IN VITRO AND *IN VIVO* EVALUATION OF THE CHEMOPREVENTIVE, GASTROPROTECTIVE AND WOUND HEALING POTENTIAL OF *ANNONA MURICATA*

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ABSTRACT

Annona muricata Linn. is a popular fruit tree growing in tropical countries. Its leaves have been extensively employed in folk medicine to treat a variety of ailments and diseases. In this study, anticancer, gastroprotective and wound healing properties of *A. muricata* leaves and their possible mechanisms of action were determined using *in vitro* and *in vivo* models. Solvent extraction yielded crude ethyl acetate extract (AMEAE), which demonstrated remarkable cytotoxicity against different cancer cell lines including A549, HT-29 and HCT-116. Hence, AMEAE anticancer property was investigated against the respective cell lines. In addition, *in vivo* chemopreventive potential of AMEAE was determined against azoxymethane-induced colonic aberrant crypt foci (ACF) in rats, and AMEAE was subjected to a bioassay-guided approach to isolate the cytotoxic compound and evaluate its apoptosis-inducing effect. AMEAE was found to induce mitochondrial-initiated events in cancer cells, as the treated cells shown disruption of mitochondrial membrane potential, cytochrome *c* leakage and elevation of Bax expression. Inversely, Bcl-2 expression was lowered in the treated cells. The following experiments suggested apoptosis induction in cancer cells, as was reflected by increase in total nuclear intensity, augmentation in sub-G_1 cells, externalization of phosphatidylserine and activation of initiator (-9) and executioner (-3/7) caspases. These findings strongly implied that exposure of AMEAE to cancer cells have resulted in apoptosis induction through the intrinsic pathway. A bioassay-guided investigation on AMEAE led to the isolation of annonaceous acetogenin, annomuricin E which induced significant apoptosis-inducing effects in HT-29 cancer cells through mitochondrial-mediated mechanism. The *in vivo* chemopreventive potential of AMEAE was examined in five groups of rats, namely negative control, cancer control and AMEAE (250, 500 mg/kg) and positive control (5-fluorouracil). Oral treatment of AMEAE at both doses

decreased the formation of colonic ACF. The expression of PCNA protein, a marker of cell proliferation, was downregulated in treated cells and associated with upregulation of Bax and downregulation of Bcl-2. These results substantiated the traditional use of *A. muricata* leaves against cancer and tumors.

The gastroprotective activity of AMEAE at two doses of 1 g/kg and 2 g/kg was examined against ethanol-induced gastric injury in rats. Gross and histological characterizations suggested the antiulcerogenic property of AMEAE. Immunostaining revealed upregulation of Hsp70 protein and downregulation of Bax protein. This activity was associated with attenuation in oxidative stress evidenced by an increase in the level of enzymatic antioxidants and nitric oxide and decrease in the level of malondialdehyde. These findings revealed promising gastroprotective potential for AMEAE, which was mediated through antioxidant and anti-inflammatory mechanisms.

Wound healing potential of AMEAE (5% w/w and 10% w/w) was evaluated against excisional wound models in rats. Significant wound healing activity was observed after topical treatment with AMEAE, assessed by macroscopic and microscopic analyses. This was associated with a decrease in the number of inflammatory cells, supported by upregulation in the expression of Hsp70 protein. In addition, level of enzymatic antioxidants showed augmentation which led to the attenuation in the malondialdehyde formation.

ABSTRAK

Annona muricata Linn. merupakan pokok berbuah yang ditanam di negara tropikal. Daun pokok ini telah digunakan secara meluas sebagai ubat tradisional bagi mengubati pelbagai penyakit. Dalan kajian ini, ciri-ciri antikanser, perlindungan gastro dan pemulihan luka oleh daun *Annona muricata* dan tindakan mekanisma yang berkemungkinan telah dikenalpasti dalam model *in vivo* dan *in vitro*. Pengekstrakan menggunakan pelarut menghasilkan ekstrak mentah etil asetat (AMEAE) yang menunjukkan tindakan sitotoksisiti yang amat berkesan terhadap sel-sel kanser seperti A549, HT-29 dan HCT-116. Oleh demikian, ciri-ciri antikanser AMEAE terhadap sel-sel tersebut dikaji. Selain itu, potensi kemopreventif *in vivo* AMEAE menentang induksi 'aberrant crypt foci' (ACF) kolon yang didorong oleh azoxymetana ke atas tikus turut dikaji. Di samping itu, pengasingan sebatian sitotoksik AMEAE dijalankan melalui pendekatan pengasingan berpandukan bioesei dan kesan induksi apoptosis disiasat. Sel-sel yang dirawat dengan AMEAE menunjukkan induksi program diaruhkan oleh mitokondria seperti gangguan terhadap potensi membran mitokondria, pelepasan sitokrom *c* dan peningkatan ekspresi Bax yang diikuti dengan penurunan ekspresi Bcl-2. Seterusnya, AMEAE memulakan induksi apoptosis dalam sel kanser seperti peningkatan keamatan jumlah nuklear, penumpuan sel pada fasa sub-G_1, pendedahan fosfatidilserin dan pengaktifan caspase pemula-9 dan pelaksana (-3/7). Penemuan ini menguatkan implikasi AMEAE dalam mengaruhkan tindakan apoptosis ke atas sel kanser melalui laluan intrinsik. Pendekatan pengasingan berpandukan bioesei terhadap AMEAE menghasilkan pengasingan sebatian annonaceous acetogenin, annomuricin E yang menunjukkan kesan tindakan apoptosis yang signifikan terhadap sel kanser HT-29 melalui mekanisme pengantaraan mitokondria. Potensi kemopenghalang *in vivo* bagi AMEAE dikaji dalam lima kumpulan tikus iaitu kawalan negatif, kawalan kanser dan AMEAE (250, 500 mg/kg) dan kawalan

positif (5-fluorouracil). Rawatan AMEAE secara oral pada kedua-dua dos menunjukkan pengurangan pembentukan ACF kolon. Ekspresi protein PCNA yang merupakan penanda bagi poliferasi sel juga diturunkan dan diiringi dengan peningkatan kawalatur Bax dan penurunan kawalatur Bcl-2 dalam sel yang dirawat. Keputusan ini seterusnya menyokong kegunaan tradisional daun *A. muricata* dalam rawatan kanser dan tumor.

Aktiviti perlindungan gastro oleh AMEAE pada dua dos, 1 g/kg dan 2 g/kg menentang kecederaan gastrik yang diaruhkan oleh etanol terhadap tikus juga turut diperiksa. Pencirian secara kasar dan kaedah histologi mencadangkan ciri-ciri antiulserogenik AMEAE. Kaedah pewarnaan imunosasi juga menunjukkan peningkatan kawalatur protein Hsp70 dan penurunan kawalatur protein Bax. Aktiviti ini seterusnya dikaitkan dengan tindakan penghalangan tekanan oksidatif yang dibuktikan dengan peningkatan aras enzim antioksida dan nitrik oksida bersama penurunan aras malondialdehid. Penemuan ini membuktikan potensi perlindungan gastro oleh AMEAE adalah berpandukan tindakan mekanisme antioksida dan antikeradangan.

Potensi pemulihan luka bagi AMEAE (5% w/w and 10% w/w) dinilai dalam model luka eksisional pada tikus. Aktiviti penyembuhan luka yang signifikan didapati selepas rawatan secara topikal dengan AMEAE yang dinilaikan melalui kaedah analisasi makroskopik dan mikroskopik. Pemerhatian ini dikaitkan dengan penurunan jumlah sel-sel keradangan yang disokong oleh peningkatan kawalatur protein Hsp70. Di samping itu, peningkatan aras enzim antioksida juga bertanggungjawab dalam menurunkan kadar pembentukan malondialdehid.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

1D: One Dimensional

13C NMR: Carbon Nuclear Magnetic Resonance

1H NMR: Proton Nuclear Magnetic Resonance

5-FU: 5-Fluorouracil

A549: Human Lung Carcinoma Cell Line

ACF: Aberrant Crypt Foci

AEU: Animal Experimental Unit

AGE: Annonaceous Acetogenin

AIC: Analytical Ion Chromatogram

AIF: Apoptosis Inducing Factor

ALK: Alkaloid

ALT: Alanine Aminotransferase

AMEAE: *Annona muricata* Leaves Ethyl Acetate Extract

ANOVA: Analysis of Variance

AO: Acridine Orange

AOM: Azoxymethane

AP: Alkaline Phosphatase

Apaf-1: Apoptotic protease-activating factor 1

APES: 3-Aminopropyltriethoxysilane

Apo2L: Apo2 Ligand

Apo3L: Apo3 Ligand

AST: Aspartate Aminotransferase

ATCC: American Type Cell Culture

ATP: Adenosine Tri Phosphate

BAD: BCL2 Antagonist of Cell Death

BAG: BCL2 Associated Athanogene

BAK: BCL2 Antagonist Killer 1

Bax: Bcl-2 Associated X Protein

Bcl-2: B-cell Lymphoma Protein 2

Bcl-10: B-cell Lymphoma Protein 10

Bcl-x: BCL2 like 1

Bcl-XL: BCL2 Related Protein, Long Isoform

Bcl-XS: BCL2 Related Protein, Short Isoform

Bcl-w: BCL2 like 2 Protein

Bid: BH3 Interacting Domain Death Agonist

Bim: BCL2 Interacting Protein BIM

Bik: BCL2 Interacting Killer

BL: Blebbing of the Cell Membrane

BPH-1: Benign Prostatic Hyperplasia

CAD: Caspase-Activated DNAse

Caspase: Cysteine-Dependent Aspartate-Directed Protease

Cat: Catalase

CC: Column Chromatography

CC: Chromatin Condensation

CCD841: Normal Human Colon Epithelial Cell Line

$CDCl_3$: Deuterated Chloroform

CDKs: Cyclin Dependent Kinases

CFA: Complete Freund's Adjuvant

CKIs: Cyclin-Dependent Kinase Inhibitors

$CHCl_3$: Chloroform

cm: Centimeter

CO_2: Carbon dioxide

COX: Cyclooxygenase

c-FLIP: FLICE-Inhibitory Protein

DAPI: (4',6-diamidino-2-phenylindole)

DEH: Dihydroethidium

DETC: Dendritic Epidermal T-Cells

DMBA: 7,12-Dimethylbenzene Anthracene

DMH: 1,2-Dimethyl Hydrazine

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribose Nuclei Acid

DISC: Death Inducing Signaling Complex

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

DR3: Death Receptor 3

DR4: Death Receptor 4

DR5: Death Receptor 5

DRSA: DPPH Radical Scavenging Activity

et al: and others

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic Acid

EEAM: Ethyl Acetate Extract of *Annona muricata* Leaves

EEAML: Ethyl Acetate Extract of *Annona muricata* Leaves

EGF: Epidermal Growth Factor

ELISA: Enzyme Linked Immunosorbent Assay

F: Fraction

FasL: Fatty Acid Synthetase Ligand

FasR: Fatty Acid Synthetase Receptor

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

FITC: Fluorescein Isothiocyanate

Fig: Figure

FRAP: Ferric Reducing Antioxidant Property

FTG: Flavonol Triglycoside

g: Gram

G1 phase: Gap 1 Phase

G2 phase: Gap 2 Phase

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GC-MS: Gas Chromatography Mass Spectrometry

GC-MS-TOF: Gas Chromatography–Time-of-Flight Mass Spectrometry

GGT: Gamma-Glutamyl Transferase

GPx: Glutathione Peroxidase

GR: Glutathione Reductase

GSH: Glutathione

GST: Glutathione-S-Transferase

GT: Granulation Tissue

GWM: Gastric Wall Mucus

h: Hour/s

H_2S: Hydrogen Sulphide

HCl: Hydrochloride

HCT116: Human Colorectal Carcinoma Cell Line

HCS: High Content Screening

H&E: Hematoxylin and Eosin

HepG2: Human Hepatocellular Carcinoma Cell Line

HPLC: High-performance Liquid Chromatography

HRSA: Hydroxyl Scavenging Activity

HSP: Heat Shock Protein

HtrA2/Omi: High-Temperature Requirement

HT-29: Human Colorectal Carcinoma Cell Line

IAP: Inhibitors of Apoptosis Proteins

IC_{50}: 50% Inhibitory Concentration

IgG: Immunoglobulin G

IL-1β: Interleukin-1 beta

IN: Inflammatory Cell

K562: Human Leukemic Cell Line

kg: Kilogram

LA: Late Apoptosis

LCMS: Liquid Chromatography Mass Spectrometry

LD_{50}: Lethal Dose 50

LDH: Lactate Dehydrogenase

M phase: Mitotic Phase

MCF-7: Human Breast Carcinoma Cell Line

MDA-MB-231: Human Breast Carcinoma Cell Line

MDA: Malondialdehyde

MeOH: Methanol

MG: Megastigmane

mg: Milligram

min: Minutes

ml: Milliliter

MMP: Mitochondrial Membrane Potential

mM: Millimolar

MPF: Mitosis-Promoting Factor

mRNA: Messenger Ribonucleic Acid

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

m/z: Mass to charge ratio

NADH: Nicotinamide Adenine Dinucleotide

NADPH: Nicotinamide Adenosine Dinucleotide Phosphate

NF-κB: Nuclear Factor-kappa B

nm: Nanometer

NMR: Nuclear Magnetic Resonance

nmol: Nanomolar

NO: Nitric Oxide

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs

PACA-2: Pancreatic Carcinoma Cell Line

PAS: Periodic Acid–Schiff

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Nuclear Antigen

PDA: Photodiode Array

PDGF: Platelet-Derived Growth Factor

PGE-2: Prostaglandin E2

PI: Propidium Iodide

PL: Phenolic

pRb: Retinoblastoma Protein

PPIs: Proton-Pump Inhibitors

ppm: Parts Per Million

PS: Phosphatidylserine

PTLC: Preparative Thin Layer Chromatography

Q-PCR: Quantitative Polymerase Chain Reaction

RIPA: Radio Immuno Precipitation Assay

RNA: Ribonucleic Acid

RNase: Ribonuclease

ROS: Reactive Oxygen Species

RPMI: Roswell Park Memorial Institute

Rpm: Rounds per Minute

HRP: Horseradish Peroxidase

S phase: DNA Synthesizing Phase

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

S.E.: Standard Error

SEM: Standard Error of Mean

Smac/DIABLO: Second mitochondrial activator of caspases/direct IAP binding protein with low PI

SOD: Superoxide Dismutase

TB: Total Bilirubin

TBA: Thiobarbituric Acid

TBARS: Thiobarbituric Acid Reactive Substances

TLC: Thin Layer Chromatography

TGF: Transforming Growth Factor

TNF-α: Tumor Necrosis Factor alpha

TNFR1: Tumor necrosis factor receptor 1

Triton X-100: Polyethylene Glycol Tert-octyphenyl Ether

TUNEL: Terminal deoxynucleotidyl Transferase dUTP Nick End Labeling

VC: Viable Cells

VEGF: Vascular Endothelial Growth Factor

WRL-68: Human Hepatic Cell Line

w/w: Weight to Weight Ratio

%: Percent

µg: Microgram

µl: Microliter

ºC: Degree Celsius

CHAPTER 1: INTRODUCTION

The prevalence of cancer remains high worldwide despite the extensive international attempts to treat it, triggering the need for further effective action to minimize its growth (Atawodi, 2011). The increasing resistance to available chemotherapeutic drugs is a major obstacle to cancer therapy, as a remarkable proportion of the tumor deteriorates and expands its resistance, ultimately leading to multidrug resistance after exposure to different pharmaceuticals with prevailing compositions and cellular targets (Perez, 2009). Moreover, preferred anticancer pharmaceuticals are expected to exclusively target cancerous cells, although the various chemotherapeutic treatments currently employed for cancer cases cause substantial side effects, such as diarrhea, bleeding, immunosuppression and hair loss (Kranz & Dobbelstein, 2012). Consequently, discovering novel natural products and metabolites derived from animals, plants and microorganisms with high efficiency against malignant cells with no cytotoxicity towards normal cells is a huge breakthrough in scientific research. Apoptosis is a type of regulated programmed cell death that has attracted a great deal of interest in oncology and cancer treatment owing to the high potential of diverse anticancer agents to provoke apoptosis in different cancer cells (Elmore, 2007). Hence, extensive research on natural products with the ability to induce apoptosis in cancer cells that can be used individually or combined with other chemotherapeutic drugs has been developing, seeking to promote the therapeutic impacts and diminish the side effects in cancer therapy (Gurib-Fakim, 2006).

The treatment of ailments and diseases related to gastrointestinal disorders with natural products is prevalent in folk medicine worldwide. Medicinal plants are extensively employed against gastric ulcers as decoctions, infusions or macerates, either in alcoholic beverages or in water (Schmeda-Hirschmann & Yesilada, 2005). Plants, spices and herbs

are recognized as an arsenal of natural antioxidants that can be gastroprotective against diseases affected by oxidative stress and lipid peroxidation (Repetto & Llesuy, 2002). It is also well established that the endogenous and exogenous production of free radicals and reactive oxygen species (ROS) can cause severe mucosal damage and lead to gastrointestinal inflammation (Yoshikawa et al., 1989). Therefore, traditional plants with antioxidant and anti-inflammatory activities can be promising candidates against gastrointestinal disorders.

Natural products possess the remarkable potential to be used for the treatment and management of wounds. In various countries, folklore and tribal sources employ a variety of plants to treat burns and wounds. The induction of healing and reconstruction of the damaged tissue are mediated through various mechanisms (Thakur, Jain, Pathak, & Sandhu, 2011). The availability and affordability of these traditional plants in combination with their safety have enhanced their role as popular choices for wound healing (Nayak & Pereira, 2006). The presence of various functional ingredients in plants has stimulated significant scientific interest in the examination of these plants with a view to exploring new wound healing agents with elevated therapeutic effects (Schmidt et al., 2009).

Natural products, particularly ones obtained from plants, have been employed to benefit humankind and preserve its well-being since the advent of medicine. Since the twentieth century, phytoconstituents have been an essential source for therapeutic discoveries (Newman & Cragg, 2012). The significance of phytochemicals in medicine and agriculture has attracted substantial scientific interest in the biological potential of these ingredients (Karim & Azlan, 2012). Despite these investigations, however, only a limited series of plant species has undergone comprehensive scientific assessment, and our understanding is relatively inadequate regarding their potential function and abilities in nature. Therefore, achieving a rational recognition of natural products demands wide-

ranging studies and fieldwork on the biological effects of these plants and their pivotal phytochemicals (Jothy et al., 2012). Regarding therapeutic prospects, plants with a long historical background of use in ethnological medicine represent a valuable source of effective phytoconstituents that provide curative or health advantages against numerous diseases and ailments (Duraipandiyan, Ayyanar, & Ignacimuthu, 2006). One such plant with prevalent ethnomedicinal usage is *Annona muricata*.

The effect of *A. muricata* leaves on various cancer cells has only been explored by basic cytotoxic investigations, and no detailed molecular mechanisms have hitherto been reported. *In vivo* evaluations of the possible anticancer and antitumor potential of *A. muricata* leaves have also been neglected in previous studies. Therefore, this study was designed to investigate *in vitro* and *in vivo* the cytotoxic properties of different extracts of the *A. muricata* leaves, followed by the determination of a bioactive compound responsible for the induction of apoptosis in human cancer cells. In addition, based on the marked antioxidant and anti-inflammatory activities reported for *A. muricata* leaves, this study sought to investigate its gastroprotective and wound healing properties and the factors involved.

The specific objectives of the study were:

1. To determine the *in vitro* cytotoxic activity of different extracts of the *A. muricata* leaves against various human cancer cell lines and investigate the apoptotic effects against human A549 lung cancer cells.

2. To evaluate the apoptosis-inducing potential of the *A. muricata* leaves and their inhibitory effects on migration and invasion against human HCT-116 and HT-29 colon cancer cells.

3. To investigate the chemopreventive potential of the *A. muricata* leaves against azoxymethane-induced colonic aberrant crypt foci in rats and to isolate, purify, and elucidate the structure of a bioactive compound responsible for the induction of apoptosis

in HT-29 cells through fractionation, applying various methods of chromatography, nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) in a bioassay-guided approach.

4. To evaluate the gastroprotective potential and the possible mechanism of *A. muricata* leaves against ethanol-induced gastric injury model in rats.

5. To determine the wound healing effect and the possible mechanism of *A. muricata* leaves against excisional wound model in rats.

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

In the fifth century BC, Hippocrates noted the intractable growth and spread of tissues throughout the body in a family of diseases with eventual outcome of death. This observation was later described by the term cancer, which means "crab" in Latin (Hajdu, 2004). Depending on the source of cells, cancers are categorized into different types, including carcinomas, leukemia, lymphomas and sarcomas. The uncontrollable division of cancer cells leads to an exponential augmentation in the number of dividing cells, which can form a mass of growing tissue referred to as a tumor or neoplasm. The pivotal factor in tumor progression is the perturbation in the homeostasis of normal tissues and unbalanced cell proliferation and cell differentiation, rather than the faster replication rate (Danial & Korsmeyer, 2004).

Detailed scientific inspections of the underlying steps of cancer development have resulted in a growing consensus of opinion that changes in various biological processes are required to transform cells into a fully malignant state. Essential features related to oncogenic conversion include the avoidance of immune surveillance and apoptosis, cell cycle dysregulation, genomic instability, independence from growth factor signaling and the induction of angiogenesis (Hanahan & Weinberg, 2000). Although most or all of these respective traits are necessary for various stages of cancer and tumors, the bases of the mutations causing these changes can differ greatly with every individual case of malignant cancer, which progressively complicates cancer molecular mechanisms by orders of magnitude. Hence, thorough perspectives are necessary in studying the multi-sequence process of cancer development (Kirienko, Mani, & Fay, 2010). However, the process is generally divided into three stages: initiation, promotion and progression (Balkwill, 2006).

Carcinogenesis or cancer development is triggered by non-lethal forms of DNA mutations that generate altered cells, known as the initiation stage (Taylor & Turnbull, 2005). For instance, the excessive production of ROS elevates the level of intracellular Ca (II), which activates calcium-dependent endonucleases and subsequent DNA mutations (Wiseman, Kaur, & Halliwell, 1995). A variety of factors, including the insertion of retrovirus, radiation, chemical carcinogens and pollution, random DNA mutations and cleavage during replication can induce the required genetic deficiency for the initiation stage. However, heritable genetic changes are responsible for certain cases (Irigaray et al., 2007).

The promotion stage requires the activation of uncontrollable cell division and subsequent generation of recognizable focal lesion. In this reversible stage, a relentless source of tumor stimulus is required to promote the mutated cells to the progression stage. The initial carcinogenic changes of a proto-oncogene to an oncogene may be intensified by subsequent alterations involving tumor promoters (Wiseman et al., 1995). These promoters are not exclusively foreign agents, and internal substances such as hormones and growth factors can reinforce the mutated cells that have already survived the initiation stage (Becker, Kleinsmith, Hardin, & Raasch, 2003).

Progression, the final stage of cancer, is generally characterized by the tumor becoming aggressive and aberrant. The key traits of this stage include apoptosis evasion, the ability to survive in the blood stream, elevated growth rate, and drug and immune killing resistance, among others. Induction of the transformation stage from benign to malignant due to the excessive build-up of genetic alterations makes this stage irreversible (Friedl & Wolf, 2003; Ikushima & Miyazono, 2010). At this stage, the cell surface molecules and connections show various alterations that can lead to metastasis. Parting from the tumor and resisting the immune system allows the malignant cells to move and metastasize in other exposed areas of the body (Nguyen & Massagué, 2007).

2.1.1 Treatment of Cancer

Extensive research over the past two decades has changed the patterns of cancer therapy from nonselective therapeutics to mechanism-based and specific treatments. The initial approach to the identification of new anticancer agents was screening for drugs with the strong potential to kill dividing cells (Vanneman & Dranoff, 2012). Although this mode of treatment remains the backbone of contemporary therapy, varying degrees of failure due to the notable toxicities, high acquired resistance and poor therapeutic index cause insufficient survival benefits (Gieseler, Rudolph, Kloeppel, & Foelsch, 2003). For instance, the prevalent anticancer drugs, including doxorubicin, irinotecan, platinum derivatives and 5-fluorouracil, nonselectively interfere with RNA and DNA metabolism and arrest the cell cycle in the S phase, while taxol derivatives function through mitotic arrest (Longley & Johnston, 2005).

Plants have a long history of application in cancer therapy, and natural compounds of plant origin have provided a number of clinically principal anticancer drugs. Some of the plant-derived anticancer agents that have been employed clinically include camptothecin derivatives, docetaxel, elliptinium, etoposide, teniposide, irinotecan, topotecan, taxol, vincristine and vinblastine. In addition, there are other plant-derived drugs under clinical development that function against different molecular targets in the malignant cells, such as combretastatin A4 phosphate and flavopiridol (Cragg & Newman, 2005).

Over recent years, a better understanding of molecular oncology has triggered the introduction of new modes of single or combined treatments, such as cancer immunotherapy and targeted drugs. Immunotherapy stimulates the immune system to annihilate tumor progression, while targeted agents aim at vital molecular targets in the cancer machinery in a more specific manner (Vanneman & Dranoff, 2012). For example, growth factor-related antibodies such as bevacizumab and cetuximab or small molecule kinase suppressors such as imatinib, everolimus and sorafenib directly target molecular

pathways involved in cell survival or death and demonstrate promising results in terms of survival rates for different types of cancer. More recently, the growing body of knowledge on programmed cell death or apoptosis has stimulated significant scientific interest in inhibiting malignant cells through specific targets with higher efficiency and attenuated adverse side effects (Ocker & Höpfner, 2012).

2.1.2 Apoptosis

In 1980th, Currie, Kerr and Wyllie coined the term "apoptosis", although certain elements of this mode of cell death had been explained beforehand (Kerr, Wyllie, & Currie, 1972). The general description of apoptosis in mammalian cells was elicited from the scientific study of *Caenorhabditis elegans* (Horvitz, 1999). Invariant elimination of a certain number of cells during the growth cycle of this nematode provided highly accurate surveillance of apoptosis, which is genetically regulated by a variety of factors (Norbury & Hickson, 2001). In mammalian species, the role of apoptosis is more highlighted during the phase of development and aging; however, homeostatic and immunological mechanisms tightly correlate with this mode of cell death. A variety of stimuli and factors, both pathological and physiological, can trigger the induction of apoptosis; however, the response of different cells may vary based on the nature of an individual stimulation. A signal that kills certain cells can leave others unaffected (Elmore, 2007).

2.1.2.1 Morphological Changes in Apoptosis

The morphological changes of apoptosis have been clarified by various forms of microscopy. Light microscopy illustrates cell shrinkage and pyknosis as early characteristic features of apoptosis (Kerr et al., 1972). Cell shrinkage is defined by compact cell size, dense cytoplasm and the close packing of organelles, and as the most important characteristic feature of apoptosis, chromatin condensation is responsible for pyknosis. In the next stage, there is a budding phase in which, as a result of considerable blebbing of the plasma membrane accompanied by cell fragmentation and karyorrhexis,

apoptotic bodies are formed containing closely packed organelles, cytoplasm and probable nuclear fragments (Figure 2.1). In the process of apoptosis, the cellular integrity is preserved until the apoptotic bodies are digested by phagolysosomes, parenchymal cells, macrophages and neoplastic cells. Due to the maintenance of cellular integrity, digestion of the apoptotic cells and non-secretion of anti-inflammatory cytokines by the engulfing cells, the process of apoptosis is not generally accompanied by inflammatory reactions (Kurosaka, Takahashi, Watanabe, & Kobayashi, 2003; Savill & Fadok, 2000).

2.1.2.2 Biochemical Changes in Apoptosis

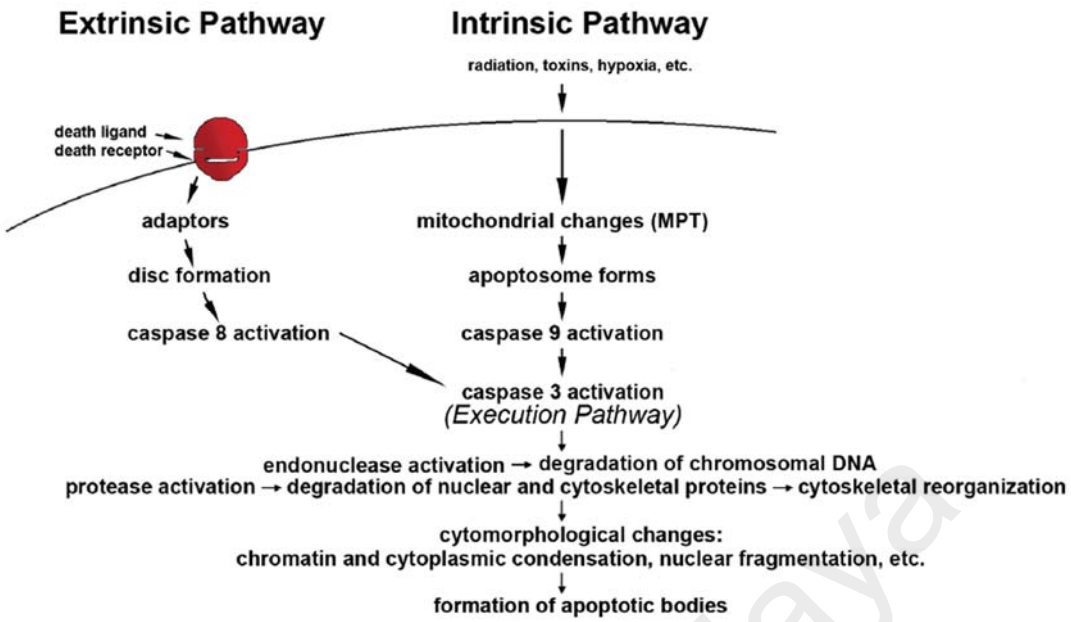
Apoptotic cells undergo various biochemical alterations, including DNA fragmentation, protein digestion, the cross-linking of proteins and phagocytic markings that make them structurally distinguishable (Figure 2.1) (Hengartner, 2000). Endonucleases dependent on Mg^{2+} and Ca^{2+} mediate DNA cleavage leading to pieces of 180 to 200 base pairs, which can be detected as a DNA ladder by agarose gel electrophoresis (Zhang & Ming, 2000). Meanwhile, tissue transglutaminase has the main role for protein cross-linking (Nemes Jr et al., 1996). Phagocytosis of the apoptotic cells is mediated by the expression of cellular ligands and diagnosis by neighboring cells. The most recognized cellular marker is attributed to the externalization of inward-facing phosphatidylserine on the plasma membrane; however, there are other known markers such as calreticulin and Annexin I (Bratton et al., 1997). As a recombinant protein with high affinity for phosphatidylserine, Annexin V is widely employed to detect phosphatidylserine externalization and the subsequent induction of apoptosis. On the surrounding cells, the complex of LDL-receptor-related protein and calreticulin interacts with outward-facing phosphatidylserine and produces a diagnostic marker (Arur et al., 2003; Brumatti, Sheridan, & Martin, 2008; Gardai et al., 2005).

As cysteinyl aspartate proteinases, caspases essentially mediate these biochemical apoptotic changes. These proteolytic enzymes are highly abundant in inactive proforms

inside cells and, after activation, can trigger a caspase cascade, leading to apoptotic biomodification (H. H. Park et al., 2007). Therefore, it is believed that after the activation of this cascade, cell death becomes an irreversible procedure. There are three main types of caspases based on their activities, namely inflammatory caspases (-1, -4, -5), executioners or effectors (-3, -6, -7) and initiators (-2, -8, -9, -10). In addition, other caspases including -11, -12, -13 and -14 have been identified (Kang, Wang, Kuida, & Yuan, 2002; Nakagawa et al., 2000; Rai, Tripathi, Sharma, & Shukla, 2005).

2.1.2.3 Apoptosis Pathways

The complex molecular cascades of apoptosis basically include two main apoptotic pathways, namely the mitochondrial or intrinsic pathway and the death receptor or extrinsic pathway. However, extensive research shows the linkage of the two pathways and a shared execution or common process. This process is triggered by caspase-3 activation and continued by DNA breakdown, the degradation of nuclear and cytoskeletal proteins, the production of apoptotic bodies and finally the expression of cellular signals for phagocytosis (Figure 2.1) (Igney & Krammer, 2002; Martinvalet, Zhu, & Lieberman, 2005).



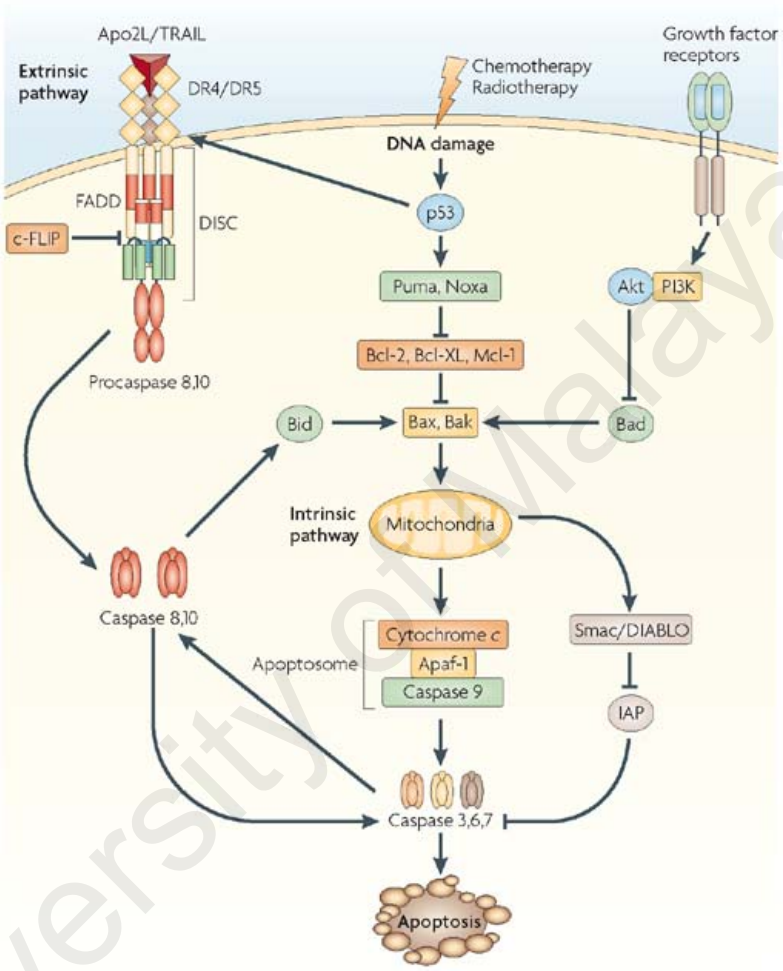
Adopted from (Elmore, 2007)

Figure 2.1: Scheme representing the main steps of two apoptosis pathways, namely extrinsic and intrinsic, associated with the execution process.

2.1.2.4 Extrinsic Pathway

The interactions between death receptors and special ligands at the cell surface primarily trigger the extrinsic machinery of apoptosis (Figure 2.2) (Locksley, Killeen, & Lenardo, 2001). The death receptor superfamily of tumor necrosis factor (TNF) is located inside the cell membrane, with the cytoplasmic death domain containing 80 amino acids and the extracellular domains containing extra cysteine residues. The death domain plays a pivotal role in the transformation of apoptotic signals from the cell membrane to intracellular structures (Ashkenazi & Dixit, 1998). The well-known receptors and their cognate ligands include DR5/Apo2L, DR4/Apo2L, DR3/Apo3L, TNFR1/TNF-α and FasR/FasL. Detailed molecular studies based on the TNFR1/TNF-α and FasR/FasL models reveal that upon the interaction of ligands and death receptors, a dimer of the death domain intervenes in the recruitment of adaptor molecules and results in the formation of a death-inducing signaling complex (DISC). As part of DISC, procaspase-8

is then autocatalyzed and triggers the execution phase (Ocker & Höpfner, 2012). This process can be aborted by a protein named c-FLIP through the neutralization of caspase-8 and the death domain of the Fas ligand (Scaffidi, Schmitz, Krammer, & Peter, 1999).



Adopted from (Ashkenazi, 2008)

Figure 2.2: Schematic representation of the main molecules involved in apoptosis pathway.

2.1.2.5 Intrinsic Pathway

In the intrinsic or mitochondria-dependent pathway, non-receptor-mediated factors play the essential role (Figure 2.2). A variety of negative or positive factors can trigger this

pathway. The lack of certain cytokines, hormones and growth factors is defined as negative stimuli, while positive stimuli include excessive exposure to certain factors, including ROS, viral infections, hyperthermia, radiation, hypoxia and toxins. After exposure to either type of factor, the inhibition of cell death is attenuated, and the apoptosis machinery is activated through mitochondrially initiated events (Elmore, 2007). This course of events is started by attenuation of the mitochondrial membrane potential through the mitochondrial permeability transition pores and the leakage of two groups of pro-apoptotic factors (Saelens et al., 2004).

The serine protease HtrA2/Omi, Smac/DIABLO and cytochrome *c* form the first pro-apoptotic group of proteins, which is primarily responsible for activating the caspase cascade. The combination of cytochrome *c* with procaspase-9 and Apaf-1 produces the apoptosome complex, which subsequently autocatalyzes the initiator caspase (Hill, Adrain, Duriez, Creagh, & Martin, 2004). HtrA2/Omi and Smac/DIABLO suppress inhibitors of apoptosis proteins (IAPs). There are also other mitochondrial proteins that bind to IAPs and inhibit them, but gene knockout studies have demonstrated that the interaction between IAPs and these other proteins does not include them as pro-apoptotic factors (Ekert & Vaux, 2005; Schimmer, 2004).

After cells are condemned to die, the role of a second group of released factors, including CAD, AIF and endonuclease G, begins with stage I of condensation. In this step, the translocation of AIF to the nucleus results in the cleavage of DNA into large parts and some nuclear condensation (Joza et al., 2001). Next, endonuclease G translocates to the nucleus and breaks down the large DNA parts into smaller oligonucleosomal pieces. Endonuclease G and AIF function caspase-independently; however, CAD is dependent on caspase-3 proteolytic activity for stage II of condensation. This later stage is accompanied by more definite DNA cleavage and chromatin condensation (Susin et al., 2000).

The mitochondrially initiated events are tightly regulated by the Bcl-2 family of proteins under the function and regulation of the p53 tumor suppressor protein. Approximately 25 members of this family are either anti-apoptotic or pro-apoptotic protein (Schuler & Green, 2001). Anti-apoptotic members include BAG, Bcl-XS, Bcl-x, Bcl-2, Bcl-w, Bcl-XL, while Blk, Bim, Bid, Bax, Bik, Bad, Bcl-10 are pro-apoptotic proteins. These respective proteins substantially help to determine whether cells undergo apoptosis or suppress the procedure. This role is believed to be mediated through the control of cytochrome *c* leakage from the mitochondria. There are several proposed mechanisms for this process; however, none of them have been definitively substantiated (Cory & Adams, 2002).

As a one good illustration of cross-talk between the intrinsic and extrinsic pathways of apoptosis, the Bid protein can cause mitochondrial disruption through the proteolytic activity of caspase-8 in the Fas pathway (Igney & Krammer, 2002). Bad is generally located in the cytosol in its phosphorylated form, but after being unphosphorylated, it relocates to the mitochondria to mediate cytochrome *c* leakage (Yang et al., 1995; Zha, Harada, Yang, Jockel, & Korsmeyer, 1996). The heterodimerization of Bad with Bcl-2 and Bcl-Xl nullifies their inhibitory roles and encourages apoptosis induction. In the absence of Bad, Bcl-2 and Bcl-Xl suppress the cytochrome *c* release and manage the caspase activation. In addition, there is the Aven protein, which interacts with Apaf-1 and Bcl-Xl and prevents the pro-caspase-9 activity. The upregulation of either Bcl-Xl or Bcl-2 results in the reduced expression of the other protein, demonstrating the opposing correlation pattern between their expression levels (Newmeyer et al., 2000).

Noxa and Puma are also considered to be important pro-apoptotic proteins. As an associate factor of p53-induced apoptosis, Noxa localization to the mitochondria and interaction with anti-apoptotic proteins triggers the pro-caspase-9 activity (Oda et al., 2000). An i*n vitro* study has shown that after the mediation of cell death by p53, Puma

overexpression is followed by the upregulation of Bax and subsequent characteristic alterations, collapse of the mitochondrial membrane potential and leakage of cytochrome *c* (Liu, Newland, & Jia, 2003). The dependence of Noxa and Puma on p53 activity suggests their potential role in apoptosis induction after oncogene stimulation and genotoxic disruptions. In addition, the oncoprotein Myc also plays an important role in mediating apoptosis in both p53-independent and -dependent manners (Elmore, 2007).

2.1.2.6 Common Pathway

In the last step, both the extrinsic and intrinsic pathways end in the execution phase. Various proteases and endonucleases are activated in this phase by effector caspases to cleave cytoskeletal and nuclear proteins and DNA, respectively. The effector caspases, including -7, -6 and -3, react with different molecular targets such as nuclear protein NuMA, PARP, cytokeratins and the actin-binding protein α-fodrin and subsequently mediate biochemical and morphological alterations (Slee, Adrain, & Martin, 2001). As the most pivotal effector caspase, caspase-3 can be activated by different initiator caspases and cleave the complex of CAD endonuclease and its suppressor (ICAD) to free the CAD protein. This endonuclease is responsible for chromatin condensation and DNA breakdown in the nucleus. In addition, the induction of apoptotic body formation and cytoskeletal rearrangement is attributed to caspase-3 (Nagata, 2000). Activation of the actin-binding protein gelsolin is also mediated by caspase-3. In healthy cells, gelsolin plays an essential role in the modulation of the actin cytoskeleton. After being cleaved by caspase-3, the gelsolin parts break down actin filaments calcium-independently and throw cellular transportation and signaling into disarray. The final step of apoptosis is the phagocytosis of apoptotic cell material (Brentnall, Rodriguez-Menocal, De Guevara, Cepero, & Boise, 2013). The essential mark of phagocytic digestion is considered to be phosphatidylserine translocation on the outer membrane and subsequent membrane phospholipid asymmetry. The presence of phosphatidylserine on outward-facing sites of

the cell surface promotes the identification of apoptotic cells for further non-inflammatory phagocytosis and cellular clearance (Fadok, de Cathelineau, Daleke, Henson, & Bratton, 2001).

2.1.3 Apoptosis and Carcinogenesis

Resistance to apoptosis or its attenuation has a pivotal role in human malignancies. Cancer cells can develop apoptosis resistance or decrease through different mechanisms. There are three main factors for the apoptosis deficiency, namely attenuated caspase activity, defective death receptor signaling and perturbation in the balance of anti-apoptotic and pro-apoptotic proteins. As one of the key players in the initiation and execution of apoptosis, it is evident than any impaired function or reduced activity of these proteins may result in the serious defect in the apoptosis progression and subsequently carcinogenesis (Fink & Cookson, 2005; Wong, 2011). Death receptor signaling can be also disrupted through different abnormalities, which leads to abortion of the extrinsic pathway of apoptosis. Such dysregulations include reduction in the level of death signals, underexpression of the receptor and defect in receptor functions. All of these contributing factors strengthen cancer development through disruption of the extrinsic pathway (Fulda, 2010; Wong, 2011).

Among various proteins involved in apoptosis induction, the ratio between anti-apoptotic and pro-apoptotic proteins plays a pivotal role in the apoptosis progression, rather than the absolute quantity of proteins. However, changes in the expression of some genes and their corresponding proteins can lead to carcinogenesis through suppression of apoptosis in malignant cells (Hanahan & Weinberg, 2000; Wong, 2011). In the Bcl-2 family of proteins, the imbalance between pro-apoptotic and anti-apoptotic members results in the dysregulation of apoptosis in the aberrant cells. This perturbation can be due to an underexpression of one or few pro-apoptotic proteins or an overexpression of one or few anti-apoptotic proteins or their combination (Wong, 2011).

At the short arm chromosome 17, the tumor suppressor gene TP53 encodes one of the most important tumor suppressor proteins, tumor protein 53 or p53, which is named after its molecular weight. After its identification in 1979, extensive research has been performed on the underlying role of p53 in carcinogenesis. Besides apoptosis, p53 is a key player in different cellular mechanisms, including chromosomal segregation, DNA recombination, gene amplification, cell cycle regulation and development. Deficiency in the p53 function has been detected in more than 50% of human malignancies (Bai & Zhu, 2006; Levine, Momand, & Finlay, 1991; Oren & Rotter, 1999).

2.1.4 Nuclear Factor kB (NF-kB) and Carcinogenesis

As a family of transcription factors, NF-kB has a pivotal role in the modulation of different cellular processes such as inflammation, immune responses and oncogenesis. Expression of a variety of genes involved in different facets of cancer, including apoptosis, migration and proliferation, is rigorously regulated by NF-kB. Perturbation in the NF-kB activity has been reported in various types of cancer. The growing body of experimental evidence supporting the functional roles of NF-kB activation in molecular oncology has stimulated significant scientific interest in characterizing NF-kB as a therapeutic target for the cancer treatment (Dolcet, Llobet, Pallares, & Matias-Guiu, 2005).

NF-kB family of transcription factors contains five genes, namely RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100) with a mutual sequence of Rel Homology Domain (RHD), and produces seven proteins. The RHD is responsible for dimerization and attachment to DNA and specific inhibitors. NF-κB1 (p50/p105) and NF-κB2 (p52/p100) are synthetized in an initial structure, while RelA, RelB and c-Rel are produced in their complete structures and their transactivation domain mediates binding to transcriptional factors. The proteins of p52 and p50 are matured through proteolysis of p105 and p100 at C-terminal ankyrin repeats by the proteasome. Although p52 and p50

have the DNA binding domain, but do not include transactivation domain (Karin & Ben-Neriah, 2000).

NF-kB can inhibit both extrinsic and intrinsic pathways of apoptosis through activation of some cellular factors, which subsequently suppress apoptosis proteins. The extrinsic pathway can be interfered through upregulation of some proteins by NF-kB. One such a protein is c-FLIP, which have a structural similarity with caspase-8 and compete with this protein for binding to DISC. However, c-FLIP does not have any protease activity and suppress the caspase cascade. Extensive research into the upregulation of c-FLIP in numerous tumors has led to the general consensus that this protein may be responsible for the resistance to death receptor apoptosis in certain types of cancer. In addition, NF-kB can also target other TNF-α-related proteins like TRAF6 and TRAF2 and activate pro-survival factors (Olsson et al., 2001; Panka, Mano, Suhara, Walsh, & Mier, 2001; Thomas et al., 2002; Wang, Mayo, Korneluk, Goeddel, & Baldwin, 1998).

Upregulation of certain anti-apoptotic Bcl-2 family members and IAPs by NF-kB can also affect the quality of apoptosis induction. IAPs (XIAP, c-IAP1 and c-IAP2) directly suppress executioner caspases and subsequently abort apoptosis through both intrinsic and extrinsic pathways. Meanwhile, expression of anti-apoptotic proteins of the Bcl-2 family neutralizes the activity of pro-apoptotic proteins and only suppress the mitochondrial dependent pathway. Furthermore, interaction between NF-kB and p53 transcriptional activity may lead to the suppression of p53-induced apoptosis. This process is mediated by downregulation of p53 and expression of certain anti-apoptotic genes (Deveraux et al., 1998; Dolcet et al., 2005; Wang et al., 1998).

2.1.5 Cell Cycle

Cell replication is mediated through a chain of actions known as the cell cycle. This process contains two main phases, namely S and M, whereby chromosome synthesis (duplication) and cell mitosis are performed and result in two daughter cells. Cells are

separated and prepared for the respective phases by other two phases, namely G1 and G2, to form the full cycle of M-G1-S-G2 as demonstrated in Figure. 2.3 (Kinjyo, Weninger, & Hodgkin, 2015). In mammalian cells, a variety of antiproliferative and proliferative signals strictly regulate the quality of cell division at specific cell cycle check points. The checkpoints have a pivotal role to assure the precise replication of nuclear DNA through a detailed assessment of DNA damage (Thornton & Rincon, 2009). The cell cycle progression relies on the physical interactions of small serine/threonine kinases known as cyclin dependent kinases (CDKs) and their activating cyclin subunits. In the presence of unfixable DNA damages, suppression or expression of certain regulators aborts cell division through cell cycle arrest and apoptosis induction (Malumbres et al., 2009).

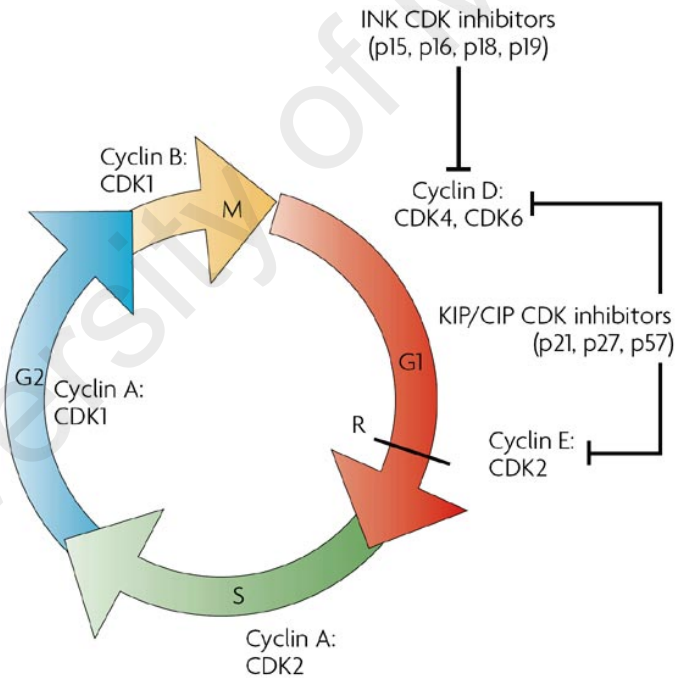


Figure 2.3: The different phases in cell cycle.

2.1.5.1 Mechanisms of Cell Cycle

The cyclin dependent kinases (CDKs) contain at least two important subunits, namely a cyclin and a kinase, and are the core of the regulatory apparatus. Changes in the structures

of these complexes drive the cells from passing through their checkpoints and moving to the next stages. Phosphorylation of different proteins by CDKs leads to the inactivation or activation of the respective proteins and directs the cell cycle progression. In mammalian cells, a constellation of cyclins (D, E, A and B) is expressed along with a constellation of kinases (CDK4, CDK6, CDK2 and CDC2) to promote cell cycle progression from G1 phase to M phase. In response to growth factors or other cellular signals, there is accumulation of CDKs (CDK4 and CDK6) and several D-type cyclins (D1, D2 and D3) in the early entry of G1 phase. In the late G1 phase, expressed cyclins A and E establish complexes with CDK2 to mediate the G1 to S transition and DNA duplication (M.-T. Park & Lee, 2003).

Since the regulation of the cell cycle progression is pivotal for appropriate proliferation and homeostasis, CDKs are subjected to a wealth of controls, including the dephosphorylation and phosphorylation of key sites on CDKs, the proteosomal degradation of selected regulatory proteins and transcriptional regulation of cyclin genes (Kirienko et al., 2010). While CDKs and their corresponding cyclins positively regulate cell cycle progression, there are suppressive factors, such as cyclin-dependent kinase inhibitors (CKIs), which inhibit this process in response to different cellular signals. Physical interactions between CKIs and CDKs can suppress CDKs activity. There are two main families of CKIs, namely INK family and CIP/KIP family. Members of the INK family, namely INK4D (p19), INK4C (p18), INK4A (p16) and INK4B (p15) negatively regulate cell cycle progression through direct association with CDK4 and CDK6, and prevention of their attachment with D-type cyclins. Members of the CIP/KIP family, namely KIP1 (p27), KIP2 (p57) and CIP1 (p21) mediate their negative regulatory effects through complex formation with the G1/S CDKs (Figure 2.3). Generally, KIP1 (p27) is highly expressed in quiescent cells. As one of the effectors of p53, CIP1 (p21) plays an important role in DNA damage checkpoint (Massagué, 2004; Sherr, 2000; Sherr &

Roberts, 1999). In the late G1 phase, there is the restriction point, which is the pivotal point of cell cycle regulation and irreversibly mediates the next stage of the cell cycle. Cyclin E-dependent kinases and cyclin D are responsible for the regulation of the restriction point (Adams, 2001).

As a timer of transcriptional events and a tumor suppressor protein, the retinoblastoma protein (pRb) functions after being phosphorylated by activated G1-phase CDK complexes. Hyper- or hypo-phosphorylated pRb can suppress or activate the E2F family of transcription factors. There are a host of genes, including cyclin A, cyclin E, CDK1, dihydrofolate reductase and thymidylate synthase, which are regulated by E2Fs and regulate the progression of cells through S, G2 and M phases (Harbour & Dean, 2000).

Completion of the DNA duplication process prepares the cell to move to the next stage through G2 phase. A noted elevation in cyclin B expression facilities this process and interaction of cyclin B with CDK1 in the presence of CDC2 forms the complex of mitosis-promoting factor (MPF). Activation of MPF is mediated by phosphorylation of CDK 1 on a threonine residue (Thr 161) by CDC25 phosphatase. Later, the MPF complex causes cyclin B degradation and the initiation of anaphase through induction of the ubiquitin proteasome pathway. At the end, dephosphorylation of CDK1 at Thr 161 resets the cell cycle clock (King, Deshaies, Peters, & Kirschner, 1996; King, Jackson, & Kirschner, 1994).

2.1.6 Cell Cycle Control and Cancer Treatment

Perturbation in the cell cycle regulation in different types of cancer has highlighted an auspicious therapeutic approach. In fact, the quiescence of cancer cells can be mediated by regulating appropriate restriction point control. In addition, excessive proliferation of cancer cells can be also employed for facilitation of apoptosis and selective treatment with chemotherapeutic agents (Y.-N. P. Chen et al., 1999).

Due to their important role in the cell cycle machinery, CDKs have been potential targets, and development of their selective inhibitors is the most promising paradigm. Extensive research has led to the establishment and modification of potent CDK suppressors. There are three main properties, which make CDK suppressors interesting therapeutic agents. Firstly, they can abort cell proliferation through cell cycle arrest at G1 or G2/M phases (Soni et al., 2001). Second, their administration alone or in combination with other anticancer agents can mediate apoptosis induction (Edamatsu, Gau, Nemoto, Guo, & Tamanoi, 2000). Third, in some cases, CDK suppression can contribute to the cell differentiation (Matushansky, Radparvar, & Skoultchi, 2000).

Promising gene therapeutic approaches have been also developed, which target negative regulator of the cell cycle apparatus, including KIP1 (p27), CIP1 (p21) and INK4A (p16), to suppress cell transformation and malignancy development. If the origin of cancer is fully illustrated, gene therapy can provide the highest opportunity for tumor treatment. However, the gene delivery system should be delicately optimized for maximum transfer of the target gene to the specific tissue (M.-T. Park & Lee, 2003).

2.2 Peptic Ulcer

Duodenal and gastric ulcers give rise to peptic ulcer disease, which has been a critical threat to the human population worldwide since the nineteenth century, with heightened outbreaks and extensive fatality. Epidemiological statistics for this disorder and its difficulties have revealed remarkable geographic diversities in prevalence and frequency. Ulcer disease development and the resultant death have been correlated with the emergence of population growth and urbanization and were construed as a birth-cohort incident while the disease was at its peak in newborns during the late 19th century (Sonnenberg, 2006; Susser & Stein, 1962). Our knowledge of the illness improved significantly with the discovery of *Helicobacter pylori* in 1982 by Warren and Marshall (Warren & Marshall, 1983). This breakthrough changed the perspective on the peptic

ulcer disease from an acid-induced disorder to an infectious disease, revealing an enormous area for thorough investigation that led to the more detailed explanation of formerly proposed pathogenic processes (Peter Malfertheiner, Chan, & McColl, 2009).

The current therapeutic principle is based on the theory of the collapse of acid secretion, which had obtained undeniable approval during and after the advent of histamine H_2-receptor antagonists. The extensive use of acid inhibitory treatment for gastric damage, which resolved previous surgical difficulties, was steadily replaced by a provisional antibiotic treatment aimed at the extermination of *H. pylori* infection (Peter Malfertheiner et al., 2007). The eradication of *H. pylori* to treat peptic ulcers received its utmost credit when Warren and Marshall were granted the Nobel Prize for Medicine and Physiology in 2005. Yet, this discovery has not ended the challenges of peptic ulcer complications. Low doses of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) are increasingly common initiators of ulcers and their difficulties even in *H. pylori*-negative cases. Nonetheless, in addition to aspirin, NSAIDs and *H pylori*, there are also other uncommon causes of ulcer disorder (Rodríguez & Tolosa, 2007).

2.2.1 Clinical Symptoms and Diagnosis

The main sign of the basic peptic ulcer is epigastric ache probably accompanied by further dyspeptic manifestations such as nausea, a sensation of early fullness and bloating. In patients suffering from intestinal ulcer, the epigastric pain generally strikes during the night or in the food abstinence state and can typically be alleviated by food ingestion or acid-counteracting mediators. Approximately one-third of these cases also suffer from heartburn, mainly without erosive esophagitis. However, symptomless progression has been reported for chronic ulcers (P Malfertheiner et al., 2002). In particular, this lack of symptoms is observed in NSAID-driven ulcers, where perforation and upper gastric and intestinal bleeding could be the initial medical indication of the ailment. Bleeding is the

most prevalent and harsh adversity of peptic ulcers, reportedly occurring in 50–170 cases per 100,000, and individuals older than 60 years have the highest risk. Perforation, of course, occurs less frequently than bleeding, with a rate of approximately 7-10 cases per 100,000. The infiltration of retroperitoneal organs is accompanied by continuous acute stinging and pain but is fortunately uncommon. Likewise, gastric outlet obstruction caused by ulcer-driven fibrosis rarely occurs and would promote the concept of a latent invasive disease (J. P. Gisbert & Pajares, 2003; Longstreth, 1995; Peter Malfertheiner et al., 2009).

Endoscopy is employed for the diagnosis of a peptic ulcer, identified as a mucosal wall break of a least 5 mm in diameter, while the term "erosion" is used for a mucosal break of less than 5 mm. The 5-mm diameter is specifically used in clinical experiments and could vary for other considerations, as its correlation with the clinical standard of mucosal penetration is not yet fully clarified. The distinctive site of the intestinal ulcer is in the duodenal bulb, where the digestive contents infiltrate into the small intestine, and the number of ulcers can be notably different. Although capable of occurring at any location from the pylorus to the cardia, the position of preference for gastric ulcers is the angulus of the lesser curvature. Infrequently, kissing ulcers are detected facing the posterior and anterior of the duodenal bulb walls. The observation of ulcers in the more distal duodenum strongly implies latent ischemia, Crohn's disease or the uncommon Zollinger-Ellison syndrome. To diagnose gastrointestinal ulcer endoscopically and detect infection by *H. pylori*, biopsy collection should be performed from the antral and fundus or body mucosa using quick urease kits and histological examinations (Peter Malfertheiner et al., 2009).

In most developed countries, symptoms similar to ulcer in patients older than 55 years are normally not examined by endoscopic checkup but by non-aggressive examination

for *H. pylori,* and positive cases must be treated with effective antibiotics. The respective test-and-treat strategy can be rationalized based on the idea that symptoms in a number of patients are the result of unrevealed ulcer disorder that can be treated by *H. pylori* eradication. Furthermore, invasive disorder is rarely seen in the youthful population in the absence of manifestations including vomiting, weight loss, anemia and loss of appetite (Kenneth McColl, 2000; KEL McColl et al., 2002).

2.2.2 Pathogenesis

The pathogenesis of gastrointestinal ulcers has been described as a complicated process that mainly involves deficiency in the protective activity of mucosal wall, perturbation in gastric acid secretion and over-activation of pepsin. There are certain environmental factors that facilitate ulcer production through the augmentation of gastric acid secretion and attenuation of the gastric wall. These contributing factors include drug consumption, inordinate alcohol intake and smoking; however, except for NSAIDs, none of them have been described as a primary pathogenic factor (Lau et al., 2011; Rosenstock, Jørgensen, Bonnevie, & Andersen, 2003). In addition, the intensifying role of psychosocial difficulties and social problems cannot be neglected in ulcer formation, although duodenal ulcers have been primarily attributed to these factors only in limited cases. One clarifying illustration here was the notable hemorrhagic ulcers in the aged after Great East Japan Earthquake. Therefore, to be more accurate, the role of stress can be narrowed to the bleeding ulcer (Gratrix, Enright, & O'Beirne, 2007; Rosenstock et al., 2003).

2.2.3 *H. pylori*-positive ulcer

Epidemiological data have shown a close correlation between infection with *H. pylori* and gastric and duodenal ulcers. The most cogent substantiation of *H. pylori* as the critical contributing factor in ulcer development was the irreversible treatment of peptic ulcers after the deracination of bacterial infections. Less than 10% of *H. pylori* infections on the mucosal wall become ulcers, although over half of the world's population is infected with

this bacteria. Determining factors of whether the infection will promote disease are the histological framework for the gastritis induction; inheritable factors; ulcerogenic strains; perturbations in acid secretion and gastric hormone homeostasis; interplay of *H. pylori* with the mucosal wall; and immunopathogenesis and gastric metaplasia in the duodenum (Peter Malfertheiner, Leodolter, & Peitz, 2000; Rauws & Tytgat, 1990).

2.2.4 Mechanisms of Peptic Ulcer and Gastroprotection

Preservation of the gastric mucosal barrier is highly dependent on the homeostasis between healthy cells and aged, damaged or death cells. It is well established that the gastric mucosa normally has a fast rate of exfoliation and proliferation with a three to five day regeneration rate. Cell homeostasis is regulated in each part of the stomach through apoptosis induction and involves 2% to 3% of all epithelial cells, although apoptosis substantially affects the higher regions of the gastric glands (Szabo & Tarnawski, 2000). *H. pylori* infection is among the critical factors that can influence the apoptotic process in gastric cells (Backert et al., 2000). If the gastrotoxicity of contributing factors overcomes the gastroprotective approaches of the human body, acute inflammation and the production of different proinflammatory cytokines will result (Xu et al., 2010). Then, the induction of acute inflammation subsequently causes neutrophil infiltration through the gastric mucosal barrier (La Casa, Villegas, De La Lastra, Motilva, & Calero, 2000).

As a conserved family of proteins, heat shock proteins play a pivotal role in preserving cells from stressful and destructive states. The folding of new proteins and refolding of structurally flawed proteins is the main responsibility of these molecular chaperones (Rokutan, 2000). Hsp70 (70 kDa), a member of the heat shock protein family, is plentifully expressed in multiple cellular regions such as the nucleus, cytosol, endoplasmic reticulum and mitochondria to protect cells against various types of stress, including heat shock, oxidative damage, infection, toxic factors, heavy metals and ischemia. The expression of Hsp70 can be reduced during the peptic ulcer formation,

intensifying inflammatory responses (Konturek et al., 2001; Robert, Ménoret, Basu, Cohen, & Srivastava, 2001).

As the rate-limiting enzyme, cyclooxygenase (COX), with its two isoforms COX-1 and COX-2, is responsible for the production of prostaglandin H_2 from arachidonic acid. To preserve physiological homeostasis and contribute to prostaglandin production, the regular expression of COX-1 in various tissues is required, while the role of COX-2 is more highlighted in inflammatory reactions through the stimulation of different factors such as growth factors, hormones, cytokines and mitogens (Church, Fleshman, & McLeod, 2003). It is believed that COX-1 suppression contributes to ulcerogenic side effects (Kurumbail, Kiefer, & Marnett, 2001).

2.2.5 Etiology and Management of Ulcers

Previously, it was believed that the induction of ulcers by NSAID was mediated through the attenuation of mucosal surface hydrophobicity and ion trapping. More recently, it was demonstrated that the inhibition of prostaglandin production is a contributing factor in NSAID-induced ulcers. The identification of COX-1 and COX-2 greatly encouraged pharmacological companies to create new NSAIDs that can selectively target COX-2. However, while these NSAIDs cause less gastrointestinal damage, the possibility has not been totally eliminated (Lanas et al., 2007; Wallace, 2008).

