CHEMOPREVENTIVE EFFECTS OF *PHALERIA MACROCARPA* (SCHEFF.) BOERL AQUEOUS EXTRACT ON HT29 COLORECTAL CANCER CELLS

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by

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LIST OF ABBREVIATIONS

°	Degree Celsius
%	Percentage
g	Gram
g/ml	Gram per milliliter
mg/ml	Milligram per milliliter
ml	Milliliter
µg	Microgram
µg/ml	Microgram per millimeter
µl	Microliter
APS	Ammonium Persulfate
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
Cdc	Cell division cycle protein
CDK	Cyclin dependent kinase
CEN	Chicken erythrocyte nuclei
CO_2	Carbon Dioxide
CTN	Calf thymocyte nuclei
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
PBS	Phosphate Buffered Saline
PI	Propidium iodide
PMSB	*Phaleria macrocarpa* (Scheff.) Boerl
RIPA	Radioimmunoprecipitation assay
rpm	Revolutions Per Minute
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBEA	Trypan Blue Exclusion Assay
TBS	Tris Buffer Saline
TEMED	Tetramethylethylenediamine

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KESAN KEMOPENCEGAHAN EKSTRAK AKUAS *PHALERIA MACROCARPA* (SCHEFF.) BOERL KE ATAS SEL KANSER KOLORETAL HT29

ABSTRAK

Sebatian semulajadi atau pemakanan dipercayai mempunyai aktiviti antikanser yang kuat, ketoksikan yang rendah dan menyebabkan sedikit kesan buruk. *Phaleria macrocarpa* (Scheff.) Boerl, (PMSB), yang biasanya dikenali sebagai Mahkota Dewa dipercayai mempunyai sebatian fitokimia yang dapat mengurangkan risiko pelbagai jenis kanser sehingga berpotensi membunuh dan menghalang pertumbuhan sel-sel adenokarsinoma kolorektal iaitu sel HT29. Tujuan kajian ini adalah untuk mengkaji aktiviti kemoprevensi daripada isi buah mentah PMSB, (WF) dan biji benih (WS) ekstrak pada kanser adenokarsinoma kolorektal, sel-sel HT29 dengan mengkaji dos konsentrasi rencat (IC_{50}), melihat perubahan morfologi, kesannya terhadap apoptosis dan peraturan kitaran sel serta protein yang terlibat. Sel kanser adenokarsinoma kolorektal dipilih kerana ia merupakan kanser yang menyebabkan kematian yang tinggi serta kurang informasi yang berkaitan pokok PMSB. Dos IC_{50} daripada PMSB, WF dan WS ekstrak dinilai oleh *trypan blue exclusion assay* (TBEA) dan perubahan morfologi diperhatikan di bawah mikroskop. Ekstrak WS dan WF menunjukkan keupayaan yang kuat untuk menghalang pertumbuhan sel pada dos IC_{50} 40 μg/ml ($p<0.05$) dan 1500 μg/ml ($p<0.05$). Pemerhatian morfologi ringkas di bawah mikroskop cahaya menunjukkan berlakunya apoptosis. Ciri morfologi apoptosis yang boleh dilihat ialah pembentukan vakuol pengembungan, putik-putik pada membran sel, pengecutan sitoplasma dan konvolusi di sekeliling pada sel yang telah dirawat.

Jasad apoptotik juga boleh dilihat di sekeliling sel yang pecah selepas berlakunya fragmentasi nuklear. Seterusnya, bagi pembuktian berlakunya apoptosis dan seterusnya menghentikan kitaran sel, teknik *flow cytometry* telah dijalankan. Hasil kajian menunjukkan bahawa kedua-dua WS dan WF memberi tindak balas apoptotik dengan ketara. Sebaliknya, hanya WS menunjukkan kesan yang signifikan terhadap peraturan kitaran sel yang menyebabkan pengumpulan sel terutama pada fasa S dan G_2/M. Keputusan analisa westen blot menunjukkan bahawa WS dan WF meningkatkan aktiviti caspase-3 dan *cyclin dependent kinase inhibitor* iaitu p27 dalam sel-sel HT29. Kesimpulannya, penemuan ini menunjukkan bahawa ekstrak mentah isi buah PMSB (WF) dan terutama benih (WS) mempunyai alternatif yang berpotensi untuk pengurusan kanser kerana sifat anti kansernya.

CHEMOPREVENTIVE EFFECTS OF *PHALERIA MACROCARPA* (SCHEFF.) BOERL AQUEOUS EXTRACT ON HT29 COLORECTAL CANCER CELLS

ABSTRACT

Natural or dietary compounds are believed to have potent anticancer activity, low toxicity and cause very few adverse effects. *Phaleria macrocarpa* (Scheff.) Boerl (PMSB), commonly known as Mahkota Dewa is believed to have phytochemical compounds that can decrease the risk of various cancers thus having the potential to kill and inhibit proliferation of the human colorectal adenocarcinoma, HT29 cells. The aim of the study was to assess the chemopreventive activities of PMSB crude water fruit flesh (WF) and seed (WS) extracts on human colorectal adenocarcinoma, HT29 cell line by studying the inhibitory concentration dose (IC_{50}), looking at the morphological changes, its effect on apoptosis and cell cycle regulation as well as the proteins involved. Colorectal adenocarcinoma was chosen because it causes high mortality rate but not much information found on its association with PMSB plant. The IC_{50} of PMSB WF and WS extracts were assessed by trypan blue exclusion assay (TBEA) and morphological changes were observed under microscope. The WS and WF extracts showed strong ability to inhibit cell growth in dose-dependent manner with IC_{50} of 40 µg/ml ($p<0.05$) and 1500 µg/ml ($p<0.05$), respectively. Brief morphological observation under light microscope at different magnifications demonstrated presence of apoptosis. Morphologic features of apoptosis can be seen especially the vacuolization of the cells, cell blebbing, budding, shrinkage of the cytoplasm and convolution of outlines in the treated cells. Apoptotic bodies can also be seen surrounding the burst cells following nuclear fragmentation. To further

