ASSESSMENT OF ANTITUMOR ACTIVITY OF STANDARDIZED POLYALTHIA LONGIFOLIA LEAF EXTRACT IN HELA CELL TUMOR XENOGRAFT MOUSE MODEL

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by

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LIST OF SYMBOLS

%	Percent
<	Less than
>	Greater than
µg	Microgram
µL	Microlitre
µm	Micrometre
℃	Degree Celsius

LIST OF ABBREVIATIONS

ADC	Autophagy-dependent cell death
ADP	Adenosine diphosphate
AFP	Alpha-fetoprotein
AGE	Advanced glycation end product
ALL	Acute lymphocytic leukemia
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ARM	Artemether
ARRIVE	Animal research reporting of *in vivo* experiments
ARS	Artemisinin
ART	Artesunate
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAD	BCL2 associated agonist of cell death
BALB/c	Albino, laboratory-bred strain mouse
BAX	BCL-2-Associated X Protein
BCA	Bicinchoninic acid
BCL-2	B-Cell Lymphoma 2
BCL-w	BCL-2-like protein 2
BID	BH3 interacting-domain death agonist

BSA	Bovine serum albumin
BUN	Blood urea nitrogen
C57-BL/6	Common inbred strain of laboratory mouse
CA 15-3	Cancer antigen 15-3
Caco-2	Cancer coli-2; Human colon adenocarcinoma
CBC	Complete blood count
CDK	Cyclin-dependent kinase
CDK1	Cyclin-dependent kinase 1
CDX	Cell line derived xenograft
CEA	Carcinoembryonic antigen
CNS	Central nervous system
CO_2	Carbon dioxide
COLO201	Colorectal Dukes' type D Adenocarcinoma 201
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
Cyp-D	Cyclophilin D
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DEN	N- nitrosodiethylamine
MST	Mean survival time
DLA	Dalton’s lymphoma ascites
DMBA	Dimethylbenz(a)-anthracene
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Du145	Human prostate cancer cell line

EAC	Ehrlich’s ascites carcinoma
EC50	Half maximal effective concentration
ECGC	Epigallocatechin-3-gallate
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EVn-50	Lignan compounds; mixture of vitexins
FADD	Fas-associated protein with death domain
FAS	Cell surface death receptor
FasL	Fas Ligand; type II membrane protein that belongs to the TNF superfamily
FBS	Fetal Bovine Serum
FC	Fold change
FKC	Flavokawain C
FRGS	Fundamental Research Grant Scheme
g	Gram
GPx	Glutathione peroxidases
GR	Glucocorticoid receptor
GSH	Glutathione
GST	Glutathione S-transferase
h	Hour
H&E	Hematoxylin & Eosin
H460	Human large-cell lung carcinoma line
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HEPE	Hydroxyeicosapentaenoic acid

HETE	5-Hydroxyeicosatetraenoic acid
HeLa	Cervical adenocarcinoma cells
HIER	Heat induced epitope retrieval
HIF-1α	Hypoxia inducible factor-1alpha
HL60	Human leukemia cell line
HMGB1	High mobility group box 1
HPV	Human Papilloma Virus
HRP	Horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
IAP	Inhibitors of Apoptosis
IC_{50}	Inhibition Concentration by half
IHC	Immunohistochemistry
IgG	Immunoglobulin G
IP	Intraperitoneal
IV	Intravenous
IVC	Individual ventilated cages
kDa	Kilodalton
kg	Kilogram
Ki-67	Nuclear protein that is associated with cellular proliferation
LC-ESI-MS/MS	Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry
LC3-II	Light chain 3 -phosphatidylethanolamine conjugate
LD50	Lethal dose which causes death by 50 percent
LDL	Low density lipoprotein

m	Molecular mass
M	Molar
mg	Milligram
mL	Millilitre
MCF-7	Breast cancer cell line
miRNA	MicroRNA
MLKL	Mixed Lineage Kinase Domain-like
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MVD	Microvessel Density
n	Sample size of a particular group
NCI	National Cancer Institute
NCI/ADR	Multidrug-resistant cell line that is a model for the study of drug resistance in ovarian cancer
NCI-460	Cellosaurus cell line
NF-kB	NF-kappaB; nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
nm	Nanometre
NOD	Non-obese diabetic
NSCLC	Non-small cell lung cancer
NU/NU	Nu nude mouse strain
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
p21	Cyclin-dependent kinase inhibitor

p27	Cyclin-dependent kinase inhibitor 1B
p53	Tumor suppressor protein
PANC-1	Human pancreatic cancer cell line
P	Statistical probability of the hypothesis
PARP	Poly (ADP-ribose) polymerase
PARP1	Poly (ADP-ribose) polymerase 1
PB	Phenobarbital
PBS	Phosphate buffered Saline
PC-3	Human prostate cancer cell line
PDX	Patient derived xenografts
PDTX	Patient derived tumor xenografts
PECAM-1	Platelet endothelial cell adhesion molecule
PGA2	Prostaglandin A2
PGAM5	Phosphoglycerate mutase 5; mitochondrial Serine (Ser)/Threonine (Thr) phosphatase
PLA2	Phospholipase A2
PUMA	p53 upregulated modulator of apoptosis
QTOF-MS	Quadrupole time-of-flight mass spectrometer
RBC	Red blood cells
rpm	Revolutions per minute
RIP1	Receptor-interacting Protein 1
RIP3	Receptor-interacting Protein 3
RM-1	Cells that promote the proliferation of osteoblast
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RT	Room temperature
RT4	Cell line exhibiting epithelial morphology
SCID	Severe combined Immunodeficiency
S.D	Standard deviation
SK-Hep1	Human Hepatic Adenocarcinoma Cell Line
SLE	Systemic lupus erythematosus
SMAC	Second Mitochondria-derived Activator of Caspases
SOD	Superoxide dismutase
SPSS	Statistical package for the social sciences
SV-HUC-1	SV40-immortalized human urinary tract epithelial cells
SW480	Best characterized of a large number of established colon cancer cell lines
T47D	Human breast cancer cells
TG	Triglyceride
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TNF-R1	Tumor necrotic factor receptor 1
TNF-R2	Tumor necrotic factor receptor 2
TNF-α	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptors
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
UHPLC	Ultra High Performance Liquid Chromatography
VEGF	Vascular endothelial growth factor

v/v	Volume per volume
VG1	Vascular endothelial growth factor antibody
w	Weight
WBC	White blood cells
XDB-C18	Zorbax Eclipse XDB column 18
XIAP	X chromosome-linked IAP
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide
z	Charge number

