SYNERGISTIC CYTOTOXIC EFFECT OF THYMOQUINONE IN COMBINATION WITH CISPLATIN AGAINST MDA-MB-231 TRIPLE NEGATIVE CELL LINE

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DEDICATION

This thesis is dedicated to my parents and siblings

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ABSTRACT

The purpose of this study is to investigate the application of genetic algorithm (GA) in modelling linear and non-linear dynamic systems and develop an alternative model structure selection algorithm based on GA. Orthogonal least square (OLS), a gradient descent method was used as the benchmark for the proposed algorithm. A model structure selection based on modified genetic algorithm (MGA) has been proposed in this study to reduce problems of premature convergence in simple GA (SGA). The effect of different combinations of MGA operators on the performance of the developed model was studied and the effectiveness and shortcomings of MGA were highlighted. Results were compared between SGA, MGA and benchmark OLS method. It was discovered that with similar number of dynamic terms, in most cases, MGA performs better than SGA in terms of exploring potential solution and outperformed the OLS algorithm in terms of selected number of terms and predictive accuracy. In addition, the use of local search with MGA for fine-tuning the algorithm was also proposed and investigated, named as memetic algorithm (MA). Simulation results demonstrated that in most cases, MA is able to produce an adequate and parsimonious model that can satisfy the model validation tests with significant advantages over OLS, SGA and MGA methods. Furthermore, the case studies on identification of multivariable systems based on real experiment t al data from two systems namely a turbo alternator and a continuous stirred tank reactor showed that the proposed algorithm could be used as an alternative to adequately identify adequate and parsimonious models for those systems. Abstract must be bilingual. For a thesis written in Bahasa Melayu, the abstract must first be written in Bahasa Melayu and followed by the English translation. If the thesis is written in English, the abstract must be written in English and followed by the translation in Bahasa Melayu. The abstract should be brief, written in one paragraph and not exceed one (1) page. An abstract is different from synopsis or summary of a thesis. It should states the field of study, problem definition, methodology adopted, research process, results obtained and conclusion of the research. The abstract can be written using single or one and a half spacing. Example can be seen in Appendix 1 (Bahasa Melayu) and Appendix J (English).

ABSTRAK

Kajian ini dilakukan bertujuan mengkaji penggunaan algoritma genetik (GA) dalam pemodelan sistem dinamik linear dan tak linear dan membangunkan kaedah alternatif bagi pcmilihan struktur model menggunakan GA. Algorithma kuasa dua terkecil ortogon (OLS), satu kaedah penurunan kecerunan digunakan sebagai bandingan bagi kaedah yang dicadangkan. Pcmilihan struktur model mengunakan kaedah algoritma genetik yang diubahsuai (MGA) dicadangkan dalam kajian ini bagi mengurangkan masalah konvergens pramatang dalam algoritma genetik mudah (SGA). Kesan penggunaan gabungan operator MGA yang berbeza ke atas prestasi model yang terbentuk dikaji dan keberkesanan serta kekurangan MGA diu t arakan. Kajian simulasi dilakukan untuk membanding SGA, MGA dan OLS. Dengan meggunakan bilangan parametcr dinamik yang setara kajian ini mendapati, dalam kebanyakan kes, prestasi MGA adalah lebih baik daripada SGA dalam mencari penyelesaian yang berpotensi dan lebih berkebolehan daripada OLS dalam menentukan bilangan sebutan yang dipilih dan ketcpatan ramalan. Di samping itu, penggunaan carian tcmpatan dalam MGA untuk menambah baik algorithma tersebut dicadang dan dikaji, dinamai sebagai algoritma mcmetic (MA). Hasil simulasi menunjukkan, dalam kebanyakan kes, MA berkeupayaan menghasilkan model yang bersesuaian dan parsimoni dan mcmenuhi ujian pengsahihan model di samping mcmperolehi beberapa kelebihan dibandingkan dengan kaedah OLS, SGA dan MGA. Tambahan pula, kajian kes untuk sistcm berbilang pcmbolehubah menggunakan data eksperimental sebenar daripada dua sistem iaitu sistem pengulang-alik turbo dan reaktor teraduk berterusan menunjukkan algoritma ini boleh digunakan sebagai alternatif untuk mcmperolehi model termudah yang memadai bagi sistem tersebut.

