THE ROLE OF *Punica granatum* AND POLYAMINES IN REGULATING CELL CYCLE AND CELL DEATH AGAINST HUMAN LUNG ADENOCARCINOMA CELLS, A549

BY

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ABSTRACT

Cancer is a complex disease to treat and has poor survival rate. Natural product has shown a promising effect in cancer treatments. *Punica granatum* or pomegranate has a potential to be a useful cancer preventive agent, however the mechanism of action is unclear. To understand how effective the pomegranate in preventing cancer growth, it is crucial to identify the signaling pathways affected. One possibility is the polyamine pathway which is important in many cells function and its up-regulation of this pathway in cancer makes it a logical target for cancer prevention. Therefore, this study was aimed to evaluate the role of pomegranate and polyamines in regulating cell cycle distribution and cell death mechanisms as well as its anti-proliferative effect in human lung adenocarcinoma cells, A549. The classification of polyamines in pomegranate juice was determined by HPLC analysis. The anti-proliferative effect of pomegranate juice was tested by using MTT assay and the effect of 2% pomegranate juice on A549 cells growth and viability were evaluated by trypan blue exclusion assay. The cellular protein and polyamines content in A549 cells were determined using Lowry assay and HPLC, respectively. The cell cycle distribution and cell death mechanisms were evaluated using flow cytometer. The gene expression was evaluated using quantitative real - time PCR. The study found that pomegranate juice was classified under low polyamine diet. At the concentration range from 0 to 3%, pomegranate juice caused inhibition of A549 cells growth. The inhibitory concentration (IC_{50}) was 1.4% ± 0.23, 1.5% ± 0.14 and 1.4% ± 0.12 after 48, 72 and 96 h exposure, respectively. At the concentration of 2%, inhibition of growth was observed by showing decreased in viable A549 cell number and protein content after 48 h ($p<0.05$), 72 h ($p<0.001$) and 96 h ($p<0.001$) exposure compared to untreated A549 cells. In contrast, the percentage of cells viability remains high and constant. The results also showed a positive correlation between total viable A549 cells number with protein content where the R^2 values for untreated and treated were 0.9248 and 0.6523. There were no significant differences in total polyamines content in untreated and treated A549 cells. The study also found that pomegranate juice induced cell cycle arrest at G_0/G_1 phase and apoptosis via intrinsic pathway following 24 h treatment. Pomegranate juice caused loss of mitochondrial membrane permeability after 48 h ($p<0.05$) exposure and a release of cytochrome *c* in cytosol after 24 h ($p<0.05$) and 48 h ($p<0.01$) exposure in treated A549 cells. In caspases analysis, it was showed that there was activation of caspase-3 following 72 h ($p<0.01$) treatment and caspase-9 after 48 ($p<0.01$) and 72 h ($p<0.05$) exposure in treated A549 cells. Lastly, gene expression study found that pomegranate juice inhibited the expression of ODC gene after 24 h and 48 h exposure ($p<0.001$) and SSAT gene after 48 h exposure ($p<0.05$) in treated A549 cells. From the study, it can be deduced that the suppression of A549 cell growth might not due to modulation of polyamine metabolism. However, pomegranate juice able to cause A549 cell growth inhibition by inducing cell cycle arrest and apoptosis through mitochondrial pathway.