PENILAIAN AKTIVITI ANTITUMOR EKSTRAK PIAWAI DAUN POLYALTHIA LONGIFOLIA DALAM MODEL TIKUS XENOGRAFT TUMOR SEL HELA

ABSTRAK

P. longifolia ialah tumbuhan ubatan tradisional dengan sumber biologi yang kaya dengan fitokimia aktif. Penyiasatan terhadap ekstrak metanol daunnya dilaporkan memberi kesan antikanser *in vitro* yang kuat pada sel-sel kanser serviks HeLa manusia. Strategi rawatan kanser serviks yang terkini menghadapi cabaran seperti peningkatan ketoksikan dadah, kemo-rintangan, dan pilihan rawatan terhad, justeru menjadikan penemuan agen antikanser hijau yang selamat dan berkesan adalah penting. Walaupun *P. longifolia* adalah tumbuhan antikanser yang berkesan, penemuan *in vitro* sahaja adalah tidak mencukupi untuk diterjemahkan kepada kegunaan manusia dan oleh itu memerlukan ujian praklinikal *in vivo*. Pada masa ini, tiada laporan mengenai penyiasatan lanjut ekstrak daun *P. longifolia* pada model tumor haiwan *in vivo*, yang mengehadkan kegunaan klinikal tumbuhan ini. Oleh itu, kajian ini dijalankan untuk menilai kesan *in vivo* antitumor ekstrak daun metanol *P. longifolia* pada sel HeLa tumor xenograf yang dibangunkan dalam tikus tiada timus. Ekstrak daun metanol *P. longifolia* dalam kajian ini menunjukkan 23 fitokimia sebatian yang dikenal pasti oleh LC-ESI-MS/MS. Selanjutnya, potensi sitotoksik ekstrak telah ditentukan dengan ujian MTT pada sel HeLa dan menunjukkan nilai IC_{50} sebanyak 21.68 µg/mL. Tikus xenograf tumor sel HeLa telah dibangunkan dan sifat perencatan tumor dos tinggi (1000 mg/kg bw) dan dos rendah (500 mg/kg bw) rawatan ekstrak daun *P. longifolia* dibandingkan dengan kumpulan kawalan dan kumpulan kawalan positif etoposide (n = 6 per kumpulan). Penilaian jumlah tumor menunjukkan perencatan ketara tumor

yang bergantung kepada dos, dengan 85.07 ± 11% dan 31.12 ± 7.6% pengurangan dalam dos tinggi dan rendah rawatan ekstrak daun *P. longifolia* selama 5 minggu berbanding kumpulan kawalan. Selanjutnya, indeks organ dan pemerhatian histologi tidak menunjukkan ketoksikan kepada organ penting. Analisis histopatologi bahagian tumor yang diwarnakan dengan H&E menunjukkan apoptosis sebagai mekanisme utama kematian sel dengan rawatan *P. longifolia*. Selanjutnya, laluan apoptosis yang dikawal telah dikenal pasti menggunakan susunan protein apoptosis manusia pada tumor sampel yang menunjukkan peningkatan tahap dalam sepuluh protein pro-apoptosis (BAD, BAX, BID, caspase-3, caspase-8, p21, p27, p53, SMAC, danCytochrome c) dan penurunan dalam dua protein anti-apoptosis (BCL-2, BCL-w) semasa rawatan *P. longifolia*. Akhirnya, analisis imunohistokimia bahagian tumor menunjukkan pengurangan yang ketara pada dos yang bergantung kepada penanda proliferasi Ki-67 dan protein angiogenik CD31 dan VEGF. Kesimpulannya, keputusan kajian xenograf tumor *in vivo* ini membuktikan bahawa ekstrak daun *P. longifolia* mempunyai sifat antitumor yang kuat terhadap tumor serviks sel HeLa dengan mengaktifkan laluan apoptosis BCL2/BAX dan p53 bersama-sama dengan kesan antiproliferatif dan antiangiogenik.

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ABSTRACT

P. longifolia is a traditional medicinal plant with a rich source of biologically active phytochemicals. Investigations on methanolic leaf extract were reported to exert potent *in vitro* anticancer effects on HeLa human cervical cancer cells. Current treatment strategies for cervical cancer are facing challenges such as increased drug toxicity, chemoresistance, and limited treatment options, thus making it imperative for the discovery of safe and effective green anticancer agents. Though *P. longifolia* is a promising anticancer plant, the *in vitro* findings by themselves are insufficient to translate to human use and therefore require *in vivo* preclinical testing. Currently, there are no reports on further investigation of *P. longifolia* leaf extracts on *in vivo* animal tumor models, which limits the clinical utility of this plant. Hence this study was conducted to evaluate the *in vivo* antitumor effects of *P. longifolia* methanolic leaf extract on HeLa cells xenografted tumors developed in athymic nude mice. *P. longifolia* leaf extract in this study showed 23 phytochemical compounds identified by LC-ESI-MS/MS. Further, the cytotoxic potency of the methanolic leaf extract was determined by MTT assay on HeLa cells showed IC_{50} values of 21.68 µg/mL. HeLa cells tumor xenograft mice were developed and tumor inhibition properties of high dose (1000 mg/kg bw) and low dose (500 mg/kg bw) *P. longifolia* leaf extract treatment were compared with vehicle control and etoposide positive control (n = 6 per group). Evaluation of tumor volume showed significant dose-dependent tumor inhibition with 85.07 ± 11% and 31.12 ± 7.6% reduction in high and low dose *P.*

longifolia leaf extract treatment for 5-weeks compared to vehicle control. Further organ index and histological observations exhibited no toxicity to vital organs. Histopathological analysis of Hematoxylin & Eosin (H&E) stained tumor sections showed apoptosis as the major cell death mechanism with *P. longifolia* treatment. Further, the apoptotic pathway regulated was identified using a human apoptotic protein array on tumor samples which showed increased levels of ten pro-apoptotic proteins (BAD, BAX, BID, caspase-3, caspase-8, p21, p27, p53, SMAC, and Cytochrome c) and a decrease in two anti-apoptotic proteins (BCL-2, BCL-w) upon *P. longifolia* treatment. Finally, immunohistochemical analysis of tumor sections revealed a significant dose-dependent reduction in proliferation marker Ki-67 and angiogenic proteins CD31 and VEGF. In conclusion, the results of this *in vivo* tumor xenograft study prove that *P. longifolia* leaf extract has potent antitumor properties against HeLa cell cervical tumors by activating BCL2/BAX and p53 apoptotic pathways along with robust antiproliferative and antiangiogenic effects.

CHAPTER 1

INTRODUCTION

1.1 Overview and rationale of the study

Cancer is the second biggest cause of death worldwide, with 19.3 million new cases every year and 10.0 million deaths as per Globocan 2020 which is projected to increase rapidly. A similar increase in new cases and deaths is observed even in Malaysia (Sung *et al*., 2021). Previously, cancer incidence and mortality were high in developed countries, but the landscape of geographical distribution of cancer is changing rapidly beyond economic status and ethnicity. Moreover, cancer has become the most significant burden to highly populated developing countries due to the difficulties in resources for early detection, treatment, and management, increasing cancer-related mortality in these countries. Over the decades, though there have been improvements over cancer treatment and control, the cost and side effects remain high without any definitive treatment. Furthermore, poor and middle-income countries account for 85 percent of the world's population; thus, treating cancer will be the biggest challenge in the coming years. Moreover, there seems to be a lack of strategy and preparedness to tackle this problem (Shah *et al*., 2019). Since this could affect millions of patients and their families both economically and psychosocially, there is an urgency and greater responsibility to find alternative treatment options such as using natural products that are effective, readily available, inexpensive, and with minimum side effects.

