THE EFFECT OF *PHYLLANTHUS DEBILIS* EXTRACT ON DNA METHYLATION AND miRNA REGULATION IN HT-29 COLORECTAL CANCER CELL LINE

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by

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LIST OF SYMBOLS

µL	microlitre
Mg	miligram
µg	microgram
Ml	mililitre
G	gram
M	Meter
mm	milimeter
nM	nanomolar
µM	micromolar
mM	Millimolar
µg/ mL	Microgram per mililitre
mg/ mL	Milligram per mililitre
mL/min	Mililitre per minute
µL/min	Microlitre per minute
^{o}C	Degree Celsius
%	Per cent
Δ	Delta
v/v	Volume per volume
β	beta

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AlCl3	Aluminium (III) chloride
Alu	Alu element
cDNA	Complementary DNA
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1- Diphenyl-2- picryl- hydrazyl
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
GAE	Gallic acid equivalent
GCMS	Gas chromatography- mass spectrometry
HPLC	High performance liquid chromatography
HT-29	Human Colorectal Adenocarcinoma.
IC50	Half minimal inhibitory concentration
LCMS	Liquid chromatography- mass spectrometry
LINE-1	Long interspersed nuclear element
MCF-7	Human Breast cancer cell line
MCF-10A	Human mammary epithelial cell
MiRNA	microRNA
mRNA	Messenger RNA
PCR	Polymerase chain raction
PBS	Phosphate buffer saline
RT-qPCR	Reverse transcription real time PCR

KESAN EKSTRAK *PHYLLANTHUS DEBILIS* PADA METILASI DNA DAN REGULASI miRNA DALAM SEL KANSER KOLOREKTAL HT-29

ABSTRAK

Phyllanthus adalah salah satu tanaman herba yang memiliki sifat antikanser. Namun tidak banyak laporan mengenai kesan produk semula jadi ini terhadap epigenetik barah. Oleh itu, kajian ini dilakukan untuk mengetahui kesan ekstrak *Phyllanthus debilis* terhadap metilasi DNA dan regulasi miRNA dalam sel kanser kolorektal HT-29. Kajian ini dimulakan dengan menentukan spesies *Phyllanthus* yang paling sesuai untuk digunakan dalam menentukan regulasi epigenetik pada sel HT-29. Tiga spesies *Phyllanthus* iaitu *Phyllanthus urinaria, Phyllanthus niruri* dan *Phyllanthus debilis* digunakan. Setiap spesies *Phyllanthus* kemudian diekstrak dengan menggunakan 100% metanol dan 100% air. Ekstrak kemudian diuji untuk Jumlah Kandungan Fenolik (TPC) dan Jumlah Kandungan Flavonoid (TFC) dan aktiviti antioksidan ektrak dengan menggunakan ujian DPPH dan ABTS. Seterusnya, ekstrak disaring dengan HPLC untuk mengesan kehadiran komponen yang disasarkan. Indeks terapeutik setiap ekstrak *Phyllanthus* kemudian diukur pada sel barah (sel payudara dan kolorektal) berbanding sel normal (sel payudara normal). Ekstrak metanol *P. debilis* menunjukkan indeks terapeutik tertinggi jika dibandingkan dengan ekstrak lain. Ekstrak metanol *P. debilis* kemudian digunakan untuk mengkaji mekanismenya dalam mengatur metilasi DNA, ekspresi gen dan miRNA dalam sel HT-29 yang menyebabkan sel mati. Dalam ekstrak air *P. debilis*, asid kafeik dan asid *p*-coumaric dijumpai. TPC ditemui dalam julat 107.09 hingga 308.71 mg GAE/g DW sementara TFC ditemui antara 7.07 hingga 35.86 mg QE/g DW. Ekstrak metanol *P. urinaria* mempunyai TPC dan TFC tertinggi dan

merupakan pengurai radikal bebas yang paling berkesan terhadap ujian DPPH dan ABTS. Analisis HPLC menunjukkan bahawa ekstrak metanolik *P. urinaria* menunjukkan adanya asid kafeik, asid *p*-coumaric dan myricetin sementara ekstrak air spesies ini hanya menunjukkan adanya myricetin. Ekstrak metanol *P. niruri* menunjukkan terdapat kandungan asid *p*-coumaric dan naringenin dan ekstrak air spesies ini menunjukkan adanya asid kafeik, asid *p*-coumaric, myricetin dan naringenin. Komponen yang dikesan dalam ekstrak air *P. niruri* juga dapat dikesan dalam ekstrak metanol *P. debilis*. Indeks terapeutik menurun mengikut urutan berikut; ekstrak metanol *P. debilis* (4.6581)> ekstrak air *P. debilis* (2.8308)> ekstrak air *P.niruri* (2.3572)> ekstrak metanol *P. niruri* (0.9819)> ekstrak air *P. urinaria* (0.8971)> ekstrak metanol *P. urinaria* (0.8356. Dalam metilasi global *Alu* dan *LINE-1,* ekstrak metanol *P. debilis* menunjukkan peningkatan yang ketara pada metilasi DNA pada status metilasi Alu ($P < 0.05$) dan peningkatan ketara dalam status metilasi *LINE-1* ($p < 0.01$). Dalam metilasi spesifik gen *TAC1*, ekstrak metanol *P. debilis* menunjukkan penurunan metilasi DNA yang ketara di lokasi 1, lokasi 2, dan di lokasi 3 ($p < 0.05$). Penurunan status metilasi *TAC1* tidak menunjukkan adanya hubungan yang ketara dengan ekspresi gen *TAC1* ($p > 0.05$). Walau bagaimanapun, ekstrak metanol *P. debilis* telah meningkatkan ekspresi miR-125a-5p dengan perubahan 2.255 kali ganda dan miR-320a dengan perubahan 1.629 kali ganda. Kesimpulannya, *Phyllantus sp*. mempunyai antioksidan tinggi yang mengandungi sebatian polifenolik. Sebatian ini mempengaruhi aktiviti sitotoksisiti *Phyllanthus sp.*, metilasi DNA, ekspresi gen dan miRNA bagi sel HT-29.

