IN VITRO AND *IN SILICO* ANTICANCER ACTIVITIES OF *ANNONA MURICATA* LINN LEAVES EXTRACTS ON LUNG CANCER CELLS

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DEDICATION

A little gift.

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ABSTRACT

Lung cancer specifically non-small cell lung cancer (NSCLC) is one of the most devastating cancers. Despite having diverse treatment methods such as surgery, chemotherapy, radiation and targeted therapies, the overall 5-year survival rate for NSCLC is accounted for only 18.2 %. The high mortality rates for NSCLC are partially due to the lack of effective prognostic factors such as biomarkers. Nowadays, plant-derived bioactive substances have been making way as potential anticancer agents. One such plant is *Annona muricata* Linn, which is also known as soursop or graviola. This plant has been widely reported to contain valuable phytochemical substances that could be developed as chemopreventive agents. The antiproliferative and anticancer activities of this tropical plant have been demonstrated in *in vitro* cell culture studies as well as in *in vivo* studies of animals. It has been discovered that *A. muricata* L. extract exerts inhibition against various number of cancer cells, involving multiple mechanism of actions. Nonetheless, the mode of action and the molecular interactions of the plant have not yet been unveiled for most of these mechanisms. In the current study, response surface methodology was utilized to optimize the ultrasonic-assisted extraction (UAE) and maceration extraction (ME) of *A. muricata* L. leaves. Central composite design was applied to optimize the antioxidant activity of both extracts. It has been found that ME extract showed high antioxidant activity as compared to UAE extract with 83.3 % and 31.6 % respectively. However, through cytotoxicity study that was done by using MTT assay, UAE extract exhibited significant cytotoxicity effects in NSCLC cell line (HLFa) with IC_{50} of 139.6 μg/mL as compared to ME extract with IC_{30} of 108.4 μg/mL. The effect of the extracts on nitric oxide (NO) production in HLFa cells was also evaluated using Griess reagent system assay. Both extracts reduced the release of nitrite in the cell supernatant which indicated the reduction in NO production. Caspase 3/7 apoptosis assay was used to detect the presence of apoptotic machinery in HLFa cells after incubation. The mRNA expression of several genes namely *HMGB1*, *BCL2* and *BAX* were quantified. The mechanistic evalution of the results showed the possibility of involvement of these genes in *A. muricata* L. anticancer effects. At the end of this study, *in silico* molecular docking interaction of several phytoconstituents of *A. muricata* L. was analysed against Bcl-2 antiapoptotic proteins namely Bcl-2, Bcl-w and Mcl-1. The stability of complexes formed was evaluated using molecular dynamic simulation. Anonaine was also detected in UAE and ME extracts through HPLC screening process with 10.6 ppm and 10.7 ppm, respectively.