Cancers are mainly classified based on the tissue of origin into carcinoma, sarcoma, lymphoma, and leukemia. Carcinomas are cancers of epithelial tissues that account for 80-90% of all cancers, with lung, colorectal, breast, and cervical being some significant types. Among these, cervical cancer is the fourth leading cause of death in women worldwide (Sung *et al*., 2021). Current treatment of choice includes

chemotherapy, radiotherapy, and surgery; however, all these treatment options have severe side effects with a higher risk of secondary malignancies, which again adds to the current global cancer burden. Despite having a battery of treatment options, there are still many additional challenges in current cervical cancer treatment, such as chemoresistance, poor treatment outcomes, high treatment cost, poor health care access, and a rapid increase in the number of cases. The possible solution is to search for new anticancer drugs of plant origin.

Cells usually function by highly regulated cell signalling pathways. Perturbations in molecular mechanisms that regulate cell survival, proliferation, and death are the significant determinants of tumorigenesis. Therefore, all anticancer chemotherapy and immunotherapy drugs target molecular pathways such as DNA damage, apoptosis, and blocking proliferation signals to check cancer cells, causing off-target adverse effects. Medicinal plant-based natural products are a promising alternative to treat different diseases, including cancer, because of their unique ability to interfere in specific molecular pathways without causing damage to normal cells. As most of these are novel, research needs to be conducted to discover their therapeutic and cancer prevention abilities.

Many plants rich in phytochemicals with various medicinal properties offer a higher chance of discovering new anticancer agents. However, finding anticancer agents from plants is laborious and resource-demanding. It is essential to identify a plant with anticancer properties by rigorous *in vitro* and *in vivo* experimentation to tackle this issue. Recently, the exploration of anticancer phytochemicals from medicinal plants has accelerated due to technological advancements in separation, purification, chemical analysis, and characterization of the lead bioactive compounds in plants. Phytochemicals are plant-derived natural chemicals with diverse biological effects,

including anticancer properties. Phytochemicals have been shown to exert better cancer prevention and cure with fewer side effects; therefore, there is increased attention to exploring the role of phytochemicals as potential anticancer drugs. Further, advances in *in vitro* screening offer an advantage in testing many plant extracts for their anticancer effects and identifying targeted molecular mechanisms. Promising medicinal plants with convincing anticancer effects is the goal of selecting a lead plant-based candidate for preclinical development. Medicinal plants, either as standardized extracts, active fractions, or pure compounds, could be a promising source of a novel anticancer drug. Moreover, natural anticancer compounds can also provide the blueprint for producing synthetic analogues. Some of the first chemotherapy drugs, namely Vinblastine and Vincristine, were synthesized based on natural compounds blueprint with anticancer/ antitumor effects.

Though 250,000 plants are reported to have medicinal properties, only a handful of them have made it into clinical trials (Amin *et al*., 2009). This lag is due to limitations on complete knowledge of the pharmacological and toxicological properties, safety issues, adverse drug reactions, efficacy, and consistency in therapeutic outcomes. It is estimated that for one plant product to reach a clinic, it takes about ten years of research because it involves multiple validation processes such as *in vitro* cell culture-based assays and *in vivo* preclinical animal experiments. In addition, this also requires the purification and characterization of bioactive compounds while maintaining their potency. Therefore, *in vitro* and *in vivo* studies have been the gold standard for identifying medicinal plants with therapeutic properties. It is important to understand safety, toxicity, and dosage before administering the plant extract to a patient to understand the therapeutic dose and its effect (Ashraf, 2020).

P. longifolia is a commonly available garden tree with well-documented medicinal usage in the traditional Ayurvedic therapeutic system. *P. longifolia* has been demonstrated *in vitro* cytotoxicity effects against cancer cells and is emerging as a promising candidate with antitumor effects (Verma *et al*., 2008; Manjula *et al*., 2010; Vijayarathna, 2016; Vijayarathna *et al*., 2017; Shanmugapriya, 2019). However, the *in vitro* assays could not justify this plant's usage on humans due to lack of details on pathological physiological effects, and only possible after preclinical *in vivo* animal experimentation. The information obtained from an animal model is crucial to avoid possible misinterpretations and translate the *in vitro* findings to human applications. So far, *P. longifolia* leaf extracts have not been tested in animal tumor models to evaluate the detailed *in vivo* antitumor effects. Preclinical evaluation of medicinal plants with cytotoxicity properties in an animal model is pivotal in the anticancer research on medicinal plants. Antitumor models such as xenograft mouse models are widely used to study medicinal plants' preclinical efficacy and tumor-suppressive properties against specific human tumors developed in immune-suppressed athymic nude mice (Lawrence *et al*., 2013). Interestingly, Jothy *et al*. (2013) investigated the genotoxic potential of *P. longifolia* leaf against H_2O_2-radical-mediated DNA damage and acute oral toxicity. The *in vitro* genotoxicity experiments revealed that the leaf extract of *P. longifolia* had no significant genotoxic effect on the tested DNA. Furthermore, the results of the *Allium cepa* assay revealed that, when used in lower quantities, *P. longifolia* leaf extract could be critical for maintaining the organism's genetic stability. *P. longifolia* leaf was shown to be safe following oral administration of up to 5000 mg/kg body weight to female albino Wistar rats in an acute oral toxicity investigation, which was an excellent attribute for the green anticancer agent (Jothy *et al*., 2013). Therefore, the current study is conducted to evaluate the *in vivo* antitumor activity of nontoxic *P. longifolia* extract

and determine the chemical composition of *P. longifolia* leaf extract. Since *in vitro* anticancer effects of *P. longifolia* leaf extract was previously shown in HeLa cells by Vijayarathna *et al*. (2017a), in this study, a human HeLa cell line-derived xenograft mouse model has been developed, and the antitumor effects of *P. longifolia* have been studied for the first time.

1.2 Objectives

The general objective of this study is as follows:

- To evaluate the cytotoxicity effect of methanolic *P. longifolia* leaves extract against HeLa cervical cancer cells

The main objectives of this study are as follows:

I. To determine the chemical composition of *P. longifolia* leaf extract using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

II. To develop the human xenografted tumor derived from HeLa cervical cancer cells to study the *in vivo* activity of *P. longifolia* leaf extract.

III. To evaluate the antitumor activity of nontoxic *P. longifolia* leaf extract in female athymic NU/NU nude mice carrying xenografted tumor derived from HeLa cervical cancer cells.

IV. To study the underlying molecular mechanisms of action of the antitumor activity of *P. longifolia* leaf extract in female athymic NU/NU nude mice carrying xenografted tumor derived from HeLa cervical cancer cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

The National Cancer Institute (NCI) of USA, Dictionary defines cancers as a term for diseases in which abnormal cells divide without control and invade nearby tissues. Cancer is one of the most significant causes of the death of the world's human population, with an estimated 10 million deaths in 2020 (Zaorsky *et al*., 2017). Cancer exists in various human anatomies through the critical aspects of evading cell death and non-stop cell divisions. The durability of cancer cells is ensured simply by its mechanism in inhibiting apoptosis while maintaining good angiogenesis formation within the cancerous tissue (Kumar *et al*., 2013).

