GENE AND PROTEIN ANALYSES OF SELECTED DRUG-RESISTAN BREAST CANCER CELL LINES TREATED WITH THYMOQUINONE-NANOPARTICLE

by

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LIST OF ABBREVIATIONS AND SYMBOLS

ABCB1	ATP-binding cassette sub-family B member 1
ATCC	American type culture collection
ATR-IR	Attenuated Total Reflectance-Infrared Spectroscopy
BCL2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
CO_2	Carbon dioxide
DCIS	Ductal Carcinoma in Situ
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EE	Entrapment Efficiency
ER	estrogen receptor
FBS	Fetal Bovine Serum
HER2	Human Epidermal Growth Factor Receptor 2
IC_{50}	Half maximal inhibitory concentration
MCF-7	Michigan Cancer Foundation-7
MDR1	Multidrug Resistance Protein 1
MRP4	Multidrug Resistance-associated Protein 4
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	Sodium chloride
PBS	Phosphate Buffered Saline
PDI	Polydispersity Index
PGA	Polyglutamic acid
PLA	Polylactic acid
PLGA-PEG	Poly(lactic-co-glycolic acid)- Polyethylene glycol
PMSF	Phenylmethane Sulfonyl Fluoride
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction

RIPA	Radio immunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SERM	Selective Estrogen Receptor Modulators
TAM	Tamoxifen
TamR MCF-7	MCF-7 resistance to tamoxifen
TBST	Tris-Buffered Saline and Tween 20
TEM	Transmission Electron Microscope
TQ	Thymoquinone
UACC 732	Resistance breast cancer cell lines
UV-VIS	Ultraviolet-visible spectrophotometry
β-actin	beta actin

ANALISA GEN DAN PROTEIN KE ATAS TITISAN SEL PAYUDARA TERPILIH RINTANG-DRUG YANG DIRAWAT DENGAN NANOPARTIKEL-THYMOQUINONE

ABSTRAK

Kemoterapi merupakan pilihan penting untuk rawatan kanser payudara. Walau bagaimanapun, rintangan terdapat drug merupakan salah satu halangan utama. Terdapat keperluan untuk menangani cabaran ini dengan drug baharu menggunakan teknologi nano. Nanoperubatan digunakan untuk penyampaian drug berpotensi dalam terapi kanser kini. Thymoquinone (TQ) bebas adalah kompaun aktif yang terkandung di dalam ekstrak biji *Nigella sativa* yang mempamerkan nilai perubatan. Oleh itu, kajian ini bertujuan untuk mensintesis dan mencirikan, nanozarah poli (dl-laktida-ko-glikolida) polietilena glikol (PLGA-PEG) yang mengandungi TQ untuk memulihara bioketersediaan TQ dan juga menghasilkan alur sel barah payudara MCF-7 rintang terhadap tamoxifen (TAM). Seterusnya, sel barah yang rintang terhadap drug dirawat dengan TQ-nanozarah untuk menentukan perubahan terhadap modulasi penyataan protein dan gen sasaran. TQ-nanozarah disediakan dengan menggunakan teknik sesaran pelarut. Ia seterusnya dicirikan dengan beberapa ujian melibatkan imbasan mikroskopi elektron tranmisi (TEM), spektroskopi ultraviolet terlihat (UV-VIS), serakan cahaya dinamik (DLS), spektroskopi inframerah transformasi (ATR-IR), kecekapan pemerangkapan dan analisa pelepasan drug. Dalam kajian ini, dua jenis titisan sel kanser payudara digunakan iaitu MCF-7 dan UACC 732 untuk mengkaji keberkesanan TQ-nanozarah. MCF-7 adalah titisan sel positif terhadap reseptor estrogen (ER+) dari subjenis luminal A. Pembangunan sel MCF-7 rintang terhadap TAM dilakukan dengan kaedah denyut. Indeks rintangan

telah ditentukan selepas rawatan dengan TAM menggunakan analisis MTS. Titisan sel UACC 732 mempamerkan reseptor faktor pertumbuhan epidermis manusia 2 (HER2) secara berlebihan, dan rintang terhadap trastuzumab dan lapatinib. Modulasi gen dan protein disebabkan oleh TQ-nanozarah dalam subjenis kanser rintang drug telah dikaji. Pencirian struktur TQ-nanozarah melalui oleh DLS dan TEM menunjukkan diameter zarah di antara 50-100 nm. Anggaran potensi zeta menunjukkan bahawa formulasi TQ-nanozarah adalah bercas negatif (-25 mV). Ia memberikan pengambilan sel secara mudah. Kejayaan pengkapsulan drug di dalam polimer telah disahkan dengan teknik ATR-IR. Kecekapan pengkapsulan TQ ialah 79.9% dan pelepasan maksimum TQ adalah 50.3%. Pembangunan sel MCF-7 rintang drug menunjukkan bahawa sel 8 μM TamR MCF-7 mencapai indeks rintangan tertinggi berbanding dengan sel induk dan sel lain yang terdedah dengan kepekatan berbeza. Ia seterusnya disokong oleh analisa protein dimana ekspresi protein MDR1 meningkat secara bererti ($p<0.05$). Kajian mendapati bahawa TQ-nanozarah mengurangkan ekspresi protein MDR1, HER2, dan MRP4 secara bererti dalam sel UACC 732 ($p<0.05$). Begitu juga sel TamR MCF-7menunjukkan pengurangan ekspresi tetapi ia adalah tidak bererti ($p>0.05$). Analisis ekspresi gen dilakukan bagi membandingkan kesan di antara TQ-nanozarah dengan doxorubicin. Kajian menunjukkan penurunan bererti dalam ekspresi dua jenis gen rintang drug (*ABCC5* dan *HER2/ERBB2*) dan gen pencambahan kanser (*BCL2*). Sebagai kesimpulan, kajian ini menunjukkan TQ-nanozarah berasaskan PLGA-PEG dapat menurunkan ekspresi gen dan protein rintangan drug. Aplikasi TQ-nanozarah berpotensi digunakan sebagai rawatan baharu untuk kanser payudara HER2.