Animal studies have demonstrated that the interaction of gastric microcirculation with neutrophils plays a pivotal role in triggering NSAID damage. Neutrophil adherence to the vascular endothelium mediates gastric damage through ROS

production, protease liberation and the disturbance of gastric blood flow. The alleviation of ulcer formation after the suppression of neutrophil adherence in animal studies substantiated this pathogenic mechanism of NSAIDs (Fiorucci, Santucci, & Distrutti, 2007; Wallace, Keenan, & Granger, 1990). The growing body of experimental evidence supports the protective role of hydrogen sulfide (H_2S) and nitric oxide (NO) for the gastric

mucosal barrier. H_2S and No encourage mucus production, suppress neutrophil adherence and elevate gastric blood flow. Therefore, NSAIDs that promote the release of H_2S and No were found to have greatly reduced destructive effects. In humans, the role of neutrophils in the initiation of NSAID-induced injury remains under investigation (Fiorucci et al., 2007).

The alleviation of acid secretion is the most essential approach to managing NSAID-mediated gastric damage. Acid production apparently intensifies gastric ulcers through its aggravation of mucosal lesions, interference in platelet activity and disruption of the healing process (Papatheodoridis, Sougioultzis, & Archimandritis, 2006). The chance of ulcer difficulties such as bleeding is four-fold higher in cases consuming NSAIDs than in normal patients. Other contributing factors include the high-dose intake of NSAIDs; the simultaneous consumption of anticoagulants, aspirin or corticosteroids; comorbidities; a history of gastric related problems; and old age (Huang, Sridhar, & Hunt, 2002; Peter Malfertheiner et al., 2009).

In 1910, Karl Schwarz began the innovation of the first generation of antiulcer drugs based on targeting the molecular systems of the mucosal barrier and acid production, but only an exiguous number of agents were medically approved to date (Schwarz, 1910). The healing and management of ulcers were completely transformed after the introduction of H_2-receptor antagonists. However, these antagonists were gradually replaced by other therapeutic agents named PPIs (proton-pump inhibitors), which also function through the suppression of acid production. PPIs were established in 1989 and target parietal cells for the selective inhibition of H^+K^+ ATPase. Based on the close correlation between the severity of ulcers and the level of acid production, PPIs were widely acknowledged as the standard ulcer treatment. However, the reoccurrence of ulcers after the treatment period convinced pharmacologists to ensure the continuation of the drug consumption and suppression of the acid production even for years. Then, the

revolution of ulcer therapy by the introduction of antibiotic treatment against *H. pylori* changed this pattern (Fellenius et al., 1981; J. Gisbert et al., 2001; Peter Malfertheiner et al., 2009).

Another group of antiulcer pharmaceutical agents was designed to function against aspirin and NSAIDs and reinforce the mucosal wall. In this group, as a prostaglandin analogue, misoprostol was commonly employed; however, its adverse side effects have restricted its consumption. In addition, bismuth salts and sucralfate mediate ulcer treatment through the strength of the mucosal barrier. The attenuation of *H. pylori* infection and acid production also partially contributes to the therapeutic effect of sucralfate (F. K. Chan et al., 2002; Ford, Delaney, Forman, & Moayyedi, 2004). The limited antibiotic effect of bismuth salts against *H. pylori* enables them to be employed as complementary treatment with antibiotics. In the last few decades, typical cytoprotective agents were replaced by a new efficient therapy that applies PPIs and antibiotic treatment against ulcers induced by gastrotoxic agents and *H. pylori*, respectively (Peter Malfertheiner et al., 2009).

2.3 Wound Healing

Wounds, especially chronic wounds, are devitalizing afflictions that affect a patient's recuperation process and severely reduce quality of life. Recent research on the Western pharmacopeia revealed that only 1%–3% of the recorded drugs are acceptable for skin-related afflictions involving wounds. In contrast, almost one-third of herbal medicines may be efficiently employed for such a purpose (Hayouni et al., 2011). Hence, novel medications derived from natural products with the capability of healing wounds have become a thriving approach as a means to improve curative impacts and accelerate the wound-healing process (Kumar, Vijayakumar, Govindarajan, & Pushpangadan, 2007).

2.3.1 Mechanisms of Wound Healing

Wound healing is a dynamic procedure involving four overlapping, nonstop, and

accurately organized phases, namely coagulation, inflammation, proliferation and maturation (Gouin & Kiecolt-Glaser, 2011; Vidinský et al., 2006). Every phase must occur in an accurate and structured routine. Prolongation, disruption or deviation of the procedure can result in long-lasting wound healing or an incurable chronic wound. In optimum wound healing, these four phases consist of the following processes in adult individuals: (I) instant hemostasis; (II) inflammatory responses; (III) migration, reproduction and differentiation of mesenchymal cells to the wound area; (IV) appropriate angiogenesis; (V) rapid re-epithelialization (re-development of epithelial tissue around the wound area); and (VI) suitable synthesis, cross-linking, and association of collagen to strengthen the tissue in recovery (Gosain & DiPietro, 2004). Immediately after wounding, the first stage of hemostasis instigates straightaway with vascular compression and expansion of the fibrin clot. Consequently, the clot and adjacent wound tissue secrete growth factors and pro-inflammatory cytokines such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, epidermal growth factor (EGF) and fibroblast growth factor (FGF). After control of the bleeding, chemotaxis occurs, which is the migration of inflammatory cells into the wound leading to promotion of the inflammatory stage, which is marked by the successive permeation of neutrophils, macrophages and lymphocytes. The removal of intruding microbes and cellular detritus in the wound site is a crucial role of neutrophils; however, these cells also generate materials such as ROS and proteases, which result in some further side damage (Campos, Groth, & Branco, 2008; Gosain & DiPietro, 2004).

Macrophages perform multiple functions in the wound healing process. In the early phase of wound incidence, macrophages produce cytokines that stimulate the inflammatory reaction by employing and triggering supplementary leukocytes. In addition, macrophages can control the induction and removal of apoptotic cells (involving neutrophils), thereby preparing the pathway for inflammation activity. While

macrophages remove these apoptotic cells, they undergo a phenotypic change to a recovering condition that activates fibroblasts, angiogenesis and keratinocytes to induce tissue reinforcement. Thus, macrophages support the transformation to the proliferative state of healing (Meszaros, Reichner, & Albina, 2000; Mosser & Edwards, 2008).

After the inflammatory cells and macrophages, T-lymphocytes drift towards wounds, and their presence peaks throughout the late proliferation and initial maturation phases. The function of T-lymphocytes is not fully understood and is currently an area of rigorous scrutiny. Numerous investigations indicate that overdue T-cell permeation and reduced T-cell aggregation in the wound area are correlated with debilitated wound healing, as other studies have reported that CD 4+ cells (helper T cells) play a beneficial role in wound healing, while CD 8+ cells (suppressor-cytotoxic T cells) exhibit a preventive function in wound healing (J. E. Park & Barbul, 2004; Swift, Burns, Gray, & DiPietro, 2001). Remarkably, an *in vivo* study performed by Gawronska-Kozak et al. (Gawronska-Kozak, Bogacki, Rim, Monroe, & Manuel, 2006) in mice lacking in both T- and B-cells revealed that the maturation phase is devitalized in the absence of lymphocytes. Furthermore, epidermis gamma-delta T-cells control several facets of wound healing, with functions that include preserving tissue structure, shielding against pathogens and adjusting inflammation. Due to their distinctive dendritic (branched form) morphology, these cells are also known as dendritic epidermal T-cells (DETCs). DETCs are stimulated by agitated, impaired, or distorted keratinocytes and generate keratinocyte growth factors, fibroblast growth factor 7 (FGF-7), and insulin-like growth factor-1 to maintain keratinocyte reproduction and cell vitality. Additionally, DETCs produce chemokines and cytokines that participate in the induction and balancing of the inflammatory reaction in the course of wound healing. While interference between keratinocytes and epidermis gamma-delta T-cells is a factor in the preservation of ordinary skin and wound restoration,

mice with the absence of skin gamma-delta T-cells reveal interruption in wound closing and a reduction in the propagation of keratinocytes at the wound area (Jameson & Havran, 2007; Mills, Taylor, Podshivalova, McKay, & Jameson, 2008).

The proliferation phase, in general, follows and overlaps with the inflammation phase and is identified by epithelial division and movement over the temporary matrix surrounding the wound (re-epithelialization). In the recovering dermis, the most prominent cell types are endothelial cells and fibroblasts, which sustain collagen production, capillary development and the construction of granulation tissue at the injury area. Within the site of the wound, fibroblasts give rise to collagen in addition to proteoglycans and glycosaminoglycans, which happen to be main factors of the extracellular matrix (ECM). After potent proliferation and ECM production, the ultimate maturation phase of wound healing begins, which can continue for years. The restoration of numerous newly produced blood vessels happens in this phase, allowing the vascular density of the wound to return to normal. One of the essential characteristics of the maturation phase is the transformation of the ECM to a structure that matches the normal tissue. In addition, the wound undergoes physical constriction throughout the wound-healing procedure, which is evidently facilitated by flexible fibroblasts (myofibroblasts) that are developed in the wound (Campos et al., 2008; Gosain & DiPietro, 2004).

2.3.2 Wound Healing Therapeutic Potential of Phytochemicals

The investigation of wound healing medications is an ongoing process in modern biomedical studies, and the exploration of plant-derived compounds represents a major chapter of this research. The utilization of herbs and their extracts dates back to the prehistoric cultures of the East as well as Africa and of Indigenous American and Indigenous South American civilizations. Over 50% of all medications in experimental use with a natural product source reveal the significance of plants as an origin of

medicinal agents (Davis & Perez, 2009). Wound healing stimulation by plant-derived products is mainly characterized by high admissibility and desirable toleration (Jagetia, Venkatesha, & Reddy, 2003). Although various natural products have been declared to possess therapeutic effects, many of them do not represent well-conducted scientific records and must be analyzed with regard to the active chemical combinations and clarification of the molecular process of their activities (Dulak, 2005).

The wound healing mechanism is stimulated by a number of natural products composed of active components such as flavonoids, triterpenes and alkaloids (Sumitra, Manikandan, & Suguna, 2005). Curcumin derived from *Curcuma longa*, asiaticoside from *Centella asiatica*, quercetin, isorhamnetin and kaempferol derivatives of *Hippophae rhamnoides*, phenolic acids from *Chromolaena odorata*, β-sitosterol and glycoprotein from the gel of *Aloe vera*, fukinolic acid and cimicifugic acids from *Cimicifuga sps*., proanthocyanidins and resveratrol derived from *Vitis vinifera*, oleanolic acid from *Anredera diffusa*, acylated iridoid glycosides extracted from *Scrophularia nodosa* and xyloglucan from *Tamarindus indicus* are a few of the significant plant-derived compounds effective in wound healing, which have been examined through *in vivo* trials (Ayyanar & Ignacimuthu, 2009).

While *in vitro* investigations are exceedingly critical to determine the primary suitable amount of possible compounds, further *in vivo* examinations are required to determine the impact of different factors such as the antimicrobial component in wound exudate, antimicrobial peptides and growth factors. Alongside the limited clinical experiments certifying their application for therapeutic involvement in wound remedies, further investigations are required with meticulous and reproducible compound preparations (Davis & Bouzari, 2004).

2.4 *Annona muricata*

A. muricata L., locally known as sirsak, paw-paw, guanabana, graviola and soursop, is a member of the Annonaceae family, which contains approximately 2,300 species and 130 genera (Leboeuf, Cavé, Bhaumik, Mukherjee, & Mukherjee, 1980; Mishra, Ahmad, Kumar, & Sharma, 2013). *A. muricata* belongs originally to the tropical countries in North and South America but currently has a wide distribution throughout subtropical and tropical areas of the world, including Nigeria, Malaysia and India (S. O. Adewole & Caxton-Martins, 2006). *A. muricata* is an erect, terrestrial evergreen tree of 5-8 m in height and shows a roundish, open canopy with dark green, glossy, large leaves. The heart-shaped green fruits of the tree are edible and reach 15-20 cm in diameter (Figure 2.4) (de Souza, Benassi, da Silva, Afonso, & Scarminio, 2009). There are numerous data in the literature showing various biological activities of *A. muricata*, which exhibits anti-arthritic (P. Chan, Ah, & Mh, 2010), anticancer (Asare et al., 2015), anticonvulsant (N'gouemo, Koudogbo, Tchivounda, Akono-Nguema, & Etoua, 1997), antidiabetic and hypolipidemic (Adeyemi, Komolafe, Adewole, Obuotor, & Adenowo, 2009), anti-inflammatory and anti-nociceptive (Hamid, Foong, Ahmad, & Hussain, 2012), antioxidant (George, Kumar, Suresh, & Kumar, 2014), antihypertensive (Nwokocha et al., 2012), antiparasitic (Osorio et al., 2007), hepatoprotective and bilirubin-lowering (Arthur, Terlabi, Larbie, & Woode, 2012), insecticidal (Ribeiro, Akhtar, Vendramim, & Isman, 2014) and molluscicidal activities (Dos Santos & Sant'Ana, 2001).

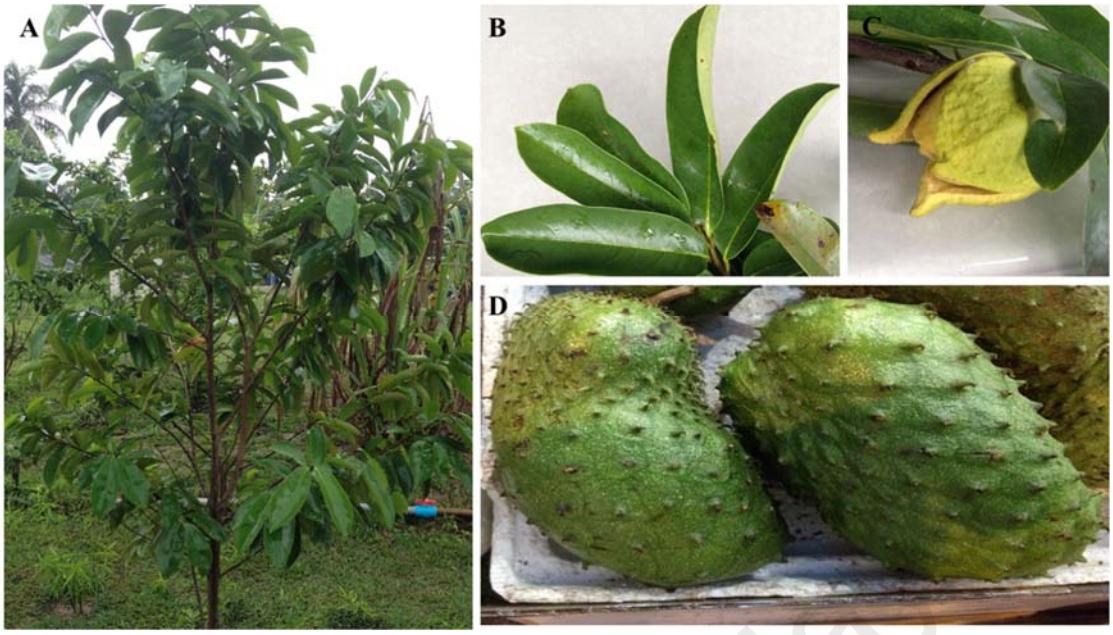


Figure 2.4: (A) Whole tree, (B) leaves, (C) flowers and (D) fruits of *A. muricata*.

2.4.1 Ethnomedicinal Usage of *A. muricata*

Different parts of the *A. muricata* tree, like other *Annona* species such as *A. reticulata* and *A. squamosa,* are prevalently employed in ethnomedicine for various diseases and ailments, especially parasitic infections and cancer. The leaves are used to treat headaches, diabetes, insomnia and cystitis. In addition, neuralgic and anti-rheumatic effects have also been attributed to the internal administration of the leaf's decoction, while topical administration of the cooked leaves is used against rheumatism and abscesses. As a natural medicine, the fruits are used for skin rushes and parasites, and they are also consumed to augment a mother's milk after childbirth (S. O. Adewole & Caxton-Martins, 2006; De Sousa, Vieira, De Pinho, Yamamoto, & Alves, 2010; Mishra et al., 2013). The crushed seeds are considered to possess anthelmintic effects against parasites, internal and external worms. In tropical Africa, the plant is used as an astringent, insecticide and piscicide agent and to treat coughs, pain and skin diseases. In tropical areas of Africa such as Nigeria and in South America, *A. muricata* leaves are

used as a traditional medicine against cancer and tumors (S. Adewole & Ojewole, 2009). In Malaysia, the combination of *A. muricata* crushed leaves with *Hibiscus rosa-sinensis* and *A. squamosa* is employed to protect against fainting (Ong & Norzalina, 1999). In India, the leaves and root-bark are believed to possess anthelmintic and antiphlogistic effects, while the flower and fruit are used as a catarrh treatment (S. Adewole & Ojewole, 2009; Watt & Breyer-Bnodwijk, 1962). Moreover, hypotensive, sedative, antispasmodic, smooth muscle relaxant, hypoglycemic and anti-inflammatory effects have also been reported for the roots, leaves and bark of *A. muricata* (S. O. Adewole & Caxton-Martins, 2006; Mishra et al., 2013). In addition to its traditional usage, there is widespread use of the fruits in the production of shakes, ice creams, syrups, candy and beverages (Jaramillo-Flores & Hernandez-Sanchez, 2000; Wu, Gu, et al., 1995).

2.4.2 Phytochemicals of *A. muricata*

Thorough phytochemical research on all portions of the *A. muricata* tree has revealed the existence of different classes of compounds, namely phenolics (PLs) (Jiménez, Gruschwitz, Schweiggert, Carle, & Esquivel, 2014), alkaloids (ALKs) (Leboeuf et al., 1980; Leboeuf et al., 1981), flavonol triglycosides (FTGs) (Nawwar et al., 2012), megastigmanes (MGs) (Matsushige, Matsunami, Kotake, Otsuka, & Ohta, 2012), essential oils and cyclopeptides (CPs) (Figure 2.4, Table 2.1) (Kossouoh, Moudachirou, Adjakidje, Chalchat, & Figuérédo, 2007; Pélissier et al., 1994). However, like other *Annona* species, *A. muricata* is well-known to possess a variety of acetogenin compounds (AGEs) (Rupprecht, Hui, & McLaughlin, 1990). In addition, as a rich source of minerals, including Fe, Na, K, Mg and Cu, the *A. muricata* fruit can supply certain required elements and essential nutrients for the human body (Gyamfi et al., 2011).

2.4.3 Essential Oil of *A. muricata*

A phytochemical investigation on *A. muricata* leaves gathered from Vietnam reported major volatile oil constituents of *β*-elemene (9.1%), α-pinene (9.4%), *ρ*-mentha-2,4(8)-diene (9.8%), germacrene D (18.1%) and *β*-pinene (20.6%) (Thang, Dai, Hoi, & Ogunwande, 2013). In addition, α-cadinol, epi-α-cadinol and δ-cadinene have been identified as other significant phytoconstituents in the leaf oil extracts (Kossouoh et al., 2007). GC analysis of *A. muricata* leaves gathered from Cameroon revealed the existence of mostly sesquiterpenes, with β-caryophyllene as the major compound (Fekam Boyom, Amvam Zollo, Menut, Lamaty, & Bessière, 1996). The essential oil of the fruit pulp contained esters of aliphatic acids with significant phytoconstituents of 2-hexenoic acid ethyl ester and 2-hexenoic acid methyl ester. Moreover, high concentrations of mono- and sesquiterpenes such as linalool, 1,8-cineole and β-caryophyllene were identified in the fruit pulp (Jirovetz, Buchbauer, & Ngassoum, 1998).

2.4.4 Annonaceous Acetogenins of *A. muricata*

AGEs are a unique class of C-35/C37 secondary metabolites derived from long chain (C-32/C34) fatty acids in the polyketide pathway. They are usually characterized by a combination of fatty acids with a 2-propanol unit at C-2 that forms a methyl-substituted α,β-unsaturated γ-lactone (Alali, Liu, & McLaughlin, 1999). Since the isolation of uvaricin from *Uvaria accuminata* in 1982, over 500 AGEs have been characterized from various members of the Annonaceae family (McLaughlin, 2008; Tempesta, Kriek, & Bates, 1982). Due to their broad biological activities and unique chemical structures, AGEs have attracted substantial scientific attention over the past few decades. A wide array of biological activities such as pesticidal, antiparasitic and antimalarial activity have been reported for AGEs (Alali et al., 1999; Carmen Zafra-Polo, Figadère, Gallardo, Tormo, & Cortes, 1998). However, the biological characterization of AGEs is generally evaluated based on suppressive activity against the mitochondrial complex I and cancer

cells (Chih, Chiu, Tang, Chang, & Wu, 2001; Zafra-Polo, González, Estornell, Sahpaz, & Cortes, 1996). Extensive biological and phytochemical research on *A. muricata* has led to the isolation of more than 100 AGEs, as shown in Table 2.1 and Figure 2.5.

Table 2.1: *Annona muricata* isolated chemical compounds. CP: cyclopeptide; PL: phenolic; AGE: annonaceous acetogenin; ALK: alkaloid; FTG: flavonol triglycoside; MG: megastigmane.

Plant part	Compound	Class	Biological activity	References
Fruits	annonaine	ALK	anti-depressive	(Hasrat, Bruyne, BACKER, Vauquelin, & Vlietinck, 1997; Hasrat, Pieters, De Backer, Vauquelin, & Vlietinck, 1997)
Fruits	nornuciferine	ALK	anti-depressive	(Hasrat, Bruyne, et al., 1997; Hasrat, Pieters, et al., 1997)
Fruits	asimilobine	ALK	anti-depressive	(Hasrat, Bruyne, et al., 1997; Hasrat, Pieters, et al., 1997)
Fruits	epomusenin-A	AGE	-	(Melot, Fall, Gleye, & Champy, 2009)
Fruits	epomusenin-B	AGE	-	(Melot et al., 2009)
Fruits	epomurinin-A	AGE	-	(Melot et al., 2009)
Fruits	epomurinin-B	AGE	-	(Melot et al., 2009)
Fruits	*cis*-annoreticuin	AGE	-	(Ragasa, Soriano, Torres, Don, & Shen, 2012)
Fruits	muricin J	AGE	toxicity against PC-3 cancer cells	(Sun, Liu, Kadouh, Sun, & Zhou, 2014)
Fruits	muricin K	AGE	toxicity against PC-3 cancer cells	(Sun et al., 2014)
Fruits	muricin L	AGE	toxicity against PC-3 cancer cells	(Sun et al., 2014)
Fruits	cinnamic acid derivative	PL	-	(Jiménez et al., 2014)
Fruits	coumaric acid hexose	PL	-	(Jiménez et al., 2014)
Fruits	5-caffeoylquinic acid	PL	-	(Jiménez et al., 2014)
Fruits	dihydrokaempferol-hexoside	PL	-	(Jiménez et al., 2014)
Fruits	*p*-coumaric acid	PL	-	(Jiménez et al., 2014)

Fruits	caffeic acid derivative	PL	-	(Jiménez et al., 2014)
Fruits	dicaffeoylquinic acid	PL	-	(Jiménez et al., 2014)
Fruits	feruloylglycoside	PL	-	(Jiménez et al., 2014)
Fruits	4-feruloyl-5-caffeoylquinic acid	PL	-	(Jiménez et al., 2014)
Fruits	*p*-coumaric acid methyl ester	PL	-	(Jiménez et al., 2014)
Leaves, Pericarp	annomuricin A	AGE	toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Jaramillo, Arango, Gonzalez, Robledo, & Velez, 2000; Wu, Gu, et al., 1995)
Leaves	annomuricin B	AGE	toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Wu, Gu, et al., 1995)
Leaves	annomuricin C	AGE	toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Wu, Zeng, et al., 1995b)
Leaves	annomuricin E	AGE	toxicity against HT-29 and PACA-2 cancer cells	(Kim, Zeng, Alali, Rogers, Wu, McLaughlin, et al., 1998)
Leaves	annomutacin	AGE	toxicity against A549 cancer cells	(Wu, Zhao, et al., 1995)
Leaves	(2,4-*cis*)-10*R*-annonacin-A-one	AGE	toxicity against A549 cancer cells	(Wu, Zhao, et al., 1995)
Leaves	(2,4-*trans*)-10*R*-annonacin-A-one	AGE	toxicity against A549 cancer cells	(Wu, Zhao, et al., 1995)
Leaves	annohexocin	AGE	toxicity against brine shrimp and different cancer cells	(Zeng, Wu, & McLaughlin, 1995)
Leaves	muricapentocin	AGE	toxicity against HT-29 and PACA-2 cancer cells	(Kim, Zeng, Alali, Rogers, Wu, McLaughlin, et al., 1998)
Leaves	(2,4-*cis*)-isoannonacin	AGE	-	(Wu, Zeng, et al., 1995a)
Leaves, Seeds	(2,4-*trans*)-isoannonacin	AGE	-	(D.-Y. Li et al., 2001; Wu, Zeng, et al., 1995a)
Leaves	muricatocin A	AGE	toxicity against A549 cancer cells	(Wu, Zeng, et al., 1995a)
Leaves	muricatocin B	AGE	toxicity against A549 cancer cells	(Wu, Zeng, et al., 1995a)
Leaves	muricatocin C	AGE	toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Wu, Zeng, et al., 1995b)

Leaves, Seeds	gigantetronenin	AGE	-	(D.-Y. Li et al., 2001; Wu, Zeng, et al., 1995b)
Leaves, Seeds, Pericarp	annonacin A	AGE	-	(Jaramillo et al., 2000; Wu, Zeng, et al., 1995a; Yu, Gui, Luo, & Sun, 1998)
Leaves	annopentocin A	AGE	toxicity against PACA-2 cancer cells	(Zeng, Wu, Oberlies, McLaughlin, & Sastrodihadjo, 1996)
Leaves	annopentocin B	AGE	toxicity against A549 cancer cells	(Zeng et al., 1996)
Leaves	annopentocin C	AGE	toxicity against A549 cancer cells	(Zeng et al., 1996)
Leaves	*cis*-annomuricin-D-one	AGE	toxicity against PACA-2, HT-29 and A549 cancer cells	(Zeng et al., 1996)
Leaves	*trans*-annomuricin-D-one	AGE	toxicity against PACA-2, HT-29 and A549 cancer cells	(Zeng et al., 1996)
Leaves	murihexocin A	AGE	toxicity against different cancer cells	(Zeng, Wu, Gu, & McLaughlin, 1995)
Leaves	murihexocin B	AGE	toxicity against different cancer cells	(Zeng, Wu, Gu, et al., 1995)
Leaves	murihexocin C	AGE	toxicity against different cancer cells	(Kim, Zeng, Alali, Rogers, Wu, Sastrodihardjo, et al., 1998)
Leaves	muricoreacin	AGE	toxicity against different cancer cells	(Kim, Zeng, Alali, Rogers, Wu, Sastrodihardjo, et al., 1998)
Leaves	*cis*-corossolone	AGE	toxicity against human hepatoma cells	(Liaw et al., 2002)
Leaves	annocatalin	AGE	toxicity against human hepatoma cells	(Liaw et al., 2002)
Leaves	annocatacin B	AGE	toxicity against human hepatoma cells	(Chang et al., 2003)
Leaves	anonaine	ALK	neurotoxic	(Fofana, Ziyaev, Abdusamatov, & Zakirov, 2011; Matsushige, Kotake, et al., 2012)
Leaves	isolaureline	ALK	-	(Fofana et al., 2011)

Leaves	xylopine	ALK	-	(Fofana et al., 2011)
Leaves	Quercetin 3-*O*-α-rhamnosyl-(1→ 6)-β-sophoroside	FTG	-	(Nawwar et al., 2012)
Leaves	gallic acid	FTG	-	(Nawwar et al., 2012)
Leaves	epicatechine	FTG	-	(Nawwar et al., 2012)
Leaves	quercetin 3-*O*-rutinosid	FTG	-	(Nawwar et al., 2012)
Leaves	quercetin 3-*O*-neohispredoside	FTG	-	(Nawwar et al., 2012)
Leaves	quercetin 3-*O*-robinoside	FTG	-	(Nawwar et al., 2012)
Leaves	catechine	FTG	-	(Nawwar et al., 2012)
Leaves	chlorogenic acid	FTG	-	(Nawwar et al., 2012)
Leaves	argentinine (1-N,N-dimethylethanyl-4,6-dimethoxy-3,8-dihydroxy-phenanthrene)	FTG	-	(Nawwar et al., 2012)
Leaves	kaempferol 3-*O*-rutinoside	FTG	-	(Nawwar et al., 2012)
Leaves	quercetin 3-*O*-glucoside	FTG	-	(Nawwar et al., 2012)
Leaves	quercetin	FTG	-	(Nawwar et al., 2012)
Leaves	kaempferol	FTG	-	(Nawwar et al., 2012)
Leaves	annonamine	ALK	-	(Matsushige, Kotake, et al., 2012)
Leaves	(*S*)-norcorydine	ALK	-	(Matsushige, Kotake, et al., 2012)
Leaves	(*R*)-4′-*O*-methylcoclaurine	ALK	-	(Matsushige, Kotake, et al., 2012)
Leaves	(*R*)-*O*,*O*-dimethylcoclaurine	ALK	-	(Matsushige, Kotake, et al., 2012)
Leaves	annoionol A	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	annoionol B	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	annoionol C	MG	-	(Matsushige, Matsunami, et al., 2012)

Leaves	annoionoside	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	vomifoliol	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	roseoside	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	turpinionoside A	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	citroside A	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	blumenol C	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	(+)-epiloliolide	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	loliolide	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	(1*S*,2*S*,4*R*)-*trans*-2-hydroxy-1,8-cineole *β*-D-glucopyranoside	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	(Z)-3-hexenyl *β*-D-glucopyranoside	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	rutin	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	kaempferol 3-*O*-rutinoside	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	kaempferol 3-*O*-robinobioside	MG	-	(Matsushige, Matsunami, et al., 2012)
Leaves	kaempferol 3-*O*-*β*-D-(2″-*O*-*β*-D-glucopyranosyl,6″-*O*-α-L-rhamnopyranosyl)glucopyranoside	MG	-	(Matsushige, Matsunami, et al., 2012)
Roots	montecristin	AGE	-	(C Gleye et al., 1997)
Roots	cohibin A	AGE	-	(Christophe Gleye et al., 1997)
Roots	cohibin B	AGE	-	(Christophe Gleye et al., 1997)
Roots	*cis*-solamin	AGE	-	(Gleye, Duret, Laurens, Hocquemiller, & Cavé, 1998)

Roots	*cis*-panatellin	AGE	-	(Christophe Gleye et al., 1998)
Roots	*cis*-uvariamicin IV	AGE	-	(Christophe Gleye et al., 1998)
Roots	*cis*-uvariamicin I	AGE	-	(Christophe Gleye et al., 1998)
Roots	*cis*-reticulatacin	AGE	-	(Christophe Gleye et al., 1998)
Roots	*cis*-reticulatacin-10-one	AGE	-	(Christophe Gleye et al., 1998)
Roots	chatenaytrienin 1	AGE	-	(Christope Gleye et al., 1998)
Roots	chatenaytrienin 2	AGE	-	(Christope Gleye et al., 1998)
Roots	chatenaytrienin 3	AGE	-	(Christope Gleye et al., 1998)
Roots	muridienin 3	AGE	-	(Christope Gleye et al., 1998)
Roots	muridienin 4	AGE	-	(Christope Gleye et al., 1998)
Roots	muricadienin	AGE	-	(Christope Gleye et al., 1998)
Roots	coronin	AGE	-	(Gleye, Akendengue, Laurens, & Hocquemiller, 2001)
Roots, Fruits	sabadelin	AGE	-	(Gleye, Laurens, Laprévote, Serani, & Hocquemiller, 1999; Ragasa et al., 2012)
Seeds	murisolin	AGE	-	(MYINT et al., 1990)
Seeds	muricatacin	AGE	toxicity against HT-29, MCF-7 and A549 cancer cells	(Rieser, Kozlowski, Wood, & McLaughlin, 1991)
Seeds, Leaves, Pericarp	annonacin	AGE	neurotoxic, molluscicidal, inhibitor of mitochondrial complex I	(Champy et al., 2004; Escobar-Khondiker et al., 2007; Jaramillo et al., 2000; Luna et al., 2006; Rieser et al., 1991; Wu, Gu, et al., 1995)
Seeds, Leaves	corossolone	AGE	toxicity against KB cancer cells and brine shrimp larva, antileishmanial	(Cortes et al., 1991; Liaw et al., 2002; Vila-Nova et al., 2013; Vila-Nova et al., 2011)
Seeds	corossolin	AGE	toxicity against KB cancer cells and brine shrimp larva	(Cortes et al., 1991)

Seeds, Roots, Leaves	solamin	AGE	toxicity against KB and VERO cancer cells	(Christophe Gleye et al., 1998; Hla Myint et al., 1991; Liaw et al., 2002)
Seeds	corepoxylone	AGE	-	(Gromek, Figadère, Hocquemiller, Cavé, & Cortes, 1993)
Seeds, Leaves	annonacin-10-one	AGE	-	(Rieser, Fang, Rupprecht, et al., 1993; Wu, Gu, et al., 1995)
Seeds	isoannonacin	AGE	molluscicidal, anticancer	(Luna et al., 2006; Rieser, Fang, Rupprecht, et al., 1993)
Seeds	isoannonacin-10-one	AGE	-	(Rieser, Fang, Rupprecht, et al., 1993)
Seeds, Leaves	goniothalamicin	AGE	molluscicidal	(Luna et al., 2006; Rieser, Fang, Rupprecht, et al., 1993; Wu, Gu, et al., 1995)
Seeds	gigantetrocin	AGE	-	(Rieser, Fang, Rupprecht, et al., 1993)
Seeds, Leaves	gigantetrocin A	AGE	toxicity against HT-29 cancer cells	(D.-Y. Li et al., 2001; Rieser, Fang, Anderson, et al., 1993; Wu, Gu, et al., 1995)
Seeds	gigantetrocin B	AGE	toxicity against HT-29 cancer cells	(D.-Y. Li et al., 2001; Rieser, Fang, Anderson, et al., 1993; Wu, Gu, et al., 1995)
Seeds, Leaves	muricatetrocin A	AGE	toxicity against HT-29 cancer cells	(Rieser, Fang, Anderson, et al., 1993)
Seeds, Leaves	muricatetrocin B	AGE	toxicity against HT-29 cancer cells	(Rieser, Fang, Anderson, et al., 1993)
Seeds, Leaves	epomuricenin A	AGE	-	(Melot et al., 2009; Roblot, Laugel, Lebœuf, Cavé, & Laprévote, 1993)
Seeds, Leaves	epomuricenin B	AGE	-	(Melot et al., 2009; Roblot et al., 1993)
Seeds	annomuricatin A	CP	-	(Chao-Ming et al., 1998; C.-M. Li et al., 1995)
Seeds	annocatacin A	AGE	toxicity against human hepatoma cells	(Chang et al., 2003)

Seeds	annomuricatin C	CP	-	(Wélé et al., 2004)
Seeds	*cis*-annonacin	AGE	crown gall tumor inhibition, toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Rieser et al., 1996)
Seeds	*cis*-annonacin-10-one	AGE	crown gall tumor inhibition, toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Rieser et al., 1996)
Seeds	*cis*-goniothalamicin	AGE	crown gall tumor inhibition, toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Rieser et al., 1996)
Seeds	arianacin	AGE	crown gall tumor inhibition, toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Rieser et al., 1996)
Seeds	javoricin	AGE	crown gall tumor inhibition, toxicity against brine shrimp, HT-29, MCF-7 and A549 cancer cells	(Rieser et al., 1996)
Seeds	murihexol	AGE	-	(Yu et al., 1998)
Seeds	donhexocin	AGE	-	(Yu et al., 1998)
Seeds	cohibin C	AGE	-	(Gleye et al., 2000)
Seeds	cohibin D	AGE	-	(Gleye et al., 2000)
Seeds	muricatenol	AGE	-	(De Yu, YU, Xiu Zhen, Lan, & YANG, 2000; D.-Y. Li et al., 2001)
Seeds	2,4-*cis*-gigantetrocinone	AGE	-	(D.-Y. Li et al., 2001)
Seeds	2,4-*trans*-gigantetrocinone	AGE	-	(D.-Y. Li et al., 2001)
Seeds	2,4-trans-isoannonacin-10-one	AGE	-	(D.-Y. Li et al., 2001)
Seeds	annomontacin	AGE	-	(D.-Y. Li et al., 2001)
Seeds	longifolicin	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)
Seeds	muricin A	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)

Seeds	muricin B	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)
Seeds	muricin C	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)
Seeds	muricin D	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)
Seeds	muricin E	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)
Seeds	muricin F	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)
Seeds	muricin G	AGE	toxicity against human hepatoma cells	(Chang & Wu, 2001)
Seeds	muricin H	AGE	toxicity against human hepatoma cells	(Liaw et al., 2002)
Seeds	muricin I	AGE	toxicity against human hepatoma cells	(Liaw et al., 2002)
Seeds	*cis*-annomontacin	AGE	toxicity against human hepatoma cells	(Liaw et al., 2002)
Seeds, Leaves	annonacinone	AGE	-	(Liaw et al., 2002)
Seeds	xylomaticin	AGE	-	(Liaw et al., 2002)
Seeds	*N*-fatty acyl tryptamines	ALK	-	(D.-Y. Li et al., 2001)
Seeds	annoreticuin-9-one	AGE	-	(Ragasa et al., 2012)
Stem barks	epoxymurin A	AGE	-	(Hisham et al., 1993)
Stem barks	epoxymurin B	AGE	-	(Hisham et al., 1993)
Leaves, Roots, Stems, Barks	reticuline	ALK	-	(Leboeuf et al., 1981)
Leaves, Roots, Stems, Barks	coclaurine	ALK	-	(Leboeuf et al., 1981)
Leaves, Roots, Stems, Barks	coreximine	ALK	-	(Leboeuf et al., 1981)
Leaves, Roots, Stems, Barks	atherosperminine	ALK	-	(Leboeuf et al., 1981)

Leaves, Roots, Stems, Barks	stepharine	ALK	-	(Leboeuf et al., 1981)
Leaves, Roots, Stems, Barks	anomurine	ALK	-	(Leboeuf et al., 1981)
Leaves, Roots, Stems, Barks	anomuricine	ALK	-	(Leboeuf et al., 1981)

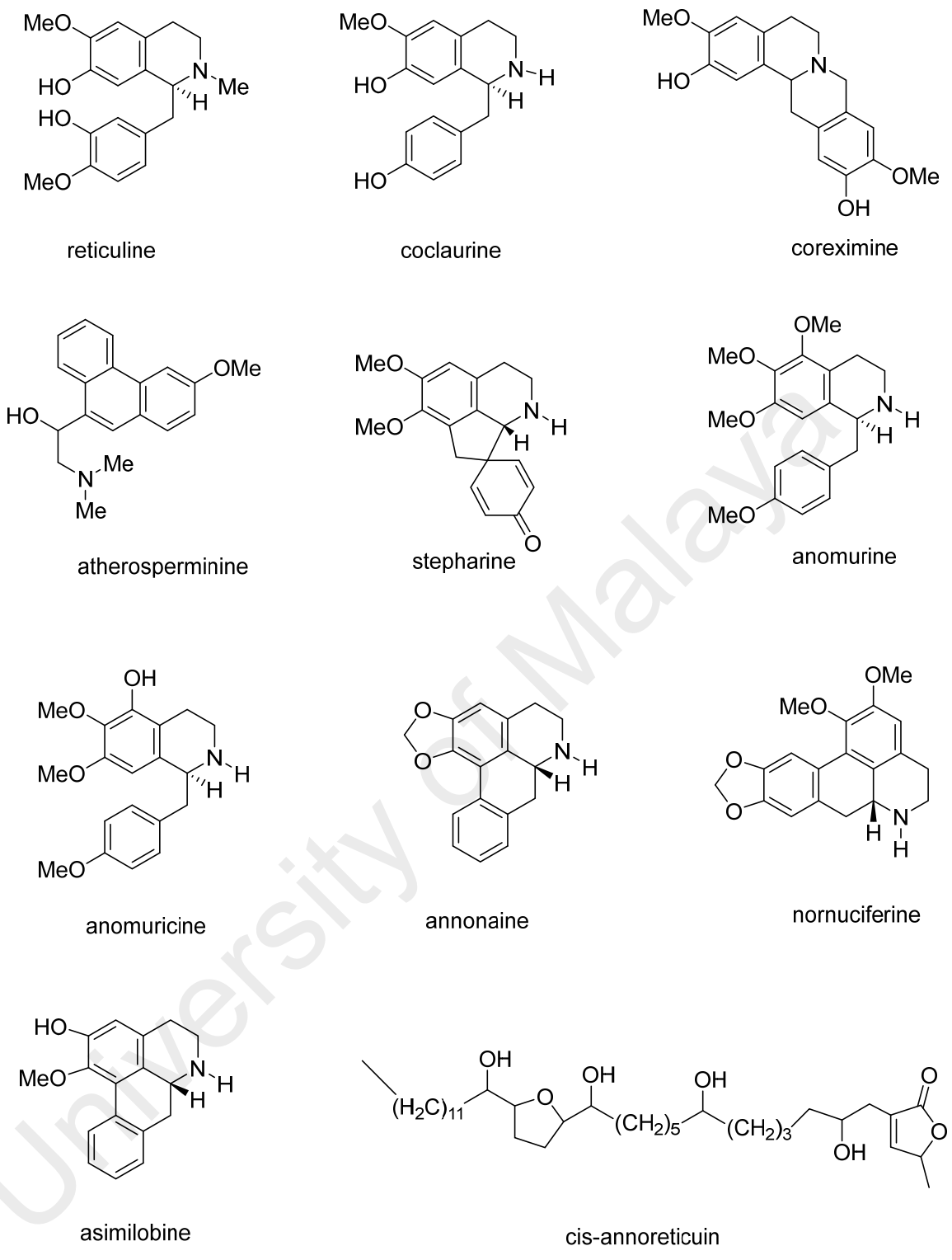
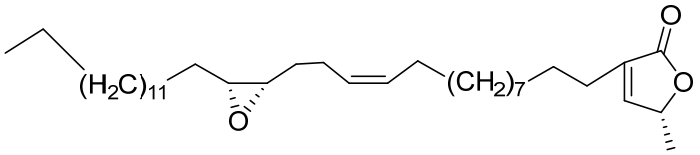
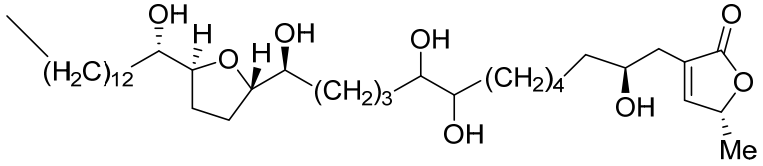


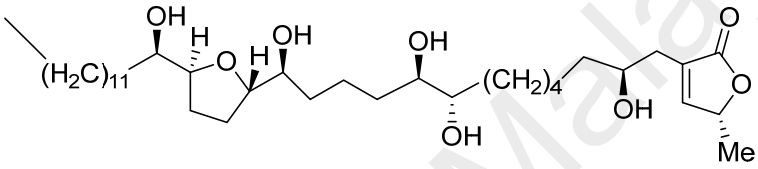
Figure 2.5: Structures of the major chemical compounds of *Annona muricata*.



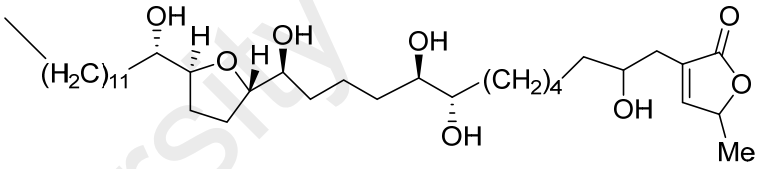
sabadelin



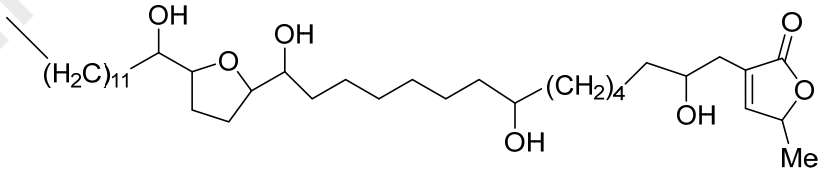
annomuricin A



annomuricin B

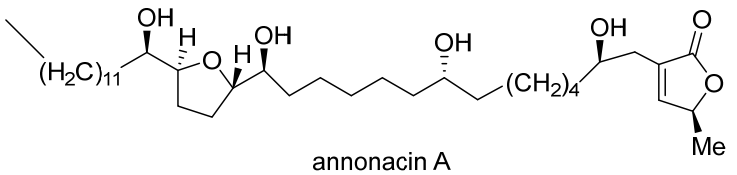


annomuricin E

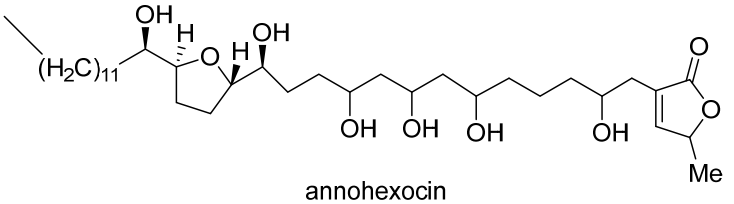


annomutacin

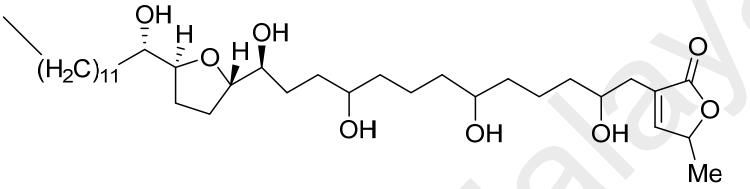
Figure 2.5, continued



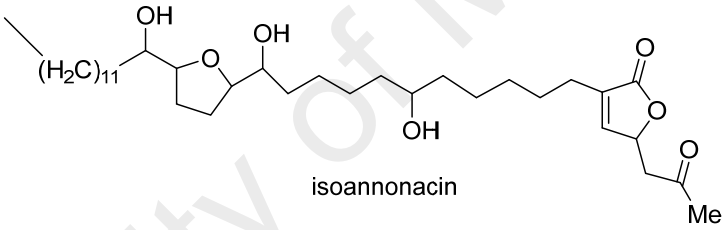
annonacin A



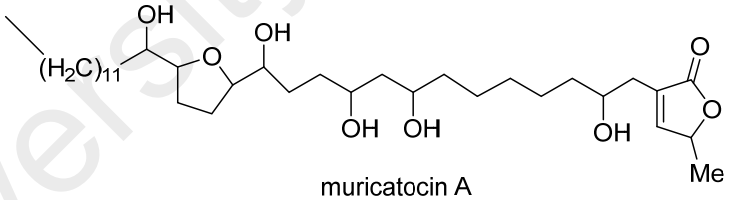
annohexocin



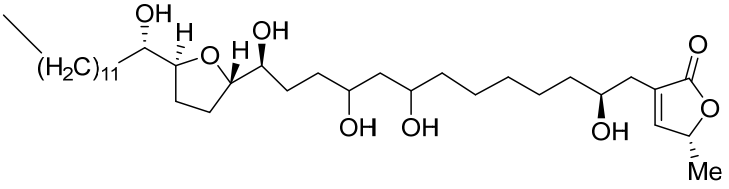
muricapentocin



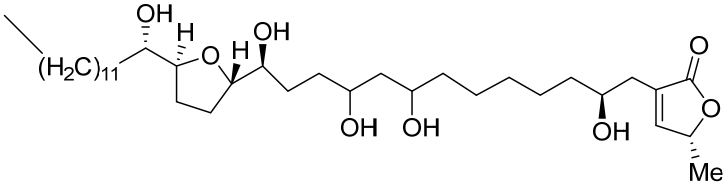
isoannonacin



muricatocin A

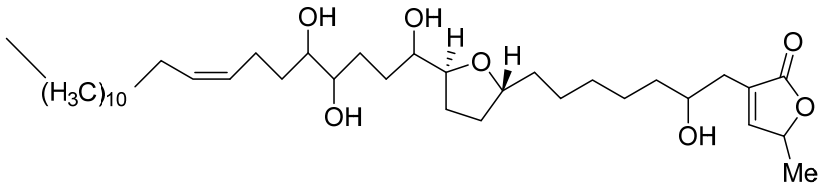


muricatocin B

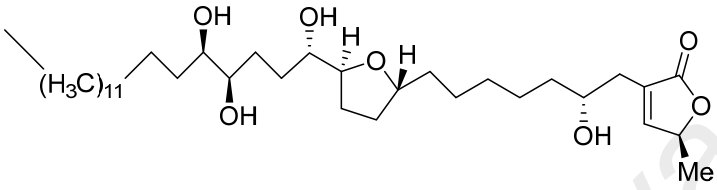


muricatocin C

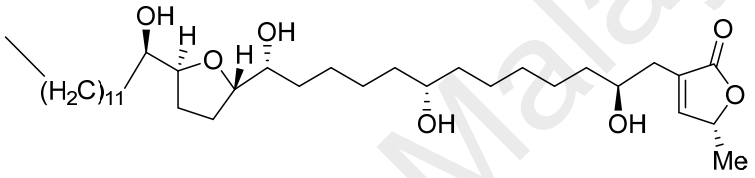
Figure 2.5, continued



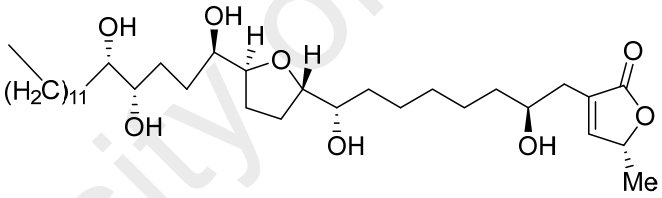
gigantetronenin



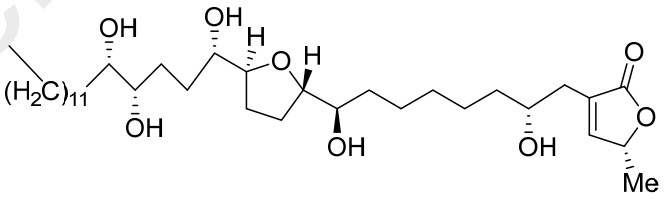
gigantetrocin A



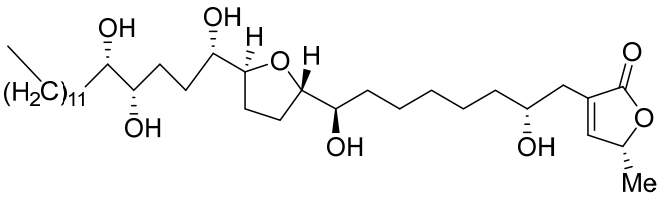
annonacin A



annopentocin A

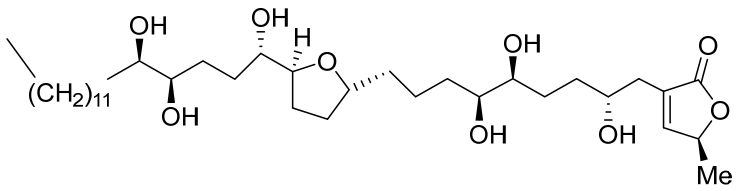


annopentocin B

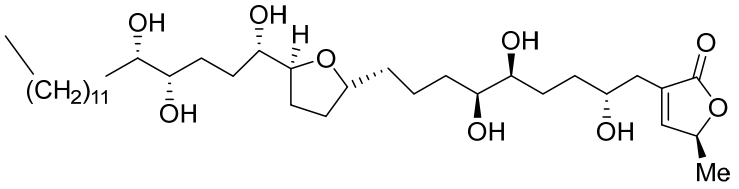


annopentocin C

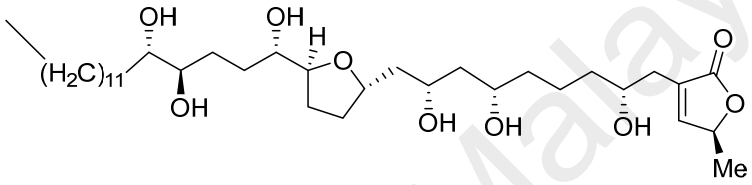
Figure 2.5, continued



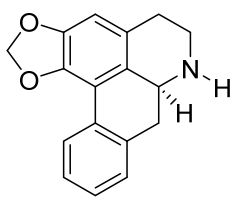
murihexocin A



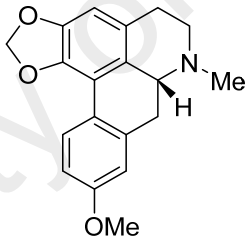
murihexocin B



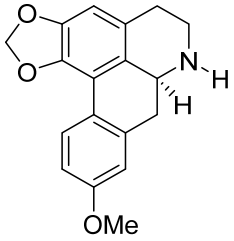
muricoreacin



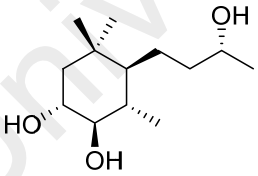
anonaine



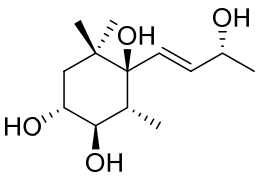
isolaureline



xylopine



annoionol A



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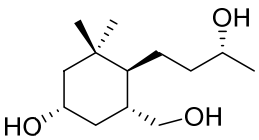

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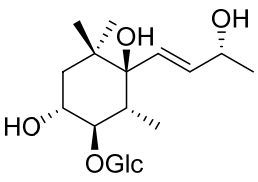
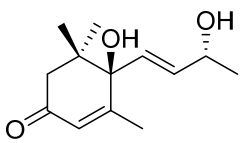
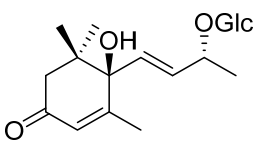


annoionoside

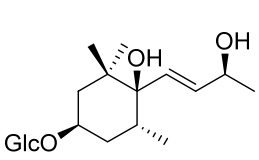


vomifoliol

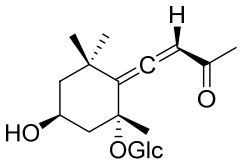


roseoside

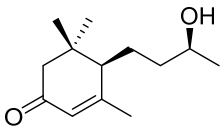
Figure 2.5, continued



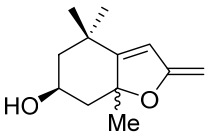
turpinionoside A



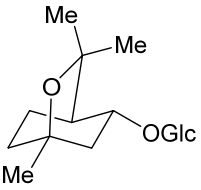
citroside A



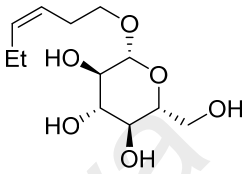
blumenol C



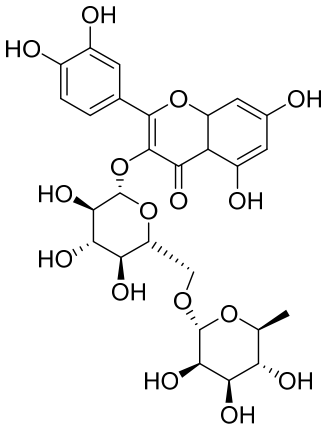
(+)-epiloliolide



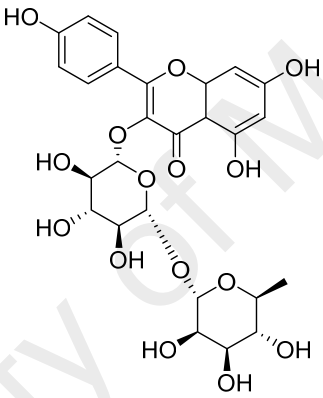
(1S,2S,4R)-trans-2-hydroxy-1,8-cineole D-glucopyranoside



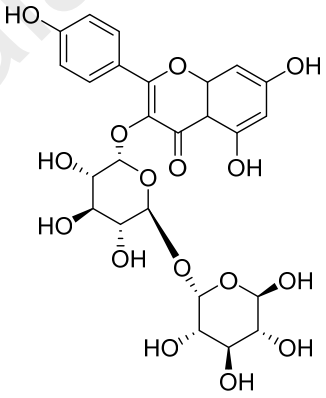
(Z)-3-hexenyl -D-glucopyranoside



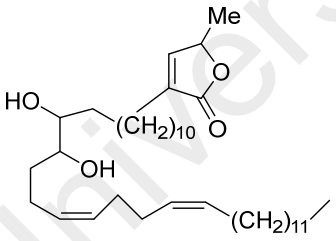
rutin



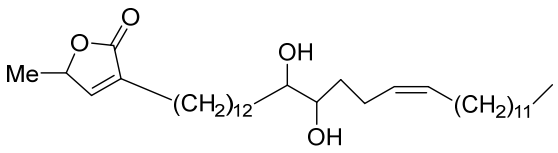
kaempferol 3-O-rutinoside



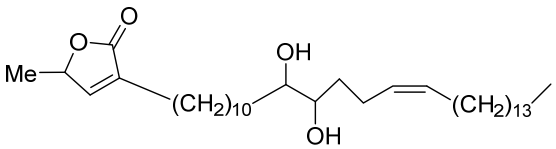
kaempferol 3-O-robinobioside



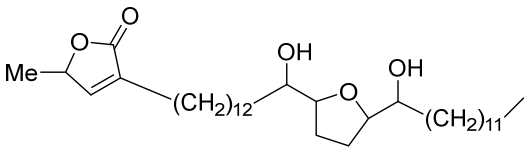
montecristin



cohibin A



cohibin B



cis-solamin

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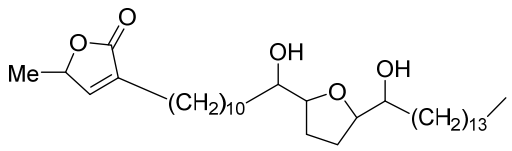
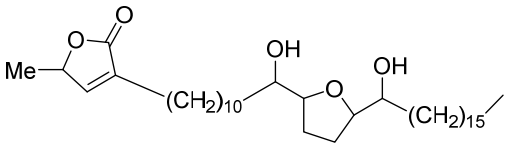
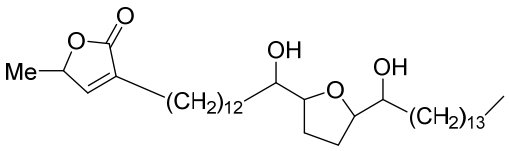


cis-panatellin



cis-uvariamicin IV



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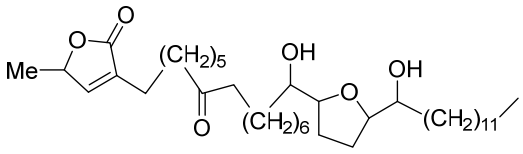
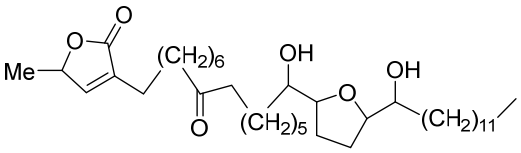


cis-reticulatacin



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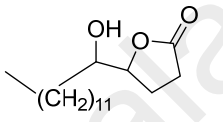
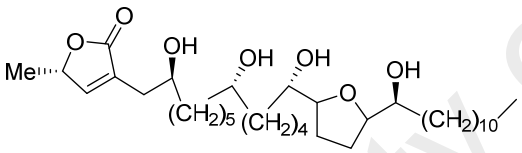
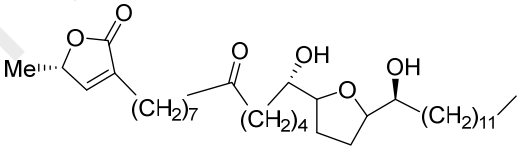


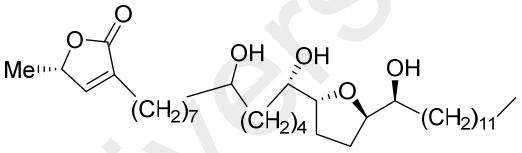
muricatacin



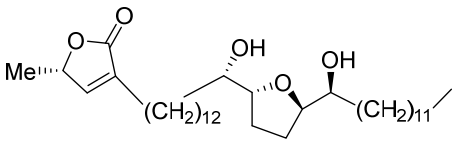
annonacin



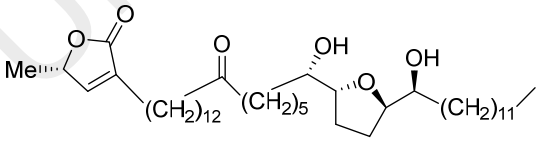
corossolone



corossolin



solamin



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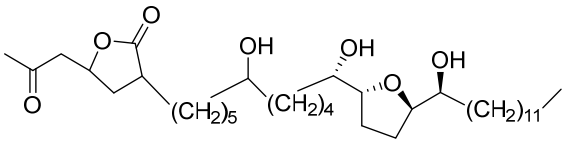
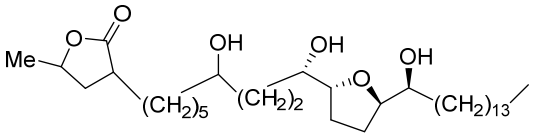


isoannonacin

Figure 2.5, continued



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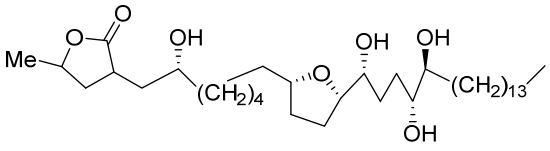
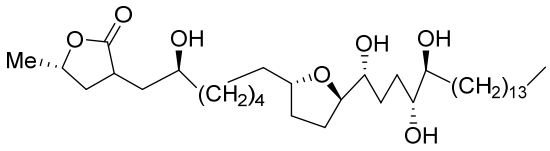


gigantetrocin



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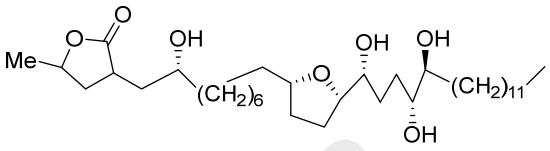
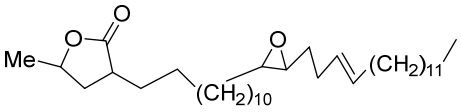


muricatetrocin A



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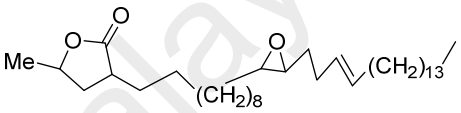
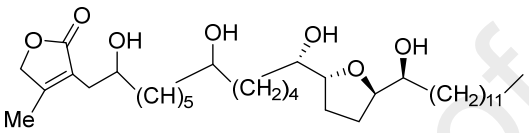


epomuricenin B



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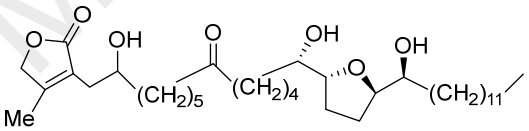

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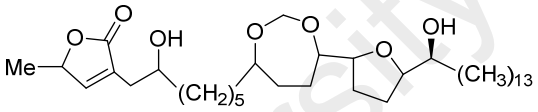

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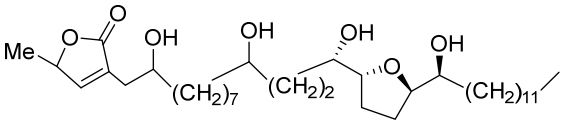

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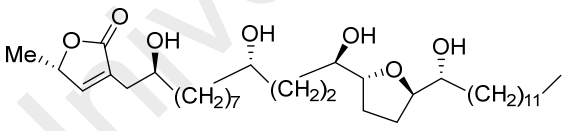

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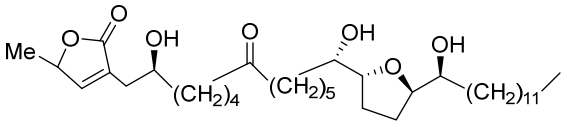

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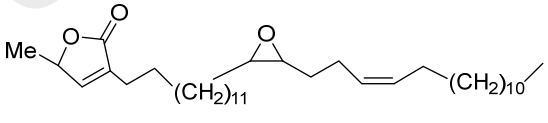

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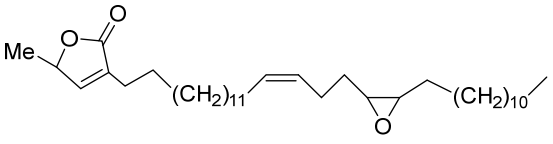

epoxymurin B

Figure 2.5, continued

2.4.5 Anticancer Activity of *A. muricata*

Describing *A. muricata* as "the cancer killer" highlights the marked anticancer potential of this plant and AGEs (Mishra et al., 2013). Extensive research has reported the substantial cytotoxic activity of *A. muricata* and its identified AGEs against different cancer cells (Arroyo, Prashad, Vásquez, Li, & Tomás, 2005; Astirin, Artanti, Fitria, Perwitasari, & Prayitno, 2013; Gavamukulya, Abou-Elella, Wamunyokoli, & AEl-Shemy, 2014; George, Kumar, Rajkumar, Suresh, & Kumar, 2012; Jaramillo et al., 2000). Nonetheless, only limited investigations have tried to clarify the molecular mechanisms of this anticancer potential (Table 2.2). A recent study on the ethanolic extract of *A. muricata* leaves showed caspase-3 activation upon treatment in myelogenous leukemic K562 cells, suggesting apoptosis induction, which was substantiated by the TUNEL assay (Ezirim et al., 2013). The anticancer potential of the *A. muricata* leaf water extract was also determined against rat prostates and the benign prostatic hyperplasia (BPH-1) cell line. The *in vitro* findings demonstrated an IC_{50} value of 1.36 mg/ml against BPH-1 cells after 72 hours. The extract also caused the down-regulation of Bcl-2 and up-regulation of Bax, as assessed by gene analysis. Upon *in vivo* testing, prostate size in rats was reduced after administration of the extract (30 and 300 mg/ml), which was proposed to occur through apoptosis induction (Asare et al., 2015). Another investigation on 7, 12-dimethylbenzene anthracene (DMBA)-induced cell proliferation in mouse breast tissues also confirmed this anticancer activity, implying notable antitumor potential against breast carcinogenesis (Minari & Okeke, 2014). In addition, skin papillomagenesis induced by croton oil and DMBA in mice was inhibited by *A. muricata* leaves (30 mg/kg) at the initiation and promotion stages (Hamizah et al., 2012). *A. muricata* anticancer and antitumor research has not been restricted solely to *in vitro* and *in vivo* studies. A case study on a 66-year old woman demonstrated that the concomitant intake of Xeloda and boiled *A. muricata* leaves stabilized metastatic breast cancer (Hansra, Silva, Mehta, &

Ahn, 2014). The promising antitumor and anticancer potential of *A. muricata* leaves has resulted in tablet formulations to be consumed as a complementary therapy against cancer (Elisya, Kardono, & Simanjuntak, 2014).

