQ. These results indicated a higher sensitivity of Dend-TQ in MDA-MB-231 cells rather than MCF-7 cells.
Figure 4.7 The graph represents fluorescence intensities obtained from two samples viz. pristine TQ and Dend-TQ normalized against the untreated cells and plotted as function of change in ROS generation. Live cells were treated with Rhodamine123 to observe the change in DΨM in different treatments: Untreated, pristine TQ, Dend-TQ. Data are mean ± Standard error from three independent experiments.

Uncontrolled ROS generation is injurious to the cells, since it can cause damage to DNA and other organelles in the cells leading to cell death [38, 39]. TQ has been reported for both anti and pro-oxidant activity [30, 40]. Since cancer cells already have higher levels of ROS, there is a very delicate and highly sensitive balance of ROS species generated in these cells. Hence, any disruption to this delicate ROS balance could lead to apoptosis. Indeed, there was a significant increase in ROS generation with Dend-TQ observed in both cell lines viz. MCF-7 and MDA-MB-231. This study not only help us to understand the DNA damage occurring due to oxidative stress but also depicts the increase in the therapeutic efficacy of TQ with decrease in the phytochemical payload at low/sub-toxic concentration of TQ. With increase in the efficacy it also solves the issue involving the occurrence of EMT as noticed in the previous chapter (Chapter 2).
The increase in ROS level abnormally is known to disturb the equilibrium required in order to maintain the mitochondrial membrane potential (DΨM). Hence, it was an essential step to investigate the change in the DΨM in the cells as trace the effect of TQ and Dend-TQ.

4.3.5.3 Mitochondrial Membrane Potential (DΨM)

The change in ROS levels in the cell is also associated with change in the membrane potential. Rh123 is a cell-permeant, cationic, fluorescent dye that is readily sequestered by active mitochondria without inducing cytotoxic effects [41, 42]. In unaffected conditions, mitochondria is able to retain Rh123 giving a strong green fluorescent signal unlike the damaged or permeabilised state [42-45].

In order to assess the change in DΨM, the MCF-7 and MDA-MB-231 cells were treated with TQ, Dend-TQ. After the 24 h incubation, the cells were washed and treated with Rhodamine 123 (Rh123) checked for fluorescence for both qualitative and quantitative analysis. Results from the Figure.4.8 suggested steady decline in the fluorescence intensity in the confocal micrographs of both cell lines with treatment of TQ and Dend-TQ. However, there is a significant loss of fluorescence in the sample containing Dend-TQ in comparison to pristine TQ sample at the same concentration.

A similar pattern was observed in the graph (Figure 4.9) when plotted as a function of change in mitochondrial membrane potential (DΨM) when compared to untreated cells. These observations clearly suggested increase in efficacy due to encapsulation of TQ within the PAMAM dendrimers, making it an attractive drug delivery model without changing the mode of action of TQ. Some studies upon extensive investigation suggested that increase in ROS activity and rupturing of the mitochondrial membrane could lead release of factors/ molecules that can lead DNA damage or breakage as well as activation different cascades leading to programmed cell death/ apoptosis [43, 45]. The cross talk between ROS and DΨM plays a vital
role in triggering the activation of apoptosis [42, 46]. Hence, TQ acts as a pro-oxidant in the above studies leading destruction and damage of breast cancer cell lines.

Figure 4.8 Confocal micrographs of live cells treated with Rhodamine123 to observe the change in $\Delta \Psi M$ at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and Dend-TQ (Scale bar: 50 μm)

Figure 4.9 The graph represents fluorescence intensities obtained from two different samples treated with TQ and Dend-TQ normalized against the untreated cells and plotted as function of change in $\Delta \Psi M$. Data are mean ± Standard error from three independent experiments
4.3.5.4 Apoptosis Assay

Apoptosis is a process of genetically programmed cell death in normal cells to maintain homeostasis which is impaired in cancerous cells [4, 39]. Hence, the cells keep proliferating endlessly. In order to analyse apoptosis induction due to TQ and Dend-TQ, Annexin V/FITC-PI staining assay was performed. Annexin V is known to bind to those cells that express phosphatidyl serine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis [47]. This is a sign for early apoptosis. Staining with Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) for identification of early and late apoptotic cells. Cells that are viable have intact membranes which act as a barrier excluding PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, both Annexin V and PI negative cells are considered viable, while cells that are Annexin V positive and PI negative are considered in early apoptosis and cells that are in late apoptosis or already dead are both Annexin V and PI positive.

The flow cytometry results in Figure 4.10 demonstrated that Dend-TQ induced apoptosis showed an increase in number of cells entering in apoptosis. After 24 h of treatment, the percentage of Annexin V-stained cells was 24.9% and 32.3% for TQ and Dend-TQ in MCF-7 cells, respectively, which suggests there is an increase in the population of cells at early apoptotic stage in both types of treatment.

However, MDA-MB-231 showed improvement inefficacy by 10 times with rise in apoptotic cells from ~ 6% to 52.6% (from Figure 4.11). Therefore, this increase in the early apoptotic cells in case of Dend-TQ, suggests an increase in therapeutic efficacy with encapsulation of TQ in dendrimer. A number of recent studies have reported TQ’s oxidant activity as one of the causes for DNA damage and ultimately leading to apoptosis [30, 48, 49].
Figure 4.10 Flow cytometry analysis of Annexin V/ FITC Assay for determination of apoptosis in MCF-7 and MDA-MB-231 with TQ and Dend-TQ treatment for 24 h

Figure 4.11 Representation of a percentage of apoptotic cells quantitated by Annexin V-FITC/PI analysis of MCF-7 and MDA-MB-231 cells treated with pristine TQ and Dend-TQ for 24 h
In fact, a study by Arafa et al., found that TQ mediated cell death occurred via an intrinsic/mitochondrial pathway, where it triggered the caspase activation leading to apoptosis due to TQ induced reactive oxygen species which caused oxidative stress [50]. Hence, the above results are in support with the literature, suggesting that TQ mediated apoptosis occurs via a mitochondrial pathway [29, 33, 49-51]

4.4 Conclusions

In conclusion, a dendrimer based formulation encapsulating TQ and its biological activity was tested in vitro in this study. From the above study, it can be concluded that dendrimer formulation helps to increase the stability of TQ compared to pristine TQ in DMSO. Simultaneously, it also enhances the therapeutic efficacy of TQ with the help of controlled release up to 24 h. With help of characterisation techniques, it was found that G4 PAMAM dendrimers (0.4% w/v) showed the highest loading of 1332 μg/mL. The UV-vis spectroscopy showed that TQ did not change in its form but was encapsulated within the dendrimer. However, for further understanding the interaction between the TQ and dendrimer, FTIR and NMR was performed on samples. The results revealed the encapsulation of TQ in its nanoparticulate form within the hydrophobic pockets of the dendrimer by host-guest interaction. The nano-formulation was then exposed to an array of cancer cell lines which showed in the improvement of cytotoxicity in profiles of all cell lines with best results in a MDA-MB-231 (breast cancer cell lines) (Figure 4.5). To further assess the reasons for toxicity, in vitro apoptosis assay, Annexin V/FITC assay was conducted. The results showed increase in the ratio of early apoptotic cells in both the cell lines (Figure 4.10). To understand the effect of TQ on DNA damage, the level of ROS and change in mitochondrial membrane potential (DΨM) was measured in both cell lines. Results suggest an increase in levels of ROS with a significant change in DΨM, upon application of Dend-TQ in comparison to pristine TQ. The
disruption of mitochondrial membrane could lead to release of catabolic hydrolases responsible for DNA, lipids and protein damage along with activation of apoptosis (Figure 4.7 and 4.9).