GENE AND PROTEIN ANALYSES OF SELECTED DRUG-RESISTANCE BREAST CANCER CELL LINES TREATED WITH THYMOQUINONE-NANOPARTICLE

ABSTRACT

Chemotherapy is an important option for the treatment of breast cancer. Nevertheless, drug resistance is one of the major obstacles. There is a need to address this challenge with a new drug using nanotechnology. Nanomedicine is used for drug delivery in the current cancer therapy. Free thymoquinone (TQ) is a potential active compound of *Nigella sativa* seed extract that exhibits medicinal value. Therefore, present study aimed to synthesise and characterise the TQ encapsulated poly (d l-lactide-co-glycolide) polyethylene glycol (PLGA-PEG) nanoparticles to conserve its bioavailability and also to develop MCF-7 cell line resistance to tamoxifen (TAM). Subsequently, the resistance breast cancer cell line were treated with TQ-nanoparticle to determine the modulation of targeted protein, and gene expression. TQ-nanoparticles were prepared by solvent displacement technique. It was then characterised with different techniques which involved Transmission Electron microscopy (TEM), ultraviolet-visible spectroscopy (UV-VIS), dynamic light scattering (DLS), attenuated total reflectance infrared (ATR-IR), entrapment efficiency and drug release analysis. In this study, two type of breast cancer cell lines are used which were MCF-7 and UACC 732 to study effectiveness of TQ-nanoparticle. The MCF-7 is estrogen receptor positive (ER +) cell line from the luminal A subtype. Development of Tamoxifen (TAM) resistance MCF-7 was done

using pulse method. Resistance index was determined after treatment with TAM using MTS assay. The UACC-732 cell line is human epidermal growth factor receptor 2 (HER2) overexpress and resistance to trastuzumab and lapatinib. Genetic and proteomic modulation caused by TQ-nanoparticles in drug-resistant cancer subtypes were investigated. TQ-nanoparticle structure characterisation by DLS and TEM revealed particle diameter between 50-100 nm. Zeta potential estimation revealed that the TQ-nanoparticle formulations were negatively charged (-25 mV). This would confer facilitated cellular uptake. The success of drug encapsulation within polymer was confirmed with ATR-IR technique. Encapsulation efficiency of TQ was 79.9% and drug release showed a maximum release of TQ at 50.3 %. Development of drug resistant MCF-7 cells showed that the 8 μM TamR MCF-7 cells achieved the highest resistance index compared to parental MCF-7 and cells exposed to other concentrations. It was further supportted by proteomic analysis, in which MDR1 expression was significantly upregulated ($p<0.05$). Findings indicated TQ-nanoparticle significantly reduced expression of MDR1, HER2 and MRP4 protein in UACC 732 cell ($p<0.05$). Similarly, resistant TamR MCF-7 cells showed reduction in expression, however they were not significant ($p>0.05$). Gene expression analysis was done to compare effect of TQ-nanoparticles with doxorubicin. Study showed significant decrease in expression of two drug resistant genes (*ABCC5* and *HER2/ERBB2)* and cancer proliferation gene (*BCL2*). In conclusion, this study show that TQ-nanoparticle based on PLGA-PEG can reduce the expression of drug resistant gene and protein. Application of TQ-nanoparticle potentially can be used as new treatment to HER2 breast cancer.

CHAPTER 1

INTRODUCTION

1.1 Cancer prevalence

Cancer is among major causes of morbidity and mortality around the world and mostly in developing countries (Ferlay et al., 2015). It is predicted that within the next 2 decades, the annual cancer cases will increase from 14 million to 22 million (Qian, 2015). The National Cancer Registry of Malaysia (NCR) year reported each year, more than 21,000 cases and almost 10,000 cases are unregistered. Generally by the age of 75 year-old, one out of four Malaysians (1:4) is expected to be diagnosed with cancer (Qian, 2015).

Cancer is a disease that causes cells to change and exhibit uncontrolled growth. It may occur at any site of the body. Majority of cancers would lead to development of lump which is called tumor (Rick Alteri et al., 2015). They exhibit different characteristics, where they can be classified as benign or malignant. Benign tumor could be removed and it may not metastasize unlike malignant tumor. Malignant tumors proliferate and could invade to other parts of the body. Cancer can be divided into five categories which include carcinomas, sarcomas, leukemia, lymphomas and gliomas. Carcinomas begin in the skin or tissues that line the internal organs. The subtypes are adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transition cell carcinoma. Whereas sites sarcoma growth are at bone, cartilage, fat, muscle or other connective or supportive tissues. Leukemia begins in the blood and bone marrow. On the other hand, lymphomas begin at the lymph glands. Central nervous system cancer or gliomas involve the brain and spinal cord (Mohamad et al., 2015). Ministry of Health Hospitals report stated that about 13% of deaths were due

to cancer. Cancer is the 3rd most common cause of death following cardiovascular diseases (22.77%) and respiratory diseases (18.54%) (Mohamad et al., 2015).

1.1.1 Breast cancer epidemiology

Breast cancer initiation sites are mostly at lobules and ducts. The other parts of this organ has fatty tissue, connective, and lymphatic tissues (Rick Alteri et al., 2015). The new cases and cause of death were among women in 140 of 184 countries worldwide (Torre et al., 2017). In Malaysia, breast cancer is the most prevalent malignancy followed by colorectal and lung cancer (Lim & Azura, 2008). The 50-59 year-old was found to be peak age cohort for breast carcinoma (Lim & Azura, 2008). Besides that, cumulative life-time risk based on ethnicity was highest among Chinese (1:16) followed by Indians (1: 17) and lowest in Malays (1:28) (Zainal Ariffin & Nor Saleha, 2011). These ethnic variances may suggest an etiological role of the genetic factors in cancer (Sung et al., 2005)

1.1.2 Breast cancer subtype

Breast cancer generally refers as one disease, but it is important to know that breast cancer is differentiated to 21 distinct histological subtypes and 4 molecular subtypes. The four molecular subtypes differ in presentation, treatment outcome and their associated risk factors (Barnard et al., 2015; Dieci et al., 2014; Koboldt et al., 2012; Tamimi et al., 2012). They are classified based on biological markers that include estrogen or progesterone receptors (HR+/HR-) and overexpression of human epidermal growth factor receptor 2 (HER2+/HER2-), a growth-promoting protein (Anderson, 2014b; Cheang et al., 2015; Kohler et al., 2015).

a) Luminal A (HR+/HER2-).