Table 2.2: Anticancer studies on *A. muricata*.

Plant part	Subject of study	Effect	Reference
water extract of the leaves	rat's prostate	reduction of prostate size	(Asare et al., 2015)
ethanolic extract of the leaves	breast tissues of mice	prevention of DMBA-induced DNA damage	(Minari & Okeke, 2014)
ethanolic extract of the leaves	DMBA/croton oil induced mice skin papillomagenesis	suppression of tumor initiation and promotion	(Hamizah et al., 2012)
ethanolic extract of the leaves	DMH induced colon cancer	reduction of ACF formation	(Eggadi et al., 2014)
ethanolic extract of the leaves	K562	induction of apoptosis	(Ezirim et al., 2013)
leaves boiled in water	metastatic breast cancer	stabilization of disease	(Hansra et al., 2014)

2.4.6 Anti-inflammatory and Anti-nociceptive Activities of *A. muricata*

Oral treatment in rats with *A. muricata* ethanolic leaf extracts (10, 30, 100 and 300 mg/kg) significantly reduced carrageenan-induced edema in rat paws by 79% in a dose-dependent manner, demonstrating its anti-inflammatory activities (Roslida, Tay, Zuraini, & Chan, 2010). This anti-inflammatory effect was accompanied by reductions in leukocyte migration and exudate volume (De Sousa et al., 2010). Oral administration of the same extract in mice showed significant suppression of abdominal contortions induced with acetic acid (0.6% v/v), exhibiting a powerful anti-nociceptive activity (Hamid et al., 2012; Roslida et al., 2010). In addition, the formalin test, paw licking and hot-plate responses also corroborated the marked analgesic effect of *A. muricata* leaves (De Sousa et al., 2010; Hamid et al., 2012; Roslida et al., 2010). The protective effect of *A. muricata* leaves against Complete Freund's adjuvant (CFA)-induced arthritis in rats and xylene-induced ear edema in mice was associated with attenuation in TNF-α and IL-1β protein

expression, demonstrating that the leaves could be used against both acute and chronic inflammation (Hamid et al., 2012). The same assays have shown anti-inflammatory and analgesic activities for *A. muricata* fruits, which were demonstrated to be induced through the suppression of inflammatory mediators and interactions with the opioidergic pathway, respectively (Ishola, Awodele, Olusayero, & Ochieng, 2014). These findings demonstrated the anti-nociceptive and anti-inflammatory effects of *A. muricata* and substantiated its traditional consumption as a pain killer.

2.4.7 Antioxidant Activity of *A. muricata*

The immoderate generation of intracellular ROS is a precursor of oxidative stress, which subsequently catalyzes metabolic deficiency and cellular death through biochemical and physiological lesions (Chance, Sies, & Boveris, 1979). The identification of antioxidants from natural products has become a matter of great interest in recent studies for their noteworthy role in nullifying the destructive effects of ROS (W. Chen, Weng, & Tseng, 2003; Liao et al., 2012). DRSA, FRAP and HRSA tests on the aqueous and methanolic leaf extracts of *A. muricata* revealed the marked antioxidative activities of both extracts, accompanied by DNA protective effects against H_2O_2-induced toxicity (George et al., 2014). The antioxidant activity of *A. muricata* leaves was found to be stronger than for the *A. squamosa* and *A. reticulata* species, as shown in different *in vitro* models, such as ABTS, nitric oxide and hydroxyl radicals (Baskar, Rajeswari, & Kumar, 2007). The seeds and leaves of the plant are reported to possess enzymatic antioxidants, including catalase and superoxide dismutase, and non-enzymatic antioxidants, including vitamin C and E (Vijayameena, Subhashini, Loganayagi, & Ramesh, 2013). Padma and colleagues showed that the ethanolic extract of *A. muricata* stem bark caused a reduction in lipid peroxidation induced by cold immobilization stress in the brain and liver of rats, indicating the adaptogenic potential of this plant (Padma, Chansauria, Khosa, & Ray, 2001; Padma, Chansouria, & Khosa, 1997). The stem bark extract (200 mg/kg) also

showed protective effects against oxidative stress induced by carbon tetrachloride in rats and significantly increased the oxidant levels and serum enzyme activities to near-normal levels. The DPPH test showed the antioxidant activity of the stem bark (Olakunle, Onyechi, & James, 2014). These findings strongly suggest the potential use of *A. muricata* as a natural source of antioxidants.

CHAPTER 3: PUBLISHED PAPERS

Published Paper 1: *Annona muricata* leaves induced apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF-κB

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Published: Journal of BMC Complementary and Alternative Medicine, 2014, 14: 299 (*ISI*-Cited Publication).

Statement of contributions of joint Authorship

S. Z. Moghadamtousi: (Candidate)

Conceived and designed the study, performed the experiments, analyzed the data and wrote the manuscript. Main author of the manuscript.

H. A. Kadir: (Principal Supervisor)

Conceived and designed the study, supervised and assisted in experiments, edited the manuscript. Co-author of the manuscript.

M. Paydar: (Research collaborator)

Assisted in performing the experiments and analyzing the data. Co-author of the manuscript.

E. Rouhollahi: (Research collaborator)

Assisted in performing the experiments. Co-author of the manuscript.

H. Karimian: (Research collaborator)

Assisted in performing the experiments and analyzing the data. Co-author of the manuscript.

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http://www.biomedcentral.com/1472-6882/14/299

BMC Complementary & Alternative Medicine

RESEARCH ARTICLE Open Access

Annona muricata leaves induced apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF-κB

Soheil Zorofchian Moghadamtousi[1], Habsah Abdul Kadir[1*], Mohammadjavad Paydar[2], Elham Rouhollahi[2] and Hamed Karimian[3]

Abstract

Background: *Annona muricata* leaves have been reported to have antiproliferative effects against various cancer cell lines. However, the detailed mechanism has yet to be defined. The current study was designed to evaluate the molecular mechanisms of *A. muricata* leaves ethyl acetate extract (AMEAE) against lung cancer A549 cells.

Methods: The effect of AMEAE on cell proliferation of different cell lines was analyzed by MTT assay. High content screening (HCS) was applied to investigate the suppression of NF-κB translocation, cell membrane permeability, mitochondrial membrane potential (MMP) and cytochrome *c* translocation from mitochondria to cytosol. Reactive oxygen species (ROS) formation, lactate dehydrogenase (LDH) release and activation of caspase-3/7, −8 and −9 were measured while treatment. The western blot analysis also carried out to determine the protein expression of cleaved caspase-3 and −9. Flow cytometry analysis was used to determine the cell cycle distribution and phosphatidylserine externalization. Quantitative PCR analysis was performed to measure the gene expression of Bax and Bcl-2 proteins.

Results: Cell viability analysis revealed the selective cytotoxic effect of AMEAE towards lung cancer cells, A549, with an IC_{50} value of 5.09 ± 0.41 μg/mL after 72 h of treatment. Significant LDH leakage and phosphatidylserine externalization were observed in AMEAE treated cells by fluorescence analysis. Treatment of A549 cells with AMEAE significantly elevated ROS formation, followed by attenuation of MMP via upregulation of Bax and downregulation of Bcl-2, accompanied by cytochrome *c* release to the cytosol. The incubation of A549 cells with superoxide dismutase and catalase significantly attenuated the cytotoxicity caused by AMEAE, indicating that intracellular ROS plays a pivotal role in cell death. The released cytochrome *c* triggered the activation of caspase-9 followed by caspase-3. In addition, AMEAE-induced apoptosis was accompanied by cell cycle arrest at G_0/G_1 phase. Moreover, AMEAE suppressed the induced translocation of NF-κB from cytoplasm to nucleus.

Conclusions: Our data showed for the first time that the ethyl acetate extract of *Annona muricata* inhibited the proliferation of A549 cells, leading to cell cycle arrest and programmed cell death through activation of the mitochondrial-mediated signaling pathway with the involvement of the NF-kB signalling pathway.

Keywords: Annona muricata, Lung cancer, Apoptosis, Caspase, Mitochondria, NF-κB

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Background

Lung cancer as one of the critical causes of cancer death throughout the world has the prevalent complication of apoptosis resistance against different anticancer agents [1]. Due to the typical asymptomatic progression of lung cancer at an early stage, it is normally diagnosed at an advanced stage (56%). In spite of all the development in chemoradiation and surgical techniques, the 5-year survival rate for patients with advanced stage disease is still 3.6% [2]. In addition, numerous lung cancer survivors suffer from lung dysfunction, particularly patients with the lung surgical history [3]. Thereby, continued research into the development of safe and efficient new anticancer agents against lung cancer cells is urgently necessary for further improvements in cancer therapy.

Apoptosis is a critical physiological process responsible for the homeostatic mechanism and maintenance of cell populations in tissues [4]. Due to the close correlation between the mechanism of apoptosis and the effect of anticancer agents, extensive research has been done on this mode of cell death [5]. The accumulation of reactive oxygen species (ROS) in cancer cells is a critical factor for the induction of apoptosis by natural products [6,7], since it will result in oxidative DNA damage following by a collapse in mitochondrial membrane potential (MMP) and leakage of cytochrome *c*, which lead to the activation of the caspase cascade [8]. Furthermore, the perturbation in the expression level of Bax and Blc-2 proteins is an important factor to determine the susceptibility of tumor cells to anticancer agents [9]. Previous anticancer studies also proved that constitutive activation of the ubiquitous transcription factor of NF-κB (nuclear factor-kappa B) is involved in governing the promoting tumor progression of solid and hemopoietic malignancies [10,11]. Therefore, anticancer agents with the ability to suppress the NF-κB translocation are effectively induce the apoptosis in cancer cells.

Annona muricata L. known as gravel, guanabana and soursop is a member of Custard-Apple plants in the Annonaceae family due to a custard-like texture of its fruit. It is a small deciduous tree with a height of 5–8 m and roundish canopy [12]. This popular fruit tree has been widely cultivated in many tropical countries and traditionally used for an array of diseases and ailments [13]. Previous studies demonstrated a significant cytotoxicity for *A. muricata* leaves against various cancer cell lines without affecting the normal cells [14,15]. Due to this tremendous antiproliferative effect, *A. muricata* was described as "the cancer killer" [15]. Ethanolic extract of *A. muricata* leaves was suggested to have apoptosis-inducing potential against myelogenous leukemic K562 cells, although the detailed mechanism of action has not been explained [16]. Amongst constituents isolated from *A. muricata* leaves, namely annonaceous acetogenins, alkaloids and essential oils, annonaceous acetogenins are strongly implied to be responsible for the promising anticancer effect [17]. The principle objective of this study was to examine how *A. muricata* leaves affecting A549 lung cancer cells, and to investigate the possible mechanism of action involved in this effect.

Methods

Plant material and extraction procedures

The plant species (*Annona muricata*) collected from Ipoh, Malaysia, was authenticated by Dr. Yong Kien Thai, an ethnobotanist from the department of Biological Sciences, University of Malaya. The voucher specimen number for this plant is KLU47978. The air-dried leaves of *A. muricata* (1 kg) were cut into fine pieces using a mill grinder and soaked in n-hexane (1500 mL, three times) in conical flasks for four days at room temperature (25–27°C). The n-hexane extract was filtered and the residues were sequentially re-extracted with ethyl acetate (1500 ml, three times) and methanol (1500 ml, three times) using the same method. The resultant filtrate was concentrated to dryness by a Buchi R110 Rotavapor (Buchi Labortechnik AG, Flawil, Switzerland) at 40°C and stored at − 30°C until use. The isolated extracts were dissolved in dimethyl sulfoxide (DMSO) for further experiments.

Cell culture and MTT assay

MCF-7 (human breast cancer cells), MDA-MB-231 (human breast cancer cells), A549 (human lung cancer cells), HepG2 (human hepatoma cells) and WRL-68 (human hepatic cells) cell lines were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (PAA, Pasching, Austria), 100 U/mL penicillin (PAA) and 50 μg/mL amphotericin B (PAA) at 37°C with 5% CO_2. The negative control for all the assays was represented by the untreated medium containing vehicle DMSO (0.1%).

The cytotoxicity of the extracts was determined using the MTT assay as originally described by Mossman [18]. Briefly, cells were treated with different concentrations (1.56, 3.12, 6.25, 12.5, 25, 50 and 100 μg/mL) of three isolated extracts (hexane, ethyl acetate and methanol) and curcumin (positive control) in 96-well plates and incubated for 72 h. After the incubation time, MTT dye (20 μL, 5 mg/mL, Sigma) was added to the cells for 4 h followed by incubation with DMSO for 10 min. The colorimetric assay was measured at the absorbance of 570 nm using a microplate reader (Asys UVM340, Eugendorf, Austria). The antiproliferative potential of the extracts was expressed as IC_{50} values. As an ethyl acetate extract of the leaves (AMEAE) demonstrated the lowest IC_{50} value against lung cancer A549 cells,

we used only AMEAE to continue this study against A549 cells.

LDH release assay

To confirm the cytotoxicity of AMEAE, we carried out lactate dehydrogenase (LDH) release assay using Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific™, Pittsburgh, PA, USA). In brief, A549 cells were treated with AMEAE at different concentrations for 48 h. The supernatant of treated A549 cells was transferred into 96-well plate to assess the LDH activity. Triton X-100 (2%) served as a positive control was used to completely lyse the cells and release the maximum LDH. Next, the LDH reaction solution (100 μl) was added to the cells for 30 min. The red color intensity presenting the LDH activity was measured by the absorbance at 490 using a Tecan Infinite®200 Pro (Tecan, Männedorf, Switzerland) microplate reader. The level of released LDH from treated cells was expressed as a percentage of positive control.

Acridine orange/propidium iodide (AO/PI) double staining assay

Morphological changes induced by AMEAE in A549 cells were analyzed using Acridine orange/Propidium iodide (AO/PI) double staining assay. Briefly, A549 cells were seeded in 60 mm^2 culture dishes followed by treatment with AMEAE (10 μg/mL) for 24, 48 and 72 h. After the incubation time, extract-untreated and treated A549 cells were harvested and washed with PBS. Then, the pellets were stained with 10 μg/mL of AO/PI (1 mg/mL). The stained cells were then observed under a BX51 UV-fluorescent microscope (Olympus, Tokyo, Japan) within 30 min.

Annexin-V-FITC assay

Induction of the early and late apoptosis by AMEAE was further studied via Annexin-V/PI staining assay. Briefly, A549 cells (1×10^6) were plated in 60 mm^2 culture dishes and treated with vehicle DMSO and AMEAE (10 μg/mL) for 24, 48 and 72 h. After harvest of adherent and suspension cells and washing them twice with PBS, they were resuspended in Annexin-V binding buffer (BD Biosciences, San Jose, CA, USA) and stained with Annexin-V-FITC (BD) and PI (Sigma) according to the manufacturer's instructions. The fluorescence intensity of A549 cells was then analyzed by flow cytometry (BD FACSCanto™ II, San Jose, CA, USA) through quadrant statistics for necrotic and apoptotic cell populations. PI was used for detection of the late apoptosis and necrosis while Annexin-V was consumed for the detection of the early and late apoptosis.

Cell cycle assay

A flow cytometry analysis was carried out to determine the cell cycle distribution in treated A549 cells with AMEAE. In brief, A549 cells (5×10^4 cells/mL) were treated with AMEAE (10 μg/mL) for 24, 48 and 72 h. After fixation with cold ethanol, cells were washed with PBS and stained with PI (50 μl, 10 mg/mL) for 1 h at 37°C. In addition, RNase A (10 mg/mL) was also used to limit the ability of the PI to bind only to DNA molecules. The stained cells were analyzed for DNA content using flow cytometer (BD FACSCanto™ II).

Reactive oxygen species (ROS) assay

The effect of AMEAE on the ROS formation in A549 cells was determined by ROS assay. Briefly, treated lung cancer cells with AMEAE at different concentrations in 96-well plates were incubated for 24 h. After the incubation time, the treated cells were stained with dihydroethidium (DEH) at 2.5 μg/mL and Hoechst 33342 (500 nM) dyes for 30 min. Then, cells were fixed with paraformaldehyde (3.5%) for 15 min and washed with PBS twice. The Cellomics ArrayScan HCS reader was used to measure the ROS generation in treated A549 cells.

To further determine the role of ROS generation in AMEAE-induced antiproliferative effect, A549 cells were treated with antioxidants prior to treatment with AMEAE and the cell viability was measured after 24 h. In brief, A549 cells in the exponential phase of growth were supplemented with antioxidants superoxide dismutase (SOD, 300 U/mL) and catalase (400 U/mL) for 1 h prior to AMEAE (20 μg/mL) treatment for 24 h. After incubation time, the cell viability analysis was carried out using a microplate reader (Asys UVM340, Eugendorf, Austria).

Multiple cytotoxicity assay

To simultaneously determine the crucial apoptotic events in A549 cells after treatment with AMEAE, we used Cellomics Multiparameter Cytotoxicity 3 Kit (Thermo Scientific™, Pittsburgh, PA, USA). Briefly, lung cancer A549 cells were seeded into 96-well plates for 24 h. The cells were treated with AMEAE at different concentrations prior to staining the cells with cell permeability and mitochondrial membrane potential (MMP) dyes. Then, cells were fixed and blocked with 1X blocking buffer according to the manufacture's protocol. Next, primary cytochrome *c* antibody and secondary DyLight 649 conjugated goat antimouse IgG were added for 1 h. Nuclei of treated cells were also stained with Hoechst 33342 dye. Stained A549 cells in 96-well plates were analyzed using ArrayScan high content screening (HCS) system.

Bioluminescent assays for caspase-8, −9 and −3/7 activities

A dose-dependent study on the caspase-8, −9 and −3/7 activation was carried out using Caspase-Glo® 3/7, 8 and 9 kit (Promega, Madison, WI, USA). In brief, a total of

5×10^3 A549 cells were seeded per well in a white 96-well microplate and incubated with different concentrations of AMEAE for 24 h. Then, caspase-Glo reagent (100 μl) was added to the cells for 30 min. The induced activation of caspases was measured using a Tecan Infinite®200 Pro (Tecan, Männedorf, Switzerland) microplate reader.

To determine the protein expression of cleaved caspase-3 and −9, western blot analysis was carried out as previously described in detail [19]. In brief, A549 cells treated with vehicle DMSO or AMEAE at different concentrations were washed with PBS and lysed in ice-cold Radio Immuno Precipitation Assay (RIPA) buffer. Cell extracts (80 μg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membrane, probed with anti-β-actin, anti-cleaved caspase-9 and anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA). HRP-conjugated secondary antibodies were used followed by the detection of protein expression using the ECL plus chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Quantitative PCR analysis

The expression of the Bax and Bcl-2 in treated A549 cells was analyzed by quantitative PCR analysis. After treatment of A549 cells with the AMEAE extract at different concentrations for 24 h, Zymo Research Quick-RNA™ MiniPrep kit (Zymo Research, Freiburg, Germany) was used to isolate total RNAs according to the manufacture's protocol. Then, High Capacity RNA-to-cDNA™ kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize complementary DNAs. Quantitative PCR was carried out with TaqMan® Gene Expression Assays and TaqMan® Fast Advanced Master Mix using the Applied Biosystems StepOnePlus™ system. GAPDH was used to normalize all data. The IDs for TaqMan® Gene Expression Assays used in this study are GAPDH: Hs02758991_g1, Bcl-2: Hs00608023_m1 and Bax: Hs00180269_m1.

Measurement of NF-κB activity

The Cellomics ArrayScan HCS system was used to analyze the suppressive effect of AMEAE on the nuclear translocation of NF-κB induced by TNF-α. The experiment was carried out using Cellomics nucleus factor-κB (NF-κB) activation kit (Thermo Scientific) as previously described [20]. In brief, A549 cells (1.0×10^4 cells/well) were treated with AMEAE at different concentrations in a 96-well plate for 3 h. The treated A549 cells were stimulated by TNF-α (1 ng/mL) for 30 min. Then, cells were fixed and stained according to the manufacturer's protocol and analyzed using Array Scan HCS Reader and Cytoplasm to Nucleus Translocation Bioapplication software.

Statistical analysis

Data are presented as mean ± SEM of three individual experiments. Statistical analysis was performed with a one-way ANOVA analysis using the Prism statistical software package (GraphPad Software, USA). Differences were considered as being significant at $^*p < 0.05$.

Results and discussion

AMEAE inhibited the proliferation of cancer cells

We first examined the cytotoxic effect of three extracts (hexane, ethyl acetate and methanol) against MCF-7 (human breast cancer cells), MDA-MB-231 (human breast cancer cells), A549 (human lung cancer cells), HepG2 (human hepatoma cells) and WRL-68 (human hepatic cells) cell lines by using MTT assay. Treatment with AMEAE for 72 h significantly reduced the cell proliferation in cancer cells. As shown in Table 1, the results showed the significant cytotoxic activity of ethyl acetate extract towards all cancer cells. The ethyl acetate extract (IC_{50} ranged from 5.09 ± 0.41 to 11.36 ± 0.67 μg/mL) showed the highest cytotoxicity against all cancer cells as compared to others. Furthermore, the ethyl acetate extract exhibited the highest cytotoxic activity towards A549 cells with the IC_{50} of 5.09 ± 0.41 compared to 11.32 ± 1.54 for the positive control curcumin. After 24 and 48 h treatment with AMEAE, A549 cells elicited the IC_{50} values of 17.542 ± 0.92 and 10.612 ± 1.34 μg/mL. It is worth noting that normal human hepatic WRL-68 cells were not noticeably affected by AMEAE treatment. The cell viability results suggested that the AMEAE inhibitory effect is selective for cancer cells.

Induction of LDH release by AMEAE

A stable cytosolic enzyme of lactate dehydrogenase (LDH) catalyzes the oxidation of L-lactate to pyruvate. Upon membrane damage in cells, LDH enzyme is released into the culture medium, suggesting the loss of membrane integrity [21]. Therefore, to further confirm the cytotoxic effect of AMEAE on A549 cells, LDH assay was also performed as another indicator of A549 cytotoxicity. As shown in Figure 1, LDH leakage of A549 cells was significantly increased with the presence of AMEAE by 5.9 folds.

Quantification of apoptosis using fluorescence microscope and AO/PI double-staining

Energy-dependent biochemical pathways and distinct morphological features are the main characteristics of apoptosis, or programmed cell death. During the early apoptosis, pyknosis and cell shrinkage are characterized in apoptotic cells [22]. Cell shrinkage is a result of condensation of organelles and the density of cytoplasm, and chromatin condensation or pyknosis is the most critical feature of early apoptosis [23]. The process of

Table 1 IC_{50} values of *A. muricata* leaves extracts on five different cell lines after 72 h treatment

Extract	IC_{50} (μg/mL)				
	MCF-7	MDA-MB-231	A549	HepG2	WRL-68
Hexane	49.92 ± 2.23	38.72 ± 0.99	21.05 ± 0.42	77.92 ± 2.23	89.53 ± 3.93
Ethyl acetate	6.39 ± 0.43	11.36 ± 0.67	5.09 ± 0.41	9.3 ± 0.91	47.10 ± 1.23
Methanol	85.58 ± 3.55	> 100 *	> 100	> 100	> 100
Curcumin	7.65 ± 0.55	9.34 ± 0.76	11.32 ± 1.54	17.66 ± 1.21	54.24 ± 2.21

The data represent the means ± SEM of three independent experiments.
* > 100 represents the IC_{50} values greater than 100 μg/mL.

late apoptosis is accompanied by budding which contains extensive blebbing of plasma membrane with tightly packed organelles [24]. In the present study, AMEAE was examined for its apoptotic-inducing activity by fluorescence microscopy analysis. Morphological changes in control and treated A549 cells were observed after treatment with AMEAE at 24, 48 and 72 h. It is noteworthy that at 72 h the control cells remained intact and displayed normal structures with a green intact nuclear structure (Figure 2A). After 24 h of treatment, clear signs of apoptosis, such as cytoplasmic shrinkage and membrane blebbing, were observed at a treatment concentration of 10 μg/mL. The early apoptotic cells were detected via the binding of AO within the fragmented DNA emanating a bright green fluorescence. At 24 h treatment with AMEAE, moderate apoptosis was seen by nuclear chromatin condensation and blebbing (Figure 2B). In addition, the late stages of apoptosis as indicated by changes such as the presence of a reddish-orange color because of the binding of PI to denatured DNA were observed after 48 and 72 h of treatment (Figure 2C and D). The results showed that AMEAE generated morphological features that are associated with apoptosis in a time-dependent manner.

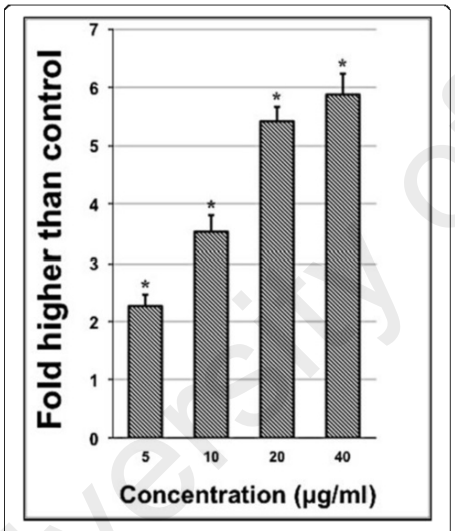

Figure 1 Lactate dehydrogenase (LDH) assay showed the cytotoxicity of AMEAE against A549 cells. The result revealed significant cytotoxicity at concentrations of 5 to 40 μg/mL, in a dose-dependent manner. The data represent the means ± SEM of three independent experiments. *$p < 0.05$ compared with the untreated group.

Induction of early and late apoptosis using flow cytometry analysis

Our investigations thus far revealed the typical morphological features of apoptosis in AMEAE-treated A549 cells. The morphological changes in apoptotic cells are accompanied by several biochemical modifications, including DNA breakdown, protein cross-linking and protein cleavage which lead to the phagocytic recognition of apoptotic cells by adjacent cells [25]. One such biochemical modification is the expression of cell surface markers such as inward-facing phosphatidylserine which translocates to the outer side of plasma membrane during the early apoptosis [26]. To investigate the biochemical characterization of apoptosis, we examined the externalization of phosphatidylserine (PS) in A549 cells by Annexin-V-FITC assay. Annexin-V as a recombinant phosphatidylserine-binding protein with a high affinity for externalized PS is used for detection of early apoptosis [27]. PI was used for detection of the late apoptosis and necrosis, while Annexin-V was for the detection of the early and late apoptosis. Accordingly, to evaluate whether A549 cells undergo apoptosis, untreated and AMEAE-treated cells were stained with Annexin-V and PI. Flow cytometry analysis of stained cells can distinguish the cells into four categories, namely viable (Annexin-V and PI negative), early apoptosis (Annexin-V positive, PI negative), late apoptosis (Annexin-V and PI positive) and necrotic (Annexin-V negative, PI positive) cells. As illustrated in Figure 3, AMEAE treatment at different time periods (24, 48 and 72 h) resulted in a time-dependent increment of early apoptotic and late apoptotic

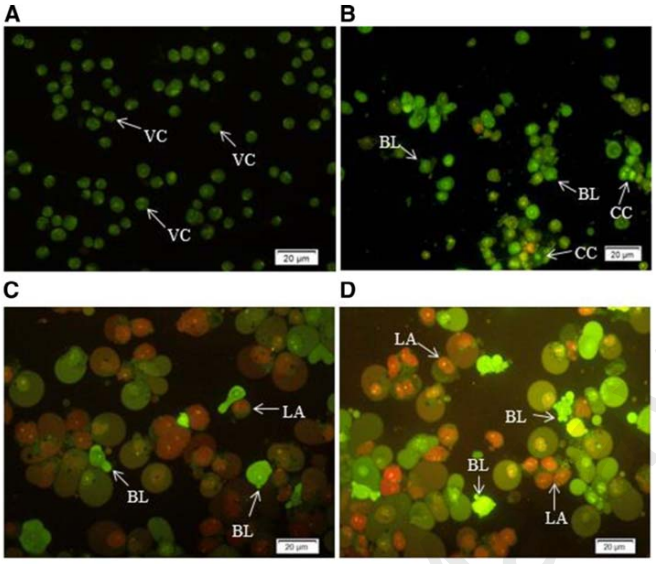

Figure 2 Fluorescent micrographs of AO/PI double stained A549 cells. (A) Untreated A549 cells after 72 h depict healthy structures. Early apoptosis features such as chromatin condensation and blebbing were observed after **(B)** 24, **(C)** 48 and 72 h **(D)** of treatment with AMEAE. Late apoptosis was observed at the late stage of treatment (magnification: 200×). VC: Viable cells; CC: Chromatin condensation; BL: Blebbing of the cell membrane; LA: Late apoptosis.

populations which peaked at 34.1% and 42.6%, respectively, after 72 h treatment.

AMEAE-induced G_1 cell cycle arrest

Cancer is often considered as a disease of cell cycle deregulation. Cell size, extracellular growth signals and DNA integrity are tightly regulated by multiple checkpoints in cell cycle progression [28]. Cancer can originate from perturbation in the expression of positive or negative regulators of cell cycle machinery leading to abnormal proliferation of cancer cells [29]. Thus, induction of cell cycle arrest in cancer cells is considered to be one of the crucial cancer treatment strategies. Phytochemicals with the ability to modulate the cell cycle progression are gaining extensive attention because of the supporting evidences of the concomitant involvement of cell cycle suppression and apoptosis [30,31]. Thus, flow cytometry analysis was performed to evaluate the effect of AMEAE on the DNA content at various cell cycle checkpoints of A549 cells by cell cycle phase distribution (G_0, G_1, S, G_2 and M) after treatment for 24, 48 and 72 h. As shown in Figure 4, there was a significant G_0/G_1 phase arrest in a time-dependent manner demonstrating that AMEAE arrested cell cycle progression at the G_0/G_1 phase (Figure 4). Moreover, the increase in the sub-G_1 phase confirms the apoptosis findings.

ROS generation induced by AMEAE

Reactive oxygen species are produced as the result of normal metabolism during the reduction of oxygen to water. Oxidative stress due to increase in the level of intracellular ROS leads to a variety of biochemical and physiological lesions following to metabolic impairments and cell death [32]. The stimulus of free radicals and oxidative stress can trigger the mitochondrial initiated events leading to the activation of intrinsic pathway [33]. Thus, there is an upsurge of interest in cancer studies to use this major role for induction of apoptosis in cancer cells. To investigate this association, the levels of intracellular ROS in A549 cells treated with AMEAE was examined by flow cytometry analysis. In the present study, it was found that AMEAE has noteworthy potential in the induction of ROS generation in A549 cells as shown in Figure 5. As a result of ROS generation, non-fluorescent dihydroethidium (DHE) convert to the fluorescent dye of ethidium, which can bind to DNA. A dose-dependent elevation in ethidium fluorescence in the nucleus was detected in treated A549 cells (Figure 5A). As shown in

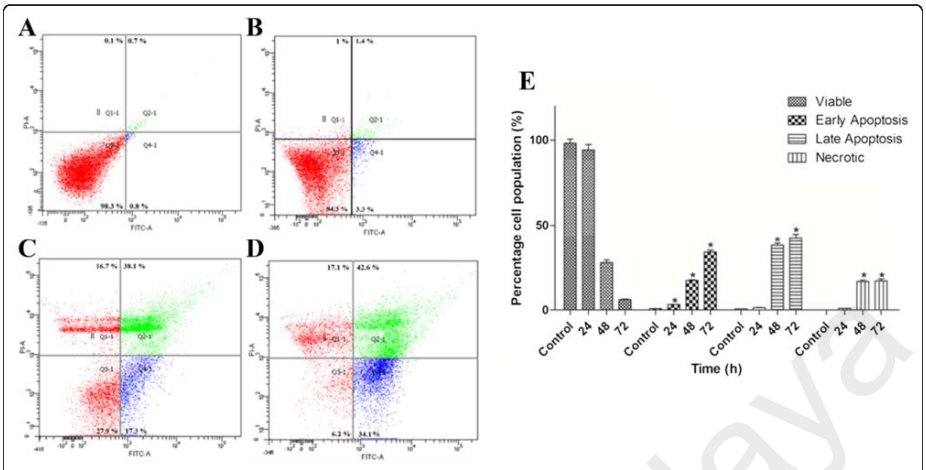

Figure 3 Time-dependent induction of early and late apoptosis by AMEAE indicated the percentage of early apoptotic, late apoptotic and necrotic cells after (B) 24, (C) 48 and (D) 72 h of treatment. **(A)** shows the cells treated with vehicle DMSO. The early and late apoptotic events are depicted in the lower right and upper left quadrant of each panel. **(E)** Representative bar chart demonstrated significant elevation in a number of early and late apoptotic cells after 48 h of treatment. The data represent the means ± SEM of three independent experiments. *$p < 0.05$ compared with the untreated group.

Figure 5B, ROS production was at the basal level in control untreated A549 cells. In contrast, treatment with AMEAE (24 h) resulted in dose-dependent increase of ROS production as shown by increased ethidium staining in the nucleus (Figure 5A and B). However, pretreatment of A549 cells with antioxidants, namely SOD and Cat significantly increased the cell viability after 24 h compared to the treatment with AMEAE alone (Figure 5C). The viable cells was 62% and 78% by pretreatment with SOD and Cat, respectively, compared with only about 43% by treatment with AMEAE alone. This result supports the close correlation between ROS burst and cytotoxic effect of AMEAE, which suggests to be through activation of the mitonchondrial initiated events.

Mitochondria-initiated events induced by AMEAE

During apoptosis, mitochondrial membrane potential is frequently disrupted due to the formation of permeability transition pores or the insertions of proapoptotic proteins, such as, Bid or Bax in the mitochondrial membrane [34]. A variety of non-receptor mediated stimuli, including free radicals, radiation, hypoxia and toxin can trigger the intrinsic signaling pathway [26]. The stimuli produce intracellular signals that cause loss in MMP and the opening of the mitochondrial permeability transition pore. These changes in the inner mitochondrial membrane lead to the release of various proteins from the intermembrane space into the cytosol [35]. The main group of released proteins consists of serine protease HtrA2/Omi, cytochrome *c* and Smac/DIABLO. Cytochrome *c* forms apoptosome through binding to pro-caspase-9 as well as Apaf-1. Apoptosome activates caspase-9 following with activation of caspase-3 [36,37].

It is well established that extensive ROS production could result in the disruption of the plasma membrane and mitochondrial damage, thus we further examined the cell membrane permeability, MMP and cytochrome *c* translocation [38]. To determine the function of mitochondria, we applied MMP fluorescent probe. As shown in Figure 6A, untreated A549 cells were strongly stained with red MMP dye, while treated A549 cells with AMEAE were not markedly stained after 24 h. The dose-dependent reduction in fluorescence intensities of MMP was associated with significant elevation in cell membrane permeability which is shown by green fluorescence intensities in Figure 6A. Furthermore, AMEAE significantly elevated the translocation of cytochrome *c* from mitochondria to cytosol in A549 cells after 24 h. In the treated A549 cells, the cyan fluorescence intensities of cytochrome *c* dye (Figure 6A) were markedly increased presenting the significant cytochrome *c* release from mitochondria. The present findings demonstrated that treatment with AMEAE revealed a concentration-dependent increase in membrane permeability, attenuation of MMP, and increase in cytochrome *c* in the cytosol when compared to the control.

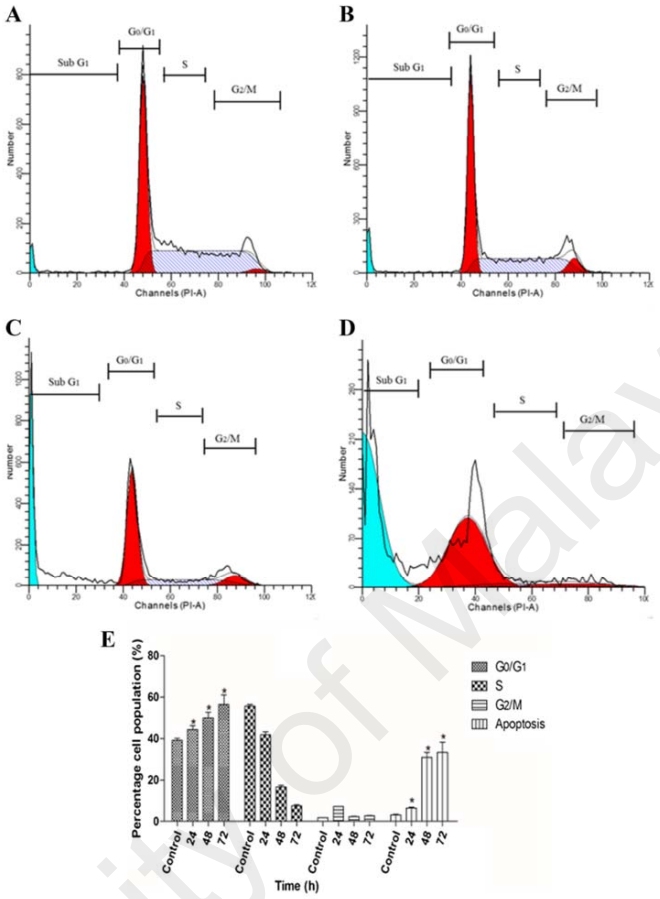

Figure 4 Flow cytometry analysis on cell cycle progression in A549 cells was carried out after incubation with AMEAE for (A) 0, (B) 24, (C) 48 and (D) 72 h. (E) Representative bar chart demonstrated significant cell cycle arrest at the G_1 phase. The data represent the means ± SEM of three independent experiments. *$p < 0.05$ compared with the no-treatment group.

AMEAE induced caspase-8, −9 and −3/7 activation

Caspase activation with proteolytic effect at aspartic acid residues are involved in an energy-dependent cascade of molecular events towards apoptosis. To date, two major groups of caspases have been identified to be involved in apoptosis pathways, including executioners or effectors (caspase-3, −6, −7) and initiators (caspase-2, −8, −9, −10) [36]. Intrinsic and extrinsic pathways are activated by their own initiator caspases, namely caspase-9 and −8, respectively. The activation of initiator caspases will, in turn, lead to activation of executioner caspases. The most critical of executioner caspases is considered to be caspase-3 [26]. The execution pathway leads to cytomorphological changes, namely chromatin condensation, cell shrinkage, formation of apoptotic bodies followed by phagocytosis of the apoptotic cell [39]. In the present study, we investigated whether AMEAE can activate the caspases by examining A549 cells treated with different concentrations for 24 h. The activity of both caspase-9 and caspase-3/7 were significantly elevated at 10, 20 and

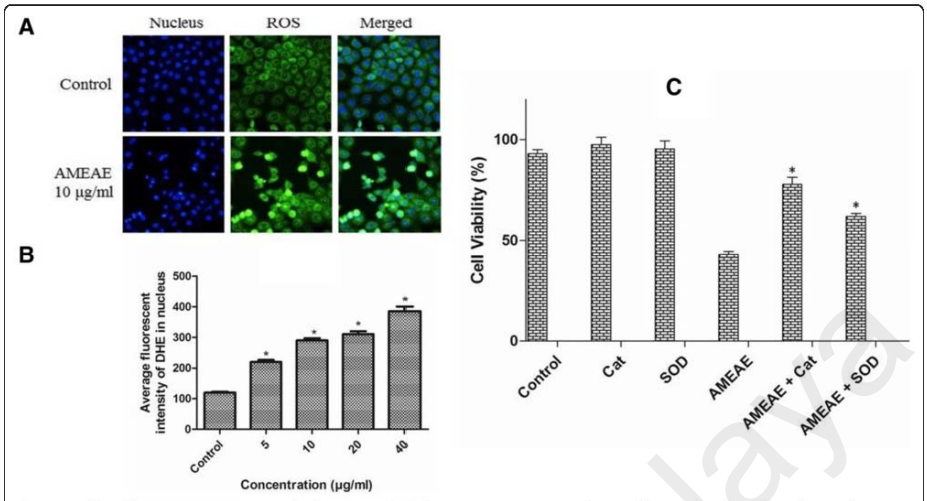

Figure 5 Effect of AMEAE on ROS generation in A549 cells. (A) Representative images of A549 cells treated with medium alone and 10 µg/mL of AMEAE for 24 h and stained with dihydroethidium and Hoechst 33342 dyes for 30 min. **(B)** Representative bar chart indicated dose-dependent elevation in ROS formation in treated A549 cells. The level of ROS was significantly elevated at 5 to 40 µg/mL concentrations. **(C)** Pretreatment of A549 cells with Cat and SOD for 1 h prior to the incubation with AMEAE caused a significant elevation in cell viability compared to the treatment alone with AMEAE. The data represent the means ± SEM of three independent experiments. *$p < 0.05$ compared with the untreated group.

40 µg/mL AMEAE treatment, while activation of caspase-8 was only triggered at higher concentrations of 20 and 40 µg/mL, suggesting that AMEAE induced apoptosis predominantly through mitochondrial-mediated intrinsic pathway (Figure 7). The western blot analysis of caspases also showed that the protein expression of cleaved caspase-3 and −9 was dose-dependently increased upon treatment with AMEAE. The caspase-3 and −9 protein expressions were significantly up-regulated at 10 to 40 µg/mL concentrations. These results suggest the involvement of caspase cascade in AMEAE-mediated apoptosis.

AMEAE induced upregulation of Bax and downregulation of Bcl-2 at the gene expression level

The mitochondria-initiated events are tightly regulated by the Bcl-2 family of proteins, including pro-apoptotic and anti-apoptotic proteins. Progression and abortion of apoptosis are governed by 25 genes in this family of proteins [40]. The pro-apoptotic protein of Bax is involved in the cytochrome *c* release from mitochondria to cytosol via dimerization and translocation to the outer mitochondrial membrane [41]. Meanwhile, anti-apoptotic proteins such as Bcl-2 suppress the translocation of cytochrome *c* (5). The levels of both Bcl-2 and Bax mRNA expression were estimated by using quantitative PCR analysis. The results demonstrated that Bcl-2 expression decreased significantly when A549 cells were treated with 20 and 40 µg/mL of AMEAE after 24 h as compared with the control cells (Figure 8). The expression of the proapoptotic Bax, however, increased significantly when treated with 20 and 40 µg/mL of AMEAE (Figure 8). Therefore, downregulation of Bcl-2 and upregulation of Bax upon AMEAE treatment could lead to loss of MMP, which facilitated cytochrome *c* release and activation of the caspase cascade. These changes in the gene expression of Bcl-2 and Bax confirmed the induction of apoptosis via mitochondrial-mediated intrinsic pathway.

NF-κB translocation suppressed by AMEAE

The ability of nuclear factor-κB (NF-κB) to inhibit the induction of apoptosis is considered to be involved in resistance against cytotoxic therapies. A variety of stimuli namely, anticancer agents and cellular stress can trigger NF-κB activation, which has been linked to extrinsic signaling pathway and inducible chemoresistance [42]. Thus, suppression of NF-κB translocation in conjunction with chemotherapy can effectively elevate the effect of cancer therapy [43]. In this study, the ArrayScan HSC system was used to determine the role of AMEAE in the

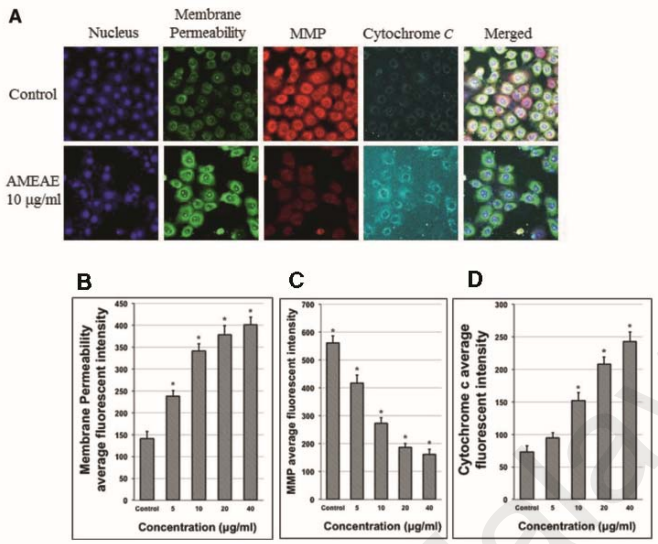

Figure 6 Effect of AMEAE on cell membrane permeability, MMP and cytochrome *c* release. (A) Representative images of A549 cells treated with medium alone and 10 µg/mL of AMEAE, and stained with MMP, cell membrane permeability, Hoechst 33342 and cytochrome *c* dyes. AMEAE caused a marked elevation in cytochrome *c* leakage and cell membrane permeability, and a noteworthy decrease in MMP. Representative bar charts indicating dose-dependent increased **(B)** cell permeability and reduced **(C)** MMP in treated A549 cells at 5 to 40 µg/mL concentrations. Meanwhile, **(D)** cytochrome *c* was significantly released at 10 to 40 µg/mL concentrations. The data represent the means ± SEM of three independent experiments. *$p < 0.05$ compared with the untreated group.

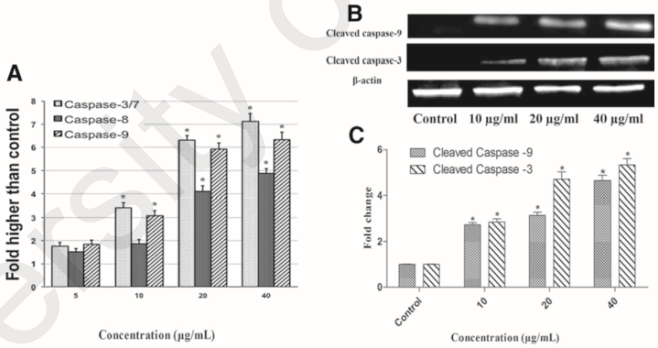

Figure 7 Effect of AMEAE of caspases activation. (A) Relative luminescence dose-dependent activation of caspase-8, −9 and −3/7 in A549 cells treated with various concentrations of AMEAE. Caspase-9 and −3/7 were significantly activated at 10 to 40 µg/mL concentrations. Meanwhile, caspase-8 was activated at higher concentrations (20 and 40 µg/mL). **(B)** A549 cells were treated with the indicated concentrations of AMEAE for 24 h. The cell lysates were evaluated for levels of cleaved caspase-3 and −9. AMEAE induced the up-regulation of cleaved caspase-3 and −9. **(C)** The quantitative analysis was expressed as a ratio to the expression of β-actin. The data represent the means ± SEM of three independent experiments. *$p < 0.05$ compared with the untreated group.

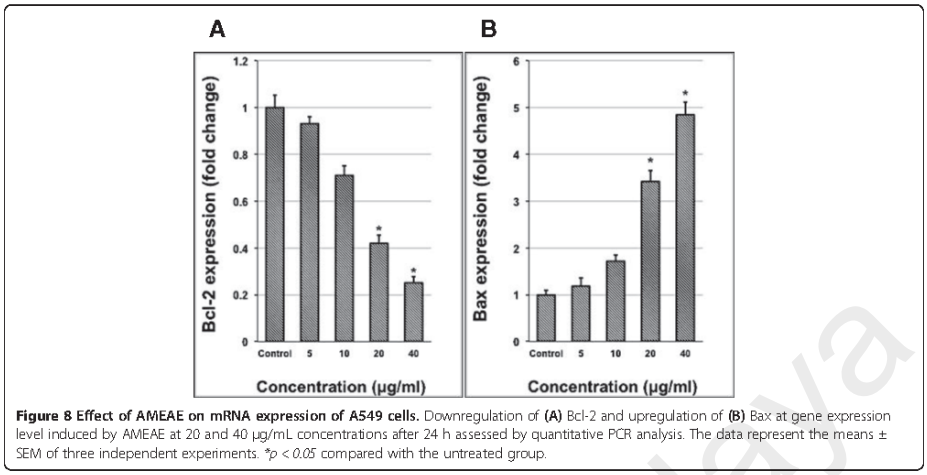

Figure 8 Effect of AMEAE on mRNA expression of A549 cells. Downregulation of **(A)** Bcl-2 and upregulation of **(B)** Bax at gene expression level induced by AMEAE at 20 and 40 μg/mL concentrations after 24 h assessed by quantitative PCR analysis. The data represent the means ± SEM of three independent experiments. **p < 0.05* compared with the untreated group.

suppression of activated NF-κB. In control cells, a high NF-κB fluorescence intensity was detected in the cytoplasm of A549 cells compared to the nuclei (Figure 9A). After treatment with AMEAE, nuclear NF-κB fluorescent intensity was dose-dependently reduced (Figure 9B). Stimulation of A549 cells by TNF-α resulted in a significant elevation in NF-κB fluorescence intensity in the nuclei, while, A549 cells treated with AMEAE elicited significant suppressive effects on the activation of NF-κB (Figure 9A and B), at 10, 20 and 40 μg/mL in a concentration-dependent manner. The present findings demonstrated that AMEAE treatment can effectively reduce the activation of NF-κB signaling pathway in A549 cells. We have shown here that the NF-kB translocation can be suppressed by AMEAE, which suggests the involvement of an NF-κB inhibition mechanism in apoptosis.

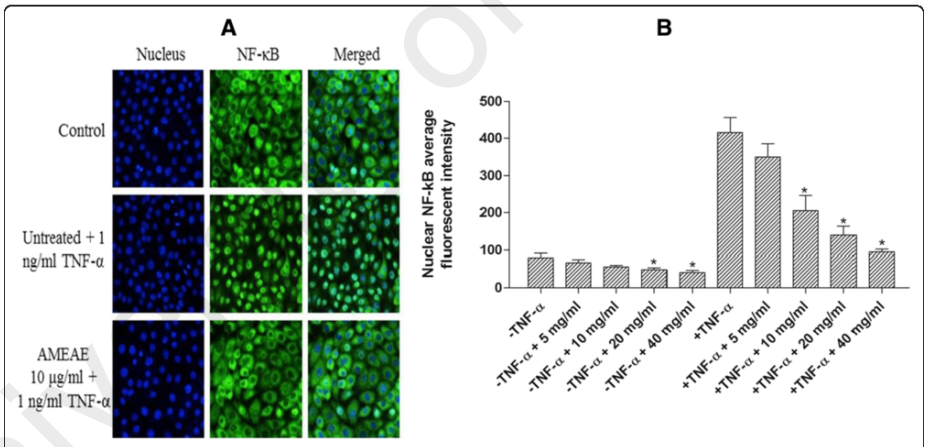

Figure 9 Effect of AMEAE on NF-κB activation. (A) Representative images of the intracellular targets of stained A549 cells that were exposed to AMEAE (10 μg/mL) and then stimulated with TNF-α. **(B)** Representative bar chart revealed the translocation of NF-κB nuclear in treated A549 cells with AMEAE. The data represent the means ± SEM of three independent experiments. **p < 0.05* compared with the untreated control group.

Conclusions

In conclusion, the anticancer potential of ethyl acetate extract of *A. muricata* leaves was supported by the evidence provided in the present study, including lactate dehydrogenase leakage, reactive oxygen species generation, loss in mitochondrial membrane potential, increase in the level of cytochrome *c*, upregulation of Bax, downregulation of Bcl-2 and activation of initiator and executioner caspases. The antiproliferative effect of AMEAE was accompanied by cell cycle arrest at G_1 phase and suppression of NF-κB translocation. The results confirmed the involvement of intrinsic pathways in induced apoptosis.

Abbreviations

AMEAE: *A. muricata* leaves ethyl acetate extract; HCS: High content screening; NF-κB: Nuclear factor-kappa B; MMP: Mitochondrial membrane potential; LDH: Lactate dehydrogenase; ROS: Reactive oxygen species (ROS); Bax: Bcl-2 associated X protein; Bcl-2: B-cell lymphoma protein 2; DHE: Dihydroethidium.

Competing interests

The authors declare that there is no conflict of interests.

Authors' contributions

SZM and HAK conceived and designed the study. SZM, MP, ER and HK performed the experiments. SZM, MP and HK analysed the data. SZM and HAK contributed reagents/materials/analysis tools. SZM wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Ramezanpour M, da Silva KB, Sanderson BJ: **Venom present in sea anemone (heteractis magnifica) induces apoptosis in non-small-cell lung cancer A549 cells through activation of mitochondria-mediated pathway.** *Biotechnol Lett* 2012, **36**(3):1–7.
2. Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A, Ward E: **Cancer treatment and survivorship statistics, 2012.** *CA-Cancer J Clin* 2012, **62**(4):220–241.
3. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, Seto T, Satouchi M, Tada H, Hirashima T, Asami K, Katakami N, Takada M, Yoshioka H, Shibata K, Kudoh S, Shimizu E, Saito H, Toyooka S, Nakagawa K, Fukuoka M: **Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial.** *Lancet Oncol* 2010, **11**(2):121–128.
4. Kim SS, Cho HJ, Kang JY, Kang HK, Yoo TK: **Inhibition of androgen receptor expression with small interfering RNA enhances cancer cell apoptosis by suppressing survival factors in androgen insensitive, late stage LNCaP cells.** *Sci World J* 2013, **2013**. doi:10.1155/2013/519397.
5. Ocker M, Höpfner M: **Apoptosis-modulating drugs for improved cancer therapy.** *Eur Surg Res* 2012, **48**(3):111–120.
6. Schumacker PT: **Reactive oxygen species in cancer cells: live by the sword, die by the sword.** *Cancer Cell* 2006, **10**(3):175–176.
7. Moghadamtousi SZ, Goh BH, Chan CK, Shabab T, Kadir HA: **Biological activities and phytochemicals of *swietenia macrophylla* king.** *Molecules* 2013, **18**(9):10465–10483.
8. Simon H-U, Haj-Yehia A, Levi-Schaffer F: **Role of reactive oxygen species (ROS) in apoptosis induction.** *Apoptosis* 2000, **5**(5):415–418.
9. Martinou J-C, Youle RJ: **Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics.** *Dev Cell* 2011, **21**(1):92–101.
10. Yang F, Jove V, Chang S, Hedvat M, Liu L, Buettner R, Tian Y, Scuto A, Wen W, Yip MLR, Meter TV, Yen Y, Jove R: **Bortezomib induces apoptosis and growth suppression in human medulloblastoma cells, associated with inhibition of AKT and NF-kB signaling, and synergizes with an ERK inhibitor.** *Cancer Biol Ther* 2012, **13**(6):349–357.
11. Van Waes C: **Nuclear factor-κB in development, prevention, and therapy of cancer.** *Clin Cancer Res* 2007, **13**(4):1076–1082.
12. Adewole SO, Caxton-Martins EA: **Morphological changes and hypoglycemic effects of *annona muricata* Linn.(annonaceae) leaf aqueous extract on pancreatic β-cells of streptozotocin-treated diabetic rats.** *Afr J Biomed Res* 2006, **9**(3):173–187.
13. Adewole SO, Ojewole JA: **Protective effects of *annona muricata* Linn. (annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats.** *Afr J Tra Compl Alter Med* 2009, **6**(1):30.
14. George VC, Kumar DN, Rajkumar V, Suresh P, Ashok R: **Quantitative assessment of the relative antineoplastic potential of the n-butanolic leaf extract of annona muricata Linn. In normal and immortalized human cell lines.** *Asian Pac J Cancer P* 2012, **13**(2):699–704.
15. Mishra S, Ahmad S, Kumar N, Sharma BK: ***Annona muricata* (the cancer killer): a review.** *Glob J Pharma Res* 2013, **2**(1):1613–1618.
16. Ezirim AU, Okachi VI, James AB, Adebeshi OA, Ogunnowo S, Odeghe OB: **Induction of apoptosis in myelogenous leukemic K562 cells by ethanolic leaf extract of *annona muricata*.** *Indian J Drugs Dis* 2013, **2**(3):142–151.
17. Zeng L, Wu F-E, Oberlies NH, McLaughlin JL, Sastrodihadjo S: **Five new monotetrahydrofuran ring acetogenins from the leaves of *annona muricata*.** *J Nat Prod* 1996, **59**(11):1035–1042.
18. Mosmann T: **Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.** *J Immunol Methods* 1983, **65**(1):55–63.
19. Liew SY, Looi CY, Paydar M, Cheah FK, Leong KH, Wong WF, Mustafa MR, Litaudon M, Awang K: **Subditine, a new monoterpenoid indole alkaloid from bark of naudea subdita (korth.) steud. Induces apoptosis in human prostate cancer cells.** *PLoS One* 2014, **9**(2):e87286.
20. Looi CY, Moharram B, Paydar M, Wong YL, Leong KH, Mohamad K, Arya A, Wong WF, Mustafa MR: **Induction of apoptosis in melanoma A375 cells by a chloroform fraction of *centratherum anthelminticum* (L.) seeds involves NF-kappaB, p53 and Bcl-2-controlled mitochondrial signaling pathways.** *BMC Complem Altern M* 2013, **13**(1):166.
21. Chan FK-M, Moriwaki K, De Rosa MJ: **Detection of Necrosis by Release of Lactate Dehydrogenase Activity.** In *Immune Homeostasis*. Edited by Snow AL, Lenardo MJ. New York: Humana Press; 2013:65–70.
22. Häcker G: **The morphology of apoptosis.** *Cell Tissue Res* 2000, **301**(1):5–17.
23. Ovadje P, Chatterjee S, Griffin C, Tran C, Hamm C, Pandey S: **Selective induction of apoptosis through activation of caspase-8 in human leukemia cells (Jurkat) by dandelion root extract.** *J Ethnopharmacol* 2011, **133**(1):86–91.
24. Bajagopalan V, Hannun YA: **Sphingolipid Metabolism and Signaling as a Target for Cancer Treatment.** In *Cell Death Signaling in Cancer Biology and Treatment*. Edited by Johnson DE. New York: Springer press; 2013:205–229.
25. Ding X, Zhu F-S, Li M, Gao S-G: **Induction of apoptosis in human hepatoma SMMC-7721 cells by solamargine from *solanum nigrum* L.** *J Ethnopharmacol* 2012, **139**(2):599–604.
26. Elmore S: **Apoptosis: a review of programmed cell death.** *Toxicol Pathol* 2007, **35**(4):495–516.
27. Arur S, Uche UE, Rezaul K, Fong M, Scranton V, Cowan AE, Mohler W, Han DK: **Annexin I is an endogenous ligand that mediates apoptotic cell engulfment.** *Dev Cell* 2003, **4**(4):587–598.
28. Park M-T, Lee S-J: **Cell cycle and cancer.** *J Biochem Mol Biol* 2003, **36**(1):60–65.
29. Pucci B, Kasten M, Giordano A: **Cell cycle and apoptosis.** *Neoplasia* 2000, **2**(4):291–299.
30. Zorofchian Moghadamtousi S, Karimian H, Khanabdali R, Razavi M, Firoozinia M, Zandi K, Kadir HA: **Anticancer and antitumor potential of fucoidan and**

fucoxanthin. Two main metabolites isolated from brown algae. *Sci World J* 2014, **2014**. doi:10.1155/2014/768323.
31. Li T, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, Baer R, Gu W: **Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence.** *Cell* 2012, **149**(6):1269–1283.
32. Adams DJ, Boskovic ZV, Theriault JR, Wang AJ, Stern AM, Wagner BK, Shamji AF, Schreiber SL: **High-throughput screening identifies small-molecule enhancers of reactive oxygen species that are nontoxic or cause genotype-selective cell death.** *ACS Chem Biol* 2013, **8**(5):923–929.
33. Circu ML, Aw TY: **Reactive oxygen species, cellular redox systems, and apoptosis.** *Free Radic Biol Med* 2010, **48**(6):749–762.
34. Kushnareva Y, Andreyev AY, Kuwana T, Newmeyer DD: **Bax activation initiates the assembly of a multimeric catalyst that facilitates Bax pore formation in mitochondrial outer membranes.** *PLoS Biol* 2012, **10**(9):e1001394.
35. Saelens X, Festjens N, Walle LV, Grup MV, Loo GV, Vandenabeele P: **Toxic proteins released from mitochondria in cell death.** *Oncogene* 2012, **23**(16):2861–2874. 2012.
36. Hill MM, Adrain C, Duñez PJ, Creagh EM, Martin SJ: **Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes.** *EMBO J* 2004, **23**(10):2134–2145.
37. Mondragón L, Orzáez M, Gortat A, Sancho M, Messeguer A, Vicent MJ, Perez-Paya E: **Molecules That Bind a Central Protein Component of the Apoptosome, Apaf-1, and Modulate Its Activity.** In *Apoptosome*. Edited by Cecconi F, D'Amelio M. Netherlands: Springer press; 2010:75–94.
38. Callus B, Vaux D: **Caspase inhibitors: viral, cellular and chemical.** *Cell Death Differ* 2006, **14**(1):73–78.
39. Delgado ME, Olsson M, Lincoln FA, Zhivotovsky B, Rehm M: **Determining the contributions of caspase-2, caspase-8 and effector caspases to intracellular VDVADase activities during apoptosis initiation and execution.** *BBA-Mol Cell Res* 2013, **1833**(10):2279–2292.
40. Cory S, Adams JM: **The Bcl2 family: regulators of the cellular life-or-death switch.** *Nat Rev Cancer* 2002, **2**(9):647–656.
41. Zamzami N, Kroemer G: **The mitochondrion in apoptosis: how Pandora's box opens.** *Nat Rev Mol Cell Bio* 2001, **2**(1):67–71.
42. Bhardwaj A, Sethi G, Vadhan-Raj S, Bueso-Ramos C, Takada Y, Gaur Y, Nair AS, Shishodia S, Aggarwal BB: **Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-κB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells.** *Blood* 2007, **109**(6):2293–2302.
43. Baldwin AS: **Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB.** *J Clin Invest* 2001, **107**(3):241–246.

