THE EFFECT OF POLYAMINES IN SELECTED PROPHETIC FRUITS ON HUMAN LUNG ADENOCARCINOMA CELL LINE, A549

BY

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ABSTRACT

Polyamines are vital in maintaining human health because they perform certain functions that are necessary for cell development. However, extensive intracellular polyamines may promote unwarranted cell proliferation and to a certain extent, stimulates cancer initiation. Therefore, this study aimed to investigate the polyamines deficient diet in chemoprevention strategy using selected fruits recommended by the prophet against human lung adenocarcinoma cells, A549. Five prophetic fruits were selected, including *Phoenix dactylifera* (ajwa dates), *Beta vulgaris* (beetroot), *Ficus auriculata* (fig), *Ziziphus jujube* (jujube) and *Vitis vinifera* (raisins). Initially, they were freeze dried and stored in -80°C until analysis. The polyamines in each selected prophetic fruit was quantified and classified by High Performance Liquid Chromatography (HPLC). Subsequently, their anti-proliferative effect on A549 cells growth was evaluated using MTT assay and Trypan blue exclusion assay. Protein content was measured using Lowry assay. The activity of genes that regulate polyamine metabolic pathway particularly ornithine decarboxylase (ODC) and spermine/spermidine (N1)-acetyltransferase (SSAT) was elucidated using qPCR. Finally, cell cycle profile and apoptosis assay were conducted with flow cytometer, while caspase assay was done using colorimetric method. It has been found that jujube showed the highest polyamines concentration (219.6 ±4.4 nmoles/ml) while fig was the lowest (39.3±3.0 nmoles/ml). MTT assay suggested IC_{50} of fruits recommended by the prophet was ranged from 15 mg/ml to 30 mg/ml. All selected prophetic fruits showed anti-proliferative effect against A549 cells. Protein content was significantly lower after 72 h of treatment with selected prophetic fruits, and total elimination of intracellular spermidine and spermine were observed in all treated A549 cells. The polyamines metabolism was found to be altered in which the intracellular polyamines depletion was mainly contributed by the downregulation of ODC in A549 cells treated with ajwa dates and jujube. Meanwhile, beetroot and fig induced the upregulation of SSAT. Cell cycle profile displayed significant cell cycle arrest at G_2/M after 48 h of exposure to jujube and raisins. It is demonstrated that beetroot, fig, jujube and raisins induced apoptosis after 48 h exposure while ajwa dates might induced other type of cell death. Caspase assay revealed significant activation of caspase 3, 8 and 9 in beetroot treated cells while no caspase activation was identified in other prophetic fruits treated cells. Considering the classification of polyamines, the anti-proliferative effect and the type of cell death, it was concluded that fig, raisins and beetroot are the promising candidates for nutritional cancer therapy and preventive approaches for cancer.