demonstrate the apoptosis mechanism and subsequently, cell cycle arrest, the flow cytometry technique was conducted. Results showed that both WS and WF significantly induced apoptotic response ($p<0.05$). On the contrary, only WS showed significant effect on cell cycle regulation which caused accumulation of cells especially at S and G_2/M phases. Western blot analysis results showed that WS and WF upregulated caspase 3 and Cyclin dependent kinase inhibitor p27 ($p<0.05$), in HT29 cells. To conclude, these findings suggested that the crude extract of PMSB fruit flesh (WF) and especially the seed (WS) to have a potential alternative for cancer management because of its anti-cancer properties.

CHAPTER 1

INTRODUCTION

1.1 Research background

Worldwide, cancer is the leading cause of death. World Health Organisation reported about 14.1 million new cases of cancer recorded annually (Ferlay et al., 2019). Common forms of cancer include breast, cervical, colorectal and lung cancers. Cancer of the large intestines, colorectal cancer, which develop especially in the colon or rectum is the third most common form of cancer (Siegel et al., 2019). It contributes about 10% to all cases of cancer (Wild and Stewart, 2014). Management of colorectal cancer include chemotherapy, radiotherapy and surgery; however, most of these treatments are expensive and do not provide permanent solutions thus alternative therapies are explored (Cassileth and Deng, 2004). One of the such new treatment is chemoprevention by the utilisation of natural products and their compounds in the management of cancer. Although chemopreventive treatment using natural dietary compounds and/or synthetic chemicals is a relatively recent method of combating cancer, it cannot be denied as one of a promising strategy. The application of these compounds and substances are by blocking, inhibiting, reversing, or retarding the process of carcinogenesis (Steward and Brown, 2013). Various researches have the evidence that most of these compounds can be used on its own or in combination with other chemotherapeutic agents to prevent or treat cancer (Steward and Brown, 2013).

Since ancient civilization existed, natural product has been used as therapeutic. In ancient Chinese medicine, plants such as sweet wormwood *(Artemisia annua*) was used for managing fevers (Miller and Su, 2011); this led to the discovery of artemisinin used in treating malaria (Miller and Su, 2011). Similarly, in the Indian Ayurvedic medicine, plants such as *Centella asiatica* are used for memory enhancement (Gohil et

al., 2010). Many of the plants are used because of their antioxidant properties thus they are proposed to have anticancer properties. This can be demonstrated in a study of several Chinese herbs, Di yu (*Sanguisorba officinalis Linn*.), Xian he cao (*Agrimonia pilosa Ledeb*.) and Da huang (*Rheum officinale Baill*.). The water extracts of these plants have high antioxidant activity and correlated with its high growth inhibitory effect on cancer cell lines, MCF-7 and A549 cells (Li et al., 2007). Another study in berries also indicates that the presence of phytochemicals that are high in antioxidants associated with strong anticancer activity (Baby et al., 2018). It is suggested that the phytochemical such as phenolics, has antioxidants that able to prevent lipid peroxidation and protein damage caused by ROS or reactive oxygen species, that subsequently prevent various degenerative diseases, including cancer. One of the plants, *Phaleria macrocarpa* (Scheff.) Boerl (PMSB), has been reported to possess potent antioxidant source (Anggraini and Lewandowsky, 2015) hence, its selection in this study.

Locally known as Mahkota Dewa, PMSB is indigenous to the Southeast Asia especially Indonesia, Malaysia and Brunei (Altaf et al., 2013). The plant is grown in commercial herbal plantation because of its economic potential. In folk medicine, the plant is frequently used in treating numerous diseases, for instance gout, hepatitis, dysentery, diabetes mellitus, psoriasis, rheumatic arthritis and of course cancer (Altaf et al., 2013; Nadri et al., 2014). In rural parts of Indonesia, integrated traditional clinics are being developed to set forth the use of PMSB as main treatment regime. Most consumption of PMSB is by drinking as tea and boiling in water.

Natural or plant-based compounds are presumed to have potent anticancer activity, with low toxicity and limited adverse side effects. Antioxidant properties have

also been reported in this Mahkota Dewa plant which have made scientists to study it for possible anti-cancer potential (Nadri et al., 2014). There is scanty scientific information on the effects of the plant especially the fruits and the seeds on colorectal cancer.

PMSB is believed to have phytochemical compounds such as phenolic compound and antioxidant activity that can decrease the risk of various cancers thus having potential to kill and inhibit proliferation of the HT-29 cells. The chemopreventive effects produced by this natural compound are believed to include disturbance of the cell cycle arrest and apoptosis. Thus, the study involves investigating the chemopreventive effects of aqueous extract of PMSB fruit flesh and seed on colorectal cancer cell line (HT-29). The main significance of the study is to elucidate the potential of PMSB fruit flesh and seed in becoming chemopreventive agent using *in vitro* model. This work seeks to determine the chemoprevention potential of PMSB fruit flesh and seed on apoptosis and cell cycle using *in vitro* model.