Prevalence of Estrogen Recaptor positive (ER+) and/or Progestron Receptor positive (PR+) with HER2- is about 74%. It has low levels of the protein Ki-67, which control cancer cell growth and it provides indication of active cell division (Parise & Caggiano, 2014). These cancers progress slowly. They are associated better prognosis because of hormone receptor expressions (Anderson, 2014a; F. M. Blows et al., 2010) MCF-7 cell line that used in this studies are luminal A subtype (Zhang et al., 2016).

b) Luminal B (HR+/HER2+).

This subtype of breast cancer has ER+ and/or PR+ and either HER2 positive or HER2 negative with high levels of Ki67. However, only 10% of patients show ER+ and/or PR+ and HER2+. It is of higher grade and found to be more aggressive compared to luminal A. Furthermore, their prognosis is slightly poorer (Parise & Caggiano, 2014).

c) Triple negative (TN).

It is hormone-receptor negative where it exhibits estrogen receptor negative (ER-), progesterone receptor negative (PR-) and HER2 negative. It is more prevalent among premenopausal group and those with mutation of *BRCA1* gene. Prevalence of TN is around 12%. It is reported to be twice higher among young women as well as African-American women (Perou & Børresen-Dale, 2011). Furthermore, 75% of TN breast cancer is basal-like subtype. They show poor prognosis because of in-availability of targeted therapy (Adrada et al., 2014; Blows et al., 2010).

d) HER2-enriched (HR-/HER2+)

Overexpression of HER2 is reported to be between 15-20% among breast cancer patients. They lack of hormone receptors and could progress rapidly. Prognosis among those with this subtype is also reported to be poor compared to ER+ breast cancers (Blows et al., 2010). Targeted therapy approach for HER2 overexpression has reversed most of the adverse prognosis effects (Rick Alteri et al., 2015). The UACC 732 drug resistance cell line used in this study was the HER2-enriched subtype due to overexpression of HER2 protein (Kalous et al., 2012).

1.1.3 Breast cancer treatments

Cancer development can be described as growing and spreading process of cancer cells in the body. There are 5 stages of cancer as show in Table 1.1. Stage I is classified when cancer cells are localised in one part of organ and relatively small. Stage II and III indicate the tumour is larger and has grown into nearby tissues or lymph nodes while stage IV indicate the disease has metastasised to other organs or throughout the body. Each of these stages has its own treatment system (Naim & Renée, 2017).

Table 1.1: Stages of cancer (Naim & Renée, 2017)

Stage	Sites
0	Abnormal cells in an area of body and may develop into cancer in future, also known as Carcinoma In Situ
I	Cancer is relatively small and contained within the organ.
II	Cancer has not spread into surrounding tissues but it may spread into lymph nodes close to the tumour.
III	Cancer may have spread to surrounding tissues and lymph nodes.
IV	Cancer has spread to other organs.

Treatment plan include local treatment, systemic treatment or a combination of the both. Local therapies used for breast cancer treatment are surgery and radiation therapy. Surgery is performed to remove growth or cancer from the body and radiation therapy kills the cancer cells and cause tumor shrinkage (Society, 2017). Whereas systemic treatment involve use of agents (cytotoxic, hormonal, and immunotherapeutic) to treat breast cancer (Gonzalez et al., 2007). This mode of treatment can reach cancer cells almost anywhere in the body and can be given either orally intravenously. Different types of treatment might be used depending on the type of breast cancer. These treatments are given in the adjuvant, neoadjuvant, and metastatic clinical settings. Adjuvant therapy refers to treatment after patients undergo surgery and patients with potential risk of relapse (Burstein et al., 2016). Neoadjuvant therapy is given to patients with locally advanced and also with inflammatory cancers (Green & Hortobagyi, 2002). Chemotherapy, hormonal therapy and targeted therapy

are type of systemic treatment and most of breast cancer patient get more than one type of treatment (Gonzalez et al., 2007). Chemotherapy can be taken orally or through intravenous (Eek et al., 2016). Hormonal therapy is given after surgery as adjuvant therapy in reducing risk of relapse (Abraham & Staffurth, 2016).Targeted therapy are drug or other element that can prevent cancer proliferation through interference with specific molecules (Wu et al., 2006).

Hormone therapy is a systemic therapy, where it circulates in the entire system (Kaseb et al., 2007). Tamoxifen (TAM) is an endocrine therapy for ER-positive cancer (Abe et al., 2005; Rick Alteri et al., 2015). It blocks estrogen from binding to receptor in cancer cells (Clarke et al., 1998). TAM is called a selective estrogen receptor modulator (SERM). It has the ability to compete with estrogen in binding to the receptor (Clarke et al., 1998; Kabel et al., 2016). It is usually given to women with ER+ after undergoing surgery or those diagnosed with ductal carcinoma in situ (DCIS). It can help lower the chance of recurrence and usually consumed for 5 to 10 years (Ali et al., 2016).