THE EFFECT OF *PHYLLANTHUS DEBILIS* EXTRACT ON DNA METHYLATION AND miRNA REGULATION IN HT-29 COLORECTAL CANCER CELL LINE

ABSTRACT

Phyllanthus is one of the herbal plants that have been found to have anticancer property. Unfortunately, there is lack of study on the effect of the plant natural products on the epigenetics of cancer. Hence, this study was carried out to determine the effect of *Phyllanthus debilis* extract on DNA methylation and miRNA regulation in HT-29 colorectal cancer cell line. The study was started by determining the most suitable *Phyllanthus* species to be used in the epigenetic regulation of HT-29 cell line. Three species of *Phyllanthus* named *Phyllanthus urinaria, Phyllanthus niruri* and *Phyllanthus debilis* were used. The phytochemicals of each *Phyllanthus* species were then extracted using 100% methanol and 100% water. The extracts were then tested for Total Phenolic Content (TPC) and Total Flavonoid content (TFC) and the antioxidant activities of the plant extracts using DPPH and ABTS assays. Then, the extracts were screened with HPLC to detect the presence of targeted compounds. The therapeutic index of each *Phyllanthus* extract was then measured on cancer cell lines (breast and colorectal cell lines) versus normal cell line (normal breast cell line). *P. debilis* methanolic extract showed highest therapeutic index when compared to other extracts. *P. debilis* methanolic extract was then used for further study to investigate its mechanisms in regulating DNA methylation, gene and miRNA expressions in HT-29 cell line that led to the cancer cell death. The TPC of the extracts ranged from 107.09 to 308.71 mg GAE/g DW while their TFC ranged from 7.07 to 35.86 mg QE/g DW. *P. urinaria*

methanolic extract had the highest TPC and TFC and it was the most potent scavenger of DPPH and ABTS radicals. HPLC analysis revealed that *P. urinaria* methanolic extracts had caffeic acid, *p*-coumaric acid and myricetin while the water extract of this species only had myricetin. *P. niruri* methanolic extract had *p*-coumaric acid and naringenin, and water extract of this species had caffeic acid, *p*-coumaric acid, myricetin and naringenin. Similarly, the compounds found in *P. niruri* water extract were also found in *P. debilis* methanolic extract. In *P. debilis* water extract, caffeic acid and *p*-coumaric acid were found. The therapeutic index decreased in the following order *P. debilis* methanol extract (4.6581) > *P. debilis* water extract (2.8308)>*P. niruri* water extract (2.3572)> *P. niruri* methanol extract *(*0.9819*)*> *P. urinaria* water extract *(*0.8971)>*P. urinaria* methanol extract (0.8356). In *Alu* and *LINE-1* global methylation, *P. debilis* methanolic extract caused a significant increase of DNA methylation at *Alu* repeat sequence ($P < 0.05$) and significant increase in *LINE-1* methylation status ($p < 0.01$). In *TAC1* gene specific methylation, *P. debilis* methanolic extract caused a significant reduction of DNA methylation at site 1, site 2, and site 3 ($p < 0.05$). The decrease of *TAC1* methylation status did not have any significant correlation with the expression of *TAC1* gene ($p>0.05$). However, *P. debilis* methanolic extract did significantly increase the expression of miR-125a-5p by 2.255-folds and miR-320a by 1.629-folds. In conclusion, *Phyllanthus sp.* possesses high antioxidants which include polyphenolic compounds. These compounds exert cytotoxicity activity of *Phyllanthus sp.* and regulate DNA methylation, gene expression and miRNA expression of HT-29 cell.

CHAPTER 1

INTRODUCTION

1.1 1.1 Background of the study

Phyllanthus is the flowering plant belongs to *Euphorbiaceae* family *(Narendra* et al., 2012). *Phyllanthus* species are small herbal plants which are native to India but can also be found in the tropical region worldwide (Sabir and Rocha, 2008). It grows 50–70 cm tall, and bears herbaceous branches ascending, and the bark is smooth. It bears several pale green flowers that are often flushed with red. The fruits are small, smooth capsules which contain seeds (Mamat & Yaacub., 2015). In Malaysia, these species are referred to as 'dukung anak' (to hold a child) as its fruit is located beneath the leaf petiole (Mediani *et al.,* 2015). Many species of plant *Phyllanthus* are regarded as weeds. They are well suited to unfavourable growing conditions and can flourish almost anywhere, including on the roadside and unattended flowerpots (Mamat & Yaacob., 2015). *Phyllanthus* species such as *P.debilis, P. pulcher, P.acidus, P. reticulatus, P. myrtifolius, P. urinaria* and *P. niruri* are traditionally used for treatment of several diseases in many tropical countries. The three most common *Phyllanthus* species that are traditionally used for medicinal purposes are *P. urinaria, P.niruri* and *P. debilis* (Mediani *et al.,* 2015).

Phyllanthus is commonly used in traditional medicine and recently became one of the most well-studied plants for the development of pharmaceutical products. In traditional practices, *Phyllanthus* species are used for the treatment of cancer (Huang *et al*., 2010), neonatal jaundice, intestinal infections, kidney stones, diabetes and hepatitis B (Calixto *et al*., 1998; Kumaran and Karunakaran, 2006). Due to its secondary metabolites, *Phyllanthus* sp. was shown to have strong medicinal properties. *Phyllanthus sp.* had been shown to possess hepatoprotective, anti-carcinogenic, anti-

bacterial, anti-viral and anti-inflammatory activity (Kalidas and Mohan, 2009). Many herbs of the *Phyllanthus* genus had been shown to contain different combinations of secondary metabolites that make them to have medicinal properties. The common compounds found in *Phyllanthus* were phyllanthin, hypophyllanthin, and niranthin which had been proven to have anti-cancer and antitumor properties (Islam et al., 2008). Other compounds found in the plant species such as rutin, quercetin and corilangin possess antioxidant and antiviral properties (Notka *et al.,* 2004;Londhe *et al*., 2008).