The sum total of all the above results suggest an improvement in the therapeutic efficacy of the TQ based dendrimer formulation due to small size of dendrimers, increase in the TQ stability and slow sustained release of TQ for up to 24 h.

## 4.5 References


Chapter 5: Self-assembled nanostructures of triblock copolymer-aspartate gold nanoparticles as TQ delivery vehicles with improved stability and therapeutic efficacy

5.1 Introduction

In the previous chapters, it was shown that nanosuspension of TQ and dendrimer encapsulated TQ exhibit significant anti-cancerous activity as a result of its encapsulation, which can protect TQ from degradation as well as its transport across the hydrophilic medium. Therefore, the drug delivery systems that contain hydrophobic domains are the desired candidates of delivering hydrophobic drugs such as TQ to the target site. There are several amphiphilic drug delivery systems that include surfactants, soluble polymers, lipoproteins, liposomes, and amphiphilic polymer based micellar systems, which have been extensively used for the drug delivery applications [1-4]. These amphiphilic carriers have the hydrophobic domains, which can be used to solubilize the hydrophobic drug for the targeted delivery and the needed biological action [5]. However, simple surfactants based systems, due to their high critical micellar concentrations (CMC), tend to be unstable under in vivo biological conditions [6]. On the other hand, lipoproteins and liposomes have the disadvantages of competitive binding with other receptors and low encapsulation and rapid release of drugs [7]. Among the aforementioned carriers, polymer micelles based drug delivery systems are considered as promising due to their higher loading of drugs, stability in blood stream, therapeutic potential and long term storage stability [1]. In simple terms, polymeric micelles possess hydrophobic core and hydrophilic shell in which the hydrophobic core provides the space for the encapsulation of hydrophobic drugs such as TQ.

Among the different amphiphilic co-polymers, Pluronic tri-block copolymers have been studied extensively for different biological applications due to their excellent biocompatibility [1, 7-15]. These tri block copolymers generally have the structure of PEO–PPO–PEO block
copolymers, wherein PEO and PPO stands for poly(ethylene oxide) and poly(propylene oxide) blocks, respectively, as shown in the following figure.

![Figure 5.1 Structure of PEO-PPO-PEO tri-block copolymer](image)

The PEO units are hydrophilic in nature, while the PPO units are hydrophobic in nature. The number of units (denoted as $x$ and $y$ in Figure 5.1) are different for different kind of pluronic polymers because the number of units provide the hydrophilicity/hydrophobicity balance of the tri-block copolymer [1]. The choice of this Pluronic block copolymer for this drug delivery application is mainly due to its propensity to self-assemble in aqueous solutions into multimolecular aggregates having spherical, rod-like or lamellar morphologies which is specifically useful to drug delivery applications [4]. In their micellar structure in water, micelles core consist of hydrophobic PPO blocks and hydrophilic PEO blocks on the surface. The core hydrophobic PPO units in these polymeric micelles will encapsulate the hydrophobic drugs like TQ and can demonstrate high loading capacity, controlled release profile for the incorporated drug, and good compatibility between the core forming block and incorporated drug. The PEO corona provides an effective steric protection for the micelles and determines the micelle hydrophilicity, charge, the length and surface density of hydrophilic blocks. These properties control important biological characteristics of a micellar carrier, such as its pharmacokinetics, biodistribution, biocompatibility, longevity, surface adsorption of biomacromolecules, adhesion to biosurfaces and targetability [6]. It is also reported that Pluronic block copolymers promote active membrane transport of numerous compounds and
thus can help in overcoming multiple drug resistance (MDR) phenomena in cancer therapy [10].

Among the different kinds of tri-block copolymers, PEO$_{20}$-PPO$_{70}$-PEO$_{20}$ (denoted as P123 from now onwards) has low critical micelle concentration and the hydrophobic-hydrophilic blocks ratio is suitable for the sensitization of MDR cells [12]. Batrakova et al. demonstrated that number of hydrophobic propylene oxide (PO) units is the critical factor for efficient sensitisation to chemotherapeutic drugs in KBV cell line [7]. However, these polymers do not have any additional functional groups for targeting, not much variation in micellar size, drug loading capacity and not efficient way of controlling the release of drugs. The applicability of these P123 micelles as drug delivery vehicles for anticancer applications (though not for phytochemicals) has been well studied so-far and the aforementioned factors need to be taken care for the efficient anticancer applications.

Therefore, the aim of this work is to functionalise the self-assembled nanostructures of P123 by amino acids and gold nanoparticles by a novel method and investigate the stability of TQ and explore its therapeutic efficacy due to encapsulation. Since TQ is a hydrophobic drug, DMSO solution of TQ can be incorporated within the self-assembled structures of a biocompatible tri-block copolymer. Gold nanoparticles will provide rigidity to the polymeric micelles, therefore, they function as additional material to control the release kinetics of TQ. These polymer micelles were surface functionalized with amino acids such as aspartic acid (acidic), lysine (basic) and histidine. The major reason to use amino acids to functionalize the P123 surface is due to their hydrogen bonding capacity with the PEO blocks of the micelles [16], providing the surface PEO units with amino acid functionality that can render the surface more hydrophilic, biocompatible and control the release of the TQ adsorbed within the hydrophobic PPO core of the micelles. Additional functional role of these amino acids is their
ability to reduce gold ions into gold nanoparticles, which protect the drug further and provide rigidity to the micellar surface [5, 17].

Since TQ is a hydrophobic drug, DMSO solution of TQ exhibit very high affinity to reside within the PPO units, which is hydrophobic in nature and protects TQ from any degradation. The amino acids and gold nanoparticles increase the surface hydrophilicity and act as barrier to release the hydrophobic drug slowly.

5.2 Materials and Methods
5.2.1 Synthesis of P123 amino acid Au micelles
TQ, P123, Amino acids aspartic acid, lysine and histidine were purchased from Sigma-Aldrich and used as such without any modification. All the solutions were prepared in deionized water.

A 0.5 wt %/V of P123 solution was made by dissolving 1 g of P123 polymer in 200 mL of de-ionized water. This solution was divided into four parts containing 50 mL of P123 solution. To the first part, 100 µM solution of TQ in DMSO (100 µL) was added and dispersed uniformly

Figure 5.2 Synthesis route of Amino acid/Gold nanoparticles functionalized P123 micelles for TQ encapsulation
by ultrasonication. This solution was kept at 37°C. To each of the remaining three P123 solutions, amino acids aspartic acid, lysine or histidine were added individually, to maintain their concentration 1 mM in 50 mL. To each amino acid containing P123 solution, aqueous solution of chloroauric acid was added. The concentration of the chloroauric acid was maintained at $2 \times 10^{-4}$ M in the amino acid functionalized P123 solution. These solutions were kept at 37°C for one day until the gold nanoparticles formation. These amino acids were reported to reduce chloroaurate ions into gold nanoparticles, therefore the P123 micelles were functionalized by both amino acids and nanoparticles. Subsequently, to all the three solutions (P123-Asp-Au, P123-Lys-Au, P123-His-Au), 100 µM solution of TQ in DMSO (100 µL) was added to each and dispersed uniformly by ultrasonication. All these solutions were kept at 37°C and then placed in a dialysis bag for 6 h in dark to remove free TQ, amino acids and gold.

5.2.2 UV visible spectra

In order to investigate the presence of TQ into the P123 amino acid gold complex, the complexes scanned in the wavelength-range of 200-800 nm using UV/vis spectrophotometer.