The cellular growth and proliferation were modulated by human epidermal growth factor receptor 2 (HER2) (Hynes & Lane, 2005). About 20% of breast tumours occur due to overexpression of HER2 receptor and amplification of *HER2* gene, and it is associated with poor prognosis (Slamon et al., 2001). Trastuzumab is a type of targeted cancer drug. Its work by targeting and binding to the HER2 receptor to prevent cancer cells from growing and dividing, hence will improve disease-free and overall survival in patients (Joensuu et al., 2006). Lapatinib is the tyrosine kinase inhibitor, it is another anti-HER2 agent that is given in combination with capecitabine,

to improve progression-free survival in patients who have progressed on trastuzumab (Geyer et al., 2006). Lapatinib bind to ATP-binding pocket of the HER2 protein, inhibits the receptor signal processes, preventing self-phosphorylation and subsequent activation of signal mechanism (Nelson & Dolder, 2006). Both the anti-HER2 monoclonal antibody trastuzumab and tyrosine kinase inhibitor lapatinab have complementary mechanisms of action and synergistic antitumour activity in models of HER2-overexpressing breast cancer (Baselga et al., 2012)

1.1.4 Multi drug resistance cancer

Based on the NCI Dictionary of Cancer Terms (2017), systemic therapy is defined as treatment using medications through blood circulation and ultimately reaching entire body. Cytotoxic, hormonal therapy and immunotherapeutic agents are systemic therapies (Gonzalez et al., 2007). Limitation of systemic agents are they are effective in 90% of primary breast cancer during early of therapy and only 50% among those with metastasis (Gonzalez et al., 2007), Tumours continue to develop resistance to the agents after a variable period of treatment. TAM is often used as anti-estrogen for hormone-dependent breast cancer (Powles et al., 2007).

The challenge in treatment is relapse due to multidrug resistance (MDR). It is the cause of failure to chemotherapy (Persidis & Aris, 1999). Cancer cells that are sensitive to drugs would be killed by chemotherapy, but higher proportion of drug-resistance cancer cells are left behind. Thus leading to poor prognosis after chemotherapy and development of resistance (Persidis & Aris, 1999). Mechanisms involved in resistance include decrease drug influx, increase efflux, detoxification systems, DNA repair and evasion (Gillet & Gottesman, 2010). The presence of at least

two molecular pump in cancer cell membranes are correlated with resistance that actively expel intracellular chemotherapy drug accumulation (Persidis & Aris, 1999). This process allows cancer cells to avoid the toxic effects of chemotherapy drug within the nucleus or cytoplasm (Persidis & Aris, 1999). P-glycoprotein or Multidrug Resistance 1 protein (MDR1) and the multidrug resistance protein family are types of pumps that are commonly found to confer chemo-resistance (Gonzalez et al., 2007). These proteins has become the targets of several anticancer agents (Persidis & Aris, 1999). For hormone dependent breast cancer patient, TAM are the anti-hormonal drug that is most frequently used in treatment. It is effective in cancers that express ER (Powles et al., 2007), but almost all tumors developed resistance to TAM after treatment (Kurebayashi, 2005).

In this study two subtype of breast cancer cell lines were used and they were purchased from ATCC. The MCF-7 cell line originates from 69 year old Caucasian patient. It was isolated in 1970 (Lee et al., 2015). MCF-7 the acronym for Michigan Cancer Foundation-7. It is used in breast cancer research related to the estrogen receptor (ER) alpha. ER positive breast cancer MCF-7 is of luminal A subtype (Zhang et al., 2016). The other breast cancer cell line were UACC 732. It is from HER2-enriched subtype that overexpress of HER2 protein (Kalous et al., 2012). This cell line was isolated form 33 year old Caucasian patient with resistance to trastuzumab and lapatinib (Canonici et al., 2013).

In this study, MCF-7 and UACC 732 breast cancer cell line were selected. The highest percentage of breast cancer patient (70%) express ER positive breast cancer, followed by 20% patient overexpress HER2 with poor prognosis.

1.1.5 Cancer Biomarker

Cancer is a genetic disease and there are more that hundred distinct type of cancer. Changes of genetic and epigenetic can cause carcinogenesis or tumorigenesis (Baylin & Herman, 2000). In early 2000, cells that progress towards a neoplastic state were found to acquire distinctive capabilities (Hanahan & Weinberg, 2000). Self-sufficiency in growth signal, resistance to growth inhibitory signal and anti-cancer therapy, evasion of cell death, replication capability, sustained angiogenesis, and metastasis were the six hallmarks for every type of cancer that very useful framework to understand the tumor pathogenesis (Hanahan & Weinberg, 2000). Recent finding showed involvement of reprogramming of energy metabolism, avoiding immune destruction, tumor-promoting inflammation, and evading immune destruction as provided in Fig 1.1 (Hanahan & Weinberg, 2011).

Biomarker is the protein or gene that could either indicate normal biologic or, pathogenic processes. It would also show changes to therapeutic intervention (Strimbu & Tavel, 2010). Biomarker can indicate normal or abnormal process that occur during an underlying condition or disease. Various types of molecules can be serve a biomarkers, for example DNA (genes), protein or hormones (Strimbu & Tavel, 2010). In cancer disease, biomarker become the important tool for cancer identification and also for cancer treatment. The cancer tissue secretion molecule or specific response of cells to cancer may produce the biomarkers, and its can be found in blood, stool, urine, tumor tissue, other tissues and body fluids. It also refers to genetic, epigenetic, proteomic, glycemic, and imaging biomarkers that used for cancer treatment, prognosis, and epidemiology (Mishra & Verma, 2010).

