ANTICANCER ACTIVITY OF IONIC LIQUID GRAVIOLA FRUIT (*Annona muricata)* EXTRACT ON MCF-7 AND HT29 CANCER CELL LINES USING *IN VITRO* AND *IN VIVO* METHODS

BY

DADDIOUAISSA DJABIR

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy (Engineering)

Kulliyyah of Engineering
International Islamic University Malaysia

APRIL 2020

ABSTRACT

Cancer, one of the major public health problems, is the leading cause of death worldwide. The available protocols of treatment include surgical intervention, radiation, and chemotherapy which cause numerous side effects on cancer patients. Many phytochemicals have anticancer properties comparable to conventional drugs. The major benefit of these compounds is the non-toxicity nature to the normal tissues. *Annona muricate,* commonly known as Graviola, is a member of the Annonaceae family. It has been used for ages in traditional medicine due to its biological activities including antioxidant, anti-inflammatory, antimicrobial and cytotoxicity to tumour cells. This research investigated the antiproliferative effect of the ionic liquid-Graviola fruit extract (IL-GFE) on *in vitro* breast MCF-7 and colon HT29 adenocarcinoma cell lines and their cytokinetic behaviour. It also identified the mechanism of IL-GFE inhibition by applying a flow cytometry technique and metabolomics study and assessed its toxicity on *in vivo* zebrafish developing embryos. The process of ionic liquid-microwave assisted extraction (IL-MAE) method was optimised by Response Surface Methodology for three parameters, namely time, irradiation power and solid-liquid ratio. The optimum extraction conditions gave a yield of Graviola fruit extract up to 66.6 % and an average IC_{50} of 4.75 µg/mL for MCF-7 and 10.56 µg/mL for HT29, while it was safe toward normal VERO cell lines. The crude IL-GFE was fractionated using the combination of thin-layer chromatography and column chromatography. Six fractions were semi-purified and subjected to phytochemical screening and antiproliferative assay in which, it revealed the presence of many phytoconstituents such as acetogenins, alkaloids, phenols, flavonoids, tannins and terpenoids. Moreover, fraction B exhibited the lowest IC_{50} toward MCF-7 and HT29 cells at 12.6 and 13.56 µg/mL, respectively. However, the crude IL-GFE had a better IC_{50} value. The crude IL-GFE with GC-TOFMS analysis revealed the presence of many phytochemicals with anticancer activity such as D-psicofuranose, pentakis ether, propyldecyl cyclopropane carboxylate, tri-ruthenium dodecacarbonyl, N-acetylimino dimethylsulfurane, pyranone, carbohydrazide and benzoic acid. The cytokinetic study showed that crude IL-GFE and Taxol inhibited the growth of MCF-7 and HT29 cells and proved their antiproliferative effect when they reduced the number of cell generations of MCF-7 from 3.71 to 1.67 and 2.18, respectively, and reduced the cell generations of HT29 cells from 3.93 to 2.96 and 2.01, respectively. Furthermore, the acute toxicity of IL-GFE was assessed on *in vivo* zebrafish model in which crude IL-GFE reduced the survival of zebrafish larvae at a relatively high dose of 250 µg/mL after 96 hpf treatment, while no significant changes on morphology of the treated zebrafish were recorded. The result of the flow cytometry also indicated that the crude IL-GFE arrested the cell cycle of MCF-7 and HT29 at G0/G1 phase and increased the apoptotic and necrotic cells in a time-dependent manner compared with the control group. Finally, the metabolomics analysis of the treated MCF-7 and HT29 cells with crude IL-GFE treatment showed an alteration of many metabolic pathways in treated cancer cells. In conclusion, crude IL-GFE can be one of the promising anticancer agents due to its selective antiproliferation against breast and colon cancer cells and its safety for the healthy cells.

