ANTICANCER EFFECTS OF GINGER AND RETINOIC ACID TREATMENT ON HeLa CERVICAL CANCER CELLS

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DEDICATION

This thesis is dedicated to my beloved parents , my supervisor, Dr Praseetha Prabhakaran, and all my friends and lecturers who have been there throughout my Masters journey

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ABSTRACT

Introduction: Cervical cancer is the second most commonly diagnosed cancer in developing countries and the recurrence after primary therapy mainly due to the existence of drug-resistant cancer stem cells (CSCs) within the tumour. This study was aimed at identifying the anticancer effects of Retinoic acid-ginger extract combined therapy on HeLa cells representing an invasive form of aggressive adenocarcinoma of the cervical. Methodology: The Cell-titre-glo, CyQuant Proliferation and Caspase 3/7 assays as well as Isobologram-combination index analysis were conducted to examine the anticancer effects of RA and GE on HeLa cells viability, proliferation, ability to induce apoptosis and combined drug relationship respectively. Retinoic acid at (5-50uM), ginger extract at (25-200uM) and RA-GE combination using RA (5-50uM) and GE (IC_{50}:100uM and IC_{75}:185uM) respectively were tested on HeLa cells. Findings: The inhibition percentage of cell viability and reduction in proliferative capacity of HeLa cells resulted in dose dependent manner with $RA_{(5\text{-}50uM)}$ (8.11% - 68.14%; 80.51% - 25.91%) $GE_{(25\text{-}200uM)}$ (13.59% – 76.49%; 80.06% - 22.67%) and combined $RA_{(5\text{-}50uM)}$-$GE_{(IC50;IC75)}$ (54.67% - 97.54%; 43.23 -3.43%%) and $GE_{(IC50;IC75)}$ -$RA_{(5\text{-}50uM)}$ (51.60% - 94.80%; 45.73% - 5.13%) respectively. A clear HeLa cell viability inhibition along with reduced proliferative capacity is observed in all treatments. . In addition, the IC_{50} concentration of RA, GE as well as combined RA-GE and GE-RA in HeLa cells were achieved at 25.6uM, 100uM, 17 uM and 21.4 uM respectively. Microscopy images of Solo RA-treated HeLa cells exhibited a more differentiated phenotype up to 25uM while ginger extract-treated HeLa cells appeared apoptotic from 40uM onwards. However, the combined RA-$IC_{50ginger\ extract}$ treatment induced a significant level of apoptosis in HeLa cells from as low as 6uM RA concentration compared to solo treatment, with RA (1.04%, negligible), ginger (IC_{25}:1.54%, IC_{50}:1.56%), ginger IC_{25}-RA IC_{50} (1.41%) and ginger IC_{50}-RA IC_{50} (1.66%). The combined RA-$IC_{50ginger\ extract}$ also exhibited a clear synergistic effect (CI: 1.20-0.26) on HeLa cells. The RA IC_{50}-$IC_{50ginger\ extract}$ has the lowest metastatic index (36.78%). Conclusion: In conclusion, these findings altogether suggest that the RA-ginger extract therapeutic strategy may be potent anticancer agent for the targeted therapy of cervical cancer cells and other CSCs-enriched cancers.