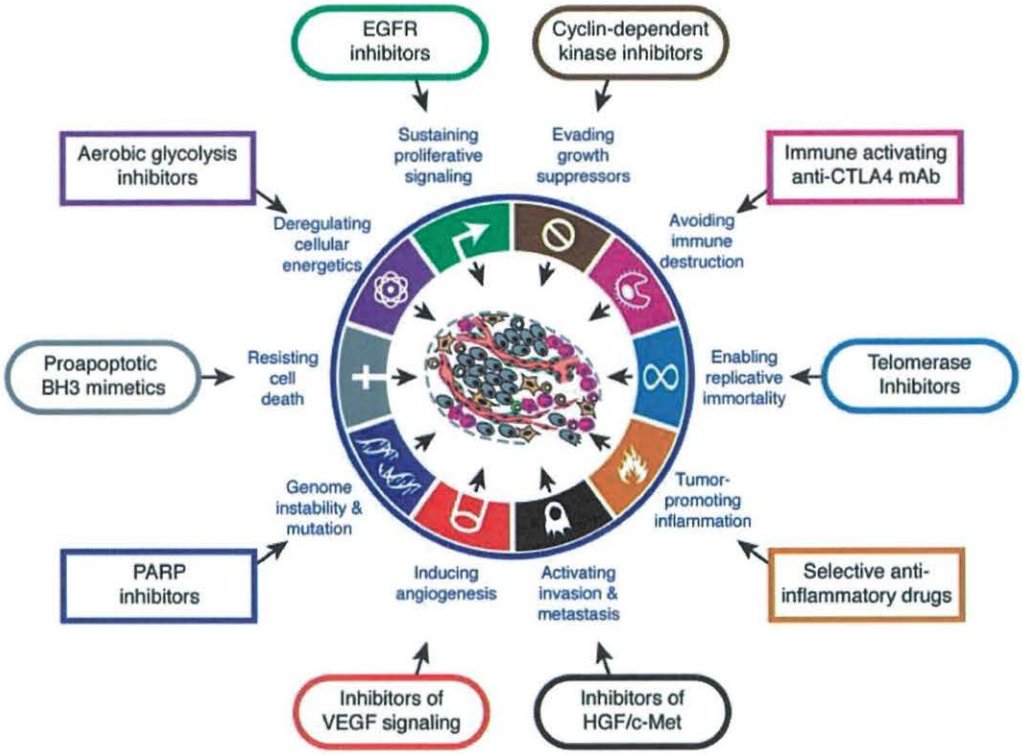

Figure 1.1: The hallmark of cancer (Hanahan & Weinberg, 2011)

1.1.5 (a) Breast cancer genes

Breast cancer is categorised as a complex disease. Classification of breast cancer show specific gene expression. Each subtype differs, where Luminal A breast cancer express *ESR1* gene, while HER 2 like breast cancer express *ERBB2* gene and Basal like or Triple negative breast cancer express *EGFR (ERBB1)* gene (Rouzier et al., 2005). Gene related to signal transduction are *BRCA1, ESR1, BCL2*, and *ERBB2* (Laakso & Hautaniemi, 2013). Some of the genes indicate DNA damage or DNA repair such as *BRCA1* and *CDKN1A.* Other gene commonly affected pathways such as angiogenesis *(ERBB2),* adhesion *(BCL2, ERBB2, EGFR)* and apoptosis *(CDKN1A, TP53, BCL2)* (Laakso & Hautaniemi, 2013).

1.1.5(b) Cancer drug resistance and drug metabolism genes

Drug resistance is indicated by the expression of gene *ABCB1, ABCC2, ABCC5, ABCG2, BCL2,* and *TP53*. Most of these genes are present in outer layer of the cell (An et al., 2017). The protein of these gene utilise the energy of adenosine triphosphate (ATP) binding and hydrolysis to translocate a wide variety of substrate and hydrophobic compounds across extra- and intracellular membranes (Kathawala et al., 2015). The predominant cause of multi drug resistance gene in cancer cells are he increased efflux of anti-cancer drug by membrane-embedded drug transporters. The most related drug transporters belongs to the ATP-Binding Cassette (ABC) superfamily (Li et al., 2016).

Apart from that, gene that relate with drug metabolism include *CYP1A1, CYP1A2, CYP2B6, CYP2C19, CYP2D6, CYP3A4, CYP3A5, NAT2,* and *SULT1E1* (An et al., 2017). *SULT1A1* and *UGT2B15* genes are part of the drug metabolism

biomarkers. Whereas, the EGFR and ERBB2 gene are biomarker for growth factor (Ohashi et al., 2013)

1.1.5(c) Cell cycle biomarkers

Cell cycle are related to DNA replication and cell separation (Williams & Stoeber, 2012). It involves 4 phases which are G1, S, G2 and M phases. The cyclins and the cyclin-dependent kinases (CDKs) are a classes of regulatory molecules that plays roles in cell cycle. Several genes are related with cell cycle regulation such as the CDKN1B gene for G1 phase and G1/S transition (Williams & Stoeber, 2012). *BRCA1, CDK2, CDKN1A, CDKN1B, CDKN2A, TYP53* genes encode for proteins that are important specific check point and cell cycle arrest (Nagata et al., 2015). Additionally *BCL2, CDK2, BRCA1, CDKN2B, TP53* genes play role in cell cycle regulation (Williams & Stoeber, 2012).

1.1.6 *Nigella sativa*

Nigella sativa (Ranunculaceae family) also known as black seed or "the Blessed Seed" is an annual herb or traditionally used for various ailments (Salem, 2005). This herb grows in countries bordering the Mediterranean Sea, Pakistan and India (B. Ali & Blunden, 2003). Recent reports state that *N. sativa* is commonly used by cancer patients as dietary supplement in complementary and alternative medicine along with chemotherapy (Jazieh et al., 2012; Mayadagli et al., 2011). In the Middle East, this plan is a part of an overall holistic approach to health as and diet everyday lifestyle even black seed is not significant component of the human diet. More than 2000 years this natural remedy has been used to promote health and treat diseases. Many studies had been done to this black seed that related to both phytochemically and

pharmacologically. The characteristic of very low degree of toxicity had been reported in numerous studies (B. Ali & Blunden, 2003). *Nigella sative* black seed contain very rich and diverse of the chemical composition, for example active ingredient such as crystalline nigellone, black seed contains 15 amino acids, proteins, carbohydrates, both fixed oils (84% fatty acids, including linolenic and oleic) and volatile oils, alkaloids, saponins, crude fiber, as well as minerals, such as calcium, iron, sodium and potassium (Gali-Muhtasib et al., 2006).

