CHEMOTHERAPEUTICS POTENTIAL OF MATCHA GREEN TEA (*Camellia sinensis* (L.) Kuntze) ON WERI-Rb-1 RETINOBLASTOMA CANCER CELLS

By

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Retinoblastoma is a childhood eye cancer that affects approximately 8,200 children each year. There is a paucity of published studies for retinoblastoma, specifically in Malaysia. Natural plants are widely used as alternative medicine in developed and developing countries. *Camellia sinensis* (matcha green tea) has been used as a traditional medicine to treat various diseases in Southeast Asia. However, their efficacy against retinoblastoma cancer has not been thoroughly investigated and characterized. The main objective of this study was to reveal the new insight on chemotherapeutics potential of matcha green tea extract (MGTE) against WERI-Rb-1, human retinoblastoma cells. Firstly, the optimized extraction condition of MGTE was determined through Response Surface Methodology (RSM) that aimed high amount of polyphenol compounds. The identification and quantification of polyphenol were performed via High-Performance Liquid Chromatography (HPLC). The highest polyphenol and antioxidant content yield were reached at a temperature of 80 °C, an extraction time of 20 min, a liquid-to-solid ratio of 100 mL/g. The HPLC analysis at the optimum extraction condition revealed 14 polyphenol compounds in MGTE.

The chemotherapeutics potential of optimized MGTE was assessed *in vitro* through two different types of cultures: two dimensional (2D) and three dimensional (3D) cultures of WERI-Rb-1. Normal epithelial retina, ARPE-19 cell lines are used as a positive control. *In vitro* analysis for 2D cultured cells was performed on cytotoxicity assay, morphological studies, cell cycle, apoptosis analysis and gene expression studies. MGTE extract showed promising chemotherapeutics effect on WERI-Rb-1 cells. The treatment of MGTE showed low cytotoxicity (IC_{50} > 200 µg/mL) toward ARPE-19 cells but high cytotoxicity on WERI-Rb-1 with IC_{50} 13.3 ± 1.40 µg/mL after 72 hours. MGTE induced apoptosis

cell death rather than necrosis and caused arrest in the sub G0 phase, probably due to DNA fragmentation. Cell cycle analysis also proved that MGTE induced apoptotic cell death in the sub G0 phase. The chemotherapeutics effect of MGTE occurred via extrinsic and intrinsic apoptosis pathway by the activation of caspase 3, caspase 8, caspase 9, Bad and Bax that culminated in the apoptosis of WERI-Rb-1 cells. The chemotherapeutics potential of MGTE was further assessed *in vitro* through the 3D culture of WERI-Rb-1. The 3D collagen WERI-Rb-1 cells were successfully developed using collagen type I as their extracellular matrix. The chemotherapeutics effect of MGTE on WERI-Rb-1 3D culture was investigated through cytotoxicity assay, morphological, gene and protein expression studies. The IC_{50} of MGTE on 3D collagen WERI-Rb-1 cells at 72 hours was higher in 3D culture cell with 64.41 ± 2.5 µg/mL. The treatment of MGTE cause a decrease in cell viability as the concentration increased as observed with DAPI/PI staining. The Scanning Electron Microscope (SEM) images showed the distinct morphological surface of 2D and 3D WERI-Rb-1 cells. MGTE and cisplatin-treated cells showed characteristics of apoptotic cell death. Treatment with MGTE on WERI-Rb-1 cells caused upregulation of pro-apoptotic proteins, thus resulting in apoptosis cell death. The gene and protein expression revealed the induction of extrinsic and intrinsic apoptosis pathways mediated by MGTE through expression of bax, caspase 3, caspase 8 and caspase 9 protein in 3D collagen WERI-Rb1 cells. However, 72 hours of MGTE treatment also induced the expression of Nrf2, HO-1, and SOD1 proteins, this likely decreased the sensitivity of WERI-Rb-1 cells toward MGTE treatment in the 3D culture system of WERI-Rb-1 cells. The upregulation of antioxidant proteins may provide cryoprotection for 3D collagen WERI-Rb-1 cells towards MGTE treatment. Despite the observed expression of Nrf2, HO-1, and SOD1 proteins, MGTE showed the ability to activate apoptosis cell death in 3D collagen WERI-Rb-1 cells. In conclusion, the findings suggest the new chemotherapeutic potential of MGTE in inducing apoptosis cell death in both 2D and 3D culture systems. This finding can be used as the fundamental understanding and new knowledge of the chemotherapeutics potential of matcha green tea extract specifically on retinoblastoma cancer cells.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