ABSTRAK

Pengenalan: Kanser serviks adalah kanser kedua yang paling sering didiagnosis di negara-negara membangun dan keberulangan selepas terapi primer terutamanya disebabkan oleh adanya sel stem kanser yang rintang drug (CSC) di dalam tumor. Kajian ini bertujuan untuk mengenal pasti kesan antikanser terapi gabungan ekstrak-halia Retinoik pada sel HeLa yang mewakili bentuk invasif adenokarsinoma agresif serviks. Metodologi: Uji Sel-Titre-Glo, CyQuant Proliferasi dan Caspase 3/7 serta analisis indeks kombinasi Isobologram dilakukan untuk memeriksa kesan antikanser RA dan GE terhadap daya maju sel, proliferasi, keupayaan untuk mendorong apoptosis dan hubungan ubat gabungan masing-masing. Asid Retinoik pada (5-50uM), ekstrak halia pada (25-200uM) dan kombinasi RA-GE menggunakan RA (5-50uM) dan GE (IC50: 100uM dan IC75: 185uM) masing-masing diuji pada sel HeLa. Keputusan: Peratusan perencatan daya maju sel dan pengurangan kapasiti proliferasi sel HeLa menghasilkan cara bergantung kepada dos $RA_{(5\text{-}50uM)}$ (8.11% - 68.14%; 80.51% - 25.91%) $GE_{(25\text{-}200uM)}$ (13.59% – 76.49%; 80.06% - 22.67%) dan kombinasi $RA_{(5\text{-}50uM)}$-$GE_{(IC50;IC75)}$ (54.67% - 97.54%; 43.23 -3.43%%) dan $GE_{(IC50;IC75)}$ -$RA_{(5\text{-}50uM)}$ (51.60% - 94.80%; 45.73% - 5.13%) masing-masing. Daya tahan sel HeLa yang jelas bersama dengan penurunan keupayaan proliferasi diperhatikan dalam semua rawatan. . Sebagai tambahan, kepekatan IC50 RA, GE serta gabungan RA-GE dan GE-RA dalam sel HeLa dicapai masing-masing pada 25.6uM, 100uM, 17 uM dan 21.4 uM. Gambar mikroskopi sel HeLa yang dirawat solo RA menunjukkan fenotip yang lebih berbeza hingga 25uM sementara sel HeLa yang dirawat dengan ekstrak halia muncul apoptotik dari 40uM dan seterusnya. Walau bagaimanapun, rawatan ekstrak RA-IC50ginger gabungan menyebabkan tahap apoptosis yang bererti pada sel HeLa dari kepekatan RA serendah 6uM berbanding dengan rawatan solo, dengan RA (1.04%, diabaikan), halia (IC25: 1.54%, IC_{50}: 1.56%), halia IC_{25}-RA IC_{50} (1,41%) dan halia IC_{50}-RA IC_{50} (1,66%). Ekstrak RA-$IC_{50halia}$ gabungan juga menunjukkan kesan sinergi yang jelas (CI: 1.20-0.26) pada sel HeLa. Ekstrak RA IC_{50}-$IC_{50ginger}$ mempunyai indeks metastatik terendah (36.78%). Kesimpulan: Sebagai kesimpulan, penemuan ini sama sekali menunjukkan bahawa strategi terapi ekstrak RA-halia mungkin merupakan agen antikanser yang kuat untuk terapi sasaran sel-sel kanser serviks dan kanser yang diperkaya dengan CSC lain.

TABLE OF CONTENTS

LISTS OF FIGURES

LISTS OF TABLES

LIST OF ABBREVIATIONS

CSCs	-	Cancer Stem Cells
RA	-	Retinoic Acid
GE	-	Ginger Extract
CI	-	Combinative Index
IC_{50}	-	50% Maximal Inhibitory Concentration
HPV	-	Human Papillomavirus
HIV	-	Human Immunodeficiency Virus
FIGO	-	Fédération Internationale de Gynécologie et d'Obstétrique (International Federation of Gynecology and Obstetrics)
ALDH	-	Aldehyde Dehydrogenase
HeLa	-	Henrietta Lacks Cells
GLOBOCAN	-	Global Cancer Incidence, Mortality and Prevalence
RAR	-	Retinoic Acid Receptor
RXR	-	Retinoid X Receptor
RARE		Retinoic Acid Response Element
ATRA		All-*trans* Retinoic Acid
CRABP	-	Cellular Retinoic Acid-Binding Protein
13-Cra	-	13-*cis* Retinoic Acid
9-cRA	-	9-cis Retinoic Acid
ROS	-	Reactive Oxygen Species
hTERT	-	Human Telomerase Reverse Transcriptase
EGFR	-	Epithelial Growth Factor Receptor
VEGF	-	Vascular Endothelial Growth Factor
CDK	-	Cyclic-Dependent Kinase
TIC	-	Tumour Initiating Cell
APL	-	Acute Promyelocytic Leukimia
LD_{50}	-	50% Maximal Lethal Dose

TBX_2	-	Thromboxane-B_2
PGE_2	-	Prostaglandin-E_2
IAP	-	Inhibitor of Apoptosis
XIAP	-	X-Chromosome-Linked Inhibitor of Apoptosis
SMAC/DIABLO	-	Second Mitochondria-Derived Activator of Caspase/ Diablo Homolog
FBS	-	Fetal Bovine Serum
DMSO	-	Dimethyl Sulfoxide
NADH	-	Nicotinamide Adenine Dinucleotide
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate
SEM	-	Standard Error of the Mean
MEE	-	Median-Effect Equation

LIST OF SYMBOLS

±	-	Plus minus
%	-	Percentage
^{o}C	-	Degree Celcius
µL	-	Microliter
mL	-	Milliliter
µM	-	Micromolar
mM	-	Millimolar
nM	-	Nanomolar
µm	-	Micrometer
µg/mL	-	Microgram per milliliter
Min	-	Minute
v/v	-	Volume per volume
rpm	-	Rotation per minute
$Mg/m^2/day$	-	Milligram of drug per meter square body surface per day
hPa	-	Hectopascal