Figure 1.2: The flower of *Nigella sativa* and its black seed. (Gali-Muhtasib et al., 2006)

1.1.7 Thymoquinone and it anti-cancer effects

Thymoquinone (TQ) is a potent compound derived from the seed of *Nigella sativa*. It is one of the major components in the essential oil of *Nigella sativa* seed extract (Burits & Bucar, 2000; Hajhashemi 2004). TQ shows many benefit and it had been reported to exhibit antioxidant (Badary et al., 2003), anti-inflammatory (El Gazzar et al., 2006) and anticancer activity (Gali Muhtasib et al., 2008). Cytotoxic effects of TQ was found in different types of malignancy such as prostate cancer (Kaseb et al., 2007), osteosarcoma (Roepke et al., 2007) , fibrosarcoma (Ivankovic et al., 2006), myeloblastic leukemia (El Mahdy et al., 2005) and colorectal carcinoma (Halagali Muhtasib et al., 2004). TQ showed the low toxicity toward healthy cell, this proved that this compound safely to use as daily intake (El Najjar et al., 2010). Studies also showed anti-cancer effects of TQ in animal model xeno-grafted with fibrosarcoma and also squamous cell carcinoma (Ivankovic et al., 2006), colon cancer (Gali Muhtasib et al., 2008) and prostate cancer (Yi et al., 2008). TQ could promote apoptosis through the p53-dependent pathway (Halagali Muhtasib et al., 2004) and p53-independent pathway (El Mahdy et al., 2005). Besides, TQ upregulated expressions of p53 and p21 in HCT116 cells and caused inhibition of Bcl-2 protein expression (Halagali Muhtasib et al., 2004).

1.2 Nanotechnology

Nanotechnology is the current approach in order to conserve the bioavailability of natural compounds. Nanomedicine is a form of nanotechnology which related to formation of nanoparticle drug-delivery (Wang et al., 2012). Nanoparticles are generally less than 100 nm and would be able to transport therapeutics to target sites (Wang et al., 2012). One of the important in use of nanoparticle is in drug delivery for

cancer treatment. Several challenges arises with the use of direct conventional chemotherapeutics, for example the drug will target cancer cells and also healthy cells (Wang et al., 2012). Nanoparticles that have better permeability and retention (EPR) would accumulate at the tumor sites (Matsumura & Maeda, 1986). Because of this character in biodistribution, nanoparticle will allow the drug to achieve higher intra-tumoral drug level and reduce healthy tissue toxicity. Commercially used pegylated liposomal doxorubicin was found to show lower dose-limiting toxicity compared to doxorubicin. Nab-paclitaxel also exhibited better therapeutic efficacy than paclitaxel (William J Gradishar et al., 2005). The ability to encapsulate and deliver poorly soluble cancer therapeutics becomes the unique property of nanoparticle. Therefore, it has gained intense interest in nanoparticle-based cancer drug industry (Wang et al., 2012).

1.2.1 PLGA-PEG and Pluronic F68

Over the years, natural and synthetic polymers have been explored to be used as formulations of nanoparticles. In which potentials of Poly(lactic acid) (PLA), Poly(glycolic acid) (PGA), and their copolymers (PLGA) have been explored mainly due to their biocompatibility and biodegradability characteristics (Bala et al., 2004). These polymers have been extensively studied as colloidal material in the synthesis of nanoparticles for drug delivery. Synthetic polymers have high purity and reproducibility compared to natural polymers. The polyester family (i.e., poly (lactic acid) (PLA), poly (ε-caprolactone) (PCL), poly (glycolic acid) (PGA)) are of interest in the biomedical industry. Moreover, poly (lactide-co-glycolide) (PLGA) has been approved by Food and Drug Administration (FDA) for use in treatment (J. M. Anderson & Shive, 1997). Advantages is that drug of interest can be delivered by

water-soluble polymer platforms (Kumari et al., 2010). Physical and chemical properties of the polymers are specially synthesised to flow through the kidney and liver without getting filtered out, thus allowing the drugs to be used more effectively (Bertrand & Leroux, 2012; Chau, 2005). Poly(lactic-co-glycolic acid)- Polyethylene glycol (PLGA-PEG) is a type of polymer that used in numerous FDA approved therapeutic agents because of the biodegradability, biocompatibility and non-toxic nature (Makadia & Siegel, 2011). It dissolves in a wide range of solvents (Makadia & Siegel, 2011). The biodegradable characteristics is when the PLGA-PEG is taken up, it will undergo hydrolysis and later forms original monomer which are lactic acid and glycolic acid. They are normal by-product of numerous metabolic process in the body (Lü et al., 2009). PLGA-PEG also has great potential in drug delivery systems as tumor-targeting carriers. It can increase the stability and prolong the circulation time of drug (Cao et al., 2016).

1.2.2 TQ-Nanoparticle

Developing TQ-nanoparticle would conserve this volatile oil and provide sustained drug release over longer duration compared to its free form. The bioavailability of drug was found to be conserved within the nanoparticles compared to free form and act gradually towards the target cells (Kumari et al., 2010). Nano-sized carriers are used in drug delivery because they are suitable for intravenous application. In addition, it has been reported that solid biodegradable nanoparticles show advantage over liposomes due to their increased stability and unique ability to create a controlled release of drugs.

1.3 Problem statement

Breast cancer is the leading cause of death among women worldwide. Despite development of new therapeutics in the last 20 years, resistance remains as a challenge in the clinical setting. TAM is a non-steroidal anti-hormonal drug and it is frequently used for the chemoprevention of breast cancer. It has been approved for treatment of women with hormone-dependent breast cancer (Ali et al., 2016). TAM treatment is very effective in tumors expressing estrogen receptors (ER) and significantly reduces the mortality of breast cancer patients. Trastuzumab is a monoclonal antibody, which is a type of targeted cancer drug (Joensuu et al., 2006). Lapatinib is the tyrosine kinase inhibitor is also used as anti-HER2 agent (Geyer et al., 2006). Even so, the benefits of cancer therapy have often been limited by resistance to this drug (Ahmed Faisal Mutee, 2012).

Approximately one-third of early-stage breast cancer patients will become resistant to TAM over the 5-year treatment period and several cellular mechanisms have been discussed to be responsible for this development (Ali et al., 2016). Up to 66% of patients exhibit resistance to trastuzumab monotherapy (Esteva et al., 2002) and development of resistance toward lapatinib stands as a major problem (Alwarawrah et al., 2015).