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LIST OF ABBREVIATIONS

ATCC	-	Americab Tissue Culture Collection
CP	-	Cisplatin
CI	-	Combination Index
DMEM	-	Dulbecco's Modified Eagle Medium
DMSO	-	Dimethylsulfoxide
DRI	-	Dose Reduction Index
ER	-	Estrogen Receptor
FBS	-	Fetal Bovine Serum
HER2	-	Human Epidermal Growth Factor Receptor 2
IC_{50}	-	Half maximal Inhibitory Concentration
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	-	Phosphate Buffer Solution
PR	-	Progesterone Receptor
TQ	-	Thymoquinone
TQ+CP	-	Thymoquinone in combination with cisplatin
TNBC	-	Triple Negative Breast Cancer

LIST OF SYMBOLS

µM	-	Micro Molar
µm	-	Micrometre
µL	-	Microliter
mL	-	Millilitre
xg	-	Centrifugal force

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CHAPTER 1

INTRODUCTION

1.1 Research background

Breast cancer is the most common cancer and the first cause of death in women worldwide. It was estimated that 2.1 million new cases and 627 thousand of breast cancer related deaths occurred in 2018 (Bray *et al.,* 2018). In Malaysia, breast cancer incidence is on the increase and at present has become the first leading cause of cancer deaths among women in all ethnics. According to the Malaysia Cancer Registry, there were 17009 new cases and 7372 deaths due to breast cancer reported from 2007 to 2011, followed up to 2016. By ethnicity, Malays (57.9%) recorded the lowest 5-year survival rate, followed by Indians (70.5%), and Chinese (76.5%) (National Cancer Registry *et al.,* 2018). One of the common risk factor associated with the differences in survival rate between ethnics in Malaysia was the identification of distinct subtypes of breast cancer (Devi *et al.,* 2012). Approximately 15-20% of breast cancer cases accounts for triple negative breast cancer (Frank *et al.,* 2013).

Triple negative breast cancer (TNBC) is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Anders *et al.,* 2013). Due to the absence of these three biomarkers, the established treatments for breast cancer including endocrine and HER2-targeted therapy are unfortunately not effective for TNBC treatment (Isakoff, 2010). Besides, TNBC appears to be more aggressive, invasive, higher in grade, and highly metastatic as compared to other subtypes (Carey *et al.,* 2010). Given the lack of therapeutic options, cytotoxic chemotherapy remains the standard treatment for TNBC patients (Peddi *et al.,* 2012). However, only 50% of TNBC patients respond to primary cytotoxic agents and most cases are associated with early relapse within 3 years of diagnosis and higher risk of mortality less than 12 months (Dent *et al.,* 2007;

Sørlie *et al.*, 2001). Therefore, studies are now focusing on other cytotoxic agents that are not routinely used for breast cancer such as platinum-based drugs in order to find the most active agent for TNBC treatment. Indeed, numerous clinical studies have shown promising results of using platinum-based drugs for treatment of TNBC in adjuvant, neoadjuvant, and metastatic settings (Sparano *et al.,* 2008; Liedtke *et al.,* 2008; Byrski *et al.,* 2012).

One of the platinum-based drugs that has been actively studied for TNBC treatment is cisplatin (CP) (Hill *et al.,* 2019). Generally, CP exerts its cytotoxic properties by binding to N7-sites of purine residues on the nucleotides to form inter- and intra-strand DNA adducts and block the RNA transcription and DNA replication, signalling DNA damage in a cell (Basu & Krishnamurthy, 2010). If not repaired, DNA-damage can induce cell-cycle arrest and ultimately cause apoptosis (Tanida *et al.*, 2012). Apoptosis is a highly regulated natural process of cell death and in cancer, the loss of apoptosis has become a promising treatment strategy for decades (Pfeffer & Singh, 2018). Despite being a potent anticancer drug, the clinical use of CP is limited due to associated toxicity and side effects in patients including nausea and vomiting, hepatotoxicity, nephrotoxicity, and cardiotoxicity (Huang *et al.,* 2017; Pratibha *et al.,* 2006; Hayati *et al.,* 2015; Hu *et al.,* 2018). These CP-related toxicities are dose-dependent, thus an overdose of this drug might cause significant deaths in patients (Tsang *et al.,* 2009). Hence, another effective strategy to reduce CP-related toxicities for better cancer treatment is urgently needed.