5.2.3 In vitro drug release

In vitro drug release from TQ, and P123 Asp-Au TQ solution formulation was evaluated using dialysis bag diffusion method. P123Asp TQ micelles, P123 Asp-Au TQ complex or TQ solution, containing TQ equivalent to 3 mg, were placed in a dialysis bag (Cellophane membrane, molecular cut off 2000 Da). After that, the dialysis bag was immersed in 100 mL of PBS in a beaker. The system was maintained at 37 ± 0.5 °C under 100 rpm speed. At predetermined time intervals, 5 mL of aliquots were collected and the same amount of fresh medium was replaced to maintain the sink condition. The collected aliquots were filtered, diluted appropriately and analysed for the absorbance between 200-800 nm using a UV-vis spectrophotometer.
5.3 Result and discussion

5.3.1 Physicochemical characterization

5.3.1.1 Dynamic light scattering and Zeta Potential

Dynamic light scattering (DLS) measurements were performed using a Malvern 4800 Autosizer employing a 7132 digital correlator.

Table 5.1 DLS and Zeta potential measurements of the P123 amino acid / Au NP functionalized micelles

<table>
<thead>
<tr>
<th>Polymer micelles</th>
<th>Hydrodynamic radius (nm)</th>
<th>Hydrodynamic radius II (nm)</th>
<th>Zeta Potential (milli volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P123</td>
<td>22</td>
<td>138</td>
<td>-5.0</td>
</tr>
<tr>
<td>P123-Asp</td>
<td>35.5</td>
<td>--</td>
<td>-3.7</td>
</tr>
<tr>
<td>P123-Lys</td>
<td>33.5</td>
<td>171</td>
<td>-9.8</td>
</tr>
<tr>
<td>P123-His</td>
<td>47</td>
<td>240</td>
<td>-12.3</td>
</tr>
<tr>
<td>P123 Asp-Au TQ</td>
<td>26.3</td>
<td>207</td>
<td>-3.34</td>
</tr>
<tr>
<td>P123-Lys-Au TQ</td>
<td>25</td>
<td>215</td>
<td>-12.4</td>
</tr>
<tr>
<td>P123-His-Au TQ</td>
<td>6</td>
<td>94</td>
<td>-12.3</td>
</tr>
</tbody>
</table>

The light source was a He–Ne laser operated at 633 nm with maximum power output of 15 mW. The correlation functions were analysed by cumulants method of analysis. DLS measurements given in Table 5.1 clearly show that bimodal distribution of P123 micelles was formed. The smaller size (22 nm) represents the individual micellar size and the large size micelle (138 nm) may be attributed to the cluster or aggregate of few micelles. However, after the addition of amino acids and formation of gold nanoparticles, the average hydrodynamic radius increased significantly for both individual micelle and aggregate of micelles size in comparison to P123 micelles. P123 micelles tend to form hydrogen bonds with polar molecules through its EO units on the surface, therefore the hydrophobic domain is known to expand [8, 10]. This may be the reason for the increase in hydrodynamic radius of the nanoparticles. Zeta
potential results showed a slightly different trend, wherein P123-Asp-Au showed decrease in the zeta potential with respect to pure P123 and P123-Lys-Au micellar systems. This may be probably due to the strong hydrogen bonding between the carboxylic units of aspartic acid with EO units of P123, thereby decreasing the surface charge of the micelles. According to Gotchev et al., zeta potential can be considered as a valid parameter only if a colloid system is electrostatically stable. However, their study suggested that the tri-block co polymers are stabilised by electrostatic and stearic forces. However, in absence of electrostatic charge the tri-block copolymers remained stable. Hence, the low zeta potential values do not necessarily indicate instability of the nanoformulation [18].

5.3.1.2 UV-Visible Spectral Results

![UV-Visible Spectral Data](image)

*Figure 5.3 UV-Visible spectral data of P123-Amino acid, P123-Amino acid-Au nanoparticles (dotted lines) and after loading of TQ (solid lines)*

Thymoquinone is known to absorb between 300-350 nm in the UV region and gold nanoparticles exhibit its strong surface plasmon resonant absorption at 520 nm; therefore the formation of nanoparticles as well as TQ incorporation can be studied using UV-Visible spectroscopic technique. Figure 5.3 shows the UV-Visible spectral data of P123 with aspartic acid, histidine and lysine amino acids (dotted lines in all three panels) before and after addition
of TQ. In all the three cases, it is clearly seen that there is a strong absorption between 300-350 nm due to the presence of thymoquinone. As mentioned earlier, P123 has hydrophobic core and addition of DMSO solution of TQ preferentially occupy these hydrophobic domains of P123-Amino acid micelles. In the case of lysine, appearance of two bands clearly says that TQ exist is in its native form as well as its isomeric form due to the basicity of lysine amino acid. Lysine is also a hydrophobic amino acid, therefore, a competitive adsorption may occur between lysine and TQ in the hydrophobic domains of P123 micelles. Since amino acids are known to reduce gold ions to form nanoparticles, P123-Amino acid micelles can form nanoparticles on their surface by reducing the gold ions. This was clearly demonstrated by the presence of strong surface plasmon resonant absorption of gold nanoparticles around 530 nm in the visible region in all three amino acid functionalized P123 micelles (dotted lines). Addition of TQ to these P123-Amino acid-Au nanoparticles, lead to the incorporation of TQ, which was confirmed by the presence of strong absorption in the region between 300-350 nm (marked with an arrow).

5.3.1.3 Transmission Electron Microscopy (TEM)

Figure 5.4 TEM images of P123-Asp-Au, P123-Lys-Au and P123-His-Au micellar systems

TEM images of these P123-Amino acids-Nanoparticles were taken using JEOL 1010 TEM instrument, operated with the 100 KV accelerating voltage. Samples were made by drop-
casting of the materials on the surface of carbon-coated copper grids. Figure 5.4 shows the TEM images of the P123-Asp-Au, P123-Lys-Au and P123-His-Au micellar systems, wherein the formation of micellar structures and the nanoparticles formation were clearly seen.

5.3.2 Biological characterization

5.3.2.1 Comparison of dose dependent toxicity of P123 Asp-Au TQ in breast cancer cell lines

In the previous Chapter 4, MCF-7 and MDA-MB-231 were found to be the most sensitive cell lines, hence MTT assay was performed with aforementioned amino acid gold nanoparticles functionalized P123 micelles loaded with TQ. Cell viability studies of MCF-7 and MDA-MB-231 cell lines were carried out using MTT assay for the period of 24 hours and the results are shown in Figure 5.5. Pure TQ was also used as reference and the cell viability was plotted against the concentration of TQ. As compared to pure TQ, all the TQ loaded amino acid gold nanoparticles functionalized micelles have shown enhanced cytotoxicity at all concentrations. The results from the Figure 5.5 suggest that P123 Asp-Au TQ showed the highest decline in cell viability in both the cell lines with respect to pristine TQ. As mentioned in the Chapter 2 section 2.3.1, 10 µM TQ concentration showed high cell viability and was responsible for the initiation of EMT in the MCF-7 cells. Hence, it was chosen as a desired concentration for further biological characterization.
Figure 5.5 Cell viability studies of the three different amino acid (aspartate, histidine and lysine) functionalized P123 micelles loaded with TQ for MCF-7 (left) and MDA-MB-231 (right). Data are mean ± Standard error from three independent experiments.