In Malaysia, colorectal cancer is the second most common cancer in males and the third most common cancer in females (Veetil *et al*., 2017). In developed Asian countries such as South Korea, Singapore and Japan, colorectal cancer is one of the most common cancer found in their population (Veetil *et al.,* 2017). One of the aetiologies of colorectal cancer has been proposed as the development of adenoma-carcinoma sequence, in which multiple genetic and epigenetic alterations had occurred, such as cancer-associated gene mutations and epigenetic modifications occurred (Leslie *et al*, 2002; Gattoliat *et al.*, 2015). The main interest in this study is the epigenetic changes that occurred in colorectal cancer. Epigenetics is the heritable gene expression changes not caused by any alteration in the DNA sequence (Esteller, 2008). These regular epigenetic changes are generally associated with an early cancer development and associated with cancer-related gene mutations. Epigenetic modifications or alterations include DNA methylation changes, microRNAs (miRNAs), and histone modifications (Leslie *et al.,* 2002; Gattoliat *et al.,* 2015). These epigenetic changes can be seen at global scale and or at specific genes promoter region.

DNA methylation occurs in the 5 position of the cytosine pyrimidine ring in the dinucleotide sequence, 5'-C–phosphate–G-3' (CpG), which forms one of the

several layers of epigenetic mechanisms that regulate and modulate gene expression via the structure of chromatin. (Tost., 2009). DNA methylation is important for the proper development of mammals, crucial for imprinting, and plays a role in maintaining genomic stability and in the compensation of dosages (Tost., 2009). DNA methylation pattern changes dramatically as cells are cancerous, due to two major phenomena. First, the tumor genome is hypomethylated globally, unlike in normal cells, primarily due to the widespread demethylation in the CpGs distributed across the tumors' body. Second, local and discrete regions situated at the promoter region of tumor-suppressor genes undergo intense hypermethylation (Esteller, 2008).

MicroRNAs (miRNAs) are short non-coding transcripts that play fundamental roles in various biological processes. miRNAs regulate gene expression by binding to target mRNAs, preventing their expression (Bartel *et al.,* 2004;Gattoliat *et al*, 2015). miRNAs are involved in the regulation of important cellular pathways, such as angiogenesis (Hua *et al.,* 2006), proliferation (Cheng *et al.,* 2005), cell death (Chan *et al.,* 2005) and the deregulation of cell growth which are all hallmarks of cancer (Hanahan & Weinberg., 2000). Thus, it is not shocking that in many different cancers, aberrant miRNA expression has been demonstrated. Also, unique miRNA profiles were shown to predict progression and outcome in a variety of cancers.

miRNAs are also dysregulated in human cancers, for example in breast and colorectal cancers. Dysregulation of miR-145 and miR-21 has been linked to tumor progression in breast cancer. Whereas in colorectal cancer, some expressions of miRNA such as (miR-125a and miR-320a) play a pivotal role in the cancer progression. (Lu-Ying *et al.,* 2014;Yang *et al.,* 2018). The tested role of miRNA in the progression of cancer highlights their potential as both novel therapeutics for cancer treatment and as prognostic markers. The miRNA expression occurs in a

developmental stage specific or tissue-specific manner and some miRNAs are imprinted, enabling gene expression to be epigenetically changed (Seit *et al*., 2003;Deo *et al.*, 2006).

The modification of these two epigenetic modifiers of the genes can be useful target for *Phyllanthus* sp. to reduce the risk of cancer. It is believed that the investigation of the potency of local resources such as *Phyllanthus* sp. could lead to the discovery of a potential anti-cancer agent, which employs molecular mechanism of regulating DNA methylation and miRNA expression.

1.2 1.2 Objectives

The aim of this study is to determine the effect P*hyllanthus debilis* extract on DNA methylation and miRNA regulation in HT-29 colorectal cancer cell line. The objectives were as follows:

1. To screen the chemical compounds in three *Phyllanthus* species by using High Performance Liquid Chromatography (HPLC) (*Phyllanthus urinaria, Phyllanthus niruri* and *Phyllanthus debilis*)
2. To determine phytochemical contents (Total Phenolic and Flavonoid Content) and evaluate antioxidant activities of different *Phyllanthus* extracts from the plant species.
3. To identify the therapeutic index of the *Phyllanthus* extracts on cancer cell lines (HT-29 and MCF-7 cells) against normal cells (MCF-10A cells).
4. To investigate the effect of *Phyllanthus* sp. on global DNA methylation (Alu and *LINE-1*) and gene specific methylation (*TAC1)* in the colorectal cancer cell lines (HT-29)
5. To investigate the effect of *Phyllanthus* sp. on miRNA-125a-5p and miRNA-320a expression in the colorectal cancer cell lines (HT-29)

CHAPTER 2

LITERATURE REVIEW

2.1 Nature of Cancer

Cancer can be defined in many ways, depending on the site, it encompasses a group of about 100 different and distinctive diseases. These diseases are characterized by an abnormal cell growth which generally results in uncontrolled proliferation. It can metastasise in other tissues and organs in some cases (Esteller, 2008). Cancer is the second leading cause of mortality worldwide (WHO., 2018), and traditional chemotherapy's inability to influence a substantial reduction in mortality suggests that new strategies are urgently needed (Sachithanandan, & Badmanaban., 2012). New and recent chemotherapeutic methods serve as alternatives to control malignancy (Kapadia *et al*., 2000).

2.2 Colorectal cancer

Colorectal cancer is the third most common cancer and the fourth leading cause of tumor related death globally (Yang et al., 2018). It is predicted that the global incidence of colorectal cancer will rise by 60% to over 2.2 million new cases and 1.1 million deaths by 2030. (Arnold *et al.*, 2017). However, in Singapore, South Korea and Japan, the incidence and mortality rates have been stable and are even declining. This phenomenon can be related to colorectal cancer screening services, reduced risk factor prevalence and/or enhanced care in these countries (Veetil *et al*., 2017). The most common methods of detection of colorectal cancer are through sigmoidoscopy and fecal occult blood test (FOBT). However, these two methods had been shown to have poor

specificity and sensitivity with the latter at risk of getting gut perforation (Ng & Wong., 2013).

In Malaysia, breast cancer is the most common cancer among women in Malaysia (Azeem *et al.,* 2015) while colorectal cancer is the second most common cancer in males and the third most common cancer in females (Veetil *et al*., 2017). Most of the colorectal cancer cases (65%) were detected at stage III and IV (Mustapha & Hassan., 2020) and the majority of patients were above 50 years old (National Cancer Patient Registry, 2010).