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Published Paper 2: *Annona muricata* leaves induce G_1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-29 colon cancer cells

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Conceived and designed the study, performed the experiments, analyzed the data and wrote the manuscript. Main author of the manuscript.

H. Karimian: (Research collaborator)

Contributed reagents/materials/analysis tools**.** Co-author of the manuscript.

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H. A. Kadir: (Principal Supervisor)

Conceived and designed the study, supervised and assisted with experiments, edited the manuscript. Co-author of the manuscript.

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Research Paper

Annona muricata leaves induce G_1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-29 colon cancer cells

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ABSTRACT

Ethnopharmacological relevance: *Annona muricata* known as "the cancer killer" has been widely used in the traditional medicine for the treatment of cancer and tumors. The purpose of this study is to investigate the anticancer properties of ethyl acetate extract of *Annona muricata* leaves (EEAM) on HT-29 and HCT-116 colon cancer cells and the underlying mechanisms.

Materials and methods: The effect of EEAM on the cell proliferation of HT-29 and HCT-116 cells was analyzed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay. High content screening system (HCS) was applied to investigate the cell membrane permeability, mitochondrial membrane potential (MMP), nuclear condensation and cytochrome *c* translocation from mitochondria to cytosol. Reactive oxygen species (ROS) formation, lactate dehydrogenase (LDH) release and activation of caspase-3/7, -8 and -9 were measured while treatment. Flow cytometric analysis was used to determine the cell cycle distribution and phosphatidylserine externalization. The protein expression of Bax and Bcl-2 was determined using immunofluorescence analysis. In addition, the potential of EEAM to suppress the migration and invasion of colon cancer cells was also examined.

Results: EEAM exerted significant cytotoxic effects on HCT-116 and HT-29 cells as determined by MTT and LDH assays. After 24 h treatment, EEAM exhibited the IC_{50} value of 11.43 ± 1.87 μg/ml and 8.98 ± 1.24 μg/ml against HT-29 and HCT-116 cells, respectively. Flow cytometric analysis demonstrated the cell cycle arrest at G_1 phase and phosphatidylserine externalization confirming the induction of apoptosis. EEAM treatment caused excessive accumulation of ROS followed by disruption of MMP, cytochrome *c* leakage and activation of the initiator and executioner caspases in both colon cancer cells. Immunofluorescence analysis depicted the up-regulation of Bax and down-regulation of Bcl-2 proteins while treated with EEAM. Furthermore, EEAM conspicuously blocked the migration and invasion of HT-29 and HCT-116 cells.

Conclusions: These findings provide a scientific basis for the use of *A. muricata* leaves in the treatment of cancer, although further *in vivo* studies are still required.

1. Introduction

Plants as a tremendous source of chemically active metabolites have received emerging attention in the area of pharmacology due to their various curative properties (Moghadamtousi et al., 2013). This critical role in progression of novel drugs is more highlighted considering the fact that natural products were involved in the development of approximately 60% of all therapeutic agents between 1981 and 2012 (Newman and Cragg, 2012). For anticancer agents, this proportion reaches roughly 75% (Newman and Cragg, 2007, 2012). Nonetheless, there are still numerous plant species that require detailed scientific scrutiny. Plants with extensive application in ethnomedicine are a potent source of active constituents for the treatment of different ailments and diseases (Duraipandiyan et al., 2006). Thus, validating the traditional use of these plants and investigating the possible mechanism of bioactivities can provide a rich source of new pharmaceutical products.

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Annona muricata L. (*Annona muricata*) as a member of the Annonaceae family, the custard apple family, is a small tropical tree widely cultivated throughout the tropical countries. *Annona* species such as *Annona squamosa* and *Annona reticulata* are well known to have traditional application in the tropics (Baskar et al., 2007). The popular fruit tree of *Annona muricata* known also as "graviola" or "soursop" has extensive ethnobotonical uses, such as sedative, astringent, piscicide, insecticide, vermifuge, hypotensive, antiparasitic and antispasmodic. Furthermore, it is traditionally used for the treatment of fevers, asthma, pain, coughs, wound and skin remedies (Adewole and Ojewole, 2009). *Annona muricata* was described as "the cancer killer" due to its remarkable cytotoxicity against various cancer cell lines. The seeds and leaves of *Annona muricata* have been widely used by the natives in South America for the treatment of cancer and tumors (Mishra et al., 2013). In tropical Africa, including Nigeria, *Annona muricata* is generally used in folk medicine as an anticancer agent (Watt and Breyer-Brandwijk, 1962; Adewole and Ojewole, 2009). The stems, barks and leaves of *Annona muricata* revealed noteworthy antiproliferative effects against cancer cells without affecting normal cells (Jaramillo et al., 2000; George et al., 2012; Mishra et al., 2013). However, detailed mechanisms of action of *Annona muricata* leaves have not been investigated thus far. The investigation on the ethanolic extract of *Annona muricata* leaves against myelogenous leukemic K562 cell line suggested the induction of apoptosis, although the possible signaling pathway and the role of cell cycle machinery in the induced-apoptosis has not been explained (Ezirim et al., 2013). According to numerous studies, alkaloids (Leboeuf et al., 1980; Leboeuf et al., 1981), acetogenins (Zeng et al., 1996; Chang et al., 2003) and essential oils (Kossouoh et al., 2007) are the main phytoconstituents isolated from *Annona muricata* leaves. Amongst isolated constituents, annonaceous acetogenins are strongly suggested to be involved in the promising antitumor and anticancer effect of *Annona muricata* leaves (Zeng et al., 1996).

The programmed cell death of apoptosis is a critical factor in tissue homeostasis and development which is generally characterized by distinct morphological changes and energy-dependent biochemical mechanisms, namely cell shrinkage, pyknosis, plasma membrane blebbing, phosphatidylserine externalization and reduction in the mitochondrial membrane potential (Peter, 2011). To date, two main apoptotic pathways of extrinsic (death receptor pathway) and intrinsic (mitochondrial pathway) are suggested for apoptosis (Elmore, 2007). A loss in mitochondrial membrane potential leads to the translocation of pro-apoptotic protein Bax to mitochondria, which results in the release of cytochrome *c* into cytosol and subsequent activation of the initiator and executioner caspases (Wyllie, 1997). The maintenance of the constant cell number in colonic mucosa is tightly regulated via balance between cell proliferation and apoptosis. Any alteration in this balance leads to an escape from the homeostasis which can result in the development of cancer cells (Hao et al., 1998; Whitfield, 2009). Thus, the key mechanisms in the prevention and suppression of colon cancer are inhibition of cell cycle machinery and induction of apoptosis in these aberrant cells. Hence, there is an urgent need and interest in the development of novel apoptosis-inducing agents showing more specificity and efficacy with minimal side-effects. In this study, we aimed to establish the possible apoptotic pathways induced in HT-29 and HCT-116 colon cancer cells by ethyl acetate extract of *Annona muricata* leaves.

2. Materials and methods

2.1. Plant materials

The leaves of *Annona muricata* were collected from Ipoh, Malaysia, in March 2013 and identified by Dr. Yong Kien Thai. A voucher specimen of this plant has been deposited at the herbarium of the University of Malaya (No. KLU47978).

2.2. Preparation of the extracts

The dried leaves of *Annona muricata* (1 kg) were first extracted with n-hexane (1500 ml, three times). Then, the extract was filtered and dried on the rotary evaporator. The residue was sequentially re-extracted with ethyl acetate (1500 ml, three times) and methanol (1500 ml, three times) and the resulting filtrates were dried under reduced pressure by a rotary evaporator at 40 °C to yield n-hexane (7 g), ethyl acetate (11 g) and methanol (5 g) extracts, respectively.

2.3. Cell culture

CCD841 (normal human colon epithelial cells), HT-29 and HCT-116 (human colon cancer cells) were obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). The cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (PAA Lab, Pasching, Austria), 50 μg/ml amphotericin B (PAA Lab) and 100 U/ml penicillin (PAA Lab) in a humidified atmosphere with 5% CO_2 in the air at 37 °C. Cells in the exponential growth phase were collected for the following experiments. The negative control for all the assays was represented by the untreated medium containing vehicle DMSO (0.1%).

2.4. Cell viability assay

Cell viability was evaluated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as previously described (Mosmann, 1983). In brief, cells (5×10^4 cells/ml) were treated with three isolated extracts at different concentrations in 96-well plate and incubated for 72 h. The colorimetric assay was measured at the absorbance of 570 nm using a microplate reader (Asys UVM340, Eugendorf, Austria). In MTT assay, 5-Fluorouracil (5-FU) (Sigma, St. Louis, MO, USA) as a standard anticancer drug was used as a positive control. The colorimetric assay was measured at the absorbance of 570 nm using a microplate reader (Asys UVM340, Eugendorf, Austria). The antiproliferative potential of the three isolated extracts was expressed as IC_{50} value, the concentration of plant extract that caused 50% inhibition of cell growth which was calculated based on the percentage of cell viability. As ethyl acetate extract of *Annona muricata* leaves (EEAM) showed the strongest IC_{50}, we used only EEAM to continue the study. The percentage of cell viability=(absorbance of treated cells/absorbance of untreated cells) × 100%

2.5. Gas chromatography of EEAM

To examine the chemical profiling of EEAM, the analysis of ethyl acetate extract was carried out using an Agilent and LECO RESTEK, Rxi-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) and a mass spectrometer Pegasus HT High Throughput TOFMS, as previously described in detail (Daferera et al., 2000). The carrier gas was helium at a flow rate of 1 ml/min. Initially, column temperature was 40 °C for 5 min, then gradually elevated to 160 °C at 4 °C/min, and finally reached 280 °C at 5 °C/min and held for 10 min. For GC–MS detection, an electron ionization system was used with ionization energy of 70 eV. The fraction was diluted 1:100 (v/v) with ethyl acetate and 1.0 μl of the diluted sample was injected automatically in splitless mode. Injector temperature was set at 250 °C. Compounds were identified from their mass spectra, by comparison of the retention times of peaks with interpretation of MS fragmentation patterns from the National

Institute of Standards and Technology (NIST147) Mass Spectral Database.

2.6. LDH release assay

LDH release assay was carried out using Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, Pittsburgh, PA, USA), as previously described (Hajrezaie et al., 2014). Measurement of lactate dehydrogenase (LDH) release in the media is an easy biomarker for determining the extent of cell death showing the elevated permeability of the plasma membrane. In brief, different concentrations of colon cancer cells were treated with EEAM and Triton X-100 as positive control for 48 h. After transferring the supernatant of the treated colon cancer cells into 96-well plates, LDH reaction solution (100 μl) was added for 30 min. Then, a Tecan Infinite®200 Pro (Tecan, Männedorf, Switzerland) microplate reader was used to measure the absorbance of red color intensity at 490 nm showing the LDH activity.

2.7. Reactive oxygen species (ROS) assay

To examine the effect of EEAM on the ROS generation, we carried out ROS assay, as previously described in detail (Peshavariya et al., 2007). In brief, treated colon cancer cells with EEAM at different concentrations in 96-well plates were incubated for 24 h prior to staining with dihydroethidium (2.5 μg/ml) for 30 min. After fixing and washing the cells, the fluorescent intensity was measured at 485 nm excitation and 520 nm emission using a Tecan Infinite®200 Pro (Tecan, Männedorf, Switzerland) microplate reader.

2.8. Annexin-V-FITC assay

Distribution of early and late apoptotic cells after treatment with EEAM was further investigated using Annexin-V/PI staining assay (Vermes et al., 1995). In brief, colon cancer cells were seeded in 60 mm^2 culture dishes and treated with vehicle DMSO and EEAM (10 μg/ml) for 24, 48 and 72 h. After incubation time, cells were harvested and washed twice with PBS. Then, cells were re-suspended in Annexin-V binding buffer (BD Biosciences, San Jose, CA, USA) and stained with Annexin-V-FITC (BD) and PI (Sigma) following the vendor's kit manual. The fluorescent intensity of colon stained cells was then determined using BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) through quadrant statistics for necrotic and apoptotic cell populations.

2.9. Cell cycle assay

Changes in cell cycle distribution induced by EEAM were analyzed using a flow cytometric analysis (Nicoletti et al., 1991). In brief, colon cancer cells (5×10^4 cells/ml) were treated with DMSO as a negative control and EEAM (10 μg/ml) for 24, 48 and 72 h. Then, the treated cells were centrifuged at 1800 rpm for 5 min and the pellet was washed twice with PBS. Then, cells were fixed by mixing 700 μl of 90% cold ethanol. The fixed cells stained with PI (50 μl, 10 mg/ml) for 1 h at 37 °C. RNase A (10 mg/ml) was also used to limit the ability of PI to bind only to DNA molecules. The stained cells were analyzed for DNA content using BD FACSCanto II flow cytometer.

2.10. Multiple cytotoxicity assay

To analyze the critical apoptotic events in colon cancer cells after treatment with EEAM, including cell permeability, mitochondrial membrane potential (MMP), cytochrome *c* release and total nuclear intensity, we used Cellomics Multiparameter Cytotoxicity 3 Kit (Thermo Scientific, Pittsburgh, PA) as previously described in detail (Lövborg et al., 2004). In brief, colon cancer cells were seeded into 96-well plates for 24, 48 and 72 h. After treatment of cells with EEAM (10 μg/ml), cell permeability and MMP dyes were added to live cells prior to fixing and blocking the cells with 1 × blocking buffer. Then, primary cytochrome *c* antibody and secondary DyLight 649 conjugated goat anti-mouse IgG were added for 1 h. Staining solution was also supplemented with Hoechst 33342 dye to stain nucleus. Stained cells in 96-well plates were analyzed using ArrayScan high content screening (HCS) system.

2.11. Bioluminescent assays for caspase-8, -9 and -3/7 activities

To determine the pathways involved in induced apoptosis, we carried out a dose-dependent study on caspase-8, -9 and -3/7 activation using Caspase-Glo-3/7, -8 and -9 kit (Promega, Madison, WI, USA) according to the manufacture's protocols. In brief, colon cancer cells (1×10^4 cells/well) were seeded into white 96-well microplate for 24 h and treated with 10 μg/ml of EEAM or DMSO vehicle control. After 24, 48 and 72 h of incubation, caspase-Glo reagent (100 μl) was added to the wells following with incubation for 30 min. The induced activation of caspases was determined using a Tecan Infinite 200 Pro (Tecan, Männedorf, Switzerland) microplate reader (Muhammad Nadzri et al., 2013).

2.12. Immunofluorescence analysis for Bax and Bcl-2

Colon cancer cells (5×10^4 cells/ml) were cultured in 96-well plates and treated with EEAM (10 μg/ml) for 24, 48 and 72 h. The treated cells were fixed with 3.5% paraformaldehyde for 15 min and supplemented with blocking buffer followed by 1 h of incubation in 0.03% Triton X-100/PBS and normal serum. Then, blocking buffer was aspirated prior to adding diluted primary antibody solution containing 1 × PBS/1% BSA/0.3% Triton X-100. Cells were incubated at 4 °C for 24 h. Bcl-2 and Bax fluorochrome-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in antibody dilution and added to the cells following with incubation for 1 h. After every step, cells were gently washed three times with PBS. Colon cancer cells were supplemented with DAPI prior to being examined using CellReporter, Molecular Devices, USA (Karimian et al., 2014).

2.13. Migration assay

To determine the suppressive effect of EEAM on migration of colon cancer cells, HCT-116 and HT-29 cells were seeded in a 6-well plate for 24 h. Then, cells were scratched with 100 μl pipette tip following with treatment with EEAM (10 μg/ml). After washing the cells with PBS twice to remove nonadherent cells, photomicrographs were captured in 0, 24, 48 and 72 h after wounding using a microscope Olympus BX51 (Olympus, Tokyo, Japan) (Sims et al., 2011).

2.14. Invasion assay

We used the commercial kit of Cultrex96 Well BME Cell Invasion Assay (Trevigen, Gaithersburg, MD, USA) according to the vendor's instructions. In brief, basement membrane extract coating solution (100 μl) was used at 37 °C for 4 h to evenly coat a 96-well unit containing 8 μm polycarbonate nucleopore filters (Corning). Colon cancer cells (2×10^5 cells/ml) in RPMI medium were placed in the upper compartment, and the lower compartment was also filled with the medium. After treatment with EEAM (10 μg/ml) for 24, 48 and 72 h, a cotton swab was used to remove the cells that had not invaded. At the end, invasion of treated colon cancer cells to the lower surface was measured using a microplate

reader (Infinite M200PRO, Tecan, Männedorf, Switzerland) (Sims et al., 2011).

2.15. Statistical analysis

All values were presented as mean ± SEM of three different experiments. A one-way analysis of variance (ANOVA) was performed using the Prism statistical software package (GraphPad Software, USA). Differences were considered as being significant at $^{*}p < 0.05$.

3. Results

3.1. EEAM suppressed the proliferation of colon cancer cells

The isolated extracts of *Annona muricata* elicited different suppressive effects on colon cancer cells. The growth inhibitory effect on the proliferation of cell lines are shown in Table 1 with IC_{50} values presented. After treatment with hexane and ethyl acetate extracts for 72 h, both HT-29 and HCT-116 cell numbers reduced dose-dependently, whereas the methanol extract did not affect the proliferation of colon cancer cells. IC_{50} values revealed that EEAM seemed to be the most active extract with IC_{50} values of 3.91 ± 0.35 μg/ml and 4.29 ± 0.24 μg/ml against HCT-116 and HT-29 cells, respectively, which were comparable with 5-FU cytotoxic potential as a standard anticancer drug. EEAM exhibited the IC_{50} values of 11.43 ± 1.87 μg/ml and 8.98 ± 1.24 μg/ml against HT-29 and HCT-116 cells after 24 h, respectively. IC_{50} values towards HT-29 and HCT-116 cells after 48 h treatment with EEAM were found to be 6.89 ± 0.70 μg/ml and 5.41 ± 0.44 μg/ml, respectively. It is worth noting that three isolated extracts revealed no noteworthy growth inhibitory potential towards normal human colon CCD841 cells compared to colon cancer cells. DMSO (0.1%) did not show any sign of toxicity, which was used as a vehicle control. The MTT result suggests that EEAM suppressive effect is selective for colon cancer cells with the safety index of 7.98 and 8.75 towards HCT-116 and HT-29 cells, respectively.

Table 1
Suppressive effect of EEAM and 5-FU on the proliferation of human colon cancer cells. IC_{50} values of *Annona muricata* leaves extracts on HT-29, HCT-116 and CCD841 cell lines after 72 h treatment with EEAM.

Extract	Cell lines, IC_{50} value (μg/ml)		
	HT-29	HCT-116	CCD841
Hexane extract	14.93 ± 0.64	12.26 ± 0.42	42.19 ± 4.66
Ethyl acetate extract	4.29 ± 0.24	3.91 ± 0.35	34.24 ± 2.12
Methanol extract	> 100	> 100	> 100
5-FU	1.10 ± 0.11	0.90 ± 0.09	–

3.2. Gas chromatography profile of EEAM using GC–MS-TOF analysis

As depicted in Fig. 1, chemical profiling of EEAM was investigated by use of GC–MS-TOF. The chromatogram showed the presence of 11 different compounds (Table 2) and the major compound of EEAM was found to be 1,2,3-Propanetriol, 1-acetate.

3.3. EEAM induced LDH leakage from colon cancer cells

A stable cytosolic enzyme of LDH which catalyzes the oxidation of L-lactate to pyruvate is released from cytosol after any irreversible membrane damage to cells (Furtado et al., 2012). After exposure to EEAM, LDH release was dose-dependently elevated in colon cancer cells compared to the untreated cells. At 10–80 μg/ml concentrations, LDH leakage was significantly increased, confirming the observed cytotoxicity in MTT assay (Fig. 2 B), while EEAM at 5 μg/ml concentration did not cause the significant release of the enzyme. The result suggested that EEAM has roughly the same cytotoxic effect on both HCT-116 and HT-29 cells, although the MTT result showed the stronger cytotoxic effect on HCT-116 cell.

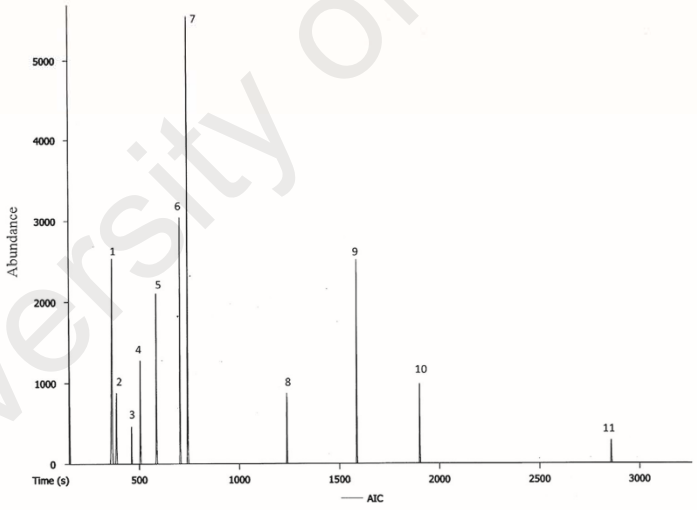

Fig. 1. GC–MS-TOF analysis of EEAM.

3.4. ROS formation induced by EEAM

Oxidative stress in cancer cells because of various biochemical and physiological lesions results in metabolic impairments and cell death (Cairns et al., 2011). ROS-induced oxidative injury leading to apoptotic death is a critical factor involved in the cytotoxic effects of the various phytoconstituents (Ziech et al., 2011). Thus, we measured the effect of EEAM on the production of ROS in the colon cancer cells. As illustrated in Fig. 2, there was a dose-dependent elevation in the level of generated ROS in the treated colon cancer cells compared to untreated cells. EEAM caused significant ROS production at 10–80 μg/ml concentrations (Fig. 2A).

3.5. Annexin-V externalization induced by EEAM

To examine the effect of EEAM on the apoptosis of colon cancer cells, Annexin-V-FITC probe was used to determine the percentage of cells undergoing apoptosis. Apoptotic cells were examined by counting the percentage of early apoptotic cells (Annexin-V positive and PI negative) and late apoptotic cells (both Annexin-V and PI positive). After treatment with EEAM, both the percentages of early and late apoptotic cells were elevated time-dependently (Fig. 3). After 72 h of treatment, the late apoptotic percentage of HCT-116 cells was elevated to 46.21 ± 3.13%, while the percentage of HT-29 cells increased to 12.53 ± 1.65%. The early apoptotic population was increased to 27.23 ± 4.65% and 16.65 ± 2.15% for HCT-116 and HT-29 cells, respectively. After 72 h treatment, the percentage of viable HCT-116 cells was reduced more effectively compared to the HT-29 cells. The result implies that EEAM can effectively induce apoptosis in both colon cancer cells.

Table 2
The detected compounds in EEAM were characterized using GC–MS-TOF analysis.

Peak no.	Name of compounds	Retention time (s)	Similarity (%)
1	3-Hexenoic acid	364	90
2	Hexenoic acid	386	81
3	2-Pyrrolidinone	460	58
4	1,2,3-Propanetriol, 1-acetate	503	86
5	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	144	88
6	Phenol, 4-ethenyl-, acetate	703	85
7	1,2,3-Propanetriol, 1-acetate	741	84
8	Fumaric acid, 4-chlorophenyl ethyl ester	1236	78
9	Phytol acetate	1584	86
10	Phytol	1899	82
11	Tocopherol	2856	63

3.6. Cell cycle arrest at G_1 induced by EEAM

As depicted in Fig. 4, there is a significant G_1 phase arrest in treated colon cancer cells in a time-dependent manner. After 72 h of treatment, the number of cells at G_1 phase significantly elevated from roughly 40% (untreated control) to 62.45% for HCT-116 and 70.22% for HT-29 cells. The number of cells in sub-G_0/G_1 phase with hypodiploid DNA in DNA histogram considered as apoptotic cells were significantly elevated after 72 h of treatment. Subsequently, the number of cells in the S phase also significantly decreased promoting cell cycle arrest at G_1 phase.

3.7. Mitochondria-initiated events induced by EEAM

To further confirm the induction of apoptosis as suggested by Annexin-V-FITC assay, we examined the critical apoptotic markers in colon cancer cells. Treatment with EEAM led in a dose-dependent elevation in cell membrane permeability (Fig. 6) shown by a noteworthy increase in green fluorescent intensity of colon cancer cells (Fig. 5). The red fluorescent intensity showing MMP was significantly decreased after EEAM (10 μg/ml) treatment. Quantitative measurement of MMP perturbations showed the significant collapse in MMP after EEAM (10 μg/ml) treatment (Fig. 6). Assessment of cytochrome *c* release from mitochondria using cyan fluorescent probes demonstrated the significant translocation of cytochrome *c* to the cytosol (Fig. 5). Release of cytochrome *c* was found elevated in a time-dependent manner (Fig. 6). These results further confirmed that EEAM suppressed colon cancer cell proliferation by induction of apoptosis.

3.8. Caspase activation induced by EEAM

The increase in the level of ROS and reduction in MMP may trigger the caspase cascade. Therefore, the activation of caspase-3/7, -8 and -9 in colon cancer cells upon treatment with EEAM was investigated through measuring the bioluminescent intensities. As shown in Fig. 7, all the caspases under investigation in both HCT-116 and HT-29 cells found to be induced while treatment. At the concentration of 10 μg/ml, the activity of caspases was elevated in a time-dependent manner. The activity of caspase-9 was significantly increased after 12 h of treatment, while caspase-8 and caspase-3/7

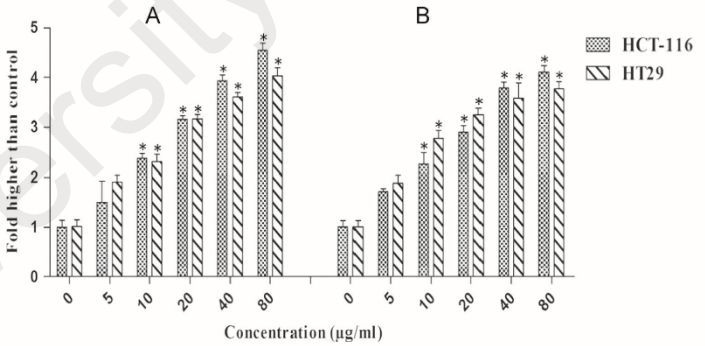

Fig. 2. The result of (A) ROS and (B) LDH assays on HCT-116 and HT-29 cells demonstrated significant LDH release and ROS generation at concentrations of 10–80 μg/ml. The data represent the means ± SEM of three independent experiments. *$P < 0.05$ compared with the untreated group.

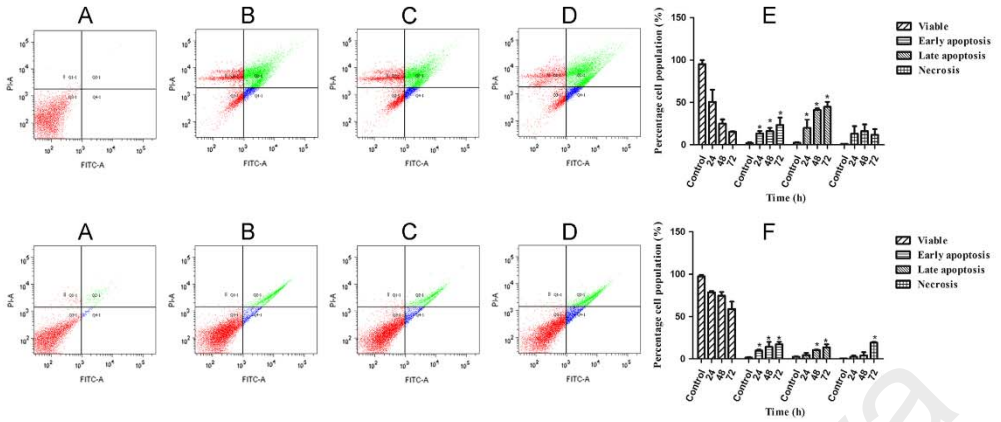

Fig. 3. Time-dependent apoptosis rates of colon cancer cells treated with EEAM detected by flow cytometry after (A) 0 h, (B) 24 h, (C) 48 h and (D) 72 h. The bar charts illustrated the percentage of (E) HCT-116 and (F) HT-29 cells undergoing early and late apoptosis. The data represent the means _ SEM of three independent experiments. $^{*}P < 0.05$ compared with the untreated group.

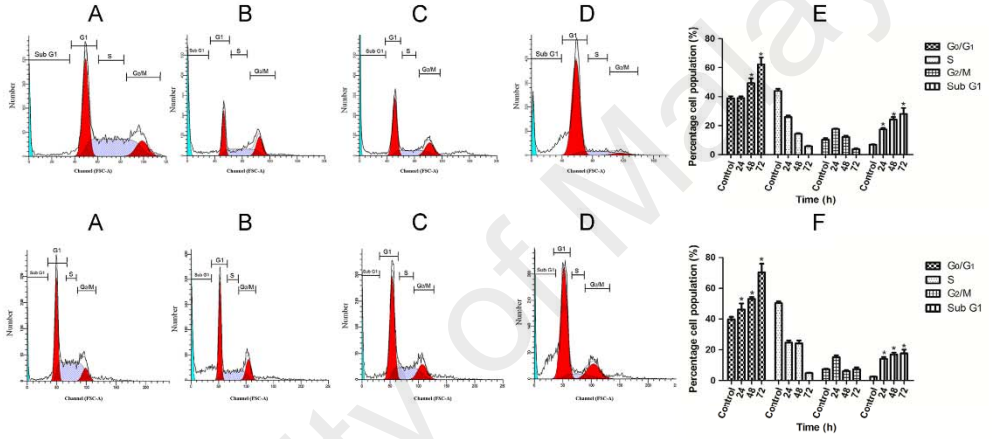

Fig. 4. Flow cytometric analysis of cell cycle arrest of colon cancer cells treated with EEAM (10 μg/ml) in a time-dependent manner, (A) control, (B) 24, (C) 48 and (D) 72 h. The representative bar charts for (E) HCT-116 and (F) HT-29 cells depicted the significant cell cycle arrest in the G_1 phase after 72 h of treatment. The data represent the means ± SEM of three independent experiments. $^{*}P < 0.05$ compared with the untreated group.

was significantly activated only after 24 h of treatment (Fig. 7). Our present findings demonstrated that EEAM induced apoptosis in both colon cancer cells is predominantly through the intrinsic pathway.

3.9. *Bax up-regulation and Bcl-2 down-regulation induced by EEAM*

To determine whether EEAM induced apoptosis in colon cancer cells by altering the expression of Bax and Bcl-2 proteins, levels of these proteins following EEAM treatment were examined by immunofluorescence analysis. Treatment of HCT-116 (Fig. 8) and HT-29 (Fig. 9) cells with EEAM (10 μg/ml) significantly induced the time-dependent elevation in the expression level of Bax and reduction in the expression level of Bcl-2 (Fig. 10). After 72 h of treatment, the numbers of cells markedly reduced shown by DAPI staining, which identifies all cell nuclei (blue) (Figs. 8 and 9). The fluorescent intensity of FITC (green) conjugated to the Bax antibodies was time-dependently increased, while the fluorescent intensity of Bcl-2 protein was decreased after 72 h of treatment. These results implied that EEAM can induce apoptosis of colon cancer cells through the perturbation in the expression of Bax and Bcl-2 proteins.

3.10. *Inhibitory effect of EEAM on the migration and invasion of colon cancer cells*

In the present study, the wound healing effect of EEAM was determined against colon cancer cells, which are known to be highly metastatic. The migration assay elicited that EEAM

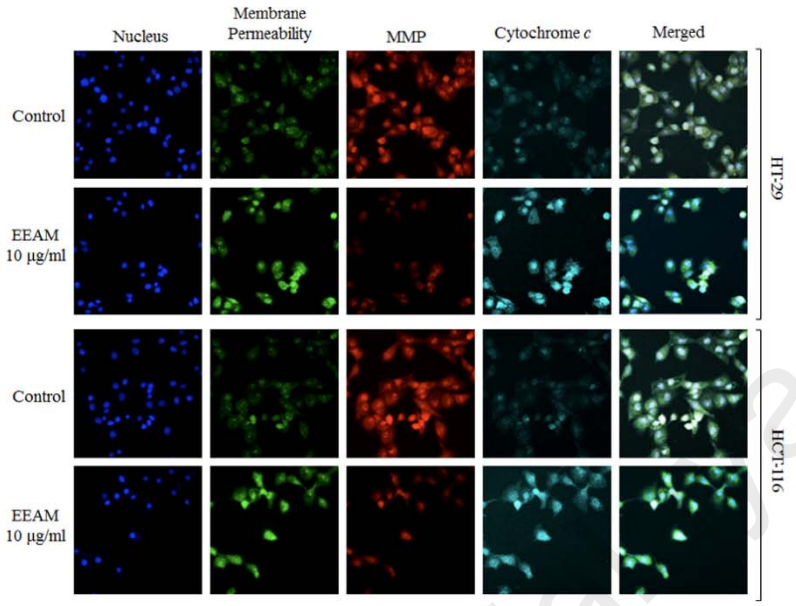

Fig. 5. Representative images of colon cancer cells treated with EEAM (10 μg/ml), stained with Hoechst 33342, cell permeability, MMP and cytochrome *c* dyes. EEAM treatment caused a noteworthy elevation in fluorescent intensity of cytochrome *c* and membrane permeability dyes associated with reduction in fluorescent intensity of MMP.

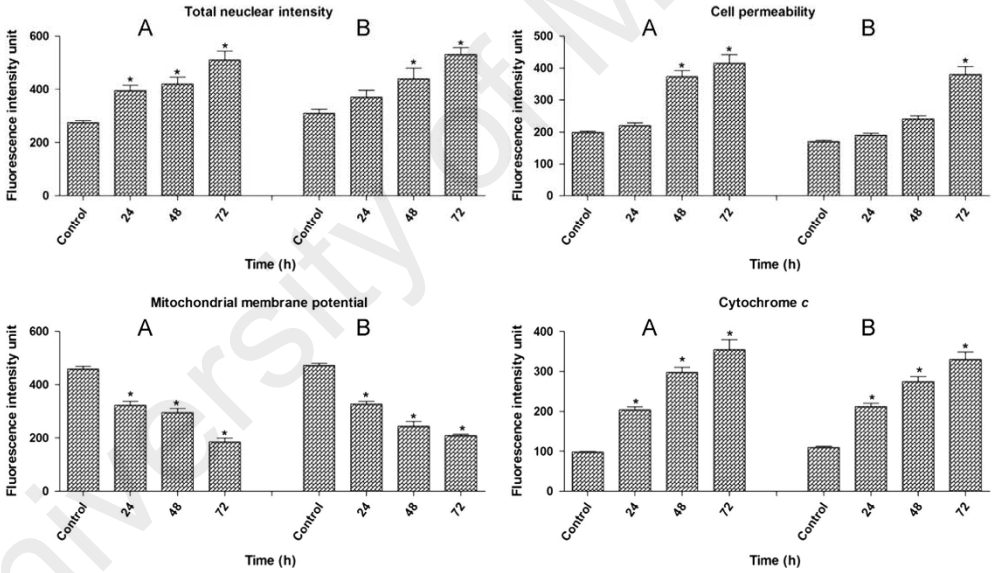

Fig. 6. Representative bar charts of EEAM mediated apoptosis markers for (A) HCT-116 and (B) HT-29 cells. Changes in cell membrane permeability, MMP and cytochrome *c* translocation were simultaneously quantified in colon cancer cells. Following treatment with EEAM, quantitative analysis of the images demonstrated the significant collapse in MMP, elevation in cell membrane permeability and cytochrome *c* release from mitochondria to cytosol, in a time-dependent manner. The data represent the means ± SEM of three independent experiments. $^{*}P < 0.05$ compared with the untreated group.

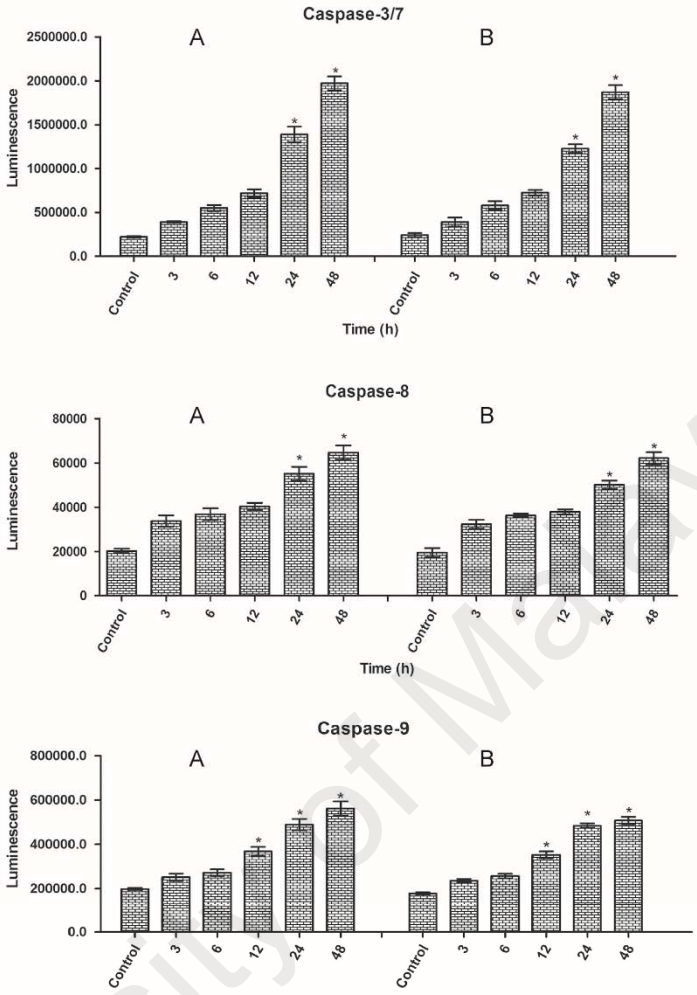

Fig. 7. Relative luminescence expression of caspase-3/7, -8 and -9 in (A) HCT-116 and (B) HT-29 cells treated with EEAM (10 μg/ml) for 3, 6, 12, 24 and 48 h. The data represent the means + SEM of three independent experiments. $^{*}P < 0.05$ compared with the untreated group.

markedly inhibited the migration of colon cancer cells (Fig. 11). The representative bar charts of the EEAM suppressive effect depicted more than 70% inhibition of treated colon cancer cells compared to the untreated cells after 72 h. The inhibitory effect of EEAM against the invasion of colon cancer cells was determined using a chamber assay and measuring the number of migrated HCT-116 and HT-29 cells through the barrier. After 72 h of treatment, the invasion of HCT-116 cells and HT-29 cells was decreased to 8.13% and 17.25% compared to the control, respectively (Fig. S1). The present findings showed that EEAM can effectively suppress the invasion of colon cancer cells. These results suggested that EEAM can effectively suppress the migration and invasion of colon cancer cells besides the induction of apoptosis.

4. Discussion

The growing body of molecular and histological experiments supporting the correlation between apoptosis and anticancer activity of pharmaceutical agents has attracted many researchers to focus on the development of new anticancer agents with potent apoptotic-inducing effects (Khan et al., 2010; Zorofchian Moghadamtousi et al., 2014). In the present study, we have investigated the apoptosis-inducing capability of *Annona muricata* leaves. Our studies using two colon cancer cell lines imply that irrespective of the type of colon cancer cells, EEAM could induce anti-proliferative effects as determined by MTT and LDH assays. However, EEAM was found to induce cytotoxicity in the normal

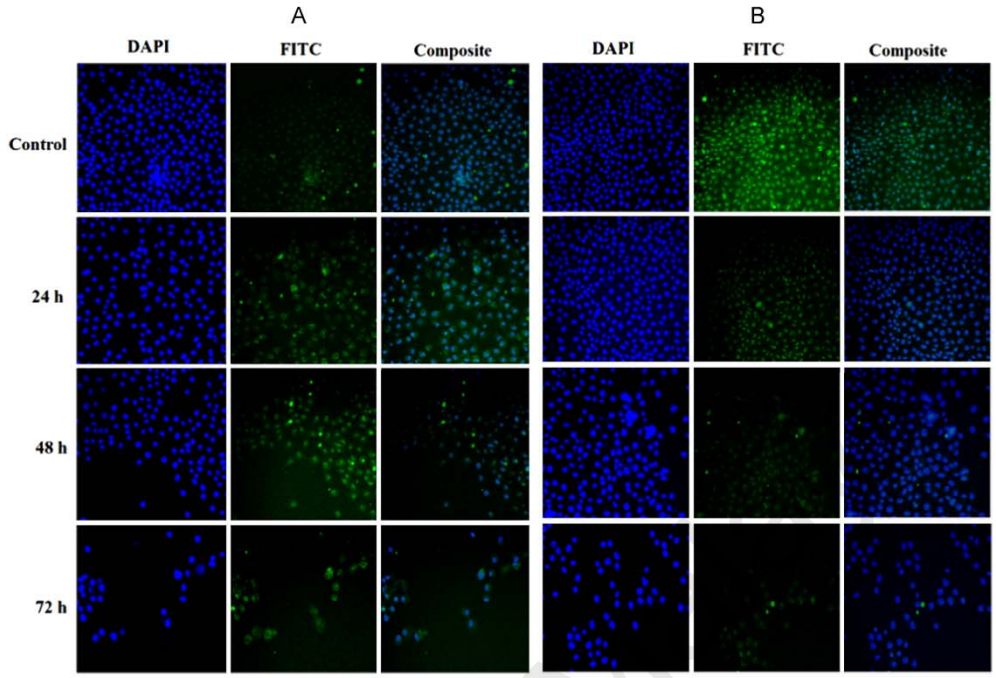

Fig. 8. Immunofluorescent detection of Bax and Bcl-2 in HCT-116 cells. The fluorescent intensity of (A) Bax and (B) Bcl-2 antibodies conjugated to FITC was increased and decreased, respectively, in a time-dependent manner. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

colon cells at markedly higher doses than the cancer cells. Previously, it has been reported that *Annona muricata* leaves induced cytotoxicity in other cancer cell lines, such as MDA-MB-435S and HeLa, with the results comparable to our study (George et al., 2012; Heri Suselo and Jusuf, 2012; Mishra et al., 2013). However, the detailed mechanism by which the *Annona muricata* leaves induce apoptosis has not been identified.

Cancer is generally well known as a disease of the cell cycle dysfunction. Deregulation of the molecular machinery of the cell cycle is one of the most critical alterations during tumor progression (Williams and Stoeber, 2012). The ability to block the cell cycle progression in cancer cells can effectively elevate the anticancer potential of natural products (Mantena et al., 2006). To investigate whether EEAM induced growth suppression is mediated by the cell cycle arrest, we examined the cell cycle distribution of colon cancer cells using flow cytometric analysis. The present study demonstrated that EEAM arrests the cell cycle of colon cancer cells at G_1 phase.

The results of our present study using flow cytometric analysis of Annexin V/PI staining demonstrated that EEAM elicited a markedly induced apoptosis in colon cancer cells. This finding was confirmed by HCS multiple cytotoxicity analysis that revealed critical characterizations of apoptosis, including nuclear condensation, decrease in MMP, cytochrome *c* leakage and perturbation in the membrane symmetry. Among the various initiating stimuli for disruption in the MMP, ROS has a critical role in the induction of apoptosis by natural products, since excessive accumulation of ROS cause oxidative DNA damage (Schumacker, 2006). The present study demonstrated that treatment with EEAM significantly increased the generation of ROS in colon cancer cells, in a concentration dependent manner. These results suggested that disruption in the MMP was caused by excessive accumulation of ROS in cancer cells. Collapse in MMP as an early apoptotic event leads to the release of pro-apoptotic molecules, mainly cytochrome *c* (Ly et al., 2003). Collectively, these findings revealed pro-apoptotic molecules involvement in the activation of the caspase cascade and subsequent demise of the cell (Liu et al., 2010).

To date, intensive molecular studies prove that two main apoptotic pathways, namely extrinsic via death receptor and mitochondrial-mediated intrinsic pathways can be triggered by the caspases (Ocker and Höpfner, 2012). Intrinsic pathway triggered by cytochrome *c* activates the caspase-9, while the extrinsic pathway activates the caspase-8 (Mishra and Kumar, 2005). Once these initiator caspases are activated, the execution phase of apoptosis via activation of the caspase-3, -6 and -7 is triggered. These executioner caspases are responsible for the later morphological changes in the apoptotic cells, such as chromatin condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies (Hengartner, 2000). The present findings showed that treatment with EEAM elevated the activities of caspase-3/7 and caspase-9 in HT-29 and HCT-116 cells. It is postulated that the induction of apoptosis in colorectal cancer cells is via a mitochondrial-mediated pathway. Pro-caspase-8 is an important factor in the formation of a death-inducing signaling complex (DISC) and its activation in the EEAM-treated colon cancer cells

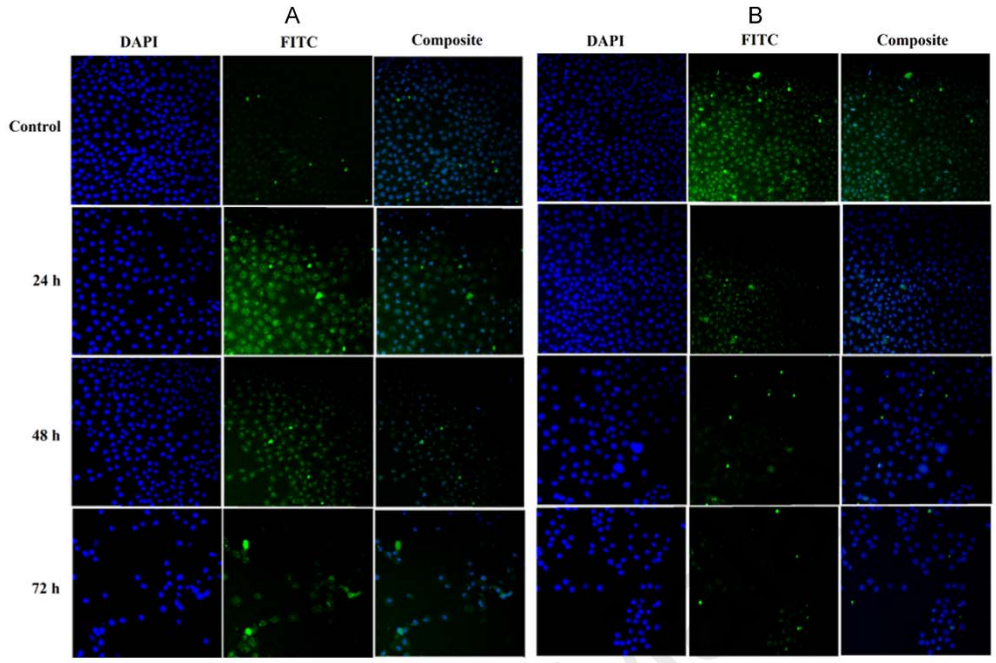

Fig. 9. Immunofluorescent detection of Bax and Bcl-2 in HT-29 cells. The result elicited the marked elevation in the fluorescent intensity of (A) Bax and reduction in the fluorescent intensity of (B) Bcl-2 antibodies conjugated to FITC, in a time-dependent manner. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

implies that apoptosis is also induced via the death receptor mediated pathway (Young et al., 2012). Activation of both pathways of apoptosis by natural products such as curcumin has been reported in the earlier studies (Karunagaran et al., 2005; Lee et al., 2011).

The apoptotic mitochondrial events are tightly regulated by the members of the Bcl-2 family of proteins (Cory and Adams, 2002). Pro-apoptotic and anti-apoptotic proteins in this family govern the mitochondrial membrane permeability. Pro-apoptotic protein of Bax via conformational changes and translocation to the mitochondria mediates the cytochrome *c* release (Liu et al., 2003). Meanwhile, anti-apoptotic protein of Bcl-2 suppresses the cytochrome *c* leakage from mitochondria (Martinou and Youle, 2011). The present findings indicated that EEAM treatment elevated the protein expression of Bax and decreased the expression of Bcl-2 proteins in colon cancer cells, which confirmed the involvement of the intrinsic pathway of apoptosis in colon cancer cells. The potential to suppress the invasive and migratory activity of tumor cells provides an effective approach to the treatment of cancers.

Migration and invasion of cancer cells allow them to penetrate and attach to the surrounding tissues. Metastatic tumors due to uncontrollable spread to different tissues and organs are the main cause of death in cancer patients (Hood and Cheresh, 2002). The most prevalent sites for the metastasis of the rectal and colon cancer are liver and lung (Mills and Stamos, 2014). Furthermore, it was reported earlier that HT-29 and HCT-116 cells are highly metastatic (Ishizu et al., 2007; Murakami et al., 2013). The present study demonstrated that EEAM treatment could conspicuously block the migration and invasion of both colon cancer cells.

Some of the phytoconstituents detected in EEAM by GC–MS-TOF analysis when isolated from various plants have shown apoptosis inducing effects in different cancer cell lines. For example, phytol and phytol acetate are well established to have apoptotic potential and anticancer activity in several plants such as *Kedrostis foetidissima* (Kalaisezhiyen and Sasikumar, 2012), *Lolium multiflorum* (Komiya et al., 1999) and *Typhonium flagelliforme* (Lai et al., 2008). Tocopherol and its derivatives have demonstrated an extensive range of apoptotic effects against various cancer cells, including erythroleukemia, prostate and breast cancer cells (Sigounas et al., 1997; Yu et al., 1999; Gysin et al., 2002; Jiang et al., 2004). The apoptotic activity of the compounds detected in EEAM is closely correlated with the findings of our present investigation. However, further studies on the pure compounds isolated from *Annona muricata* leaves through bioassay guided approach are still required in order to certainly explore the major active compounds responsible for the promising anticancer activity of the leaves.

5. Conclusions

In summary, these findings demonstrate that *Annona muricata* leaves has noteworthy pro-apoptotic potential, irrespective of the type of colon cancer cells. Our study shows that EEAM induced mitochondrial-mediated apoptosis in colon cancer cells associated

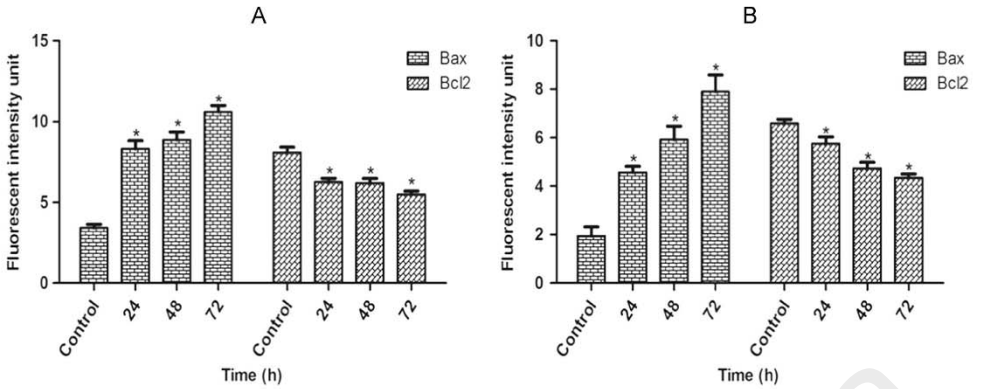

Fig. 10. Quantitative analysis of Bax and Bcl-2 expression in (A) HCT-116 and (B) HT-29 cells. The bar charts depicted a time-dependent elevation in the Bax expression and reduction in the Bcl-2 expression at the protein level. The data represent the means ± SEM of three independent experiments. $^{*}P < 0.05$ compared with the untreated group.

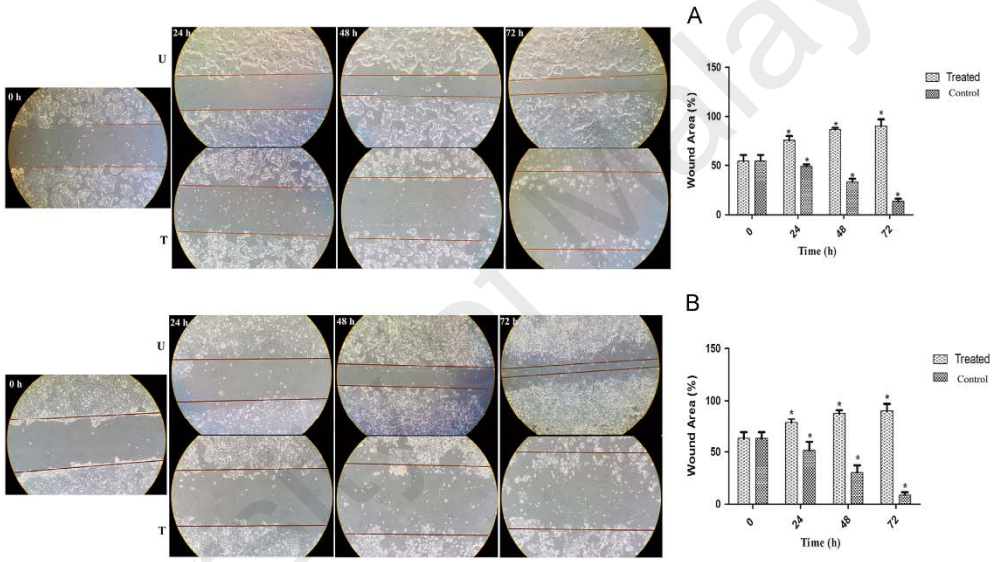

Fig. 11. EEAM inhibited migration of colon cancer cells. Seeded colon cancer cells in 6-well plates were wounded and imaged (0 h). Treated (A) HCT-116 and (B) HT-29 cells with EEAM (10 μg/ml) (T) and untreated (U) cells were incubated for 72 h. Untreated colon cancer cells significantly migrated within 72 h, while EEAM treatment effectively suppressed the migration of colon cancer cells. The representative bar charts demonstrated the percentage of migrated (A) HCT-116 and (B) HT-29 cells. The data represent the means ± SEM of three independent experiments. $^{*}P < 0.05$ compared with the untreated group.

with the G_1 cell cycle arrest. EEAM induces apoptosis by generating ROS and down-regulating anti-apoptotic Bcl-2 while up-regulating pro-apoptotic Bax. These processes subsequently lead to attenuation of MMP and cytochrome *c* release. Release of cytochrome *c* activates caspase cascade that triggers execution of apoptosis through DNA fragmentation. Furthermore, migration and invasion of colon cancer cells were blocked by EEAM where constitutive metastasis of tumor cells remains a critical obstacle in cancer treatment. These findings suggest the potential therapeutic value of EEAM and *in vivo* antitumor investigation of the leaves extract is underway.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.08.011.

References

Adewole, S.O., Ojewole, J.A., 2009. Protective effects of *Annona muricata* Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats. African Journal of Traditional, Complementary, and Alternative Medicines 6, 30–41.

Baskar, R., Rajeswari, V., Kumar, T.S., 2007. *In vitro* antioxidant studies in leaves of *Annona* species. Indian Journal of Experimental Biology 45, 480–485.

Cairns, R.A., Harris, I.S., Mak, T.W., 2011. Regulation of cancer cell metabolism. Nature Reviews Cancer 11, 85–95.

Chang, F.-R., Liaw, C.-C., Lin, C.-Y., Chou, C.-J., Chiu, H.-F., Wu, Y.-C., 2003. New adjacent bis-tetrahydrofuran Annonaceous acetogenins from *Annona muricata*. Planta Medica 69, 241–246.

Cory, S., Adams, J.M., 2002. The Bcl2 family: regulators of the cellular life-or-death switch. Nature Reviews Cancer 2, 647–656.

Daferera, D.J., Ziogas, B.N., Polissiou, M.G., 2000. GC–MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. Journal of Agricultural and Food Chemistry 48, 2576–2581.

Duraipandiyan, V., Ayyanar, M., Ignacimuthu, S., 2006. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. BMC Complementary and Alternative Medicine 6, 35.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicologic Pathology 35, 495–516.

Ezirim, A., Okachi, V., James, A., Adebeshi, O., Ogunnowo, S., Odeghe, O., 2013. Induction of apoptosis in myelogenous leukemic K562 cells by ethanolic leaf extract of *Annona muricata*. Indian Journal of Drugs and Diseases 2, 241–247.

Furtado, C.M., Marcondes, M.C., Sola-Penna, M., de Souza, M.L., Zancan, P., 2012. Clotrimazole preferentially inhibits human breast cancer cell proliferation, viability and glycolysis. PloS One 7, e30462.

George, V.C., Kumar, D.N., Rajkumar, V., Suresh, P., Ashok, R., 2012. Quantitative assessment of the relative antineoplastic potential of the n-butanolic leaf extract of *Annona Muricata* Linn. in normal and immortalized human cell lines. Asian Pacific Journal of Cancer Prevention 13, 699–704.

Gysin, R., Azzi, A., Visarius, T., 2002. γ-Tocopherol inhibits human cancer cell cycle progression and cell proliferation by down-regulation of cyclins. The FASEB Journal 16, 1952–1954.

Hajrezaie, M., Paydar, M., Moghadamtousi, S.Z., Hassandarvish, P., Gwaram, N.S., Zahedifard, M., Rouhollahi, E., Karimian, H., Looi, C.Y., Ali, H.M., 2014. A Schiff base-derived copper (II) complex is a potent inducer of apoptosis in colon cancer cells by activating the intrinsic pathway. The Scientific World Journal 2014, 12. http://dx.doi.org/10.1155/2014/540463, Article ID 540463.

Hao, X., Du, M., Bishop, A.E., Talbot, I., 1998. Imbalance between proliferation and apoptosis in the development of colorectal carcinoma. Virchows Archiv 433, 523–527.

Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770–776.

Heri Suselo, Y., Jusuf, S.A., 2012. The selective cytotoxicity of ethanolic extract of *Annona muricata* leaf on HeLa cervical cancer cells, 24–27.

Hood, J.D., Cheresh, D.A., 2002. Role of integrins in cell invasion and migration. Nature Reviews Cancer 2, 91–100.

Ishizu, K., Sunose, N., Yamazaki, K., Tsuruo, T., Sadahiro, S., Makuuchi, H., Yamori, T., 2007. Development and characterization of a model of liver metastasis using human colon cancer HCT-116 cells. Biological and Pharmaceutical Bulletin 30, 1779–1783.

Jaramillo, M., Arango, G., Gonzalez, M., Robledo, S., Velez, I.D., 2000. Cytotoxicity and antileishmanial activity of *Annona muricata* pericarp. Fitoterapia 71, 183–186.

Jiang, Q., Wong, J., Fyrst, H., Saba, J.D., Ames, B.N., 2004. γ-Tocopherol or combinations of vitamin E forms induce cell death in human prostate cancer cells by interrupting sphingolipid synthesis. Proceedings of the National Academy of Sciences of the United States of America 101, 17825–17830.

Kalaisezhiyen, P., Sasikumar, V., 2012. GC–MS evalution of chemical constituents from methanolic leaf extract of *Kedrostis foetidissima* (Jacq.) cogn. Asian Journal of Pharmaceutical and Clinical Research 5, 77–81.

Karimian, H., Mohan, S., Moghadamtousi, S.Z., Fadaeinasab, M., Razavi, M., Arya, A., Kamalidehghan, B., Ali, H.M., Noordin, M.I., 2014. *Tanacetum polycephalum* (L.) Schultz-Bip. induces mitochondrial-mediated apoptosis and inhibits migration and invasion in MCF7 Cells. Molecules 19, 9478–9501.

Karunagaran, D., Rashmi, R., Kumar, T., 2005. Induction of apoptosis by curcumin and its implications for cancer therapy. Current Cancer Drug Targets 5, 117–129.

Khan, N., Adhami, V.M., Mukhtar, H., 2010. Apoptosis by dietary agents for prevention and treatment of prostate cancer. Endocrine-related Cancer 17, R39–R52.

Komiya, T., Kyohkon, M., Ohwaki, S., Eto, J., Katsuzaki, H., Imai, K., Kataoka, T., Yoshioka, K., Ishii, Y., Hibasami, H., 1999. Phytol induces programmed cell death in human lymphoid leukemia Molt 4B cells. International Journal of Molecular Medicine 4, 377–457.

Kossouoh, C., Moudachirou, M., Adjakidje, V., Chalchat, J.-C., Figuérédo, G., 2007. Essential oil chemical composition of *Annona muricata* L. leaves from Benin. Journal of Essential Oil Research 19, 307–309.

Lai, C.-S., Mas, R.H., Nair, N., Majid, M., Mansor, S., Navaratnam, V., 2008. *Typhonium flagelliforme* inhibits cancer cell growth *in vitro* and induces apoptosis: An evaluation by the bioactivity guided approach. Journal of Ethnopharmacology 118, 14–20.

Leboeuf, M., Cavé, A., Bhaumik, P., Mukherjee, B., Mukherjee, R., 1980. The phytochemistry of the Annonaceae. Phytochemistry 21, 2783–2813.

Leboeuf, M., Legueut, C., Cavé, A., Desconclois, J., Forgacs, P., Jacquemin, H., 1981. Alcaloïdes des Annonacées XXIX: Alcaloïdes de l'*Annona muricata* L. Planta Medica 42, 37–44.

Lee, S.-T., Wong, P.-F., Cheah, S.-C., Mustafa, M.R., 2011. Alpha-tomatine induces apoptosis and inhibits nuclear factor-kappa B activation on human prostatic adenocarcinoma PC-3 cells. PloS One 6, e18915.

Liu, F.-T., Newland, A.C., Jia, L., 2003. Bax conformational change is a crucial step for PUMA-mediated apoptosis in human leukemia. Biochemical and Biophysical Research Communications 310, 956–962.

Liu, T., Wu, L.Y., Choi, J.K., Berkman, C.E., 2010. Targeted photodynamic therapy for prostate cancer: inducing apoptosis via activation of the caspase-8/-3 cascade pathway. International Journal of Oncology 36, 777–784.

Lövborg, H., Nygren, P., Larsson, R., 2004. Multiparametric evaluation of apoptosis: effects of standard cytotoxic agents and the cyanoguanidine CHS 828. Molecular Cancer Therapeutics 3, 521–526.

Ly, J., Grubb, D., Lawen, A., 2003. The mitochondrial membrane potential ($\Delta\Psi m$) in apoptosis; an update. Apoptosis 8, 115–128.

Mantena, S.K., Sharma, S.D., Katiyar, S.K., 2006. Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. Molecular Cancer Therapeutics 5, 296–308.

Martinou, J.-C., Youle, R.J., 2011. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Developmental Cell 21, 92–101.

Mills, S., Stamos, M.J., 2014. Metastatic colorectal cancer. Complexities in Colorectal Surgery, 91–104.

Mishra, N.C., Kumar, S., 2005. Apoptosis: a mitochondrial perspective on cell death. Indian Journal of Experimental Biology 43, 25–34.

Mishra, S., Ahmad, S., Kumar, N., Sharma, B.K., 2013. *Annona muricata* (The cancer killer): a review. The Global Journal of Pharmaceutical Research 2, 1613–1618.

Moghadamtousi, S.Z., Goh, B.H., Chan, C.K., Shabab, T., Kadir, H.A., 2013. Biological activities and phytochemicals of *Swietenia macrophylla* King. Molecules 18, 10465–10483.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Muhammad Nadzri, N., Abdul, A.B., Sukari, M.A., Abdelwahab, S.I., Eid, E.E., Mohan, S., Kamalidehghan, B., Anasamy, T., Ng, K.B., Syam, S., 2013. Inclusion complex of zerumbone with hydroxypropyl-β-cyclodextrin induces apoptosis in liver hepatocellular HepG2 cells via caspase 8/BID cleavage switch and modulating Bcl2/Bax ratio. Evidence-Based Complementary and Alternative Medicine 2013, 16. http://dx.doi.org/10.1155/2013/810632, Article ID 810632.