Cervical cancer is the fourth leading cause of cancer-related deaths in women, while breast cancer is the top killer (Sung *et al*., 2021). Human papillomavirus infection is the leading cause of cervical cancer. Though preventing HPV infection and early screenings should reduce the number of cases, cervical cancer is still a significant public health issue for middle-aged women, specifically in countries with limited resources (Arbyn *et al*., 2020). As cancer encroachment becomes more and more aggressive, the advancement areas for treatment and preventive therapies also intensify globally. Current treatment options involve chemotherapy, radiotherapy, surgery, and immunotherapy but still pose a high risk of cancer recurrence and chemoresistance (Talib *et al*., 2020). Cancer cell heterogeneity poses a more significant challenge when establishing or identifying a typical cancer cell survival target. Discovering a drug that hinders multiple survival paths of cancer cells may increase the competence window

for a potent anti-cancer approach. Imperatively, this might also encourage pragmatic strategy over a single target in heterogeneous tumors (Swanton *et al*., 2011).

Cancer illness is increasing worldwide, signifying that the current cancer treatments need to be revisited to improve their efficacy and the development of less adverse toxicity drugs (Adamson, 2015). Therefore, there is an urgent need to develop more effective and less toxic anticancer agents. It is estimated that up to four billion people living in third world countries depend on herbal medicinal remedies as the primary source of their healthcare (Ekor, 2014). Thus, discovering anticancer agents of plant origin contributes to goal 3 'Good Health and Well-being' of United Nations sustainable development global goals especially in resource-limited settings (Yovo, 2022).

Pharmaceutical drugs' side effects generally are preponderant with the risk it carries relative to their benefits within their primary effect (Karimi *et al*., 2015). Hence, the exploration of medicinal plants is considered less toxic and endeavors a greater alternative mode of action to treating cancer. Although the current chemotherapies are successful, they are often toxic and detrimental. Besides being inexpensive, herbal remedies are becoming well accepted throughout the world. Therefore, physicians in many countries take considerate measurements to comprehend the knowledge and consecutively integrate it into their health care system (Clement *et al*., 2005; Maha and Shaw, 2007; Awodele *et al*., 2012). The aptitude of a plant-derived drug to land as a therapeutic compound relies on its selective toxicity exertion towards cancer cells, all at the same time not interrupting the quality of those healthy non-cancerous cells (Greenwell and Rahman, 2015).

2.2 Therapeutic benefits of medicinal plant products

Humans have been using plants as herbal medicines to alleviate diseases since 3,000 B.C. (Petrovska, 2012). Herbal medicine has been extensively referenced in several

traditional medicinal systems, such as Ayurveda, throughout history. Despite widespread claims about plants' curative properties, scientists have only lately recognised the use of plants as pharmaceutical agents, leading to the isolation of active chemicals and components from plants for disease treatment. The early 19th century discovery of morphine in *Papaver somniferum* (the opium poppy) highlights the importance of identifying active chemicals and components in natural compounds for pharmacological uses.

2.2.1 Bioactive natural compounds

Natural products' contributions to the development of novel pharmaceutical medications have prompted numerous pharmaceutical industries to invest in screening more plants for treatment of various diseases. Active ingredients are identified as secondary metabolites or phytochemicals distributed in different plant parts, such as leaves, fruits, seeds, stems, bark, roots, etc. In addition, endophytes predominantly microorganisms living in symbiotic relationship within the plant tissue are also known to produce biologically active compounds of medicinal value that are similar to host plant (Jia *et al*., 2016). Plant-derived phytochemicals are broadly classified into alkaloids, polyphenols, terpenes, organosulphur derivatives. Polyphenols are the most prominent family consisting of over 2000 compounds comprising significant classes such as flavonoids, phenolic acids, stilbenes, and lignans (Figure 2.1).

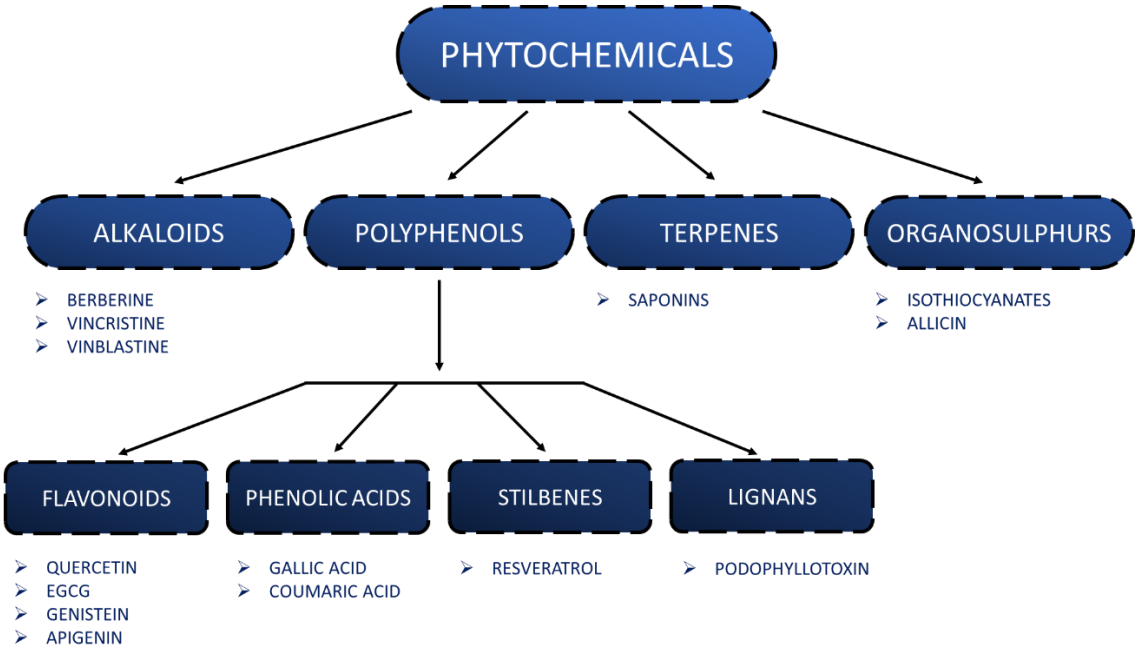

Figure 2.1: Classification of phytochemicals

Natural products, such as vinblastine and vincristine, vinca alkaloids produced from *Catharanthus roseus*, account for over 60% of anticancer drugs (Newman and Cragg, 2012). Polyphenols and their synthetic analogues, interestingly, top the list of prospective anticancer medicines in the treatment of ovarian, breast, pancreatic, and lung malignancies (Squires *et al*., 2003; Chan *et al*., 2006; Tang *et al*., 2012).

Antioxidant agents, such as polyphenols, are abundant in many medicinal plants. Plants' therapeutic capabilities are enhanced by the presence of antioxidant activity in conjunction with active phytochemicals. Polyphenols, such as flavonoids, are important in lowering the risk of disease in humans, such as cardiovascular disease, inflammation, and cancer (Hertog *et al*., 1995; Fotsis *et al*., 1997; Hollman and Katan, 1997; Agrawal, 2011; Kumar *et al*., 2014). Free radicals cause oxidative stress, which contributes to disease development (Machlin and Bendich, 1987; Uddin and Ahmad, 1995). Polyphenols are well-known antioxidants with the ability to neutralise free radicals. Polyphenols have redox characteristics that allow them to operate as potential antioxidants by reducing or blocking free radicals (Soobrattee *et al*., 2005).