خلاصة البحث

تعد عديدة الأمينات في غاية الأهمية للحفاظ على الصحة لأدائها لبعض المهام اللازمة في عملية نمو الخلية. ومع ذلك، كثرة عديدة الأمينات في داخل الخلايا قد تحفز نموا خلويا غير مرغوب به وقد تحفز أيضا في نفس الوقت نمو الخلايا السرطانية. ولذلك هدفت هذه الدراسة إلى التحقيق في الحميات الغذائية الخالية من متعددة الأمينات في استراتيجيات الوقاية الكيميائية باستخدام بعض الثمرات المختارة التي أوصى بها النبي صلى الله عليه وسلم ضد خلايا الرئة السرطانية البشرية (A549). تم اختيار خمسة ثمرات، وهي العجوة (فينيكس داكتيليفيرا)، والشمندر (بيتا فولجاريس)، والتين (فيكوس أوريكولاتا)، والعِنَّاب (زيزيفوس جوجوبي)، والزبيب (فيتيس فينفيرا). تم في البداية تجفيف هذه الثمرات بالتجميد وحفظها تحت درجة حرارة −80 °C إلى موعد التحليل. تم تصنيف وتحديد كمية متعددة الأمينات في الثمرات النبوية المختارة باستخدام الكروماتوجرافيا السائلة العالية الأداء (HPLC). في نفس الوقت، تم تقييم نشاط التثبيط الخلوي على خلايا A549 الخاص بهذه الثمرات باستخدام اختبار MTT والاختبار الاستقصائي للتريبان الأزرق. تم قياس محتوى البروتين بواسطة اختبار لاوري. تم توضيح النشاط الجيني المنظم للمسارات الأيضية للأمينات المتعددة، بالتحديد مسار الأورنيتين ديكاربوكسيليس (ODC) وسبيرمين/سبيرميدين (N1)-أسيتيلترانسفيريس (SSAT)، باستخدام qPCR. وفي النهاية تم القيام باختبار ملخص الدورة الخلوية وآلية موت الخلية باستخدام عداد الكريات التدفقي، واختبار إنزيم الكاسبيس باستخدام طريقة القياس اللوني. أظهرت النتائج أن أعلى تركيز للأمينات المتعددة كان في العناب (219.6 ± 4.4 نانو مول/مل) وأدنى تركيز كان في التين (39.3 ± 3.0 نانو مول/مل). أظهر اختبار الـ MTT أن الـ IC_{50} للثمرات المختارة التي أوصى بها النبي صلى الله عليه وسلم كانت بين 15 مغ/مل و 30 مغ/مل. أظهرت كل الثمرات نشاطا تثبيطيا لنمو الخلايا ضد خلايا A549. كان محوى البروتين منخفضا بشكل ملحوظ بعد المعالجة التي دامت 72 ساعة بالثمرات، وتم ملاحظة الاستقصاء الكلي للسبيرميدين و السبيرمين من داخل الخلايا في كل خلايا A549 المعالجة. تم اكتشاف أن المسار الأيضي للأمينات المتعددة قد غُيّر بحيث أن استنفاذ متعددة الأمينات داخل الخلايا كان سببه التنظيم التخفيضي لل ODC في خلايا الـ A549 المعالجة بالعجوة والعناب. في حين أن تأثير التين والشمندر قد حث على التنظيم الرفعي لل SSAT. أظهر ملخص الدورة الخلوية توقفا ملحوظا في الدورة الخلوية في G_2/M بعد 48 ساعة من التعريض للعناب و الزبيب. تم إثبات أن الشمندر، و التين، والزبيب قد عززوا آلية موت الخلية بعد 48 ساعة من التعريض، أما العجوة فقد أظهرت نوعا آخر من الموت الخلوي. أظهر اختبار إنزيم الكاسبيس تنشيطا ملحوظا في إنزيمات كاسبيس 3، و8، و9 في الخلايا المعالجة بالشمندر، ولكن لم يكن هناك أي نشاط ملحوظ في الخلايا المعالجة بالثمرات الأخرى. بالاعتماد على تصنيف عديدة الأمينات، ونشاط التثبيط الخلوي، ونوع آلية الموت الخلوي، فإنه بإمكاننا استنتاج أن التين، والزبيب، وجذر الشمندر هي ثمرات واعدة في العلاج السرطاني الغذائي ونهج الوقاية السرطانية.

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 (Biomedical Science)

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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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Signature Date

This thesis is dedicated to my husband, parents, and siblings

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%	Percentage
°C	Degree Celcius
µg	Microgram
µl	Microliter
µm	Micrometre
µM	Micromolar
cm^2	Square Centimetre
g	Gram
g	Gravitational force
h	Hour
kg	Kilogram
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
ng	Nanogram
nm	Nanometre
nM	Nanomolar
nmol	Nanomole
Pa	Pascal

rpm	Revolutions per minute
s	Second

LIST OF ABBREVIATIONS

4MCHA	Trans-4-methylcyclohexylamine
A549	Human Lung Adenocarcinoma Cell Line
ACTB	Beta-Actin
ADC	Arginine Decarboxylase
AdoData	S-Adenosyl-1,12-Diamio-3-Thio-Azadodecane
AdoMet	S-adenosyl-L-methionine
AdoMetDC	AdoMet Decarboxylase
AIF	Apoptosis Inducing Factor
AKT	Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
ANOVA	Ordinary Two-Way Analysis of Variance
APAF-1	Adaptor Protein Apoptotic Protease-Activating Factor 1
ATP	Adenosine Triphosphate
B(a)P	Benzo-Alpha-Pyrene
B. vulgaris	*Beta vulgaris*
BCL2	B-cell Lymphoma 2
BPA	Bisphenol A
BRAF	Proto-Oncogene B-Raf
BSA	Bovine Serum Albumine
$CaCl_2$	Calcium Chloride
Cad	Cadaverine
CDC	Centers For Disease Control And Prevention
cDNA	Complementary DNA
CO^2	Carbon Dioxide
DcAdoMet	Decarboxylated AdoMet
DEPC	Diethyl Pyrocarbonate
DFMO	Alpha-Difluoromethylornithine
DMBA	Dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ED	Extensive-Stage Disease
ED	Extensive-Stage Disease
EGFR	Epidermal Growth Factor Receptor
ESR	Electron Spin Resonance Spectroscopy
F. auriculata	*Ficus auriculata*
FAD	Flavin Adenine Dinucleotide
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
H_2O_2	Hydrogen Peroxide
$HClO_4$	Perchloric Acid
HDAC	Histone Deacetylase