1.2 Objectives of the study

The general objective of the study was to investigate the chemopreventive effects of aqueous extract of PMSB seed and fruit flesh on colorectal cancer cell line (HT-29).

The specific objectives of the research include:

1. To determine the inhibitory concentration (IC_{50}) of WS and WF extract.
2. To investigate the anti-proliferative effects of WS and WF extracts.
3. To study the effects of WS and WF extracts on the apoptosis.
4. To investigate the effects of WS and WF extracts on the cell cycle regulation.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer Incidences

Today, cancer is a significant global disease burden and represents a major obstacle to both doctors and scientists, but the aetiology and specific causes of this disease remain obscured. Colorectal cancer (CRC) is the third commonest diagnosed cancer and the second leading cause of cancer-related mortality (Ferlay et al., 2019). It is also the only cancer that occurs with approximately equal frequency in men and women (Kim et al., 2015).

There were 1,849,518 estimated new CRC cases and 880,792 CRC-related deaths in 2018, according to the World Health Organisation (WHO) GLOBOCAN 2019 database, and the 5-year prevalence of CRC was 62.8/100,000 and placed second among all cancer types (Ferlay et al., 2019). Overall, 2,595,326 men and 2,194,309 women were diagnosed with CRC all over the world in 2018 (Ferlay et al., 2019). Regional estimates show that among half of the new cases, deaths and 5-year prevalence cases were found in Asia (Wong et al., 2019). Ferlay et al., (2019) also reported that China, Japan, Korea, Malaysia, Singapore and Turkey have recorded higher 5-year prevalence rates than other Asian countries (approximately 46.5/100,000). In 2018, Asia has the highest 51.8% incidence rate and 52.4% mortality in the case of colorectal cancer, which comprises both sexes and ages per 100,000 people worldwide (Onyoh et al., 2019).

According to the 5-year report by the Malaysian National Cancer Registry, which covers all cancer cases registered by the state registries in Malaysia from 2012 to 2016, a total number of 115,238 new cancer cases were reported in Malaysia during this period with a total of 82,601 medically certified and non-medically certified cancer

deaths (Manan et al., 2019). In Malaysia, a total of 15,515 cases of colorectal cancer were registered for the period of 2012-2016 compared with 13,693 cases in 2007-2011 report. 56.1% were reported among males whereas 43.9% were among females (Manan et al., 2019). A research conducted in Malaysia, looking at the incidence and mortality by ethnicity, it was reported that in contrast with Indians and Malays, the Chinese population had the largest age-standardized occurrence (27.35/100,000) and age-standardized mortality (11.85/100,000), (Manan et al., 2019; Sung et al., 2008).

2.2 Anatomy and physiology of the colon and rectum

The colon is located in the end of the digestive system. The function of colon is to extract water and salts from the solid wastes before being disposed of from the body. The adult male colon is about 166 cm on average, while the female has a length of about 155 cm (Hounnou et al., 2002). The fermentation process of unabsorbed material take place in the colon, carried out by the microbiota (especially bacteria) present there. In contrast with the small intestine, colon does not play a major role in absorption of food and nutrients. The colon is divided into four sections which are the ascending colon, the transverse colon, the descending colon, and the sigmoid colon. The ascending and descending colon are located retroperitoneally, while the transverse colon and sigmoid colon are intraperitoneal. Figure 2.1 shows the large intestine which consists of the cecum, colon and rectum.

The rectum is the final straight portion of the large intestine in humans and other mammals (Rex et al., 2009). The rectum in human is about 12 centimetres, beginning at the end of the sigmoid colon. The rectum is the temporary storage site for faeces (Cunningham et al., 2010). When the rectal wall expands due to the filling of materials from within, stretch receptors at the rectal walls stimulate the desire to defecate

(Thompson, 1994). The rectum is located retroperitoneally. Cancer of the rectum is known as rectal cancer which is a subgroup of colorectal cancer.