POTENSI KEMOTERAPEUTIK TEH HIJAU MATCHA (*Camellia sinensis* (L.) Kuntze) KE ATAS SEL KANSER RETINOBLASTOMA, WERI-Rb-1

Oleh

NOR HAFIZA BINTI SAYUTI

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Retinoblastoma adalah satu kanser mata dalam kalangan kanak-kanak yang menjejaskan kira-kira 8,200 kanak-kanak setiap tahun. Terdapat kekurangan kajian yang diterbitkan untuk retinoblastoma, khususnya di Malaysia. Tumbuhan semula jadi menawarkan sumber yang hebat untuk dibangunkan sebagai agen kemoterapi yang baru dan berkesan. *Camellia sinensis* telah digunakan sebagai ubat tradisional di Asia Tenggara untuk merawat pelbagai penyakit. Walau bagaimanapun, keberkesanannya terhadap kanser retinoblastoma belum dikaji secara menyeluruh. Objektif utama kajian ini adalah untuk mendedahkan pandangan baharu tentang potensi kemoteraputik bagi ekstrak teh hijau matcha (MGTE) terhadap WERI-Rb-1, sel retinoblastoma. Pertama, keadaan pengekstrakan optimum MGTE ditentukan melalui kaedah permukaan tindak balas (RSM) yang menyasarkan jumlah sebatian polifenol yang tinggi. Pengenalpastian dan kuantifikasi polifenol dilakukan menggunakan analisis kromatografi cecair berprestasi tinggi (HPLC). Hasil kandungan polifenol dan antioksidan tertinggi dicapai pada suhu 80°C, masa pengekstrakan 20 minit dan nisbah cecair kepada pepejal 100 mL/g. Analisis HPLC telah menemukan 14 sebatian polifenol dalam MGTE.

Potensi kemoterapeutik MGTE dinilai secara *in vitro* melalui dua jenis kultur yang berbeza: kultur dua dimensi (2D) dan kultur tiga dimensi (3D) WERI-Rb-1. Sel sihat retina epitelium, ARPE-19 digunakan sebagai kawalan positif. Analisis *in vitro* untuk sel kultur 2D dilakukan pada ujian sitotoksisiti, kajian morfologi, analisis kitaran sel, analisis sel apoptosis dan kajian ekspresi gen. Rawatan MGTE menunjukkan sitotoksisiti rendah (IC_{50} > 200 µg/mL) terhadap sel ARPE-19 tetapi sitotoksisiti tinggi pada WERI-Rb-1 dengan IC_{50} 13.3 ± 1.40 µg/mL selepas 72 jam. MGTE menyebabkan kematian melalui apoptosis bukannya nekrosis dan menyebabkan hentian kitaran sel pada fasa sub G0, yang mungkin disebabkan oleh pemecahan DNA. Keputusan analisis kitaran sel juga

membuktikan bahawa MGTE menyebabkan kematian sel apoptosis dalam fasa sub G0. Kajian juga mendapati bahawa kesan kemoterapeutik MGTE berlaku melalui laluan apoptosis ektrinsik dan intrinsik yang dibuktikan oleh pengaktifan caspase 3, caspase 8, caspase 9, Bad dan Bax yang memuncak dalam apoptosis sel kanser retinoblastoma.