Murakami, T., Kawada, K., Iwamoto, M., Akagami, M., Hida, K., Nakanishi, Y., Kanda, K., Kawada, M., Seno, H., Taketo, M.M., 2013. The role of CXCR3 and CXCR4 in colorectal cancer metastasis. International Journal of Cancer 132, 276–287.

Newman, D.J., Cragg, G.M., 2007. Natural products as sources of new drugs over the last 25 years ⊥. Journal of Natural Products 70, 461–477.

Newman, D.J., Cragg, G.M., 2012. Natural products as sources of new drugs over the 30 years from 1981 to 2010. Journal of Natural Products 75, 311–335.

Nicoletti, I., Migliorati, G., Pagliacci, M., Grignani, F., Riccardi, C., 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. Journal of Immunological Methods 139, 271–279.

Ocker, M., Höpfner, M., 2012. Apoptosis-modulating drugs for improved cancer therapy. European Surgical Research 48, 111–120.

Peshavariya, H.M., Dusting, G.J., Selemidis, S., 2007. Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase. Free Radical Research 41, 699–712.

Peter, M.E., 2011. Programmed cell death: apoptosis meets necrosis. Nature 471, 310–312.

Schumacker, P.T., 2006. Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer Cell 10, 175–176.

Sigounas, G., Anagnostou, A., Steiner, M., 1997. dl-α-tocopherol induces apoptosis in erythroleukemia, prostate, and breast cancer cells. Nutrition and Cancer 28, 30–35.

Sims, J.D., McCready, J., Jay, D.G., 2011. Extracellular heat shock protein (Hsp) 70 and Hsp90α assist in matrix metalloproteinase-2 activation and breast cancer cell migration and invasion. PloS One 6, e18848.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutellingsperger, C., 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. Journal of Immunological Methods 184, 39–51.

Watt, J., Breyer-Brandwijk, M., 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa, 2nd ed.. E & S Livingstone Ltd, Edinburgh, London p. 1457.

Whitfield, J.F., 2009. Calcium, calcium-sensing receptor and colon cancer. Cancer Letters 275, 9–16.

Williams, G.H., Stoeber, K., 2012. The cell cycle and cancer. The Journal of Pathology 226, 352–364.

Wyllie, A.H., 1997. Apoptosis: an overview. British Medical Bulletin 53, 451–465.

Young, M.M., Takahashi, Y., Khan, O., Park, S., Hori, T., Yun, J., Sharma, A.K., Amin, S., Hu, C.-D., Zhang, J., 2012. Autophagosomal membrane serves as platform for intracellular death-inducing signaling complex (iDISC)-mediated caspase-8 activation and apoptosis. Journal of Biological Chemistry 287, 12455–12468.

Yu, W., Simmons-Menchaca, M., Gapor, A., Sanders, B.G., Kline, K., 1999. Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols. Nutrition and Cancer 33, 26–32.
Zeng, L., Wu, F.-E., Oberlies, N.H., McLaughlin, J.L., Sastrodihadjo, S., 1996. Five new monotetrahydrofuran ring acetogenins from the leaves of *Annona muricata*. Journal of Natural Products 59, 1035–1042.
Ziech, D., Franco, R., Pappa, A., Panayiotidis, M.I., 2011. Reactive Oxygen Species (ROS)-induced genetic and epigenetic alterations in human carcinogenesis. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 711, 167–173.
Zorofchian Moghadamtousi, S., Karimian, H., Khanabdali, R., Razavi, M., Firoozinia, M., Zandi, K., Abdul Kadir, H., 2014. Anticancer and antitumor potential of fucoidan and fucoxanthin, two main metabolites isolated from brown algae. The Scientific World Journal 2014, 10. http://dx.doi.org/10.1155/2014/768323, Article ID 768323.

Published Paper 3: The Chemopotential Effect of *Annona muricata* Leaves against Azoxymethane-Induced Colonic Aberrant Crypt Foci in Rats and the Apoptotic Effect of Acetogenin Annomuricin E in HT-29 Cells: A Bioassay- Guided Approach

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RESEARCH ARTICLE

The Chemopotential Effect of *Annona muricata* Leaves against Azoxymethane-Induced Colonic Aberrant Crypt Foci in Rats and the Apoptotic Effect of Acetogenin Annomuricin E in HT-29 Cells: A Bioassay-Guided Approach

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Abstract

Annona muricata has been used in folk medicine for the treatment of cancer and tumors. This study evaluated the chemopreventive properties of an ethyl acetate extract of *A. muricata* leaves (EEAML) on azoxymethane-induced colonic aberrant crypt foci (ACF) in rats. Moreover, the cytotoxic compound of EEAML (Annomuricin E) was isolated, and its apoptosis-inducing effect was investigated against HT-29 colon cancer cell line using a bioassay-guided approach. This experiment was performed on five groups of rats: negative control, cancer control, EEAML (250 mg/kg), EEAML (500 mg/kg) and positive control (5-fluorouracil). Methylene blue staining of colorectal specimens showed that application of EEAML at both doses significantly reduced the colonic ACF formation compared with the cancer control group. Immunohistochemistry analysis showed the down-regulation of PCNA and Bcl-2 proteins and the up-regulation of Bax protein after administration of EEAML compared with the cancer control group. In addition, an increase in the levels of enzymatic antioxidants and a decrease in the malondialdehyde level of the colon tissue homogenates were observed, suggesting the suppression of lipid peroxidation. Annomuricin E inhibited the growth of HT-29 cells with an IC50 value of 1.62 ± 0.24 μg/ml after 48 h. The cytotoxic effect of annomuricin E was further substantiated by G1 cell cycle arrest and early apoptosis induction in HT-29 cells. Annomuricin E triggered mitochondria-initiated events, including the dissipation of the mitochondrial membrane potential and the leakage of cytochrome *c* from the mitochondria. Prior to these events, annomuricin E activated caspase 3/7 and caspase 9. Upstream, annomuricin E induced a time-dependent upregulation of Bax and downregulation of Bcl-2 at the mRNA and

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protein levels. In conclusion, these findings substantiate the usage of *A. muricata* leaves in ethnomedicine against cancer and highlight annomuricin E as one of the contributing compounds in the anticancer activity of *A. muricata* leaves.

Introduction

The complex and multistep process of carcinogenesis generally involves three main stages: initiation, promotion and progression [1]. Perturbations in the genetic level as a result of exposure to carcinogenic agents, including chemical, physical or viral agents, can trigger the initiation phase [2]. Morphological changes and the expansion of altered cells are paramount characterizations of the promotion stage. In the progression stage, genotypic and phenotypic conversions are accompanied with malignancy and metastasis [3].

Colorectal cancer evolves through the deregulation and aberrant growth of epithelial cells in the appendix, colon or rectum [4]. Early detection is pivotal to reduce the number of colorectal cancer victims [5]. The promotion stage in this type of cancer is characterized by aberrant crypt foci (ACF), which are the earliest identifiable precancerous lesions in colon carcinogenetic models in both animals and humans [6]. Therefore, monitoring for ACF is widely employed to inspect the effects of various anticarcinogens against colorectal cancer [7]. The carcinogen azoxymethane (AOM, $C_2H_6N_2O$), an oxide of azomethane, has been widely utilized to start the initiation phase of colorectal cancer, thus stimulating AOM-induced ACF in experimental models. This carcinogenic agent is particularly effective for the induction of colorectal cancer [8].

The evasion of apoptosis is an important property of human cancers, which effectively cause tumor formation and cancer progression [9]. The resistance of cancer cells to apoptosis in response to pertinent stimuli is a critical rationale behind treatment failure [10,11]. Therefore, the majority of strategies used in cancer treatment, including chemotherapy and radiation therapy, are generally based on inducing apoptosis in cancer cells [12]. The induction of apoptosis in cancer cells is primarily triggered through two apoptosis pathways: the intrinsic (mitochondrial) pathway and the extrinsic (receptor) pathway, which both eventually lead to the executioner phase via caspase activation [13]. Caspases, including initiators and executioners, are a family of enzymes that act as death effector proteins in different types of cell death [14].

The long history of employing natural products in ethnomedicine with low-prices and limited side effects, in contrast to expensive synthetic drugs with severe adverse side effects, was the main reason for the development of new pharmaceutical drugs from natural sources [15,16]. In addition, a marked similarity between numerous plant ingredients and the compositions of the human body has evolved acceptable immunity to the majority of plant-derived products. Over the past few decades, natural compounds with apoptosis-inducing effects have attracted noteworthy interest in the area of anticancer pharmaceutical agents [15,16]. There is a growing trend towards natural products with high hopes for new anticancer drugs with similar effect to camptothecin (*Camptotheca acuminata*) and paclitaxel (*Taxus brevifolia*) [17]. Numerous plants were subjected to detailed scientific scrutiny and plenty of them, including *Allium sativum* [18], *Andrographis paniculata* [19], *Glycine max* [20], *Gynura procumbens* [21], *Panax ginseng* [22], *Zingiber officinale* [23], reported to possess noteworthy anticancer and antitumor activity. Therefore, screening for new plant-derived anticancer agents may lead to cost-effective chemotherapeutic drugs with diminished side effects while maintaining therapeutic efficacy.

Annona muricata L. (*A. muricata*), commonly named "graviola" or "soursop", is a small tropical tree from the Annonaceae family, also known the custard apple family [24,25]. This

popular fruit tree, known as "the cancer killer", has an extensive traditional history in the treatment of cancer and tumors in South America and tropical Africa, especially Nigeria [26–28]. Different studies on *A. muricata* leaves have demonstrated noteworthy cytotoxic effects against various cancer cell lines [28–30]. In our previous cytotoxicity screening, the ethyl acetate extract of *A. muricata* leaves (EEAML) was found to induce apoptosis in A549, HT-29 and HCT-116 cancer cells [28,30]. Moreover, the safety of EEAML for animal studies was proven by the acute toxicity study in rats, which showed no sign of toxicity, even at a high dose of 2 g/kg [25]. The present study was designed to evaluate the chemopreventive properties of EEAML on the development and growth of AOM-induced colorectal cancer in rats by analyzing the incidence of ACF. Moreover, EEAML was subjected to a bioassay-guided approach to isolate the cytotoxic compound annomuricin E from *A. muricata* and examine its apoptosis-inducing effects.

Materials and Methods

General Experimental Procedures

Column chromatography (CC) was run on a silica gel 60 column (40–63 μm particle size, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on an aluminum supported silica gel 60 F_{254} column (Merck). Preparative TLC (PTLC) was run on glass coated with silica gel 60 F_{254} (Merck). ^{1}H NMR and ^{13}C NMR spectra were analyzed in $CDCl_3$ on a JEOL JNM-FX500 spectrometer (Tokyo, Japan). The ultraviolet absorption spectra were obtained on a Shimadzu UV-160A spectrophotometer (Kyoto, Japan) using methanol (CH_3OH) as a solvent. The separation was performed on a HPLC machine (Gilson, Inc., Middleton, WI, USA) with a photodiode array (PDA) detector and an ODS C_{18} column (Phenomenex, Torrance, CA, USA). The mass spectra were measured with an Agilent 6530 mass spectrometer (Santa Clara, CA, USA). The infrared spectra were obtained on a Perkin Elmer Spectrum 400-FTIR spectrometer (Waltham, MA, USA) with $CHCl_3$ as a solvent.

Plant Material and Extraction

Fresh leaves of the *A. muricata* plant were collected from Ipoh, Malaysia, in March 2013. We obtained prior permission from all landowners and no endangered or protected species were sampled. Botanical identification was performed by Dr. Yong Kien Thai, an ethnobotanist from the Department of Biological Sciences at the University of Malaya. A voucher specimen (No. KLU47978) has been deposited in the herbarium of the University of Malaya. The dried powdered leaves (3 kg) of *A. muricata* were macerated with ethyl acetate (3 × 2,500 ml) three times at room temperature. The extracting solvent was decanted and concentrated to dryness using a rotary vacuum evaporator (Buchi Labortechnik AG, Flawil, Switzerland) at 40°C. The percentage yield after extraction was 3.9% (117 g). The isolated extract was dissolved in 10% Tween-20 (Sigma, St. Louis, MO, USA) to prepare 250 mg/kg and 500 mg/kg stocks for further experiments.

Animals and Ethics Statement

Healthy adult male *Sprague Dawley* rats (180–250 g weight) were provided by the Animal House of the AEU (Animal Experimental Unit, University of Malaya) in clean, sterile and polyvinyl cages. Rats were housed in a standard animal room air-conditioned at 22–24°C and 55% humidity with a normal pellet diet and water *ad libitum*. Light and dark cycles were scheduled for 12 h each. At the end of the experiment, each animal was sacrificed under ketamine/xylazine anesthesia. The animal studies were performed in the AEU after approval of the protocol by the FOM Institutional Animal Care and Use Committee, University of Malaya (FOM

IACUC, ethic No.: 2014-03-05-PHAR/R/SZM). All rats received humane care in accordance with national guidelines (Guide for the Care and Use of Laboratory Animals) [31].

Experimental Protocols

The experiment was performed as previously described in detail [32]. Thirty male rats (n = 6 per group) in five groups (negative control, cancer control, low dose of EEAML, high dose of EEAML and treatment control) were subcutaneously injected once a week for two consecutive weeks according to the "Induction" column in Table 1. Then, all of the rodents were orally fed once a day for two months based on the experimental design (Table 1), except for the treatment control group, which was intra-peritoneally injected with 35 mg/kg of 5-FU (Sigma, St. Louis, MO, USA) for five consecutive days. The condition of the animals was observed every morning throughout the experimental period.

Counting the ACF

To determine the intensity of colonic ACF formation after 10 weeks of injection with AOM, ACF counting was performed as previously described in detail [33]. In brief, rats were anesthetized with a high dose of ketamine (30 mg/kg, 100 mg/mL) and xylazine (3 mg/kg, 100 mg/mL) under aseptic conditions. The excised colon was flushed with phosphate buffered saline (PBS, Sigma), opened longitudinally and fixed flat between filter papers overnight at 4°C using 10% buffered formalin. Equal lengths of the proximal and distal portions of the fixed colons were stained with 0.5% methylene blue solution. After washing away the excess stain, topographic analysis was performed under a light microscope (Nikon, Tokyo, Japan) to score the total number of ACF, as well as the number of crypts per focus.

Immunohistochemistry

Immunohistochemical evaluation of proliferating cell nuclear antigen (PCNA), Bax and Bcl-2 proteins was performed on deparaffinized tissue sections using the commercial Dako ARK Peroxidase kit (DAKO, Carpinteria, CA, USA) according to the vendor's instructions. In brief, the antigen retrieval process of tissue sections was performed using 10 mM citrate buffer (pH 6.0); the tissue sections were then washed with PBS and blocked with peroxidase blocking buffer. Next, the tissue sections were incubated with diluted mouse PCNA (1:100, Cat: ab2426), Bax (1:100, Cat: ab7977) and Bcl-2 (1:100, Cat: ab7973) antibodies (Abcam, Cambridge, MA, USA) for 15 min. All of the slides were then incubated with the appropriate amount of streptavidin-HRP for 30 min at room temperature. The slides were developed with a diaminobenzidine (DAB) substrate-chromogen system and were counterstained in hematoxylin. The

Table 1. The experimental design and specifications.

Group	Description	Induction	Treatment
A	Negative control	normal saline (15 mL/kg)	10% Tween-20 (5 ml/kg)
B	Cancer control	AOM (15 mL/kg)	10% Tween-20 (5 ml/kg)
C	Low dose	AOM (15 mL/kg)	EEAML (250 mg/kg)
D	High dose	AOM (15 mL/kg)	EEAML (500 mg/kg)
E	Treatment control	AOM (15 mL/kg)	5-FU (35 mg/kg)

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measurement of the PCNA labeling index (PI) was calculated using the formula below [34].

$$PI = \frac{\text{number of positive cells}}{\text{total number of epithelial cells}} \times 100$$

Enzymatic Antioxidants

The colon tissue samples were homogenized in phosphate buffer solution (10% w/v) using a Teflon homogenizer (Polytron, Heidolph RZR 1, Germany). The supernatant was separated after centrifugation at 4000 rpm for 10 min at -4°C. The antioxidant enzymatic activities were assessed using catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) assay kits (Cayman Chemical, Ann Arbor, MI, USA) following the vendor's instructions.

Malondialdehyde

A commercial kit (Cayman Chemical, Ann Arbor, MI, USA) was used to measure the malondialdehyde (MDA) levels in colon tissue homogenates employing the thiobarbituric acid reactive substances (TBARS) assay as previously described in detail [35]. The TBARS assay determines the MDA level, which represents the intensity of lipid peroxidation.

Bioassay-Guided Fractionation and Isolation of Compound

Based on the results of the MTT assay from our previous study [28], the ethyl acetate extract of *A. muricata* leaves was selected for further purification. The crude ethyl acetate extract (9 g) was subjected to CC, which was performed on a silica gel 60 column. The column was eluted with hexane/ethyl acetate mixtures of increasing polarity (70:30 → 0:100). TLC analysis was performed on the collected eluates, and those samples displaying similar R_f values on the TLC were pooled to yield six fractions (designated F_1-F_6). Each fraction was subjected to an MTT assay. Because fraction 3 (F_3) elicited the strongest cytotoxic effect on HT-29 cells (Fig 1), it was used for further purification. Approximately 3.9 g of the bioactive fraction was subjected to another step of chromatography on a silica 60 micro column and were eluted stepwise with ethyl acetate/dichloromethane mixtures of increasing polarity (70:30 → 0:100), and five

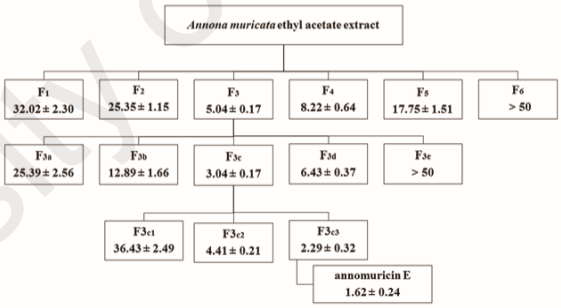

Fig 1. Schematic representation of the bioassay-guided isolation of annomuricin E from EEAML. The cytotoxic effect of each fraction was examined against HT-29 cells for 48 h using an MTT assay. The IC_{50} values (μg/ml) represent the means ± SEM of three independent experiments.

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fractions (F_{3a}-F_{3e}) were obtained. The bioactive fraction F_{3c} (1.8 g) was further fractionated on a preparative TLC using dichloromethane/methanol mixtures of increasing polarity (70:30). Another three fractions ($F3_{c1}$-$F3_{c3}$) were obtained and subjected to an MTT assay. The successive separation of $F3_{c3}$ (0.75 g) by preparative HPLC with an ODS C18 column (4.6 x 250 mm, 5.0 μm, 70 A) and the mobile system (50–100% MeOH-H2O ingredient, detection at 220 nm, 7 ml/min) yielded 5 mg of annomuricin E (0.0025%).

Annomuricin E

Oil, [α]D +13 (c = 0.5 MeOH) UV (MeOH) λ_{max}, 220 nm; IR ($CHCl_3$) ν_{max} 3401, 1704 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz), ^{13}C NMR ($CDCl_3$, 125 MHz) (see Table 2); LCMS m/z 613.4734 $[M+1]^+$—(calculated for $C_{35}H_{64}O_8$).

Cell Culture

CCD841 (normal human colon epithelial cells) and HT-29 (human colon cancer cells) were purchased from the American Type Cell Collection (ATCC, Manassas, VA, USA). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The culture medium consisted of Dulbecco's Modified Eagles medium (Sigma) that was supplemented with 10% Fetal Bovine Serum (PAA Laboratories, Pasching, Australia), 100 μg/ml streptomycin (Sigma) and 100 U/ml penicillin (Sigma). The untreated medium containing 0.1% vehicle DMSO was applied as the negative control for all of the assays in the *in vitro* study.

Table 2. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data of annomuricin E in $CDCl_3$ (δ in ppm, *J* in Hz).

Position	^1H-NMR (δ ppm)	^{13}C-NMR (δ ppm)
1		174.7
2		131.2
3	2.49 *m*	33.4
4	3.83 *m*	70.0
5	1.43 *m*	37.3
6–8	1.46 *m*	22.7
9	1.40 *m*	32.0
10	3.40 *m*	77.1
11	3.41 *m*	75.2
12–14	1.99 *m*	32.0
15	3.77 *m*	75.2
16	3.83 *m*	81.7
17–18	2.40 *m*	29.4
19	3.83 *m*	79.5
20	3.77 *m*	75.2
21–31	1.28	29.5
32	0.85 *m*	19.1
33	7.17 *d* (2.2)	152.0
34	5.04 *m*	78.1
35	1.40 *d* (2.1)	14.2

doi:10.1371/journal.pone.0122288.t002

MTT Assay

Cell viability analysis was performed using the MTT assay as described previously [36]. In brief, cells (5×10^4 cells/ml) at the exponential phase of growth were seeded in a 96-well plate and treated with serial concentrations of the tested agent (0.62, 1.25, 2.5, 5, 10, 20, 40 and 80 μg/ml) for 12, 24 and 48 h. 5-FU, a standard anticancer drug, was used as a positive control in this assay. After incubation, 20 μl of the MTT solution (5.0 mg/ml, Sigma) was loaded into each well, and the cells were further incubated at 37°C for 4 h. DMSO (150 μl) was then used to dissolve the formazan crystals. The cytotoxicity against cancer and normal cells was measured at the absorbance of 570 nm using an ELISA reader (Asys UVM340, Eugendorf, Austria). The data were then processed, and the antiproliferative potential of the tested agents was expressed as IC_{50} values, the concentration that causes a 50% inhibition of cell growth.

Lactate Dehydrogenase (LDH) Release Assay

To further confirm the cytotoxic effects of annomuricin E on HT-29 cells, the LDH release assay was performed using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, Pittsburgh, PA, USA) as previously described [28]. Briefly, HT-29 cells at the exponential phase of growth were treated with different concentrations of annomuricin E and Triton X-100 (positive control) for 24 h. After the incubation, the treated HT-29 cells were exposed to the LDH reaction solution (100 μl) for 30 min. The red color intensity, representing the level of released LDH, was then measured at 490 nm using the Tecan Infinite 200 Pro (Tecan, Männedorf, Switzerland) microplate reader. The result of LDH release was calculated as a percentage of the positive control.

Cell Cycle Assay

To determine the effect of annomuricin E on the cell cycle distribution, flow cytometric analysis was performed as described previously [37]. In brief, HT-29 cells (1×10^6 cells/ml) at the exponential phase of growth were seeded in 6-well plates and treated with annomuricin E at the IC_{50} concentration for 12, 24 and 48 h. After incubation, the treated HT-29 cells were harvested, washed twice with ice-cold PBS and fixed overnight at 4°C with 90% ethanol. The following day, the cells were washed and stained with propidium iodide (PI, 100 μl, 1 mg/ml). The cellular RNA was degraded using the enzyme RNAse A (200 μg/ml, Sigma). The stained cells were instantly examined using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) by analyzing 10,000 cells per sample. The data were processed using ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Quantitative Detection of Early and Late Apoptosis

Flow cytometric analysis was performed to quantify early and late apoptosis in treated HT-29 cells using the commercial BD Pharmingen Annexin V-FITC Apoptosis Detection kit (APOAlert Annexin V; Clontech, Mountain View, CA, USA). Briefly, HT-29 cells (1×10^5 cells/ml) at the exponential phase of growth were incubated with annomuricin E at the IC_{50} concentration for 12, 24 and 48 h. After incubation, the treated cells were harvested, washed twice with PBS and suspended in the Annexin-V binding buffer. The cells were then supplemented with Annexin-V-FITC and PI, according to the vendor's instructions. The stained cells were examined using a BD FACSCanto II flow cytometer. Early and late apoptotic cells and necrotic cells were quantitatively detected using a quadrant statistics analysis [38].

Detection of Caspases Activation

A luminescence-based analysis was performed to investigate the activity of caspase 3/7 and caspase 9 using the Caspase-Glo 9 Assay and Caspase-Glo 3/7 Assay commercial kits (Promega Corporation, Fitchburg, WI, USA) as described previously [39]. Briefly, HT-29 cells (2×10^5 cells/ml) were seeded overnight in a white-walled 96-well plate and treated with an IC_{50} dose of annomuricin E for 3, 6, 12, 24 and 48 h. After incubation, 100 μl of the caspase-Glo reagent was added to each well according to the manufacturer's protocol. Luminescence, which represents the caspase activities, was measured using a luminescence microplate reader (Tecan Infinite 200 Pro).

Multiple Cytotoxicity Assay

The simultaneous analysis of critical apoptosis markers, namely cell membrane permeability, cytochrome *c* leakage from the mitochondria, mitochondrial membrane potential (MMP) and total nuclear intensity, in HT-29 cells was performed using the Cellomics Multiparameter Cytotoxicity 3 Kit (Cellomics, Pittsburgh, PA, USA) as previously described in detail [40]. In brief, HT-29 cells (1×10^5 cells/ml) were plated overnight in a 96-well plate and were exposed to an IC_{50} dose of annomuricin E for 12, 24 and 48 h. After incubation, the treated cells were stained with a cell permeability dye (FITC), a cytochrome *c* dye (Cy3), a mitochondrial membrane potential dye (Cy5) and a nuclear dye (Hoechst 33342), according to the vendor's protocol. The plates were analyzed using a Cell Reporter cytofluorimeter system (Gentix/Molecular Devices, United Kingdom).

Gene Expression Analysis of Bcl-2/Bax

The mRNA expression of two proteins, Bcl-2 and Bax, was quantified using real-time Q-PCR analysis as described previously with some modifications [41]. In brief, HT-29 cells at the exponential phase of growth were treated with annomuricin E at the IC_{50} concentration for 12, 24 and 48 h. The total RNA of treated cells was isolated using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) followed by the synthesis of the complementary DNA using the iScript cDNA synthesis kit (Biorad, Hercules, CA, USA). Q-PCR was performed on the StepOne PLUS real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA). The β-actin housekeeping gene was used as a positive reference and was applied to normalize the target mRNA. The Q-PCR master mix was provided by Solaris Q-PCR Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA) for the gene expression analysis of Bcl-2, AX-003307-00-0100; Bax, AX-003308-00-0100; and β-actin, AX-003451-00-0100.

Immunofluorescence Analysis of Bcl-2/Bax

The perturbation in the protein expressions of Bcl-2 and Bax was investigated using immunofluorescence analysis as previously described in detail [37]. In brief, the HT-29 cells (5×10^4 cells/ml) were seeded in a 96-well plate and exposed to the IC_{50} dose of annomuricin E for 12, 24 and 48 h. After washing the cells twice with PBS, they were fixed in 4% paraformaldehyde at 25°C for 20 min prior to blocking with blocking buffer (0.03% Triton X-100/PBS and normal serum) for 1 h. The cells were then supplemented with a primary antibody solution and incubated overnight at 4°C. After incubation, the cells were treated with Bcl-2 and Bax fluorochrome-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. The cells were then washed twice with PBS prior to staining with DAPI. The stained cells were examined using the Cell Reporter cytofluorimeter system.

Statistical Analysis

Data from the rat study were reported as the means ± standard error of n animals per group. The experimental data were analyzed with one-way analysis of variance, followed by Tukey's post hoc test using the SAS 9.1 statistical program (SAS Institute Inc., Cary, NC, USA). *In vitro* results were presented as the means ± standard error of the mean from three independent experiments. Statistical analysis was performed using the statistical package GraphPad Prism Version 5 (GraphPad Software Inc., San Diego, USA). One-way analysis of variance (Dunnett's multiple comparison test) was used to distinguish the difference among groups. All values at $P<0.05$ were considered significant.

Results and Discussion

ACF Frequency

To evaluate the effect of EEAML on suppressing colon carcinogenesis, ACF were employed as a biomarker to assess early stage AOM-induced colon cancer in rats. The incidence of ACF on the proximal and distal parts of the colon mucosa were analyzed with methylene blue staining immediately after the sacrifice of animals, and these data are shown in Fig 2 and Table 3. ACF were characterized by crypts with elevated sizes, altered luminal epithelia and easily discernible pericryptal zones. Topographical views of the stained colon specimens did not elicit any microscopic changes in the negative control group (Fig 3). Meanwhile, all rats injected with AOM developed ACF containing different numbers of crypts (Table 3). In agreement with previously published findings, ACF formation in the distal colon was significantly higher than the proximal colon [20,42]. Compared with the cancer control group, the administration of 5-FU or EEAML at 250 mg/kg or 500 mg/kg significantly suppressed the formation of ACF (79.5%, 61.2% and 72.5%, respectively). The doses used in this experiment were chosen based on the previous studies on the effect of different plant extracts against AOM-induced ACF formation [21,32]. A recent investigation on anticancer activity of *A. muricata* at a single dose of 300 mg/kg confirmed that these two doses would be appropriate for this study [43]. The respective investigation reported a similar reduction in ACF formation that demonstrated a potent

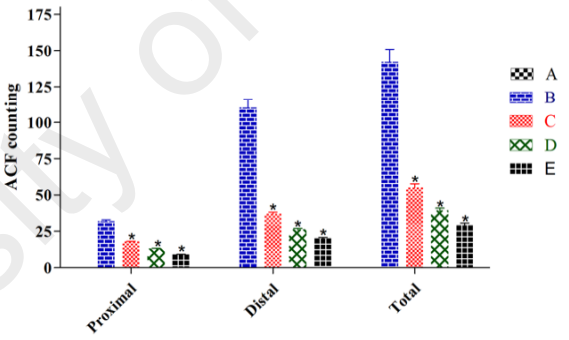

Fig 2. The number of ACF formed in proximal and distal parts of the colon. Tissue specimens were collected from five groups of rats: (A) negative control, (B) cancer control, (C) low dose of EEAML, (D) high dose of EEAML and (E) treatment control. Data are expressed as the means ± SEM of (n = 6/group). *$P<0.05$ compared with cancer control.

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Table 3. Distribution of aberrant crypt categories (1, 2, 3, 4 and more) in the colons of five groups of rats: (A) negative control, (B) cancer control, (C) low dose of EEAML, (D) high dose of EEAML and (E) treatment control.

Group	No. of crypts per ACF					
	1 crypt	2 crypt	3 crypt	4 crypt and more	Total	Inhibition (%)
A	0	0	0	0	0	-
B	33 ± 2.46	29 ± 1.89	48 ± 2.49	32 ± 2.32	142 ± 7.88	-
C	18 ± 0.92*	17 ± 0.68*	11 ± 0.66*	9 ± 0.48*	55 ± 2.32*	61.2
D	10 ± 0.65*	14 ± 0.59*	9 ± 0.52*	6 ± 0.25*	39 ± 1.48*	72.5
E	12 ± 0.26*	6 ± 0.45*	7 ± 0.78*	4 ± 0.18*	29 ± 1.40*	79.5

Data expressed as the means ± SEM of (n = 6/group).

*$P<0.05$ compared with cancer control.

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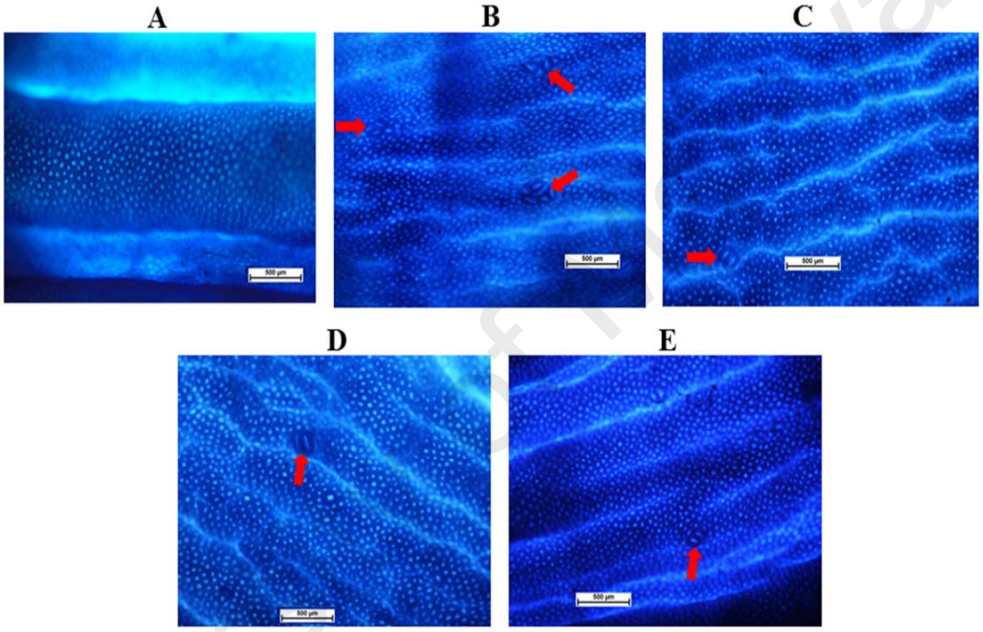

Fig 3. Topographical views of the colon mucosa. Tissue specimens were collected from five groups of rats: (A) negative control, (B) cancer control, (C) low dose of EEAML, (D) high dose of EEAML and (E) treatment control and were stained with methylene blue dye. The red arrows depict ACF in the colon mucosa. Scale bar: 500 μm.

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anticancer activity of *A. muricata* leaves against 1, 2-dimethyl hydrazine-induced colon cancer, which was associated with an elevated apoptosis index [43]. In addition, another study on the breast tissues of female albino mice demonstrated that *A. muricata* leaves exhibited preventative effects against 7, 12-dimethylbenzene anthracene-induced breast cancer cell proliferation [44]. This growing body of experimental evidence supports the preventive effects of *A. muricata* leaves against cancer development and strongly supports the ethnomedicinal application of this plant.

EEAML Induced the Down-Regulation of PCNA

Proliferating cell nuclear antigen (PCNA), originally known as DNA polymerase delta auxiliary protein, is a marker of cell proliferation because it is an indicator of a cell's replication capability [45]. In mammalian cells, PCNA is involved in several metabolic pathways, including cell cycle, chromatin remodeling, DNA methylation, DNA repair, DNA synthesis and Okazaki fragment processing [46]. A number of clinical studies evaluating the inverse correlation between PCNA expression and cancer progression have led to the suggestion that the ratio of PCNA-positive cells provides a prognostic index for cancer [47,48].

Because an elevated rate of proliferation is a critical hallmark for ACF formation in colon tissues, we investigated PCNA expression using immunostaining. Microscopic examinations of colon tissue sections clearly revealed an elevated level of PCNA-positive cells in the cancer control group (PI: 81%) compared with the negative control group (PI: 4%). The rats treated with 5-FU (PI: 24%) exhibited a significantly lower number of positive cells compared with the rats treated with AOM, and similar results were observed in rats treated with EEAML at doses of 250 mg/kg (PI: 42%) and 500 mg/kg (PI: 31%) (Fig 4).

Oncology studies have proven that aberrant proliferation of epithelial cells is one of the early indicators of pre-neoplasia [49,50]. Deschner et al. [51] earlier reported that the administration of chemical carcinogens to animals leads to an extensive proliferation zone with an elevated labeling index. The results of the present study revealed the potential role of PCNA down-regulation on the protective effects of EEAML against induced colon cancer. We found that EEAML administration caused an attenuated proliferation zone and a lower labeling index. A similar decline in PCNA expression was reported earlier in an investigation that showed effective chemoprevention against AOM-induced colon cancer in rats [52]. Based on our results, EEAML is postulated to suppress ACF formation through perturbations in cell proliferation pathways.

EEAML Induced the Up-Regulation of Bax and the Down-Regulation of Bcl-2

The Bcl-2 family of proteins, including pro-apoptotic and anti-apoptotic proteins, contains a total of 25 genes and plays a pivotal role in the control and regulation of mitochondria-mediated apoptosis [53,54]. The pro-apoptotic protein Bax mediates the leakage of pro-apoptotic factors, including cytochrome *c*, Ca^{2+} and Smac/DIABLO, into the cytosol through dimerization and translocation to the outer mitochondrial membrane [13]. Anti-apoptotic proteins, including Bcl-2, in turn suppress the function of apoptosis mediators [55].

In the present study, immunohistochemical evaluation demonstrated the up-regulation of Bax and down-regulation of Bcl-2 in colon tissues after treatment with EEAML (both doses) and 5-FU. Our results showed that an accumulation of Bax in the colon tissues of rats treated with EEAML was comparable to that of rats treated with 5-FU (Fig 5). As shown in Fig 6, Bcl-2 expression in the cancer control group was noticeably higher than the negative control group, representing the suppression of apoptosis among colon cells. In rats treated with EEAML at

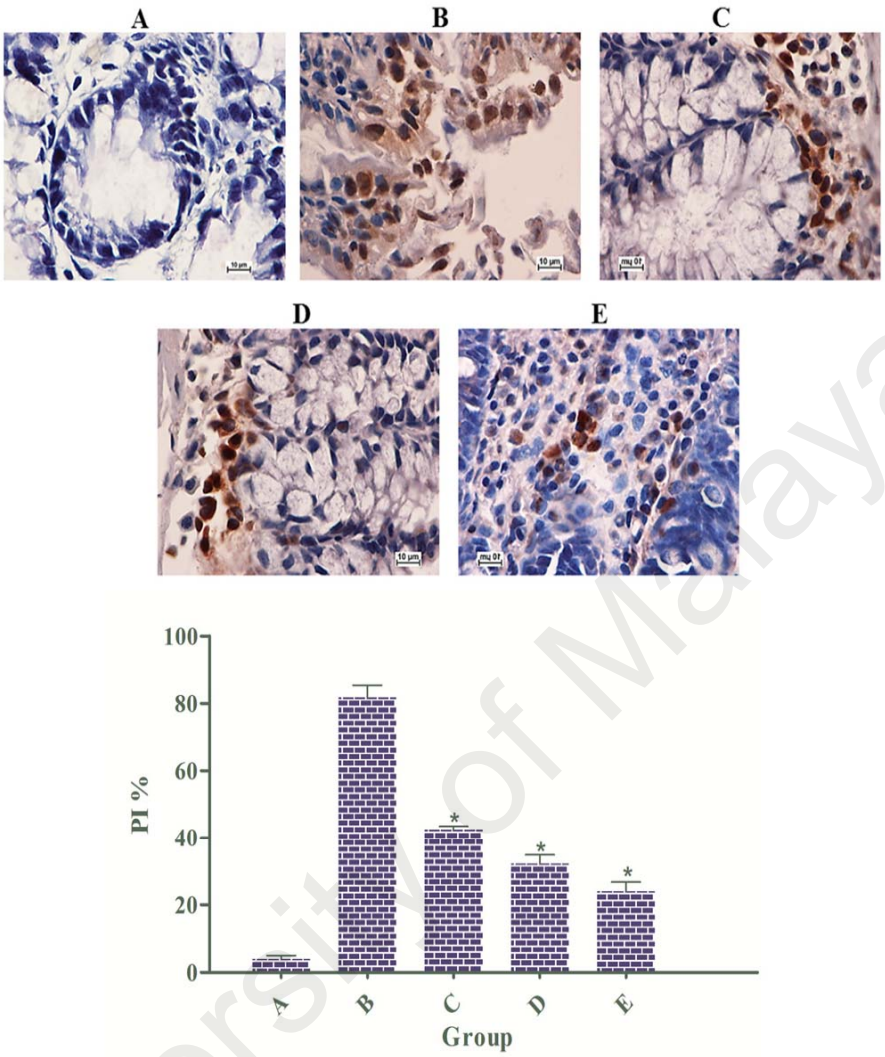

Fig 4. Immunohistochemical analysis of colon tissue sections for PCNA. Tissue specimens were collected from five groups of rats: (A) negative control, (B) cancer control, (C) low dose of EEAML, (D) high dose of EEAML and (E) treatment control. Quantitative analysis of immunopositivity shown as brown staining demonstrated a significant down-regulation of PCNA in groups C-E compared with the cancer control group. Data are expressed as the means ± SEM of (n = 6/group). *$P<0.05$ compared with the cancer control group. Scale bar: 10 μm.

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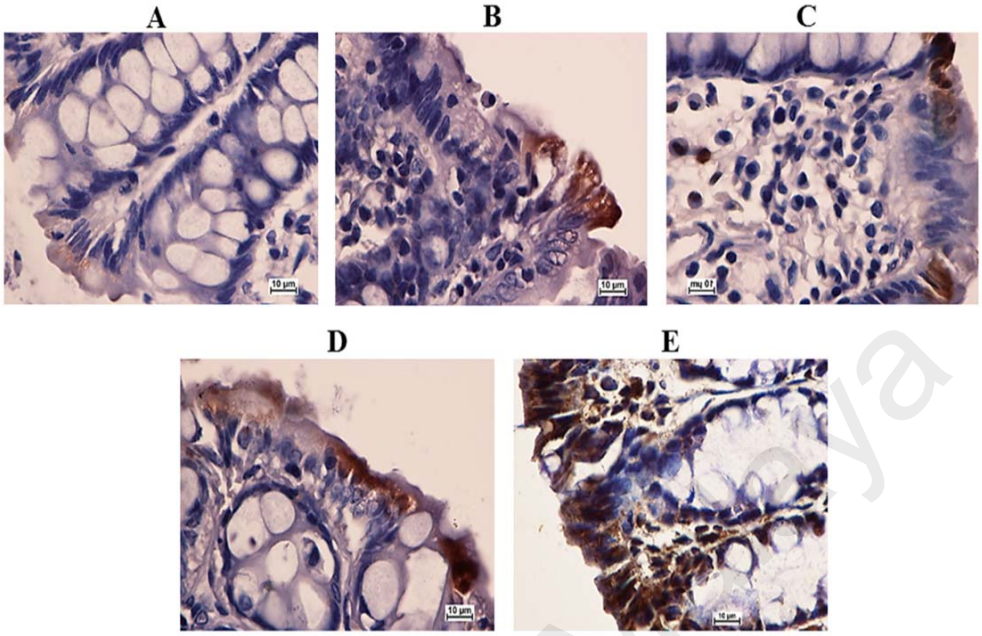

Fig 5. Expression of Bax in colon tissue sections. Tissue specimens were collected from five groups of rats (n = 6/group) and were analyzed using immunohistochemistry: (A) negative control, (B) cancer control, (C) low dose of EEAML, (D) high dose of EEAML and (E) treatment control. The up-regulation of Bax in groups C-E is shown as brown staining. Scale bar: 10 μm.

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doses of 250 mg/kg and 500 mg/kg and 5-FU, an accumulation of Bcl-2 protein in colon tissues was markedly decreased. Administration of EEAML (500 mg/kg) and 5-FU decreased Bcl-2 protein expression to approximately the level of the negative control group.

Previous studies have reported that high Bax protein expression may augment the median survival among cancer patients [56]. In addition, a deficiency in Bax protein has a strong impact on tumor clonal evolution [57]. The results of our present study demonstrate that EEAML has the potential to induce apoptosis in colon cells that are susceptible to AOM damage. This *in vivo* observation agreed with our previous *in vitro* study by illustrating the up-regulation of Bax and the down-regulation of Bcl-2 in HT-29 cells treated with EEAML [30].

EEAML Augmented Enzymatic Antioxidants Activities

As an aggressive factor, reactive oxygen species (ROS) play a pivotal role in the pathogenesis of colorectal cancer [58]. The production of reactive oxygen species (ROS) are part of the normal metabolism in the human body, and cellular antioxidants containing enzymatic and non-enzymatic scavengers maintain ROS at their physiological levels [59]. Nonetheless, an extensive generation of ROS, including hydrogen radicals, hydrogen peroxide and superoxide anions, causes oxidative stress, which leads to metabolic impairments and irreversible cell damages

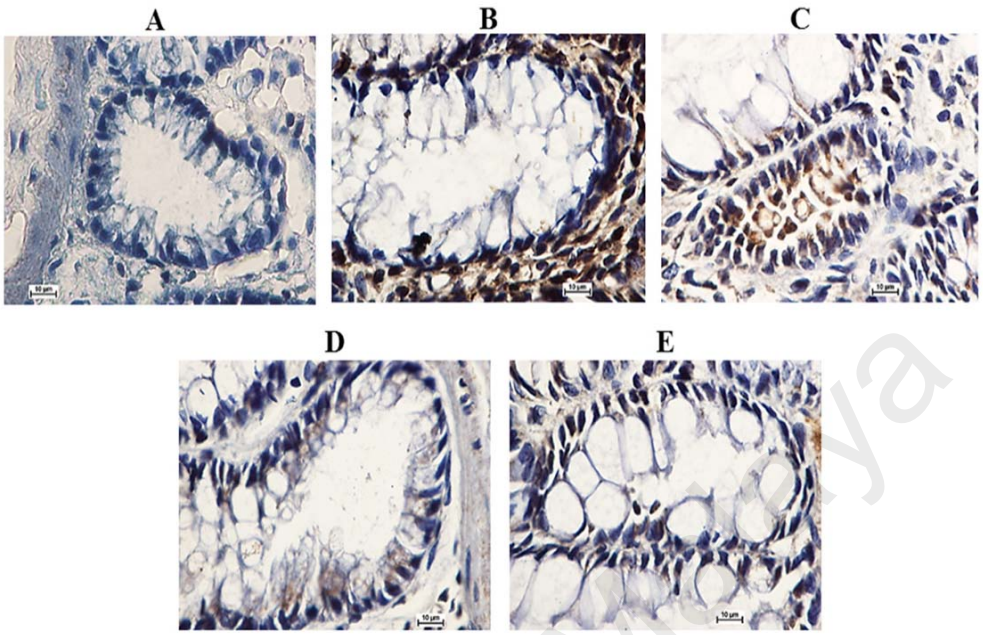

Fig 6. Immunohistochemical analysis of colon tissue sections for Bcl-2. Tissue specimens were collected from five groups of rats (n = 6/group): (A) negative control, (B) cancer control, (C) low dose of EEAML, (D) high dose of EEAML and (E) treatment control. Immunopositivity shown as brown staining revealed the down-regulation of Bcl-2 in groups C-E. Scale bar: 10 μm.

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[60]. SOD, the first scavenging barrier against ROS, converts the superoxide to hydrogen peroxide, which is subsequently degraded to water and oxygen by CAT [61]. The degradation of lipid peroxides to hydroxyl lipids and water is mediated by GPx through oxidation of glutathione to glutathione disulfide [62,63].

The activities of antioxidant enzymes were significantly reduced in the AOM-treated group compared with the negative control group (Fig 7). However, EEAML supplementation at both doses significantly restored the levels of these enzymes towards normal values. As expected, EEAML showed a greater antioxidant defense than 5-FU. A number of earlier *in vitro* and *in vivo* studies have demonstrated that the leaves of *A. muricata* possess significant antioxidant potential [64–66]. Moreover, the leaves elicited noticeable defensive activities against acute and chronic inflammation in rats through suppressive effects on the secretion of proinflammatory cytokines [67]. Immunological studies have led to the suggestion that concomitant administration of chemotherapeutic agents and antioxidant drugs counteract chemotherapy toxicity and enhance the survival rate among cancer patients [68,69]. Therefore, an establishment of anticancer agents with innate antioxidant defense may result in the discovery of new generations of anticancer drugs.

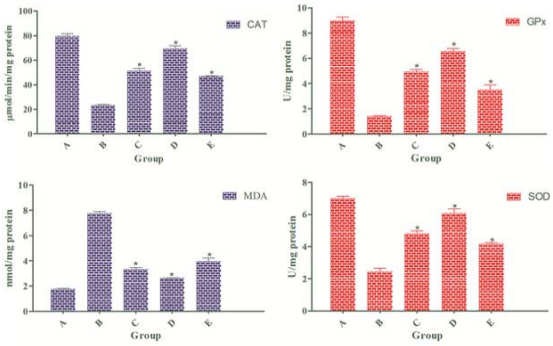

Fig 7. Level of CAT, GPx, MDA and SOD in colon tissue homogenates. Samples were collected from five groups of rats: (A) negative control, (B) cancer control, (C) low dose of EEAML, (D) high dose of EEAML and (E) treatment control. Data are expressed as the means ± SEM of (n = 6/group). *$P<0.05$ compared with cancer control.

doi:10.1371/journal.pone.0122288.g007

EEAML Suppressed Lipid Peroxidation

Excessive ROS generation results in the production of lipid radicals and rearrangements of unsaturated lipids, leading to the formation of different degraded metabolites, including alkenes, lipid hydroperoxides and MDA, which eventually disrupt the integrity of membrane lipids [70,71]. MDA, a major metabolite of this process, is an easy indicator of lipid peroxidation and oxidative stress [72]. As a carcinogenic agent, AOM causes lipid peroxidation as a result of oxidative stress [73], which was observed in our study after administration of AOM to the cancer control group (Fig 7). This result appears to be in line with previous reports that plasma and tissue MDA concentrations are markedly elevated in patients suffering from colorectal cancer [74,75]. As expected, because of the augmentation in the enzymatic and antioxidant activities, EEAML treatment at both doses significantly reduced MDA formation in colon tissues, and this reduction was stronger than the reduction found after treatment with 5-FU. This result confirmed the protective effects of EEAML against oxidative stress in colon tissues, which was reflected by reduced MDA production.

Isolation of the Bioactive Compound, Annomuricin E

The dried leaves of *A. muricata* were extracted with ethyl acetate at room temperature. After concentrating the solution until dry, the ethyl acetate extract was obtained. The ethyl acetate extract was fractionated by chromatography on a silica gel 60 column, which yielded six fractions. Fraction 3 (3.9 g) was further purified on a micro column followed by preparative TLC and finally preparative HPLC using an ODS C-18 column and a PDA detector to obtain annomuricin E (Fig 8), which was identified by 1D and 2D NMR, mass spectrometry and other physical properties that were then compared with reported data [76].

The annonaceous acetogenins, a series of C-35/C-37 fatty acid derivatives, are a class of natural products that are uniquely isolated from the Annonaceae family [77]. The isolation of more than 500 annonaceous acetogenins from different parts of plants in this family has been performed for more than 27 years [78]. This hyperbioactive group of natural products exhibits

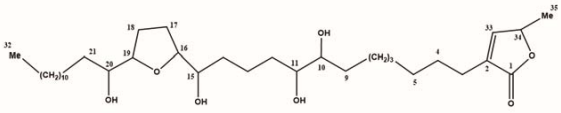

Fig 8. Chemical structure of annomuricin E.

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a variety of bioactivities, including anticancer, antiparasitic, immunosuppressive and insecticidal effects [79,80]. Due to the broad spectrum of bioactivities in annonaceous acetogenins, modified analogues and mimics of these compounds were synthesized to substantiate the ideas regarding the mechanisms of these compound [78]. Previous studies have reported that they are potent suppressors of complex I (NADH, ubiquinone oxidoreductase) in insect and mammalian mitochondrial electron transport systems and of NADH oxidase in the plasma membrane of cancer cells [81–83]. Hence, further studies on annonaceous acetogenins may lead to the establishment of new generations of anticancer drugs.

Annomuricin E Suppressed the Proliferation of HT-29 Cells

Annomuricin E was investigated for its suppressive effect against HT-29 colon cancer cells and CCD841 normal colon cells using the MTT assay. As shown in Table 4, the IC_{50} value of annomuricin E on HT-29 cells was 5.72 ± 0.41 μg/ml, 3.49 ± 0.22 μg/ml and 1.62 ± 0.24 μg/ml after 12, 24 and 48 h treatments, respectively, which were comparable with the suppressive potential of 5-FU as a standard anticancer drug. When compared with HT-29 cells, annomuricin E was far less cytotoxic to the normal cells, as revealed by the relatively high IC_{50} value on CCD841 (32.51 ± 1.18 μg/ml for 48 h). These results are in line with a previous report that showed the cytotoxic effect of annomuricin E against six different human cancer cells with selectivities toward PACA-2 (a pancreatic carcinoma cell line) and HT-29 cells [76]. In addition, earlier studies have shown that acetogenins are potentially effective against multidrug resistant cancer cell lines [84,85].

Annomuricin E Induced LDH Leakage in HT-29 Cells

Because any irreversible membrane damage to cells causes a leakage of LDH from the cytosol, the level of this stable cytosolic enzyme in cellular culture supernatants is a simple and quick assay to determine the cellular cytotoxicity [86]. The cytotoxic effect of annomuricin E against HT-29 cells was further substantiated by an LDH assay. As depicted in Fig 9, the control cells treated with 0.1% vehicle DMSO showed a low level of LDH release after 24 h of treatment. In contrast, LDH leakage modestly increased with the presence of annomuricin E at concentrations of 1 and 2 μg/ml. Meanwhile, treatment of concentrations from 4 to 16 μg/ml led to a

Table 4. Cytotoxic effects of annomuricin E and 5-FU on the proliferation of CCD841 and HT-29 cells after 12, 24 and 48 h of treatment.

Cell line	IC_{50} (μg/ml)					
	Annomuricin E 12 h	5-FU 12 h	Annomuricin E 24 h	5-FU 24 h	Annomuricin E 48 h	5-FU 48 h
HT-29	5.72 ± 0.41	4.85 ± 0.38	3.49 ± 0.22	2.96 ± 0.43	1.62 ± 0.24	1.50 ± 0.17
CCD841	64.32 ± 3.76	58.50 ± 2.09	47.10 ± 0.47	44.35 ± 2.25	32.51 ± 1.18	36.32 ± 0.43

The IC_{50} values represent the means ± SEM of three independent experiments.

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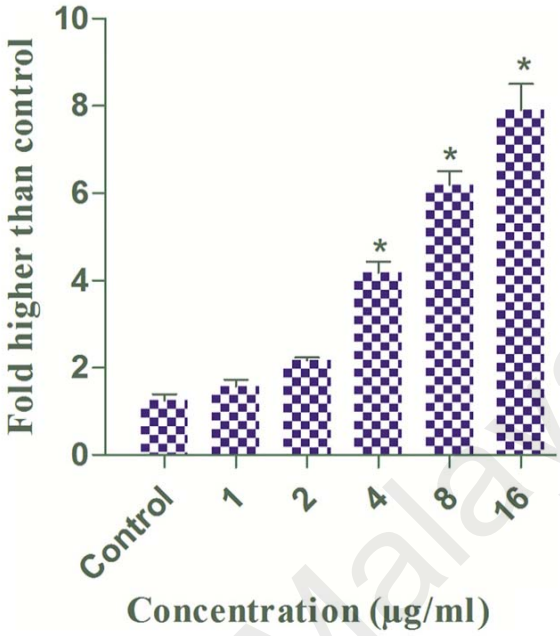

Fig 9. Effects of annomuricin E on LDH leakage formation in HT-29 cells. Cells were exposed to 0.1% vehicle DMSO (control) and annomuricin E at different concentrations for 24 h. The treated HT-29 cells showed a significant LDH release at 4 to 16 µg/ml concentrations compared with the control. The data represent the means ± SEM of three independent experiments. *$P<0.05$ compared with the control.

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significant LDH release compared with the control. The significant LDH leakage from HT-29 cells was shown at concentrations as low as 4 µg/ml, which was compatible with the 24-h IC_{50} value of annomuricin E (3.49 ± 0.22 µg/ml) against HT-29 cells.

Cell Cycle Arrest at G_1 Induced by Annomuricin E

Cancer progression is often associated with irregularities in cell cycle function [87]. A growing body of experimental evidence supporting the concomitant involvement of cell cycle suppression and apoptosis has stimulated widespread attention to phytochemicals with cell-cycle modulatory effects [15,88]. Hence, we first evaluated whether the suppressive effect of annomuricin E was accompanied by a block in the cell cycle using PI staining and flow cytometry analysis. As illustrated in Fig 10, the augmented accumulation of HT-29 cells in the G_1 phase was initiated after 12 h of treatment with annomuricin E, and this accumulation of cells in the G_1 phase continued in a time-dependent manner. After 24 and 48 h, the percentage of HT-29 cells treated with annomuricin E that were arrested at the G_1 phase reached 89.65% and 94.60%, respectively. This was accompanied by a concurrent decline in the S and G_2/M cell populations compared with the control. These results indicated that annomuricin E arrested HT-29 cells at

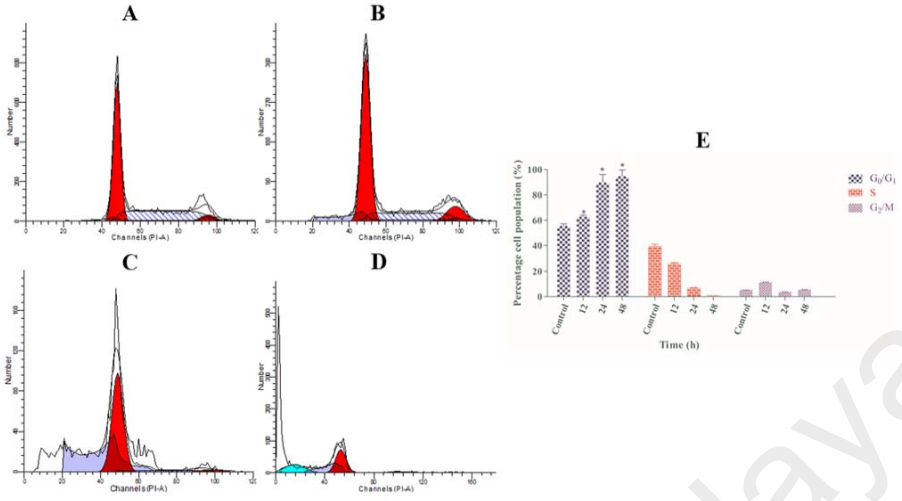

Fig 10. Effect of annomuricin E on cell cycle distribution in HT-29 cells. Cells were treated with (A) 0.1% vehicle DMSO (control) for 48 h and annomuricin E at the IC_{50} concentration for (B) 12, (C) 24 and (D) 48 h. After staining the cells with PI, the DNA contents were monitored using flow cytometry. (E) The representative bar chart shows the significant induction of G_1 cell cycle arrest by annomuricin E after 12 h of treatment. The data represent the means ± SEM of three independent experiments. *$P<0.05$ compared with the control.

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the G_1 phase. An earlier study on annonacin, an annonaceous acetogenin from the seeds of *Annona reticulata*, also showed the induction of cell cycle arrest in T24 bladder cancer cells at the G_1 phase through the activation of p21 [89].

Phosphatidylserine Externalization Induced by Annomuricin E

As one of the biochemical characterizations of apoptosis, a transverse redistribution of phosphatidylserine (PS) on the outer plasma membrane arises during early apoptosis [90]. A fluorescent probe of Annexin V-FITC is a recombinant protein with a high affinity for externalized PS [91]. To gain insight into the mechanism through which annomuricin E induces its cytotoxic effects, HT-29 cells were stained with Annexin V-FITC/PI and analyzed using flow cytometry. In cells treated with 0.1% vehicle DMSO (control), only 3.6% and 0.5% of cells were in early (Annexin V^+/PI^-) and late (Annexin V^+/PI^+) apoptosis after 48 h, respectively (Fig 11). However, the percentage of early and late apoptotic cells were significantly increased to 13.9% and 6.9%, respectively, after being treated with annomuricin E (IC_{50} concentration) for 12 h. The percentages of early and late apoptotic populations peaked at 24 h with values of 27.3% and 13.6%, respectively, and were reduced slightly at 48 h. This was reduction was associated with a significant elevation in the number of necrotic cells (Annexin V^-/PI^+) at 24 and 48 h. This increase in necrotic cells can be explained by the long exposure of annomuricin E to HT-29 cells that allowed the cells to enter secondary necrosis from primary apoptosis, increasing the number of dead cells. These data showed that annomuricin E caused its cytotoxic effects through the induction of apoptosis in HT-29 cells.

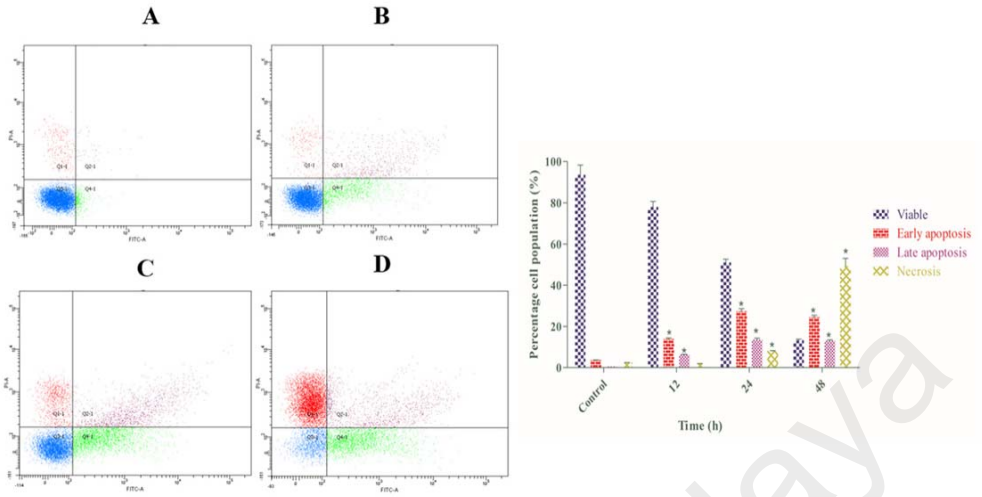

Fig 11. Effect of annomuricin E on apoptosis in HT-29 cells via quadrant statistics. After treatment with annomuricin E (IC_{50} concentration) for (B) 12, (C) 24 and (D) 48 h, the cells were double stained with Annexin V-FITC/PI and monitored using flow cytometry. Cells treated with 0.1% vehicle DMSO were employed as the (A) control treatment. (E) The representative bar chart depicted the percentages of early apoptotic, late apoptotic and necrotic cells. The data represent the means ± SEM of three independent experiments. *$P<0.05$ compared with the control.

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Caspase Activation Induced by Annomuricin E

The energy-dependent process of apoptosis relies heavily on the family of caspases, or cysteinyl aspartate proteinases, to hierarchically convert the initiating cellular stimuli to the final cell demise [14]. Caspases, which are initially synthesized as inactive proforms, consist of three main classes: inflammatory (caspase-1, -4, -5), initiator (caspase-2, -8, -9, -10) and executioner or effector caspases (caspase -3, -6, -7) [54]. A complex cascade of caspase-dependent events in the apoptosis process is triggered by initiators and is finalized by effectors that mediate the typical biochemical modifications during apoptosis execution [92]. To determine whether annomuricin E-induced apoptosis in HT-29 cells is mediated through caspases, the activities of caspase 9 and caspase 3/7 were investigated using bioluminescent analysis. In this assay, the luminescent intensity is proportional to the activation of caspases. As shown in Fig 12, both caspase 9 and caspase 3/7 were activated in a time-dependent manner after exposure to annomuricin E at the IC_{50} concentration. After 12 to 48 h of treatment, the activities of both caspases were significantly elevated, suggesting that annomuricin E-induced apoptosis occurs through the involvement of caspase 9 and caspase 3/7 activation.

Mitochondria-Initiated Events Induced by Annomuricin E

As a convergent center of internal apoptotic stimuli, mitochondria play a pivotal role in the intrinsic pathway of apoptosis. Depletion of MMP leads to the opening of mitochondria permeability transition pores and the further release of pro-apoptotic proteins, such as cytochrome *c*, from the mitochondria to the cytosol, resulting in the formation of the apoptosome and the

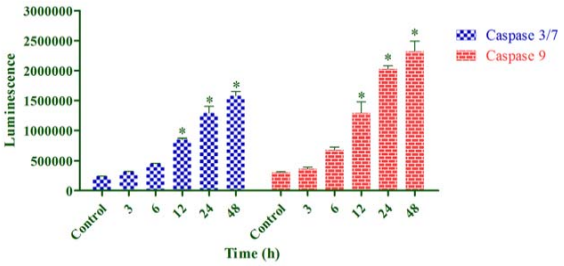

Fig 12. Effect of annomuricin E on caspase 3/7 and caspase 9 activities in HT-29 cells using bioluminescent analysis. Cells were treated with the IC_{50} concentration of annomuricin E for 3, 6, 12, 24 and 48 h. The activities of both caspase were significantly elevated after 12 h of treatment. Cells treated with 0.1% vehicle DMSO were employed as the control treatment. The data represent the means ± SEM of three independent experiments. *$P<0.05$ compared with the control.