Biologically active phytochemicals that contribute to therapeutic characteristics are frequently found in low amounts (Dhanani *et al*., 2017). Extraction, homogenization, and grinding are just a few of the ways for extracting phytochemicals and important step in learning about the therapeutic qualities of plants. The most frequent approach for extracting phytochemicals from plant materials is extraction. Furthermore, the extraction solvent has a major impact on the characteristics of the extracts as well as the percentage yield of plant materials (Gong *et al*., 2012). Due to the existence of compounds with varying chemical characteristics and polarity, the results obtained with the extracts show that the solvent of choice can impact the composition and activity of the extracts (Sultana *et al*., 2009). Polar solvents, such as

water and methanol, are typically employed to extract polyphenols from plants; as a result, water and methanol are regularly used in most extraction methods. Methanol is also employed because it is relatively affordable, can dissolve a variety of compounds, and evaporates quickly, which is important when bioactive substances must be concentrated using a rotary evaporator. Methanol is also more effective in extracting low molecular weight polyphenols, resulting in more phenolic chemicals accumulating in the methanolic extracts (Dai and Mumper, 2010).

The breakthrough in the chemical field with the advances in chemical analysis led to the isolation and characterization of various purified bioactive compounds from plants, which initiated the exploration of plant sources as chemotherapeutic candidates for cancer (Cragg and Newman, 2005). The continuous rise in cancer cases and the failure of conventional synthetic chemotherapies due to drug resistance and excessive toxicity towards healthy normal non-targeted tissues have also pushed the utilization of naturally occurring phytochemicals of plants, which is shown to improve treatment efficiency with reduced side effects. Accordingly, increasing scientific evidence confirms the remarkable anticancer activity induced by phytochemicals derived from plants. This has prompted to explore the role of phytochemicals in cancer cure.

2.2.2 Plant-derived chemotherapy for cancer treatment

Plant-derived chemotherapy agents are currently used to treat various cancers. Mainly they are attributed to one of the four classes, namely the vinca alkaloids (e.g., vincristine and vinblastine), the taxanes (e.g., Paclitaxel), the camptothecin derivatives (e.g., camptothecin and irinotecan), and the epipodophyllotoxins (e.g., etoposide and teniposide). Vinca alkaloids such as vinblastine and vincristine are commonly used to treat leukemia, non-small cell lung cancer, Hodgkin's lymphoma, breast cancer, brain cancer, bladder cancer, melanoma, and testicular cancer (Thirumaran *et al*., 2007;

Moudi *et al*., 2013). Taxanes such as Paclitaxel from plant *Taxus brevifolia* are commonly used to treat cervical, ovarian, breast, bladder, Kaposi's sarcoma, prostate, lung, and pancreatic cancers (Sharifi-Rad *et al*., 2021). Irinotecan is a semi-synthetically derived agent from the camptothecin alkaloid extracted from the *Camptotheca acuminata* (Robert and Rivory, 1998). It is commonly used to treat colon and small cell lung cancer either as a single agent or adjunct to another chemotherapy (Hamilton and Rath, 2016; Kondo *et al*., 2018). The semisynthetic derivatives of the natural substance epipodophyllotoxin, derived from the plant *Podophyllum peltatum*, etoposide and teniposide, are two clinically active compounds (Cragg and Newman, 2013; Lee and Xiao, 2005). Etoposide is commonly used as an adjuvant with other chemotherapies to treat small cell lung cancer (Jett *et al*., 2007; Rezonja *et al*., 2013) and testicular cancer (Pectasides *et al*., 2004), while teniposide is used to treat acute lymphocytic leukemia (ALL) (Bjorkholm, 1990) and non-Hodgkin's lymphoma (Tirelli *et al*., 1984).

2.3 Mechanisms of phytochemical induced cell death

Green phytochemical from Mother Nature, especially medicinal plants, is a rich source of novel therapeutics and prevention agents. Most plants yield a vast array of phytochemicals that play essential roles in cancer prevention and cure via various biological activities and unknown mechanisms of action. Since cancer has a significant impact on human health and contributes to mortality, it is appropriate to examine the role of phytochemicals in cancer prevention and cure (Vineis and Fecht, 2018). Phytochemicals are effective in cancer prevention and cancer cures via antioxidant activity, pro-oxidant activity, apoptosis induction, necrosis induction, autophagy induction, and miRNA regulation, as shown in Figure 2.2.

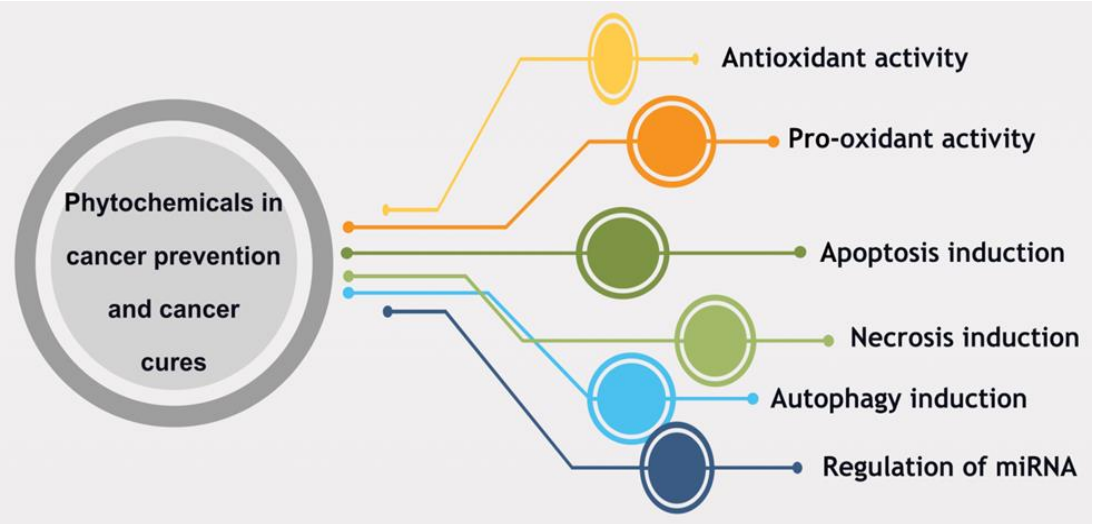

Figure 2.2: The role of phytochemicals in cancer prevention and cancer cures via various modes of action in cancer cells
Source: Cilwyn *et al.*, 2021.