Potensi kemoteraputik MGTE selanjutnya dinilai secara *in vitro* melalui kultur 3D WERI-Rb-1. Sel kultur kolagen 3D WERI-Rb-1 telah berjaya dihasilkan menggunakan kolagen jenis I sebagai matriks ekstraselularnya. Kesan kemoterapeutik MGTE pada sel kultur kolagen 3D WERI-Rb-1 telah disiasat melalui ujian sitotoksisiti, kajian morfologi, kajian ekspresi gen dan protein. Nilai IC_{50} MGTE ke atas sel kultur kolagen 3D WERI-Rb-1 pada 72 jam rawatan adalah lebih tinggi dalam sel kultur 3D dengan nilai 64.41 ± 2.5 µg/mL. Rawatan MGTE menyebabkan penurunan kadar sel hidup apabila kepekatan rawatan meningkat seperti yang diperhatikan dengan pewarnaan DAPI/PI. Imej mikroskop pengimbasan elektron (SEM) menunjukkan permukaan morfologi berbeza antara sel kultur 2D dan sel kultur kolagen 3D WERI-Rb-1. Sel yang dirawat dengan MGTE dan cisplatin menunjukkan ciri-ciri jelas kematian sel apoptosis. Rawatan dengan MGTE pada sel WERI-Rb-1 menyebabkan peningkatan regulasi protein pro-apoptosis, dengan itu mengakibatkan kematian sel secara apoptosis. Ekspresi gen dan protein mendedahkan induksi laluan apoptosis ekstrinsik dan intrinsik yang dimediasi oleh MGTE melalui ekspresi bax, caspase 3, caspase 8 dan caspase 9 protein dalam sel kultur kolagen 3D WERI-Rb1. Walau bagaimanapun, 72 jam rawatan MGTE juga menyebabkan peningkatan ekspresi protein Nrf2, HO-1, dan SOD1, expresi ini mungkin boleh mengurangkan sensitiviti sel WERI-Rb-1 terhadap rawatan MGTE dalam sistem sel kultur 3D WERI-Rb-1. Peningkatan pengawalseliaan protein antioksidan mungkin boleh memberikan perlindungan untuk sel kultur kolagen 3D WERI-Rb-1 terhadap rawatan MGTE. Walaupun ekspresi protein Nrf2, HO-1, dan SOD1 dikesan selepas rawatan, MGTE boleh mengaktifkan kematian sel secara apoptosis dalam sel kultur kolagen 3D WERI-Rb-1. Kesimpulannya, penemuan mencadangkan potensi kemoterapi baru MGTE dalam mendorong kematian sel apoptosis dalam kedua-dua sistem kultur 2D dan 3D. Penemuan ini boleh digunakan sebagai pemahaman asas dan pengetahuan baharu tentang potensi kemoterapi ekstrak teh hijau matcha khususnya pada sel-sel kanser retinoblastoma.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ARPE-19	Retinal Pigment Epithelial Cell Line
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2 protein
CASP	Caspase (cysteine-aspartic protease)
CDK	Cyclin-dependent kinase
CNS	Central composite design
cDNA	Complementary deoxyribonucleic acid
CV	Coefficient of variation
DMEM-F12	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleoside triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EHS	Engelbreth-Holm-Swarm
FAD	Food and Drug Administration
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLOBOCAN	Global Cancer Incidence, Mortality, and Prevalence
GTPs	Green tea polyphenols
HPLC	High performance liquid chromatography
IC_{50}	Inhibition concentration at 50%
IIRC	International intraocular retinoblastoma classification

ICRB	International Classification system for retinoblastoma
IVC	Intravenous chemotherapy
LDLs	Low-density lipoproteins
mg	milligram
mRNA	Messenger RNA
MTT	4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NaOH	Sodium hydroxide
NCI	National Cancer Institute
NCD	Non-communicable disease
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate buffer saline/solution
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
RSM	Response surface methodology
Rpm	Revolutions per minute
RT	Reverse transcription
TEMED	Tetramethyl ethylenediamine
TNF	Tumor necrosis factor
TME	Tumor microenvironment
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
SYBR	Synergy Brands, Inc
qPCR	Quantitative polymerase chain reaction

WERI-Rb-1	Human retinoblastoma cells
UPM	Universiti Putra Malaysia
µg	Microgram
µL	Microlitres
v/v	Volume per volume

CHAPTER 1

INTRODUCTION

1.1 Background of study

Non-communicable diseases (NCDs) remain a big concern in the 21st century, as these disorders are the world's leading cause of death. As reported by World Health Organization (WHO), four main NCDs are include cardiovascular diseases, chronic respiratory diseases, cancers, and diabetes ("WHO | Noncommunicable diseases country profiles 2018", 2018). Cancer is expected to rank as the first or second leading cause of mortality. Global cancer statistics by Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) reported the worldwide prevalence of cancer has grown to 18.1 million cases and 9.6 million deaths from cancer (Bray et al., 2018).