Among all the three amino acid functionalized P123 micelles, the aspartic acid functionalized micelles were found to exhibit higher cytotoxicity at both concentrations. Lysine and Histidine have additional basic functional groups and they may contribute or compete with the biological action of TQ. Therefore, aspartic acid functionalized P123 micelles were chosen as TQ drug delivery vehicles for the detailed studies. Both cell lines were exposed with increasing concentrations of the pristine TQ and P123 Asp-Au TQ in a dose dependent manner for 24 h. The graphs from Figure 5.6 depict cytotoxicity of only P123 Asp-Au TQ in MCF-7 (image on the left) and MDA-MB-231(image on the right) with respect to pristine TQ after 24 h treatment for a simplification of enhanced therapeutic response. The response at higher concentrations remained almost the same as that of TQ in MDA-MB-231 cells. Both graphs in the Figure 5.5 includes the toxicity of each separate component required for the synthesis of polymer complex which include P123, Aspartate (amino acid), P123 with aspartate, P123 with aspartate decorated with gold. In Figure 5.6, P123 Asp-Au TQ showed a steady decline in cell viability. In MCF-7 cells, the pristine TQ shows about 30% decrease in cell viability at 10 μM; however
P123 Asp-Au TQ showed decrease in viability to 55% in comparison to the untreated sample. Complete cell death was seen in the MCF-7 cell at 25 μM due to P123 Asp-Au TQ instead of that observed in TQ at 100 μM.

In the case of MDA-MB-231, the cell viability due to pristine TQ at 10 μM treatment shows only 58% decline in cell viability with regards to control sample. On other hand P123 Asp-Au TQ shows significant decline of about 42% with regards to. These results clearly suggest that even though both cell lines get affected due to encapsulation of TQ; MDA-MB-231 is more sensitive to P123 Asp-Au TQ in comparison to MCF-7 cells. The reduction of cell viability at most concentrations of TQ also indicates improvement in the efficacy of the TQ. The reason behind better efficacy could be maintenance of integrity of TQ by encapsulation within the polymeric capsules [8, 9].

Another observation that can be made from the two graphs is that the empty micelles with all the components except TQ, show very low toxicity in both breast cancer cell lines in comparison to P123 Asp-Au TQ. This clearly suggests that the toxicity obtained was directly from the TQ loaded in the nanomicelles and enhanced effect suggested that the stability of TQ was improved in comparison to TQ in the solution.

The cytotoxicity profile of the each individual component used in synthesis of the polymeric micelles was additionally done as seen in Figure 5.6. The aim was to check if all the components were biocompatible in the cells. Most components showed negligible cytotoxicity in MCF-7 cell and MDA-MB-231 at even high concentrations.
5.3.2.2 In vitro drug release

The graph in the Figure 5.7 shows a comparison of TQ’s release profile from P123 Asp TQ (without gold nanoparticles) and P123 Asp-Au TQ (with gold nanoparticles) plotted against pristine TQ in the solution. The results suggested that TQ from the solution was released within 4 h which meant that, it is readily available to the cells. However, there is a decrease in the release of both formulations. P123 Asp TQ is amino acid conjugated P123 micelles and P123 Asp-Au TQ comprises of TQ loaded amino acid conjugated P123 micelles with gold nanoparticles. From the graph, it is observed that P123 micelles show the slowest release with respect to micelles consisting gold nanoparticles followed by polymeric micelles with gold addition. This is a clear indication that the gold nanoparticles formed due to reduction of gold
chloride by aspartate, facilitates slow and sustained release of TQ. Furthermore, the results in the cytotoxicity graph (Figure 5.6) also reflect a possibility for increased therapeutic efficacy.

Figure 5.7 In silico release of TQ from TQ solution, P123 Asp TQ and P123 Asp-Au TQ at 37°C in PBS up to 24 h

5.3.2.3 Intracellular ROS generation:

The results from anti-proliferative assay suggested that Asp-Au-TQ is more potent and anti-cancerous towards breast cancer cells. However, the assay does not provide an insight into the mode of action for increase therapeutic efficacy. Hence the formulation was checked, if the ROS pathway was affected or not. The cells were treated at the concentration of 10 µM for TQ and P123 Asp-Au TQ and then were stained with carboxy-H₂DFFDA. After the dye crosses the cell membrane, the process of deacetylation involved with cellular esterase occurs in the cell and the non-fluorescent compound formed within the cell reacts with the carboxy-H₂DFFDA within the cell to give a fluorescent signal.
The amount of ROS generation increased in the cells in the following order:

Untreated cells < TQ < P123 Asp-Au TQ

In case of the quantitative method performed on the fluorescence spectrophotometer includes live cell measurement where the treated cells were dislodged and stained with carboxy-H$_2$DFFDA. The fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as function of increase in amount of ROS generated. The results in the Figure 5.7 (a) suggest an increase in ROS generation in both samples viz. TQ and P123 Asp-Au TQ. This result was further established with the help of quantitative analysis using a fluorescence spectrophotometer. The fluorescence intensities obtained from all three samples were normalized against the untreated cells and plotted as function of change in ROS generation. The elevation in the levels of ROS generated by P123 Asp-Au TQ compared to pristine TQ was noted higher in MDA-MB-231 cells than in MCF-7 cells (Figure 5.9). There was approximately 1.5 fold change in the intensity in MDA-MB-231 cells with respect to untreated sample whereas only 0.75 fold change was seen in case of MCF-7 cells. The results
were in agreement with the fluorescent micrographs indicating a rise in the generation of ROS in significant amounts with P123 Asp-Au TQ treatment.

![Graph](image)

**Figure 5.9** The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as function of increase in amount of ROS generated. Data are mean ± Standard error from three independent experiments.

Current literature acknowledges the presence of cross talk between the ROS and DΨM. As listed above, one of the harmful effects involve change in the membrane potential of mitochondria. Hence, the next step was to check if DΨM was affected by the treatment, as literature suggests that cancer cells tend to have higher ROS compared to normal epithelial cells.

### 5.3.2.4 Mitochondrial Membrane potential (DΨM)

The change in the mitochondrial membrane potential was assessed in MDA-MB-231 in presence of TQ and P123 Asp-Au TQ. After the 24 hour incubation the cells were washed and treated Rh123. The fluorescence micrographs and intensities were captured for qualitative and quantitative analysis respectively.
The results from the Fig 5.10 comprise of the confocal micrographs show Rh123 stained cells in presence of TQ and P123 Asp-Au TQ. The quantitative data from the graph in Figure 5.11 suggests a steady decline in the mitochondrial membrane potential upon P123 Asp-Au TQ treatment in both cell lines with about 30% decrease in MCF-7 and about 55% in MDA-MB-231. These results exhibit a change in the membrane potential of mitochondria. The dye specifically gets accumulated in the mitochondria with intact membrane.

![Confocal micrographs of live cells treated with Rhodamine123 to observe the change in DΨM at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and Asp Au-TQ (Scale bar: 50 μm)](image)

*Figure 5.10 Confocal micrographs of live cells treated with Rhodamine123 to observe the change in DΨM at different treatments in MCF-7 and MDA MB-231: Control, Pristine TQ and Asp Au-TQ (Scale bar: 50 μm)*

The decline in fluorescent intensities is a sign for change in DΨM across the mitochondrial membrane, which could lead to leaky structure of the mitochondria. The leaky structure may lead to leakage of catabolic hydrolases, proteases and nucleases that are required to tackle the reactive oxygen species generated within the mitochondria [19-21]. The ROS is usually kept in control by their specific inhibitors or by sequestration of their activators. This disruption of the mitochondrial membrane occurring due to change in the membrane potential, allows the escape of activators that exert pressure on both vital and lethal functions required for maintenance of cells.
The graph represents fluorescence intensities obtained from all three samples normalized against the untreated cells and plotted as function of change in $D\Psi_M$. Data are mean ± Standard error from three independent experiments.