خلاصة البحث

يعد مرض السرطان من بين المشكلات الأساسية للصحة العامة وهو السبب الرئيسي للوفاة في العالم. تشمل البروتوكولات العلاجية المتاحة التدخل الجراحي والإشعاعي والعلاج الكيميائي الذي يسبب آثارًا جانبية عديدة لدى مرضى السرطان. العديد من المواد الكيميائية النباتية لها خصائص مضادة للسرطان منافسة للأدوية التقليدية. الفائدة الرئيسية لهذه المركبات تكمن في طبيعتها غير السمية للأنسجة السليمة. *Annona muricata* المعروفة باسم الجرافيولا، نبتة من عائلة الأنوناسي، تم استخدامها منذ القديم في الطب التقليدي بسبب أنشطتها البيولوجية بما في ذلك النشاط المضاد للأكسدة والالتهابات والميكروبات، إضافة الى السمية الخلوية للخلايا السرطانية. يهدف هذا البحث إلى دراسة التأثير المضاد للسرطان لمستخلص السائل الأيوني لفاكهة الجرافيولا على الخلايا السرطانية للثدي MCF-7 والخلايا السرطانية للقولون HT29 وسلوكهما الخلوي، إضافة الى تحديد آلية عمل مستخلص فاكهة الجرافيولا من خلال تطبيق تقنية التدفق الخلوي ودراسة الأيض وتقييم سميته في أجنة سمكة الزرد. أولا، تم تحسين طريقة الاستخلاص بالسائل الأيوني بمساعدة الميكروويف من خلال منهجية الاستجابة السطحية لثلاثة عوامل: الوقت (دقيقة)، قدرة التشعيع (واط) ونسبة المادة الصلبة بالنسبة للسائل (غرام/مل). أعطت ظروف الاستخلاص المثلى مستخلص الفاكهة بنسبة تصل إلى 66.6٪ ومتوسط تثبيط قدره 4.75 ميكروغرام/مل بالنسبة لخلايا سرطان الثدي و10.56 ميكروغرام/مل بالنسبة لخلايا سرطان القولون، في حين كانت آمنة تجاه الخلايا VERO السليمة. بعد ذلك، تم تنقية مستخلص فاكهة الجرافيولا الخام باستخدام كل من تقنية كروماتوجرافيا الطبقة الرقيقة وكروماتوجرافيا العمود. تم اصطفاء ستة أجزاء نشطة وتعريضها للفحص الكيميائي النباتي والنشاط المضاد للسرطان، حيث كشفت الدراسة عن وجود العديد من المركبات النباتية مثل الأسيتوجينات، القلويدات، الفينولات والفلافونويدات والتانينس والتيربينويدات. علاوة على ذلك، أظهر الجزء النشط باء أدنى IC_{50} بالنسبة لخلايا سرطان الثدي والقولون عند 12.6 و13.56 ميكروغرام/مل، على التوالي، مقارنة بالأجزاء النشطة الأخرى. لكن يبقى مستخلص فاكهة الجرافيولا الخام هو الأكثر تثبيطا لخلايا سرطان الثدي والقولون مقارنة بالمركبات الفعالة الأخرى. بالإضافة الى ذلك، أظهر تحليل مستخلص فاكهة الجرافيولا الخام باستخدام الـ GC-TOFMS وجود العديد من المواد الكيميائية النباتية ذات النشاط المضاد للسرطان. أظهرت دراسة الحركية الخلوية أن مستخلص فاكهة الجرافيولا الخام والتاكسول حالت دون نمو الخلايا السرطانية للثدي والقولون وأثبتت تأثيرها المضاد للسرطان عندما خفضت عدد الأجيال لخلايا سرطان الثدي من 3.71 إلى 1.67 و2.18 على التوالي. بينما، قللت عدد أجيال الخلايا السرطانية للقولون من 3.93 إلى 2.96 و2.01، على التوالي. بعد ذلك، تم تقييم سمية مستخلص فاكهة الجرافيولا الخام على أجنة سمكة الزرد النامية، حيث تسبب المستخلص في موت يرقات سمكة الزرد بعد معالجتها بجرعة عالية نسبيًا قدرها 250 ميكروغرام/مل، بعد 96 ساعة من العلاج، في حين لم تحدث تغييرات كبيرة على شكل يرقات الزرد المعالجة. أشارت نتيجة قياس التدفق الخلوي إلى أن مستخلص فاكهة الجرافيولا الخام أوقف دورة الخلية للخلايا السرطانية الثديية والقولونية في مرحلة G0/G1 وزاد من الخلايا الميتة بسبب الموت الخلوي المبرمج والنخرية. أخيرًا، فيما يخص تحليل اللأيض للخلايا، فقد أظهر تغييرا واضحا في أيض الخلايا السرطانية المعالجة بالمستخلص. في الختام، يمكن أن يكون مستخلص فاكهة الجرافيولا الخام واحدًا من العوامل الواعدة المضادة للسرطان، نظرًا لنشاطه الانتقائي ضد الخلايا السرطانية للثدي والقولون وعدم تأثيره على الخلايا السليمة.