Malaysia's Cancer Registry report statistic data showed that ocular cancer is listed in the top 5 most frequent cancers in male children, accounting for 3.5% after leukemia, brain and nervous system, lymphoma, and bone cancers (Azizah et al., 2019). There is two primary ocular cancer, which includes retinoblastoma and uveal blastoma. Retinoblastoma is one of the most prevalent intraocular malignancies in children, with 3% of all cancers in pediatrics (Singh & Kashyap, 2018). Retinoblastoma is a world problem affecting approximately 8,200 children each year (Rao & Honavar, 2017). Retinoblastoma affects mainly children within the age of 5 years, and it is believed to be hereditary, which likely to cause death if improper treatment is given during the treatment (Harbour, 2006). It is fatal if left untreated. However, the high success rate of ocular cancer treatment is higher in developed countries. A total of 87 patients (117 eyes) were diagnosed in Malaysia between January 2004 and December 2009 (Jamalia et al., 2010). Various therapies, including chemotherapy, cryotherapy, thermotherapy, and radiation therapy, have cured retinoblastoma (Rodriguez-Galindo et al., 2008). The prescribed medications may have harmful effects on the body system, such as chemical therapy that kills cancer cells and can destroy healthy cells, resulting in side effects. These therapy give a side impact on multiple factors, including overall wellbeing, quality of life, and emotional control of the patient (Dijk et al., 2007; Belson et al., 2020).

The usage of different spices, fruit, and medicinal plants as therapeutic agents to preserve nutritional health in most developed countries was extensively tracked and examined. WHO reported that 80% of the world's population uses conventional medicine for basic healthcare needs. This alternative required the use of plant extract and its bioactive compound. Secondary metabolites from plants have a variety of biological properties beneficial to humans, such as antiallergic, anticarcinogenic, antibacterial, anti-inflammatory, antidiabetic, and antioxidant properties (Tran et al., 2020). In the normal and stable state of the

body, free radicals and natural antioxidants are regulated, and if the equilibrium is disrupted, oxidative stress may occur. Oxidative stress can cause damage/death in cellular components such as DNA, protein, and membrane lipid (Pizzino et al., 2017). Besides, chronic inflammation also leads to the development of cancer. A study by Wang & Karin (2015) estimated that 20% of cancers result from chronic inflammation and persistent infections.

In recent years, the impact of dietary polyphenols on well-being and active compounds from natural plants has been acknowledged by various scientific, epidemiological, and pharmacological studies (Rahman et al., 2021). The polyphenols constitute a large group of several dietary phytochemicals that include sub-classes such as flavonoids, stilbenes, phenolic acids, and lignans rich in antioxidant activity that may act against cancer development (Brglez., 2016). Plant secondary metabolites have proved to possess many pharmacological activities. Epidemiological evidence, *in vitro* and *in vivo* research, infer the preventive impact of their secondary metabolites on several oxidation-associated diseases like cardiovascular, neurodegenerative, and cancer, resulting in respective dietary advice (Roleira et al., 2015). The phytochemicals have several influences on intrinsic DNA repair, impairing the suppression of tumors that hinder cell proliferation mechanisms (Kotecha et al., 2016). These features are believed to lead to their possible anticancer effect and are used as part of clinical therapeutics to diagnose and treat various diseases like cancer (Greenwell & Rahman, 2015).

Camellia sinensis (L.) Kuntze tea plant from the Theaceae family is cultivated in approximately 30 countries worldwide. *C. sinensis* is probably one of the most investigated plants for its medicinal and food applications. Tea from the evergreen plant *C. sinensis* is widely consumed as a beverage worldwide. There is a range of tea forms, including black, green, white, oolong, and red teas. The most proven effects on human health have been observed with green tea consumption among other types of tea. According to Yang et al. (2014), the tea of *C. sinensis* has been used extensively for centuries as a medicinal herb since the Tang and Song Dynasties due to its cost-effectiveness and minimal side effects. Green tea is the minimized oxidize as it contains higher polyphenol than other tea types, such as black and oolong tea. The phytochemicals of *C. sinensis* are widely studied for various pharmacological usage, especially on green tea polyphenols (GTPs) (Rafieian et al., 2017).