خلاصة البحث

يعتبر السرطان من الأمراض المستعصية علاجها، ونسب الوفيات بسبب هذا المرض عالية جدا. أظهرت المركبات الطبيعية نتائج جيدة في علاج السرطان، حيث أعطت ثمرة الرمان (*Punica granatum*) دلائل مهمة على إحتوائها على مركبات واقية ضد السرطان، لكن طريقة تأثيرها مازالت غير واضحة. لفهم كيفية تاثير الرمان على منع نمو السرطان، من الواجب التعرف على المسارات الفعالة المعنية. من بين المسارات المحتملة مسار عديد الأمينات الذي يعتبر من المسارات المهمة في عمل العديد من الخلايا، وتنظيمها العالي في حالة الاصابة بالسرطان يؤهلها لأن تكون هدفا منطقيا للوقاية من السرطان. كان الهدف من هذه الدراسة هو تقييم دور عديد الأمينات في الدورة الخلوية وفي آلية موت الخلية، وتأثيرها كمضاد للتضاعف في خلايا رئة الانسان السرطانية A549. تم اختبار فاعلية السمية الخلوية لثمرة الرمان باستعمال اختبار MTT وتم دراسة تأثير التركيز 2% من عصير الرمان على نمو خلايا A549، وتم فحص حيوية الخلايا باستعمال أزرق التريبان. تم تحديد المحتوى الخلوي من البروتينات باستعمال اختبار Lowry وتحديد عديدات الأمينات بالكروموتوغرافيا من الطبقة الرقيقة تحت ضغط عالي (HPLC). وتم استعمال جهاز القياس الطيفي لتحديد مراحل الدورة الخلوية وآليات موت الخلية. تم استعمال تقنية التقدير الكمي للوقت الحقيقي لتفاعل البليمريز التتابعي لمعرفة ترجمة الجين. أظهرت الدراسة أن الرمان يصنف تحت الحميات المنخفضة في عديدات الأمينات. بينت النتائج أن لدى عصير الرمان القدرة على تثبيط الخلايا السرطانية A549 في التراكيز بين 0 الى 3% . تراكيز التثبيط (أقل تركيز قاتل لـ 50 % من الخلايا أو IC_{50}) كانت 1.4% ± 0.23 بعد 48 ساعة، و 1.5% ± 0.14 بعد 72 ساعة، و1.4% ± 0.12 بعد 96 ساعة من التعريض. مقارنة بخلايا A549 الغير المعالجة، بإمكان ملاحظة وجود تثبيط للنمو وانخفاض في كمية البروتينات وعدد الخلايا عند التركيز 2% بعد 48 ساعة ($p<0.05$)، و 72 ساعة ($p<0.001$)، و 96 ساعة ($p<0.001$) من التعريض. بالمقابل، نسبة الخلايا الحية كانت كبيرة وثابتة. بينت النتائج كذلك أن هناك علاقة إجابية بين العدد الاجمالي لخلايا A549 والكمية الاجمالية للبروتينات، حيث أن R^2 لكل من الخلايا المعالجة والغير المعالجة كان 0.9248 و 0.6523. لكن لم يكن هناك أي انخفاض مهم في تركيز عديدات البيتيد في خلايا A549 المعالجة والغير معالجة. تمت أيضا ملاحظة أن بإمكان عصير الرمان كبح الدورة الخلوية عند المرحلة G_0/G_1 كما أنه يدفع الخلية إلى آلية الموت المبرمج بسلوكها مسارا متأصلا وذلك في خلال 24 ساعة من العلاج. يسبب عصير الرمان فقدان النفاذية الاختيارية للغشاء الخلوي للميتوكوندريا بعد 48 ساعة ($p<0.05$)، و بالتالي تحرير محتواها من السايتوكروم C داخل العصارة الخلوية بعد 24 ساعة ($0.05>p$) و48 ساعة ($0.01>p$) من التعريض في خلايا A549 المعالجة. بينت دراسة سلسلة التفاعلات (Caspases) أن عصير الرمان ينشط caspase-3 بعد 72 ساعة ($p<0.01$)، بينما ينشط caspase-9 بعد 48 ساعة ($p<0.01$) و 72 ساعة من تعريض خلايا A549 لعصير الرمان. بينت الدراسات الجينية كذلك أن عصير الرمان يثبط التعبير الجيني لـ ODC بعد 24 ساعة و 48 ساعة ($p<0.001$) من التعريض، ويثبط تعبير الجين SSAT بعد 48 ساعة ($p<0.05$) من تعريض خلايا A549 المعالجة لعصير الرمان. من المستنتج من خلال الدراسة أن تعديل أيض عديد الأمينات ليس هو المساهم الفعلي في كبح نمو خلايا A549، ولكن العنصر المساهم في عصير الرمان هو حث كبح الدورة الخلوية وآلية موت الخلية من خلال المسار الميتوكوندري.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences.