APPROVAL PAGE

The thesis of Daddiouaissa Djabir has been approved by the following:

Azura Amid
Supervisor

Nassereldeen Ahmed Kabbashi
Co-Supervisor

Fazia Adyani Ahmad Fuad
Co-Supervisor

Hamzah Mohd Salleh
Internal Examiner

Noriham Abdullah
External Examiner

Fadzilah Adibah Abdul Majid
External Examiner

Mohammad Naqib Ishan Jan
Chairman

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.

Daddiouaissa Djabir

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

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

ANTICANCER ACTIVITY OF IONIC LIQUID GRAVIOLA FRUIT (*Annona muricata*) EXTRACT ON CANCER CELL LINES USING *IN VITRO* AND *IN VIVO* METHODS

I declare that the copyright holders of this thesis 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 Daddiouaissa Djabir

……..…….. ……..

Signature Date

ACKNOWLEDGEMENTS

In the Name of Allah, the Most Gracious, Most Merciful and Salam upon His Messenger, Prophet Mohammed (SAW).

All praise and thanks are due to my creator and sustainer, for guiding me to conceptualise, develop and accomplish this research work.

My sincere appreciation goes to my dear parents, who granted me the gift of their unwavering belief in my ability to accomplish this goal and my wife for her support and patience.

I would like to express the immense appreciation to my supervisors- Prof. Dr. Azura Amid, Prof. Dr. Nassereldeen Ahmed Kabbashi and Asst. Prof. Dr. Fazia Adyani Ahmad Fuad for their guidance, advice, patience, encouragement and valuable assistance that enabled me to accomplish this work smoothly and efficiently.

I wish to extend my warmest thanks to Dr. Shirwan for his time and guidance and all lab mates, Br. Oualid, Br. Ahmed, Br. Phirdaous, Br. Deni and my friends Hassen and Slimane as well as to Mr. Said Ammisaid for his proof reading and all those who have helped me go through this work.

Finally, I acknowledge the valuable inputs of the Department of Biotechnology Engineering and all lecturers and technical staff who helped me in accomplishing this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACS	American Cancer Society
ACGs	Acetogenins
AHC	Agglomeration Hierarchical Clustering
ANOVA	Analysis of Variance
Annexin-V-FITC	Apoptosis Test FITC kit
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
A-549	Human Lung Carcinoma Cells
BC	Breast Cancer
BCAAs	Branched-Chain Amino Acids
Bcl2	B Cell Lymphoma 2
BSC	Biological Safety Cabinet
BSTFA	N,O bis(trimethylsilyl)-trifluoroacetamide
[C4MIM]BF4	1-butyl-3-methylimidazolium tetrafluoroborate
[C4MIM]Cl	1-butyl-3-methylimidazolium chloride
[C4MIM]PF6	1-butyl-3-methylimidazolium hexafluorophosphate
CC	Column chromatography
CV	Coefficient of Variation
DB 3	Durian Belanda 3
DCM	Dichloromethane
DF	Dilution Factor
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOE	Design of Experiments
et al.	et alia – and others
etc.	and other types
EtOH	Ethanol
EU	European Union
FBS	Fetal Bovine Serum
FCCCD	Face-Centered Central Composite Design
FDA	Food and Drug Administration
GC-TOFMS	Gas Chromatography- Time-of-Flight Mass Spectrometry
HepG2	Human Hepatoma Cell Line
HCT-116	Human Colon Cancer Cell Line
HL-60	Human Leukemia Cell Line
HPF	Hours Post Fertilisation
HRE	Heat Reflux Extraction
Hrs	Hours
HT29	Human Colon Adenocarcinoma Cell Line
IACUC	Institutional Animal Care and Use Committee
IC_{50}	Half Maximal Inhibitory Concentration
IL-GFE	Ionic Liquid-Graviola Fruit Extract
IL-MAE	Ionic Liquid-based Microwave-Assisted Extraction
ISO	International Organization for Standardization

