CYTOTOXIC EFFECTS AND MECHANISMS OF CELL DEATH OF *Artocarpus altilis* ON HUMAN BREAST, COLON, LUNG AND SKIN CANCER CELLS

BY

TARA K. JALAL

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in Health Sciences

Kulliyyah of Allied Health Sciences
International Islamic University Malaysia

JUNE 2018

ABSTRACT

Cancer is a major cause of morbidity and mortality worldwide. In recent biomedical researches, the areas of cancer and infectious diseases have a leading position in utilization of medicinal plants as a source of drug discovery. Malaysia has a diversity and large quantity of underutilized fruits which are rich in phenolic compounds. The objective of study one was projected *in vitro* to explore natural sources of antioxidant in *Artocarpus.altilis* (breadfruit) extracts and antioxidant properties. The total phenolic content (TPC) was measured using the Folin-Ciocalteau method and the total flavonoid content (TFC) was determined by using aluminium chloride colorimetric method. Antioxidant properties were determined via the DPPH radical scavenging and β–carotene bleaching (βCB) assays. The various fruits parts Pulp (PU), peel (PE) and whole fruit (WF) were extracted with various solvents such as hexane, dichloromethane (DCM) and methanol. The methanol extracts obtained the highest yields among other solvents (hexane and DCM). The pulp (edible portion) had the highest yield ($p<0.01$). Methanol extract of pulp part revealed the highest total phenol and flavonoid content value of 781±17.32 mg (GAE)/g and 6213.33±82.24 mg (QE)/g of dry sample, respectively. IC_{50} values of methanol extract of pulp part in DPPH radical were obtained to be 0.05±0.00 mg/mL as compared to positive control (ascorbic acid) 0.06±0.00 and the antioxidant activity for the β-carotene bleaching assay was 88.34±0.75% of methanol extract of pulp part as compared to the positive control (Trolox) 90.02±0.87%. The objective of study two was to identify and quantify some phenolic compounds in the methanol extracts. By using the ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC/MSMS) based approach, a total of 9 compounds were detected and characterized on the basis of their chromatographic retention time, UV-vis spectra and mass spectra in the negative-ion mode and data from the literature. The results of the various parts of *A. altilis* fruit extracts showed promising antioxidant and potential bioactivities due to the high content of phenolic compounds. The purpose of the study three was to evaluate the cytotoxicity effects of methanol fruit extracts on four human cancer (A375, MCF-7, A549, and HT-29) cell lines. The IC_{50} of the samples were measured using trypan blue exclusion assay (TBEA). The methanol extract of pulp part showed the least inhibition concentration of 15.40±0.91 μg/mL on A375 cells. In the study four, the molecular mechanism of methanol extracts-induced apoptosis and cell cycle arrested in human cancer cells were investigated in a time dependent approach by using flow cytometry. The treated cells were stained with nexin to detect early and late apoptosis and with PI for cell cycle arrest associated with the DNA fragmentation, various cells arrests were occurred at G1/S, S and G2/M phases. Lastly the gene expression analysis by reverse transcription quantitative PCR (qPCR) method was carried out by analysing the expression of gene of interest for quantification of mRNA levels. Results after cells treated with IC_{50} were revealed by upregulating of anti-apoptotic genes/downregulated of pro-apoptotic *BCL-2* gene expressions were triggered the treated cells into *CASPASE-3*, intrinsic and extrinsic pathways. These findings suggest that the methanol extracts of three parts of *A. altilis* fruit have potential chemotherapeutic activity against human cancer cell lines mainly the pulp part of the fruit.