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to my parents, may they always be blessed by Allah S.W.T

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
ACN	Acentonitrile
Annexin V-PE	R-phycoerthrin annexin V conjugate
Apaf-1	Apoptotic protease-activating factor 1
ATCC	American Type Cell Collection
ATP	Adenosine triphosphate
Bax/Bcl-XL	Bax Bcl-2 associated X protein
BSA	Bovine serum albumin
BSC	Biological safety cabinet
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CD95	Cluster of differentiation 95
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
C_2H_6O	Ethanol
C_3H_8O	Isopropanol
CO_2	Carbon dioxide
$CuSO_4$	Copper sulphate
dATP	Deoxyadenosine triphosphate
DEPC	Diethyl pyrocarbonate
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DT	Doubling time
ELISA	Enzyme-linked immunosorbent assay
FAM	Flourescein
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FITC-LEHD-FMK	LEHD-fluromethylketone conjugated to FITC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GMP	Good manufacturing practices
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
IgG	Immunoglobulin G
JC-1	5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
MMP	Mitochondrial membrane permeability
MTT	Methylthiazole trazolium
Na_2Co_3	Sodium carbonate
NaOH	Sodium hydroxide
NCDH	Sodium carbonate decahydrate
NF-κB	Nuclear factor κB
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
PBS	Phosphate buffer solution

PCA	Perchloric acid
PCR	Polymerase chain reaction
PEE	Pomegranate fruit ethanol extract
PenStrep	Penicillin-streptomycin
PFE	Pomegranate fruit extract
PI	Propidium iodide
PS	Phosphotidylserine
qPCR	Quantitative polymerase chain reaction
R^2	Coefficient of determination
RNA	Ribonucleic acid
RNAse A	Ribonuclease A
RT-qPCR	Real-time quantitative polymerase chain reaction
SAMDC	*S*-adenosylmethionine decarboxylase
S.E.M	Standard error of the mean
SIMPs	Soluble intermembrane proteins
SMO	Spermine oxidase
SSAT	Spermidine/spermine N^1-acetyltransferase
T75	75 cm^2 tissue culture flask
TEAC	Trolox equivalent antioxidant capacity
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Z-VAD-FMK	Benzyloxycarbonylvalyl-alany-laspartyl fluromethyl ketone

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cancer is a disease which is caused by uncontrolled proliferation and growth of abnormal cells in the body (Ho *et al*., 2013). It arises as a result of accumulation of multiple mutations within genome which the process is known as carcinogenesis (Saunders and Wallace, 2010). It is a multistage process which usually takes many years to develop and leads to disruption of basic biological functions such as cell division, differentiation, angiogenesis, and migration (Turrini *et al*., 2015). World Health Organization (2015) reported that cancer is the number one cause of mortality worldwide following closely cardiovascular disease and stroke which accounting for 8.2 million cancer related deaths in 2012.

The most common forms of cancer related deaths were lung cancer (1.59 million deaths), liver cancer (745,000 deaths), stomach cancer (723,000 deaths), colorectal cancer (694,000 deaths), breast cancer (521,000 deaths) and oesophageal cancer (400,000 deaths). GLOBOCAN 2012 stated that lung cancer has been the most common cancer in the world for several decades. There are estimated to be 1.8 million new cases in 2012 (12.9% of the total) and 58% of which occurred was in the less developed regions. Cancer mortality is reported to be higher among men compared to women (207.9 per 100,000 men and 145.5 per 100,000 women) (National Cancer Institute, 2015). Last two decades, the mortality rates for all cancers have shown some improvements. However, there are still a number of cancers which has poor survival rate such as lung cancer which is 5% in the world (Cancer Research

UK, 2015). Lung cancer has been proven difficult to control with the therapeutic drugs and surgical approaches (Khan *et al*., 2007). The patient with lung cancer faces a grim prognosis because it is highly aggressive and refractory to chemotherapy. Lung cancer has increased at alarming rate since the last decade. The survival rate for lung cancer is higher in women compared to in men. About 89% of lung cancer cases occurred each year in United Kingdom is linked to major lifestyle and other risk factors (Cancer Research UK, 2015). One of the risk factors is the increasing trend in smoking among the community.

There are several conventional therapies for cancer including chemotherapy, radiotherapy as well as surgical approaches (Guan & Yang, 2014). However, these existing therapies do not efficient enough to increase the survival rate. Chemotherapy has been used for long time ago in cancer treatment but at the end of chemotherapy session, patient will experience several non-specific side effects such as nausea, vomiting, fatigue and hair loss (Coates *et al*., 1983; Griffin *et al*., 1996). As previously mentioned, lung cancer is one of the cancer which is resistant to chemotherapy. Therefore, many scientific research endeavours have focused on finding other alternative strategies to reduce the prevalence of cancer as well as to increase the cancer survival rate. Recently, there is a growing interest in consumption of natural product as an alternative strategy in reducing the risk of getting cancer and preventing the recurrence of cancer.