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activation of caspase 9 [93]. The aforementioned results revealed that caspase 9 activation occurred during the exposure of HT-29 cells to annomuricin E. Subsequently, we examined the mitochondria-initiated events in treated HT-29 cells using a cell reporter system. As shown in Fig 13, the number of cells was considerably reduced after exposure to annomuricin E at the IC_{50} concentration. Four florescent markers were used to monitor the changes in total nuclear intensity, cell membrane permeability, MMP and cytochrome *c* release of the treated cells in a time-course experiment. A 12-h treatment of annomuricin E induced a significant collapse in MMP associated with an increase in cytochrome *c* leakage from the mitochondria in a time-dependent manner (Fig 14). The total nuclear intensity represents pyknosis as a result chromatin condensation, which is the most characteristic property of apoptosis [54], and was significantly elevated at 12, 24 and 48 h (Fig 14). In addition, the cell membrane permeability of HT-29 cells was significantly increased only at the later stages of treatment (24 and 48 h). These

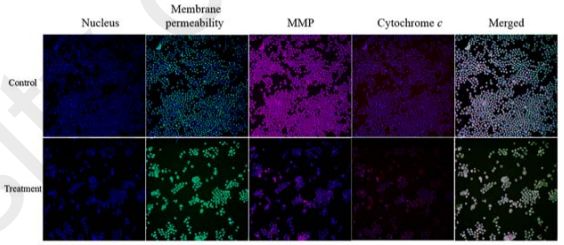

Fig 13. Images of HT-29 cells treated with annomuricin E at the IC_{50} concentration for 24 h. The treated cells were stained with different and specific dyes for the detection of total nuclear intensity, cell membrane permeability, MMP and cytochrome *c* release. Cells treated with 0.1% vehicle DMSO were employed as the control treatment.

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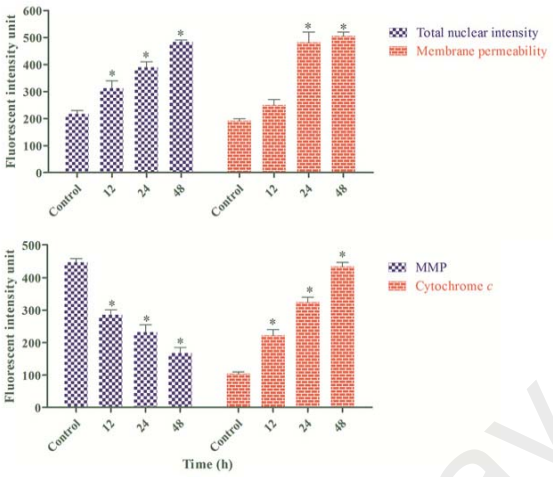

Fig 14. Representative bar charts of the multiple cytotoxicity assay. After 12 h of treatment with annomuricin E at the IC_{50} concentration, the total nuclear intensity, MMP and cytochrome *c* release were significantly elevated compared with the control. However, cell membrane permeability showed a significant increase only after 24 h. Cells treated with 0.1% vehicle DMSO were employed as the control treatment. The data represent the means ± SEM of three independent experiments. **P*<0.05 compared with the control.

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results suggest that annomuricin E caused the dissipation of MMP and the leakage of cytochrome *c* from mitochondria, which resulted in the activation of caspase 9.

Bax Up-Regulation and Bcl-2 Down-Regulation Induced by Annomuricin E

Because annomuricin E elicited the ability to interfere with MMP in HT-29 cells, we raised the possibility of Bax and Bcl-2 involvement in annomuricin E-induced apoptosis. Hence, the expression of Bax and Bcl-2 was investigated at both the mRNA and protein levels using Q-PCR and immunofluorescence analysis, respectively. As shown in Fig 15, the mRNA expression of the Bax protein was significantly and time-dependently elevated after 12 h treatment and reached an approximately 5-fold higher level after 48 h. In spite of Bax up-regulation, the mRNA expression of the anti-apoptotic protein Bcl-2 was time-dependently reduced from 12 to 48 h. Immunofluorescence analysis demonstrated that the number of HT-29 cells treated with annomuricin E decreased in a time-dependent manner after 12, 24 and 48 h, as illustrated by the blue fluorescent staining of DAPI, which identifies all cell nuclei (Fig 16). The time-dependent reduction in the number of surviving cells was accompanied with a distinct increase in the fluorescent intensity of FITC dye (green) that represented Bax protein expression, which reached a value approximately 10-fold higher than the control after 48 h (Fig 17). Bcl-2 protein expression also significantly and dose-dependently reduced compared with the control. The perturbations in Bax and Bcl-2 expression at the mRNA and protein level substantiated the idea that annomuricin E-induced apoptosis was through the mitochondria-mediated pathway.

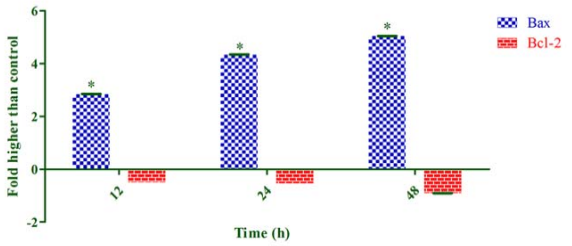

Fig 15. Effect of annomuricin E on Bax and Bcl-2 mRNA expression was assessed using Q-PCR analysis. The housekeeping gene β-actin was used for the normalization of the mRNA expression. The result depicted a time-dependent upregulation of Bax and down-regulation of Bcl-2 after treatment with annomuricin E at the IC_{50} concentration. The data represent the means ± SEM of three independent experiments. *$P<0.05$ compared with the control.

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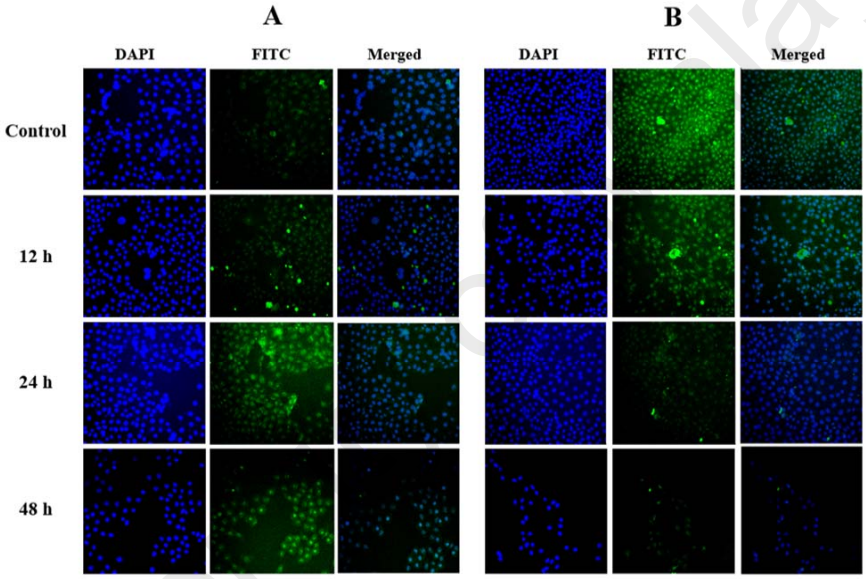

Fig 16. Immunofluorescence analysis of (A) Bax and (B) Bcl-2 protein expression in HT-29 cells. Cells were treated with annomuricin E at the IC_{50} concentration for 12, 24 and 48 h and were stained with DAPI and Bax/Bcl-2 antibodies conjugated to FITC. Cells treated with 0.1% vehicle DMSO were employed as the control treatment. As the number of cells reduced in a time-dependent manner, the fluorescent intensity showed a marked upregulation and down-regulation for Bax and Blc-2 proteins, respectively.

doi:10.1371/journal.pone.0122288.g016

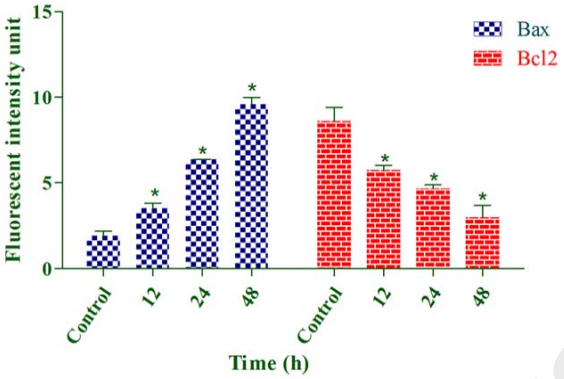

Fig 17. Representative bar charts of Bax and Bcl-2 immunofluorescence analysis. Annomuricin E at IC_{50} concentration induced significant upregulation of Bax and down-regulation of Bcl-2 after 12 h. Cells treated with 0.1% vehicle DMSO were employed as the control treatment. The data represent the means ± SEM of three independent experiments. *$P<0.05$ compared with the control.

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Conclusions

Overall, we provided evidence that the ethyl acetate extract of *A. muricata* leaves has the potential to suppress the AOM-induced development of ACF in rats. The data substantiated the traditional use of *A. muricata* leaves against cancer and tumors. The suppressed formation of ACF in rats after EEAML oral administration was accompanied with down-regulation of PCNA and Bcl-2 proteins and up-regulation of Bax protein in the colon tissue, indicating a possible mechanism at the molecular level. The reported pharmacological effect of the *A. muricata* leaves may be partially due to the presence of annomuricin E. This acetogenin suppressed the proliferation of HT-29 cells selectively and induced apoptosis that was associated with G_1 cell cycle arrest and mitochondria-mediated pathways.

Supporting Information

S1 Fig. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data of annomuricin E in $CDCl_3$ (δ in ppm, *J* in Hz).
(DOCX)

Author Contributions

Conceived and designed the experiments: SZM MAA HAK. Performed the experiments: SZM ER HK MF. Analyzed the data: SZM MF. Contributed reagents/materials/analysis tools: SZM. Wrote the paper: SZM.

References

1. Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett. 1995; 93: 55–71. PMID: 7600544
2. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. A review of human carcinogens—part B: biological agents. Lancet Oncol. 2009; 10: 321–322. PMID: 19350698

3. Kumar S, Weaver VM. Mechanics, malignancy, and metastasis: the force journey of a tumor cell. Cancer Metast Rev. 2009; 28: 113–127.
4. Giovannucci E. Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. Am J Clin Nutr. 2007; 86: 836S–842S.
5. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature. 2006; 445: 111–115. PMID: 17122771
6. Alrawi SJ, Schiff M, Carroll RE, Dayton M, Gibbs JF, Kulavlat M, et al. Aberrant crypt foci. Anticancer Res. 2006; 26: 107–119. PMID: 16475686
7. Fenoglio-Preiser CM, Noffsinger A. Review article: Aberrant crypt foci: A Review. Toxicol Pathol. 1999; 27: 632–642. PMID: 10588543
8. Raju J. Azoxymethane-induced rat aberrant crypt foci: relevance in studying chemoprevention of colon cancer. World J Gastroentero. 2008; 14: 6632. PMID: 19034964
9. Fulda S. Modulation of apoptosis by natural products for cancer therapy. Planta Med. 2010; 76: 1075–1079. doi: 10.1055/s-0030-1249961 PMID: 20486070
10. Fulda S. Tumor resistance to apoptosis. Int J Cancer. 2009; 124: 511–515. doi: 10.1002/ijc.24064 PMID: 19003982
11. Gutschner T, Diederichs S. The hallmarks of cancer. RNA Biol. 2012; 9: 703–719. doi: 10.4161/rna.20481 PMID: 22664915
12. Wong R. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Canc Res. 2011; 30: 87. doi: 10.1186/1756-9966-30-87 PMID: 21943236
13. Ocker M, Höpfner M. Apoptosis-modulating drugs for improved cancer therapy. Eur Surg Res. 2012; 48: 111–120. doi: 10.1159/000336875 PMID: 22538523
14. Fan T-J, Han L-H, Cong R-S, Liang J. Caspase family proteases and apoptosis. Acta Bioch Bioph Sin. 2005; 37: 719–727.
15. Zorofchian Moghadamtousi S, Karimian H, Khanabdali R, Razavi M, Firoozinia M, Kelvan Z, et al. Anticancer and antitumor potential of fucoidan and fucoxanthin, two main metabolites isolated from brown algae. Sci World J. 2014; 2014: doi: 10.1155/2014/768323
16. Mishra BB, Tiwari VK. Natural products: an evolving role in future drug discovery. Eur J Med Chem. 2011; 46: 4769–4807. doi: 10.1016/j.ejmech.2011.07.057 PMID: 21889825
17. Taylor P, Colman L, Bajoon J. The search for plants with anticancer activity: Pitfalls at the early stages. J Ethnopharmacol. 2014; 158: 246–254. doi: 10.1016/j.jep.2014.10.034 PMID: 25446637
18. Thomson M, Ali M. Garlic [*Allium sativum*]: A Review of its Potential Use as an Anti-Cancer Agent. Curr Cancer Drug Targets. 2003; 3: 67–81. PMID: 12570662
19. Bardi DA, Halabi MF, Hassandarvish P, Rouhollahi E, Paydar M, Moghadamtousi SZ, et al. *Andrographis paniculata* Leaf Extract Prevents Thioacetamide-Induced Liver Cirrhosis in Rats. Plos One. 2014; 9: e109424. doi: 10.1371/journal.pone.0109424 PMID: 25280007
20. Gourineni V, Verghese M, Boateng J, Shackelford L, Bhat K. Chemopreventive potential of synergy1 and soybean in reducing azoxymethane-induced aberrant crypt foci in fisher 344 male rats. J Nutr Metab. 2011; 2011: doi: 10.1155/2011/983038
21. Shwter AN, Abdullah NA, Alshawsh MA, Alsalahi A, Hajrezaei M, Almaqrami AA, et al. Chemoprevention of colonic aberrant crypt foci by *Gynura procumbens* in rats. J Ethnopharmacol. 2014; 151: 1194–1201. doi: 10.1016/j.jep.2013.12.044 PMID: 24393787
22. Shin HR, Kim JY, Yun TK, Morgan G, Vainio H. The cancer-preventive potential of *Panax ginseng*: a review of human and experimental evidence. Cancer Causes Control. 2000; 11: 565–576. PMID: 10880039
23. Abdulaziz Bardi D, Halabi MF, Abdullah NA, Rouhollahi E, Hajrezaie M, Abdulla MA. *In vivo* evaluation of ethanolic extract of *Zingiber officinale* rhizomes for its protective effect against liver cirrhosis. BioMed Res Int. 2013; 2013: doi: 10.1155/2013/918460
24. George VC, Kumar DN, Rajkumar V, Suresh P, Ashok R. Quantitative assessment of the relative antineoplastic potential of the n-butanolic leaf extract of *Annona Muricata* Linn. in normal and immortalized human cell lines. Asian Pac J Cancer Prev. 2012; 13: 699–704. PMID: 22524847
25. Moghadamtousi SZ, Rouhollahi E, Karimian H, Fadaeinasab M, Abdulla MA, Kadir HA. Gastroprotective activity of *Annona muricata* leaves against ethanol-induced gastric injury in rats via Hsp70/Bax involvement. Drug Des Dev Ther. 2014; 8: 2099–2111. doi: 10.2147/DDDT.S70096 PMID: 25378912
26. Mishra S, Ahmad S, Kumar N, Sharma BK. *Annona muricata* (The cancer killer): A review. Glob J Pharma Res. 2013; 2: 1613–1618.

27. Adewole S, Ojewole J. Protective effects of *Annona muricata* Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats. Afr J Tradit Complement Altern Med. 2009; 6: 30–41.
28. Moghadamtousi SZ, Karimian H, Rouhollahi E, Paydar M, Kadir HA, Fadaeinasab M, et al. *Annona muricata* leaves induce G_1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in Human HCT-116 and HT-29 colon Cancer cells. J Ethnopharmacol. 2014; 156: 277–289. doi: 10.1016/j.jep.2014.08.011 PMID: 25195082
29. Ezirim A, Okachi V, James A, Adebeshi O, Ogunnowo S, Odeghe OB. Induction of apoptosis in myelogenous leukemic K562 cells by ethanolic leaf extract of *Annona muricata*. Indian J Drugs Dis. 2013; 2: 241–247.
30. Moghadamtousi SZ, Kadir HA, Paydar M, Rouhollahi E, Karimian H. *Annona muricata* leaves induced apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF-kappaB. BMC Complem Altern M. 2014; 14: 299.
31. Garber JC, Barbee RW, Bielitzki JT, Clayton LA, Donovan JC, Hendriksen CFM, et al. (Guide for the care and use of laboratory animals. 2011; Washington, DC: The National Academies Press.
32. Almagrami AA, Alshawsh MA, Saif-Ali R, Shwter A, Salem SD, Abdulla MA. (2014) Evaluation of Chemopreventive Effects of *Acanthus ilicifolius* against Azoxymethane-Induced Aberrant Crypt Foci in the Rat Colon. 2014; Plos One 9: e96004. doi: 10.1371/journal.pone.0096004 PMID: 24819728
33. Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett. 1987; 37: 147–151. PMID: 3677050
34. Hajrezaie M, Hassandarvish P, Moghadamtousi SZ, Gwaram NS, Golbabapour S, NajiHussien A, et al. Chemopreventive Evaluation of a Schiff Base Derived Copper (II) Complex against Azoxymethane-Induced Colorectal Cancer in Rats. 2014; Plos One 9: e91246. doi: 10.1371/journal.pone.0091246 PMID: 24618844
35. Fraga CG, Leibovitz BE, Tappel AL. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. Free Radic Biol Med. 1988; 4: 155–161. PMID: 3356355
36. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65: 55–63. PMID: 6606682
37. Karimian H, Mohan S, Moghadamtousi SZ, Fadaeinasab M, Razavi M, Arya A, et al. *Tanacetum polycephalum* (L.) Schultz-Bip. Induces Mitochondrial-Mediated Apoptosis and Inhibits Migration and Invasion in MCF7 Cells. Molecules. 2014; 19: 9478–9501. doi: 10.3390/molecules19079478 PMID: 24995928
38. Karimian H, Moghadamtousi Zorofchian S, Fadaeinasab M, Golbabapour S, Razavi M, Hajrezaie M, et al. *Ferulago angulata* activates intrinsic pathway of apoptosis in MCF-7 cells associated with G_1 cell cycle arrest via involvement of p21/p27. 2014; Drug Des Dev Ther. 2014; 8: 1481–1497. doi: 10.2147/DDDT.S68818 PMID: 25278746
39. Hajrezaie M, Paydar M, Zorofchian Moghadamtousi S, Hassandarvish P, Gwaram NS, Zahedifard M, et al. A Schiff Base-Derived Copper (II) Complex Is a Potent Inducer of Apoptosis in Colon Cancer Cells by Activating the Intrinsic Pathway. Sci World J. 2014; 2014: doi: 10.1155/2014/540463
40. Lövborg H, Nygren P, Larsson R. Multiparametric evaluation of apoptosis: effects of standard cytotoxic agents and the cyanoguanidine CHS 828. Mol Cancer Ther. 2004; 3: 521–526. PMID: 15141009
41. Liew SY, Looi CY, Paydar M, Cheah FK, Leong KH, Wong WF, et al. Subditine, a new monoterpenoid indole Alkaloid from bark of *Nauclea subdita* (Korth.) Steud. Induces apoptosis in human prostate cancer cells. 2014; Plos One 9: e87286. doi: 10.1371/journal.pone.0087286 PMID: 24551054
42. Guo Y-W, Chen Y-H, Chiu W-C, Liao H, Lin S-H. Soy Saponins Meditate the Progression of Colon Cancer in Rats by Inhibiting the Activity of β-Glucuronidase and the Number of Aberrant Crypt Foci but Not Cyclooxygenase-2 Activity. ISRN Oncol. 2013; 2013: doi: 10.1155/2013/645817
43. Eggadi V, Gundamedi S, Sheshagiri SBB, Revoori SK, Jupally VR, Kulandaivelu U. Evaluation of Anticancer Activity of *Annona muricata* in 1, 2-Dimethyl Hydrazine Induced Colon Cancer. World Appl Sci J. 2014; 32: 444–450.
44. Minari J, Okeke U. Chemopreventive effect of *Annona muricata* on DMBA-induced cell proliferation in the breast tissues of female albino mice. Egypt J Med Hum Genet. 2014; 15: 327–334.
45. Mayer A, Takimoto M, Fritz E, Schellander G, Kofler K, Ludwig H. The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. Cancer. 1993; 71: 2454–2460. PMID: 8095852
46. Maga G, Hübscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci. 2013; 116: 3051–3060.

47. Isozaki H, Okajima K, Ichinona T, Tanimura M, Morita S, Takada Y, et al. The significance of proliferating cell nuclear antigen (PCNA) expression in cancer of the ampulla of Vater in terms of prognosis. Surg Today. 1994; 24: 494–499. PMID: 7919730

48. Naryzhny SN, Lee H. Characterization of proliferating cell nuclear antigen (PCNA) isoforms in normal and cancer cells: there is no cancer-associated form of PCNA. FEBS Lett. 2007; 581: 4917–4920. PMID: 17900571

49. Lipkin M, Blattner WE, Fraumeni JF, Lynch HT, Deschner E, Winawer S. Tritiated thymidine (φp, φh) labeling distribution as a marker for hereditary predisposition to colon cancer. Cancer Res. 1983; 43: 1899–1904. PMID: 6831425

50. De Leon MP, Roncucci L, Di Donato P, Tassi L, Smerieri O, Amorico MG, et al. Pattern of epithelial cell proliferation in colorectal mucosa of normal subjects and of patients with adenomatous polyps or cancer of the large bowel. Cancer Res. 1988; 48: 4121–4126. PMID: 3383201

51. Deschner EE, Long FC, Hakissian M, Herrmann SL. Differential susceptibility of AKR, C57BL/6J, and CF1 mice to 1, 2-dimethylhydrazine-induced colonic tumor formation predicted by proliferative characteristics of colonic epithelial cells. J Natl Cancer Inst. 1983; 70: 279–282. PMID: 6571937

52. Velmurugan B, Singh RP, Agarwal R, Agarwal C. Dietary feeding of grape seed extract prevents azoxymethane-induced colonic aberrant crypt foci formation in fischer 344 rats. Mol Carcinogen. 2010; 49: 641–652.

53. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer. 2002; 2: 647–656. PMID: 12209154

54. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007; 35: 495–516. PMID: 17562483

55. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004; 305: 626–629. PMID: 15286356

56. Sturm I, Petrowsky H, Volz R, Lorenz M, Radetzki S, Hillebrand T, et al. Analysis of p53/BAX/p16ink4a/CDKN2 in esophageal squamous cell carcinoma: High BAX and p16ink4a/CDKN2 identifies patients with good prognosis. J Clin Oncol. 2001; 19: 2272–2281. PMID: 11304781

57. Ionov Y, Yamamoto H, Krajewski S, Reed JC, Perucho M. Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. Proc Natl Acad Sci. 2000; 97: 10872–10877. PMID: 10984511

58. Nirmala P, Ramanathan M. Effect of kaempferol on lipid peroxidation and antioxidant status in 1, 2-dimethyl hydrazine induced colorectal carcinoma in rats. Eur J Pharmacol. 2011; 654: 75–79. doi: 10.1016/j.ejphar.2010.11.034 PMID: 21172346

59. Liochev SI. Reactive oxygen species and the free radical theory of aging. Free Radic Biol Med. 2011; 60: 1–4.

60. Chen W, Weng Y-M, Tseng C-Y. Antioxidative and antimutagenic activities of healthy herbal drinks from Chinese medicinal herbs. Am J Chin Med. 2003; 31: 523–532. PMID: 14587875

61. Naziroglu M. Molecular role of catalase on oxidative stress-induced Ca2+ signaling and TRP cation channel activation in nervous system. J Recept Sig Transd. 2012; 32: 134–141. doi: 10.3109/10799893.2012.672994 PMID: 22475023

62. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot. 2003; 91: 179–194. PMID: 12509339

63. Meguid NA, Dardir AA, Abdel-Raouf ER, Hashish A. Evaluation of oxidative stress in autism: defective antioxidant enzymes and increased lipid peroxidation. Biol Trace Elem Res. 2011; 143: 58–65. doi: 10.1007/s12011-010-8840-9 PMID: 20845086

64. George VC, Kumar DN, Suresh P, Kumar RA (2014) Antioxidant, DNA protective efficacy and HPLC analysis of *Annona muricata* (soursop) extracts. J Food Sci Technol. 2014; 1–8.

65. Baskar R, Rajeswari V, Kumar TS. *In vitro* antioxidant studies in leaves of *Annona* species. Indian J Exp Biol. 2007; 45: 480–485. PMID: 17569293

66. Adewole SO, Caxton-Martins EA. Morphological changes and hypoglycemic effects of *Annona muricata* linn. (annonaceae) leaf aqueous extract on pancreatic β-cells of streptozotocin-treated diabetic rats. Afr J Biomed Res. 2006; 9: 173–180.

67. Foong CP, Hamid RA. Evaluation of anti-inflammatory activities of ethanolic extract of *Annona muricata* leaves. Rev Bras Farmacogn. 2012; 22: 1301–1307.

68. Lissoni P, Barni S, Mandala M, Ardizzoia A, Paolorossi F, Vaghi M, et al. Decreased toxicity and increased efficacy of cancer chemotherapy using the pineal hormone melatonin in metastatic solid tumour patients with poor clinical status. Eur J Cancer. 1999; 35: 1688–1692. PMID: 10674014

69. Cerea G, Vaghi M, Ardizzoia A, Villa S, Bucovec R, Mengo S, et al. Biomodulation of cancer chemotherapy for metastatic colorectal cancer: a randomized study of weekly low-dose irinotecan alone versus irinotecan plus the oncostatic pineal hormone melatonin in metastatic colorectal cancer patients progressing on 5-fluorouracil-containing combinations. Anticancer Res. 2002; 23: 1951–1954.
70. Vaca C, Wilhelm J, Harms-Ringdahl M. Interaction of lipid peroxidation products with DNA. A review. Mutat Res-Rev Mutat. 1988; 195: 137–149.
71. Pandurangan AK, Dharmalingam P, Anandasadagopan S, Ganapasam S (2012) Effect of luteolin on the levels of glycoproteins during azoxymethane-induced colon carcinogenesis in mice. Asian Pac J Cancer Prev. 2012; 13: 1569–1573. PMID: 22799368
72. Demircan B, Çelik G, Süleyman H, Akçay F. Effects of indomethacin, celecoxib and meloxicam on glutathione, malondialdehyde and myeloperoxidase in rat gastric tissue. Pain Clinic. 2005; 17: 383–388.
73. Komiya M, Fujii G, Takahashi M, Iigo M, Mutoh M. Prevention and intervention trials for colorectal cancer. Jpn J Clin Oncol. 2013; 43: 685–694. doi: 10.1093/jjco/hyt053 PMID: 23613189
74. Hendrickse C, Kelly R, Radley S, Donovan I, Keighley M, Neoptolemos JP. Lipid peroxidation and prostaglandins in colorectal cancer. Br J Surg. 1994; 81: 1219–1223. PMID: 7953368
75. Skrzydlewska E, Sulkowski S, Koda M, Zalewski B, Kanczuga-Koda L, Sulkowska M. Lipid peroxidation and antioxidant status in colorectal cancer. World J Gastroenterol. 2005; 11: 403–406. PMID: 15637754
76. Kim G-s, Zeng L, Alali F, Rogers LL, Wu F-E, McLaughlin JL, et al. Two new mono-tetrahydrofuran ring acetogenins, annomuricin E and muricapentocin, from the leaves of *Annona muricata*. J Nat Prod. 1998; 61: 432–436. PMID: 9584396
77. Rupprecht JK, Hui Y-H, McLaughlin JL. Annonaceous acetogenins: a review. J Nat Prod. 1990; 53: 237–278. PMID: 2199608
78. Liaw C-C, Wu T-Y, Chang F-R, Wu Y-C. Historic perspectives on Annonaceous acetogenins from the chemical bench to preclinical trials. Planta Med. 2010; 76: 1390–1404. doi: 10.1055/s-0030-1250006 PMID: 20577943
79. Bermejo A, Figadère B, Zafra-Polo M-C, Barrachina I, Estornell E, Cortes D. Acetogenins from Annonaceae: recent progress in isolation, synthesis and mechanisms of action. Nat Prod Rep. 2005; 22: 269–303. PMID: 15806200
80. Alali FQ, Liu X-X, McLaughlin JL. Annonaceous acetogenins: recent progress. J Nat Prod. 1999; 62: 504–540. PMID: 10096871
81. Nakanishi Y, Chang F-R, Liaw C-C, Wu Y-C, Bastow KF, Lee K-H. Acetogenins as Selective Inhibitors of the Human Ovarian 1A9 Tumor Cell Line 1. J Med Chem. 2003; 46: 3185–3188. PMID: 12852747
82. James Morré D, de Cabo R, Farley C, Oberlies NH, McLaughlin JL. Mode of action of bullatacin, a potent antitumor acetogenin: inhibition of NADH oxidase activity of HeLa and HL-60, but not liver, plasma membranes. Life Sci. 1994; 56: 343–348.
83. Lewis MA, Arnason J, Philogene B, Rupprecht J, McLaughlin J. Inhibition of Respiration at Site I by Asimicin, an Insecticidal Acetogenin of the Pawpaw, *Asimina triloba*(Annonaceae). Pestic Biochem Phys. 1993; 45: 15–23.
84. Oberlies NH, Chang C-j, McLaughlin JL. Structure-activity relationships of diverse Annonaceous acetogenins against multidrug resistant human mammary adenocarcinoma (MCF-7/Adr) cells. J Med Chem. 1997; 40: 2102–2106. PMID: 9207950
85. Oberlies NH, Croy VL, Harrison ML, McLaughlin JL. The Annonaceous acetogenin bullatacin is cytotoxic against multidrug-resistant human mammary adenocarcinoma cells. Cancer Lett. 1997. 115: 73–79. PMID: 9097981
86. Abe K, Matsuki N. Measurement of cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. Neurosci Res. 2000; 38: 325–329. PMID: 11164558
87. Park M-T, Lee S-J. Cell cycle and cancer. J Biochem Mol Biol. 2003; 36: 60–65. PMID: 12542976
88. Wang Z-Y, Wang D-M, Loo TY, Cheng Y, Chen L-L, Shen J-G, et al. *Spatholobus suberectus* inhibits cancer cell growth by inducing apoptosis and arresting cell cycle at G2/M checkpoint. J Ethnopharmacol. 2011; 133: 751–758. doi: 10.1016/j.jep.2010.11.004 PMID: 21073941
89. Yuan S-SF, Chang H-L, Chen H-W, Yeh Y-T, Kao Y-H, Lin K-H, et al. Annonacin, a mono-tetrahydrofuran acetogenin, arrests cancer cells at the G1 phase and causes cytotoxicity in a Bax-and caspase-3-related pathway. Life Sci. 2003; 72: 2853–2861. PMID: 12697268
90. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. Trends Cell Biol. 2006; 16: 189–197. PMID: 16529932

91. Brumatti G, Sheridan C, Martin SJ. Expression and purification of recombinant annexin V for the detection of membrane alterations on apoptotic cells. Methods. 2008; 44: 235–240. doi: 10.1016/j.ymeth.2007.11.010 PMID: 18314054

92. Lakhani SA, Masud A, Kuida K, Porter GA, Booth CJ, Mehal WZ, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science. 2006; 311: 847–851. PMID: 16469926

93. Martinou J-C, Youle RJ. Mitochondria in Apoptosis: Bcl-2 Family Members and Mitochondrial Dynamics. Dev Cell. 2011; 21: 92–101. doi: 10.1016/j.devcel.2011.06.017 PMID: 21763611

Published Paper 4: Gastroprotective activity of *Annona muricata* leaves against ethanol-induced gastric injury in rats via Hsp70/Bax involvement

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ORIGINAL RESEARCH

Gastroprotective activity of *Annona muricata* leaves against ethanol-induced gastric injury in rats via Hsp70/Bax involvement

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Abstract: The popular fruit tree of *Annona muricata* L. (Annonaceae), known as soursop and graviola, is a widely distributed plant in Central and South America and tropical countries. Leaves of *A. muricata* have been reported to possess antioxidant and anti-inflammatory activities. In this study, the gastroprotective effects of ethyl acetate extract of *A. muricata* leaves (EEAM) were investigated against ethanol-induced gastric injury models in rats. The acute toxicity test of EEAM in rats, carried out in two doses of 1 g/kg and 2 g/kg, showed the safety of this plant, even at the highest dose of 2 g/kg. The antiulcer study in rats (five groups, n=6) was performed with two doses of EEAM (200 mg/kg and 400 mg/kg) and with omeprazole (20 mg/kg), as a standard antiulcer drug. Gross and histological features showed the antiulcerogenic characterizations of EEAM. There was significant suppression on the ulcer lesion index of rats pretreated with EEAM, which was comparable to the omeprazole effect in the omeprazole control group. Oral administration of EEAM to rats caused a significant increase in the level of nitric oxide and antioxidant activities, including catalase, glutathione, and superoxide dismutase associated with attenuation in gastric acidity, and compensatory effect on the loss of gastric wall mucus. In addition, pretreatment of rats with EEAM caused significant reduction in the level of malondialdehyde, as a marker for oxidative stress, associated with an increase in prostaglandin E2 activity. Immunohistochemical staining also demonstrated that EEAM induced the downregulation of Bax and upregulation of Hsp70 proteins after pretreatment. Collectively, the present results suggest that EEAM has a promising antiulcer potential, which could be attributed to its suppressive effect against oxidative damage and preservative effect toward gastric wall mucus.

Keywords: *Annona muricata,* annonaceae, gastric injury, antioxidants, Hsp70/Bax

Introduction

Peptic ulcer is a debilitating disease which affects 4 million people of all ages globally each year.[1,2] This prevalent gastrointestinal disorder is defined as integrity disturbance of the duodenum or gastric mucosa, which is characterized by mucosal damage due to gastric acid and pepsin secretion.[3,4] The gastrointestinal inflammation caused by a peptic ulcer can deteriorate into a perforated peptic ulcer and become a life-threatening disease, with the mortality rate of 10%–40%.[5–7] The increased incidence of contributing factors to peptic ulcer among people, including alcohol consumption, nutritional deficiencies, smoking, and stress has made this disease a major health problem of global concern.[8–10] In addition, the available therapeutic drugs for peptic ulcers have failed to meet pharmacological expectations, to a certain degree, due to arrhythmia, gynecomastia, hypersensitivity, impotence, and hematopoietic changes of drugs, including anticholinergics, H_2-receptor antagonists, and proton pump inhibitors.[11,12] Therefore, there is a surge

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of interest in developing new pharmacological agents with gastroprotective effect against peptic ulcers.

Investigation into different diseases at the cellular level and exploring the molecular pathway have provided a new approach for the development of pharmacological products.[13] In recent studies, it is well established that heat shock proteins (Hsps) have a critical role in the gastric defense mechanism at the intracellular level.[14–16] Hsp70, as a major molecular chaperone, is responsible for cellular recovery against various stimuli, through modulation of the denatured and unfolded proteins.[17,18] The protective role of this protein against gastric mucosal damage induced by ulcerogenic conditions and toxic agents has made it a key factor in gastrointestinal studies.[19–21] Therefore, studies on antiulcer agents with the potential to increase the expression of Hsp70 can lead to the development of new gastroprotective drugs with marked improvements in efficiency.

Annona muricata L., a member of the Annonaceae family, is a widely distributed plant in Central and South America and tropical countries.[22,23] Also known as soursop and graviola, this small tropical tree plant has long been cultivated by native peoples, due to its extensive applications in folk medicine and heart-shaped, edible fruits.[24,25] The lanceolate dark green leaves of *A. muricata* are traditionally used as an antispasmodic nervine for heart conditions and as a sedative. In addition, the leaves are applied to treat asthma, cough, fever, headache, hypertension, and toothache.[24,26,27] The leaves of *A. muricata* have been found to possess significant antioxidant effects, assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, ferric reducing antioxidant power, and hydroxyl-scavenging activity techniques in animal models.[23,26,28] In addition, the leaves demonstrated a notable protective effect against acute and chronic inflammations in rats, through suppression of proinflammatory cytokines.[29] Previous studies have shown that the main chemical constituents in *A. muricata* are annonaceous acetogenins, alkaloids, and essential oils.[24] Due to the significant antioxidant and anti-inflammatory features of *A. muricata* leaves, this plant may be a promising candidate for antiulcer agents. Hence, the present study was carried out to investigate the acute toxicity and gastroprotective activity of *A. muricata* leaves against ethanol-induced gastric injury in rats.

Materials and methods

Plant collection and preparation of the ethyl acetate extract

The leaves of *A. muricata* were collected from Ipoh, Malaysia, in June 2013. The plant was authenticated by Dr Yong Kien Thai, Institute of Biological Sciences, University of Malaya. A voucher specimen has been deposited at the herbarium of the University of Malaya (Number KLU47978). The leaves were dried at 35°C and subsequently powdered (1,981 g), followed by maceration at room temperature with ethyl acetate for 4 days. After filtering the extract, the solvent was removed using a rotary evaporator (Buchi, Germany) at 40°C. The percentage yield after extraction, three times with ethyl acetate was 4.1% (81.3 g).

Drugs and chemicals

In this study, omeprazole (Sigma-Aldrich, St Louis, MO, USA) was used as the reference antiulcer medicine. All substances administered to animals, including ethyl acetate extract of *A. muricata* leaves (EEAM) and omeprazole, were dissolved in the vehicle (5% Tween® 20).

Chemical analysis of EEAM

Chemical profiling of EEAM was carried out using an Agilent, a LECO Restek Rxi®-5MS capillary column (30 minutes, 0.25 mm internal diameter, 0.25 µm film thickness), and a LECO Pegasus® HT high throughput time-of-flight mass spectrometer, as previously described in detail.[30] The carrier gas was helium, at a flow rate of 1 mL per minute (min). Column temperature was initially 40°C for 5 min, then gradually elevated to 160°C at 4°C/min, and finally increased to 280°C at 5°C/min and held for 10 min. For gas chromatography–mass spectrometry detection, an electron ionization system was used, with ionization energy of 70 eV. The fraction was diluted 1:100 (by volume) with ethyl acetate, and 1.0 µL of the diluted sample was injected automatically in splitless mode. Injector temperature was set at 250°C. Compounds were identified from their mass spectra, by comparison of the retention times of peak with interpretation of mass spectroscopy fragmentation patterns from the National Institute of Standards and Technology (NIST147) mass specteral database.

Animals

Adult and healthy Sprague Dawley® strain rats (180–250 g) of both sexes were obtained from Animal House, Faculty of Medicine, University of Malaya (Kuala Lumpur, Malaysia). Animals were housed at controlled room temperature (~24°C) with free access to standard rat pellets and tap water ad libitum, under an artificial lighting system (daily ratio 1:1). The experiments were carried out after approval of the protocol by the committee for animal experimentation, Faculty of Medicine, University of Malaya (Ethic Number 2014-03-05/PHAR/R/SZM).

Toxicity evaluation of EEAM

Eighteen female rats (6–8 weeks old) were divided into three groups (n=6), namely, vehicle, low dose, and high dose, and orally administered with Tween 20 (5%), 1 g/kg EEAM, and 2 g/kg EEAM, respectively. Prior to dosing, rodents were fasted overnight (water was accessible), and fasting was continued for 3 hours after dosing. The rats were then monitored for mortality, if any, or any other sign of toxicological symptoms twice per day during a period of 2 weeks. On day 15, the rats were euthanized using an overdose of xylazine and ketamine, and tested for biochemical parameters and histological examinations of liver and kidney.

Ethanol-induced gastric injury

The experiment was performed as previously described in detail.[31] After 24 hours of fasting (water was accessible except for the last 2 hours), the 30 male rats (n=6 per group) in five groups were pretreated according to Table 1. To induce gastric injury, 1 hour after treatment, all the rodents were orally administered with absolute ethanol (5 mL/kg), except for the normal control group, which was administered with 5% Tween 20. The rats were euthanized 1 hour later with an overdose of xylazine and ketamine, and their stomachs were removed and prepared for further analysis.

Macroscopic evaluation of lesions

After rinsing the stomachs of the rats with water to remove blood clots and gastric contents, they were opened along the greater curvature and fixed to examine the gastric lesion index, as previously described.[32] Microscopic scores were calculated, based on the following parameters: edema or hemorrhage, loss of mucosal folding, mucosal discoloration (score: 1 each); ulcers less than 1 mm/cm^2 (score: number of ulcers ×2); ulcers more than 1 mm/cm^2 (score: number of ulcers ×3); perforated ulcers (score: number of ulcers ×4).

Table 1 The experimental design and specifications

Group	Description	Pretreatment	Treatment
A	Normal control	5% Tween® 20 (5 mL/kg)	5% Tween 20
B	Ulcer control	5% Tween 20 (5 mL/kg)	Absolute ethanol
C	Low dose	EEAM (200 mg/kg)	Absolute ethanol
D	High dose	EEAM (400 mg/kg)	Absolute ethanol
E	Omeprazole control	Omeprazole (20 mg/kg)	Absolute ethanol

Abbreviation: EEAM, ethyl acetate extract of *Annona muricata* leaves.

Evaluation of the loss in gastric wall mucus (GWM) and gastric juice acidity

To determine gastric juice acidity, the stomachs were dissected and the contents were drained into falcon tubes and centrifuged at 3,000 rpm for 15 min. Then, gastric acidity was measured using a digital pH meter. Perturbation in GWM was determined for each group.[33] In brief, the glandular segments of each stomach were removed prior to immersing the stomach tissue in 1% alcian blue solution (in sucrose solution, buffered with sodium acetate at pH 5). Then, the excess dye was removed by rinsing with sucrose solution. Magnesium chloride solution was used to extract the dye complexed with GWM. After mixing the extract with diethyl ether, the level of GWM in each group was determined through measurement of the absorbance at 580 nm. The quantity of GWM was expressed as µg of alcian blue per gram of tissue.

Enzymatic activities of stomach tissue homogenate

Preparation of the gastric tissue homogenate (150 mg tissue/mL phosphate buffered saline) for each rat was performed using a teflon homogenizer (Polytron, Heidolph, Germany). Then, the supernatant of tissue homogenate was isolated after centrifugation at 4,000 rpm for 10 min and used for further assessment of enzymatic activities. The levels of catalase (CAT), glutathione (GSH), nitric oxide (NO), and superoxide dismutase (SOD) were determined using commercial kits (Cayman Chemical, Ann Arbor, MI, USA), according to the vendor's instructions. In addition, determination of the levels of malondialdehyde (MDA) and prostaglandin E2 (PGE-2) was also carried out using commercial kits (Cayman Chemical), based on the protocols provided by the manufacturer.

Histopathology

Stomachs were fixed in 10% formalin. For microscopic analysis, after processing the tissues in a paraffin tissue-processing machine (Leica Microsystems Incorporated, Nussloch, Germany), 5 µm sections of tissue were stained with hematoxylin and eosin (H&E) dye and Periodic acid–Schiff (PAS) dye (Sigma-Aldrich), to examine tissue architecture, and changes in glycogen and generated mucus, respectively. Lastly, a light microscope (Nikon, Tokyo, Japan) was used to observe the gastric sections.

Immunohistochemistry evaluation

Immunohistochemistry analysis of Bax and Hsp70 was carried out, as previously described in detail.[34,35] Briefly,

after processing the specimens of gastric tissue as previously mentioned, 5 μm sections were fixed on 3-aminopropyltrimethoxysilane-(APES) treated glass slides and supplemented with Bax and Hsp70 (Abcam, Cambridge, MA, USA), for immunohistochemical staining using a streptavidin peroxidase (Abcam). The slides were then observed under a light microscope with brown color representing positive immunohistochemical staining.

Statistical analysis

All values were reported as mean ± standard error of n animals per group. Statistical evaluation of the data was carried out using one-way analysis of variance, followed by Tukey's test. In the cases of ulcer index and gastric juice acidity, data were analysed using the Kruskal–Wallis test, and Dunn's multiple comparison test, as a post hoc test. A value of $P<0.05$ was considered significant.

Results and discussion

Nutraceuticals, including dietary supplements, functional foods, and herbal products have maintained their importance in the treatment of various ailments and diseases, irrespective of the extensive development of synthetic drugs in the pharmaceutical industry.[36] In recent years, considering the prominence of gastric injury and unpredictable side effects of the long-term use of synthetic drugs, interest in the use of herbal products has markedly elevated.[37] Previous studies have illustrated that numerous plants from different families, including Annonaceae, possess promising antiulcer properties.[37,38] An in vitro and in vivo study on *A. squamosa*, a member of the Annonaceae family, showed that the twigs of this plant possess antiulcer constituents with suppressive effect on H^+K^+-ATPase activity.[39] *Uvaria chamae* leaves (Annonaceae), with extensive application in folk medicine, are an antiulcer herb, scientifically proven to have antiulcer activity in rats.[40] *Polyalthia longifolia*, belonging to the Annonaceae family, was found to have anti-inflammatory and antiulcer properties in its fresh leaves, associated with antioxidant activity.[41,42] In the current study, we investigated the antiulcer effects of EEAM against ethanol-induced gastric injury in rats.

Chemical profiling of EEAM

The chemical profiling of EEAM showed the presence of two major compounds, namely, caryophyllene and α-copaene (Figure 1). Previous studies showed that among 80 essential oil compounds detected in *A. muricata* leaves, caryophyllene was the most abundant constituent (13.6%).[43,44] A recent investigation exhibited that the antioxidant effect of β-caryophyllene can effectively protect the rat liver from carbon tetrachloride-induced fibrosis, by suppressing hepatic stellate cell activation.[45] Caryophyllene oxide isolated from the bark of *A. squamosa* demonstrated significant anti-inflammatory and analgesic activities.[46] In addition, α-copaene was also reported as one of the significant chemical constituents (2.0%–7.3%) detected in volatile oil contents of *A. muricata*.[47] Caryophyllene and α-copaene were previously found to have promising antioxidant activity when they were isolated from *A. salzmannii*.[48] Considering the anti-inflammatory and antioxidant activities reported for caryophyllene and α-copaene, as the major compounds detected in EEAM, this extract may be a potential antiulcer candidate against ethanol-induced gastric injury in rats.

EEAM safety

Examination of acute toxicity is the first step in the study of the biological activities of plants.[49] In this study, no mortality in rats was observed with two concentrations of the EEAM extract (1 g/kg and 2 g/kg). After 2 weeks of treatment with EEAM, the rats did not demonstrate important changes in body weight, behavior (ie, ataxia, hypoactivity, and hyperactivity), microscopic morphology, and functional tests of kidney and liver (Table 2 and Figure 2). From these results, it is concluded that EEAM is quite safe, even at the highest dose of 4 g/kg, with no detectable sign of acute toxicity; the oral lethal dose (LD_{50}) of EEAM in rats was higher than 2 g/kg body weight. Our result was comparable with a previous investigation on the ethanol extract from *A. muricata* leaves, which showed an LD_{50} of 1.67 g/kg in mice, presenting high

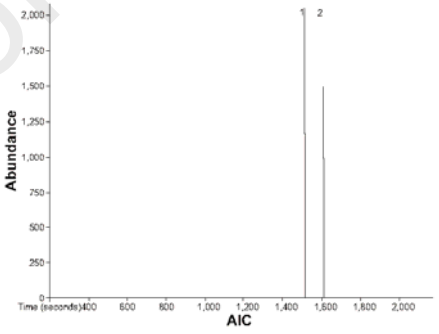

Figure 1 A chromatogram analysis of EEAM, characterized with the GC-MS-TOF.
Notes: GC-MS-TOF analysis demonstrated the presence of two major compounds, namely (1) caryophyllene and (2) α-copaene.
Abbreviations: EEAM, ethyl acetate extract of *Annona muricata* leaves; GC-MS-TOF, gas chromatography–time-of-flight mass spectrometry; AIC, analytical ion chromatogram.

Table 2 Effects of EEAM on renal and liver function tests of rats after 14 days of acute toxicity study did not show any significant differences between vehicle control and EEAM treated groups

Renal function test							
Group	**Sodium (mM/L)**	**Potassium (mM/L)**	**Chloride (mM/L)**	**CO_2 (mM/L)**	**Anion (mM/L)**	**Urea (mM/L)**	**Creatinine (μM/L)**
Vehicle	141.5±0.6	3.91±0.05	105.7±0.9	23.1±0.2	17.3±0.4	6.3±0.2	30.6±1.5
1 g/kg	140.6±0.3	4.14±0.07	104.3±1.1	24.4±0.3	16.6±0.3	5.8±0.1	30.7±1.2
2 g/kg	140.7±0.4	4.22±0.03	104.4±1.0	25.1±0.2	16.9±0.6	5.6±0.2	31.8±1.1

Liver function test								
Group	**Total protein (g/L)**	**Albumin (g/L)**	**Globulin (g/L)**	**TB (μM/L)**	**AP (U/L)**	**ALT (U/L)**	**AST (U/L)**	**GGT (U/L)**
Vehicle	70.5±0.7	35.4±0.8	27.3±0.8	3.1±0.1	160.4±2.4	27.4±0.9	25.1±2.2	1.1±0.1
1 g/kg	71.4±0.3	36.6±0.4	26.5±0.4	3.2±0.2	159.3±1.9	28.2±1.1	27.5±1.5	1.0±0.1
2 g/kg	71.7±0.8	37.1±0.2	26.1±0.2	3.2±0.1	158.5±2.5	28.9±1.5	26.3±1.4	1.2±0.1

Notes: Values expressed as mean ± SEM. A value of $P<0.05$ was considered significant.
Abbreviations: EEAM, ethyl acetate extract of *Annona muricata* leaves; TB, total bilirubin; AP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; SEM, standard error of the mean.

safety of *A. muricata* leaves.[50] However, a previous study on the acute toxicity of *A. muricata* leaf aqueous extract reported the relatively low LD_{50} value of 155 mg/kg in mice, which showed moderate safety of aqueous extract in mice.[27]

Macroscopic analysis of gross appearances of stomach and gastric ulcer index

The rodents that were administrated with absolute ethanol experienced severe macroscopic damage, compared to the normal control group (Figure 3A), which was evidenced by development of hemorrhagic ulceration (Figure 3B). The consequent hemorrhagic detriment was conspicuously attenuated by pretreatment with EEAM at 200 mg/kg and 400 mg/kg doses, with a few fields of hyperemia (Figure 3C and 3D). In addition, rats with prior administration of omeprazole (20 mg/kg) were able to suppress damage to the stomach, showing similar features to the normal control group (Figure 3E). Macroscopic analysis of the gross appearances of the stomach demonstrated that EEAM administration, especially at 400 mg/kg dose, had a protective effect against ethanol-induced gastric injury, which was comparable to the protective effect of omeprazole, as a standard antiulcer drug.

In the ethanol-induced gastric injury model, pretreatment with EEAM at 200 mg/kg and 400 mg/kg doses

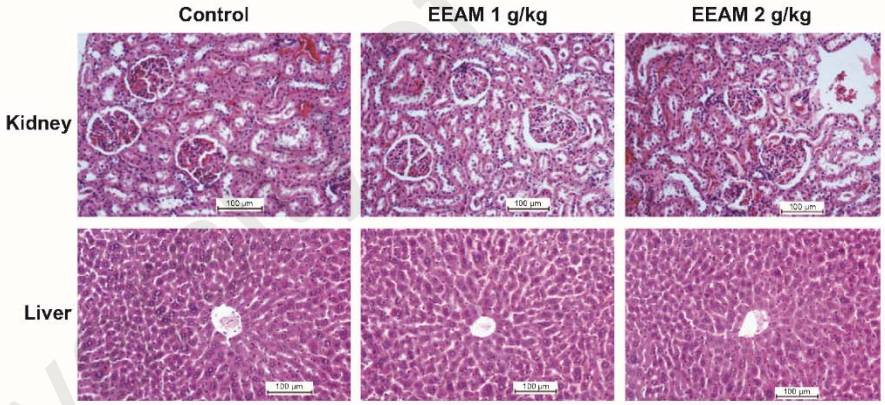

Figure 2 Histopathology analysis (H&E staining, 20×) of kidney and liver from control, EEAM at 1 g/kg, and EEAM at 2 g/kg demonstrated no significant differences in the structures of kidney and liver between vehicle control and EEAM-treated groups.
Abbreviations: EEAM, ethyl acetate extract of *Annona muricata* leaves; H&E, hematoxylin and eosin.

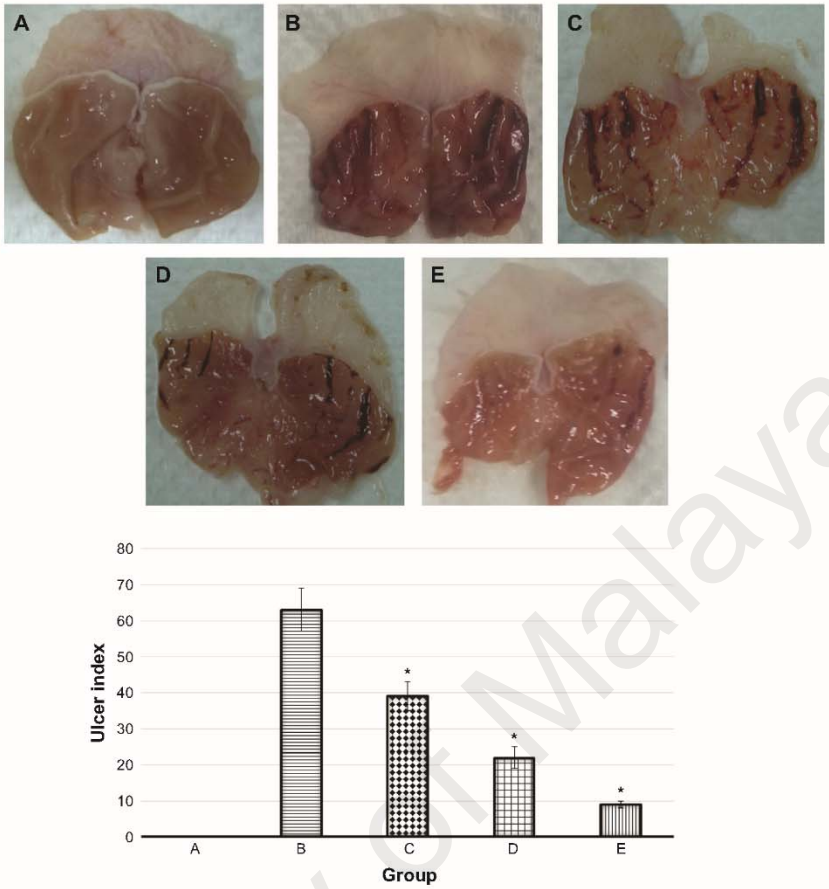

Figure 3 Gross appearances of stomach from five groups of rats, namely: (**A**) normal control, (**B**) lesion control, (**C**) low dose of EEAM, (**D**) high dose of EEAM, and (**E**) omeprazole control. Quantitative analysis of ulcer index showed significant reduction in the ulcer index after pretreatment with EEAM (200 mg/kg and 400 mg/kg), and omeprazole.

Notes: Data are reported as means ± SEM of six animals per group. Statistical comparison was performed using the Kruskal–Wallis test, followed by the Dunn test. A value of $^{*}P<0.05$ was considered significant.

Abbreviations: EEAM, ethyl acetate extract of *Annona muricata* leaves; SEM, standard error of the mean.

caused significant inhibition on the ulcer lesion index, compared to the ulcer control group (Figure 3). In addition, there was a significant reduction in all the gastric ulcer parameters after preadministration with omeprazole, as a standard antiulcer drug (Figure 3). This proton pump inhibitor drug has been extensively used to treat various ailments regarding gastric acid secretion.[51] Besides the antisecretory effect of omeprazole in acid-dependent ulcer models, it is well known to have a mucosal protective effect in non-antisecretory doses.[52] The noteworthy healing rates of omeprazole among patients suffering from peptic ulcer are mainly due to its gastric mucosa-protective effect, associated with the ability to suppress gastric juice acidity via proton pump inhibitory activity.[53] Therefore, any herbal medicine with the same protective effects may possibly show promising antiulcer potential.[54] Hence, in the next

step of this study, we investigated the effect of EEAM on GWM and gastric juice acidity.

EEAM protected the GWM and attenuated gastric juice acidity

Among the various factors involved in the intensification of lesion ulcers, exogenous gastric acid is known to be a crucial aggressive factor in the stomach.[55] The excessive production of gastric acid in patients suffering from peptic ulcers can severely intensify the gastric injury, which generally parallels reduction in the level of GWM.[56] Perturbation in the balance between aggressive factors and defensive factors, including cellular mucus, cell proliferation, cell shedding, and mucus secretion, results in gastric hyperacidity.[57] In patients with peptic ulcers, proton pump inhibitors attenuate acid secretion in the stomach and recover the production of GWM.[4] It is well established that ethanol is a potent necrotizing agent that severely damages the protective factors of the mucosa.[58] In our study, after administration of ethanol to rats, levels of GWM and pH were significantly reduced, compared to the normal control group (Figure 4). However, pretreatment of rats with two doses of EEAM caused significant attenuation in gastric acidity, compared to the ulcer control group. In addition, EEAM significantly retrieved the loss in GWM. Nonetheless, rats pretreated with omeprazole had comparatively higher protection of GWM and lower gastric acidity, compared to EEAM at both doses (Figure 4).

EEAM induced changes in enzymatic activities of stomach tissue homogenate

Administration of absolute ethanol to rodents induced significant reduction in antioxidant activities and NO level of stomach homogenate, compared to the normal control group. Meanwhile, pretreatment with EEAM at two doses, and omeprazole, significantly surged the levels of antioxidant activities and NO (Table 3). It is well established that reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radicals, and superoxide anions, as an aggressive factor, have a critical role in oxidative damage to the gastric mucosa.[59] Cellular antioxidants maintain ROS at their physiological levels to attenuate tissue damages. The higher level of GSH was found to minimize oxidative damages.[60] By converting into glutathione disulfide; this tripeptide with thiol groups reduces cellular ROS.[61] In addition, SOD has a scavenging effect against ROS, by converting the superoxide to hydrogen peroxide, which is subsequently converted to water by CAT.[62]

One of the important factors for the slow flow of gastric blood, which leads to the development of hemorrhagic lesions and subsequent solubilization of gastric mucus contents, was found to be a reduction in the level of NO. This leads to an elevation in the flow of K^+, Na^+, and pepsin secretion associated with a loss in the level of H^+ ions.[63] The protective barrier produced by NO, through the suppression of neutrophil infiltration, inhibits gastric damage from ethanol.[64] Through an inhibitory effect on neutrophil infiltration, NO also attenuates the secretion of inflammatory mediators.[65] Therefore, antiulcer agents with the ability to promote NO production can provide a more protective effect against gastric lesions.

The level of MDA, as a final product of lipid peroxidation, is an easy biomarker to determine oxidative stress.[66] Lipid peroxidation is mediated by neutrophils, via generation of superoxide anions.[65] As a necrotizing agent, ethanol caused

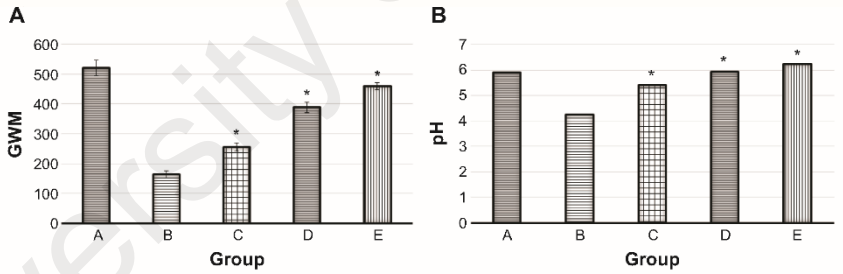

Figure 4 Measurement of (**A**) GWM (μg of alcian blue per gram of tissue) and (**B**) pH in five groups of rats, namely: (A) normal control, (B) lesion control, (C) low dose of EEAM, (D) high dose of EEAM, and (E) omeprazole control.

Notes: Data for GWM are reported as means ± SEM of six animals per group. Statistical comparison for the median pH values was performed using the Kruskal–Wallis test, followed by the Dunn test. A value of $^{*}P<0.05$ was considered significant.

Abbreviations: GWM, gastric wall mucus; EEAM, ethyl acetate extract of *Annona muricata* leaves; SEM, standard error of the mean.

Table 3 Effect of EEAM at (C) 200 mg/kg and (D) 400 mg/kg doses, and (E) omeprazole on enzymatic activities of stomach tissue homogenate. (A) and (B) are presenting the normal control and lesion control groups, respectively

Animal group	CAT nM/min/mL	GSH (μM/mg protein)	NO (μM/g protein)	SOD (U/mg protein)	MDA (μM/g protein)	PGE-2 (ng/mg protein)
A	116.11±4.17	16.49±0.44	11±0.9	19.66±0.25	72.23±1.1	4.07±0.09
B	70.14±3.21	9.19±0.38	5.7±0.09	10.19±0.55	168.23±4.98	2.12±0.03
C	81.53*±2.11	12.51*±0.18	7.7*±0.7	12.89*±0.56	123.88*±6.71	2.8*±0.1
D	103.23*±4.21	13.49*±0.12	9.1*±1.1	14.79*±0.61	95.22*±0.22	3.4*±0.7
E	113.23*±5.1	15.02*±0.33	10.1*±1	17.11*±0.7	89.23*±0.41	3.8*±0.06

Notes: Data are reported as means ± SEM of six animals per group. A value of *$P<0.05$ was considered significant.
Abbreviation: EEAM, ethyl acetate extract of *Annona muricata* leaves; CAT, catalase; GSH, glutathione; NO, nitric oxide; SOD, superoxide dismutase; MDA, malondialdehyde; PGE-2, prostaglandin E2; SEM, standard error of the mean.

an increase in the level of MDA,[67] which was observed in our study after administration of absolute ethanol to rats in the normal control group (Table 3). As is to be expected, due to the elevation in the activity of antioxidants, pretreatment with EEAM significantly suppressed MDA production in gastric tissue, which was close to the omeprazole effect (Table 3).

Prostaglandins, due to the regulatory effect on the function of the gut, have promising potential in the treatment of different gastric ailments.[68] PGE-2 with E prostanoid receptors has a critical role in the motility and secretion of the stomach. Previous studies have shown that the cytoprotective effect of PGE-2 provides noteworthy resistance to gastric mucosal cells against strong irritants, including ethanol.[69,70] In our study, the ulcer control group showed significant reduction in the level of PGE-2, while rats in groups C–E demonstrated compensatory increases. However, omeprazole administration to rats caused the highest level of PGE-2 in gastric tissue homogenates (Table 3).

Histopathological effect of EEAM on gastric lesions

As was observed in the macroscopic appearance, histological analysis of the gastric tissues also demonstrated various microscopic characterizations, as illustrated in Figures 5 and 6. H&E staining of the gastric tissues illustrated the severe superficial injury induced by ethanol (Figure 5). Apparent features of gastric ulcers, including edema and leukocyte infiltration, were observed in the lesion control group. Histological analysis indicated that oral administration of EEAM (200 mg/kg and 400 mg/kg doses), and omeprazole, markedly protected the gastric tissues from hemorrhagic lesions associated with attenuation of leukocyte infiltration

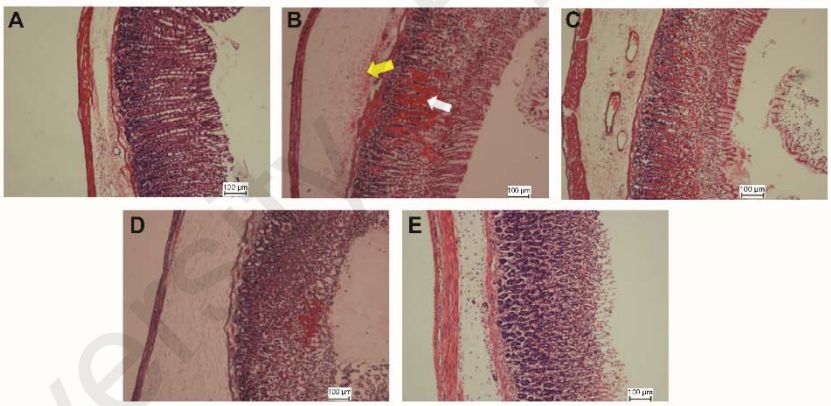

Figure 5 Histopathological staining of gastric tissues after treatment with EEAM at (**C**) 200 mg/kg and (**D**) 400 mg/kg doses, and (**E**) omeprazole, using H&E staining (20×). (**A**) and (**B**) are presenting the normal control and lesion control groups, respectively. In the lesion control group, there are clear signs of severe damage to the surface epithelium (white arrow) and leukocyte infiltration (yellow arrow).
Abbreviations: EEAM, ethyl acetate extract of *Annona muricata* leaves; H&E, hematoxylin and eosin.