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of the Problem

Cancer has emerged as one of the major threat and major global challenges to humankind. Cervical cancer is considered as major threats to woman globally with increasing incidence and mortality rate. Cervical cancer is being the most common cancer of female reproductive organ in the world (Haghshenas *et al*., 2013), second most female-specific cancer type after breast cancer (McGuire, 2016) and third leading cause of death due to cancer in female population in developing countries (Torre *et al*., 2015). By epidemiological estimate from Global Cancer Observatory, GLOBOCAN, global annual incident of cervical cancer is 569,847 new cases and 311,365 deaths in 2018 (GLOBOCAN., 2018). This has marked as more than 55% mortality cases and it also has been found that these mortality rate is originatd from developing countries (Torre *et al*., 2015). The majority cervical cancer cases are squamous cell carcinoma and followed by adenocarcinoma (Green, 2006; Guerra & Ramírez, 2017).

The prime factor causing cervical cancer is due to the Human Papillomavirus (HPV) infection (Chansaenroj *et al*., 2014). HPV has a circular double stranded DNA at the size of 8kb. This virus can be transmitted from one person to another by sexual contact. There are more than 200 types of HPV virus that have been identified to date but only HPV type 16 and 18 are oncogenic and can induce cervical cancer (Cubie, 2013; PaVE: Papillomavirus Episteme, 2018). HPV type 16 and type 18 is contributing to approximately 75% of total cervical cancer cases. HPV's cause a high-grade intraepithelial neoplastic which then leads to the formation of invasive carcinoma (Schlecht *et al*., 2001 ; Liu *et al*., 2017). Based on the origin of the disease, it is important to choose the right treatment method to treat cervical cancer according to

the stage and severity respectively. Even though HPV is the major cause of the cervical cancer, but the recurrence of the cervical cancer is due to the presence of cancer stem cell (CSCs) which leads to the metastasis and recurrence of the cervical cancer (Organista-Nava *et al*., 2019).

Currently, there are many treatments available to treat the cervical cancer according to their stages. Those treatments are radiation therapy, surgery, radiation therapy, hormone therapy, adjuvant chemotherapy as well as targeted therapy such as usage of Trastuzumab, Pertuzumab and Docetal (Swain, 2007; Pareja *et al*., 2016) can cure 80% to 95% of patients effectively of female patients only at early stage (FIGO stage IA-IIA0). Meanwhile only prolong survival at advanced stage (FIGO stage IIB-IVB) with very high chances of cancer recurrent (Vinogradov and Wei, 2012; Gadducci *et al*., 2010; Quinn *et al*., 2006). The major reason for this is that the current available treatments are being only effective in treating non-stem cell enriched cervical cancer. Cervical cancers are also known to be enriched cancer stem cells making it difficult to be treated due to resistance to therapies (Liu *et al*., 2013).

The current treatment to cure cervical cancer is evident in killing the cancer cells by shrinking the tumor, however, the crucial CSCs which is functioned in metastasis are ineffectually difficult to be eliminated due to its resistance to therapeutics (Reya *et al*., 2001; Vinogradov and Wei, 2012). The bulk population of cancer cells are differentiated cancer cells with limited proliferative potential and is not the main cause of metastasis. Whereas the surviving CSCs with high proliferative potential and self-renewal ability, are known to have high ALDH activity (Liu and Zheng, 2013). This event leads to the rise of new tumor cells, resulting in the relapse of cancer (Vinogradov and Wei, 2012). In addition, these recurrent tumors are often more malignant and fast spreading which develop drug resistance to previously treated chemotherapeutics.

Nevertheless, current studies show that the cervical cancer cells are sensitive towards differentiation-inducing drugs including retinoic acid (RA). Retinoids, the

main active metabolite are structurally related to the hormone of vitamin A. However, acquired resistance posed by retinoids remain a challenge in cancer treatment. RA is known to exert distinct biological functions depending on its interacting partner. In contrast, dietary phytochemical such as ginger (*Zingiber Officinale*) along with its bioactive compounds is known to be a useful chemopreventive and anticancer agent as exhibited in various *in vitro* and *in vivo* studies including cervical cancer. Despite that, information on the combined therapeutic effect of Retinoic Acid (RA)-ginger Extract (GE) in cervical cancer remains unexplored and is yet to be elucidated. This project will provide fundamental knowledge on a potential novel therapeutic target in the treatment and management of cervical cancer.