ABSTRAK

Kanser paru-paru terutamanya kanser paru-paru sel bukan kecil (NSCLC) merupakan salah satu kanser yang merbahaya. Walaupun terdapat kaedah-kaedah rawatan yang pelbagai seperti pembedahan, kemoterapi, terapi radiasi bersasar, namun kadar kemandirian keseluruhan dalam 5 tahun hanya mencecah sebanyak 18.2 %. Kadar kematian pesakit NSCLC yang tinggi sebahagiannya adalah disebabkan kekurangan cara yang effektif untuk mengenalpasti penyakit contohnya melalui penanda-penanda bio. Masa kini, bahan-bahan bioaktif bersumberkan tumbuh-tumbuhan berpotensi sebagai agen antikanser. Salah satu tumbuhan berpotensi tersebut ialah *Annona muricata* Linn ataupun juga dikenali sebagai durian belanda atau graviola. Tumbuhan ini telah dilaporkan dengan meluas mempunyai bahan fitokimia yang bernilai yang berkemungkinan dapat dibangunkan sebagai agen penghalang perkembangan kanser. Tumbuhan tropika ini telah menunjukkan aktiviti antiproliferatif dan antikanser melalui kajian kultur sel secara *in vitro* dan kajian terhadap binatang secara *in vivo*. Ekstrak *A. muricata* L. dilihat dapat memberi kesan perencatan terhadap pelbagai jenis sel kanser, yang meliputi pelbagai jenis mekanisma tindakbalas. Walaubagaimapun, cara tindakbalas dan interaksi molekul yang ditunjukkan oleh tumbuhan ini masih belum dapat dirungkai sepenuhnya. Dalam kajian ini, kaedah sambutan permukaan telah digunakan untuk mengoptimumkan proses pengekstrakan daun *A. muricata* L. menggunakan kaedah ultrasonik (UAE) dan kaedah penyusutan (ME). Rekabentuk komposit berpusat telah digunakan untuk mengoptimumkan aktiviti antioksida bagi kedua-dua ekstrak. ME telah menunjukkan aktiviti antioksida yang tinggi dengan berbanding UAE dengan masing-masing merekodkan bacaan 83.3 % dan 31.6 %. Namun begitu, melalui kajian tahap toksik terhadap sel yang telah dilakukan menggunakan teknik MTT, UAE menunjukkan kesan toksik yang lebih ketara terhadap sel NSCLC (HLFa) dengan IC_{50} 139.6 µg/mL berbanding dengan ekstrak ME dengan IC_{30} 108.4 µg/mL. Kesan kedua-dua ekstrak terhadap pengeluaran nitrik oksida (NO) dalam sel-sel HLFa juga telah dinilai menggunakan *Griess reagent system*. Ekstrak-ekstrak ini dilihat mengurangkan pelepasan nitrit di dalam media yang dapat disimpulkan sebagai pengurangan pengeluaran NO. Teknik apoptosis Caspase 3/7 telah digunakan untuk mengesan sel-sel HLFa yang mengalami apoptosis selepas inkubasi. Ekspresi gen mRNA seperti *HMGB1*, *BCL2* dan *BAX* telah dinilai. Penilaian keputusan ekspresi gen telah menunjukkan kemungkinan penglibatan gen-gen ini di dalam tindakbalas antikanser *A. muricata* L. Di akhir kajian ini, interaksi dok molekul in siliko antara beberapa komponen fitokimia dari *A. muricata* L. dengan protein-protein seperti Bcl-2, Bcl-w dan Mcl-1 telah dianalisa. Kompleks yang terhasil dari interaksi tersebut telah dinilai kestabilannya menggunakan proses pemilihan simulasi molekul dinamik. Kewujudan anonaine di dalam UAE dan ME telah disahkan melalui teknik HPLC masing-masing dengan kepekatan 10.6 ppm dan 10.7 ppm.

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LIST OF ABBREVIATIONS

ACGs	-	Annonaceous acetogenins
ADC	-	Adenocarcinoma
AIF	-	Apoptosis inducing factor
ANT	-	Adenine nucleotide transporter
APAF-1	-	Apoptotic inducing factor-1
C.V.	-	Coefficient of variation
CCD	-	Central composite design
CICD	-	Caspase-independent cell death
COPD	-	Chronic obstructive pulmonary disease
DAMP	-	Damage-associated molecular pattern
DISC	-	Death-inducing signal complex
DMEM	-	Dulbecco's Modified Essential Medium
DMSO	-	Dimethyl sulfoxide
DPPH	-	2, 2, diphenyl-2-picryl-hydrazyl
EndoG	-	Endonuclease G
eNOS	-	Endothelial NOS
FAD	-	Flavin adenine dinucleotide
FBS	-	Fetal bovine serum
FDA	-	Food and Drug Administration
FMN	-	Flavin mononucleotide
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
HMGB1	-	High Mobility Group Box 1 protein
HPLC	-	High Performance Liquid Chromatography
HTS	-	High-throughput screening
IAPs	-	Inhibitor of apoptosis protein
IFN-γ	-	Interferon-γ
IL-1α	-	Interleukin-1α
IL-1β	-	Interleukin-1β
IMS	-	Inter membrane space
iNOS	-	Inducible NOS

IUPAC	-	International Union of Pure and Applied Chemistry
LDH	-	Lactate dehydrogenase
LGA	-	Lamarckian Genetic Algorithm
LINCS	-	Linear Constraint
LPS	-	Lipopolysaccharide
MAE	-	Microwave-assisted extraction
ME	-	Maceration extraction
MD	-	Molecular Dynamics
MOMP	-	Mitochondrial outer membrane permeabilization
mPTP	-	Mitochondrial permeability transition pore
MTT	-	3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCR	-	National Cancer Registry
NMR	-	Nuclear magnetic resonance
nNOS	-	Neuronal NOS
NO	-	Nitric oxide
NOS	-	Nitric oxide synthase
NOS1	-	nNOS
NOS2	-	iNOS
NOS3	-	eNOS
NSCLC	-	Non-small cell lung cancer
OMM	-	Outer mitochondrial membrane
PAMP	-	Pathogen-associated molecular pattern
PARP	-	Poly (ADP-ribose) polymerase
PBC	-	Periodic boundary condition
PBS	-	Phosphate buffer saline
PDB	-	Protein Data Bank
PME	-	Particle Mesh Ewald
PS	-	Phosphatidylserine
RAGE	-	Receptor for advanced glycation end products
RAMPAGE	-	Ramachandran Plot Assessment
Rg	-	Radius of gyration
RMSD	-	Root mean square deviation
RMSF	-	Root mean square