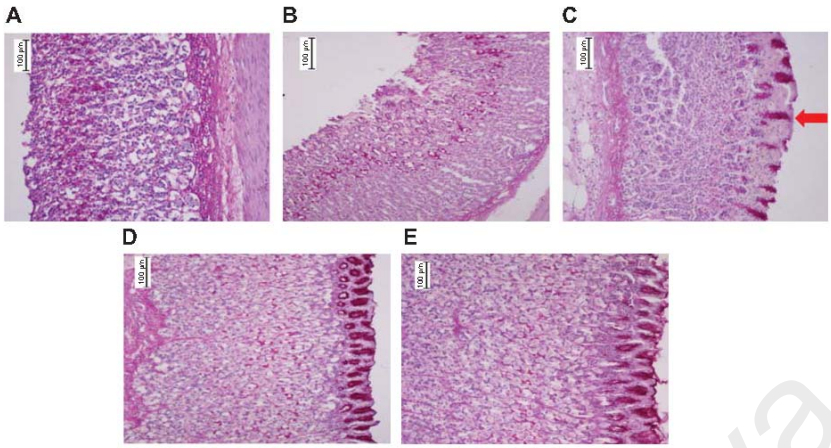

Figure 6 Histopathological staining of gastric tissues after treatment with EEAM at (**C**) 200 mg/kg and (**D**) 400 mg/kg doses, and (**E**) omeprazole, using PAS staining (20×). (**A**) and (**B**) are presenting the normal control and lesion control groups, respectively. PAS staining illustrated the glycogen accumulation (red arrow) in rats pretreated with EEAM and omeprazole.
Abbreviations: EEAM, ethyl acetate extract of *Annona muricata* leaves; PAS, Periodic acid–Schiff.

and submucosal edema. In spite of a conspicuous protective effect of EEAM, the sign of hemorrhagic lesions was clearly evident in EEAM 200 mg/kg pretreated rats. Nonetheless, administration of EEAM at 400 mg/kg dose, and omeprazole, demonstrated approximately the same characterizations as the normal control group (Figure 5).

Glycogen production in the gastric epithelium was examined using PAS staining. The glycogen content in gastric mucosa, presented as magenta color in PAS staining, showed an almost similar level in all groups of rats, except the lesion control, which suggested a collapse in glycogen production in this group (Figure 6). The PAS staining intensity was elevated in the pretreated groups of rats, with EEAM and omeprazole, compared to the lesion control group, showing the beneficial effect of EEAM on glycogen generation. Induction of glycogen formation by EEAM at 400 mg/kg dose approximates the effect of omeprazole. The findings of histopathological staining confirmed the role of EEAM, particularly at 400 mg/kg dose, in the enhancement of mucus level.

EEAM induced up-regulation of Hsp70 and down-regulation of Bax

Hsp70, a low-molecular weight chaperone and a member of the four families of heat shock proteins, has a critical role in the posttranslational characterizations of polypeptides.[71] Previous studies have shown the cytoprotective role of Hsp70 against different stress conditions.[72–74] In the current study, immunohistochemical analysis showed that mucosal expression of Hsp70 was upregulated in EEAM- and omeprazole-pretreated groups (Figure 7). However, accumulation of Hsp70 in the gastric tissue of rats pretreated with omeprazole was comparable to rats pretreated with EEAM. The normal control group also elicited a higher level of Hsp70 expression, compared to the lesion control group (Figure 7).

A Bcl-2 family of proteins, with 25 members, consists of two groups of proapoptotic and antiapoptotic proteins.[75] Bax, a proapoptotic protein, promotes the induction of apoptosis in stressed cells via mitochondrial-initiated events.[76,77] Therefore, an inhibitory effect on Bax protein expression attenuates cellular damage to the gastric tissue.[78] As illustrated in Figure 8, the expression of Bax protein in the lesion control group was markedly higher, compared to the normal control group, representing the undergoing process of apoptosis among gastric cells. In pretreated rats with EEAM at 200 mg/kg and 400 mg/kg doses, and omeprazole, accumulation of Bax protein in gastric tissue was noticeably reduced (Figure 8). Administration of EEAM (400 mg/kg), and omeprazole, reduced Bax protein expression to approximately the level of the normal control group. However, rats treated with EEAM at 200 mg/kg dose still elicited the accumulation of Bax protein in gastric tissue (Figure 8).

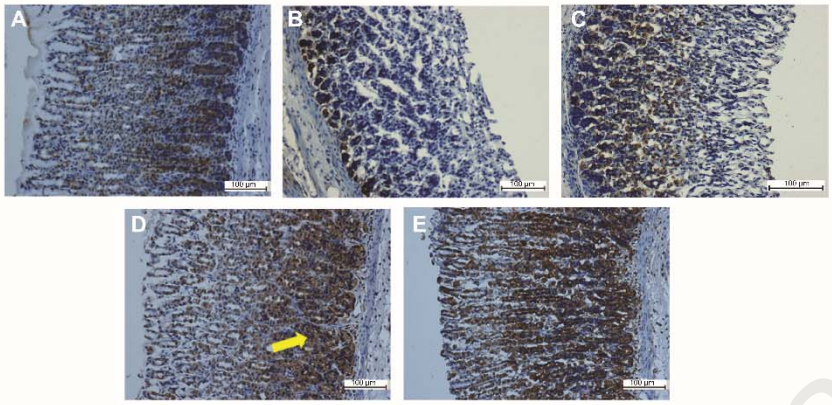

Figure 7 Immunohistochemical analysis of Hsp70 expression from five groups of rats, namely: (**A**) normal control, (**B**) lesion control, (**C**) low dose of EEAM, (**D**) high dose of EEAM, and (**E**) omeprazole control. Immunohistochemistry staining showed upregulation of Hsp70 in groups **C**–**E**. (**A**) and (**B**) are presenting the normal control and lesion control groups, respectively. The yellow arrow shows Hsp70 accumulation in gastric tissue (20×).
Abbreviation: EEAM, ethyl acetate extract of *Annona muricata* leaves.

Conclusion

The results obtained in this study showed the safety of EEAM in rats, even at the highest dose of 2 g/kg. However, further chronic toxicity testing should be conducted to confirm its safe usage. In vivo study in rats demonstrated the promising antiulcer effect of EEAM against gastric mucosal injury induced by ethanol. EEAM mediated its antiulcer potential probably through its ROS-scavenging activity and protective effect against GWM damage. In addition, Hsp70 upregulation and Bax downregulation were found to be involved in the suppression of gastric injuries. However, an investigation on the major active compound in EEAM is still required, to fully illustrate the antiulcer potential of *Annona muricata* leaves.

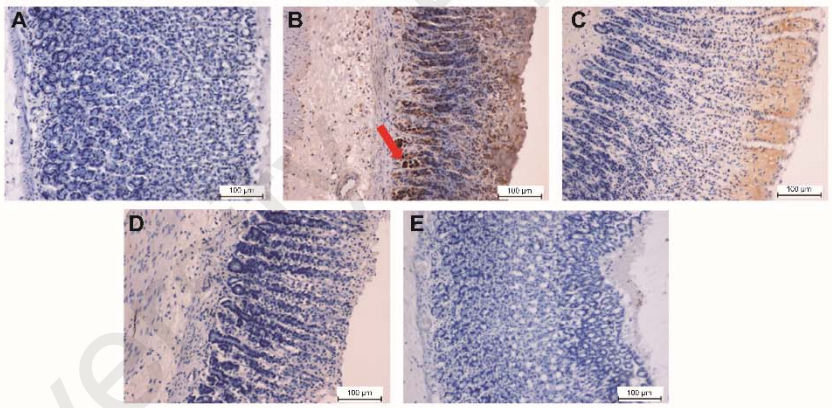

Figure 8 Immunohistochemical analysis of Bax protein expression from five groups of rats, namely: (**A**) normal control, (**B**) lesion control, (**C**) low dose of EEAM, (**D**) high dose of EEAM, and (**E**) omeprazole control. Immunohistochemistry staining showed downregulation of Bax in groups **C**–**E**. (**A**) and (**B**) are presenting the normal control and lesion control groups, respectively. The red arrow shows Bax protein accumulation in gastric tissue (20×).
Abbreviation: EEAM, ethyl acetate extract of *Annona muricata* leaves.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Zelickson MS, Bronder CM, Johnson BL, et al. Helicobacter pylori is not the predominant etiology for peptic ulcers requiring operation. *Am Surg*. 2011;77(8):1054–1060.
2. Thorsen K, Søreide JA, Kvaløy JT, Glomsaker T, Søreide K. Epidemiology of perforated peptic ulcer: age- and gender-adjusted analysis of incidence and mortality. *World J Gastroenterol*. 2013;19(3):347–354.
3. Ribeiro AR, Diniz PB, Estevam CS, Pinheiro MS, Albuquerque-Júnior RL, Thomazzi SM. Gastroprotective activity of the ethanol extract from the inner bark of Caesalpinia pyramidalis in rats. *J Ethnopharmacol*. 2013;147(2):383–388.
4. Ramakrishnan K, Salinas RC. Peptic ulcer disease. *Am Fam Physician*. 2007;76(7):1005–1012.
5. Bertleff MJ, Lange JF. Perforated peptic ulcer disease: a review of history and treatment. *Dig Surg*. 2010;27(3):161–169.
6. Svanes C. Trends in perforated peptic ulcer: incidence, etiology, treatment, and prognosis. *World J Surg*. 2000;24(3):277–283.
7. Møller MH, Adamsen S, Wøjdemann M, Møller AM. Perforated peptic ulcer: how to improve outcome? *Scand J Gastroentero*. 2009;44(1):15–22.
8. Kobayashi M, Takeuchi M, Hashimoto S, et al. Contributing factors to gastric ulcer healing after endoscopic submucosal dissection including the promoting effect of rebamipide. *Dig Dis Sci*. 2012;57(1):119–126.
9. Moriya M, Uehara A, Okumura T, Miyamoto M, Kohgo Y. Stress-induced hemorrhagic gastric ulcer after successful Helicobacter pylori eradication: two case reports. *J Med Case Rep*. 2011;5:252.
10. Andersen IB, Jørgensen T, Bonnevie O, Grønbaek M, Sørensen TI. Smoking and alcohol intake as risk factors for bleeding and perforated peptic ulcers: a population-based cohort study. *Epidemiology*. 2000;11(4):434–439.
11. Bighetti AE, Antonio MA, Kohn LK, et al. Antiulcerogenic activity of a crude hydroalcoholic extract and coumarin isolated from Mikania laevigata Schultz Bip. *Phytomedicine*. 2005;12(1–2):72–77.
12. Lakshmi V, Singh N, Shrivastva S, et al. Gedunin and Photogedunin of *Xylocarpus granatum* show significant anti-secretory effects and protect the gastric mucosa of peptic ulcer in rats. *Phytomedicine*. 2010;17(8–9):569–574.
13. Hajrezaie M, Hassandarvish P, Moghadamtousi SZ, et al. Chemopreventive evaluation of a Schiff base derived copper (II) complex against azoxymethane-Induced colorectal cancer in rats. *PloS one*. 2014;9(3):e91246.
14. Liu W, Chen Y, Lu G, Sun L, Si J. Down-regulation of HSP70 sensitizes gastric epithelial cells to apoptosis and growth retardation triggered by H. pylori. *BMC Gastroenterol*. 2011;11:146.
15. Otaka M, Odashima M, Watanabe S. Role of heat shock proteins (molecular chaperones) in intestinal mucosal protection. *Biochem Biophys Res Commun*. 2006;348(1):1–5.
16. Konturek PC, Brzozowski T, Ptak A, et al. Nitric oxide releasing aspirin protects the gastric mucosa against stress and promotes healing of stress-induced gastric mucosal damage: role of heat shock protein 70. *Digestion*. 2002;66(3):160–172.
17. Basu S, Srivastava PK. Heat shock proteins: the fountainhead of innate and adaptive immune responses. *Cell Stress Chaperon*. 2000;5(5):443.
18. Mayer MP, Bukau B. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci*. 2005;62(6):670–684.
19. Oyake J, Otaka M, Matsuhashi T, et al. Over-expression of 70-kDa heat shock protein confers protection against monochloramine-induced gastric mucosal cell injury. *Life Sci*. 2006;79(3):300–305.
20. Hirata I, Naito Y, Handa O, et al. Heat-shock protein 70-overexpressing gastric epithelial cells are resistant to indomethacin-induced apoptosis. *Digestion*. 2009;79(4):243–250.
21. Odashima M, Otaka M, Matsuhashi T, et al. Mechanical strain stress suppresses expression of HSP70 and wound restoration in gastric mucosal cells. *Dig Dis Sci*. 2007;52(11):3087–3091.
22. George VC, Kumar DR, Rajkumar V, Suresh PA, Kumar RA. Quantitative assessment of the relative antineoplastic potential of the n-butanolic leaf extract of Annona muricata Linn. in normal and immortalized human cell lines. *Asian Pac J Cancer Prev*. 2012;13(2):699–704.
23. George VC, Kumar DR, Suresh PK, Kumar RA. Antioxidant, DNA protective efficacy and HPLC analysis of Annona muricata (soursop) extracts. *J Food Sci Tech*. 2014:1–8.
24. Zeng L, Wu FE, Oberlies NH, McLaughlin JL, Sastrodihadjo S. Five new monotetrahydrofuran ring acetogenins from the leaves of Annona muricata. *J Nat Prod*. 1996;59(11):1035–1042.
25. Gleye C, Laurens A, Hocquemiller R, Cavé A, Laprévote O, Serani L. Isolation of montecristin, a key metabolite in biogenesis of acetogenins from Annona muricata and its structure elucidation by using tandem mass spectrometry. *J Org Chem*. 1997;62(3):510–513.
26. Adewole SO, Caxton-Martins EA. Morphological changes and hypoglycemic effects of Annona muricata linn. (annonaceae) leaf aqueous extract on pancreatic β-cells of streptozotocin-treated diabetic rats. *Afr J Biomed Res*. 2006;9(3):173–180.
27. Adewole SO, Ojewole JA. Protective effects of Annona muricata Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats. *Afr J Tradit, Complement Altern Med*. 2008;6(1):30–31.
28. Baskar R, Rajeswari V, Kumar TS. In vitro antioxidant studies in leaves of *Annona* species. *Indian J Exp Biol*. 2007;45(5):480.
29. Foong CP, Hamid RA. Evaluation of anti-inflammatory activities of ethanolic extract of Annona muricata leaves. *Rev Bras Farmacogn*. 2012;22(6):1301–1307.
30. Karimian H, Mohan S, Moghadamtousi SZ, et al. Tanacetum polycephalum (L.) Schultz-Bip. induces mitochondrial-mediated apoptosis and inhibits migration and invasion in MCF7 cells. *Molecules*. 2014;19(7):9478–9501.
31. Robert A, Nezamis JE, Lancaster C, Hanchar A. Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury. *Gastroenterology*. 1979;77(3):433–443.
32. Gamberini MT, Skorupa LA, Souccar C, Lapa AJ. Inhibition of gastric secretion by a water extract from Baccharis triptera, Mart. *Mem Inst Oswaldo Cruz*. 1991;86 Suppl 2:137–139.
33. Corne SJ, Morrissey SM, Woods RJ. Proceedings: A method for the quantitative estimation of gastric barrier mucus. *J Physiol*. 1974;242(2):116P–117P.
34. Hajrezaie M, Golbabapour S, Hassandarvish P, et al. Acute toxicity and gastroprotection studies of a new schiff base derived copper (II) complex against ethanol-induced acute gastric lesions in rats. *PloS one*. 2012;7(12):e51537.
35. Golbabapour S, Gwaram NS, Hassandarvish P, et al. Gastroprotection studies of Schiff base zinc (II) derivative complex against acute superficial hemorrhagic mucosal lesions in rats. *PloS one*. 2013;8(9):e75036.
36. Moghadamtousi SZ, Kamarudin MN, Chan CK, Goh BH, Kadir HA. Phytochemistry and biology of Loranthus parasiticus Merr, a commonly used herbal medicine. *Am J Chin Med*. 2014;42(1):23–35.
37. Falcão HS, Mariath IR, Diniz MF, Batista LM, Barbosa-Filho JM. Plants of the American continent with antiulcer activity. *Phytomedicine*. 2008;15(1–2):132–146.

38. Awaad AS, El-Meligy RM, Soliman GA. Natural products in treatment of ulcerative colitis and peptic ulcer. *J Saudi Chem Soc*. 2013;17(1):101–124.
39. Yadav DK, Singh N, Dev K, et al. Anti-ulcer constituents of Annona squamosa twigs. *Fitoterapia*. 2011;82(4):666–675.
40. Chilaka KC, Unekwe PC, Chilaka JU, Nweke IN. Studies on the anti-ulcer properties of ethanolic leaf extracts of Uvaria chamae. *Trop J Med Res*. 2010;14(2).
41. Chanda S, Baravalia Y, Kaneria M. Protective effect of Polyalthia longifolia var. pendula leaves on ethanol and ethanol/HCl induced ulcer in rats and its antimicrobial potency. *Asian Pac J Trop Med*. 2011;4(9):673–679.
42. Sharma RK, Mandal S, Rajani GP, Gupta N, Srivastava DP. Antiulcer and antiinflammatory activity of fresh leave extracts of Polyalthia longifolia in rats. *Int J Drug Dev Res*. 2011;3:351–359.
43. Pélissier Y, Marion C, Kone D, Lamaty G, Menut C, Bessière J-M. Volatile components of Annona muricata L. *J Essent Oil Res*. 1994;6(4):411–414.
44. Kossouoh C, Moudachirou M, Adjakidje V, Chalchat J-C, Figuérédo G. Essential oil chemical composition of Annona muricata L. leaves from Benin. *J Essent Oil Res*. 2007;19(4):307–309.
45. Calleja MA, Vieites JM, Montero-Meterdez T, et al. The antioxidant effect of β-caryophyllene protects rat liver from carbon tetrachloride-induced fibrosis by inhibiting hepatic stellate cell activation. *Br J Nutr*. 2013;109(3):394–401.
46. Costa EV, Dutra LM, de Jesus HC, et al. Chemical composition and antioxidant, antimicrobial, and larvicidal activities of the essential oils of *Annona salzmannii* and *A. pickelii* (Annonaceae). *Nat Prod Commun*. 2011;6(6):907–912.
47. Thang TD, Dai DN, Hoi TM, Ogunwande IA. Study on the volatile oil contents of Annona glabra L., Annona squamosa L., Annona muricata L. and Annona reticulata L., from Vietnam. *Nat Prod Res*. 2013;27(13):1232–1236.
48. Chavan MJ, Wakte PS, Shinde DB. Analgesic and anti-inflammatory activity of Caryophyllene oxide from Annona squamosa L. bark. *Phytomedicine*. 2010;17(2):149–151.
49. Déciga-Campos M, Rivero-Cruz I, Arriaga-Alba M, et al. Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. *J Ethnopharmacol*. 2007;110(2):334–342.
50. de Sousa OV, Vieira GD, de Jesus RG, Yamamoto CH, Alves MS. Antinociceptive and anti-inflammatory activities of the ethanol extract of Annona muricata L. leaves in animal models. *Int J Mol Sci*. 2010;11(5):2067–2078.
51. Li XQ, Andersson TB, Ahlström M, Weidolf L. Comparison of inhibitory effects of the proton pump-inhibiting drugs omeprazole, esomeprazole, lansoprazole, pantoprazole, and rabeprazole on human cytochrome P450 activities. *Drug Metab Dispos*. 2004;32(8):821–827.
52. Schneeweiss S, Maclure M, Dormuth CR, Glynn RJ, Canning C, Avorn J. A therapeutic substitution policy for proton pump inhibitors: clinical and economic consequences. *Clin Pharmacol Ther*. 2006;79(4):379–388.
53. Tang RS, Wu JC. Managing peptic ulcer and gastroesophageal reflux disease in elderly Chinese patients – focus on esomeprazole. *Clin Interv Aging*. 2013;8:1433–1443.
54. Borrelli F, Izzo AA. The plant kingdom as a source of anti-ulcer remedies. *Phytother Res*. 2000;14(8):581–591.
55. Brzozowski T, Konturek PC, Konturek SJ, et al. Role of gastric acid secretion in progression of acute gastric erosions induced by ischemia-reperfusion into gastric ulcers. *Eur J Pharmacol*. 2000;398(1):147–158.
56. Masuda Y, Tanaka T, Inomata N, et al. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun*. 2000;276(3):905–908.
57. Laloo D, Prasad SK, Krishnamurthy S, Hemalatha S. Gastroprotective activity of ethanolic root extract of Potentilla fulgens Wall. ex Hook. *J Ethnopharmacol*. 2013;146(2):505–514.
58. Wallace JL. Nonsteroidal anti-inflammatory drugs and the gastrointestinal tract. Mechanisms of protection and healing: current knowledge and future research. *Am J Med*. 2001;110(1A):19S–23S.
59. Kwiecien S, Brzozowski T, Konturek SJ. Effects of reactive oxygen species action on gastric mucosa in various models of mucosal injury. *J Physiol Pharmacol*. 2002;53(1):39–50.
60. Avci E, Akarslan ZZ, Erten H, Coskun-Cevher S. Oxidative stress and cellular immunity in patients with recurrent aphthous ulcers. *Braz J Med Biol Res*. 2014;47(5):355–360.
61. Rozza AL, de Mello Moraes T, Kushima H, Nunes DS, Hiruma-Lima CA, Pellizzon CH. Involvement of glutathione, sulfhydryl compounds, nitric oxide, vasoactive intestinal peptide, and heat-shock protein-70 in the gastroprotective mechanism of Croton cajucara Benth. (Euphorbiaceae) essential oil. *J Med Food*. 2011;14(9):1011–1017.
62. Oliveira FA, Vieira-Júnior GM, Chaves MH, et al. Gastroprotective and anti-inflammatory effects of resin from Protium heptaphyllum in mice and rats. *Pharmacol Res*. 2004;49(2):105–111.
63. Pan LR, Tang Q, Fu Q, Hu BR, Xiang JZ, Qian JQ. Roles of nitric oxide in protective effect of berberine in ethanol-induced gastric ulcer mice. *Acta Pharmacol Sin*. 2005;26(11):1334–1338.
64. Tuchinda P, Reutrakul V, Claeson P, et al. Anti-inflammatory cyclohexenyl chalcone derivatives in Boesenbergia pandurata. *Phytochemistry*. 2002;59(2):169–173.
65. Kobayashi T, Ohta Y, Yoshino J, Nakazawa S. Teprenone promotes the healing of acetic acid-induced chronic gastric ulcers in rats by inhibiting neutrophil infiltration and lipid peroxidation in ulcerated gastric tissues. *Pharmacol Res*. 2001;43(1):23–30.
66. Demircan B, Çelik G, Süleyman H, Akçay F. Effects of indomethacin, celecoxib and meloxicam on glutathione, malondialdehyde and myeloperoxidase in rat gastric tissue. *Pain Clinic*. 2005;17(4):383–388.
67. Kasdallah-Grissa A, Mornagui B, Aouani E, et al. Resveratrol, a red wine polyphenol, attenuates ethanol-induced oxidative stress in rat liver. *Life Sci*. 2007;80(11):1033–1039.
68. Konturek SJ, Konturek PC, Brzozowski T. Prostaglandins and ulcer healing. *J Physiol Pharmacol*. 2005;56 Suppl 5:5–31.
69. Hatazawa R, Tanaka A, Tanigami M, et al. Cyclooxygenase-2/prostaglandin E2 accelerates the healing of gastric ulcers via EP4 receptors. *Am J Physiol Gastrointest Liver Physiol*. 2007;293(4):G788–G797.
70. Sakata D, Yao C, Narumiya S. Prostaglandin E2, an immunoactivator. *J Pharmacol Sci*. 2010;112(1):1–5.
71. Ngosuwan J, Wang NM, Fung KL, Chirico WJ. Roles of cytosolic Hsp70 and Hsp40 molecular chaperones in post-translational translocation of presecretory proteins into the endoplasmic reticulum. *J Biol Chem*. 2003;278(9):7034–7042.
72. Santoro MG. Heat shock factors and the control of the stress response. *Biochem Pharmacol*. 2000;59(1):55–63.
73. Mosser DD, Caron AW, Bourget L, et al. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol Cell Biol*. 2000;20(19):7146–7159.
74. Suemasu S, Tanaka K, Namba T, et al. A role for HSP70 in protecting against indomethacin-induced gastric lesions. *J Biol Chem*. 2009;284(29):19705–19715.
75. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007;35(4):495–516.
76. Qiao WL, Wang GM, Shi Y, et al. Differential expression of Bcl-2 and Bax during gastric ischemia-reperfusion of rats. *World J Gastroenterol*. 2011;17(13):1718–1724.
77. Ocker M, Höpfner M. Apoptosis-modulating drugs for improved cancer therapy. *Eur Surg Res*. 2012;48(3):111–120.
78. Mahr S, Neumayer N, Gerhard M, Classen M, Prinz C. IL-1beta-induced apoptosis in rat gastric enterochromaffin-like cells is mediated by iNOS, NF-kappaB, and Bax protein. *Gastroenterology*. 2000;118(3):515–524.

Published Paper 5: *Annona muricata* leaves accelerate wound healing in rats via involvement of Hsp70 and antioxidant defence

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Original research

Annona muricata leaves accelerate wound healing in rats via involvement of Hsp70 and antioxidant defence

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HIGHLIGHTS

- This study substantiated the traditional use of *Annona muricata* upon wound injury.
- *A. muricata* leaves accelerated various stages of wound healing.
- Wound healing effects were accompanied with epithelialization and collagen synthesis.
- Immunohistochemical analysis showed the up-regulation of HSP70 protein.
- The protective mechanism was through suppression of the oxidative stress.

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ABSTRACT

Introduction: *Annona muricata*, a member of the Annonaceae family, is commonly known as soursop and graviola. The leaves of this tropical fruit tree are widely used in folk medicine against skin diseases and abscesses, however there is no scientific evidence justifying the use of *A. muricata* leaves. The aim of the present study is to evaluate the wound healing potential of ethyl acetate extract of *A. muricata* leaves (EEAM) towards excisional wound models in rats.
Methods: *Sprague Dawley* rats (24) were randomly divided into four groups, viz. (A) vehicle control, (B) low dose of EEAM (5% w/w), (C) high dose of EEAM (10% w/w) and (D) positive control with excisional wound created on the neck area. Wounds were topically dressed twice a day for 15 days. On the 15th day, animals were sacrificed and then processed for immunohistochemical and histological evaluations, including Hematoxylin & Eosin and Masson Trichrome stainings. The activity of antioxidants, namely catalase, glutathione peroxidase and superoxide dismutase, and malondialdehyde (MDA) was measured in wound tissue homogenate.
Results: Macroscopic and microscopic analysis of wounds demonstrated a significant wound healing activity shown by EEAM at two doses. Treatment of wounds with ointment containing EEAM caused significant surge in antioxidants activities and decrease in the MDA level of wound tissues compared with vehicle control. The immunohistochemical evaluation revealed conspicuous up-regulation of Hsp70 in treated wounds with EEAM, suggesting the anti-inflammatory effect of EEAM.
Conclusion: EEAM exhibited a promising wound healing potential towards excisional wound models in rats.

1. Introduction

A wound is generally defined as a loss or damage of cells which breaks the anatomic or functional continuity of the skin [1]. A dynamic process of wound healing requires an elaborate biological

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cascade of cellular, biochemical, molecular and physiological events which mainly consists of four phases, namely coagulation, inflammation, proliferation and maturation or tissue remodeling [2,3]. The constant cell-matrix and cell–cell interactions during the process of wound healing makes these phases fairly integrated and overlapped [4]. The result of this complex phenomenon leads to connective tissue recovery and fibrous scar reconstruction which result in mending of anatomical continuity and functional role of the living tissue [5].

A variety of cells and tissues with collaborative efforts are involved in the process of healing. Coagulation and inflammation together as phase 1 of the healing process requires the neutrophils migration at wound margin, developing to the fibrin clot. In phase 2 or the proliferation phase, replacement of neutrophils by macrophages is an important step [6]. In addition, the incision space is filled by the invasion of granulation tissue. In the maturation or tissue remodeling as the last step of healing, the collagen accumulation and fibroblast proliferation cause an elevation in the tensile strength of the skin associated with conspicuous decrease in edema and leukocyte infiltration [6,7]. The healing process is only finished after complete mending of disrupted surface by collagen [8]. In recent years, there has been a noteworthy surge of interest in using natural remedies with more efficient healing potential against different dermatological and skin disorders, including burns, cuts and wounds [9]. Medicinal plants have provided more affordable wound healing products with higher safety from hypersensitive reactions compared to the synthetic pharmaceutical agents [10,11].

The popular fruit tree of *Annona muricata* L. (Annonaceae) commonly known as graviola or soursop is commercially cultured in Central and South America and tropical countries [12]. The edible fruit of this small tropical tree is extensively used to produce candy, juice and sherbets [13]. In addition, the importance of this plant is also contributed to its various applications in folk medicine [14]. All parts of this plant, including the bark, fruit-seeds, leaves and root are used in natural medicines in the tropics [15]. Traditionally, the leaves are used for cystitis, diabetes, headaches, hypertension, insomnia, liver problems and as an antidysenteric, anti-inflammatory and antispasmodic [16]. The cooked leaves are topically applied against abscesses [16]. In tropical Africa, including Nigeria, leaves are traditionally used against skin diseases [15]. However, the wound healing potential of the *A. muricata* leaves has not been replicated in an experimental animal study as of yet. Therefore, the present study was designed to examine the efficacy of ethyl acetate extract of *A. muricata* leaves (EEAM) towards excisional wound healing on rats.

2. Materials and methods

2.1. Materials

A blank placebo (an aqueous semisolid cream) and intrasite gel (standard wound dressing drug, Smith & Nephew Ltd., UK) were purchased from the pharmacy of University of Malaya Medical Centre. The intrasite gel used as positive control contains modified carboxymethyl cellulose polymer (2.3%), propylene glycol (20%) and water, and promotes natural debridement by rehydrating necrotic tissue. The leaves of *A. muricata* were collected from Ipoh, Malaysia, in March 2013, and was authenticated by Dr. Yong Kien Thai at Institute of Biological Sciences, Faculty of Science Building, University of Malaya. The voucher specimen of the same has been deposited at the herbarium of the University of Malaya (voucher specimen No. KLU47978).

2.2. Preparation of the leaves extract

The leaves (1 kg) were cut into small pieces, washed with distilled water and dried in the oven at 45 °C for 5 days. After grinding the leaves, the leaf powder was soaked in ethyl acetate (1500 ml, three times) in conical flasks for 3 days at 25 °C. The residue was removed by filtration using filter papers (Whatman No. 1). Afterwards, the extract was concentrated by recovering the solvent using a rotator evaporator (Buchi, Flawil, Switzerland). After drying the extract in the vacuum oven at 40 °C, the semisolid mass of EEAM (15 g, ethyl acetate free) was homogeneously mixed with the placebo in two concentrations (5% and 10% w/w) and stored at 2–8 °C in the refrigerator for further evaluation of wound healing activity.

2.3. Animals

Healthy adult male *Sprague Dawley* rats weighing 180–250 g from the animal house of AEU (Animal Experimental Unit, University of Malaya) were used in this study. Rodents were maintained in clean, sterile, polyvinyl cages with normal pellet diet and water *ad libitum*. They were housed under standard environmental conditions of humidity and temperature (25 ± 0.50 °C) with 12 h light/dark cycle. The animal studies were carried out in AEU with due permission from the FOM Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC, ethic No.: 2014-03-05-PHAR/R/SZM).

2.4. Excision wound model

The acute wound healing activity of EEAM in this study was tested on uninfected excisional wounds. The wounding creation was performed under general anaesthesia by using 2 ml of diethyl ether (99% purity, Sigma Chemical Co., St. Louis, MO, USA). After shaving and disinfecting the skin with alcohol (70%), a local anaesthetic injection was performed by lignocaine HCl (1 ml, 2%, 20 mg/ml). After marking an oval wound, a uniform wound (approx. 500 mm^2) with 2 mm depth was excised from the nape of the dorsal neck of each rat using a pair of surgical scissors aseptically (Fig. 1). The neck area of wound was chosen to avoid any unwanted biting and stretching from the rats. Any damage to the muscle layer was carefully avoided and all the procedure was performed with the constant tension of the skin. The forceps and scissors were cleansed with alcohol (70%) after each use.

2.5. Grouping, topical treatment and sampling

Rats were grouped into four groups, viz. (A) vehicle control, (B) low dose (5% w/w), (C) high dose (10% w/w) and (D) positive control group. Each group consisted of six animals, and treatments were started 24 h after the wounding procedure. The wounds of the vehicle control group (group A) were dressed topically twice daily with blank placebo (0.2 ml). With the same procedure, the positive control group (group D) was treated with intrasite gel (0.2 ml) and two doses of EEAM (5% and 10% w/w, 0.2 ml) were used for the treatment of the low dose (group B) and high dose (group C) groups, respectively.

2.6. Determination of the wound closure percentage

The wound closure area (mm^2) of each rat was measured by tracing the wound on days 5, 10 and 15 using a permanent marker and transparent papers under light anaesthesia with ketamine and xylazine. At these days, the percent wound healing values were determined for each group by calculating the percentage of wound reduction from the original wound.

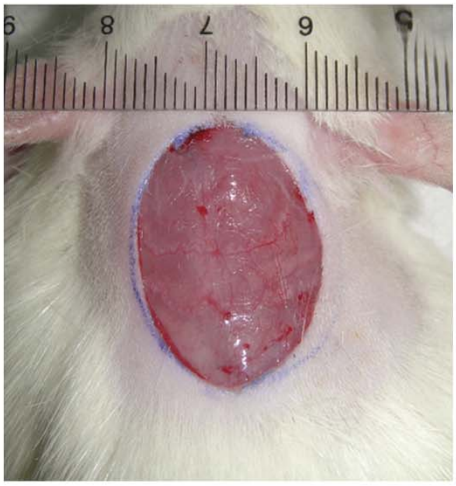

Fig. 1. Excisional skin wound on day 0, before starting treatments. A uniform wound was excised from the nape of the dorsal neck of each rat.

2.7. Histological evaluation of healed wounds

On day 15th, the rats were euthanized using an overdose of ketamine (30 mg/kg, 100 mg/ml) and xylazine (3 mg/kg, 100 mg/ml). The specimens of skin from healed wounds and surrounding tissues were excised from each rat. After processing the tissues in the paraffin tissue-processing machine (Leica Microsystems Incorporated, Nussloch, Germany), 5 μm tissue sections were proceeded with Hematoxylin & Eosin (H & E) and Masson Trichrome staining for microscopic analysis.

2.8. Immunohistochemistry

Immunohistochemical evaluation was performed as previously described in detail [17]. In brief, the EGFR pharmDx kit (DakoCytomation; Carpinteria, CA, USA) was used for immunohistochemical staining according to the vendor's instructions. After blocking the endogenous peroxidase using peroxidase block, the tissue sections were incubated with Hsp70 (1:500, Cat: ab2787, Abcam, Cambridge, MA, USA) biotinylated primary antibody for 15 min. Slides were then incubated with sufficient amounts of streptavidin–HRP for 15 min. Tissue staining was visualized using a DAB substrate chromogen solution. Then, sections were stained with hematoxylin for 5 s. The positive findings of samples were demonstrated with brown color under a light microscope.

2.9. Enzymatic activities

On day 15th after surgery, tissue specimens excised from the wounds were homogenized in 1.15% calcium chloride (1:5 w/v) using a teflon homogenizer (Polytron, Germany). After centrifuging the tissue homogenate at 5000 rpm for 5 min, the supernatant was used for the further assessment on enzymatic activities. To determine the level of glutathione peroxidase (GPx) activity in tissue homogenate, the Lawrence and Burk method was used as previously described [18]. In brief, the reaction mixture contained EDTA (0.2 ml), GSH (0.1 ml, 1 mM), NADPH (0.1 ml, 0.2 mM), phosphate buffer (2 ml, 50 mM, pH 7.0) and sodium azide (0.3 ml, 1 mM). The enzyme solution (0.2 ml) was added to the reaction mixture following incubation for 5 min at 25 °C. Then, the reaction was started by adding H_2O_2 (0.2 ml, 0.25 mM). The optical density presenting the GPx activity was determined at 340 nm. The result is expressed as nmol of NADPH oxidized/min/mg protein. The level of catalase (CAT) and superoxide dismutase (SOD) in tissue homogenate was measured using commercial kits (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.10. Lipid peroxidation

Determination of Lipid peroxidation in wound tissue homogenate was performed using the TBA reaction as previously described in detail [19]. TBA reaction measures the level of malondialdehyde (MDA) as a product of lipid peroxidation. The result is expressed as nmol of MDA formed/mg protein.

2.11. Statistical analysis

All values were reported as means ± SEM of n animals per group. Statistical evaluation of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered statistically significant when $P < 0.05$.

3. Results and discussion

3.1. Effect of EEAM on wound closure

As it is illustrated from Fig. 2, on day 5 of treatments, each group revealed different rates of wound contraction, however intrasite gel and EEAM (only at 10% concentration) caused significant reduction in the wound area, compared to the vehicle control (Fig. 3). After 10 days of topical treatments with EEAM (5% and 10%) and intrasite gel, all three groups showed significant elevation in wound contraction, compared to the vehicle control. Administration of EEAM (5% and 10%) and intrasite gel after 15 days caused 69%, 77% and 81% wound closure, respectively (Fig. 3).

3.2. Histopathological effect of EEAM in wound tissues

Histopathological survey of wound tissues after the surgery on day 15 was carried out using H & E and Masson Trichrome stainings. As illustrated in Fig. 4, tissue sections stained with H & E demonstrated that wound areas in treated rats with EEAM (5% and 10%) and intrasite gel were conspicuously smaller than those treated with the blank placebo. On day 15, EEAM treated rats at both concentrations, especially 10%, demonstrated very close profiles when compared to the positive control group. Dermis maturation and organization of collagen fibers were examined by the Masson Trichrome staining (Fig. 5). In bank placebo treated wounds, collagen fibers were characterized by poor orientation and disorganization. The scattered collections of inflammatory cells, including macrophages and neutrophils, with disorganized-oriented collagen fibers displayed the immature tissue granulation in vehicle control group. However, EEAM treatment markedly stimulated and elevated the deposition of collagen fibers which were comparable with the positive control.

3.3. EEAM induced up-regulation of Hsp70 in wound tissues

In our study, upon treatment of wounds with EEAM (5% and 10%), protein expression of Hsp70 was markedly increased in the wound tissues, which were comparable with Hsp70 up-regulation in the positive control group (Fig. 6).

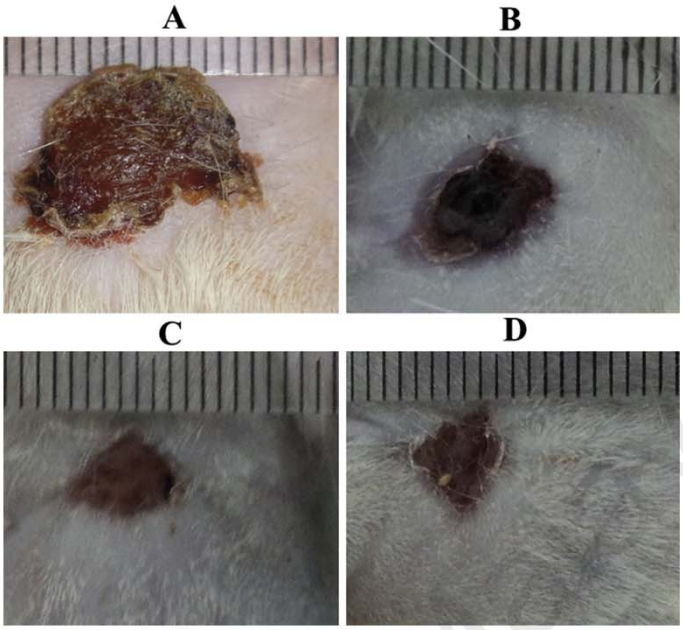

Fig. 2. Gross appearance of the healing of excisional wound in rats at day 15. The rats were topically dressed (0.2 ml) with (A) blank placebo, EEAM at two doses (B) 5%, (C) 10% w/w and (D) intrasite gel. EEAM, ethyl acetate extract of *A. muricata* leaves.

3.4. Effect of EEAM on enzymatic activities in wound tissues

Changes in the SOD activity of the wound tissue of rats after administration of EEAM and intrasite gel are shown in Table 1. Topical treatment with EEAM (5% and 10%) and intrasite gel led to significant elevated activity of the enzyme in the wound tissue compared to the vehicle control. The significant increase in SOD activity after EEAM (5% and 10%) treatment appears to be a

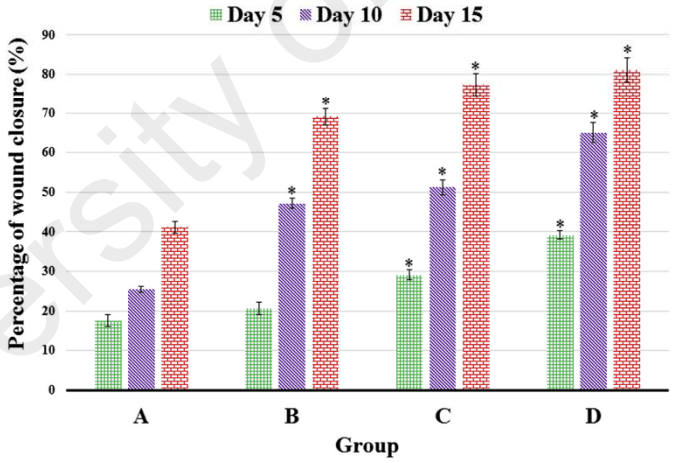

Fig. 3. Effect of topical treatments on percentage of wound closure in four groups. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Data are reported as means ± SEM of six animals per group. A value of $P < 0.05$ was considered significant. EEAM, ethyl acetate extract of *A. muricata* leaves.

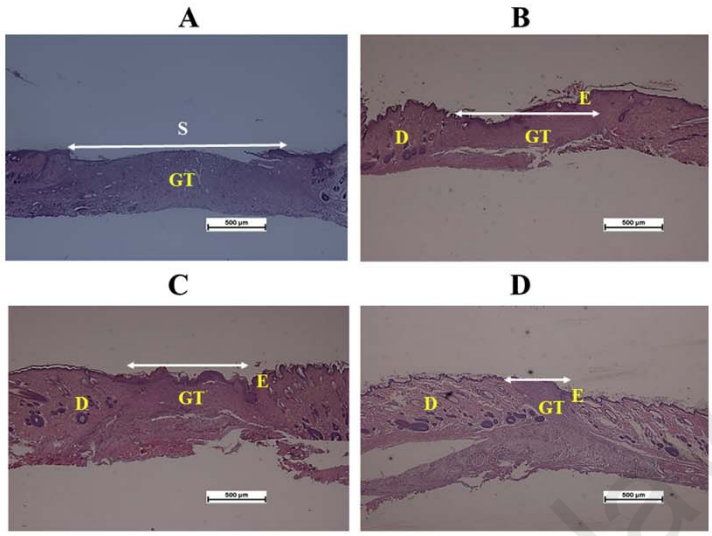

Fig. 4. Histological analysis (H & E) of wound tissues on day 15 after operation, from four groups of rats. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. The white arrows illustrate the wound area for each group. D, dermis; E, epidermis; EEAM, ethyl acetate extract of *A. muricata* leaves; GT, granulation tissue; S: scar width. Scale bar: 500 μm.

protective mechanism to promote the wound healing process in rats. The CAT activity was significantly elevated in the wound tissues of rats treated with EEAM (5% and 10%) and intrasite gel. The increased CAT activity in the wound tissue is a protective response like SOD against oxidative damages to accelerate the wound healing process. It is clearly evident from the data in Table 1 that EEAM treatment at both doses resulted in significant elevation in the GPx activity in the wound tissues compared to the vehicle control.

3.5. Effect of EEAM on MDA level in wound tissues

Levels of MDA in the topically treated rats with EEAM (5% and 10%) and intrasite gel revealed a significant reduction compared to the vehicle control group (Fig. 7). This result strongly suggests that EEAM treatment at both doses markedly attenuated the lipid peroxidation in the wound site of rats.

4. Discussion

A. muricata were previously found to possess chemical constituents of different alkaloids and essential oils [20–22]. Nonetheless, species of the Annonaceae family, including *A. muricata* are well known to have a variety of acetogenins compounds that act as major bioactive compounds in these species [23]. Previous studies have shown that *Annona* species have promising wound healing capability. A study on wound healing activity of *A. muricata* stem bark revealed conspicuous decrease in the wound area of rats after topical treatment with the alcoholic extract of stem bark [24]. In another study, *Annona squamosa* leaves demonstrated a promising wound healing effect against streptozotocin-induced diabetic rats [25]. Wound healing studies generally evaluate two simple and reproducible wound models, namely incisional and excisional [26]. Considering the promising wound healing activity of the *Annona* species, in this study, full thickness excisional wound model was applied to macroscopically and histologically investigate the wound healing potential of EEAM on rats.

The untreated wounds in vehicle control group were characterized with stiff and intact dark brown scabs (Fig. 2). The findings in the current study demonstrated that topical administration of EEAM noticeably accelerated the wound healing process in rats. New thin epidermis formed in EEAM (5% and 10%) treated wounds which provided the protection to the wounds from further injuries by covering the entire wound area (Fig. 4). Epidermis layer of wounds from EEAM (5% and 10%) treated rats was thicker than the wounds from the positive control group. However, the wounds from vehicle control group was just at the early phase of epithelialization. The tissue sections from EEAM (5% and 10%) treated rats elicited a well-advanced organization of granulation tissue and ongoing formation of new blood vessels which were comparable with positive control group. The tissue sections from the EEAM (5% and 10%) treated wounds revealed well-organized accumulation of collagen fibers with fewer inflammatory cells compared to the vehicle control group (Fig. 5). The histological analyses of rats demonstrated that treatment of wound area with EEAM extract at both doses markedly accelerated the original tissue regeneration.

Heat shock proteins (Hsps) are crucial factors for the wound healing process due to their role in cell proliferation, collagen synthesis, modulation of inflammation and wound debris clearance. Despite the abundance of Hsps in cells, their expressions are modulated at the basic levels under normal physiological conditions [27]. Under the wound healing stress, Hsps expression dramatically elevates to attenuate the inflammatory responses and to accelerate the wound healing process [28]. Therefore, any perturbation in the expression and function of Hsps in response to various cellular stress may lead to a wide variety of wound healing complexities [29]. As the abundant inducible Hsp in the wound bed,

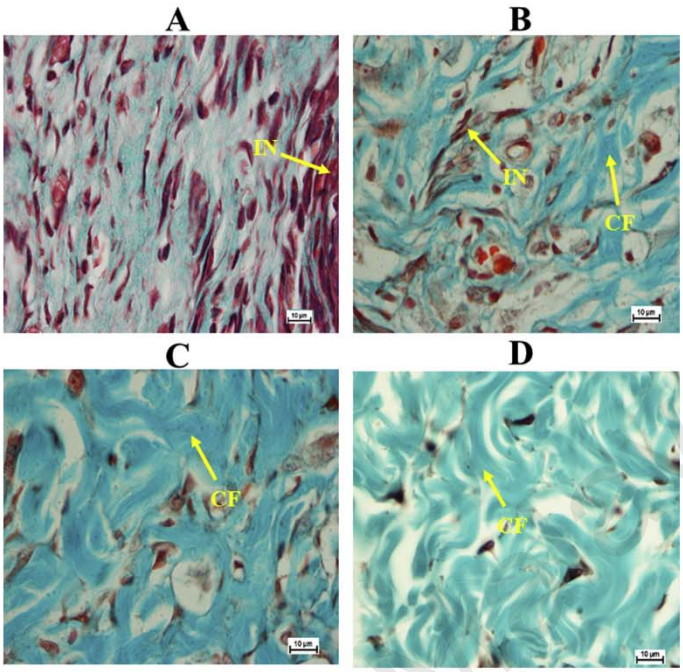

Fig. 5. Histological analysis (Masson Trichrome) of wound tissues on day 15 after operation, from four groups of rats. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. CF, collagen fiber; EEAM, ethyl acetate extract of *A. muricata* leaves; IN: inflammatory cell. Scale bar: 10 μm.

Hsp70 is effectively responsible for the cell survival and protein homeostasis within the healing wound [30]. The immunohistochemistry analysis in our study revealed that the up-regulation of Hsp70 by EEAM was comparable with the effect of intrasite gel. This result strongly implies that EEAM induced the protein expression of Hsp70 in the wound tissue which led to amelioration of the wound healing process.

The process of wound healing is accompanied with skin ischemia, which promotes the generation of reactive oxygen species by activated leukocytes in the tissue site. The more release of oxygen-derived free radicals through the positive feedback attracts more leukocytes and amplifies the oxidative damages in the wound tissue [31]. Under normal conditions, body homeostasis balances the level of free radicals using the endogenous antioxidant capacity of the human body. However, when this level exceeds the normal capacity of antioxidants to balance, highly activated radicals will cause different structural changes and further reversible or irreversible cell injuries [32,33]. Antioxidant defence systems of cells contains a variety of enzymatic and non-enzymatic scavengers. The enzymatic antioxidants of cells, including CAT, GPx, glutathione reductase (GR), glutathione-s-transferase (GST), SOD play a critical role in the attenuation of oxidative stress induced by reactive oxygen species [34]. The first defensive mechanism against reactive oxygen species is provided by SOD, which attenuates oxidative stress through dismutation of $O_2^{\cdot}$. CAT enzyme has an important role in converting the endogenous H_2O_2 to water and oxygen [35]. The accumulation of H_2O_2 in cells results in the generation of highly reactive free hydroxyl radical ($OH^{\cdot}$) through Fenton reaction, which has an important devastating role in oxidative damages [36,37]. Another important antioxidant enzyme, GPx, degrades lipid peroxides to hydroxyl lipids and waters through conversion of glutathione to glutathione disulfide [38].

The excessive production of reactive oxygen species and oxidative stress in the wound site is known to cause lipid peroxidation in the respective tissue [39,40]. Lipid peroxidation of organelle and cellular membranes, being one of the destructive effects of oxygen radicals, is responsible for the defect in endothelial cells, fibroblast and collagen metabolism and keratinocyte capillary permeability. In addition, the elevated lipid peroxidation in the wound tissue may have been a contributing factor in the impairment of vascular endothelial growth factor (VEGF) expression and subsequently deficiency of the wound healing process [41]. As the main oxidation product of peroxidized polyunsaturated fatty acids, MDA is a critical biomarker for lipid peroxidation [42].

Previous *in vitro* and *in vivo* studies also reported the antioxidant activity of *A. muricata* leaves [43,44]. Among different *Annona* species, leaves of *A. muricata* were found to have the highest antioxidant activity assessed by DRSA (DPPH radical scavenging activity), FRAP (Ferric reducing antioxidant property) and HRSA (hydroxyl scavenging activity) techniques [44]. An *in vivo* study also showed that ethyl acetate extract of *A. muricata* leaves caused an increase in the activity of CAT, glutathione and SOD on gastric cells

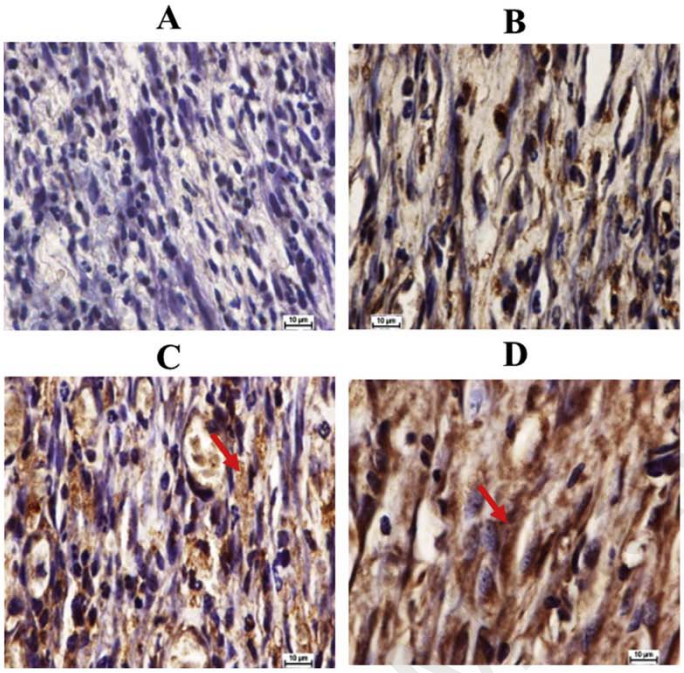

Fig. 6. Immunohistochemical analysis of Hsp70 protein expression from four groups of rats. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Immunohistochemistry staining showed up-regulation of Hsp70 in groups B–D. The red arrows depict Hsp70 protein accumulation in gastric tissue. EEAM, ethyl acetate extract of *A. muricata* leaves. Scale bar: 10 μm.

Table 1
Effect of ointment treatment on antioxidants activities.

Group	CAT (nM/min/ml)	GPx (nM/min/mg)	SOD (U/mg protein)
A	85.45 – 3.78	16.19 + 1.34	14.25 – 1.15
B	115.7 – 1.29*	23.19 + 0.94*	19.25 – 0.73*
C	131.29 – 2.44*	29.25 ± 1.4*	27.25 ± 1.13*
D	146.11 – 4.17*	31.67 + 2.02*	36.78 – 2.6*

Antioxidant enzymes include CAT, SOD and GPx from four groups of rats namely, (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Data are reported as means ± SEM of six animals per group. A value of $P < 0.05$ was considered significanL CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase.

of ethanol-treated rats [45]. Our findings in the present study strongly suggested that topical administration of EEAM on wounds of rats conspicuously increased the antioxidant capacity of the wound tissue leading to hastening of the wound healing process.

5. Conclusion

In conclusion, these findings showed that EEAM accelerates various stages of wound healing, including wound contraction, epithelialization and collagen synthesis. In addition, it also decreased the oxidative and inflammatory stresses in the wound area. Treated wounds with EEAM displayed organized generation of collagen fibers with reduced number of inflammatory cells. However, further study with bio-assay guided approach is still required to establish the principle bioactive compound responsible for the wound healing effect of EEAM.

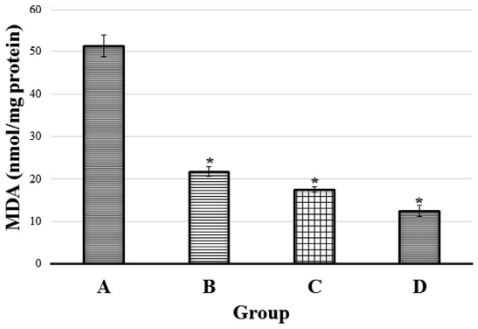

Fig. 7. Effect of ointment treatments on MDA level in wound tissue homogenate from four groups. Four groups of rats include (A) vehicle control, (B) low dose of EEAM, (C) high dose of EEAM and (D) positive control. Data are reported as means ± SEM of six animals per group. A value of $P < 0.05$ was considered significant. EEAM, ethyl acetate extract of *A. muricata* leaves.

Ethical approval

The animal studies were carried out in AEU (Animal Experimental Unit, University of Malaya) with due permission from the FOM Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC, ethic No.: 2014-03-05-PHAR/R/SZM).

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Author contribution

Soheil Zorofchian Moghadamtousi, Mahmood Ameen Abdulla and Habsah Abdul Kadir conceived and designed the study. Soheil Zorofchian Moghadamtousi and Elham Rouhollahi performed the experiments. Maryam Hajrezaie and Hamed Karimian analysed the data. Mahmood Ameen Abdulla and Habsah Abdul Kadir contributed reagents/materials/analysis tools. Soheil Zorofchian Moghadamtousi wrote the manuscript.

Disclosure

The authors declare that they have no conflict of interests.

Guarantor

Mahmood Ameen Abdulla and Habsah Abdul Kadir accept full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

References

[1] S.Y. Asadi, P. Parsaei, M. Karimi, S. Ezzati, A. Zamiri, F. Mohammadizadeh, M. Rafieian-Kopaei, Effect of green tea (*Camellia sinensis*) extract on healing process of surgical wounds in rat, Int. J. Surg. 11 (2013) 332–337.
[2] B. Vidinský, P. Gál, T. Toporcer, F. Longauer, L. Lenhardt, N. Bobrov, J. Sabo, Histological study of the first seven days of skin wound healing in rats, Acta Vet. Brno 75 (2006) 197–202.
[3] J.-P. Gouin, J.K. Kiecolt-Glaser, The impact of psychological stress on wound healing: methods and mechanisms, Immunol. Allergy Clin. 31 (2011) 81–93.
[4] V. Falanga, Wound healing and its impairment in the diabetic foot, Lancet 366 (2005) 1736–1743.
[5] P.G. Bowler, Wound pathophysiology, infection and therapeutic options, Ann. Med. 34 (2002) 419–427.
[6] R.F. Diegelmann, M.C. Evans, Wound healing: an overview of acute, fibrotic and delayed healing, Front. Biosci. 9 (2004) 283–289.
[7] D.D. Kokane, R.Y. More, M.B. Kale, M.N. Nehete, P.C. Mehendale, C.H. Gadgoli, Evaluation of wound healing activity of root of *Mimosa pudica*, J. Ethnopharmacol. 124 (2009) 311–315.
[8] B. Kumar, M. Vijayakumar, R. Govindarajan, P. Pushpangadan, Ethnopharmacological approaches to wound healing—exploring medicinal plants of India, J. Ethnopharmacol. 114 (2007) 103–113.
[9] S.C. Davis, R. Perez, Cosmeceuticals and natural products: wound healing, Clin. Dermatol. 27 (2009) 502–506.
[10] S. Zorofchian Moghadamtousi, H. Abdul Kadir, P. Hassandarvish, H. Tajik, S. Abubakar, K. Zandi, A review on antibacterial, antiviral, and antifungal activity of curcumin, Biomed. Res. Int. (2014), http://dx.doi.org/10.1155/2014/186864.
[11] R. Raina, S. Parwez, P. Verma, N. Pankaj, Medicinal plants and their role in wound healing, Online Vet. J. 3 (2008) 21.
[12] S.Z. Moghadamtousi, H.A. Kadir, M. Paydar, E. Rouhollahi, H. Karimian, *Annona muricata* leaves induced apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF-kappaB, BMC Complement. Altern. Med. 14 (2014) 299.
[13] C. Gleye, A. Laurens, R. Hocquemiller, O. Laprévote, L. Serani, A. Cavé, Cohibins A and B, acetogenins from roots of *Annona muricata*, Phytochemistry 44 (1997) 1541–1545.
[14] S.Z. Moghadamtousi, H.A. Kadir, H. Karimian, E. Rouhollahi, M. Paydar, M. Fadaeinasab, *Annona muricata* leaves induce G_1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-29 colon Cancer cells, J. Ethnopharmacol. 156 (2014) 277–289.
[15] S. Adewole, J. Ojewole, Protective effects of *Annona muricata* Linn. (Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats, Afr. J. Traditional. Cam 6 (2009) 31–40.
[16] O.V. De Sousa, G.D.-V. Vieira, J.d.J.R. De Pinho, C.H. Yamamoto, M.S. Alves, Antinociceptive and anti-inflammatory activities of the ethanol extract of *Annona muricata* L. leaves in animal models, Int. J. Mol. Sci. 11 (2010) 2067–2078.
[17] D.A. Bardi, M.F. Halabi, P. Hassandarvish, E. Rouhollahi, M. Paydar, S.Z. Moghadamtousi, N.S. Al-Wajeeh, A. Ablat, N.A. Abdullah, M.A. Abdulla, *Andrographis paniculata* leaf extract prevents thioacetamide-induced liver cirrhosis in rats, PloS One 9 (2014) e91246.
[18] R.A. Lawrence, R.F. Burk, Glutathione peroxidase activity in selenium-deficient rat liver, Biochem. Biophys. Res. Commun. 71 (1976) 952–958.
[19] E. Wills, Mechanisms of lipid peroxide formation in animal tissues, Biochem. J. 99 (1966) 667–676.
[20] M. Leboeuf, A. Cavé, P. Bhaumik, B. Mukherjee, R. Mukherjee, The phytochemistry of the Annonaceae, Phytochemistry 21 (1980) 2783–2813.
[21] M. Leboeuf, C. Legueut, A. Cavé, J. Desconclois, P. Forgacs, H. Jacquemin, Alkaloids of Annonaceae. XXIX. Alkaloids of *Annona muricata* L, Planta Med. 42 (1981) 37–44.
[22] C. Kossouoh, M. Moudachirou, V. Adjakidje, J.-C. Chalchat, G. Figuérédo, Essential oil chemical composition of *Annona muricata* L. leaves from Benin, J. Essent. Oil Res. 19 (2007) 307–309.
[23] J.K. Rupprecht, Y.-H. Hui, J.L. McLaughlin, Annonaceous acetogenins: a review, J. Nat. Prod. 53 (1990) 237–278.
[24] P.M. Paarakh, J. Chansouria, R. Khosa, Wound healing activity of *Annona muricata* extract, J. Pharm. Res. 2 (2009) 404–406.
[25] T. Ponrasu, L. Suguna, Efficacy of *Annona squamosa* on wound healing in streptozotocin-induced diabetic rats, Int. Wound J. 9 (2012) 613–623.
[26] P. Tamri, A. Hemmati, M.G. Boroujerdnia, Wound healing properties of quince seed mucilage: *In vivo* evaluation in rabbit full-thickness wound model, Int. J. Surg. 12 (2014) 843–847.
[27] M. Atalay, N. Oksala, J. Lappalainen, D.E. Laaksonen, C.K. Sen, S. Roy, Heat shock proteins in diabetes and wound healing, Curr. Protein Pept. Sci. 10 (2009) 85–95.
[28] S. Bruemmer-Smith, F. Stüber, S. Schroeder, Protective functions of intracellular heat-shock protein (HSP) 70-expression in patients with severe sepsis, Intens. Care Med. 27 (2001) 1835–1841.
[29] Y. Chen, T.S. Voegeli, P.P. Liu, E.G. Noble, R.W. Currie, Heat shock paradox and a new role of heat shock proteins and their receptors as anti-inflammation targets, Inflamm. Allergy Drug Targets 6 (2007) 91–100.
[30] M. Wagstaff, M. Shah, D. McGrouther, D. Latchman, The heat shock proteins and plastic surgery, J. Plast. Reconstr. Aesthet. 60 (2007) 974–982.
[31] D.R. Bickers, M. Athar, Oxidative stress in the pathogenesis of skin disease, J. Investig. Dermatol. 126 (2006) 2565–2575.
[32] M. Valko, D. Leibfritz, J. Moncol, M.T. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell B 39 (2007) 44–84.
[33] A. Soneja, M. Drews, T. Malinski, Role of nitric oxide, nitroxidative and oxidative stress in wound healing, Pharmacol. Rep. 57 (2005) 108–119.
[34] M. Singh, R. Sandhir, R. Kiran, Effects on antioxidant status of liver following atrazine exposure and its attenuation by vitamin E, Exp. Toxicol. Pathol. 63 (2011) 269–276.
[35] M. Naziroglu, Molecular role of catalase on oxidative stress-induced Ca2+ signaling and TRP cation channel activation in nervous system, J. Recept. Sig. Transduct. 32 (2012) 134–141.
[36] S. Park, J.A. Imlay, High levels of intracellular cysteine promote oxidative DNA damage by driving the fenton reaction, J. Bacteriol. 185 (2003) 1942–1950.
[37] J. Bai, A.I. Cederbaum, Mitochondrial catalase and oxidative injury, Neurosignals 10 (2001) 189–199.
[38] O. Blokhina, E. Virolainen, K.V. Fagerstedt, Antioxidants, oxidative damage and oxygen deprivation stress: a review, Ann. Bot. London 91 (2003) 179–194.
[39] R. Muralikrishna Adibhatla, J. Hatcher, Phospholipase A_2, reactive oxygen species, and lipid peroxidation in cerebral ischemia, Free Radic. Biol. Med. 40 (2006) 376–387.
[40] D. Gopinath, M.R. Ahmed, K. Gomathi, K. Chitra, P. Sehgal, R. Jayakumar, Dermal wound healing processes with curcumin incorporated collagen films, Biomaterials 25 (2004) 1911–1917.
[41] D. Altavilla, A. Saitta, D. Cucinotta, M. Galeano, B. Deodato, M. Colonna, V. Torre, G. Russo, A. Sardella, G. Urna, Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angiogenesis in the genetically diabetic mouse, Diabetes 50 (2001) 667–674.
[42] A. Elia, W. Waller, S. Norton, Biochemical responses of bluegill sunfish (Lepomis macrochirus, Rafinesque) to atrazine induced oxidative stress, Bull. Environ. Contam. Toxicol. 68 (2002) 809–816.
[43] S.O. Adewole, E.A. Caxton-Martins, Morphological changes and hypoglycemic effects of *Annona muricata* linn. (annonaceae) leaf aqueous extract on pancreatic β-cells of streptozotocin-treated diabetic rats, Afr. J. Biomed. Res. 9 (2006) 480–485.
[44] R. Baskar, V. Rajeswari, T.S. Kumar, In vitro antioxidant studies in leaves of *Annona* species, Indian J. Exp. Biol. 45 (2007) 480–485.
[45] S.Z. Moghadamtousi, E. Rouhollahi, H. Karimian, M. Fadaeinasab, M.A. Abdullah, H.A. Kadir, Gastroprotective activity of *Annona muricata* leaves against ethanol-induced gastric injury in rats via Hsp70/Bax involvement, Drug Des. Dev. Ther. 8 (2014) 2099–2111.