KAED	Kulliyyah of Architecture and Environmental Design
KMO	Kaiser-Meyer-Olkin
LC_{50}	50 % Lethality Concentration
LDHA	Lactic Acid Dehydrogenase-A
LLE	Liquid-Liquid Extraction
LOF	Lack of Fit
MCF-7	Human Breast Adenocarcinoma Cell Line
MCF-10A	Nontumorigenic Human Breast Epithelial
MeOH	Methanol
MMP	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
MTT	4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NADH	Nicotinamide Adenine Dinucleotide
NCI	National Cancer Institute
NEAA	Nonessential Amino Acids
NHEKN	Neonatal Normal Human Epithelial Keratinocytes
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
NMSC	Non-Melanoma Skin Cancer
OD	Optical Density, unit of Absorbance
OFAT	One-Factor-At-a-Time
PACA-2	Human Pancreatic Cancer Lines
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PC-3	Human Prostate Cancer
PI	Polarity Index
PS	Phosphatidylserine
p53	Tumor Protein 53
Rf	Retardation Factor
rpm	Rotation Per Minute
RNase A/PI	Ribonuclease A / Propidium Iodide
RSM	Response Surface Methodology
R.Time	Retention Time
SD	Standard Deviation
SE	Standard Error
SPE	Solid-Phase Extraction
TCA	Tricarboxylic Acid Cycle
TLC	Thin Layer Chromatography
TNM	Tumor Node Metastasis
UAE	Ultrasound Assisted Extraction
VERO	Normal Kidney Cell – African Green Monkey
WST	1;2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
±	Plus-Minus
<	Less than
g	Gram
G0	Gap 0 Phase
G1	Gap 1 Phase
G2/M	Gap 2 / Mitotic Phase
S	Synthesis Phase
mg/L	Milligram per Milliliter
mol/L	Mole per Liter
R^2	Coefficient of Determination
td	Doubling Time
µg/mL	Microgram per Millilitre
µ	Specific Growth Rate
X	Number of Generations

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Natural plant extracts have been used for a considerable length of time by many cultures and civilisations for the treatment of various health problems. Over 80% of the worldwide population nowadays relies on natural plant extracts through conventional therapies. Many studies have focused on the prevention, progression, and treatment of cancer using natural products. However, there is still room for improvement. Currently, the use of synthetic chemotherapeutic drugs fails to be considered as effective therapeutic agents, and this is because of the greater part of severe toxic side effects to the normal cells. According to Ioannis et al. (2015), an alternative therapeutic approach that uses natural products showed more advantages with fewer side effects.

Numerous herbs and species are used in the world such as ginger, onion, garlic, cardamon, coriander, and turmeric to treat many diseases. Among them, Annona muricata shows enormous medicinal properties. A. muricata Linn is a lowland fruit tree under the Annonaceae family. A. muricata is likewise commonly known as Graviola or Soursop or Guanabana. It is called soursop because of the sour and sweet flavour of its extensive fruit (Patel & Patel, 2016). The Graviola is native to the tropical zones, South America and Africa but is currently widely cultivated in the tropical areas around the world, including Southeast Asia and Southern Florida, from the ocean level to the altitudes of around 1150 meters. Graviola is a thin, small, and cold-intolerant tree, which achieves heights of 4 to 6 meters. Its edible fruits are large, heart-shaped, dark green in

colour, with a diameter which varies between 5-20 cm and an average weight of 0.4 to 1.0 kg (Figure 1.1) (Daddiouaissa & Amid, 2018).