ROS	-	Reactive oxygen species
RSM	-	Response Surface Methodology
RT-PCR	-	Real-time polymerase chain reaction
SASA	-	Solvent Accessible Surface Area
SAVES	-	Structural Analysis and Verification Server
SCC	-	Squamous cell carcinoma
SCLC	-	Small cell lung cancer
SEM	-	Standard error means
SMI	-	Small molecule inhibitor
SPC	-	Single Point Charge
SPE	-	Superficial fluid extraction
TNF-α	-	Tumor necrosis factor-α
UAE	-	Ultrasonic-assisted extraction
VEGF	-	Vascular endothelial growth factor
WHO	-	World Health Organization

LIST OF SYMBOLS

%	-	percent
°C	-	degree celcius
g	-	gram
g	-	relative centrifugal force
g/mol	-	gram per mol
h	-	hour
L	-	liter
mg/mL	-	milligram per mililiter
min	-	minute
mL	-	mili liter
mM	-	mili molar
nM	-	nano molar
rpm	-	revolution per minute
µg/mL	-	microgram per mililiter
µL	-	micro liter

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Lung cancer is a major healthcare problem in Malaysia and across the globe. This disease is one of the most prominent cause of cancer-related mortality; about 40% cases was reported in developing countries like Malaysia [1]. In 2012, approximately 1.8 million cases were recorded with more than 80% of the proportion succumb to this fatal disorder. In Malaysia, lung cancer is the leading cause of cancer-death among males population and the fifth cause among females with about 13.8% and 3.8% cases respectively [2,3]. Lung cancer is among the cancers that constitute more than 58% of the projected global cancer burden [4]. In 2030, the leading cancer locations include lung, prostate and melanoma for men, and breast, thyroid and uterine for women [5]. There are three major types of lung cancers: non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC) and lung carcinoid tumor. Despite numerous efforts – ranging from cancer immunotherapy to natural-derived anticancer agents – have been taken in the recent years in pursuit to puzzle out the way to combat this incurable malady, the 'war' against cancers is getting more perplexing and gripping. The hard-truth reality is that some part of cancer mechanism – up to the present time – is obscure; no individual in scientific community could authoritatively explain the 'actual' causes of cancers and how to prevent it let alone to cure it. However, in the recent years, natural-derived products such as plant-based products have gained wide interest. Reflection of this circumstances can be seen from the report of World Health Organization (WHO) which stated that more than 80% of the global population relies on plant-based traditional medicine for their primary healthcare; this has raised up the possibility of plant-derived substances to be the potential remedy everyone is looking for [6,7]. For the record, there are more than 3000 plant species being applied in the treatments of cancer-like diseases including swelling, abscesses, calluses, warts and polyps prompting the birth of about 60% of the currently available anticancer drugs

[8,9]. The application of plant-derived substances as anticancer drugs such Taxol is beneficial to consumers as these substances exert fewer adverse effects, and they are cost-effective alternative [10,11].

As one of the most arguably potential source of anticancer agent, *Annona muricata* Linn, has been the subject of extensive researches for a number of years. Groups of phytochemical compounds including flavonoids, alkaloids and annonaceous acetogenins (ACGs) and several types of extracts from *A. muricata* L. have been the candidates for anticancer drugs – widely manipulated to determine their characteristics and mode of actions. They have been reported to eliminate cancer cells and disrupt cancer cells development through numerous numbers of mechanisms in cell culture studies as well as in animal studies. For instance, *A. muricata* L. leaves ethyl acetate extract inhibited proliferation of lung cancer A549 cells via mitochondrial-mediated pathway with the involvement of NF-κB, a cell signaling regulator [12]. Likewise, commercialized leaves water extract of *A. muricata* L. minimized pancreatic metastasis and tumorigenicity of implanted pancreatic tumors [13]. A synergistic study of major bioactive phytochemicals (flavonoids and ACGs) from *A. muricata* L. leaves showed that these substances inhibited proliferation activity of prostate cancer cells and the cells' clonogenic capacity [14]. On the other hand, (-)-anonaine, an alkaloid, demonstrated dose-dependent antiproliferation, DNA damage and antimigration in human lung carcinoma (H1299) cells [15]. Furthermore, it also stimulated cell cycle arrest in the similar cell line [15]. These indicate that more studies involving lung cancer such as NSCLC are welcomed, as there are room for manipulation and improvement.