2.3.1 Role of phytochemicals in cancer prevention via antioxidant activity

A free radical is a molecule (or atom) comprising one or more unpaired electrons in outer orbit, making them unstable and highly reactive. This leads them to steal electrons from other molecules to achieve stability. Subsequently, the attacked molecule converts to a free radical to start the chain reaction cascade, eventually injuring the healthy cells (Mukherji and Singh, 1984). Free radicals consist of reactive oxygen species and reactive nitrogen species, made in the human body naturally via numerous endogenous systems according to diverse physiochemical or pathological circumstances (Valko *et al*., 2007). Free radicals are created in the human body either from usual important metabolic routes or from outside sources such as exposure to irradiation, ozone, pollutants, and various toxic chemicals (Bagchi and Puri, 1998). However, the generated free radicals can be dangerous at an excessive level and damage the main apparatuses and biomolecules of cells such as DNA, proteins, and cell membranes. The various injuries caused by excessive free radicals to human cells, particularly the damage to DNA, may lead to cancer and other diseases (Dreher and Junod, 1996). Antioxidants, also identified as “free radical scavengers,” are chemicals such as phytochemicals that neutralize the highly reactive free radicals by donating an electron to the free radical or via quenching chain-initiating catalyst prevent them from instigating harm to the healthy cells. Hence, the plants' phytochemicals react as a natural antioxidant to block free radicals' harmful action, preventing cancer development in humans (Rohman *et al*., 2006). Plant phytochemicals are the most commonly known antioxidants, including ascorbate, tocopherols, polyphenols, and terpenoids (Dimitrios, 2006).

The anticancer activity of phytochemicals via antioxidant activity may depend on the diverse inter-dependent pathway, as shown in Figure 2.3. Firstly, the phytochemical will enter the cells to start the pro-oxidant anticancer activity. The accumulation of phytochemicals in the cell will reduce the excessive amounts of free radicals, thus protecting the cell from cancer development. Conclusively, the various inter-dependent processes exhibit the beneficial effects of the antioxidant activity of phytochemicals that efficiently prevent cancer development in humans.

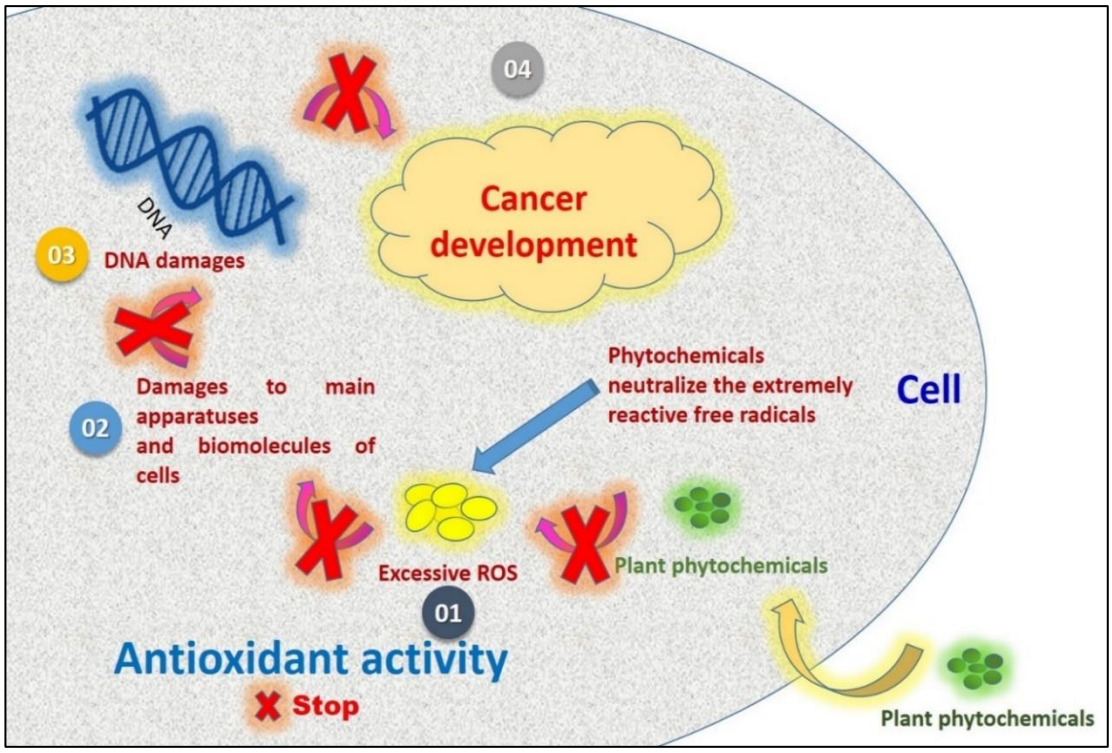

Figure 2.3: The antioxidant activity of phytochemicals in cancer prevention
Source: Cilwyn *et al.*, 2021.

2.3.2 Role of phytochemicals in cancer prevention via pro-oxidant activity

Pro-oxidant represents any phytochemicals that persuade oxidative stress either by generating free radicals or hindering the antioxidant systems. The redox level will determine how well a healthy cell is functioning. Besides, the protection of antioxidant systems is essential for the survival of the cells. Free radicals play a vital part in cancer. Excessive exposure to free radicals has been linked with increased cancer risk. Although high free radicals can be an increased risk of cancer for a normal cell, a high level of free radicals can also trigger apoptosis and cell death in various cancer cells. Decisively, the actions of free radicals in cancer development, inhibition, and treatment are tremendously complex and highly challenging to research. Alteration of redox homeostasis in cancerous and healthy cells recommends that pro-oxidant-based upregulation of cellular free radicals would target specifically against cancer cells without damaging the normal healthy cells (Wondrak, 2009). Even though the antioxidant activity of various phytochemicals is well researched and commonly applied to stop or cure cancer, multiple phytochemicals also exhibit the pro-oxidant and free radicals generating activities under unique conditions, especially in cancer cells. Accordingly, a pro-oxidant activity, a phytochemical, will attack cancerous cells, which are already at an extraordinary level of free radicals with high oxidative stress without affecting the well-tolerated non-cancerous cells (González-Bártulos *et al*., 2015). Several phytochemicals that target the cellular redox balance produce excessive reactive oxygen species (ROS) (Kruk *et al*., 2019) and eventually lead to cell death. Moreover, the transition metal-based phytochemicals could be favorable for pro-oxidant therapies (Rahal *et al*., 2014). Once the metal-based phytochemicals accumulate metals, namely iron and copper, induced the cycling redox reactions in the cancer cells, which will lead to the production of excessive amounts of free radicals, mainly the highly damaging

hydroxyl radical species via the Fenton reaction. The phytochemicals belonging to the flavonoid group, such as quercetin and kaempferol, have been reported to exhibit the pro-oxidant activity when a transition metal is available (Halliwell, 2008).