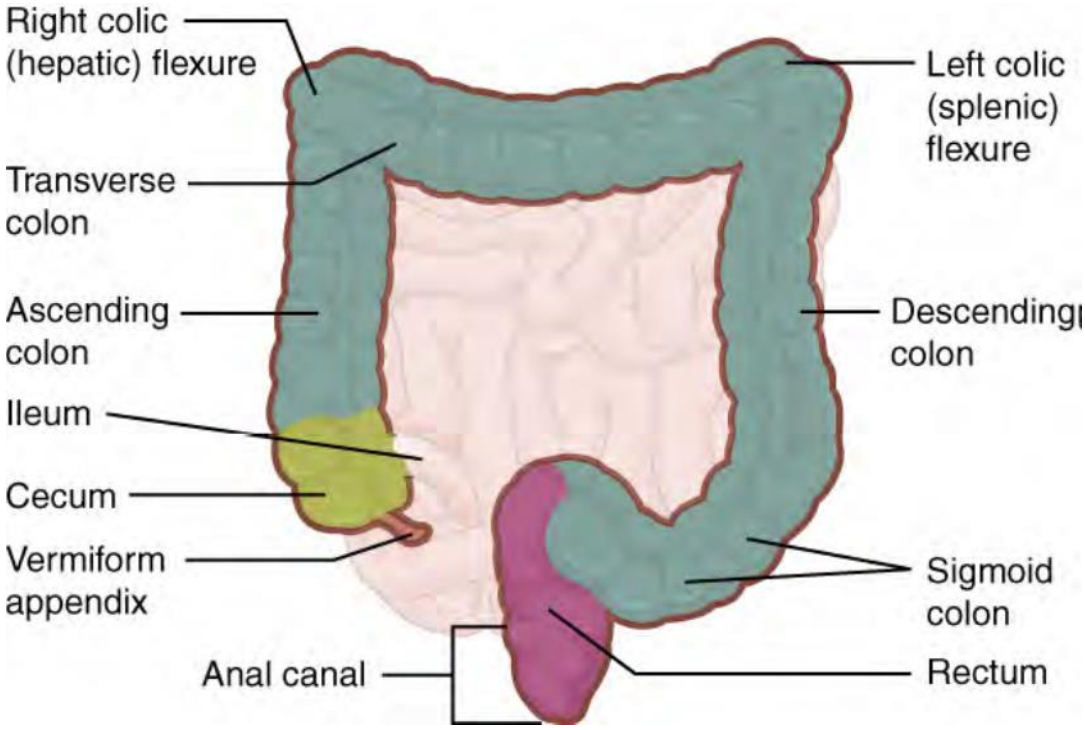

Figure 2.1 Large intestine showing the cecum, colon, and rectum. Extracted from Small and Large Intestines, Anatomy and Physiology, (2018).

2.3 Colorectal cancer

The cancer disease was discovered by the Egyptians in 1500 BC, but the name cancer was introduced by Hippocrates, the father of medicine (Hajdu, 2011). The word "cancer" is originated from "Karkinos", a Greek word which means crab (Hajdu, 2011). Cancer is a global burden including Malaysia. In Malaysia, annually, there are about 32,000 new cases of cancer. With reference to data from the International Agency for Research on Cancer, cancer is the third most common cause of death in Malaysian hospitals after heart diseases and septicaemia (IARC., 2018). In Malaysia, the five (5) most common cancers in men are colorectal, lung, prostate, lymphoma and nasopharyngeal whereas in female are breast, colorectal, cervix, ovary and lung (Manan

et al., 2019). The incidence of cancer is on the increase, this is due to several factors such as ageing and growth of the population, lifestyle changes and obesity (Jemal et al., 2011). According to the National Cancer Institute, there are about 14.5 million people had been diagnosed with cancer in 2014, this number is predicted to increase to 19 million by 2024.

Cancerous cells are cells that keep dividing leading tumour formation. Tumour formation are in two types: benign and malignant. The benign tumour is a tumour that does not expand to surrounding tissue, has a well-defined radius and is only harmful if the tumour compresses the tissues around it (Almeida and Barry, 2011). On the other hand, malignant tumour is a tumour that metastasizes across the lymphatic system, blood vessels and even infected tissues to surrounding tissues. (Liang et al., 2009).

Colorectal cancer which also known as colon cancer or bowel cancer, can be defined as the development of cancerous cell in the colon or rectum of the large intestine (Bibbins-Domingo et al., 2016). Colorectal (colon) cancer is usually due to ageing and lifestyle although there is also a genetic background (Bibbins-Domingo et al., 2016). Worldwide, colorectal cancer is the third most common type of cancer signifying about 10% of all cases of cancer (Favoriti et al., 2016). The commonest metastasis sites for colorectal cancer include the liver, lung and peritoneum (Wild and Stewart, 2014).

2.3.1 Symptom of colorectal cancer

The symptoms associated with colorectal cancer depend on the location of the cancer whether in the bowel or the cancer has metastasised to other areas of the body (Yamada et al., 2009). The common symptoms consist of constipation that becomes worse, rectal bleeding, decrease in the thickness of the stool, inappetence, weight loss nausea and vomiting in elderly (Yamada et al., 2009). Rectal bleeding is the common

sign, seen in aged people especially those over fifty years of age (Yamada et al., 2009). Other symptoms that should be concerned are weight loss and change in bowel habit associated with bleeding (Astin et al., 2011).

2.3.2 Risk factor of colorectal cancer

The incidence of colorectal cancer is associated with many risk factors. Some modifiable factors include smoking, excessive alcohol consumption, inactive way of life, high fat and high protein diet and obesity (Theodoratou et al., 2017) whereas the non-modifiable factors include age, ethnicity, body height, genetic factors, family history and gender. The consumption of processed and red meat has also been identified as another risk factor of colorectal cancer (Theodoratou et al., 2017). Family history of colorectal cancer also increases the disease risk of an individual. Inflammatory bowel disease (IBD) which are group of inflammatory diseases affecting the small intestine and colon may increase the chance of colorectal cancer (McGuire, 2016). IBD for instance ulcerative colitis or Crohn's disease has been related to the increasing of colorectal cancer risk (McGuire, 2016).

Epidemiology result indicated that there is a direct relationship between diet of an individual to the development of cancer. Previous report stated that the incidence of colorectal cancer among Africans was low when compared to the high incidence found among African-Americans (Ferlay et al., 2010). The report suggested that probably because Africans eat more vegetables, nuts and fibres when compared to fermented foods found among western populations. It was also that reported Asian populations who migrate to western countries have increased risk of cancer due to adoption of western lifestyles (Ferlay et al., 2010).