Surgery (Verweij *et al.,* 2016), chemotherapy (Tol *et al.,* 2009) and radiotherapy (Bosset *et al.,* 2006) are some of the methods used for treatment of colorectal cancer. Surgery is still an effective method for the treatment of colorectal cancer, but it may be difficult to treat elderly patients using the method. These patients have a higher incidence of post-treatment complications and mortality (Verweij *et al.,* 2016). Preoperative radiotherapy is another way of colorectal cancer treatment. Regardless of the protocol, preoperative radiotherapy reduces local rates of recurrence by 50 to 60 % compared to surgery alone (Bosset *et al.*, 2006). Given the increase in radiotherapy and chemotherapy, it does not appear that the overall survival rate of patients with colorectal cancer is significantly elevated.

The underlying mechanism for inducing this cancer and its development is largely unknown. Initially, it is believed that cancer arises not only because of the genetic variations but also because of epigenetic changes. Though genetics is concerned with the information transmitted on the basis of gene sequence, epigenetics is concerned with the inheritance of gene expression-based information (Esteller, 2008). Targeting the abnormal changes in genetic mutation and epigenetics could be the future treatment strategies in cancer. Figure 2.1 shows the stages of colorectal cancer.

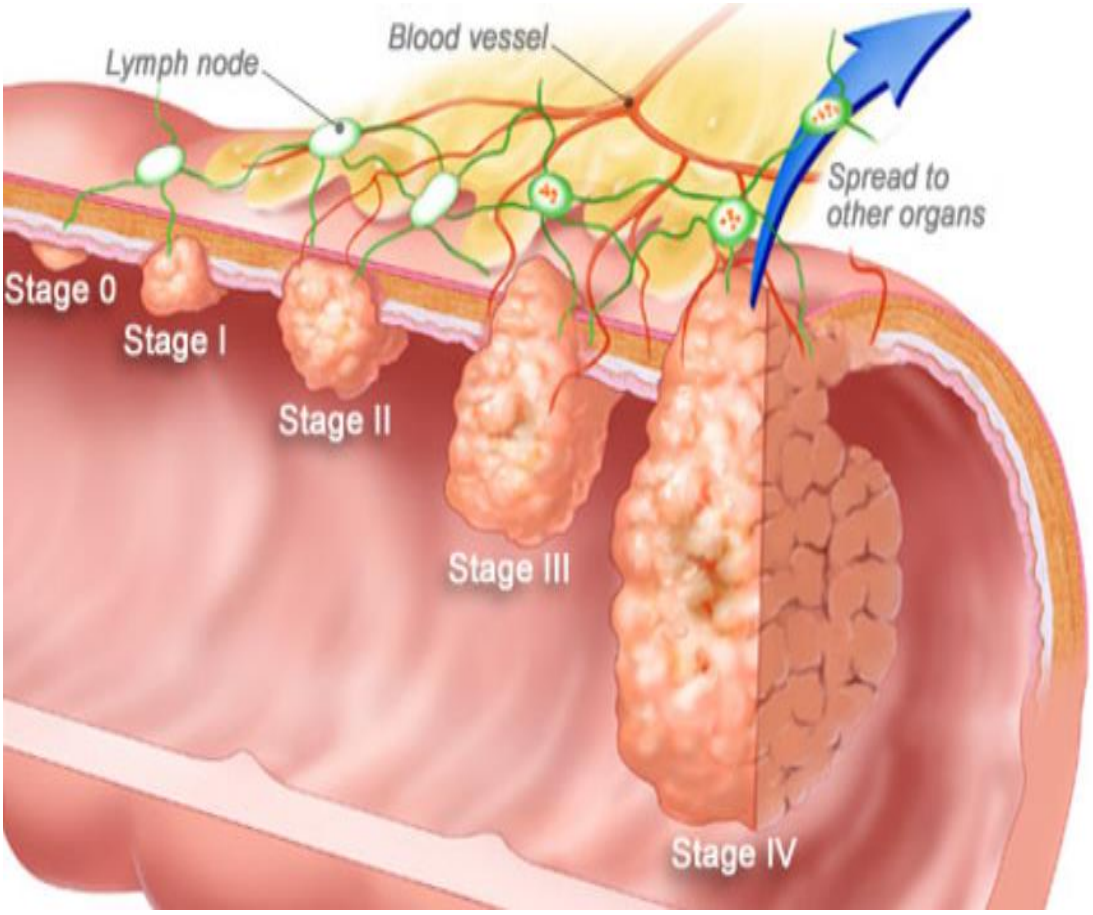

Figure 2.1. Stages of colorectal cancer. Cancer is in the innermost lining of the colon or rectum at stage 0. During Stage I, the disease has grown into the muscle layer of the colon or rectum. In stage II, cancer has grown into or through the outermost layer of the colon or rectum. The cancer had spread to one or more lymph nodes in the area at Stage III and lastly at stage IV it will spread to other parts of the body such as the liver, lungs or bones (WebMD, 2020).

2.3 Causes and Symptom of Colon Cancer

Colorectal cancers are adenocarcinoma of the colon, rectum and the appendix that commonly arise from the polyps (Skibber *et al*., 2001). As a result of the gradual accumulation of genetic and epigenetic changes, colorectal cancer develops leading to the transformation of normal colonic epithelium into colon adenocarcinoma (Grady & Markowitz., 2002). Three different pathogenetic pathways have been implicated in the development of these tumors. They were microsatellite instability, chromosomal instability and CpG island methylator phenotype (Pancione *et al.,* 2012). The loss of genomic stability is a crucial molecular and pathophysiological step in this process and helps to create a permissive environment for tumor suppressor gene and oncogene alterations to occur (Grady & Markowitz, 2002). Since colorectal cancers arise from cumulative accumulation of genetic and molecular changes over several years, the risk of developing colorectal cancer rises with age, with over 90% of new cases being diagnosed in patients over 50 years of age (Skibber *et al*., 2001). Most patients were diagnosed in the late stage and their survival rate of 5 years was lower (Veetil *et al*., 2017). Genetic alterations involve mutations of oncogenes and tumor-suppressor genes that directly control cell birth and cell death, such as KRAS, APC, and p53. Another alteration involves mutations of DNA mismatch repair genes such as MLH1 (Shen *et al.*, 2007).