There is a growing body of data that suggested drugs combination can minimize the toxicity effect towards normal cells while still providing therapeutic effect, such as cancer cell death (He *et al.,* 2019; Abe *et al.,* 2019; Monroe *et al.,* 2019). The drugs that are used in combination may interact in many unexpected ways and exhibit distinct effects for instance, synergism, additive, and antagonism (Lehar *et al.,* 2009). Among these interactions, achieving drug synergy is a highly pursued goal in anticancer drug combination studies (Yin *et al.,* 2014). Hypothetically, drug synergy could be achieved by using drugs that complement each other in terms of anticancer properties but with non-overlapping toxicity profiles. Recent experimental efforts are focusing on identifying synergistic drug

pairs by using conventional cytotoxic drug with phytochemicals since natural compound has a safer toxicity profile compared to synthetic drugs (Ndreshkjana *et al.,* 2019; Tan *et al.,* 2019). In addition, phytochemicals have been proved to show wide range of biological activity including anti-inflammatory, anti-oxidant, and anti-cancer properties (Yimer *et al.,* 2019; Ozdemir *et al.,* 2018; Singh *et al.,* 2016). The combination of selected phytochemical with conventional chemotherapeutic may provide synergistic effect at a lower dose which can results to higher anticancer effect and reduced toxicity towards normal cells (Pezzani *et al.,* 2019).

Thymoquinone (TQ) is a phytochemical derived from *Nigella sativa* and has been reported to exert numerous pharmacological activities such as such as anti-inflammatory, antimicrobial, antidiabetic, and also anticancer (Yazdi *et al.,* 2018; Chaieb *et al.,* 2011; Younus *et al.,* 2018; Asaduzzaman *et al.,* 2017). In the previous years, emerging evidence has shown anticancer activity of TQ in many types of cancer, for instance, bladder, cervical, colon, gastric, glioblastoma, and breast cancer (Iskender *et al.,* 2016; Reindl *et al.,* 2008; Kundu *et al.,* 2014; Zhu *et al.,* 2016; Gurung *et al.,* 2010; Rajput *et al.,* 2013). TQ exhibited anticancer response on cancer cell lines through many cellular mechanisms such as anti-proliferation, cell-cycle arrest, and apoptosis induction (ElKhoely *et al.,* 2015; Bhattacharya *et al.,* 2015; Woo *et al.,* 2013). Particularly in TNBC, TQ inhibited growth of both MDA-MB-468 and MDA-MB-231 cell lines by arresting G1 phase of the cell cycle and induced apoptosis through caspase-dependent and independent pathways (Sutton *et al.,* 2014). Moreover, TQ has been shown to protect normal cells from oxidative damage and prevents toxic side effects in many types of cells such as prostate epithelial cells, intestinal cells, lung fibroblasts, and breast epithelial cells (Kaseb *et al.,* 2017; El-Najjar *et al.,* 2010; Gurung *et al.,* 2010; Kabil *et al.,* 2018).

Previously, TQ has been used in combination with CP against cancer cell lines derived from colon, esophageal, oral squamous, gastric, ovarian, and also breast cancer (Zhang *et al.,* 2016; Hu *et al.,* 2018; Alaufi *et al.,* 2017; Ma *et al.,* 2017; Nessa *et al.,* 2011). Latest study by Liu *et al.* (2017) revealed that the combination of TQ and CP able to enhance the cytotoxicity against ovarian cancer cells (SK-OV-3) by upregulating BAX and downregulating BCL2 gene which contributed to

apoptotic cell death (Liu *et al.,* 2017). Moreover, Sutton *et al.* (2014) has reported that TQ able to enhance the cytotoxic effect of CP against MDA-MB-468 TNBC cells, highlighting the potential of this drugs combination for TNBC treatment. However, no further evidence on the synergism of TQ and CP against any TNBC cells is discussed in the literature. Taken the evidence that TQ in combination with CP may enhance the cytotoxic effect against cancer cells, this study was aimed to investigate the synergistic cytotoxic effect of combining TQ with CP against MDA-MB-231 TNBC cells.

1.2 Problem statement

Cisplatin (CP) is cytotoxic, thus it targets all rapidly dividing cells including cancerous and normal cells. The use of cisplatin in the cancer treatment is limited with its severe toxicities in normal cells such as dose-dependent hepatotoxicity and nephrotoxicity (Pratibha *et al.,* 2006; Sheth *et al.,* 2017). One of the strategy to reduce the toxic effect of CP is by combining CP with non-toxic phytochemical such as thymoquinone (TQ) to achieve higher therapeutic index at lower doses, avoiding the dose-limiting effect of CP against normal cells (Jafri *et al.,* 2010). The protective effect of TQ against cisplatin-induced toxicity have been well documented in many studies (Ulu *et al.,* 2012; Al-Malki & Sayed, 2014). From these findings, combining TQ with CP may serves as an effective way to reduce the dose-limiting effect of CP. Previously, the combination of TQ with CP have been found to enhance the cytotoxic effects and induced apoptosis in several cancer including colon, esophageal, gastric, oral squamous, and ovarian cancer (Zhang *et al.,* 2016; Hu *et al.,* 2018; Ma *et al.,* 2017; Alaufi *et al.,* 2017; Nessa *et al.,* 2011). Taken these findings into consideration, the present study was carried out to investigate the possible synergistic cytotoxic effect of combining TQ and CP against MDA-MB-231 TNBC cell line.