The termination of mitochondria’s biological redox function occurs with release of catabolic enzymes that lead to cell death. As mentioned in the previous Chapters 3 and 4, the particular stage is also known as “a point of no return”. The initiation of apoptosis in the cancer cells occur which eventually leads to DNA damage and finally destruction of the cell [22]. It is also evident from the above results that cross talk between ROS and $D\Psi_M$ is very important to maintain functional mitochondria in the cells [4, 22, 23]. If changes in $D\Psi_M$ are seen in the cells due to TQ and its nano-formulation, it would be ideal to investigate the initiation of early apoptosis in order to approve the mitochondrial dependent pathway for cell death.

5.3.2.5 Apoptosis Assay:

With enhanced ROS levels and change in $D\Psi_M$ and results from Chapters 3 and 4 suggesting an increase in levels of apoptosis upon usage of drug delivery vehicle. The relationship between cell proliferation and apoptosis is important for maintaining normal tissue homeostasis and development [24, 25]. However, cancer cells strongly depict an anti-apoptotic feature leading
to uncontrolled proliferation of cells. The disruption caused within genetically defined programmed cell death would be a major cause for endless proliferation of cancerous cells. There a number of reasons for apoptosis to occur in a cell [17, 25, 26]. One such reason can be associated with increase in ROS levels, which lead to change in DΨM [27-29]. Hence cells were checked for apoptosis using Annexin V/FITC-PI staining assay as discussed previously in Chapter 3. The degree of apoptosis induced by the P123 Asp-Au TQ on MCF-7 and MDA-MB-231 cell lines investigated by Annexin V/Propidium Iodide (PI) dual staining facilitates the detection of live cells (Q4; AV-/PI-) early apoptotic cells (Q3; AV+/PI-), late apoptotic cells (Q2; AV+/PI+) and necrotic cells (Q1; AV-/PI+). In MCF-7 cells (Figure 5.13), apoptotic cell population rose from 0 to 24.9 % after pristine TQ treatment (10 µM). However after the treatment of P123 Asp-Au TQ (10 µM equivalent TQ) the apoptotic cells population rose to 58%, indicating 2 fold rise in comparison to the untreated.

The results were in agreement with the levels of ROS and change in DΨM observed in both cell lines. From previous Chapters 3 and 4, MDA-MB-231 was found most sensitive which is similar to the results in this Chapter as well. This not only indicates Asp Au-TQ shows an increase in efficacy when compared to that of pristine TQ but also indicated that the cell death due to nano-formulation might be due to initiation of apoptosis via a mitochondria based pathway.
Figure 5.12 The flow analysis of Annexin V/FITC Assay for determination of apoptosis in MCF-7 and MDA-MB-231 upon treatment with 10 µM TQ and P123 Asp Au-TQ treatment for 24 h.

Figure 5.13 Graphical representation of percentage apoptotic cells (Annexin V +ve) with respect to different treatments.
5.5 Conclusion:

In summary, a novel polymer micelles based on aspartate-polymer conjugate coated with gold have been prepared, partially characterised and evaluated for their cytotoxic activity against two human breast cancer cell lines. The TEM images confirm the formation of capsules coated with gold nanoparticles. The results showed that nanoformulation exhibited remarkable cell growth inhibition activities on different cancer cells. In addition, they enhance the degree of apoptosis in both cell lines by causing a significant elevation in ROS production which in turn disrupts the mitochondrial membrane releasing catabolic enzymes responsible for initiation of apoptosis. Overall, these results demonstrate that the P123 Asp-Au TQ has the potential to be developed as nano-carrier for encapsulating TQ and other phytochemicals to enhance their therapeutic effect and their stability.

5.6 References


Chapter 6: Comparative analysis of different drug delivery platforms for TQ

6.1 Introduction (re-visiting the problems of TQ):

In vitro studies have shown that TQ has strong anticancer effect [1-9], which was also evident in the case of in vivo models such as mice and rats [3, 10]. However, the sole human cancer clinical trial using pristine TQ did not show any significant therapeutic effect [11]. This is possibly due to the several challenges mentioned in Chapter 1, such as the lipophilic nature of TQ, which creates a problem of solubility that could lead to low bioavailability. In the case of oral administration of TQ, it bio-transforms to a hydroquinone due to the activation of phase 2 enzymes in the liver, making it less bioavailable to the body [12]. The issue of bioavailability suggested that the concentration of TQ reaching a tumour in the body, might be much lower than the dose administered [13-15]. Hence, this study was conducted to understand the effect of sub-toxic concentrations of TQ on MCF-7 cells.

In Chapter 2, it was observed that low concentrations of TQ reduce E-cadherin (E-cad) expression along with destruction of the cadherin-catenin complex. This leads to the translocation of β-catenin to the nucleus, which activates the transcription of CdK2. This, in turn, triggers increased proliferation of MCF-7 cells and is also associated with activation of epithelial-mesenchymal transition (EMT). This poses a serious problem, as it increases the aggressiveness of cancer and activates metastasis. To mitigate this problem, three different nanoformulations were used to increase the effectiveness of TQ against cancer cells.

The objective of this chapter is to provide a comparative analysis of the three different nanoformulations used in this thesis, to understand the most effective nanoformulation against
breast cancer. This chapter also highlights the effect of TQ nanoformulations on E-cad reduction in association with EMT activation.

6.2 Materials and Methods

The materials and method used for flow cytometric analysis of E-cad expression in MCF-7 cells is as described in Chapter 2 section 2.2.6.

6.2.1 Calculating IC50 and IC80 for each of the nanoformulations

The concentration at which the compound (TQ) reduces the cell viability by half (50%) is known as inhibitory concentration (IC50). Initially the IC50 was calculated using Probit software (Israel) that utilised the cytotoxicity results obtained in Chapters 2-5. In order to generate IC80 values, a trend line was plotted to generate an equation of the line for the three nanoformulations in each cell line. The slope was determined from the equation:

\[ y = mx + c \]

Where m: slope of the line, c: intercept of (y, x)

The slope values and their respective IC50 values obtained were used as inputs to determine the IC50/EC80 (inhibitory concentration) using calculator from Graphpad® quickcalcs (GraphPad Software, Inc. USA) to calculate IC50. The IC80 values were also calculated in similar manner for all the nanoformulations.

6.3 Results and discussion

6.3.1 Comparative analysis on the basis of physico-chemical characterisation

Table 6.1 enlists the characteristics of each of the nanoformulations used for delivering TQ. According to the results, TQ nanosuspension had the smallest hydrodynamic radius and amino acid functionalised polymer micelles were the largest.
The surface charge is known to play an important role with regards to uptake of nanoformulations in cells, hence zeta potential is an important characterisation measurement used for determination of the surface charge. It is also an important parameter for predicting the long term colloidal stability of the nanoformulation [16]. Based on zeta potential measurements, nanosuspensions showed better stability in comparison to dendrimers and amino-acid polymeric micelles.