خلاصة البحث

السرطان هو سبب رئيسي للأمراض والوفيات في جميع أنحاء العالم. في البحوث الطبية الحيوية الأخيرة، دراسات السرطان والأمراض المعدية لها مكانة رائدة في استخدام النباتات الطبية كمصدر للكشف عن أدوية علاجية. ماليزيا لديها تنوع وكمية كبيرة من الفواكه غير المستغلة وهي غنية في المركبات الفينولية. وكان الهدف الأول من هذه الدراسة في المختبرايجاد المصادر الطبيعية لمضادات الأكسدة في لمستخلصات فاكهة *A. altilis*. تم قياس المحتوى الفينولي الكلي (TPC) باستخدام طريقة Folin-Ciocalteau وتم تحديد محتوى الفلافونويد الكلي (TFC) باستخدام طريقة كلوريد الألمنيوم اللونية. تم تحديد خصائص مضادات الأكسدة عن طريق تشيط الجذور الحره (DPPH) وطريقة ابيضاض البيتا كاروتين (βCB). تم استخراج مختلف أجزاء الفواكه بما يتضمن اللب (PU)، القشر (PE) والفاكهة كاملة (WF) مع مختلف المذيبات مثل الهكسان، ثنائي كلورو ميثان (DCM) والميثانول. كانت مستخلصات الميثانول أعلى نشاطا من المذيبات الأخرى (هيكسان و ثنائي كلورو ميثان). وكان لب الفاكهة (الجزء الصالح للأكل) الأعلى نشاطا ($p<0.01$). كشف مستخلص الميثانول لجزء اللب أعلى قيمة من الفينول والفلافونويد الكلي من 781±17.32 ملغ (GAE)/g و 6213.33±82.24 ملغ (QE)/g من العينة الجافة، على التوالي. تم الحصول على قيم IC_{50} لمستخلص الميثانول من اللب في DPPH جذر لتكون 0.05±0.00 مغ / مل بالمقارنة مع التحكم الإيجابي (حمض الاسكوربيك) 0.06±0.00 وكان النشاط المضاد للأكسدة ل βCB 88.34±0.75٪ من مستخلص الميثانول لجزء اللب بالمقارنة مع التحكم الإيجابي (ترولوكس) 90.02±0.87 %. وكان الهدف الثاني من الدراسة هو تحديد كمية بعض المركبات الفينولية في مستخلصات الميثانول. وهو ما تم تحديده باستخدام مقاييس الطيف الكتلي السطحي اللوني السائل عالي الأداء (UHPLC/MSMS)، تم الكشف عن 9 مركبات وتمييزها على أساس وقت الاحتفاظ الكروماتوغرافي، والأطياف فوق البنفسجية والأطياف الكتلية، ووضع الأيونات. وأظهرت نتائج الأجزاء المختلفة من مستخلصات الفاكهة نشاطا بيولوجيا مضادا للأكسدة وإمكانات حيوية محتملة بسبب المحتوى المرتفع للمركبات الفينولية. وكان الغرض الثالث من الدراسة هو تقييم التأثيرات السامة علي الخلايا من مستخلص الميثانول للفاكهة على أربعة خلايا سرطانية بشرية (A375, MCF-7, A549, and HT-29). تم قياس IC_{50} من العينات باستخدام مقايسة استبعاد التريبان الأزرق (TBEA). أظهر مستخلص الميثانول من جزء اللب أقل تثبيط 0.91±15.40 ميكروغرام /مل على خلايا A375 .في الهدف الرابع من الدراسة، تم التحقيق في الآلية الجزيئية لمستخلص الميثانول المستمدة من موت الخلايا المبرمج ودورة انحسار الخلية في الخلايا السرطانية البشرية في نهج يعتمد على الوقت باستخدام التدفق الخلوي. تم صبغ الخلايا المعالجة مع نيكسين للكشف عن موت الخلايا المبرمج في وقت مبكر ومؤخر ومع PI للكشف عن انحسار دورة الخلية المرتبطة بتجزئة الحمض النووي، وانحسار الخلايا المختلفة في مراحلG1/S ، S وG2/M. وأخيرا تم تحليل الجينات للتعبير عن طريق النسخ العكسي الكمي بطريقة PCR (qPCR) من خلال تحليل التعبير عن الجينات لتقدير مستويات mRNA. تم الكشف عن النتائج للخلايا التي عولجت مع IC_{50} عن طريق زيادة التنظيم الايجابي من الجينات المضادة لموت الخلايا المبرمج وانخفاض التنظيم الايجابي من تعبيرات الجينات المانعة لموت الخلايا المبرمج مثل *BCL-2* تم تنشيط ال *CASPASE-3* في الخلايا المعالجة عند كل من المسارات الداخلية والخارجية لموت الخلايا. وتشير هذه النتائج إلى أن مستخلصات الميثانول المكون من ثلاثة أجزاء من فاكهة *A. Altilis* لها نشاط العلاج الكيميائي المحتمل ضد الخلايا السرطانية البشرية وخصوصا جزء اللب من الفاكهة.

APPROVAL PAGE

The thesis of Tara K. Jalal has been approved by the following:

_____
Ridhwan Abdul Wahab
Supervisor

_____
Muhammad Nor Omar
Co-Supervisor

_____
Norlelawati A. Talib
Internal Examiner

_____
Muhhammad Mahdi Abdul Jamil
External Examiner

_____
Tengku Sifzizul Tengku Muhammad
External Examiner

_____
Zarina Zainuddin
Chairperson

DECLARATION

I hereby declare that this dissertation 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.

Tara K. Jalal

Signature ..... Date .....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

CYTOTOXIC EFFECTS AND MECHANISMS OF CELL DEATH OF *Artocarpus altilis* ON HUMAN BREAST, COLON, LUNG AND SKIN CANCER CELLS

I declare that the copyright holders of this dissertation are jointly owned by the student and IIUM.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.

2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.