CHAPTER 4: CONCLUSION

In summary, these results from the current investigation have yielded insight into the chemopreventive potential of *Annona muricata*. The apoptosis-inducing effect of *A. muricata* on three different cancer cell lines and subsequent bio-assay-guided investigation on AMEAE have resulted in the isolation of annomuricin E. Annomuricin E was demonstrated to possess cytotoxic effects towards HT-29 cells. The exposure of annomuricin E to HT-29 cells triggered mitochondrial-mediated events and altered the balance of the mitochondrial membrane potential in favor of cytochrome *c* release through the elevation of Bax expression and the attenuation of Bcl-2 expression. These changes were accompanied by G_1 cell cycle arrest in the treated cells. An *in vivo* study on azoxymethane-induced colonic aberrant crypt foci in rats also revealed similar promising chemopreventive effects. The decrease in the number of aberrant crypt foci and PCNA expression associated with changes in Bax and Bcl-2 expression levels strongly substantiated the *in vitro* results.

The *in vivo* gastroprotective potential of AMEAE was tested against ethanol-induced gastric mucosal injury in rats. Our findings showed a promising antiulcer effect of the plant extract. Hsp70 protein expression was down-regulated after the oral administration of AMEAE, which was associated with elevation in the activities of enzymatic antioxidants. The gastroprotective potential of *A. muricata* leaves is strongly attributed to the anti-inflammatory activity of AMEAE and inhibitory effect against oxidative damage, leading to the preservation of the gastric wall mucus.

Wound healing studies of AMEAE have revealed that the plant extract accelerates different stages of wound healing, such as collagen synthesis, epithelialization and wound contraction. The topical application of AMEAE to the wounds led to the attenuation of inflammatory cells and the arranged formation of collagen fibers. The wound healing

effect of AMEAE is probably mediated through the anti-inflammatory and anti-oxidative activities of *A. muricata* leaves.

In addition to being an important source for the food industry and an indigenous medicinal plant, *A. muricata* has been proven to possess a wide spectrum of biological activities. Because the majority of previous studies were focused on the biological activities of the plant extract, further investigations on the biochemical and physiological functions of active compounds and the more detailed mechanisms underlying these activities are pivotal for the development of nutraceutical products.

REFERENCES

Adams, P. D. (2001). Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdks. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1471*(3), M123-M133.

Adewole, S., & Ojewole, J. (2009). Protective effects of *Annona muricata* Linn.(Annonaceae) leaf aqueous extract on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats. *African Journal of Traditional, Complementary and Alternative Medicines, 6*(1), 30-41.

Adewole, S. O., & Caxton-Martins, E. A. (2006). Morphological changes and hypoglycemic effects of *Annona muricata* linn.(annonaceae) leaf aqueous extract on pancreatic β-cells of streptozotocin-treated diabetic rats. *African Journal of Biomedical Research, 9*(3), 173 - 187.

Adeyemi, D. O., Komolafe, O. A., Adewole, O. S., Obuotor, E. M., & Adenowo, T. K. (2009). Anti hyperglycemic activities of *Annona muricata* (Linn). *African Journal of Traditional, Complementary, and Alternative Medicines, 6*(1), 62-69.

Alali, F. Q., Liu, X.-X., & McLaughlin, J. L. (1999). Annonaceous acetogenins: recent progress. *Journal of Natural Products, 62*(3), 504-540.

Arroyo, J., Prashad, M., Vásquez, Y., Li, E., & Tomás, G. (2005). Actividad citotóxica in vitro de la mezcla de Annona muricata y Krameria lappacea sobre células cancerosas de glándula mamaria, pulmón y sistema nervioso central. *Revista Peruana de Medicina Experimental y Salud Pública, 22*(4), 247-253.

Arthur, F. K., Terlabi, E. O., Larbie, C., & Woode, E. (2012). Evaluation of hepatoprotective effect of aqueous extract of *Annona muricata* (Linn.) leaf against carbon tetrachloride andacetaminophen-induced liver damage. *Journal of Natural Pharmaceuticals, 3*(25-30).

Arur, S., Uche, U. E., Rezaul, K., Fong, M., Scranton, V., Cowan, A. E., . . . Han, D. K. (2003). Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Developmental Cell, 4*(4), 587-598.

Asare, G. A., Afriyie, D., Ngala, R. A., Abutiate, H., Doku, D., Mahmood, S. A., & Rahman, H. (2015). Antiproliferative Activity of Aqueous Leaf Extract of Annona muricata L. on the Prostate, BPH-1 Cells, and Some Target Genes. *Integrative Cancer Therapies, 14*(1), 65-74.

Ashkenazi, A. (2008). Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. *Nature Reviews Drug Discovery, 7*(12), 1001-1012.

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science, 281*(5381), 1305-1308.

Astirin, O. P., Artanti, A. N., Fitria, M. S., Perwitasari, E. A., & Prayitno, A. (2013). *Annonaa muricata* Linn Leaf Induce Apoptosis in Cancer Cause Virus. *Journal of Cancer Therapy, 4*(07), 1244-1250.

Atawodi, S. E. (2011). Nigerian foodstuffs with prostate cancer chemopreventive polyphenols. *Infectious Agents and Cancer, 6*(Suppl 2), S9.

Ayyanar, M., & Ignacimuthu, S. (2009). Herbal medicines for wound healing among tribal people in Southern India: Ethnobotanical and Scientific evidences. *International Journal of Applied Research in Natural Products, 2*(3), 29-42.

Backert, S., Ziska, E., Brinkmann, V., Zimny-Arndt, U., Fauconnier, A., Jungblut, P. R., . . . Meyer, T. F. (2000). Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cellular Microbiology, 2*(2), 155-164.

Bai, L., & Zhu, W.-G. (2006). p53: structure, function and therapeutic applications. *Journal of Cancer Molecules, 2*(4), 141-153.

Balkwill, F. (2006). TNF-α in promotion and progression of cancer. *Cancer and Metastasis Reviews, 25*(3), 409-416.

Baskar, R., Rajeswari, V., & Kumar, T. S. (2007). *In vitro* antioxidant studies in leaves of *Annona* species. *Indian Journal of Experimental Biology, 45*(5), 480-485.

Becker, W. M., Kleinsmith, L. J., Hardin, J., & Raasch, J. (2003). *The World of the cell* (Vol. 6): Benjamin Cummings San Francisco.

Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A., & Henson, P. M. (1997). Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *Journal of Biological Chemistry, 272*(42), 26159-26165.

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E., & Boise, L. H. (2013). Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biology, 14*(1), 32.

Brumatti, G., Sheridan, C., & Martin, S. J. (2008). Expression and purification of recombinant annexin V for the detection of membrane alterations on apoptotic cells. *Methods, 44*(3), 235-240.

Campos, A. C., Groth, A. K., & Branco, A. B. (2008). Assessment and nutritional aspects of wound healing. *Current Opinion in Clinical Nutrition & Metabolic Care, 11*(3), 281-288.

Carmen Zafra-Polo, M., Figadère, B., Gallardo, T., Tormo, J., & Cortes, D. (1998). Natural acetogenins from annonaceae, synthesis and mechanisms of action. *Phytochemistry, 48*(7), 1087-1117.

Champy, P., Höglinger, G. U., Féger, J., Gleye, C., Hocquemiller, R., Laurens, A., . . . Lombès, A. (2004). Annonacin, a lipophilic inhibitor of mitochondrial complex I, induces nigral and striatal neurodegeneration in rats: possible relevance for atypical parkinsonism in Guadeloupe. *Journal of Neurochemistry, 88*(1), 63-69.

Chan, F. K., To, K., Wu, J. C., Yung, M., Leung, W., Kwok, T., . . . Hui, E. (2002). Eradication of *Helicobacter pylori* and risk of peptic ulcers in patients starting long-term treatment with non-steroidal anti-inflammatory drugs: a randomised trial. *The Lancet, 359*(9300), 9-13.

Chan, P., Ah, R., & Mh, K. (2010). Anti-arthritic activities of *Annona muricata* L. leaves extract on complete Freund's adjuvant (CFA)–induced arthritis in rats. *Planta Medica, 76*(12), P166.

Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews, 59*(3), 527-605.

Chang, F.-R., Liaw, C.-C., Lin, C.-Y., Chou, C.-J., Chiu, H.-F., & Wu, Y.-C. (2003). New adjacent Bis-tetrahydrofuran Annonaceous acetogenins from *Annona muricata*. *Planta Medica, 69*(3), 241-246.

Chang, F.-R., & Wu, Y.-C. (2001). Novel Cytotoxic Annonaceous Acetogenins from *Annona muricata*. *Journal of Natural Products, 64*(7), 925-931.

Chao-Ming, L., Ning-Hua, T., Hui-Lan, Z., Qing, M., Xiao-Jiang, H., Yi-Neng, H., & Jun, Z. (1998). Cyclopeptide from the seeds of *Annona muricata*. *Phytochemistry, 48*(3), 555-556.

Chen, W., Weng, Y.-M., & Tseng, C.-Y. (2003). Antioxidative and antimutagenic activities of healthy herbal drinks from Chinese medicinal herbs. *The American journal of Chinese Medicine, 31*(04), 523-532.

Chen, Y.-N. P., Sharma, S. K., Ramsey, T. M., Jiang, L., Martin, M. S., Baker, K., . . . Kaelin, W. G. (1999). Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. *Proceedings of the National Academy of Sciences, 96*(8), 4325-4329.

Chih, H.-W., Chiu, H.-F., Tang, K.-S., Chang, F.-R., & Wu, Y.-C. (2001). Bullatacin, a potent antitumor annonaceous acetogenin, inhibits proliferation of human hepatocarcinoma cell line 2.2. 15 by apoptosis induction. *Life Sciences, 69*(11), 1321-1331.

Church, R., Fleshman, J., & McLeod, H. (2003). Cyclo-oxygenase 2 inhibition in colorectal cancer therapy. *British Journal of Surgery, 90*(9), 1055-1067.

Cortes, D., Myint, S. H., Laurens, A., Hocquemiller, R., Leboeuf, M., & Cavé, A. (1991). Corossolone et corossoline, deux nouvelles γ-lactones mono-tétrahydrofuraniques cytotoxiques. *Canadian Journal of Chemistry, 69*(1), 8-11.

Cory, S., & Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews Cancer, 2*(9), 647-656.

Cragg, G. M., & Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology, 100*(1), 72-79.

Danial, N. N., & Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell, 116*(2), 205-219.

Davis, S. C., & Bouzari, N. (2004). Development of antimicrobials for wound care: *in-vitro* and *in-vivo* assessments. *Wounds, 16*(11), 344-347.

Davis, S. C., & Perez, R. (2009). Cosmeceuticals and natural products: wound healing. *Clinics in Dermatology, 27*(5), 502-506.

De Sousa, O. V., Vieira, G. D.-V., De Pinho, J. d. J. R., Yamamoto, C. H., & Alves, M. S. (2010). Antinociceptive and anti-inflammatory activities of the ethanol extract of Annona muricata L. leaves in animal models. *International Journal of Molecular Sciences, 11*(5), 2067-2078.

de Souza, R., Benassi, E., da Silva, R. R., Afonso, S., & Scarminio, I. S. (2009). Enhanced extraction yields and mobile phase separations by solvent mixtures for the analysis of metabolites in *Annona muricata* L. leaves. *Journal of Separation Science, 32*, 4176-4185.

De Yu, L., YU, J. G., Xiu Zhen, L., Lan, S., & YANG, S. L. (2000). Muricatenol, a linear acetogenin from *Annona muricata* (Annonaceae). *Chinese Chemical Letters, 11*(3), 239-242.

Deveraux, Q. L., Roy, N., Stennicke, H. R., Van Arsdale, T., Zhou, Q., Srinivasula, S. M., . . . Reed, J. C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *The EMBO Journal, 17*(8), 2215-2223.

Dolcet, X., Llobet, D., Pallares, J., & Matias-Guiu, X. (2005). NF-kB in development and progression of human cancer. *Virchows Archiv, 446*(5), 475-482.

Dos Santos, A., & Sant'Ana, A. (2001). Molluscicidal properties of some species of *Annona*. *Phytomedicine, 8*(2), 115-120.

Dulak, J. (2005). Nutraceuticals as anti-angiogenic agents: hopes and reality. *Journal of Physiology and Pharmacology. Supplement, 56*(1), 51-69.

Duraipandiyan, V., Ayyanar, M., & Ignacimuthu, S. (2006). Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. *BMC Complementary and Alternative Medicine, 6*(1), 35.

Edamatsu, H., Gau, C.-L., Nemoto, T., Guo, L., & Tamanoi, F. (2000). Cdk inhibitors, roscovitine and olomoucine, synergize with farnesyltransferase inhibitor (FTI) to induce efficient apoptosis of human cancer cell lines. *Oncogene, 19*(27), 3059-3068.

Eggadi, V., Gundamedi, S., Sheshagiri, S. B. B., Revoori, S. K., Jupally, V. R., & Kulandaivelu, U. (2014). Evaluation of Anticancer Activity of *Annona muricata* in 1, 2-Dimethyl Hydrazine Induced Colon Cancer. *World Applied Sciences Journal, 32*(3), 444-450.

Ekert, P. G., & Vaux, D. L. (2005). The mitochondrial death squad: hardened killers or innocent bystanders? *Current Opinion in Cell Biology, 17*(6), 626-630.

Elisya, Y., Kardono, L. B., & Simanjuntak, P. (2014). Tablet Formulation of The Ethyl Acetate Soluble Extract of Soursop (*Annona muricata* L.) Leaves. *Asian Journal of Applied Sciences, 2*(3), 323-329.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology, 35*(4), 495-516.

Escobar-Khondiker, M., Höllerhage, M., Muriel, M.-P., Champy, P., Bach, A., Depienne, C., . . . Yagi, T. (2007). Annonacin, a natural mitochondrial complex I inhibitor, causes tau pathology in cultured neurons. *The Journal of Neuroscience, 27*(29), 7827-7837.

Ezirim, A., Okachi, V., James, A., Adebeshi, O., Ogunnowo, S., & Odeghe, O. (2013). Induction of apoptosis in myelogenous leukemic K562 cells by ethanolic leaf extract of *Annona muricata*. *Indian Journal of Drugs and Diseases, 2*(3), 142-151.

Fadok, V. A., de Cathelineau, A., Daleke, D. L., Henson, P. M., & Bratton, D. L. (2001). Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *Journal of Biological Chemistry, 276*(2), 1071-1077.

Fekam Boyom, F., Amvam Zollo, P., Menut, C., Lamaty, G., & Bessière, J. (1996). Aromatic plants of tropical Central Africa. Part XXVII. Comparative study of the volatile constituents of five Annonaceae species growing in Cameroon. *Flavour and Fragrance Journal, 11*(6), 333-338.

Fellenius, E., Berglindh, T., Sachs, G., Olbe, L., Elander, B., Sjöstrand, S.-E., & Wallmark, B. (1981). Substituted benzimidazoles inhibit gastric acid secretion by blocking ($H^+ + K^+$) ATPase. *Nature, 290*, 159–161.

Fink, S. L., & Cookson, B. T. (2005). Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and Immunity, 73*(4), 1907-1916.

Fiorucci, S., Santucci, L., & Distrutti, E. (2007). NSAIDs, coxibs, CINOD and H 2 S-releasing NSAIDs: what lies beyond the horizon. *Digestive and Liver Disease, 39*(12), 1043-1051.

Fofana, S., Ziyaev, R., Abdusamatov, A., & Zakirov, S. K. (2011). Alkaloids from *Annona muricata* leaves. *Chemistry of Natural Compounds, 47*(2), 321-321.

Ford, A. C., Delaney, B. C., Forman, D., & Moayyedi, P. (2004). Eradication therapy in *Helicobacter pylori* positive peptic ulcer disease: systematic review and economic analysis. *The American Journal of Gastroenterology, 99*(9), 1833-1855.

Friedl, P., & Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature Reviews Cancer, 3*(5), 362-374.

Fulda, S. (2010). Evasion of apoptosis as a cellular stress response in cancer. *International Journal of Cell Biology, 2010*, doi: 10.1155/2010/370835.

Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., . . . Henson, P. M. (2005). Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell, 123*(2), 321-334.

Gavamukulya, Y., Abou-Elella, F., Wamunyokoli, F., & AEl-Shemy, H. (2014). Phytochemical screening, anti-oxidant activity and *in vitro* anticancer potential of ethanolic and water leaves extracts of *Annona muricata* (Graviola). *Asian Pacific Journal of Tropical Medicine, 7*, S355-S363.

Gawronska-Kozak, B., Bogacki, M., Rim, J. S., Monroe, W. T., & Manuel, J. A. (2006). Scarless skin repair in immunodeficient mice. *Wound Repair and Regeneration, 14*(3), 265-276.

George, V. C., Kumar, D., Rajkumar, V., Suresh, P., & Kumar, R. A. (2012). Quantitative assessment of the relative antineoplastic potential of the n-butanolic leaf extract of *Annona muricata* Linn. in normal and immortalized human cell lines. *Asian Pacific Journal of Cancer Prevention, 13*(2), 699-704.

George, V. C., Kumar, D. N., Suresh, P., & Kumar, R. A. (2014). Antioxidant, DNA protective efficacy and HPLC analysis of *Annona muricata* (soursop) extracts. *Journal of Food Science and Technology*, 1-8.

Gieseler, F., Rudolph, P., Kloeppel, G., & Foelsch, U. (2003). Resistance mechanisms of gastrointestinal cancers: why does conventional chemotherapy fail? *International Journal of Colorectal Disease, 18*(6), 470-480.

Gisbert, J., Gonzalez, L., Calvet, X., Roque, M., Gabriel, R., & Pajares, J. (2001). Proton pump inhibitors versus H2-antagonists: a meta-analysis of their efficacy in treating bleeding peptic ulcer. *Alimentary Pharmacology & Therapeutics, 15*(7), 917-926.

Gisbert, J. P., & Pajares, J. M. (2003). Helicobacter pylori infection and perforated peptic ulcer prevalence of the infection and role of antimicrobial treatment. *Helicobacter, 8*(3), 159-167.

Gleye, C., Akendengue, B., Laurens, A., & Hocquemiller, R. (2001). Coronin from Roots of *Annona muricata*, a Putative Intermediate in Acetogenin Biosynthesis (1). *Planta Medica, 67*(06), 570-572.

Gleye, C., Duret, P., Laurens, A., Hocquemiller, R., & Cavé, A. (1998). cis-Monotetrahydrofuran Acetogenins from the Roots of *Annona muricata* 1. *Journal of Natural Products, 61*(5), 576-579.

Gleye, C., Laurens, A., Hocquemiller, R., Cavé, A., Laprévote, O., & Serani, L. (1997). Isolation of montecristin, a key metabolite in biogenesis of acetogenins from *Annona muricata* and its structure elucidation by using tandem mass spectrometry. *The Journal of Organic Chemistry, 62*(3), 510-513.

Gleye, C., Laurens, A., Hocquemiller, R., Laprévote, O., Serani, L., & Cavé, A. (1997). Cohibins A and B, acetogenins from roots of *Annona muricata*. *Phytochemistry, 44*(8), 1541-1545.

Gleye, C., Laurens, A., Laprévote, O., Serani, L., & Hocquemiller, R. (1999). Isolation and structure elucidation of sabadelin, an acetogenin from roots of *Annona muricata*. *Phytochemistry, 52*(8), 1403-1408.

Gleye, C., Raynaud, S., Fourneau, C., Laurens, A., Laprévote, O., Serani, L., . . . Hocquemiller, R. (2000). Cohibins C and D, Two Important Metabolites in the Biogenesis of Acetogenins from *Annona muricata* and *Annona nutans*. *Journal of Natural Products, 63*(9), 1192-1196.

Gleye, C., Raynaud, S., Hocquemiller, R., Laurens, A., Fourneau, C., Serani, L., . . . Fournet, A. (1998). Muricadienin, muridienins and chatenaytrienins, the early precursors of annonaceous acetogenins. *Phytochemistry, 47*(5), 749-754.

Gosain, A., & DiPietro, L. A. (2004). Aging and wound healing. *World Journal of Surgery, 28*(3), 321-326.

Gouin, J.-P., & Kiecolt-Glaser, J. K. (2011). The impact of psychological stress on wound healing: methods and mechanisms. *Immunology and Allergy Clinics of North America, 31*(1), 81-93.

Gratrix, A., Enright, S., & O'Beirne, H. (2007). A survey of stress ulcer prophylaxis in intensive care units in the UK. *Anaesthesia, 62*(4), 421-422.

Gromek, D., Figadère, B., Hocquemiller, R., Cavé, A., & Cortes, D. (1993). Corepoxylone, a possible precursor of mono-tetrahydrofuran γ-lactone acetogenins: biomimetic synthesis of corossolone. *Tetrahedron, 49*(24), 5247-5252.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine, 27*(1), 1-93.

Gyamfi, K., Sarfo, D., Nyarko, B., Akaho, E., Serfor-Armah, Y., & Ampomah-Amoako, E. (2011). Assessment of elemental content in the fruit of Graviola plant, *Annona Muricata*, from some selected communities in Ghana by instrumental neutron activation analysis. *Elixir Food Science, 41*, 5671-5675.

Hajdu, S. I. (2004). Greco-Roman thought about cancer. *Cancer, 100*(10), 2048-2051.

Hamid, R. A., Foong, C. P., Ahmad, Z., & Hussain, M. K. (2012). Antinociceptive and anti-ulcerogenic activities of the ethanolic extract of *Annona muricata* leaf. *Revista Brasileira de Farmacognosia, 22*(3), 630-641.

Hamizah, S., Roslida, A., Fezah, O., Tan, K., Tor, Y., & Tan, C. (2012). Chemopreventive potential of *Annona muricata* L leaves on chemically-induced skin papillomagenesis in mice. *Asian Pacific Journal of Cancer Prevention, 13*(6), 2533-2539.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hansra, D. M., Silva, O., Mehta, A., & Ahn, E. (2014). Patient with Metastatic Breast Cancer Achieves Stable Disease for 5 Years on Graviola and Xeloda after

Progressing on Multiple Lines of Therapy. *Advances in Breast Cancer Research, 3*(03), 84-87.

Harbour, J. W., & Dean, D. C. (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes & Development, 14*(19), 2393-2409.

Hasrat, J., Bruyne, T. D., BACKER, J. P., Vauquelin, G., & Vlietinck, A. (1997). Isoquinoline Derivatives Isolated from the Fruit of *Annona muricata* as 5-HTergic 5-HT_{1A} Receptor Agonists in Rats: Unexploited Antidepressive (Lead) Products. *Journal of Pharmacy and Pharmacology, 49*(11), 1145-1149.

Hasrat, J., Pieters, L., De Backer, J.-P., Vauquelin, G., & Vlietinck, A. (1997). Screening of medicinal plants from Suriname for 5-HT_{1A} ligands: Bioactive isoquinoline alkaloids from the fruit of *Annona muricata*. *Phytomedicine, 4*(2), 133-140.

Hayouni, E., Miled, K., Boubaker, S., Bellasfar, Z., Abedrabba, M., Iwaski, H., . . . Hamdi, M. (2011). Hydroalcoholic extract based-ointment from *Punica granatum* L. peels with enhanced *in vivo* healing potential on dermal wounds. *Phytomedicine, 18*(11), 976-984.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Hill, M. M., Adrain, C., Duriez, P. J., Creagh, E. M., & Martin, S. J. (2004). Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *The EMBO Journal, 23*(10), 2134-2145.

Hisham, A., Sreekala, U., Pieters, L., Bruyne, T. D., Van den Heuvel, H., & Claeys, M. (1993). Epoxymurins A and B, two biogenetic precursors of annonaceous acetogenins from *Annona muricata*. *Tetrahedron, 49*(31), 6913-6920.

Hla Myint, S., Cortes, D., Laurens, A., Hocquemiller, R., Lebęuf, M., Cavé, A., . . . Quéro, A.-M. (1991). Solamin, a cytotoxic mono-tetrahydrofuranic γ-lactone acetogenin from *Annona muricata* seeds. *Phytochemistry, 30*(10), 3335-3338.

Horvitz, H. R. (1999). Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer Research, 59*(7 Supplement), 1701s-1706s.

Huang, J.-Q., Sridhar, S., & Hunt, R. H. (2002). Role of Helicobacter pylori infection and non-steroidal anti-inflammatory drugs in peptic-ulcer disease: a meta-analysis. *The Lancet, 359*(9300), 14-22.

Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis. *Nature Reviews Cancer, 2*(4), 277-288.

Ikushima, H., & Miyazono, K. (2010). TGFβ signalling: a complex web in cancer progression. *Nature Reviews Cancer, 10*(6), 415-424.

Irigaray, P., Newby, J., Clapp, R., Hardell, L., Howard, V., Montagnier, L., . . . Belpomme, D. (2007). Lifestyle-related factors and environmental agents causing cancer: an overview. *Biomedicine & Pharmacotherapy, 61*(10), 640-658.

Ishola, I. O., Awodele, O., Olusayero, A. M., & Ochieng, C. O. (2014). Mechanisms of Analgesic and Anti-Inflammatory Properties of *Annona muricata* Linn.(Annonaceae) Fruit Extract in Rodents. *Journal of Medicinal Food, 17*(12), 1375-1382.

Jagetia, G. C., Venkatesha, V., & Reddy, T. K. (2003). Naringin, a citrus flavonone, protects against radiation-induced chromosome damage in mouse bone marrow. *Mutagenesis, 18*(4), 337-343.

Jameson, J., & Havran, W. L. (2007). Skin γδ T-cell functions in homeostasis and wound healing. *Immunological Reviews, 215*(1), 114-122.

Jaramillo-Flores, M., & Hernandez-Sanchez, H. (2000). Thermal diffusivity of soursop (*Annona muricata* L.) pulp. *Journal of Food Engineering, 46*(2), 139-143.

Jaramillo, M., Arango, G., Gonzalez, M., Robledo, S., & Velez, I. D. (2000). Cytotoxicity and antileishmanial activity of *Annona muricata* pericarp. *Fitoterapia, 71*(2), 183-186.

Jiménez, V. M., Gruschwitz, M., Schweiggert, R. M., Carle, R., & Esquivel, P. (2014). Identification of phenolic compounds in soursop (*Annona muricata*) pulp by high-performance liquid chromatography with diode array and electrospray ionization mass spectrometric detection. *Food Research International, 65*, 42-46.

Jirovetz, L., Buchbauer, G., & Ngassoum, M. B. (1998). Essential oil compounds of the *Annona muricata* fresh fruit pulp from Cameroon. *Journal of Agricultural and Food Chemistry, 46*(9), 3719-3720.

Jothy, S. L., Torey, A., Darah, I., Choong, Y. S., Saravanan, D., Chen, Y., . . . Sasidharan, S. (2012). Cassia spectabilis (DC) Irwin et Barn: A promising traditional herb in health improvement. *Molecules, 17*(9), 10292-10305.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., . . . Ravagnan, L. (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature, 410*(6828), 549-554.

Kang, S., Wang, S., Kuida, K., & Yuan, J. (2002). Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock. *Cell Death Differ, 9*, 1115-1125.

Karim, A. A., & Azlan, A. (2012). Fruit pod extracts as a source of nutraceuticals and pharmaceuticals. *Molecules, 17*(10), 11931-11946.

Karin, M., & Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annual Review of Immunology, 18*(1), 621-663.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer, 26*(4), 239-257.

Kim, G.-s., Zeng, L., Alali, F., Rogers, L. L., Wu, F.-E., McLaughlin, J. L., & Sastrodihardjo, S. (1998). Two new mono-tetrahydrofuran ring acetogenins, annomuricin E and muricapentocin, from the leaves of *Annona muricata*. *Journal of Natural Products, 61*(4), 432-436.

Kim, G.-S., Zeng, L., Alali, F., Rogers, L. L., Wu, F.-E., Sastrodihardjo, S., & McLaughlin, J. L. (1998). Muricoreacin and murihexocin C, mono-tetrahydrofuran acetogenins, from the leaves of *annona muricata* in honour of professor GH Neil Towers 75th birthday. *Phytochemistry, 49*(2), 565-571.

King, R. W., Deshaies, R. J., Peters, J.-M., & Kirschner, M. W. (1996). How proteolysis drives the cell cycle. *Science, 274*(5293), 1652-1659.

King, R. W., Jackson, P. K., & Kirschner, M. W. (1994). Mitosis in transition. *Cell, 79*(4), 563-571.

Kinjyo, I., Weninger, W., & Hodgkin, P. D. (2015). Shedding light on cell cycle control by T and B lymphocytes. *Cell Cycle, 14*(15), 2381-2382.

Kirienko, N. V., Mani, K., & Fay, D. S. (2010). Cancer models in *Caenorhabditis elegans*. *Developmental Dynamics, 239*(5), 1413-1448.

Konturek, J., Fischer, H., Konturek, P., Huber, V., Boknik, P., Luess, H., . . . Hahn, E. (2001). Heat shock protein 70 (HSP70) in gastric adaptation to aspirin in *Helicobacter pylori* infection. *Journal of Physiology and Pharmacology, 52*(1), 153–164.

Kossouoh, C., Moudachirou, M., Adjakidje, V., Chalchat, J.-C., & Figuérédo, G. (2007). Essential oil chemical composition of *Annona muricata* L. leaves from Benin. *Journal of Essential Oil Research, 19*(4), 307-309.

Kranz, D., & Dobbelstein, M. (2012). A killer promoting survival: p53 as a selective means to avoid side effects of chemotherapy. *Cell Cycle, 11*(11), 2053-2054.

Kumar, B., Vijayakumar, M., Govindarajan, R., & Pushpangadan, P. (2007). Ethnopharmacological approaches to wound healing—exploring medicinal plants of India. *Journal of Ethnopharmacology, 114*(2), 103-113.

Kurosaka, K., Takahashi, M., Watanabe, N., & Kobayashi, Y. (2003). Silent cleanup of very early apoptotic cells by macrophages. *The Journal of Immunology, 171*(9), 4672-4679.

Kurumbail, R. G., Kiefer, J. R., & Marnett, L. J. (2001). Cyclooxygenase enzymes: catalysis and inhibition. *Current Opinion in Structural Biology, 11*(6), 752-760.

La Casa, C., Villegas, I., De La Lastra, C. A., Motilva, V., & Calero, M. M. (2000). Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. *Journal of Ethnopharmacology, 71*(1), 45-53.

Lanas, A., Baron, J. A., Sandler, R. S., Horgan, K., Bolognese, J., Oxenius, B., . . . Schoen, R. (2007). Peptic ulcer and bleeding events associated with rofecoxib in a 3-year colorectal adenoma chemoprevention trial. *Gastroenterology, 132*(2), 490-497.

Lau, J. Y., Sung, J., Hill, C., Henderson, C., Howden, C. W., & Metz, D. C. (2011). Systematic review of the epidemiology of complicated peptic ulcer disease: incidence, recurrence, risk factors and mortality. *Digestion, 84*(2), 102-113.

Leboeuf, M., Cavé, A., Bhaumik, P., Mukherjee, B., & Mukherjee, R. (1980). The phytochemistry of the Annonaceae. *Phytochemistry, 21*(12), 2783-2813.

Leboeuf, M., Legueut, C., Cavé, A., Desconclois, J., Forgacs, P., & Jacquemin, H. (1981). Alkaloids of Annonaceae. XXIX. Alkaloids of *Annona muricata* L. *Planta medica, 42*(05), 37-44.

Levine, A. J., Momand, J., & Finlay, C. A. (1991). The p53 tumour suppressor gene. *Nature, 351*(6326), 453-456.

Li, C.-M., Tan, N.-H., Lu, Y.-P., LIANG, H.-L., Mu, Q., Zheng, H., . . . Zhou, J. (1995). Annomuricatin A, a new cyclopeptide from the seeds of *Annona muricata*. *Acta Botanica Yunnanica, 17*, 459-462.

Li, D.-Y., Yu, J.-G., Zhu, J.-X., Yu, D.-L., Luo, X.-Z., Sun, L., & Yang, S.-L. (2001). Annonaceous acetogenins of the seeds from *Annona muricata*. *Journal of Asian Natural Products Research, 3*(4), 267-276.

Liao, J.-C., Deng, J.-S., Chiu, C.-S., Huang, S.-S., Hou, W.-C., Lin, W.-C., & Huang, G.-J. (2012). Chemical compositions, anti-inflammatory, antiproliferative and radical-scavenging activities of *Actinidia callosa* var. *ephippioides*. *The American journal of Chinese Medicine, 40*(05), 1047-1062.

Liaw, C.-C., Chang, F.-R., Lin, C.-Y., Chou, C.-J., Chiu, H.-F., Wu, M.-J., & Wu, Y.-C. (2002). New Cytotoxic Monotetrahydrofuran Annonaceous Acetogenins from *Annona muricata*. *Journal of Natural Products, 65*(4), 470-475.

Liu, F.-T., Newland, A. C., & Jia, L. (2003). Bax conformational change is a crucial step for PUMA-mediated apoptosis in human leukemia. *Biochemical and Biophysical Research Communications, 310*(3), 956-962.

Locksley, R. M., Killeen, N., & Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell, 104*(4), 487-501.

Longley, D., & Johnston, P. (2005). Molecular mechanisms of drug resistance. *The Journal of Pathology, 205*(2), 275-292.

Longstreth, G. F. (1995). Epidemiology of hospitalization for acute upper gastrointestinal hemorrhage: a population-based study. *The American Journal of Gastroenterology, 90*(2), 206-210.

Luna, J. D. S., De Carvalho, J., De Lima, M., Bieber, L., Bento, E. D. S., Franck, X., & Sant'Ana, A. (2006). Acetogenins in *Annona muricata* L.(Annonaceae) leaves are potent molluscicides. *Natural Product Research, 20*(3), 253-257.

Malfertheiner, P., Chan, F. K., & McColl, K. E. (2009). Peptic ulcer disease. *The Lancet, 374*(9699), 1449-1461.

Malfertheiner, P., Dent, J., Zeijlon, L., Sipponen, P., Veldhuyzen Van Zanten, S., Burman, C. F., . . . Lonovics, J. (2002). Impact of Helicobacter pylori eradication on heartburn in patients with gastric or duodenal ulcer disease—results from a randomized trial programme. *Alimentary Pharmacology & Therapeutics, 16*(8), 1431-1442.

Malfertheiner, P., Leodolter, A., & Peitz, U. (2000). Cure of Helicobacter pylori-associated ulcer disease through eradication. *Best Practice & Research Clinical Gastroenterology, 14*(1), 119-132.

Malfertheiner, P., Megraud, F., O'Morain, C., Bazzoli, F., El-Omar, E., Graham, D., . . . Kuipers, E. J. (2007). Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut, 56*(6), 772-781.

Malumbres, M., Harlow, E., Hunt, T., Hunter, T., Lahti, J. M., Manning, G., . . . Wolgemuth, D. J. (2009). Cyclin-dependent kinases: a family portrait. *Nature Cell Biology, 11*(11), 1275-1276.

Martinvalet, D., Zhu, P., & Lieberman, J. (2005). Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity, 22*(3), 355-370.

Massagué, J. (2004). G1 cell-cycle control and cancer. *Nature, 432*(7015), 298-306.

Matsushige, A., Kotake, Y., Matsunami, K., Otsuka, H., Ohta, S., & Takeda, Y. (2012). Annonamine, a new aporphine alkaloid from the leaves of *Annona muricata*. *Chemical and Pharmaceutical Bulletin, 60*(2), 257-259.

Matsushige, A., Matsunami, K., Kotake, Y., Otsuka, H., & Ohta, S. (2012). Three new megastigmanes from the leaves of *Annona muricata*. *Journal of Natural Medicines, 66*(2), 284-291.

Matushansky, I., Radparvar, F., & Skoultchi, A. I. (2000). Reprogramming leukemic cells to terminal differentiation by inhibiting specific cyclin-dependent kinases in G1. *Proceedings of the National Academy of Sciences, 97*(26), 14317-14322.

McColl, K. (2000). Should Non-Invasive *Helicobacter pylori* Testing Replace Endoscopy in Investigation of Dyspepsia? *Helicobacter, 5*(S1), 11-15.

McColl, K., Murray, L., Gillen, D., Walker, A., Wirz, A., Fletcher, J., . . . Dickson, A. (2002). Randomised trial of endoscopy with testing for Helicobacter pylori compared with non-invasive H pylori testing alone in the management of dyspepsia. *BMJ, 324*(7344), 999-1002.

McLaughlin, J. L. (2008). Paw Paw and Cancer: Annonaceous Acetogenins from Discovery to Commercial Products⊥. *Journal of Natural Products, 71*(7), 1311-1321.

Melot, A., Fall, D., Gleye, C., & Champy, P. (2009). Apolar Annonaceous acetogenins from the fruit pulp of *Annona muricata*. *Molecules, 14*(11), 4387-4395.

Meszaros, A. J., Reichner, J. S., & Albina, J. E. (2000). Macrophage-induced neutrophil apoptosis. *The Journal of Immunology, 165*(1), 435-441.

Mills, R. E., Taylor, K. R., Podshivalova, K., McKay, D. B., & Jameson, J. M. (2008). Defects in skin γδ T cell function contribute to delayed wound repair in rapamycin-treated mice. *The Journal of Immunology, 181*(6), 3974-3983.

Minari, J., & Okeke, U. (2014). Chemopreventive effect of *Annona muricata* on DMBA-induced cell proliferation in the breast tissues of female albino mice. *Egyptian Journal of Medical Human Genetics, 15*(4), 327-334.

Mishra, S., Ahmad, S., Kumar, N., & Sharma, B. K. (2013). *Annona muricata* (The cancer killer): A review. *The Global Journal of Pharmaceutical Research, 2*(1), 1613-1618.

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology, 8*(12), 958-969.

MYINT, S. H., Laurens, A., Hocquemiller, R., Cavé, A., Davoust, D., & Cortes, D. (1990). Murisolin: a new cytotoxic mono-tetrahydrofuran-γ-lactone from *Annona muricata*. *Heterocycles, 31*(5), 861-867.

N'gouemo, P., Koudogbo, B., Tchivounda, H. P., Akono-Nguema, C., & Etoua, M. M. (1997). Effects of ethanol extract of *Annona muricata* on pentylenetetrazol-induced convulsive seizures in mice. *Phytotherapy Research, 11*(3), 243-245.

Nagata, S. (2000). Apoptotic DNA fragmentation. *Experimental cell research, 256*(1), 12-18.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., & Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. *Nature, 403*(6765), 98-103.

Nawwar, M., Ayoub, N., Hussein, S., Hashim, A., El-Sharawy, R., Wende, K., . . . Lindequist, U. (2012). Flavonol triglycoside and investigation of the antioxidant and cell stimulating activities of *Annona muricata* Linn. *Archives of Pharmacal Research, 35*(5), 761-767.

Nayak, B., & Pereira, L. M. P. (2006). *Catharanthus roseus* flower extract has wound-healing activity in Sprague Dawley rats. *BMC Complementary and Alternative Medicine, 6*(1), 41.

Nemes Jr, Z., Friis, R., Aeschlimann, D., Saurer, S., Paulsson, M., & Fésüs, L. (1996). Expression and activation of tissue transglutaminase in apoptotic cells of involuting rodent mammary tissue. *European Journal of Cell Biology, 70*(2), 125-133.

Newman, D. J., & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products, 75*(3), 311-335.

Newmeyer, D., Bossy-Wetzel, E., Kluck, R., Wolf, B., Beere, H., & Green, D. (2000). Bcl-xL does not inhibit the function of Apaf-1. *Cell Death and Differentiation, 7*, 402-407.

Nguyen, D. X., & Massagué, J. (2007). Genetic determinants of cancer metastasis. *Nature Reviews Genetics, 8*(5), 341-352.

Norbury, C. J., & Hickson, I. D. (2001). Cellular responses to DNA damage. *Annual Review of Pharmacology and Toxicology, 41*(1), 367-401.

Nwokocha, C. R., Owu, D. U., Gordon, A., Thaxter, K., McCalla, G., Ozolua, R. I., & Young, L. (2012). Possible mechanisms of action of the hypotensive effect of *Annona muricata* (soursop) in normotensive Sprague-Dawley rats. *Pharmaceutical Biology, 50*(11), 1436-1441.

Ocker, M., & Höpfner, M. (2012). Apoptosis-modulating drugs for improved cancer therapy. *European Surgical Research, 48*(3), 111-120.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., . . . Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science, 288*(5468), 1053-1058.

Olakunle, S., Onyechi, O., & James, O. (2014). Toxicity, anti-lipid peroxidation, invitro and invivo evaluation of antioxidant activity of *Annona muricata* ethanol stem bark extract. *American Journal of Life Sciences, 2*(5), 271-277.

Olsson, A., Diaz, T., Aguilar-Santelises, M., Osterborg, A., Celsing, F., Jondal, M., & Osorio, L. (2001). Sensitization to TRAIL-induced apoptosis and modulation of FLICE-inhibitory protein in B chronic lymphocytic leukemia by actinomycin D. *Leukemia, 15*(12), 1868.

Ong, H., & Norzalina, J. (1999). Malay herbal medicine in Gemencheh, Negri Sembilan, Malaysia. *Fitoterapia, 70*(1), 10-14.

Oren, M., & Rotter, V. (1999). Introduction: p53–the first twenty years. *Cellular and Molecular Life Sciences CMLS, 55*(1), 9-11.

Osorio, E., Arango, G. J., Jiménez, N., Alzate, F., Ruiz, G., Gutiérrez, D., . . . Robledo, S. (2007). Antiprotozoal and cytotoxic activities *in vitro* of Colombian Annonaceae. *Journal of Ethnopharmacology, 111*(3), 630-635.

Padma, P., Chansauria, J., Khosa, R., & Ray, A. (2001). Effect of *Annooa muricata* and *Polyalthia cerasoides* on Brain Neurotransimitters and Enzyme Monoamine Oxidase Following Cold Immobilization Stress. *Journal of Natural Remedies, 1*(2), 144-146.

Padma, P., Chansouria, J., & Khosa, R. (1997). Effect of alcohol extract of *Annona muricata* on cold immobilization stress induced tissue lipid peroxidation. *Phytotherapy Research, 11*(4), 326-327.

Panka, D. J., Mano, T., Suhara, T., Walsh, K., & Mier, J. W. (2001). Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *Journal of Biological Chemistry, 276*(10), 6893-6896.

Papatheodoridis, G. V., Sougioultzis, S., & Archimandritis, A. J. (2006). Effects of Helicobacter pylori and nonsteroidal anti-inflammatory drugs on peptic ulcer disease: a systematic review. *Clinical Gastroenterology and Hepatology, 4*(2), 130-142.

Park, H. H., Lo, Y.-C., Lin, S.-C., Wang, L., Yang, J. K., & Wu, H. (2007). The death domain superfamily in intracellular signaling of apoptosis and inflammation. *Annual Review of Immunology, 25*, 561-586.

Park, J. E., & Barbul, A. (2004). Understanding the role of immune regulation in wound healing. *The American Journal of Surgery, 187*(5), S11-S16.

Park, M.-T., & Lee, S.-J. (2003). Cell cycle and cancer. *Journal of Biochemistry and Molecular Biology, 36*(1), 60-65.

Pélissier, Y., Marion, C., Kone, D., Lamaty, G., Menut, C., & Bessière, J.-M. (1994). Volatile components of *Annona muricata* L. *Journal of Essential Oil Research, 6*(4), 411-414.

Perez, E. A. (2009). Impact, mechanisms, and novel chemotherapy strategies for overcoming resistance to anthracyclines and taxanes in metastatic breast cancer. *Breast Cancer Research and Treatment, 114*(2), 195-201.

Ragasa, C. Y., Soriano, G., Torres, O. B., Don, M.-J., & Shen, C.-C. (2012). Acetogenins from *Annona muricata*. *Pharmacognosy Journal, 4*(32), 32-37.

Rai, N. K., Tripathi, K., Sharma, D., & Shukla, V. K. (2005). Apoptosis: a basic physiologic process in wound healing. *The International Journal of Lower Extremity Wounds, 4*(3), 138-144.

Rauws, E., & Tytgat, G. (1990). Cure of duodenal ulcer associated with eradication of *Helicobacter pylori*. *The Lancet, 335*(8700), 1233-1235.

Repetto, M., & Llesuy, S. (2002). Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Brazilian Journal of Medical and Biological Research, 35*(5), 523-534.

Ribeiro, L. P., Akhtar, Y., Vendramim, J. D., & Isman, M. B. (2014). Comparative bioactivity of selected seed extracts from Brazilian *Annona* species and an acetogenin-based commercial bioinsecticide against *Trichoplusia ni* and *Myzus persicae*. *Crop Protection, 62*, 100-106.

Rieser, M. J., Fang, X.-P., Rupprecht, J. K., Hui, Y.-H., Smith, D. L., & McLaughlin, J. L. (1993). Bioactive single-ring acetogenins from seed extracts of *Annona muricata*. *Planta Medica, 59*(1), 91-92.

Rieser, M. J., Fang, X. P., Anderson, J. E., Miesbauer, L. R., Smith, D. L., & McLaughlin, J. L. (1993). Muricatetrocins A and B and Gigantetrocin B: Three New Cytotoxic

Monotetrahydrofuran-Ring Acetogenins from *Annona muricata*. *Helvetica Chimica Acta, 76*(7), 2433-2444.

Rieser, M. J., Gu, Z.-M., Fang, X.-P., Zeng, L., Wood, K. V., & McLaughlin, J. L. (1996). Five novel mono-tetrahydrofuran ring acetogenins from the seeds of *Annona muricata*. *Journal of Natural Products, 59*(2), 100-108.

Rieser, M. J., Kozlowski, J. F., Wood, K. V., & McLaughlin, J. L. (1991). Muricatacin: A simple biologically active acetogenin derivative from the seeds of *annona muricata* (annonaceae). *Tetrahedron Letters, 32*(9), 1137-1140.

Robert, J., Ménoret, A., Basu, S., Cohen, N., & Srivastava, P. K. (2001). Phylogenetic conservation of the molecular and immunological properties of the chaperones gp96 and hsp70. *European Journal of Immunology, 31*(1), 186-195.

Roblot, F., Laugel, T., Lebœuf, M., Cavé, A., & Laprévote, O. (1993). Two acetogenins from *Annona muricata* seeds. *Phytochemistry, 34*(1), 281-285.

Rodríguez, L. A. G., & Tolosa, L. B. (2007). Risk of upper gastrointestinal complications among users of traditional NSAIDs and COXIBs in the general population. *Gastroenterology, 132*(2), 498-506.

Rokutan, K. (2000). Role of heat shock proteins in gastric mucosal protection. *Journal of Gastroenterology and Hepatology, 15*(s1), 12-19.

Rosenstock, S., Jørgensen, T., Bonnevie, O., & Andersen, L. (2003). Risk factors for peptic ulcer disease: a population based prospective cohort study comprising 2416 Danish adults. *Gut, 52*(2), 186-193.

Roslida, A., Tay, C., Zuraini, A., & Chan, P. (2010). Anti-inflammatory and Anti-nociceptive Activities of the Ethanolic Extract of *Annona muricata* Leaf. *Journal of Natural Remedies, 10*(2), 97-104.

Rupprecht, J. K., Hui, Y.-H., & McLaughlin, J. L. (1990). Annonaceous acetogenins: a review. *Journal of Natural Products, 53*(2), 237-278.

Saelens, X., Festjens, N., Walle, L. V., Van Gurp, M., van Loo, G., & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene, 23*(16), 2861-2874.

Savill, J., & Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature, 407*(6805), 784-788.

Scaffidi, C., Schmitz, I., Krammer, P. H., & Peter, M. E. (1999). The role of c-FLIP in modulation of CD95-induced apoptosis. *Journal of Biological Chemistry, 274*(3), 1541-1548.

Schimmer, A. D. (2004). Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Research, 64*(20), 7183-7190.

Schmeda-Hirschmann, G., & Yesilada, E. (2005). Traditional medicine and gastroprotective crude drugs. *Journal of Ethnopharmacology, 100*(1), 61-66.

Schmidt, C., Fronza, M., Goettert, M., Geller, F., Luik, S., Flores, E., . . . Laufer, S. (2009). Biological studies on Brazilian plants used in wound healing. *Journal of Ethnopharmacology, 122*(3), 523-532.

Schuler, M., & Green, D. (2001). Mechanisms of p53-dependent apoptosis. *Biochemical Society Transactions, 29*(6), 684-687.

Schwarz, K. (1910). Über penetrierende Magen-und Jejunalgeschwüre. *Beitr Klin Chir, 67*, 96–128.

Sherr, C. J. (2000). The Pezcoller lecture: cancer cell cycles revisited. *Cancer Research, 60*(14), 3689-3695.

Sherr, C. J., & Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Development, 13*(12), 1501-1512.

Slee, E. A., Adrain, C., & Martin, S. J. (2001). Executioner caspase-3,-6, and-7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *Journal of Biological Chemistry, 276*(10), 7320-7326.

Soni, R., O'Reilly, T., Furet, P., Muller, L., Stephan, C., Zumstein-Mecker, S., . . . Chaudhuri, B. (2001). Selective in vivo and in vitro effects of a small molecule inhibitor of cyclin-dependent kinase 4. *Journal of the National Cancer Institute, 93*(6), 436-446.

Sonnenberg, A. (2006). Causes underlying the birth-cohort phenomenon of peptic ulcer: analysis of mortality data 1911–2000, England and Wales. *International Journal of Epidemiology, 35*(4), 1090-1097.

Sumitra, M., Manikandan, P., & Suguna, L. (2005). Efficacy of Butea monosperma on dermal wound healing in rats. *The International Journal of Biochemistry & Cell Biology, 37*(3), 566-573.

Sun, S., Liu, J., Kadouh, H., Sun, X., & Zhou, K. (2014). Three new anti-proliferative Annonaceous acetogenins with mono-tetrahydrofuran ring from graviola fruit (*Annona muricata*). *Bioorganic & Medicinal Chemistry Letters, 24*(12), 2773-2776.

Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., . . . Prévost, M.-C. (2000). Two distinct pathways leading to nuclear apoptosis. *The Journal of Experimental Medicine, 192*(4), 571-580.

Susser, M., & Stein, Z. (1962). Civilisation and peptic ulcer. *The Lancet, 279*(7221), 116-119.

Swift, M. E., Burns, A. L., Gray, K. L., & DiPietro, L. A. (2001). Age-related alterations in the inflammatory response to dermal injury. *Journal of Investigative Dermatology, 117*(5), 1027-1035.

Szabo, I., & Tarnawski, A. (2000). Apoptosis in the gastric mucosa: molecular mechanisms, basic and clinical implications. *Journal of Physiology and Pharmacology, 51*(1).

Taylor, R. W., & Turnbull, D. M. (2005). Mitochondrial DNA mutations in human disease. *Nature Reviews Genetics, 6*(5), 389-402.

Tempesta, M. S., Kriek, G. R., & Bates, R. B. (1982). Uvaricin, a new antitumor agent from *Uvaria accuminata* (Annonaceae). *The Journal of Organic Chemistry, 47*(16), 3151-3153.

Thakur, R., Jain, N., Pathak, R., & Sandhu, S. S. (2011). Practices in wound healing studies of plants. *Evidence-Based Complementary and Alternative Medicine, 2011*, doi:10.1155/2011/438056.

Thang, T., Dai, D., Hoi, T., & Ogunwande, I. (2013). Study on the volatile oil contents of Annona glabra L., Annona squamosa L., Annona muricata L. and Annona reticulata L., from Vietnam. *Natural Product Research, 27*(13), 1232-1236.

Thomas, R. P., Farrow, B. J., Kim, S., May, M. J., Hellmich, M. R., & Evers, B. M. (2002). Selective targeting of the nuclear factor-κB pathway enhances tumor necrosis factor–related apoptosis-inducing ligand-mediated pancreatic cancer cell death. *Surgery, 132*(2), 127-134.

Thornton, T. M., & Rincon, M. (2009). Non-classical p38 map kinase functions: cell cycle checkpoints and survival. *International Journal of Biological Sciences, 5*(1), 44-52.

Vanneman, M., & Dranoff, G. (2012). Combining immunotherapy and targeted therapies in cancer treatment. *Nature Reviews Cancer, 12*(4), 237-251.

Vidinský, B., Gál, P., Toporcer, T., Longauer, F., Lenhardt, Ľ., Bobrov, N., & Sabo, J. (2006). Histological study of the first seven days of skin wound healing in rats. *Acta Veterinaria Brno, 75*(2), 197-202.

Vijayameena, C., Subhashini, G., Loganayagi, M., & Ramesh, B. (2013). Phytochemical screening and assessment of antibacterial activity for the bioactive compounds in *Annona muricata*. *International Journal of Current Microbiology and Applied Sciences 2*(1), 1-8.

Vila-Nova, N. S., de Morais, S. M., Falcão, M. J. C., Alcantara, T. T. N., Ferreira, P. A. T., Cavalcanti, E. S. B., . . . Wilson, M. (2013). Different susceptibilities of *Leishmania* spp. promastigotes to the *Annona muricata* acetogenins annonacinone and corossolone, and the *Platymiscium floribundum* coumarin scoparone. *Experimental Parasitology, 133*(3), 334-338.

Vila-Nova, N. S., Morais, S. M. d., Falcão, M. J. C., Machado, L. K. A., Beviláqua, C. M. L., Costa, I. R. S., . . . Andrade Júnior, H. F. d. (2011). Leishmanicidal activity and cytotoxicity of compounds from two Annonacea species cultivated in Northeastern Brazil. *Revista da Sociedade Brasileira de Medicina Tropical, 44*(5), 567-571.

Wallace, J. L. (2008). Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? *Physiological Reviews, 88*(4), 1547-1565.

Wallace, J. L., Keenan, C. M., & Granger, D. N. (1990). Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 259*(3), G462-G467.

Wang, C.-Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., & Baldwin, A. S. (1998). NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science, 281*(5383), 1680-1683.

Warren, J. R., & Marshall, B. (1983). Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *The Lancet, 321*(8336), 1273-1275.

Watt, J. M., & Breyer-Bnodwijk, M. (1962). The medicinal and poisonous plants of southern and eastern Africa: being an account of their medicinal and other uses, chemical composition, pharmacological effects aod toricotogy in man and annimal: Edinburgh & London : E. & S. Lívingstone Ltd.

Wélé, A., Zhang, Y., Caux, C., Brouard, J.-P., Pousset, J.-L., & Bodo, B. (2004). Annomuricatin C, a novel cyclohexapeptide from the seeds of *Annona muricata*. *Comptes Rendus Chimie, 7*(10), 981-988.

Wiseman, H., Kaur, H., & Halliwell, B. (1995). DNA damage and cancer: measurement and mechanism. *Cancer Letters, 93*(1), 113-120.

Wong, R. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research, 30*(1), 87.

Wu, F.-E., Gu, Z.-M., Zeng, L., Zhao, G.-X., Zhang, Y., McLaughlin, J. L., & Sastrodihardjo, S. (1995). Two new cytotoxic monotetrahydrofuran Annonaceous acetogenins, annomuricins A and B, from the leaves of Annona muricata. *Journal of Natural Products, 58*(6), 830-836.

Wu, F.-E., Zeng, L., Gu, Z.-M., Zhao, G.-X., Zhang, Y., Schwedler, J. T., . . . Sastrodihardjo, S. (1995a). Muricatocins A and B, two new bioactive monotetrahydrofuran Annonaceous acetogenins from the leaves of *Annona muricata*. *Journal of Natural Products, 58*(6), 902-908.

Wu, F.-E., Zeng, L., Gu, Z.-M., Zhao, G.-X., Zhang, Y., Schwedler, J. T., . . . Sastrodihardjo, S. (1995b). New bioactive monotetrahydrofuran Annonaceous acetogenins, annomuricin C and muricatocin C, from the leaves of *Annona muricata*. *Journal of Natural Products, 58*(6), 909-915.

Wu, F.-E., Zhao, G.-X., Zeng, L., Zhang, Y., Schwedler, J. T., McLaughlin, J. L., & Sastrodihardjo, S. (1995). Additional bioactive acetogenins, annomutacin and (2, 4-trans and cis)-10R-annonacin-A-ones, from the leaves of *Annona muricata*. *Journal of Natural Products, 58*(9), 1430-1437.

Xu, S., Kojima-Yuasa, A., Azuma, H., Kennedy, D. O., Konishi, Y., & Matsui-Yuasa, I. (2010). Comparison of glutathione reductase activity and the intracellular glutathione reducing effects of 13 derivatives of 1′-acetoxychavicol acetate in Ehrlich ascites tumor cells. *Chemico-Biological Interactions, 185*(3), 235-240.

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., & Korsmeyer, S. J. (1995). Bad, a heterodimeric partner for Bcl-x L and Bcl-2, displaces Bax and promotes cell death. *Cell, 80*(2), 285-291.

Yoshikawa, T., Ueda, S., Naito, Y., Takahashi, S., Oyamada, H., Morita, Y., . . . Kondo, M. (1989). Role of oxygen-derived free radicals in gastric mucosal injury induced by ischemia or ischemia-reperfusion in rats. *Free Radical Research, 7*(3-6), 285-291.

Yu, J.-G., Gui, H.-Q., Luo, X.-Z., & Sun, L. (1998). Murihexol, a linear acetogenin from *Annona muricata*. *Phytochemistry, 49*(6), 1689-1692.

Zafra-Polo, M. C., González, M. C., Estornell, E., Sahpaz, S., & Cortes, D. (1996). Acetogenins from Annonaceae, inhibitors of mitochondrial complex I. *Phytochemistry, 42*(2), 253-271.

Zeng, L., Wu, F.-E., Gu, Z.-m., & McLaughlin, J. L. (1995). Murihexocins A and B, two novel mono-THF acetogenins with six hydroxyls, from *Annona muricata* (Annonaceae). *Tetrahedron Letters, 36*(30), 5291-5294.

Zeng, L., Wu, F.-E., & McLaughlin, J. L. (1995). Annohexocin, a novel mono-THF acetogenin with six hydroxyls, from *Annona muricata* (Annonaceae). *Bioorganic and Medicinal Chemistry Letters, 5*(16), 1865-1868.

Zeng, L., Wu, F.-E., Oberlies, N. H., McLaughlin, J. L., & Sastrodihadjo, S. (1996). Five new monotetrahydrofuran ring acetogenins from the leaves of *Annona muricata*. *Journal of Natural Products, 59*(11), 1035-1042.

Zha, J., Harada, H., Yang, E., Jockel, J., & Korsmeyer, S. J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X L. *Cell, 87*(4), 619-628.

Zhang, J. H., & Ming, X. (2000). DNA fragmentation in apoptosis. *Cell Research, 10*(3), 205-211.

List of Publications and Papers Presented

Publications

1. **Moghadamtousi, S. Z.**, Kadir, H. A., Paydar, M., Rouhollahi, E., & Karimian, H. (2014). *Annona muricata* leaves induced apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF-kappaB. *BMC Complementary and Alternative Medicine, 14*(1), 299 (*ISI*-Cited Publication).

2. **Moghadamtousi, S. Z.**, Karimian, H., Rouhollahi, E., Paydar, M., Fadaeinasab, M., & Kadir, H. A. (2014). *Annona muricata* leaves induce G 1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-29 colon cancer cells. *Journal of Ethnopharmacology, 156*, 277-289 (*ISI*-Cited Publication).

3. **Moghadamtousi, S. Z.**, Rouhollahi, E., Karimian, H., Fadaeinasab, M., Firoozinia, M., & Ameen Abdulla, M. (2015). The Chemopotential Effect of *Annona muricata* Leaves against Azoxymethane-Induced Colonic Aberrant Crypt Foci in Rats and the Apoptotic Effect of Acetogenin Annomuricin E in HT-29 Cells: A Bioassay-Guided Approach. *PloS One, 10*(4), e0122288 (*ISI*-Cited Publication).

4. **Moghadamtousi, S. Z.**, Rouhollahi, E., Karimian, H., Fadaeinasab, M., Abdulla, M. A., & Kadir, H. A. (2014). Gastroprotective activity of *Annona muricata* leaves against ethanol-induced gastric injury in rats via Hsp70/Bax involvement. *Drug Design, Development and Therapy, 8*, 2099-2111 (*ISI*-Cited Publication).

5. **Moghadamtousi, S. Z.**, Rouhollahi, E., Hajrezaie, M., Karimian, H., Abdulla, M. A., & Kadir, H. A. (2015). *Annona muricata* leaves accelerate wound healing in rats via involvement of Hsp70 and antioxidant defence. *International Journal of Surgery, 18*, 110-117 (*ISI*-Cited Publication).

6. **Moghadamtousi S.Z.**, Fadaeinasab, M., Nikzad, S., Mohan, G., Hapipah, M. A., & Kadir, H. A. (2015). *Annona muricata* (Annonaceae): A review of its traditional uses, isolated acetogenins and biological activities. *International Journal of Molecular Sciences*, accepted (*ISI*-Cited Publication).

Papers presented

1) **Moghadamtousi S.Z.** & Kadir, H. A., *Annona muricata* Leaves Induced Mitochondrial-Mediated Apoptosis in A549 Cells. ICPPM 2014: International

Conference on Pharmacology and Pharmaceutical Medicine. 25th – 26th August, 2014, Kuala Lumpur, Malaysia (International).

2) **Moghadamtousi S.Z.** & Kadir, H. A., Chemopreventive effect of *Annona muricata* leaves against azoxymethane-induced colonic aberrant crypt foci in rats. ICBENS 2015: International Conference on Biological Engineering and Natural Sciences, 19th – 21th January, 2015, Singapore (International).