Table 6.1 Comparison of different nanoparticulate systems used for delivering TQ

<table>
<thead>
<tr>
<th></th>
<th>Nanosuspension (TQ–NSP B)</th>
<th>Dendrimers (Dend-TQ)</th>
<th>Amino acid polymer micelles (P123 Asp Au-TQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS diameter (nm)</td>
<td>11.2±0.78</td>
<td>15.97±1.7</td>
<td>104.86±1.3</td>
</tr>
<tr>
<td>Zeta Potential (mV)</td>
<td>-16.3±2.1</td>
<td>-14.1±0.9</td>
<td>0.603 ± 6.93</td>
</tr>
<tr>
<td>Type of Interaction with TQ</td>
<td>Hydrophobic (Non-polar)</td>
<td>Host-guest (Electrostatic)</td>
<td>Hydrophobic (Non-polar)</td>
</tr>
<tr>
<td>Drug loading</td>
<td>N/A</td>
<td>333 µg/mL</td>
<td>800 µg/mL</td>
</tr>
<tr>
<td>% Drug release in 12 h</td>
<td>N/A</td>
<td>81%</td>
<td>86.3%</td>
</tr>
</tbody>
</table>

In the case of drug loading studies, it can be observed that the results correspond to above size distribution data. Since dendrimer has a smaller hydrodynamic radius in comparison to P123 Asp-Au TQ micelles the amount loaded in dendrimers was much lower than the micelles. The drug loading and release studies were not applicable for nanosuspension as it was the pure/pristine drug that was transformed to nanometre sized particles.

6.3.2 Comparative analysis on the basis of cytotoxicity profile

In order to perform a comparative analysis of all three nanoformulations on the basis of anti-proliferative effect and to understand the kinetics of TQ degradation after encapsulation, cell viability assay of each nanoparticle with equivalent amount of loaded TQ was performed.
Figure 6.1 shows a comparison of the dose dependent cytotoxicity of each nanoformulation in comparison to pristine TQ, in two breast cancer cell lines - MCF-7 and MDA-MB-231 after 24 h. It should be noted that in the absence of TQ, the dendrimers (~85% viability) and P123 Asp-Au micelles (~80% viability) showed no significant toxicity to both cell lines tested.

In MCF-7 cells (Figure 6.1 a), at relatively lower concentrations (50 µM) all three TQ nanoformulations showed complete cell death within 24 h, which was only seen at 100 µM in case of pristine TQ. Both cell lines were found to be most susceptible to P123 Asp-Au TQ formulation with ~65-70% decrease in viability at 10 µM equivalent TQ concentration. Dend-TQ and P123 Asp-Au TQ, however, showed similar effectiveness at 10 µM equivalent TQ concentration (Figure 6.1 a).

Another interesting finding observed in Figure 6.1 is, that while TQ-NSP B proved to be less effective in MCF-7 cells up to 25 µM equivalent TQ, it showed significant toxicity in MDA-MB-231 cells. TQ-NSP B showed the highest decline in the cell viability of MDA-MB-231 by 80% at a 10 µM TQ equivalent concentration, suggesting it is the most effective formulation in case of MDA-MB-231 cells.

The resistance to TQ-NSP B is not well understood in case of MCF-7, however, one reason could be the morphology of MCF-7 cells. As discussed earlier in Chapter 2 section 2.4, the morphology of the cells plays an influential role in cancer therapy. MCF-7 cells are known to preserve their characteristics of differentiated mammary epithelium in \textit{in vitro} conditions. As seen in Chapter 2 Figure 2.3, MCF-7 cells grow in domed shaped colonies, that tightly adhered to each other, allowing less surface area exposed for the penetration of certain nanoformulations [17]. However, this was not the case for MDA-MB-231 cells with regards to cell morphology; hence this cell line was mose susceptible to TQ-NSP B. These results also
suggest TQ-NSP B as a better drug delivery platform for highly metastatic cells such as MDA-MB-231 cells.

Taken together, all the three nanoformulations showed an enhanced response in comparison to pristine TQ. The decline in cell viability was observed in each system at almost all TQ concentrations with complete cell death at ~50 µM TQ.

Keeping in mind the outcomes from the above analysis, an experiment was devised in order to explore if the TQ in its nanoparticulate form, at low concentrations, could lead to activation of EMT, eventually leading to metastasis.

![Figure 6.1 Dose dependent cytotoxicity of drug delivery systems viz. dendrimer (Dend-TQ), P123 based amino acid capsules (P123 Asp-Au TQ) and nanosuspension (TQ-NSP B) in (a) MCF-7 and (b) MDA-MB-231 cells after 24 h. Data are mean ± Standard error from three independent experiments](image)

6.3.3 Flow cytometric analysis for E-cad expression in MCF-7 cells

The cytotoxicity assay indicated an improvement in the stability and efficacy of TQ, especially at lower concentrations, as reflected in Figure 6.1. However, it is not known if low TQ
concentrations within the encapsulated nanoformulations would show activation of EMT, similar to lower concentrations of pristine TQ (Chapter 2, Figures 2.2.6 and 2.2.7). In order to address this, a 10 µM concentration of pristine TQ in MCF-7 cells, which showed 80% viability with a marked EMT activity, was selected as a reference point. The next part of the investigation involved determining the relative concentration of TQ, in each nanoformulation, at which the cells showed 80% viability (IC80). The value for each of the nanoformulation were calculated as follows: Dend-TQ (3.5 µM), TQ NSP-B (9.72 µM) and P123 Asp-Au TQ (4.25 µM). After the IC80 values were generated for each nanoformulation, MCF-7 cells were treated and assessed for viability to confirm the IC80 values obtained from the above mentioned calculations.

The results showed ~80% viability (from Figure 6.2) for each of the nanoformulation, which was similar to that of pristine TQ, indicating that the calculations were accurate. The next step was to assess the expression of E-cad via flow cytometry. The histograms in Figure 6.3 consist of mean fluorescence intensities (FITC-A) plotted against the cell counts. The histograms typically show two peaks which are designated for two separate populations of E-cad, namely FITC-A (+ve) or FITC-A (-ve). The FITC A+ve population represents an E-cad positive populations and the FITC A–ve represents an E-cad negative population. The populations are represented by their respective peaks in the histograms, indicating the percentage of cells in each population.
Figure 6.2 The bar graph represents the cell viability after treatment with in MCF-7 cells after treatment with TQ along with an untreated control, 10 μM TQ treatment (green) and at titrated TQ concentrations of the three nanoparticles viz. Dend-TQ (3.5 μM), TQ NSP-B (9.72 μM) and P123 Asp-Au TQ (4.25 μM). Data are mean ± Standard error from three independent experiments.

The result from the first histogram [Figure 6.3 (a)] represent cells with no treatment of TQ, showing 87% of positive and 13% of negative E-cad populations. These cells are considered to exhibit stable E-cadherin expression.

After the treatment with 10 μM pristine TQ, the cells showed substantial changes in both populations [Figure 6.3 (b)] with a rise in the negative (FITC A–ve) population from 13% to 64% and a decrease in the positive population from 87% to 36%. This suggests that there is an increase in the number of cells with very low or negligible amounts of E-cad expression and is discussed in detail in Chapter 2, section 2.2.6.

TQ-NSP B [Figure 6.3 (d)] showed a negative population of 59% and a positive population of 41%, which was similar to pristine TQ [Figure 6.3 (b)]. If the cell viability results were to be taken into consideration, it would be observed that MCF 7 cells showed the same viability with
both, TQ-NSP B and pristine TQ (Figure 6.1 (a). This could explain the similar trend in E-cad population between these two treatments.

Figure 6.3 The flow cytometery graphs obtained by flow cytometery analysis represents the mean fluorescence intensity of in MCF-7 cells after treatment with TQ along with an (a) untreated control, (b) 10 μM TQ treatment and at calculated TQ concentrations of the three nanoparticles viz. (c) Dend-TQ (3.5 μM), (d) TQ NSP-B (9.72 μM) and (e) P123 Asp-Au TQ (4.25 μM).