3. The IIUM library will have the right to make, store in a retrieved system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Tara K. Jalal

……..…….. ……..
Signature Date

ACKNOWLEDGEMENTS

All praise to ALLAH (S.W.T), a big chapter of my life is to be turned. However i cannot start a new one without acknowledging those who helped me through this one. I also ask His Peace and Blessings upon Prophet Muhammad (PBUH), who is forever a torch of guidance and knowledge for humanity as a whole.

I would like to express my gratitude to my supervisor Asst.Prof. Dr. Ridhwan bin Abdul Wahab for his continuous guidance and support throughout my PhD study. It is an honour for me to have been his student. I certainly learnt a lot from him. I would like to thank him for his patience and humble character. I cannot thank you enough for everything you did for me, but I pray that Allah will reward you and may HE bless you and your family.

I am indebted to my co-supervisors Prof. Dr. Muhammad Nor bin Omer for his continued support and encouragement throughout my postgraduate career including my MSc and PhD My special thanks and appreciation also Asst. Prof. Dr. Mohammad Syaiful Bahari Abdul Rasad for steering my efforts in the right direction to complete this study.

I also extend my heartfelt gratitude to my beloved late parents (my loving father Kamal Jalal and my inspiring gorgeous mother Ronak Baker Sami). I am a lucky person and blessed from Allah to have wonderful siblings for their support and patience and also for always being there when I am upset and feel down. I really thank you all and love you all so much (Dr. Mustafa, Namo, Media, Sara, Sali, Muhammad and Huda).

I am deeply indebted to my husband. I do not know where I would be without the support and love of my husband, Ahmed. I have never in my life met a man as brilliant, loving, kind, tolerant, patient and exciting as he is. He has been there for me through years of self-doubt, and sometimes self-loathing - and for that, I am eternally grateful. Please do not ever doubt my love for you. I would also like to acknowledge my deepest affection to my children, Mariam my lovely advisor girl, Zainab my fragile girl and Zahra my adorable and caring girl. No one will ever take your place in my life, no one could ever be more important than you. My darling, my children, I love you all.

It is also a pleasure for me to thank Br. Ahmad Muzzamil, science officer (Kulliyah of Scince Biotechnology Department) for his valuable technical support. Thank you for being there when needed.

Much of my spiritual hardships were eased by my dear friends Lailuma Momand, Mariam Abimbola, Alaina, Vegnish, Hassan and everyone else, and for that I am really grateful. Thank you from the bottom of my heart and May ALLAH ease your study; wish you all the success and happiness insha' Allah. All praise to Allah for everything HE has given me.

This project is dedicated to Allah and then His beloved Prophet and Messenger Muhammad (S.A.W.), and to my beloved late mother Ronak Baker Sami and father Kamal Jalal Ahmed

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AOAC	Association of Official Agricultural Chemists
ATCC	American type culture collection
AIF	Apoptosis inducing factor
APAF-1	Aapoptosis protease-activating factor-1
BLAST	Basic local alignment search tool
CASPASE	Cysteine aspartic acid protease
*CDK*s	Cyclin-dependent kinases
CDNA	Complementary DNA
CIP/KIP	CDK Interacting protein/kinase inhibitor protein
DCM	Dichloromethane
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESI	Electrospray ionization
FADD	Fas-associated death domain protein
IC_{50}	Inhibition concentration (reduces the effect by 50%)
IAP	Iinhibitor of apoptosis
FDA	Food and drug administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine-cytosine (DNA base pairing)
KIP	Kinase inhibitory proteins

LCMS/MS	Liquid chromatography-tandem mass spectrometry
MDR	Multi-drug resistance
mRNA	messenger RNAs
MtPTP	Mitochondrial permeability transition pores
NCBI	National center for biotechnology
OFR	Oxygen-free radicals
OMM	Outer mitochondrial membrane
QPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Real-Time
SPF	S-phase promoting factor
SMAC	Second mitochondria-derived activator of caspase
PI	Propidium Iodide
TFC	Total Flavonoids Content
TPC	Total Phenol Content
VS.	Versus
UHPLC	Ultra-high-performance liquid chromatography
WHO	World health organization
7AAD	7-Aminoactinomycin D
PU	Pulp part
PE	Peel part
WF	Whole fruit

LIST OF SYMBOLS

α	Alpha
β	Beta
ΔΔ	Delta-delta
Cq	Quantification cycle
g	Gram
G	Gap
M	Mitosis
S	Synthesis
mg GAE/g	Milligrammes of gallic acid equivalent per gram of dry weight
mg QE/g	Milligrammes of quercetin equivalent per gram of dry weight
µl	Microliter
°C	Degree Celsius
%	Percent
-	To
>	More than
<	Less than
±	Plus-minus
x	Times
=	Equal to