Sign and symptoms of colorectal cancer include blood in the stool or a change in bowel habits. Other symptoms such as diarrhea, constipation, or feeling that the bowel does not empty all the way, stools that are narrower than usual, frequent gas pains, bloating, fullness, or cramps, weight loss for no known reason, feeling very tired and vomiting are also some of the symptoms that may be caused by colon cancer of colorectal cancer (National Cancer Institute, 2020).

2.4 Epigenetics

Epigenetics was first introduced by C.H. Waddington in 1939, which is a term used to name "the causal interactions between genes and their products, which bring the phenotype into being,". It was then identified as those heritable changes in gene expression which are not caused by any alteration in the DNA sequence (Esteller, 2008). Epigenetic modification includes changes in histone modifications, DNA methylation and microRNAs (miRNAs) (Leslie *et al.,* 2002; Gattolliat *et al.*,2014). Epigenetic modifications or alterations are common in colorectal development sequences of adenocarcinoma and sometimes followed by genetic mutations (Leslie *et al.,* 2002; Gattolliat *et al*.,2014). Epigenetic changes are often influenced by many factors as depicted by Figure 2.2.

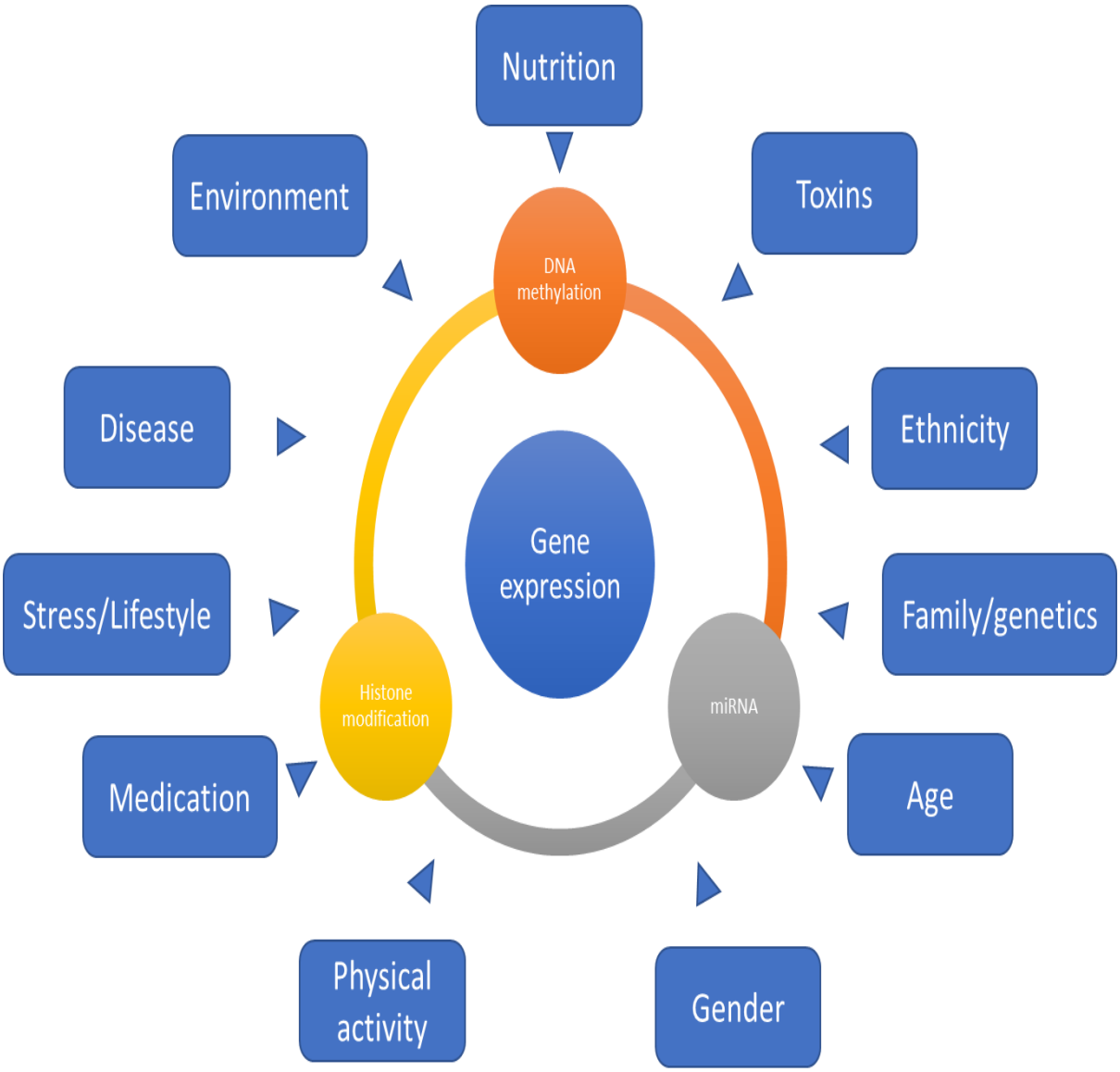

Figure 2.2. Modulation and interaction of epigenetic mechanisms. In general, epigenetics is influenced by endogenous and exogenous factors. Gene regulation depends on a complex interplay between posttranslational histone modifications and DNA methylation. miRNA either directly affects gene expression or modulates other epigenetic mechanisms (Bursh *et al.*, 2015).

2.4.1 DNA Methylation

DNA methylation is the best-known epigenetic marker (Esteller, 2008). It plays a crucial role in gene expression regulation and in maintaining chromatin integrity (Deaton & Bird, 2011). DNA methylation occurring on the 5 position of the pyrimidine ring of cytosines in the context of the dinucleotide sequence CpG, forms one of the multiple layers of epigenetic mechanisms controlling and modulating gene expression through chromatin structure (Tost., 2009). DNA demethylation is a process involving removal of a methyl group from a nucleotide in DNA (Tollefsbol., 2012). Some nuclear enzymes, known as DNA methyltransferases (DNMTs), conduct DNA methylation (Dawood & Efferth., 2015). Figure 2.2 showed the methylation of Cytosine on the 5 position of the pyrimidine ring.