The histogram obtained after the treatment with Dend-TQ and P123 Asp-Au TQ (Figure 6.3 c and e) showed a similar response, where the negative populations rose to 73% and 77% respectively and the positive populations decreased to 27% and 23%, respectively. This
suggests that low concentrations of TQ could be responsible for the decline in E-cad, which could activate the process of EMT and thereby metastasis in cancer cells.

6.4 Conclusion:
The overall results obtained from this chapter help us to evaluate the three nanoformulations on the basis of their physicochemical characterisations and biological activity on two breast cancer cell lines. The characteristics that were evaluated included DLS, zeta-potential and drug loading and release. The size of the nanoformulations increased in the following order:

**TQ-NSP B < Dend-TQ < P123 Asp-Au TQ**

The smallest of all the nanoformulations was TQ-NSP-B followed by Dend-TQ and P123 Asp-Au TQ. The dendrimer formulation showed the best results in term of drug release after 12 h. The *in vitro* release for all the nanoformulations was much better when compared to pristine TQ dissolved in solution. This suggests that encapsulation not only provided greater stability to TQ, but a slow sustainable release of TQ would be more effective for a relatively longer time.

In MCF-7 cells, P123 Asp-Au TQ and Dend-TQ showed higher efficacy in comparison to TQ-NSP B. However, in case of MDA-MB-231, TQ-NSP B showed a higher efficacy from 10 µM onwards in comparison to other nanoformulations. The cytotoxic profile of Dend-TQ and P123 Asp-Au TQ in cancer cells were consistent in both cell lines with effective results being observed at lower concentration than 10 µM TQ.

From the overall results, it can be deduced that at sub-lethal concentration of TQ, in any condition, whether encapsulated or in pristine form, can lead to decline in E-cadherin expression and in-turn lead to transition of epithelial cancer cells into mesenchymal phenotype.
6.5 References


Chapter 7: Summary, conclusion and future prospects

7.1 Summary and conclusion

With advancement in cancer medicine and therapy in last few decades, Cancer still remains the leading cause of death across the world. Since last four decades breast cancer therapy has encountered some important changes in screening, treatment and prevention but there is still a large room for improvement in the field [1-3].

Currently, a combination of chemotherapy (streamline therapy) and radiation (adjuvant therapy) is still the treatment of choice for breast cancer; however, it is often accompanied by adverse effects which significantly reduce the quality of patient's life. Hence, there is a need for an alternative for chemo-drugs in order to mitigate the above mentioned problems.

One such alternative involves the use of natural dietary phytochemicals which have been nominated for epidemiological and pre-clinical, early clinical studies for the treatment of cancer such as curcumin, epigallocatechin, capsaicin, thymoquinone and many more [4-7]. Phytochemicals usually exhibit low side effects and exhibit pleiotropic behaviour which makes them a suitable candidate for use as an alternative to chemotherapy which can eventually lead to the betterment of patient’s health and for improvement of therapeutic efficacy simultaneously.

As mentioned above the pleiotropic nature of these phytochemicals makes them a good candidate for cancer therapy. Thymoquinone (TQ) is one such phytochemical that forms a major constituent of the volatile oils obtained from the black seed or black cumin. It has been more than 50 years since the identification of thymoquinone in the oils [8, 9]. Several reports suggest TQ to be anticancer and anti-inflammatory in nature. Most of the literature exhibits TQ as a cytotoxic agent or pro-apoptotic agent at high concentrations [10-17].
The key aspect of this thesis has been to increase the understanding of the mechanistic aspects of TQ and make an attempt to shed light on the changes in the biochemical pathway of TQ’s activity at lower concentrations in cancer cells.

In Chapter 2, cytotoxicity assay was used to identify the cell line most sensitive to TQ, by performing dose dependent toxicity studies on four cancer cell lines. The results obtained suggested that TQ was effective against all four cancer cell lines; however, both the breast cancer cell lines (MCF-7 and MDA-MB-231) showed the higher sensitivity to TQ.

For a better understanding of TQ and its effect at low concentrations in cancer cells, MTT assay was performed in dose and time dependent manner. Even though the results indicate that TQ was toxic to cancer cells at higher concentrations at all the three time points viz. 24, 48 and 72 h, at lower concentrations, it exhibited time dependent recovery. Therefore, it was not only important to study/explore the above effect but also develop an understanding at the molecular level in the cancer cells. For evaluation of TQ toxicity in a qualitative manner, a morphological assay of MCF-7 and MDA-MB-231 was performed with 3 concentrations of TQ (viz. 10, 25 and 50 μM). The cells changed in morphology upon TQ treatment. At 10 μM the cells started to round off at the edges due to membrane ruffling. This indicated that TQ might be affecting the extracellular adhesion protein as well as reorganizing the cytoskeleton. At high concentration it was clearly seen that the cells were separated from colonies and rounded with a few floating cells. MCF-7 is an adherent cell line and rounding off and getting detached from the substrate indicates cell death. This is considered as one of the hallmarks of cell death in epithelial cancer cells [18, 19].

The change in morphology is associated with reorganization of the cytoskeletal proteins. The cytoskeletal reorganization within the cells occurring at low concentrations of TQ was observed by probing F-actin fibre (polymeric actin fibres involved in structure and motility of
the cells) and vinculin (focal adhesion protein). Generally, F-actin fibres in control/untreated MCF-7 cells were randomly distributed and vinculin was localized towards the edges of cells at focal adhesion points [20]. However, after TQ treatment there was a visible reorganization of both proteins with change in the morphology of cells which showed bipolar characteristics. The F-actin was aligned towards the two poles of the cells and amount of vinculin had less localization at the focal adhesion points. The above evidence suggested that the cells were in a migratory state. The feature for cells becoming bipolar also suggests that possibility of cells becoming metastatic in nature [20, 21].

In order to understand the effect of change in the morphology and the reorganization of cytoskeletal proteins; it was necessary to evaluate the expression of E-cad (highly expressed in MCF-7 cells), a cell surface transmembrane glycoprotein which is a key protein for Ca^{2+} dependent cell-cell adhesion. It plays an important role as an inhibitor for migration and motility in cells [21, 22]. Since the loss of this protein is associated with the beginning of migratory pathway for the cells, it was considered as good indicator for migration. In order to determine the effect of TQ on the motility and migration of MCF-7 cells, confocal microscopy and flow cytometry studies suggested the loss of E-cad from the cells. The results clearly indicated that E-cad was affected due to TQ and its degradation was further confirmed by validating the translocation of β-catenin into nucleus with the help of chromatin immuno precipitation (ChIP) assay. ChIP assay results suggested that β-catenin, was localised in the nucleus after the destruction of the CCC. The results also confirmed that sub-lethal doses of TQ lead to an increase in CdK2 production, which results from in β-catenin translocation into the nucleus, leading to the degradation of the CCC. Since CdK2 is known to increase cell proliferation [23-25], the ChIP assay results indicate that low concentrations of TQ could exhibit a pro-cancerous effect.
Destruction of cadherin-catenin complex (CCC) has not only been associated with loss of cell polarity leading to cell death, but also with activation of the migratory machinery leading to epithelial to mesenchymal transition (EMT) of the cells. This phenomenon is known to enhance cancer progression and metastasis [18, 21, 26, 27]. Hence, the above results suggested that a sub-lethal dose of TQ may have a pro-cancerous effect through EMT of cancer cells.

All of the above led to the identification of a new mode of action of TQ in breast cancer cells, which lead to lysosomal degradation of E-cad followed by destruction of CCC.

If low concentration of TQ could possibly initiate EMT, the question that would arise from this study is would it be possible to increase the efficacy of these phytochemicals for better therapeutic effective treatment against breast cancer cells?