MDR in cancer is a phenomenon of resistance to a number of structurally different chemotherapeutic agents after exposure anti-cancer agent. It is associated with overexpression of drug resistance gene and its product. This protein plays an important role in limiting cancer drug efficacy. Therefore, better understanding of the

molecular mechanisms of drug-resistant in breast cancer with the application of TQ-nanoparticles will be investigated in this study to MDR.

1.4 General objective

To synthesise and characterise TQ-nanoparticles for the treatment of drug resistant breast cancer subtypes.

Specific objectives:

1. To synthesise of TQ-nanoparticles using two formulations. Of drug to polymer ratio (1:7 and 1:20)
2. To characterise the TQ-nanoparticle .
3. To develop TAM resistant MCF-7 cell lines using pulse technique.
4. To determine drug resistant and cancer protein expressions in different breast cancer subtypes with free TQ and TQ-nanoparticles treatment using Western Blot analysis
5. To determine of drug-resistance and cancer relative gene expression between Doxorubicin (Dox) and TQ-nanoparticles treatments.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

In this study, TQ (99% purity) and Pluronic F68 (molecular weight of 8,500 Da) were purchased from Sigma-Aldrich (Missouri, U.S). Acetonitrile is of analytical grade and it was purchased from Fisher Scientific (Hampton,U.S). Milli-Q grade water was used for all the preparation of solutions. Phosphate buffer solution (PBS) was purchased from Fisher Scientific and sterilised prior to use.

2.2 Development of TQ nanoparticle by entrapment method

The first part of this study involved synthesis of PLGA PEG TQ nanoparticles using of two different formulations. Nanoparticles with a matrix structure containing TQ were optimised with the solvent displacement techniques as described by Ravindran et al., (2010) and Vega et al., (2013) as shown in Table 2.1.

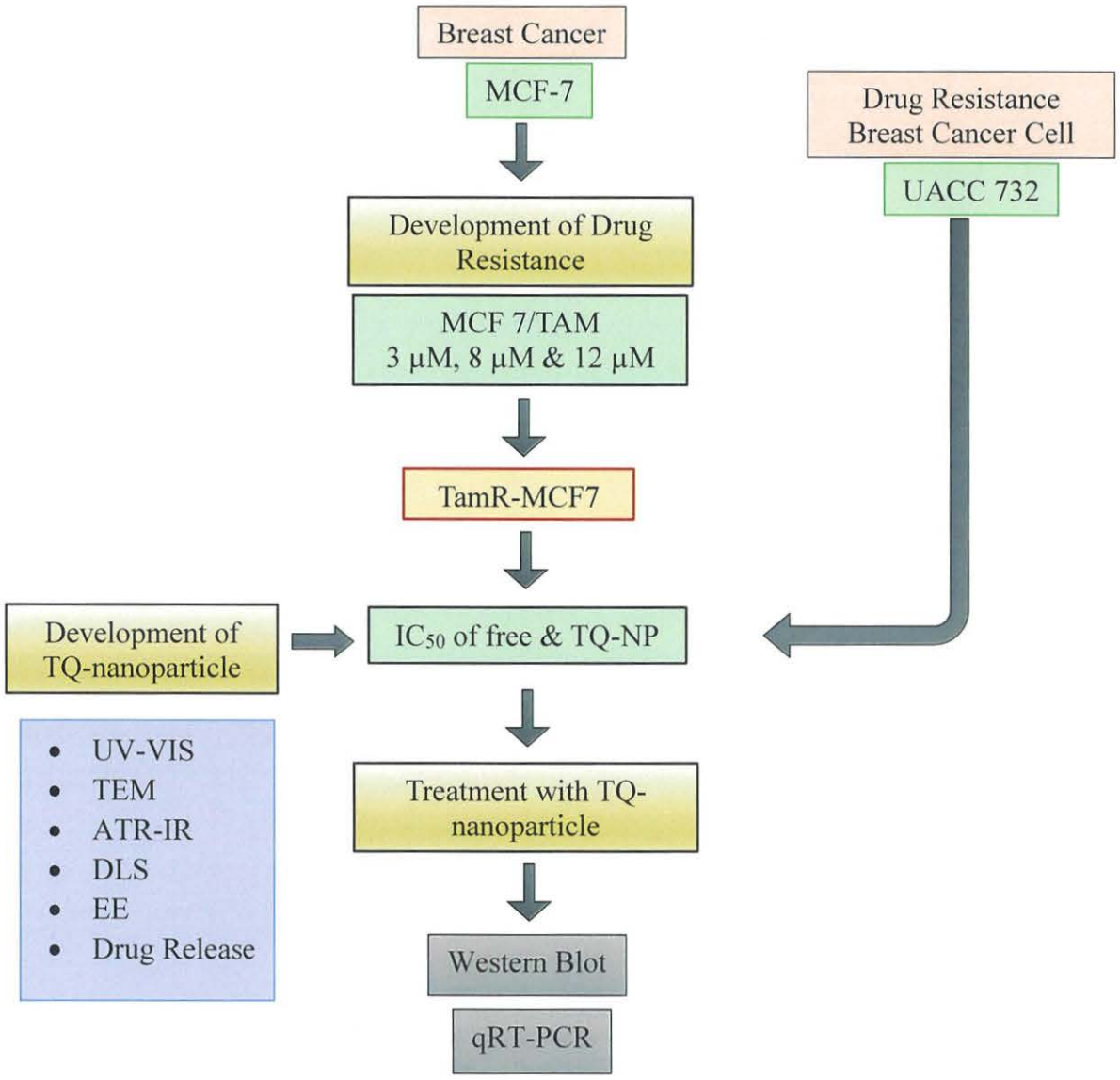

Figure 2.1 : The flow chart of this study is divided into 3 parts. The first part involved development of drug resistant MCF-7 with tamoxifen, second part was the synthesis and characterisation of thymoquinone-nanoparticle, followed by third part which was treatment of resistant cells with thymoquinone-nanoparticel.