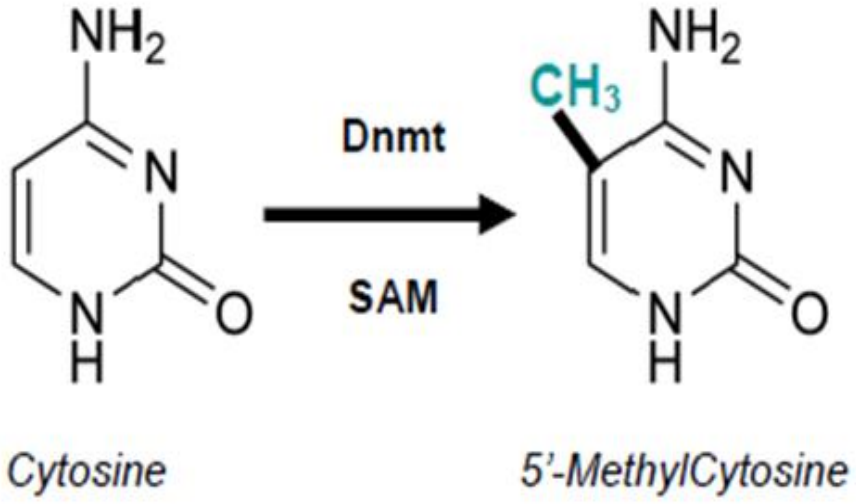

Figure 2.3. **Methylation of Cytosine to 5'-MethylCytosine. Methylation process catalyzed by the DNA methyltransferases enzyme family.** This process requires S-adenosylmethionine as a methyl donor. S-adenosyl-L-homocysteine, a by-product of catalyzed methylation process of SAM (Lisanti., 2013).

Repetitive genomic sequences are heavily methylated in healthy cells, while most CpG islands are nonmethylated (Baylln *et al*., 1997). In certain areas of the genome, CpG dinucleotide is always densely clustered, often in the promoter region and these clusters are called "CpG islands" (Takai & Jones, 2002). This allows for the expressing of genes in the presence of the required transcription activators. In particular cases, however, gene-promoting regions are methylated in human cells as part of natural growth processes (Baylln *et al*., 1997).

According to Tost (2009), the identification of aberrant changes in DNA methylation has occurred in several diseases, especially cancer. Methylation patterns of DNA can be used to diagnose cancer at very early stages, to identify tumors, and to predict and monitor the anti-cancer response. DNA methylation is a promising biomarker for many applications as it is a stable nucleic acid-based modification with restricted dynamic range that is technically simple to manage (Tost., 2009). Human cancer cells obtained from clinical tissue specimens frequently show genome-wide (global) DNA hypomethylation and gene-specific DNA hypermethylation (Sharma *et al*.,2010).

2.4.1(a) Global Methylation

Aberrant DNA hypomethylation can induce activation of oncogenes and loss of imprinting (Sharma *et al.*,2010). The cancer cells caused by carcinogen showed a decrease in overall genomic methylation of about 20–40% compared to normal cells. Compared with normal tissue, 20–60% of 5-methylcytosine content (demethylation) was lost in a cancer cell's genome and repetitive sequence demethylation accounts for 20–30% of the human genome **(**Ehrlich., 2002). In addition, genome hypomethylation is an early event in the development of cancer and accumulates in all tumorigenic stages, from benign proliferation to invasive cancer (Fraga *et al.*, 2004).

While gene-specific demethylation occurs in the sense of global hypomethylation of DNA, many of the effects are thought to occur by triggering the transposable elements and endogenous retroviruses present in the human genome, and through loss of imprinting (Whitelaw & Martin, 2001). Potentially, the reactivation of strong promoters associated with transposable elements can globally alter the rates of expression of transcription factors and/or gene expression of the growth regulatory genes in which those factors reside (Whitelaw & Martin, 2001).

2.4.1(b) Gene Specific Methylation

The presence of CpG island promoter hypermethylation affects genes regulation in almost all cellular functions, such as hormonal response, apoptosis, cell cycle, cell adherence and invasion, DNA repair and carcinogen metabolism (Esteller *et al*., 2001). Hypermethylation of DNA at the CpG islands of promoter region will result in silencing of specific genes, including tumor-suppressor genes (Sharma *et al.*,2010). In normal tissue, these CpG islands are unmethylated, but often become hypermethylated in cancer cells, resulting in gene silencing (Esteller, 2008). The specific genes which are hypermethylated in tumor cells are strongly specific to the tissue of origin (Costello *et al.,* 2000; Esteller *et al.,* 2001). For example, *BRCA1* hypermethylation is a biomarker for breast and ovarian tumors (Esteller *et al*., 2001) but does not occur in other tumor types. Ropero *et al.,* (2004) reported that hMLH1 methylation-mediated silencing is typical of gastric, colorectal and endometrial neoplasm but is almost unmethylated in other solid tumors.

2.4.2 microRNA (miRNA)

MicroRNAs (miRNAs) are essential to many cellular functions and are often dysregulated during the development of cancer (Tollefsbol., 2012). miRNAs act as endogenous gene silencers within cells, repressing target mRNA at a translational level. miRNAs are strongly evolutionarily preserved, and are found in virus, plant and animal genomes. It is now known that miRNAs may represent anywhere in the region of 1-3% of the entire human genome (Bentwich *et al.*,2005; Sontheimer & Carthew., 2005) and estimates of the number of miRNA targets indicate that they may play an important role in regulating as many as 30% of mammalian genes (Lewis *et al.,* 2005).

The data available suggest that miRNA expression profiles varied between normal and tumor tissues and also between various types of tumors. MicroRNA expression levels (miRNAs) are reduced globally in cancer relative to matched normal tissues and miRNA activity has recently been involved in tumorigenesis (Diederichs & Haber,2006). Volinia *et al.,* (2006) profiled miRNA expression in more than 500 samples of tumor and normal tissue of colon, lung, pancreatic, gastrointestinal, breast and prostate tissues. The results revealed that miRNA expressions were commonly dysregulated in the malignant tissues. A study by Lu *et al.,* (2005) profiled the expression of 217 miRNAs in 334 samples comprised of primary tumors, tumor cell-lines and normal non-cancer tissue, from 20 different cancer types. It was found that the miRNA profiles accurately differentiate between normal and malignant tissue, separate cancer type, categorize differentiation state and cluster samples according to their embryonic lineage. Such miRNA profiles may be appropriate for the detection of different cancers or for stratification of tumors in addition to serve as prognostic or therapeutic markers (Tollefsbol., 2012). Previous studies that showed some miRNAs involved in the cancer development are displayed in Table 2.1.