The anticancer activity of phytochemicals via pro-oxidant activity may depend on diverse inter-dependent routes, as shown in Figure 2.4. Firstly, the phytochemical will enter the cells to start the pro-oxidant anticancer activity. Secondly, the accumulation of phytochemicals will trigger the cell to produce excessive free radicals. Subsequently, the excess free radicals will trigger DNA fragmentation and DNA damage via oxidative mechanisms. An excessive amount of free radicals in the cells will react with the cellular DNA, thus altering its structure and disturbing the normal function of the DNA. This is one of the main reasons for DNA damage induced by pro-oxidant activity of the phytochemical (Beckman and Ames, 1997). Even though the DNA molecule is intact, free radicals can act against the DNA. They can cause various harms, namely alteration of nucleotide bases, single and double-strand DNA molecule disruptions, loss of purines in the DNA, destruction to the deoxyribose sugar, cross-link between DNA and protein, and destruction of the standard DNA repair systems (Srinivas *et al*., 2019). The DNA fragmentation will lead to the induction of cell cycle arrest. Eventually, this will lead to apoptotic cell death. Conclusively, the various inter-dependent processes exhibit the beneficial effects of the pro-oxidant activity of phytochemicals that efficiently kills the cancer cells.

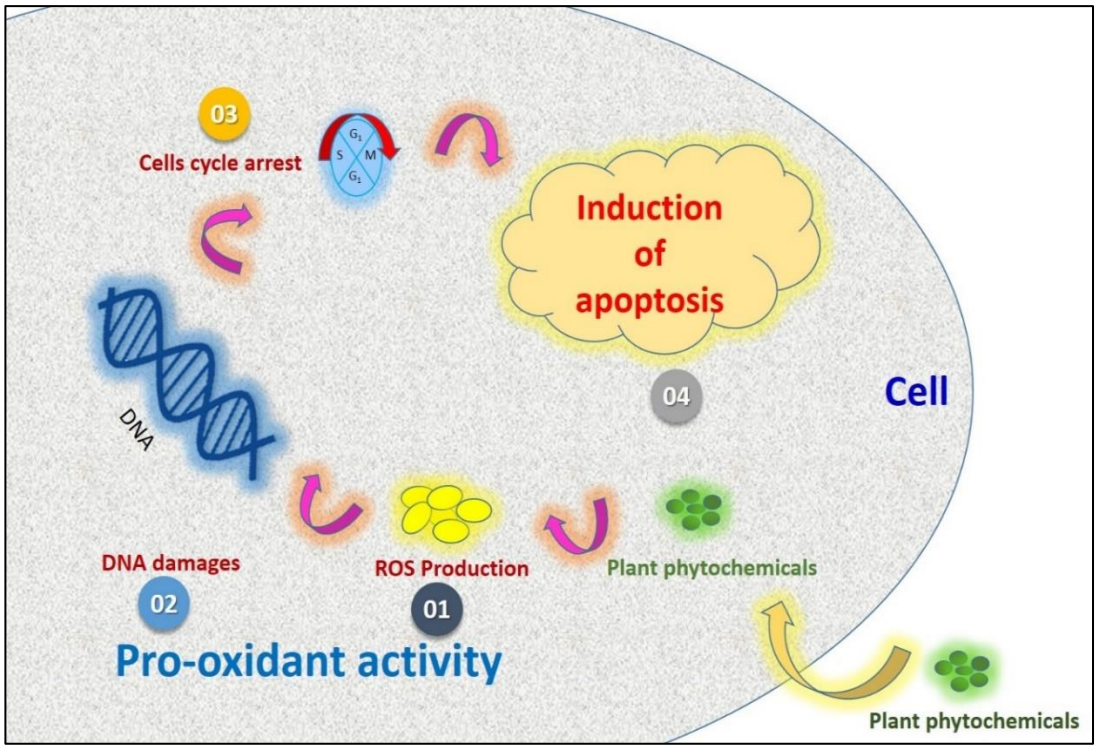

Figure 2.4: The pro-oxidant activity of phytochemicals in cancer prevention
Source: Cilwyn *et al.*, 2021.

2.3.3 Role of phytochemicals in cancer cure via apoptosis induction

Major plant-based phytochemicals such as terpenoids, phenolic acids, and alkaloids have shown potentially promising anticancer properties by fine-tuning the ROS signalling pathways (Chirumbolo *et al*., 2018). In line with the anti-oxidative and ROS-scavenging properties numerous phytochemicals have been studied and scientifically demonstrated to induce apoptosis through ROS generation. An established plant-based anticancer agent can exemplify this, resveratrol, which was shown to induce caspase-8/caspase-3-dependent apoptosis in human colon cancer cells, HCT29 and COLO201, significantly increasing the intracellular ROS levels (Miki *et al*., 2012). Another commonly known phytochemical exhibiting anticancer activity, quercetin, was also shown to inhibit cancer growth by inducing apoptosis via cyclooxygenase-2 (COX-2)-dependent ROS generation.

Furthermore, numerous phytochemicals showed potentially promising anticancer activity by inducing apoptosis via up-regulating the expression of caspases. This can be exemplified by a phytochemical called hyperforin which was reported to promote caspase-dependent apoptosis in various leukemia cell lines by up-regulating caspase-9, caspase-8, and caspase-3 (Hostanska *et al*., 2003). One such phytochemical is corosolic acid, which promotes caspase activation, leading to mitochondria-mediated signalling pathways to induce cell death in HeLa cells (Xu *et al*., 2009). The purified bioactive compound, pyranocycloartobiloxanthone A, was also reported to play an imperative role in inducing apoptosis in breast cancer cells by up-regulating Bcl-2 expression and down-regulating Bax expression, eventually leading to the release of Cytochrome c, initiating the caspase cascade (Mohan *et al*., 2012).

Interestingly, another phytochemical from the class of phenolics, known as Scutellarin, has been proven to promote apoptosis by activating the p53 pathway (Yang

et al., 2017). Scutellarin was found to suppress the anti-apoptotic protein Bcl-2, which eventually activates the pro-apoptotic protein, p53, leading to the up-regulation of Bax protein to induce caspase-3 dependent apoptosis in human colon cancer (Yang *et al*., 2017). Another such phenolic compound, gallic acid, was also reported inducing apoptosis in cancer cells via the up-regulation of the p53. This, in turn, depolarizes the mitochondrial membrane potential and facilitates the release of caspase-activator, Cytochrome c, and induces an intrinsic apoptotic pathway (Yang *et al*., 2018). The role of another important phytochemical capsaicin to induce p53-mediated apoptosis in various cancer cells has been well-elucidated in previous studies (Jin *et al*., 2014; Clark and Lee, 2016; Garufi *et al*., 2016).

Various other phytochemicals have been reported to play an essential role in cancer cure by targetting nuclear factor kappa B (NF-kB) to promote cancer cell death (Kumar. M *et al*., 2016). The fact that NF-kB is highly expressed in cancer cells is inevitable due to its regulation of anti-apoptotic and apoptotic genes (Tse *et al*., 2007; Manu and Kuttan, 2008; Oh *et al*., 2012; Kumar. M *et al*., 2016). Intriguingly, various phytochemicals, including alkaloids and flavonoids, induce apoptosis in cancer cells by targeting the NF-kB signalling pathway. For instance, known phytochemicals including xanthohumol (Colgate *et al*., 2007), magnolol (Tse *et al*., 2007), morusin (Lee *et al*., 2008), ursolic acid (Manu and Kuttan, 2008), corilagin (Gambari *et al*., 2012) were demonstrated to significantly down-regulate the expression of NF-kB in various cancer cells. The suppression of this apoptosis-inhibitor, NF-kB, eventually leads to tumor necrotic factor-α (TNF-α)-induced apoptosis. A recent review collectively elucidated TNF-α induced activation of NF-kB in mitochondria to stimulate programmed cell death by releasing Cytochrome c to the cytoplasm, then activating a caspase cascade (Albensi, 2019).