1.3 Research objectives

This project aims to investigate the possible synergistic cytotoxic effect of thymoquinone (TQ) in combination with cisplatin (CP) against MDA-MB-231 triple negative breast cancer (TNBC) cell line and the underlying mechanism of cell death induction. The specific objectives of this research were:

(a) To evaluate the effect of TQ, CP, and their combination on the cell viability of MDA-MB-231 TNBC cells and WRL-68 liver cells using MTT colorimetric assay.

(b) To determine the synergistic cytotoxic effect of TQ in combination with CP using Combination Index (CI) method by Chou-Talalay.

(c) To determine the apoptotic effect of TQ, CP, and their synergistic combination on the cell nuclei morphology and cellular apoptosis/necrosis using fluorescent DAPI staining and Annexin-V FITC/PI staining assay respectively.

(d) To evaluate the activity of caspase-8 and caspase-9 that are respectively responsible for initiation of extrinsic and intrinsic apoptosis and executioner caspase-3/7 using Caspase-Glo assay.

(e) To evaluate the mRNA expression of target genes associated with transcription factor (p53, p63, and p73), cell cycle arrest (p21), extrinsic apoptosis (TNF and Fas), and intrinsic apoptosis signaling (PUMA, NOXA, Bak, Bax, and BCL2) using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

1.4 Research scopes

In order to achieve the objectives of this study, the effect of TQ, CP, and their combination on the cell viability of MDA-MB-231 TNBC cells and WRL-68 liver cells were first evaluated using MTT assay. The synergistic combination dose of TQ and CP was determined using Chou-Talalay Combination Index (CI) method. The CI method utilized CompuSyn software for automated simulation of the CI values

and dose reduction index (DRI) together with generation of other graphs including dose-effect curves, median-effect plot, and isobologram. The cytotoxic effect of TQ, CP, and their synergistic combination on the cell nuclei morphology and cellular apoptosis/necrosis were determined using blue-fluorescent DAPI staining and Annexin-V FITC/PI staining assay. The photograph of DAPI stained cell nuclei was collected using Eclipse Ti Series inverted microscope equipped with Nikon's NIS elements imaging software (Nikon Instruments Inc., Tokyo, Japan). Whereas, the relative fluorescence unit (RFU) of Annexin V FITC/PI stained cells were measured using GloMax®-Multi Detection System (Promega Corp., Madison, Wisconsin, United States). Next, the apoptotic effect of the treatments on the activity of initiator caspases (caspase-8 and -9) and executioner caspases (caspase-3/7) were evaluated using luminescent Caspase-Glo assays. The results were recorded in the form of relative luminescent unit (RLU) using GloMax®-Multi Detection System (Promega Corp., Madison, Wisconsin, United States). The caspase-8 and -9 used in this study distinguished the involvement of the extrinsic and intrinsic apoptosis induced by the treatment. Further evaluation on the mRNA expression of the downstream proteins involved in the transcription factors, cell cycle, extrinsic, and intrinsic signalling pathways was performed using qRT-PCR. The markers used in this study were p53, p63, and p73 for transcription factor, p21 for cell cycle, TNF and Fas for extrinsic, and PUMA, NOXA, Bax, Bak, and BCL2 for intrinsic signalling. The qRT-PCR was performed using BioRad CFX96 Real Time PCR System (Bio-Rad Laboratories, Inc., Hercules, California, United States). The fold gene expression of the sample was calculated using the delta-delta CT ($2^{-\Delta\Delta Ct}$) method.

1.5 Research significance

The study of the cytotoxicity of TQ and CP can provide more therapeutic potential for cancer treatment. The synergistic combination dose of TQ with CP can possibly solve the toxicity issue of CP towards normal cells. Their synergistic interactions can provide more understanding about signalling pathways such as apoptosis induction in the treatment of cancer.

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LIST OF PUBLICATION

Non-Indexed Conference Proceeding

1 **Zolkemri, Z.,** Kabi, K., Baba, S., & Hamdan, S. (2018). Optimization of spheroid formation of HeLa cells using combination of hanging drop method and 96-well plate coated agarose. In 2018 *The 7th International Graduate Conference on Engineering, Science & Humanities.* (pp 491-493). ISBN: 978-967-2171-27-0