Approximately 10% of patients suffering from colorectal cancer are associated with insufficient physical activity (Lee et al., 2012). In respect to alcohol, alcohol drinking increases the risk especially drinking a bottle per day (Fedirko et al., 2011). The risk of colorectal cancer can be reduced by drinking at least 5 glasses of water per day (Boleij et al., 2011). Smoking is a major risk factor for all types of cancer including colorectal cancer. This is because the components of cigarette are harmful. People who smoke are more susceptible to develop colorectal cancer. Research has shown that the substances in cigarette can cause DNA damage leading to tumour formation. Furthermore, smoking could affect the immune system and expose the smoker to the risk of cancer (Theodoratou et al., 2017). The colorectal cancer risk is also enhanced with bacterial infections especially *Streptococcus gallolyticus*, *Streptococcus bovis* and *Gallolyticus bacteremia* (Abdulamir et al., 2011).

Even though numerous individuals are exposed to these risk factors, colorectal cancer is only developed in some of the exposed individuals, meaning that genetic variance partially determines human susceptibility to colorectal tumorigenesis. In Malaysia, the highest incidence of colorectal cancer was reported in the Chinese population (Manan et al., 2019). Several studies have associated the effect of genetic polymorphism to the risk of colorectal cancer in Chinese population. Previous studies have shown, that single nucleotide polymorphism (SNP) of NFkB1-94(ins/ins+del/ins), NFkBIA, rs6695837, MEG3 and XPG Asp1104His, potentially contribute to the incidence of colorectal cancer in Chinese populations (Cao et al., 2016; H. Du et al., 2014; Lou et al., 2017; Song et al., 2011) . This factor has also been widely researched in genome-wide association studies (GWASs) conducted by various groups of researchers (J. Dai et al., 2017; Jiang et al., 2015, 2020) where they found the association of genetic variants and colorectal cancer.

2.3.3 Staging of colorectal cancer

Colorectal cancer staging is the strongest predictor for patient survival and determining the stages is critical for appropriate patient management (Compton and Greene, 2004). Staging requires doctors to establish the cancer extent and the coverage of the of the spread. The standard staging of colorectal cancer that being recognized by National Cancer Institute and most pathologist association, is the tumour, node, metastasis (TNM) staging system by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC).

Large intestines consist of two main components which are the colon and rectum. The wall of the intestines comprises of multiple layers of tissues. Mucosa is the inner layer which includes the epithelium, the connective tissue and muscularis mucosa which is a thin muscle layer. Beneath the mucosa is the fibrous tissue or submucosa, muscularis propria, a thick muscle layer and the outermost layers of connective tissue, subserosa and serosa as shown in Figure 2.2.

In order to strategize the treatment plan, the extent of the disease need to be determined by doctors. This is known as staging and staging is done by looking at the degree of cancer invasion. There are a few different ways to stage colorectal cancer. The Dukes staging is the classic method of colorectal staging. When the cancer is confined to the inner lining of the bowel or slightly growing into the muscle layer, it is classified as Dukes A. When the cancer has invaded the muscle layer of the bowel, it is classified as Dukes B. When the cancer spreads to the mesenteric lymph nodes, it is classified as Dukes C while Dukes D is classified when the cancer spreads to other organ such as liver, lung and bones (Figure 2.3).

With reference to the 2018 American Joint Committee on Cancer (AJCC), on colorectal cancer staging, colorectal cancer is staged based on the examination of tissue

removed during surgery for better accuracy on top of clinical staging that was based on physical examination, biopsies and imaging results as in the exhibit in the TNM staging system (Figure 2.4).

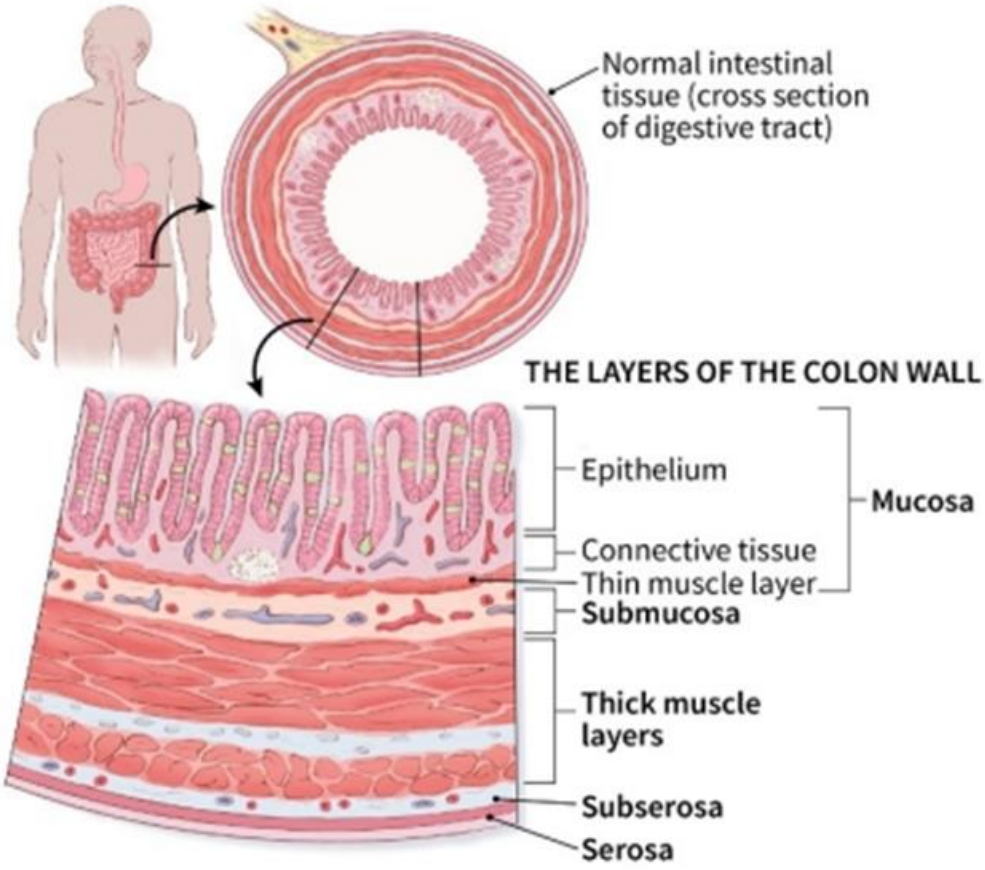

Figure 2.2 Layers of the colon wall. Extracted from American Cancer Society website.