Besides, several plant-based secondary metabolites are reported to induce the extrinsic-pathway of apoptosis in cancer cells. A form of flavonol can depict this, kaempferol, which has been previously reported to up-regulate the expression of FasL, leading to the activation of caspase-8 in colon cancer cells (Lee *et al*., 2014). Bid protein, which is cleaved by the activated caspase-8 in the extrinsic apoptosis pathway, is translocated into the mitochondria, promoting intrinsic apoptosis (Lim *et al*., 2014). A phenolic compound called hispidin has been proven to increase the level of death receptor 3 in colon cancer cells, leading to activation of the caspases involved in the extrinsic apoptosis pathway, namely caspase-1 and caspase-8, along with the cleavage of PARP to induce cell death (Hengartner, 2000). Cumulatively, it can be suggested that these plant-derived biologically active compounds induce apoptosis mainly through the mitochondria-dependent mechanism. In short, phytochemicals promote ROS generation in cancer cells, causing polarization of mitochondria membrane potential leading to the release of various toxin proteins, including Cytochrome c. Eventually, Cytochrome c attaches to apoptotic protease activating factor-1 (Apaf-1), which activates caspase-9, forming the Cytochrome c/Apaf-1/caspase nine complexes known as the apoptosome, which activates the caspase-3, ending in apoptosis (Hengartner, 2000). On the other hand, phytochemicals were also found to be inducing the extrinsic apoptotic pathway by up-regulation of death ligands such as FasL, TNF-α, TRAIL. These ligands are responsible for activating caspase-8 by binding to death receptors such as FAS, TNFR, and other death receptors. Active caspase-8 cleaves Bid protein into tBid, which is then translocated to mitochondria to promote BAX and BAK proteins, allowing the intrinsic pathway to occur by activating the caspase cascade. Figure 2.5 shows the intrinsic and extrinsic apoptotic pathways induced by phytochemicals.

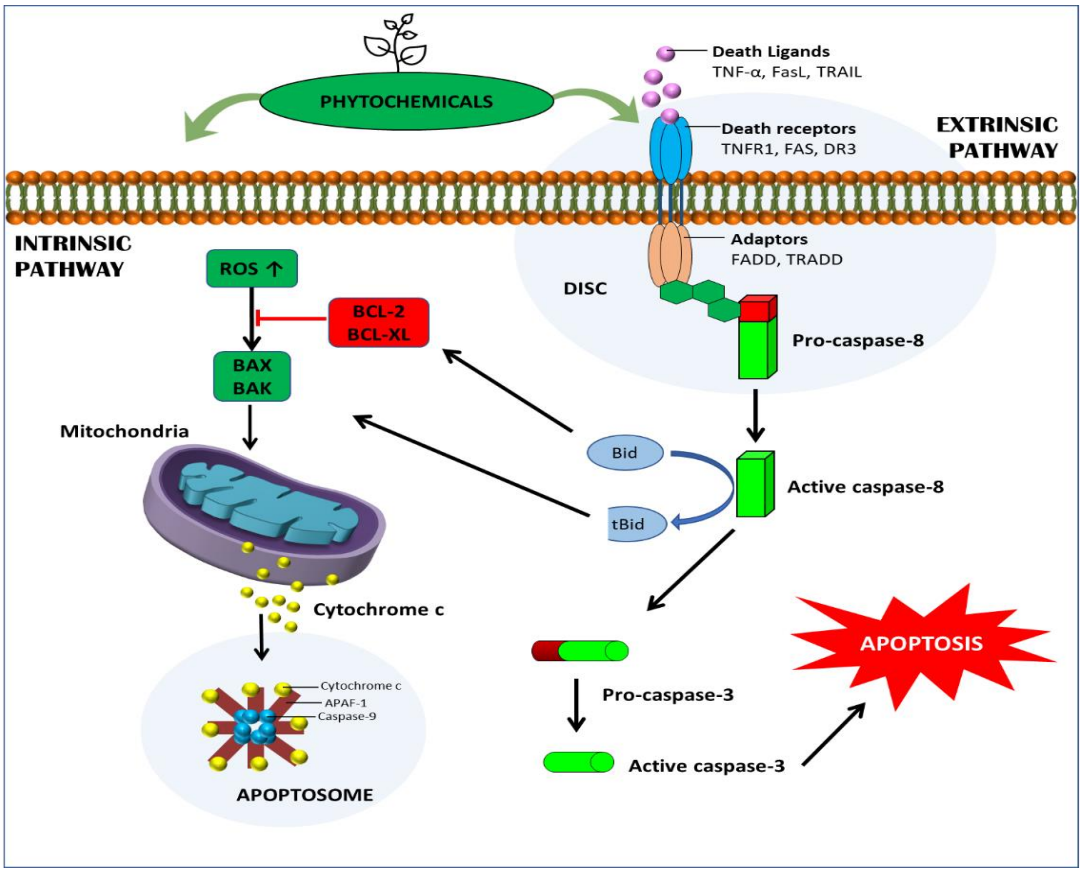

Figure 2.5: Intrinsic and extrinsic apoptosis mechanisms induced by phytochemicals
Source: Cilwyn *et al.*, 2021.

2.3.4 Role of phytochemicals in cancer cure via necrosis induction

Phytochemicals have been shown to protect cells by interfering with their molecular pathways that regulate the cell cycle, survival, angiogenesis, and cell death. These properties made phytochemicals an essential source of drugs for the prevention and treatment of cancer. Notably, most of these compounds target the apoptotic mechanism mainly by interfering with caspase-dependent pathways (Ashraf, 2020). However, other non-apoptotic cell death pathways such as necrosis also play an essential role in checking neoplastic cells and destroying tumor cells. Inducing necrosis is a known mechanism of some anticancer drugs, such as DNA-alkylating agents, in treating human cancers (Cho and Park, 2014). Therefore, understanding the role of phytochemicals in signalling cascades involved in the induction of necrotic cell death will allow to develop a novel drug to treat cancer.

Depending on the physiological and pathological conditions, a cell would either take the apoptotic or necrotic pathway. Unlike apoptosis, necrosis does not have a dedicated molecular pathway. Instead, it overlaps with many of those caspase-independent apoptotic pathways, which culminates in disruption of organelle and loss of membrane integrity resulting in the spillover of cellular contents (Lee *et al*., 2018). In a tumor microenvironment, induction of the necrotic pathway would cause more damage as it destroys the cells. The contents released from these cells create a pro-inflammatory environment (Lee *et al*., 2018). Phytochemicals with enhanced necrosis may help to exert more effective tumor suppression properties.

A programmed and well-controlled form of necrosis is necroptosis, mainly triggered by extracellular stimuli similar to the extrinsic apoptotic pathway. Necroptosis is primarily orchestrated by serine/threonine kinase receptor-interacting protein 1/3 (RIP1 and RIP3) to induce necrotic cell death. RIP1 and RIP3 can be activated by