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cancer is defined as the uncontrolled or unregulated growth of cells. It is currently the leading cause of death worldwide. It has been reported to be the second highest cause of death in developing and developed countries (Siegel et al, 2016). According to the National Cancer Registry 2017 report, over 21,000 new cancer cases were detected in Peninsular Malaysia with age-standardized incidence rate (ASR) equal to 131.3 per 100,000 of the population. Breast, colorectal, lung and cervix cancers were found to be the most common types reported in the hospitals in Peninsular Malaysia (Omar et al., 2006). Torre et al., (2015) reported that there were 32.6 million new cancer cases and 8.2 million deaths due to cancer condition globally. In Asia alone, which represents 60% of the world total population, the incidence of cancer cases is projected to increase from 6.1 million in 2008 to 10.6 million in 2030 (Sankaranarayanan et al., 2014). Surgery, radiation, and chemotherapy are among the modalities used in cancer treatment, whose goal is to either cure the disease or prolong and improve the patient's quality of life. Though chemotherapy has led to improvement in this part, drug resistance and toxicities remains a significant challenge (Gottesman, 2002). Thus, there is a crucial need to identify safer but equally effective agents to be used in cancer treatments, which can be found in natural agents.

Tamimi et al., (2002) stated that there are numerous chances to intervene in this process, which could reveal more significant methods reduce the occurrence of cancer. On the other hand, antioxidants play an important role in the protection of the

human body against damage by reactive oxygen species (Govindarajan, 2005). Previous studies have shown that intake of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases related to cellular oxidative damage probably over 100 associated diseases in total (Valko et al., 2007; Fisher-Wellman et al., 2009). Therefore, the use of natural products for pharmaceutical purposes has gradually increased. Hence, such natural products from plants should investigate to understand their properties, safety, and efficacy. This will result in the minimal use of most common cancer treatments such as radiation therapy, surgery and chemotherapy. Each of these treatment methods has significant limitations, but chemotherapy by cytotoxic drugs offers the only approach to treating the metastasized cases (Lyons, 2007). However, the development of cancer cell resistance to chemotherapy has demonstrated the growing needs for discovering new cytotoxic drugs that act by distinctive mechanisms.

Some anticancer agents derived from plants are (vincristine, vinblastine, etoposide, paclitaxel, camptothecin, topotecan and irinotecan) from marine organisms several compounds identified are (cytarabine, alpine and dolastatin 10) and from microorganisms are (dactinomycin, bleomycin, and doxorubicin). There are numerous agents identified from fruits and vegetables used in anticancer therapy. These agents are curcumin (turmeric resveratrol, red grapes, peanuts and berries), genistein (soybean), diallyl sulfide (allium), S-allyl cysteine (allium), allicin (garlic), lycopene (tomato), capsaicin (red chilli), diosgenin (fenugreek), 6-gingerol (ginger), ellagic acid (pomegranate), ursolic acid (apple, pears, prunes), silymarin (milk thistle), anethole (anise, camphor, and fennel), catechins (green tea), eugenol (cloves), indole-3-carbinol (cruciferous vegetables), limonene (citrus fruits), beta carotene (carrots) and

dietary fiber. These natural compounds are great opportunity to evaluate not only very new chemical classes of anticancer agents, but also novel leads compound and potentially relevant mechanisms of action (Abhishek et al., 2011). *Artocarpus artilis* belongs to the family Moraceae found widely throughout tropical and temperate regions (Southeast Asia) and has the potential to be a source of bioactive compounds.

1.2 STUDY JUSTIFICATION

The global market for traditional medicines was estimated at US$ 83 billion annually in 2008. With an exponential rate of increment, more than 80% of the world's populations depend on traditional medicine for their main health care needs (WHO, 2011). Cragg et al., 2005 reported that more than 60% of currently used anti-cancer agents come from natural products. Medicinal plants that are commonly used with the antioxidant activity known worldwide belong to plants from several families, especially Lamiaceae (rosemary, sage, oregano, marjoram, basil, thyme, mints, balm), Apiaceae (cumin, fennel, caraway), and Zingiberaceae (turmeric, ginger). In addition, composition and concentration of antioxidants that are present, such as phenolic compounds are related to antioxidant effect (Masuda et al., 2015). Natural products remain an important source of new drugs. Natural products played an important role in contributing to the drugs resources. Aproximately 60 cancer chemotherapeutic drugs are in the market. For instance, in the United States, there are now four structural classes of plant anticancer agents available. These are constituted by the catharanthus (vinca) alkaloids (vinblastine, vincristine, vinorelbine), the epipodophyllotoxins (etoposide, etoposide phosphate, teniposide), the taxanes (paclitaxel and docetaxel), and the camptothecin derivatives (irinotecan and topotecan) (Cragg et al., 1997). Natural products are an attractive source of new therapeutic compounds as