The use of TQ has some limitations which include poor stability and bioavailability within the human body; hence TQ has not been a front runner for application in chemotherapy. In an in vitro environment, TQ comes in direct contact with the target cells, however, in an in vivo environment, it has to cross several barriers such as membrane permeability, low pH in stomach and insufficient time for absorption thereby losing its activity due to degradation, before reaching the target site and this in turn leads to low availability of TQ at the target site [28-30].

The therapeutic efficacy of drugs and phytochemicals is most often reduced due to its incapability to gain access to the site of action due to its insolubility in aqueous environment or degradation before reaching the target site [28, 31-33].

To address low bioavailability and improved therapeutic efficacy, three different nanoformulations were synthesized and characterized, wherein TQ was either encapsulated/conjugated to an appropriate carrier to prevent degradation, thereby ensuring delivery of TQ to the target in its intact form, enhancing its therapeutic efficacy.
Nanosuspension was the nanoformulation of TQ that was employed to increase the efficacy in the breast cancer cells. TQ, in presence of a surfactant d-α-tocopherol polyethylene glycol 1000 succinate (TPGS), was converted into its nanosuspension (Chapter 3). This formulation when tested on breast cancer cell lines showed increase in therapeutic efficacy at low concentrations of TQ.

Dendrimers are monodisperse, macromolecular structures with globular polymeric architecture having several unique advantages over conventional drug delivery systems [34, 35]. In Chapter 4, the dendrimers were loaded with TQ and tested for efficient delivery of biologically active TQ to the breast cancer cells. The dendrimers loaded with TQ showed increase in therapeutic efficacy at lower concentrations of TQ which suggested that increase the half-life of the TQ due to encapsulation within the dendrimer pockets. The results suggested an improvement in the therapeutic efficacy of the TQ based dendrimer formulation due to small size of dendrimers, increase in the TQ stability and slow sustained release of TQ for up to 24 h.

In Chapter 5, another drug delivery system was based on Pluronic 123 micelles were used in order to increase stability and explore its therapeutic efficacy due to encapsulation. The aim was to functionalise the self-assembled nanostructures of P123 by amino acids and gold nanoparticles by a novel method. The reason behind functionalisation was to obtain a slow and sustained release profile of TQ over a longer period of time. Overall results demonstrated that the P123 Asp-Au TQ has the potential to be developed as a biocompatible nano-carrier for encapsulating TQ and other phytochemicals to enhance their therapeutic effect and their stability making them a successful line of treatment with low side effects.

When pristine TQ was administered to cells at 10 μM, viability was decreased by ~20% and expression of E-cad was reduced very significantly. When the concentrations of the nanoformulation were titrated on the basis of viability from levels of free TQ at 10 μM, a reduction
in expression was visible, with cell death remaining almost same as free TQ. The results indicated that low concentrations of TQ (low bioavailability) induced EMT in the cells. The thesis further suggested that the problem of TQ stability can be resolved by the use of different nano-formulations to increase its therapeutic efficacy against breast cancer.

The overall study in this thesis, investigates a new mode of interaction of pristine TQ due to low stability and bioavailability while creating a path towards increasing its stability and efficacy using different types of nanoformulations.

7.2 Future prospects

In last few decades phytochemicals have gained a significant attention as natural products for treatment of various diseases. The interaction of natural phytochemicals in biological system has been extensively studied for anticancer activity and numerous signalling pathways have been identified. This thesis encompasses two major aspects which include elucidating the mechanistic aspect of TQ interaction in cancer cells at low or sub-toxic concentration and using nanoformulations to address the issue of low stability and bioavailability.

The mechanistic aspect of TQ was studied with regards to reorganization of cytoskeleton and destruction of cadherin-catenin complex. However, phytochemicals such as TQ affect multiple signalling transduction pathways; hence in-depth evaluation at molecular and genetic level would be ideal to understand the effect of TQ.

In order to improve the understanding on TQ’s mechanism, it requires genomic and proteomic profiling of cells after treatment of TQ. The main outcome from both can be used to deduce a systematic network of molecules affected by the application of TQ. Further knowledge could be enhanced by using a systems biology approach which involves the use of integration of large
data sets obtained from genomics, proteomics and transcriptomics to obtain a holistic view of complex interaction of molecules occurring in the cells [36-38].

According to literature, quinones are known to exhibit mutagenicity. They are also known to cause irreversible/reversible genetic alterations thereby disrupt the functioning of DNA or cause mutations in the DNA [2, 39]. These mutations represent a threat to the human race with regards to cancer. Hence, epigenetic studies would help to reveal the useful markers affected by TQ which could provide the insight into new signalling pathways as well as identify the useful markers required for cancer diagnosis and pharmacological treatment.

The second main aspect covered in the thesis is use of nanoformulations to address the issue of low bioavailability to improve its stability by encapsulation. The improvement in therapeutic efficacy of TQ not only depends upon TQ loading and release kinetics but also on the uptake of nanoformulations in the cells. In order to understand the TQ loaded nanoparticle interaction with cancer cells and delivery of its cargo, uptake studies could be performed. This could be done either by loading with fluorescent dye and tagging a fluorophore on the surface of the nanoparticle as rhodamine B or fluorescein isothiocyanate (FITC) [35, 40]. In case of P123 amino acid micelles, the uptake studies can be performed by using transmission electron microscopy (TEM) as the polymeric micelles consist of gold nanoparticles formed on the surface. Gold nanoparticles are known to be promising contrasting agent and are known for their use in tagging antibodies in order to provide detection in TEM samples of cells.

Recently, gold nanoparticles have attained a lot of importance as contrasting agents for computed tomography as they exhibit biocompatibility and high x-rays absorption coefficient [41, 42]. Hence, the P123 amino acid micelles applicability should be explored in medical imaging. With this added feature the micelles could be exploited for diagnostics and therapeutic
capabilities at the same time [43-45]. Thus, emerging as a versatile tool for exploitation in the field of theranostics.

The future work can be focussed on the unique approach of conjugating one or more ligands specific to the receptors on cancer cells on either dendrimers or P123 amino acid micelles which can further increase the uptake, specificity and selectivity for cancer cells.

Dendrimers and P123 amino acid micelles showed promising results in case of MCF-7 and MDA-MB-231 cells with much higher increase in therapeutic efficacy. It would be interesting to gain insight and further evaluate their bio-distribution in vivo in order to translate the drug delivery technology from bench to bedside.

Overall work summarized in this thesis offers a vision to understand the mechanistic aspects of thymoquinone at sub-toxic concentration during low bioavailability and extenuating by using drug delivery carriers.

7.3 References:


Appendix

Publications

The research in this thesis will be published in several articles, detailed below:

Articles under preparation

Chapter 2

“Thymoquinone mediated lysosomal degradation of E-cadherin in breast cancer cell”
Vishal Mistry, Ravi Shukla, Vipul Bansal.

Chapter 3

“Nanosuspension of Thymoquinone: Formulation, characterization and bio-evaluation”
Vishal Mistry, Hitesh Kulhari, Deep Pooja, Ravi Shukla and Vipul Bansal.

Chapter 4

“Thymoquinone encapsulation in dendrimers enhances its efficacy towards breast cancer”
Vishal Mistry, Hitesh Kulhari, Ravi Shukla and Vipul Bansal.

Chapter 5

“Self-assembled nanostructures of triblock copolymer-aspartate gold nanoparticles as TQ delivery vehicles with improved stability and therapeutic efficacy”
Vishal Mistry, S.Periasamy, Hitesh Kulhari, Ravi Shukla and Vipul Bansal.