Table 2.1 Formulation of nanoparticle using different drug to polymer ratio.

	1:20 drug to polymer ratio formulation (Ravindran et al. 2010)	1:7 drug to polymer ratio formulation (Vega et al. 2013)
TQ (mg)	5 mg	12.5 mg
PLGA-PEG (mg)	100 mg	90 mg
Ratio polymer:drug	20:1	7:1
PLGA-PEG (%)	10%	3.6 %
Acetonitril (ml)	10 ml	25 ml
Pluronic F68 (mg)	10 mg	50 mg
miliQ water (ml)	10 ml	50 ml (pH 3.5)
Percentage of pluronic F68 (%)	0.1%	0.1%

2.2.1 Synthesis of PLGA-PEG TQ nanoparticles

(a) Method 1:20 drug to polymer ratio formulation (Ravindran et al., 2010)

A total of 100 mg of PLGA-PEG and 5 mg TQ were mixed in 10 mL acetonitrile. The mixture was then added in a drop wise manner to an aqueous solution rotating at 1500 rpm containing 0.1% Pluronic F-68 as surfactant. The resulting dispersion of micelle was vacuum evaporated at 39°C for 1 hour to eliminate the organic solvent. This was followed by centrifugation at 12000 rpm for 20 min to remove undissolved TQ. Micelles formed were subsequently washed with deionized water for 3 times. Later the sample was freeze dried with 5% sucrose as a cryoprotectant. The final polymer concentration was 5 mg/mL of TQ-nanoparticles and it was stored at 4°C prior use.

(b) Method 1:7 drug to polymer ratio formulation (Vega et al., 2013).

Nanoparticles with a matrix structure containing TQ were prepared by the solvent displacement technique as described by Vega et al (2013). A total of 90 mg of PLGA-PEG and 12.5 mg of TQ were mixed in 25 mL of acetonitrile. The mixture was then added drop wise manner into 50 mL of an aqueous pH 3.5 solution rotating at 1500 rpm which contained 10 mg/mL pluronic F-68 surfactant. Optimization was performed in duration of evaporation, where the resulting dispersion of nanoparticles were vacuum evaporated was compared between an hour at 50^0C and 3 hours at 50^0C. This step was done to eliminate by evaporation phase of the organic solvent. Subsequent, step was done with an hour at 50^0C because the volume of the final evaporation solution were same. The samples were later stored at 4°C until further use in experiments.

2.3 Characterisation of TQ encapsulation in the polymeric micelles

2.3.1 Attenuated total reflectance infrared spectroscopic study

Attenuated total reflectance infrared (ATR-IR) spectroscopy was carried out to determine qualitative identification of organic and inorganic compounds and for identifying the bio-groups that were bound distinctively. During this analysis, a spot of the sample was subjected to a modulated infrared (IR) beam. The samples transmittance and reflectance of the infrared rays at different frequencies were translated into an IR absorption plot consisting of reverse peaks. The TQ-nanoparticles were examined by ATR-IR Spectrometer Frontier Perkin Elmer (Massachusetts, U.S) to confirm the encapsulation. The range of the scan was between 650 cm^{-1} to 4000 cm^{-1}.

2.3.2 Ultraviolet-visible spectrophotometry (UV-VIS) for qualitative analysis

Chemical structure of free TQ and TQ-nanoparticle were analyses using visible light and ultraviolet of UV-VIS spectrophotometer. Special type of spectrometer are used to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. The basic principle is that each compound absorbs or transmits light over certain range of wavelength. The spectrophotometric analyses were performed on UV-VIS Spectrophotometer Shimadzu UV 2600 (Kyoto, Japan) to determine the maximum wavelength (λ_{max}) of TQ. The sample were prepared by dilute the free TQ and TQ-nanoparticle in PBS solution. The sample than loaded 1 ml into the cuvette and placed the cuvette into the spectrophotometer. The samples were scanned between 200-400 nm.

2.4 Physicochemical characterisation

2.4.1 Transmission electron microscopy (TEM)

Transmission electron microscopy was used to observe TQ-nanoparticle morphology, shape and size range. The images of nanoparticle were taken from Philips CM12 transmission electron microscope (Philips Electron Optics, Eindhoven, Netherlands). The morphology was determined at an accelerating voltage of 200 KV with 1K × 1K digital images captured using an AMT CCD camera. Samples were prepared by adding single drop of the nanoparticle suspension to copper grid and left to dry completely at room temperature. The samples were then negatively stained with 2% uranyl acetate prior to imaging.

2.4.2 Measurement of particle size and zeta potential

Particle size, size distribution and zeta potential (particle charge) in colloidal suspensions can be determined by measuring the random changes (Brownian motion) in the intensity of light scattered from the suspension or solution. This technique call dynamic light scattering (DLS), measured by using Malvern Zetasizer (Malvern, UK). Sample of nanoparticles suspension was diluted 10 times in miliQ water. Particle size distribution of nanoparticles is reported as polydispersity index (PDI), a measure of the distribution broadness of the particle size. One milliliter of sample was placed into a cuvette and analyzed at 25 ^{0}C with an angle of 90°. Each sample was measured in triplicates. Analysis was performed using Zetasizer software.

2.5 Measurement of entrapment efficiency and drug release

2.5.1 Study of entrapment efficiency

Entrapment efficiency (EE) refers to the capability of the drug to be entrapped into the nanoparticle. The percentage of EE will calculated by (total drug added – free non-entrapped drug) divided by the total drug added.

The samples were first suspended in PBS solution, then sonicated for 3 minutes and vortexed for a few seconds. TQ was separated by ultracentrifugation at 14,000 rpm at room temperature for 30 minutes. The resulting supernatant was collected and quantified spectrophotometrically using UV-VIS Spectrophotometer Shimadzu, (Kyoto, Japan) at 257 nm. The amount of drug entrapped was calculated as in following equation:

$$\% \text{ EE} = \frac{\text{Amount of TQ in nanoparticles}}{\text{Total amount of TQ}} \times 100\%$$