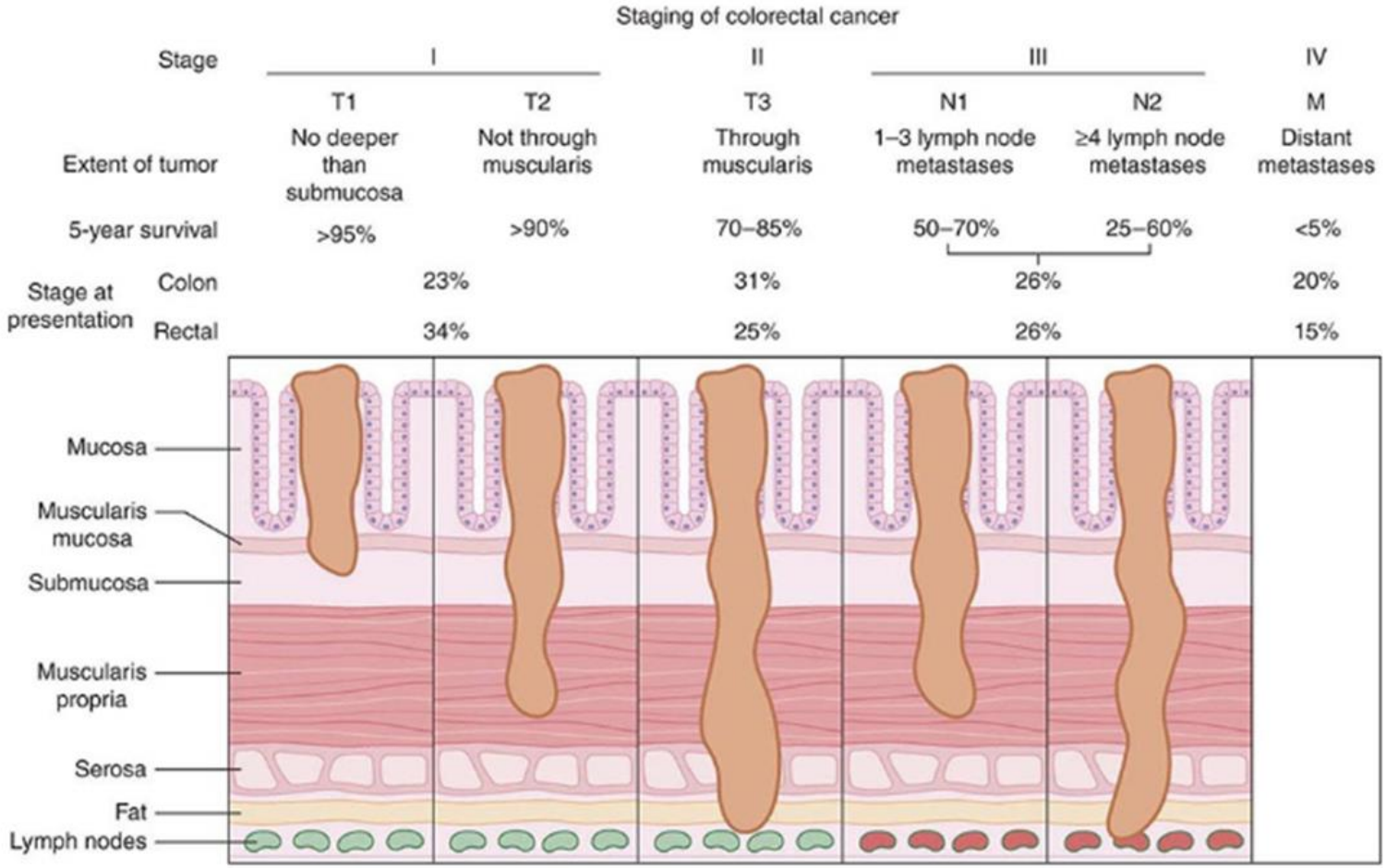

Figure 2.3 Modified Dukes' staging of colorectal cancer. Extracted from clinicalgate.com website

Stage	TNM Classification	Five-Year Survival
		%
I	T1–2, N0, M0	>90
IIA	T3, N0, M0	60–85
IIB	T4, N0, M0	
IIIA	T1–2, N1, M0	25–65
IIIB	T3–4, N1, M0	
IIIC	T (any), N2, M0	
IV	T (any), N (any), M1	5–7

Primary tumor (T)

TX: Primary tumor cannot be assessed

Tis: Carcinoma in situ

T1: Tumor invades submucosa

T2: Tumor invades muscularis propria

T3: Tumor penetrates muscularis propria and invades subserosa

T4: Tumor directly invades other organs or structures or perforates visceral peritoneum

Nodal status (N)

NX: Regional lymph nodes cannot be assessed

N0: No metastases in regional lymph nodes

N1: Metastases in one to three regional lymph nodes

N2: Metastases in four or more regional lymph nodes

Distant metastases (M)