Table 2.1: The miRNA, its target genes and type of cancer involved.

miRNA	Target	Cancer type	References
miR-146a	*TGF-β* and *IL-10,*	Colorectal	Khorrami et al., 2017
miRNA-21	*PTEN*	Breast	Wang et al., 2011
miR-587	*PPP2R1B*	Colorectal	Zhang et al., 2016
miR-513a-3p	*GSTP1*	Lung	Zhang et al., 2012
miR-591	*ZEB1*	Ovarian	Huh et al., 2013
miR- 23a	*APAF-1*	Colorectal	Shang et al., 2014
miR-148a	*MSK1*	Prostate	Fujita et al., 2010
miR-125a	*VEGFA*	Colorectal	Yang et al., 2018
miR-320	*CDK6*	Colorectal	Tadano et al., 2016

2.5 Herbs, a Phytotherapeutic Potential Source for Cancer Treatment

Plants have played a significant role as a source of potential anticancer agents and it is notable that 60 percent of currently used anticancer agents are derived from natural sources including micro-organisms, marine organisms and plants (Newman *et al.*, 2003; Cragg *et al*., 2011). Plant-based medicine undoubtedly played a role in the treatment of cancer (chemotherapy), and the mechanisms of interactions between several phytochemicals and cancer cells are extensively studied (Kaufman *et al*., 1998). In particular, there is increasing demand in the pharmacological evaluation of the various plants used for cancer treatment. Herbal treatment has been used in traditional systems of medicine such as Traditional Chinese medicine and Ayurverdic medicine as a primary prevention of cancer as it strengthens the immune system, decreases side effects of chemotherapy, prevents cancer recurrence and it serves as alternative option to conventional medicines. Herbal plants had been used extensively for treatment of cancers. Wu *et al*., (2002) reported that *Hemsleya amabilis* had anticancer effect towards breast cancer cell. Panax ginseng had been reported by Chang *et al.*, (2003) and Kiefer & Pantuso (2003) to have anticancer effect on ovarian cancer cell. Adlay seed was reported to have antiproliferative and chemopreventive effects against lung cancer *in vitro* and *in vivo* (Chang *et al.,* 2003).

2.6 Natural Compounds in Natural Products as Epigenetic Regulators

The epigenetic inactivation of tumor suppressor genes is a basic factor in human cancer pathogenesis (Damman *et al.*, 2017). Since epigenetic modifications are reversible, the development of drugs that regulate epigenetic regulation is a very promising and attractive way to treat or prevent cancers, including the development of functional foods or supplements as epigenetic modulators for cancer based on nutrition (Issa & Kantarjian, 2009; Link et al., 2010). The restoration of various tumor suppressor genes that are repressed by aberrant epigenetic alterations can be achieved by dietary epigenetic modifications induced by phytochemicals (Lee *et al*., 2011).

Many secondary plant metabolites derived from teas, spices, nuts, vegetables and traditional medicinal herbs as natural products have regulatory effects on the epigenetic machinery. These products have been found to regulate biomarkers involved in multiple cancer-related pathways such as activator protein 1 (*AP-1*), *NFkB*, peroxisome proliferator-activated receptor-*y* (*PPARy*), signal transducers and activators of transcription (*STAT3*), *Nrf2,* estrogen receptor, liver X receptor (*LXR*) and hypoxia inducible factor-1 (*HIF-1*) and epigenetic cofactors (miRNAs) as observed in *in vitro* and *in vivo* studies (Berghe, 2012; Wang *et al.*, 2012; Lee *et al.*, 2013).

In order to reverse the epigenetic silencing of *RASSF1A* expression in cancer cells, through demethylation, natural supplements such as natural methyl donors, vitamins and polyphenols were used (Damman *et al.*, 2017). Apart from methyl donors such as folate and vitamin B12 which play an important role on the regulation of methylation levels of *RASSF1A*, Damman *et al*. (2017) also stated that vitamin A and vitamin D may also regulate the expression of DNMT and reverse the epigenetic silencing of tumor suppressor gene. Epigallocatechin-3-O-gallate (EGCG), green tea derived flavonoids and genistein isolated from *Genista tinctoria* was found to regulate DNMTIs, which are

responsible for the maintenance of methylation (Dawood & Efferth., 2015). Meanwhile, extract from *Vitex rotundifolia* was shown to be able to re-express *ZIC1*, a tumor suppressor gene that is silenced in SW620 colon cancer cells by promoter hypermethylation and the extract was considered a potential source of demethylation agent for the gene *ZIC1* (Sohn *et al*., 2013).

Natural compounds were also shown to regulate miRNA expression. According to Ozbey *et al*., (2018), apigenin, a plant derived flavonoid efficiently controlled oncogenic and tumor suppressor miRNAs in different cancers. It acted synergistically with different miRNA inhibitors and miRNA mimics to inhibit cancer cell growth and proliferation. Apigenin was noted for upregulating miR-520b and miR-101 to suppress tumor growth in different cancers. (Ozbey *et al.,* 2018).

Brown propolis that has higher content of flavones was reported to regulate more miRNAs than green propolis, which has lesser flavone content (Zaccharia *et al.,* 2017). Zaccharia *et al*., (2017) also proposed that when the content of flavanone increase, the epigenetic activity will also increase, which will then result in the modulation of miRNA expressions such as- miR-203a 3p, miR-17 3p, miR-19a 3p and miR-27a-3p.