Figure 1.1 Graviola fruit (*Annona muricata* L)

Phytochemical analysis of the plant reveals the presence of alkaloids, phenols, terpenoids, flavonoids and it has an enormous potential anticancerous compound coined as acetogenins which assume to play a vital role towards numerous types of cancer. Acetogenins are potent inhibitors of NADH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) of the membrane mitochondrial of cancer cells. The fruit is of economic value and consequently cultivated and used broadly as expendable food (Patel & Patel, 2016).

Cancer is a group of diseases that are still considered as one of the leading causes of morbidity and mortality in the world. This disease can be defined as an abnormal growth of cells with the potential to spread or invade to the other tissues of the body. It is caused by mutations in gene expression leading to disequilibrium of cell proliferation and cell death (Ruddon, 2007).

Breast adenocarcinoma is one of the most cancer incidents among women, with an estimated 268,600 new cases, and 41,760 breast cancer (BC) deaths estimated to occur in the United States' women in 2019 (Siegel, Miller, & Jemal, 2019). Therefore,

BC is a challenge among research communities around the world; this is because of the BC incidence rate keep increasing by 0.4 % annually worldwide according to the final report conducted by Jemal and co-workers (2017). On another hand, 101,420 new cases of colon adenocarcinoma were estimated to occur for both sexes and 51,020 estimated deaths in the United States in 2019. Current protocols of treatment include radiation therapy, surgical intervention, and chemotherapy which induce numerous side effects including nausea, fatigue, vomiting, weak of the immune system and hair loss (Griffin et al., 1996). Thus, the search for alternative treatment is necessary.

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF THE STUDY

Cancer is among the most common causes of mortality in the world, with an estimation of 18.1 million new cancer cases and 9.6 million cancer deaths to occur in 2018 worldwide, projected to rise by at least 70% by 2030 (Antoni et al., 2016; Bray et al., 2018). Among of command cancer treatments are surgery, chemotherapy and radiotherapy. However, more people are suffering every day from the effect of chemotherapy which causes various kinds of undesirable side effects such as hair loss, weakness of the immune system, loss of appetite, hormonal fluctuation, anxiety, depression and some even die not because of cancer but because not able to cope with the side effects. In addition to that, these drugs are costly and not affordable for everyone especially for the patients from low-income families. The development of new alternative anticancer drugs from plants remains one of the most challenging areas of research. Graviola has various pharmacological properties namely anti-cancerous properties, hepatoprotective, antioxidant, antidiabetic, insecticidal and pesticidal properties and anti-microbial activity resulted from its phytoconstituents. Most Graviola bio-compounds were extracted through solvent extraction methods which present many

disadvantages including a large amount of extractant waste, use of hazardous and flammable organic solvents, potential toxicity emissions during extractions. To our knowledge, there are no phytochemicals extracted by ionic liquid microwave-assisted extraction method (IL-MAE) from Graviola fruit. IL-MAE method may be a better choice in producing safe compounds which are solvent-free for human consumption.

Thus, this research aimed to investigate the therapeutic potential of the crude IL-GFE and its isolated active fractions as an anticancer agent. It is strongly believed that the discovery of anticancer properties from Graviola fruit could lead to the development of a new generation of anticancer drugs that possess both chemopreventive and chemotherapeutic properties which are safer and more influential without weakening the patients' health.

1.3 RESEARCH HYPOTHESIS

Natural products can be consumed as alternative medicine that may not have any side effects on individuals. Nowadays, much interest in maintaining health care and efforts are made to accomplish this through the extraction of plants' compounds and evolution in medicinal use. Today, traditional knowledge and practices have contributed to our modern medicine which is attested by more than 40% of commonly prescribed medications throughout the world. Most of them found their origins directly or indirectly in plants or animals (Ahmad & Ismail, 2003).

Different parts of Graviola have been reported and used traditionally to treat many types of diseases. Graviola fruits contain many useful phytochemicals therefore, phytochemicals from Graviola fruit may be the suitable candidate to be used as a chemopreventive medicine in treating cancer.