MX: Presence or absence of distant metastases cannot be determined

M0: No distant metastases detected

M1: Distant metastases detected

Figure 2.4 TNM Staging System for Colorectal Cancer. Extracted from The American Joint Committee on Cancer (AJCC) tumour/node/metastasis (TNM) classification and staging system for colorectal cancer

2.3.4 Current treatment of colorectal cancer

Many cases of colorectal cancer may be treated if detected early. The prognosis depends on several factors especially the cancer stage. In general, most patients survive at least 5 years after their diagnosis if treatment starts at early stage. However, if the cancer spread, the 5-year survival rate drops significantly (Wild and Stewart, 2014). Colorectal cancer, at stage I, II, and III are believed to be curable. In most cases, stage IV cancer is incurable (Cunningham et al., 2010). At this stage, there are several potential complications such as colon blockage, metastasis to organs or tissues and development of a second primary colorectal cancer (Cunningham et al., 2010).

The mainstay of treatment for colorectal cancer is surgical resection which is also the current practice in Malaysia according to Clinical Practice Guideline: Management of Colorectal Carcinoma (MOH, 2019). The level of metastasis usually determines the long-term prognosis after surgery. According to report, the risk of metastasis is correlated to the depth of cancer penetration into the intestinal wall (Böckelman et al., 2015). Patients undergoing colorectal carcinoma surgery will be supplemented with antibiotic and venous thromboembolism prophylaxes (MOH, 2019). Surgery may be continued with chemotherapy to reduce recurrent or metastasis (Böckelman et al., 2015) and radiotherapy as adjuvant therapies and for palliative purposes (MOH, 2019). These treatments can affect the quality of life of the patients. Because of these common cancer treatments, there is a need to find less invasive or non-invasive alternative way.

Major contributor to the limited effectiveness of treatment is the cancer cell resistance to current chemotherapeutic agents. Inhibition or avoidance of drug-induced apoptosis is most significant mechanism that contributes to chemo-resistance. Therefore, the chemoprevention that suppresses, delaying or reversing of

carcinogenesis using pharmacologic intervention especially with naturally-occurring or synthetic agents, has been the main focus (Steward and Brown, 2013). The ultimate aim of cancer chemoprevention is the discovery of natural agents with verifiable efficacy against specific molecular targets. However, to date, in South East Asia inadequate studies available on chemopreventive activities on cancerous cells even though tropical plants in South East Asia could have greater potential for chemoprevention than those found in temperate zones.

The high consumption of plant source can provide health benefits due to their phytoconstituents such as carotenoids, flavonoids, phenolic compounds and vitamins (Kumar and Pandey, 2013; Treml and Šmejkal, 2016). Metabolites such as Vitamin C, E and β-carotenes are well known as powerful anti-oxidant properties (Kumar and Pandey, 2013; Treml and Šmejkal, 2016). However, studies have shown that another group of naturally-occurring antioxidants such as phenolics and polyphenolics are better scavengers of free radicals than vitamin C and E (Treml and Šmejkal, 2016). Most of natural product interest in plant phenolics are usually flavonoid. Flavonoids are some of the most powerful antioxidant known. However, humans are unable to produce flavonoid themselves (Kumar and Pandey, 2013). They must get the nutritional values of flavonoid from the food that they eat and from supplements. PMSB have been reported to be a good source of antioxidant which was demonstrated in this study.

2.3.5 Screening and diagnostic tools

The use of biomarker is gaining wide acceptance in diagnosing cancer. In colon cancer diagnosis, several diagnostic techniques have been devised. Seroprevalence of *Streptococcus bovis* and *Streptococcus gallolyticus* were considered as potential biomarkers for the early prediction of an underlying bowel lesion especially among high

risk population (Abdulamir et al., 2011). The presence of antibodies to the antigens *Streptococcus bovis* and *Streptococcus gallolyticus* or the antigens themselves in the bloodstream has been indicated to serve as markers for carcinogenesis in the colon (Abdulamir et al., 2011).

The diagnosis of colorectal cancer can also be performed by sampling the areas of the colon for possible tumour growth (Cunningham et al., 2010). Sampling usually taken when performing sigmoidoscopy or colonoscopy. Most of it depends the location of the lesion. Adenocarcinoma are the most common form of colorectal cancer. Other rare type found to be more aggressive include adenosquamous and squamous cell carcinoma (Mansour et al., 2015).

2.3.6 Colorectal cell lines

The use of cell line in cancer research is common. This is because it helps to mimic the cancer microenvironment. Cell culture using cell lines present unique advantages in biomedical research because of the readily available supply of live cells, the capacity to control experimental factors and the most important is act as collective reference model systems (Ahmed et al., 2013). Cancer cell lines are used in preclinical studies such as drug testing and biomarker discovery (Ahmed et al., 2013). There are several types of cell lines which have been developed to mimic colorectal cancer for research use. They include HT-29, DLD-1, SW48, CaCo-2, SW620, HCT-116, LoVo, IS1, RKO, IS2, SW1116, NCI-H508, LS-174T, WiDr, ALA, CO-115, HCT-15, FRI COLO 320, V9P, EB, SW948, IS3, TC7, TC71, SW480 and LS1034. These cell lines have different characteristics emanating from origins and capability (Ahmed et al., 2013). In this study, HT-29 was used because of their ability to simulate real colon tissue *in vitro* by differentiation (Le Bivic et al., 1988).