Chemoprevention and cancer treatment using natural product has gained attention at all levels and developed as a major field of scientific investigation. It is because cancer is a preventable disease. Adopting a healthy lifestyle such as having a healthy diet, being physically active and reducing the use of tobacco would help to reduce the prevalence of cancer as well as prevent recurrence of cancer. According to

Gullet *et al*. (2010), chemoprevention is defined as pharmacological intervention either with synthetic or naturally occurring compounds that may prevent carcinogenesis or the development of invasive cancer.

Chemoprevention using natural compounds had been shown to be safer than synthetic compounds because it can be found in the diet, wide availability and tolerability (Gullet *et al*., 2010). Natural compounds from fruits and vegetables had been reported to exert anticancer effect and have a potential to reduce the risk of cancer. One of the fruits which possess several medicinal properties had been selected in this study which was *Punica granatum* or pomegranate. Pomegranate was known to exert anticancer effect and provide the best protection against many diseases. Pomegranate contains a number of bioactive compounds which may contribute to chemopreventive property. Usually, chemopreventive agents are found as a complex mixture, not in isolation. So, most probably the chemopreventive action of pomegranate will be resulted from a number of different bioactive compounds which affect multiple pathways to produce a cumulative chemopreventive effect. In many cases, the mechanism of action is still unclear and the signaling pathway affected is not known.

There are several pathways which are well known to be affected by cancer such as NF-kB signaling (Dolcet *et al*.,2005), Wnt signaling (Reya & Clevers, 2005) and hormone receptor signaling (Saunders & Wallace, 2010). However, there is another pathway which has not been explored in detail yet which is polyamine pathway. Polyamine is found in almost species and have an important role in normal and malignant cells proliferation and death pathways which induced programmed cell death known as apoptosis (Moschou & Roubelakis-Angelakis, 2014). Polyamines are highly regulated and dependent on the activity of its rate limiting enzyme, ornithine

decarboxylase (ODC) which involved in polyamine biosynthesis (Ray *et al*., 1999). In cancer cells, polyamines present in high level (Soda, 2011). The up-regulation of the polyamines and their involvement in the regulation of cell growth makes them as a logical target for the cancer treatment. In order to ensure the effectiveness of the pomegranate as anti-proliferative agent, the signaling pathways affected should be known and the mechanism of action should be elucidated.

The ultimate goal of cancer treatment is to promote cancer cells death without causing too much damage to normal cells (Gerl & Vaux, 2005). The existing cancer treatments have been known to cause cell death by two modes which are induction of programmed cell death, apoptosis and direct toxicity. In cancer treatment, using anticancer agents that induced apoptosis are highly recommended due to its ability to not causing death of normal cells. Besides, cell cycle arrest also becomes the target in cancer treatment. Generally, cancer occurs as a result of dysregulation of cell cycle which serves to protect from DNA damage. Whenever DNA damage occurs, cell cycle arrest provides tumor cells to undergo repairs mechanism. However, failure to repair the DNA damage will cause activation of apoptotic cascade which leads to cell death. Recently, there are many cancer treatments that focus on apoptosis induction as well as cell cycle arrest as the mean of cancer cell death. One of the focuses in this study is to investigate the effect of pomegranate in cell cycle distribution as well as cell death mechanism in human lung adenocarcinoma cells, A549.

1.2 RESEARCH OBJECTIVES

The general aim for this study is to investigate the role of pomegranate juice and polyamines in regulating cell cycle distribution and cell death mechanisms in human lung adenocarcinoma A549 cells. The specific objectives of this study are:

1- To quantify and classify the polyamines content in the pomegranate juice.

2- To evaluate the anti-proliferative effect of the pomegranate juice on the growth of human lung adenocarcinoma A549 cells.

3- To determine the human lung adenocarcinoma A549 cells cycle profile changes induced by pomegranate juice.

4- To identify the type of cell death induced by pomegranate juice and the mechanisms involved.

5- To identify the effect of pomegranate juice on the gene expression of ornithine decarboxylase (ODC), enzyme involved in the polyamine synthesis and spermidine/spermine N^1-transferase (SSAT), enzyme involved in the polyamine catabolism.