HeLa	Cervical Cancer Cells
HepG2	Human Hepatoma Cells
hFSE	Human Foreskin Surface Epithelial
HPLC	High Performance Liquid Chromatography
HRPC	Hormone-Refractory Prostate Cancer
IARC	International Agency For Research On Cancer
IC_{50}	Half Maximal Inhibitory Concentration
IIUM	International Islamic University Malaysia
LCCs	Large Cell Carcinomas
LD	Limited-Stage Disease
LKB1	Serine/Threonine Kinase 11
LPSs	Lipopolysaccharides
MAP	(2R,5R)-δ-Methylacetylenicputrescine
MCF-7	Human Breast Cancer Cells
MGBG	Methylglyoxal Bis
MOMP	Mitochondrial Outer Membrane Permeabilization
mRNA	Messenger RNA
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCR	The National Cancer Registry Of Malaysia
NMR	Nuclear Magnetic Resonance
NSCLCs	Non-Small Cell Lung Carcinomas
ODC	Ornithine Decarboxylase
P. dactylifera	*Phoenix dactylifera*
P53/TP53	Tumor Protein P53
PAHs	Polycyclic Aromatic Hydrocarbons
PAO	Polyamine Oxidase
PBS	Phosphate Buffered Saline
PC-3	Human Prostate Cancer Cell
PCBs	Polychlorinated biphenyls
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDD	Polyamine Deficient Diet
PGG	Pentagalloylglucose
PI	Propidium Iodide
PKB	Protein Kinase B
pNA	Peptide Para-Nitroaniline
PPE	Personal Protective Equipment
PRD	Polyamine Reduced Diet
PRST2	Human Prostate Cancer Cell
PS	Phosphatidylserine
Put	Putrescine
RB	Retinoblastoma Protein
RNA	Ribonucleic Acid
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
SCC	Squamous Cell Carcinoma

SCLCs	Small Cell Lung Carcinomas
SD	Standard Deviation
SEM	Standard Error of Mean
SMAC	Second Mitochondria-Derived Activator of Caspases
SMO	Spermine Oxidase
Spd	Spermidine
SPDS	Spermidine Synthase
Spm	Spermine
SPMS	Spermine Synthase
SSAT	Spermidine/Spermine N-Acetyltransferase
TAC	Total Antioxidant Capacity
TCM	Traditional Chinese Medicine
TNF	Tumor Necrosis Factor
TP	Total Polyphenol
U937	Lymphoma Cells
UV	Ultraviolet
V. vinifera	*Vitis vinifera*
VALCSG	Veterans Administration Lung Cancer Study Group
w/v	Weight/Volume
WHO	World Health Organization
XIAP	X-Linked Inhibitor of Apoptosis Protein
Z. jujube	*Ziziphus jujube*
Z-VAD	Inhibitor Benzyloxycarbonyl-Val-Ala-Asp

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cancer is a major public health problem around the globe accounting for top cause of morbidity and mortality (World Health Organization [WHO], 2015) second to cardiovascular diseases (Centers for Disease Control and Prevention [CDC], 2014). The latest global cancer data available in 2012 revealed that 32.6 million people suffered from cancer and 8.2 million deaths was reported ever since. Lung cancer contributes to the highest (13%) cancer burden worldwide, followed by breast cancer (11.9%) and colorectal cancer (9.7%). These intensifying figures are expected to increase one fold for the next two decades (World Cancer Report, 2014) as a result of challenges and limitations associated with current cancer treatments.

Standard cancer therapies which consist of chemotherapy, radiotherapy and surgery are inadequate where there are surplus demands for improvement of present technologies (Wicki et al., 2015). Challenges of existing anti-cancer treatments include non-selective targeting, resistance to anti-cancer drugs and side effects following administration. Due to this reasons, an effective preventive cancer strategy was introduced to complement cancer treatment; which is the chemoprevention. Chemoprevention employs natural or synthetic agents that are able to inhibit, suppress or reverse cancer initiation and progression (Steward and Brown, 2013). It is not limited to cancer patients but also caters the high risk populations; of those who survive from cancers, people with inherited cancer syndromes and individual with family history of cancer. Plenty of researches have demonstrated positive outcomes in

chemoprevention therapy using medicinal plants, indicating them as a rational and appealing strategy (Mehta et al., 2010). *Ayurveda*, one of the traditional practices that use natural products, is proven in managing minor and major neoplasms, as well as benign and malignant tumour (Premalatha and Rajgopal, 2005). Other practices such as Traditional Chinese Medicine (TCM) and medicines of Mayan are also recognized to maintain health and prevent diseases (Mehta et al., 2010).

Nutritional cancer therapy is one of the chemopreventive strategies that utilize nutrition to prevent metastasis of existing neoplasia tissues as well as to increase life span, improve performance and quality of health. In this study, we particularly interested in polyamines deficient diet (PDD) as nutritional cancer therapy. Polyamines (putrescine, spermidine and spermine) are present in all eukaryotes where they are typically derived from endogenous biosynthesis and exogenous supplies through intestinal microorganisms and diet (Kalač, 2009). Polyamines are involved in various physiological functions varying from cell growth and proliferation to cell survival and death (Cohen, 1998). The intracellular polyamines level is tightly regulated by a systematic mechanism (Pegg et al., 1981) of polyamines biosynthesis, interconversion and degradation. However, the polyamines metabolism is frequently dysregulated in cancer resulting in escalation of intracellular polyamine pools.