NSCLC is the most common type of lung cancers accounting for about 80-85%, including adenocarcinoma (ADC) and squamous cell carcinoma (SCC) [16]. NSCLC is a type of aggressive cancer in which cancer cells form in the lung tissues. Risk factors for NSCLC include smoking, secondary smoking, radiation, environmental factors, genetic (family history) and HIV. Despite diverse treatment methods such as surgery, chemotherapy, radiation and targeted therapies, the overall 5-year survival rate for NSCLC is accounted for only 18.2% [17]. The high mortality rates for NSCLC are partially due to the lack of effective prognostic factors such as

biomarkers. NSCLC is associated with several symptoms including shortness of breath, chest pain, lost of appetite and tiredness. To date, there is no cure or preventive treatment for NSCLC; however, several factors – including the stage of cancer, type of mutations in cancer and patients' health – may affect the chance of recovery and treatment. A meta-analysis study involving 2651 NSCLC patients has detected the higher High Mobility Group Box 1 (HMGB1) expression in NSCLC cells than that in the healthy cells [16]. Thus, identifying the expression and behavior of novel prognostic factors like HMGB1, as biomarkers, may be a clinically useful tool for early detection of NSCLC and for potential cancer therapeutic target.

HMGB1, an extracellular damage-associated molecular pattern (DAMP), is a vital regulator of cell death and cell survival. It possesses several important functions in many diseases especially inflammatory diseases and cancers [18–20]. Its overexpression is linked to the hallmarks of cancer including angiogenesis capacity, apoptosis evasion, insensitivity to growth inhibitors, tissue invasion and metastasis, inflammation, self-sufficiency in growth signals and unlimited replicative potential [21]. The expression of vascular endothelial growth factor (VEGF), one of the main angiogenic factors, in ovarian carcinoma (SKOV3) cells was reduced with the interference of HMGB1 [22]. During cell death mechanisms, including apoptosis and necrosis, HMGB1 release was observed in pancreatic cancer (Panc-1) and cervical cancer (HeLa) cell lines [23]. Overexpression of HMGB1 in colorectal cancers had contributed significantly in tumor progression and tumors' ability to metastasize [24]. While in NSCLC, HMGB1 enhanced the increase in cancer cells migration ability through the activation of TLR4/NF-κB signaling – inducing metastasis [25]. It was revealed that the protein level of HMGB1 in patients with NSCLC of TNM Stages III-IV was significantly higher as compared to TNM Stages I-II, indicating that HMGB1 plays a crucial role in the progression of NSCLC [26]. These also demonstrated that HMGB1 expression could be regarded as important prognostic biomarker in cancers development specifically for NSCLC diagnosis, thus making it particularly interesting as potential therapeutic targets for cancer drug discovery.

Many studies have examined the relationship HMGB1 expression and NSCLC. Even though most of the results remain unsettled, some study has shown significance

correlation [26]. Hence, the present study focuses to investigate the mechanism involved in the effect of *A. muricata* L. leaves extract on the expression level of HMGB1 and several other biomarkers involved in regulating apoptosis in NSCLC. This study would provide additional information to the present anticancer knowledge and future research.

1.2 Problem Statement

A. muricata L. has long been postulated to possess anticancer properties against various types of cancer. The plant bioactive constituents such as ACGs have been identified to be vital contributors to anticancer effects, leading to its characterization and isolation. Even though the researches involving ACGs have been carried out for few decades, these compounds have never been commercialized or developed as anticancer drug for clinical test. These circumstances prompt a wave of questions debating the idea with hope that the premise relating to the anticancer effect of *A. muricata* L. is not just another scientific blunder.

From a local perspective, there have been a growing number of products based on *A. muricata* L. such as health supplement capsule, pill, juice and ice cream. The common conception among the public in Malaysia is that by consuming these products, their detrimental health could be ameliorated thus preventing them from succumbing to cancer. Therefore, there is huge responsibility lies within scientific community to clear the air to avoid any further misunderstanding that is not beneficial for the society in large.