1.2 Statement of the Problem

Cervical cancer is the one of the most well-known malignant cancer of female reproductive organs in the world (Haghshenas *et al*. 2013). Although numerous types of effective treatments such as radical surgery, chemotherapy, radiotherapy, or any combination of therapeutic strategies are available today, up to one-third cases of cancer recurrence were still found in population of female patients after receiving primary treatment, with pelvis being the most common site of failure (Gadducci *et al*., 2010; Bellone *et al*., 2007; Leitao, 2002; Friedlander, 2002). For example, the cervical cancer relapse rate in FIGO stages IB-IIA were found around 11% to 22%, whereas 28% to 64% in FIGO stages IIB-IVA (Gadducci *et al*., 2010; Quinn *et al*., 2006). CSCs theory has been believed as the main reason contributing to tumor recurrence (Vinogradov and Wei, 2012). CSCs is a small subpopulation of cancerous cells with pluripotency and self-renewal ability inside tumors. CSCs can develop drug resistance resiliently and proliferate to drug-resistant cancer cells and even develop into more malignant tumors, causing the relapse of cancer after treatment (Vinogradov and Wei, 2012). Therefore, the effort has been focused on permanent cure of recurrent cervical cancer, however, no promising therapeutic strategies have been invented today albeit research on drug discovery of potential naturally derived drugs for cervical cancer had been commenced since 40 years ago (Karikas *et al*., 2010). Many studies are being

conducted to study the effectiveness of retinoic acid (RA) in treating other cancers as well. RA is a metabolite of Vitamin A and it plays important roles in cell proliferation and differentiation (Zanotto-Filho *et al*., 2008 ; Schultz & Harringto., 2018). Furthermore, extracts of ginger had also shown efficient anticancer activities against HeLa cells (Ansari *et al*., 2016) . However, the *in vitro* studies on co-treatment of retinoic acid and ginger extract have not been extensively studied. It has been Therefore, further assessment on anticancer effect and the synergistic effect of this co-treatment on HeLa cervical cancer cells were the focus of the research.

1.3 Objectives of the Study

The objectives of the research are:

1) To determine anticancer effect of combined retinoic acid-ginger treatment on HeLa cervical cancer cells in comparison to RA and GE alone.
2) To determine the synergism of the combined treatment of RA-GE on HeLa cells.
3) To evaluate the ability of RA, GE, and RA-GE treatment to induce apoptosis in HeLa cervical cancer cells.
4) To examine the prohibitive effect of RA, GE and RA-GE treatment on metastasis of HeLa cells.

1.4 Scope of the Study

The research project was carried out at Cancer Research Laboratory in block T02, Faculty of Science, UTM Johor. The goal of this research was to determine anticancer effects of RA, GE, and combined RA-GE on cell viability of HeLa cells via

cell viability assay and proliferation assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay and CyQuant NF assay). Furthermore, synergism effect of RA-GE on the HeLa cells was performed out using CompuSyn and Microsoft Excel software. In the study, we also evaluate the Moreover, drug dosages of all treatments against HeLa cell line in order to determine 50% maximal inhibitory concentration and ability to induce apoptosis via apoptosis assay (Caspase-Glo® 3/7 assay). After that, drug treatment of different dosages was tested on HeLa cells to examine the anti-metastatic effect. Finally, statistical analysis of results from both cell viability assay, apoptosis assay, and cell migration assay were achieved using t-test and GraphPad Prism software.

1.5 Significance of the Study

To date, cervical cancer at stage IIB is still incurable as no applicable therapeutic strategies for permanent cure as well as the high cancer recurrence (Gadducci *et al*.,2010; Bellone *et al*., 2007; Quinn *et al*., 2006). Nonetheless, the efficacy of co-treatment of retinoic acid and ginger extract might become the potential solution for cervical cancer treatment. The combination treatment using two sequence of drugs RA+GE and GE+RA on HeLa is the first to be done. In addition, the ginger extraction method used in this study shown better anti-cancer effect compared to other ginger extraction method used in previous studies(Romero *et al.,* 2017).Therefore, this research contributes to further understanding on anticancer effect of combined treatment of retinoic acid- ginger extract on cell differentiation, apoptosis, and metastasis of HeLa cervical cancer cell line. Furthermore, these findings also provided fresh insights for non-chemotherapeutic treatment in cancer therapy, especially tumors enriched with CSCs. Therefore, this research established the basis for researchers to further explore, optimize, and finally incorporate this pharmacological understanding in cancer therapeutic strategies in foreseeable future.

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