Significant association between intracellular polyamines and cancer development has been observed in various types of cancer such as in colon, skin, prostate (Babbar and Gerner, 2011), lung, stomach, and breast cancer (Criss, 2003). The increase of intracellular polyamines is able to promote unwarranted cell growth and proliferation. Since exogenous polyamines from dietary components contribute to the largest source of intracellular polyamines pool, it is essential to conduct polyamines analysis in food to provide basis for PDD in cancer patients and high-risk

population such as cancer survivor and heredity cancer. The practicality of PDD therapy has been observed in metastatic hormone-refractory prostate cancer (HRPC) patients where it improved patients' quality of life and pain control (Cipolla et al., 2010).

Up to this day, polyamines analysis in medicinal fruits has not much being elaborated. The potential risks of consuming high polyamines medicinal fruits by cancer patients and high risk populations are often ignored. Since high polyamines diet might lead to increase intracellular polyamines pool as well as rise chances for cancer initiation and progression, assessing polyamines content and evaluating the selected fruits' ability to exterminate cancer cells might be useful. The prophetic fruits therefore were chosen because the health benefits associated with their consumptions were vastly mentioned in Quran and Hadith. Only a few studies were done on lung cancer in relation to polyamines, hence, assessing the anti-cancer prophetic fruits on A549 cells are our main interest. Thus, present works have extended polyamines study in *Phoenix dactylifera* (ajwa dates), *Beta vulgaris* (beetroot), *Ficus auriculata* (fig), *Zizyphus jujube* (jujube) and *Vitis vinifera* (raisins) as well as evaluation of their roles as a chemopreventive agent against cancer in A549 cells.

1.2 PURPOSE OF THE STUDY

The aim of this study was to assess polyamines in selected prophetic fruits (ajwa dates, beetroot, fig, jujube and raisins) and elucidate the role of polyamines in human lung adenocarcinoma cell line, A549 as a chemoprevention target for therapy.

1.3 RESEARCH OBJECTIVES

The specific objectives for this study are:

1. To quantify and classify the ajwa dates, beetroot, fig, jujube and raisins based on their total polyamines.
2. To assess anti-proliferative effect of selected prophetic fruits on human lung adenocarcinoma cells, A549.
3. To measure protein and intracellular polyamines following treatments with selected prophetic fruits in A549 cells.
4. To identify the effect of selected prophetic fruits on the polyamines metabolic pathway based on ornithine decarboxylase (ODC) and spermidine/spermine N-acetyltransferase (SSAT) gene expression.
5. To determine type of cell death and the mechanism induces by selected prophetic fruits.

1.4 RESEARCH QUESTIONS

1. What is the concentration and classification of polyamines in selected prophetic fruits?
2. Is there any anti-proliferative effect exerted by these fruits on human lung adenocarcinoma cells, A549?
3. Do the fruits reduce protein and intracellular polyamines content?
4. Do the fruits change the expression of ODC and SSAT?
5. Do the fruits cause cell death and what is the mechanism?

1.5 RESEARCH HYPOTHESES

Polyamines (putrescine, spermidine and spermine) are detected in selected prophetic fruits with varying concentrations. Significant anti-proliferative effect and growth inhibitory effect against human lung adenocarcinoma cells, A549 are observed following treatments. The anti-proliferative effect of prophetic fruits is correlated with protein reduction and positive correlation is observed between cell number and protein content after induction of prophetic fruits as treatments. There are noticeable changes in intracellular polyamines via modification of polyamines metabolism through ODC and SSAT activities. Apoptosis is expected to be the main types of cell death via a cascade of caspases.

1.6 SIGNIFICANCE OF THE STUDY

This study will contribute to the significant approach in cancer chemoprevention strategies via nutritional therapy. Knowledge of polyamines content in selected prophetic fruits and the roles of polyamines in cancer cells allow proper selection of food as dietary intake by high risk population and cancer patients. This complementary approach presumably would minimize chances for cancer development and progression. These were achieved by quantifying polyamines concentration in selected anti-cancer prophetic fruits followed by *in vitro* assessments that consist of evaluating prophetic fruits anti-proliferative effect and measuring protein and intracellular polyamines changes following induction of prophetic fruits. This study proceeded further with identifying the effect of prophetic fruits on polyamine metabolic pathway and determining types and mechanisms of cell death induced by selected anti-cancer prophetic fruits. From all of the above, recommendations as diet were determined.