In hope that *A. muricata* L. might display significant effect against lung cancer cells at the end of this study, the current research adds fresh and deepening information to the current anticancer knowledge. Even though NSCLC is the leading contributor of cancer mortality, there is lack of *A. muricata* L. anticancer study on this type of lung cancer cell. It was reported that the high 5-year survival rate for NSCLC (18.2%) are due to the inadequacy of functional prognostic biomarkers and proper treatment [27]. This shortfall is due to difficulties in diagnosing NSCLC; patients are diagnosed at

advanced stages with distant and local metastases. The discovery of HMGB1 as potential effective markers for early diagnosis of NSCLC has allowed this biomarker to be the subject of thorough experimentations. *In silico* molecular docking and molecular dynamic (MD) simulation were also applied to investigate the intriguing possibility of *A. muricata* Linn's bioactive compounds namely annonaceous acetogenins (ACGs) to possess Bcl-2 antiapoptotic inhibitory properties. Through molecular docking approach, the affinity of the compounds of interest towards Bcl-2 antiapoptotic proteins would be determined and possible interaction between amino acids and compounds would be crucial information for development of anticancer drug using ACGs.

Thus, the understanding upon the underlying mechanism triggered by potential anticancer agent such as *A. muricata* L. extract in NSCLC would certainly ignite a slight hope in this long-running battle. This study is the first to evaluate the correlation between *A. muricata* L. effect and release of HMGB1 during cell death mechanism in NSCLC.

1.3 Research Objectives

The main objective of this study was to investigate the mechanistic effect induced by *A. muricata* L. optimized leaves extracts in NSCLC. The objective is further separated into several other objectives as listed below:

(a) To optimize the extraction parameters for high yield of *A. muricata* L. extracts

(b) To determine the antioxidant activity of the optimized extracts

(c) To investigate the effect of the optimized extracts on HMGB1 gene expression in NSCLC cells

(d) To characterize the *in silico* molecular interactions of the plant's bioactive compounds

1.4 Scope of the Study

The scope of this research are as listed below:

1. Antioxidant-response optimization of ultrasonic-assisted extraction parameters (time, amplitude, ratio) and maceration extraction parameters (time, ratio, speed) of *A. muricata* L. leaves by using response surface methodology.
2. Determination of the antioxidant activity of the extracts by using 2, 2, diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay.
3. Cytotoxicity and anti-proliferation activities determination of the extracts by using 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) against human lung epidermoid carcinoma (HLFa) cells and human fibroblast cells (HSF1184).
4. Nitric oxide (NO) determination by using nitrite concentration assay.
5. Determination of apoptotic activity by using apoptosis detection assay.
6. Quantification of gene expression of *BCL2*, *BAX* and *HMGB1* by using real-time polymerase chain reaction (RT-PCR).
7. Characterization of binding interactions involving acetogenins of *A. muricata* L. and Bcl-2 antiapoptotic proteins, by using *in silico* molecular docking software, AutoDock 4.2 and molecular dynamics (MD) simulation.
8. Screening of bioactive compounds (ACGs) in the extracts by using High Performance Liquid Chromatography (HPLC).

1.5 Significances of the Study

The current study presents several novel contributions as well as additional knowledge in the field of *A. muricata* L anticancer research. The present study emphasizes the effect of extraction method on antioxidant effect response of *A. muricata* L and their correlation to antioxidant activities. This study is among a few

studies, which have been done to optimize the ultrasonic-assisted and maceration extractions, and comparison of anticancer effects between both optimized extracts provide new information to this particular field of research. This study also highlights the potential of *A. muricata* L. extracts to be further developed as anticancer agent against lung cancer especially NSCLC. Furthermore, as the effect of plant extracts on HMGB1 has never been explored before, this study would be the first to offer novel report upon *A. muricata* L. mechanistic activity against HMGB1 in NSCLC, in addition to other regulators such as Bcl-2 and Bax that could be a steppingstone to future anticancer studies as well as provides insight on the novel understanding of therapeutic potential of HMGB1 as prognostic biomarker.

1.6 Thesis Structure and Organization

This thesis comprises five chapters. The first chapter serves as a backbone of the whole thesis. It covers the underlying premise that led to the initiation of this study. The idea was presented in summarized language, of which consists of research background, problem statement, objective, scope and significances of the study.

Chapter 2 gives thorough reviews and discussions on newest literatures available. In addition, it discusses the arguable potential of *A. muricata* L. as candidate for anticancer agent. It also reviews the prevalence of lung cancer.

Chapter 3 elucidates the selected experimental-methodologies, of which were conducted to present the evidences for the hypothesis of the present study.

Chapter 4 puts forward the observed results together with the discussion of the findings that covers the anti-oxidative characteristics of *A. muricata* L. extract, the gene expression analysis of *BCL2*, *BAX* and *HMGB1* in lung cancer cells and the *in silico* virtual screening analysis.

Chapter 5 concludes the current study and summarizes the outcome. It also recommends suggestions for future researches.

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