1.3 RESEARCH QUESTIONS

1. Does pomegranate contain polyamines? If yes, what is the classification of polyamine diet for pomegranate juice?
2. Does pomegranate juice inhibit the proliferation and growth of human lung adenocarcinoma A549 cells?
3. Does pomegranate juice amend the human lung adenocarcinoma A549 cells cycle profile?

4. What is the type of cell death induced by the pomegranate juice and the mechanisms involved?
5. Does the gene expression of ornithine decarboxylase (ODC), enzyme involved in the polyamine synthesis and spermidine/spermine N1-transferase (SSAT), enzyme involved in the polyamine catabolism affected by pomegranate juice?

1.4 RESEARCH HYPOTHESES

The hypotheses are as follows:

1. Polyamines present in pomegranate in low level. Thus pomegranate is classified as low polyamines diet.
2. The pomegranate juice possesses anti-proliferative activity against human lung adenocarcinoma A549 cells by showing growth inhibition.
3. The pomegranate juice induces the cell cycle profile changes in human lung adenocarcinoma A549 cells.
4. The pomegranate juice induces cell death on human lung adenocarcinoma A549 cells via apoptosis.
5. The pomegranate juice inhibits the expression of ornithine decarboxylase (ODC) and induces the expression of spermidine/spermine N1-acetyltranferase (SSAT) which leads to depletion of polyamines level.

CHAPTER TWO

LITERATURE REVIEW

2.1 POLYAMINES: AN OVERVIEW

2.1.1 Structure and Properties

The basic natural polyamines known as putrescine, spermidine and spermine are widely distributed and present in almost all living organisms such as prokaryotes, eukaryotes, plants and animals. Aliphatic polyamines have a low molecular weight (Schipper *et al*., 2000) and simple chemical structure (Figure 2.1). Polyamines are water soluble and are highly charged cations at physiological conditions. They can bind to numerous macromolecules including DNA, RNA, proteins, enzymes and many negatively charged phosphorylated molecules in the cytoplasm and nucleus (CRISS, 2003).

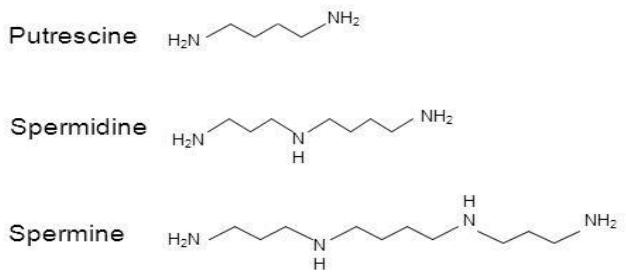

Figure 2.1 Chemical structures of the polyamines, putrescine, spermidine and spermine (Minois *et al*., 2011)

2.1.2 Physiological Roles of Polyamines

Polyamines are essential for physiological as well as pathological condition in human. Polyamines play a crucial role in cell proliferation (CRISS, 2003) and differentiation (Schipper *et al*., 2000). Under physiological conditions, polyamines are flexible polycations that are able to interact with negatively charged macromolecules which led to the stabilization of DNA, RNA and some proteins. This interaction determines polyamines are the essential factors for the growth, maintenance and function of normal cells (Kalač, 2014) as well as malignant cells (Davidson *et al*., 1999). In cancer cells, polyamines present in high level (Soda, 2011). Enhanced level of polyamines biosynthesis in cancer tissues led to increased polyamine availability which enhanced the growth of cancer cells. Therefore, the growth of tumor cells is accelerated with the presence of polyamines.

2.1.3 Metabolism of Polyamines

Polyamine metabolism involves synthesis of polyamine and catabolism of polyamines. The polyamines biosynthesis is started with the decarboxylation of ornithine by the rate-limiting enzyme, ornithine decarboxylase (ODC) (Ramani *et al*., 2014) producing putrescine (Figure 2.2). Putrescine is subsequently converted to spermidine by addition of aminopropyl groups. The reaction involves *S*-adenosylmethionine decarboxylase (SAMDC) and spermidine synthases (Thomas & Thomas, 2001). The conversion of spermidine to spermine also requires SAMDC along with spermine synthase (Childs *et al*., 2003). The decarboxylation reaction is reversible process. Hence, the higher polyamines can be converted back to putrescine (Jänne *et al*., 2004).