2.7 Overview of *Phyllanthus*

Phyllanthus is a genus that is widespread in both tropical and subtropical regions. This plant can also be found in the rainforests of Amazonia (Prakash, 2001). The species recently became of interest in research because of their use in traditional medicine and were shown to possess strong medicinal properties (Kalidas & Mohan., 2009). Various therapeutic properties of this genus have been reported, including being anti-viral (HIV and hepatitis B), anti-hypertensive, anti-hepatotoxic and anti-metastatic (Naik & Juvekar., 2003; Lin et al., 2008; Sabir & Roca., 2008; Lee et al., 2011). There were also some reported anticarcinogenic activity of various *Phyllanthus* plants (Lee *et al.*,2011). *Phyllanthus* species such as *P. niruri, P. debilis, P. myrtifolius P. reticulatus, P. acidus, P. pulcher* and *P. urinaria* are traditionally used for treatment of several diseases in many tropical countries (Kumaran & Karunakaran., 2006). Whole plant or parts of it were used for the treatment of diabetes, kidney stones, intestinal infections, hepatitis B and neonatal jaundice (Calixto *et al.*,1998; Kumaran & Karunakaran., 2006).

Plant species of the *Phyllanthus* genus have traditionally been recognized as part of medicinal applications to counter degenerative diseases and they have been reported to be useful in the natural treatment of diseases, as it has been substantiated by numerous scientific studies showing that many species of this genus have different nutraceutical properties that may have positive effects on human health (Summanen, 1999). The efficacy of these plants in treating a wide spectrum of diseases may be attributed to their active compounds (Mahdi *et al.*, 2011).

The major active compounds of most *Phyllanthus* species are tannins, ellagitannins and flavonoids (Mahdi *et al*., 2011). Other phytochemical compounds that were isolated from *Phyllanthus* species include furanolactone, alkaloid, benzenoid, diterpene and triterpene (Summanen., 1999). The roots and leaves of *P. gomphocarpus*

tested positive for saponin, tannins, flavonoids and terpenoids (Ebby-Anuar *et al.*, 2014). Ebby-Anuar *et al*., (2014) also suggested that *P. gomphocarpus* roots and leaves are potential sources of nutrients and natural antioxidants for the treatment of various diseases induced by free radicals. A study by Kumaran & Karunakaran (2006) showed that five species of *Phyllanthus,* namely *P. maderaspatensis, P. debilis, P.virgatus, P. urinaria* and *P. amarus* have significant antioxidant activity. These extracts were found to have different levels of antioxidant activity in all the systems tested. Luo *et al.*, (2011) revealed that all the *P. embilica* phenolics tested showed strong radical scavenging capacity, good chelating activity for Fe2 + and good lipid peroxidation inhibition capability. Three common *P.*species that were traditionally used for treatment in Malaysia are *P. urinaria*, *P. niruri* and *P. debilis.*

2.7.1 *Phyllanthus urinaria*

P. urinaria is widely used to treat fever, inflammation, constipation, hepatotoxicity, vision impairment, urinary disease among others and it is also used to detoxify poison from the body (Kumaran and Karunakaran, 2007; Amin *et al*., 2012). In China, *P. urinaria* was mainly used for cancer treatment (Huang *et al*., 2010; Tang *et al*., 2010). Huang *et al*., (2006) reported that *P. urinaria* extract is an antiangiogenic and anti-tumor agent, which can be used safely in animals. Giridharan *et al*., (2002) stated that 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxide, a lignan isolated from *P. urinaria* ethyl acetate extract was shown to exert anticancer activity by inducing apoptosis, which was influenced by inhibiting telomerase activity and expression of Bcl-2. More significantly, *P. urinaria* extract's tumor-specific cytotoxic effect was demonstrated by the fact that *P. urinaria* did not inhibit the proliferation and viability of non-tumor/normal cells (Huang *et al.*, 2004). *P. urinaria* extract also possesses other bioactive compounds such as coumarin, ellagitannin and sterol, which can act as antiviral, antioxidant and anti-inflammatory agents (Sarin *et al*., 2014).

2.7.2 *Phyllanthus niruri*

P. niruri is the most common *Phyllanthus* species used as medicine in most of tropical countries (Wong *et al*., 2013). There are many important conventional uses of *P. niruri* for treatment and management of a wide variety of diseases. Some of the medicinal uses in experimental models were endorsed, indicating that the plant extracts possess various pharmacological properties (Junior., 2012). Some of the most promising medicinal properties the plant has include anti-hypertensive, anti-HIV, antilithic, anti-hepatotoxic, and anti-hepatitis B activities (Bagalkotkar *et al.*, 2006). The analysis of its extracts showed the plant has many bioactive compounds, which had diverse pharmacological and therapeutic potentials such as tannins, ricinolic acid, phyllanthin, hypophyllanthin, lignans, phenyl propanoids, niruriside, triterpenes, and phyltetralin (Calixto *et al*., 1998; Rajeshkumar *et al*., 2002; Bagalkotkar *et al*., 2006; Van and Ha, 2007; Xiang-Rong, 2007; Narendra *et al*., 2012). For example, lignans showed an excellent hepatoprotective effects, whereas the terpenes group of compounds exhibited anti-microbial and anti-cancer properties (Bagalkotkar *et al*., 2006).

2.7.3 *Phyllanthus debilis*

P. debilis is a fewer species of *Phyllanthus* and is generally used as a replacement for other common species of *Phyllanthus* such as *P. amarus (*Kumaran and Karunakaran, 2007). This herb shows anti-inflammatory (Chandrashekar *et al.,* 2005) and antihepatotoxic (Ahmed *et al.,* 2009) properties. *P. debilis* is commonly used for treatment such as jaundice, sores, diarrhoea, ulcers, scabies and ringworms (Wanniarachchi *et al*., 2009). The aqueous extract of the plant shows antihyperglycemic property (Wanniarachchi *et al*., 2009). Although known as a substitute for *P. amarus*, this species has been found to have a better hepatoprotective activity than *P. amarus* (Sane *et al*., 1995). *P. debilis* has been shown to possess higher antioxidant activity compared to *P. urinaria, P. amarus, P. maderaspatensis* and *P. virgatus* (Kumaran and Karunakaran, 2007). Bioactive compounds found in *P. debilis* include; (1) debelalactone, which acts as antihepatotoxic, (2) phyllanthin and (3) hypophyllanthin which were shown to exhibit anti-cancer activity (Sarin *et al*., 2014). Phytoesterols found in *P. debilis* such as β-sitosterol act as anti-inflammatory and analgesic agents (Sarin *et al*., 2014).