1.2 Significant and research problem

Retinoblastoma is a rare type of cancer that is difficult to treat and diagnose due to tumor-suppressor gene mutations and a lack of focused, efficient, and cost-effective therapy, indicating a significant need for alternate approaches to these issues. To protect vision and avoid enucleation, chemotherapy is currently considered to be an important treatment for retinoblastoma. Nevertheless, drug resistance and relapses remain a major problem in retinoblastoma's treatment.

Thus, the new chemotherapeutics agent is needed to improve the efficiency and reduce side effect of chemotherapy drugs.

Green tea has been reported to exert various anticancer effect of various cancer cells. However, the anticancer potential of matcha green tea on retinoblastoma is not well studied. Numerous prior research has demonstrated that dietary phytochemicals, including epigallocatechin gallate (EGCG) from green tea, have a variety of positive benefits, including decreasing inflammatory processes, enhancing antioxidant activity, encouraging tumor cells to undergo apoptosis, and protecting cells from tumor growth. Due to its low cost and natural abundance, it is an interesting substance to explore. Catechins, the active component of green tea, are well-known natural antioxidants. Consumption of green tea extract has been suggested to be beneficial to the eye (Chu et al., 2010). A published study also indicates that EGCG may have a protective effect against retinal disorders connected with H_2O_2-induced oxidative stress (Cia et al., 2014). However, little study has been conducted on the chemotherapeutic activity of matcha green tea extract, particularly in retinoblastoma cells.

In cancer treatment, the use of plant-based products can minimize the adverse side effects of chemotherapy or other therapies (Seca & Pinto, 2018). Some chemotherapy agents are toxic in healthy tissue, thus indicating the need for new preventive and therapeutic drugs with no or low toxicity (Falzone et al., 2018). Approximately 60% of cancer medications come from herbal plants and other natural sources (Sharifi-Rad et al., 2019). However, several plants have anti-cancer capabilities but not yet extensively examined (Ghagane et al., 2017). Continuously study for better and more efficient chemotherapeutic and treatment is undoubtedly required to increase effectiveness and reduce cancer care treatment costs. Understanding the molecular mechanism of natural plants is essential to discover new potential bioactive compounds specific for ocular cancer.

Today, two-dimensional (2D) cell culture models are still employed to evaluate drug candidate cellular responses. While 2D cell culture is widely recognized and has contributed to a greater knowledge of pharmacological modes of action, it does have limitations. The three-dimensional (3D) culture is becoming more popular in tumor cell biology research since it may imitate *in vivo* microenvironments. The 3D cell culture is more beneficial as it allows drug tolerance and efficacy test *in vivo* conditions than conventional 2D cell culture (Fang & Eglen, 2017). The 3D culture models of commonly used retinoblastoma cancer cell lines such as WERI-Rb-1 or Y79 are still not widely studied. Moreover, the 3D collagen model of human-derived retinoblastoma cancer cell lines has yet to be established.

Thus, the current study proposes to investigate the chemotherapeutics potential of matcha green tea extract on the 2D and 3D collagen cell culture model of WERI-Rb-1 cells which provide more realistically mimic the *in vivo* cell behaviors and provide more predictable results. This study will examine the effect of

aqueous matcha green tea extract as a new chemotherapeutics agent on retinoblastoma (*in vitro*). *In vitro* models allow for studying of cellular processes such as proliferation, apoptosis, and cancer progression in a controlled environment. (Louzada et al., 2012), which will hopefully improve the prognosis of patients with retinoblastoma. As a result, it may reveal a new application for the sample. Hence, the findings from this study can serve as a baseline and shed light for future research to study the new chemotherapeutics potential of matcha green tea for treatment of retinoblastoma cancer particularly.

1.3 Objectives

The general objective of this study was to evaluate the chemotherapeutics potential of matcha green tea, *Camellia sinensis* on retinoblastoma cells (WERI-Rb-1).

The specific objectives of this study are described as follow:

I. To optimize the aqueous extraction parameters of polyphenols and antioxidant contents from *Camellia sinensis* leaves (matcha green tea) and identifying possible bioactive compounds.
II. To evaluate the cytotoxicity, cell cycle arrest, and apoptosis induction by optimized matcha green tea extract (MGTE) on retinoblastoma, WERI-Rb-1 cells.
III. To develop a three-dimensional (3D) culture system for WERI-Rb-1 and to evaluate the cytotoxicity activity, genes, and proteins expression involved in the regulation of apoptosis in MGTE treated 3D culture.

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