2.4 Apoptosis and cell cycle in colorectal cancer

Cell proliferation is closely associated with apoptosis for the reason that the cell cycle dysregulation directly affects the sensitivity of apoptosis. p53, the tumour suppressor gene has an important role in understanding DNA damage, apoptosis and cell cycle. Current studies have shown that p53 is able to detect DNA damage causing cell cycle arrest at G_1 phase to allow for repair. Unrepairable damage will lead to the triggering of apoptotic cell death (Mendoza-Rodríguez and Cerbón, 2001). Chemopreventive agents usually act by altering one of the abnormal processes in cell cycle resulting in inhibition of apoptosis.

2.4.1 Induction of apoptosis in colorectal cancer

In order to restrain the development of cancer, chemoprevention approach this issue by looking at the mechanism-based approach. Apoptosis is one of the critical cellular events in cancer chemoprevention (De Flora and Ferguson, 2005). Essentially, multicellular organisms develop, maintains tissue homeostasis and eliminate unwanted or damaged cells with the help from internal mechanism called apoptosis (De Flora and Ferguson, 2005). Apoptosis can be clarified as a process of cell death related to fragmentation of genomic DNA (Green, 2011). Furthermore, apoptosis can be characterized morphologically by condensation of the cytoplasmic content, chromatin condensation, nuclear pyknosis, cytoskeletal collapse, rounding of cell, membrane blebbing and the formation of membrane-bound apoptotic bodies. These apoptotic bodies will be phagocytosed rapidly and digested by macrophages or neighbouring cells without triggering the immune response (Green, 2011).

The morphological and biochemical changes in apoptosis mechanism is defined by a family of cysteine proteases called caspases (Shalini et al., 2015). Caspases

contribute importantly in most apoptotic cell death, as well as in cell migration, cell mortality, and in certain cell enucleation (Shalini et al., 2015). Therefore, in this study we choose the caspases proteins as the biomarkers for screening the chemopreventive activities from the crude extracts of PMSB fruit and seed. This study is one of the steps to accelerate the understanding of the molecular mechanisms and effects of phytochemicals in anti-tumour promotion and progression. The research data that will be produced and critically analysed may reveal vital determination of molecular foundation of the potential chemoprevention agents. In addition, it is also important to determine its long-term effectiveness and safety as a part of cancer treatment.

2.4.2 Cell cycle arrest in colorectal cancer

The progression and division process of cells, known as the cell cycle, plays an important role in the development of cancer. The normal cells control the cell cycle by a complex series of signalling pathways. This process regulates cell growth and division, to produce two daughter cells. In mammals, the cell cycle consists of four distinct phases. The phase where two gap occurs, G_1 and G_2, consist of RNA synthesis and protein synthesis, while the DNA replication happens at the S-phase, and during the M-phase the cells undergo mitosis and cytokinesis (Vermeulen et al., 2003).

If the cells are not dividing, they remain in a resting state or quiescent known as G_0 phase. G_1, S, and G_2 phases are referred to collectively as interphase (Garrett, 2001). This process also includes mechanisms to ensure errors are corrected, and if not, the cells goes through cell death (apoptosis). In cancer, because of genetic mutations, this regulatory process malfunctions, resulting in uncontrolled cell proliferation.

2.5 Cancer chemoprevention

Over the years, compounds originated from plants have contributed as an important source of anticancer therapies because of its availability. Most importantly, compounds from plants are comparatively less toxic than most chemotherapy drugs. More than thirty plant-derived compounds have been isolated and established for cancer clinical trials (Gali-Muhtasib et al., 2015). This figure is estimated from 3000 varieties of plant that have been reported to treat cancer. Anti-cancer drugs that were developed from plants may induce apoptosis via several mechanisms whether extrinsic or intrinsic and whether that mechanism involves caspases, with the dependency on p53 or not. Cell death by plant-derived anticancer drugs have also demonstrated other modes such as necrosis-like programmed cell death, senescence, mitotic catastrophe and mainly autophagy. (Gali-Muhtasib et al., 2015).

Although studies have shown that plant materials have an important role in the management of colorectal cancer, much is not known about the mechanism of action and how they develop resistance to the development and progression of cancer. This is the basis of chemoprevention. Although, epidemiological data have shown inconsistent results regarding the roles of plant materials in cancer prevention, experimental observation have shown that they can play an important role (Naithani et al., 2008; Steward and Brown, 2013).

The cancer prevention potentials in plants and other natural products have been numerously reported due to the presence of phytochemicals that possess health protective benefits (Pinakin et al., 2020). *In vitro* and *in vivo* studies have evidences that plant materials such as polyphenols, alkaloids and phenols found in herbs, vegetables and fruits can inhibit cancer growth (Naithani et al., 2008).

These chemopreventive agents have several mechanisms by which they prevent the development of cancer such as suppressing the proliferation of cancerous cells, inducing apoptosis, inhibiting the formation of blood vessels (angiogenesis), suppression of expression of antiapoptotic proteins and inhibiting the signalling of the growth factor pathways (De Flora and Ferguson, 2005).

Finally, the use of natural products especially plant materials for preventing cancer is considered non-toxic, cost-effective and efficient strategy for reducing the burden of cancer (De Flora and Ferguson, 2005). Examples of natural products that have been reported to have high potential for the prevention of cancer include *Curcuma longa*, *Anacardium occidentale* and *Moringa oleifera* (Altemimi et al., 2017; Handa et al., 2008).

2.6 *Phaleria macrocarpa* (Scheff.) Boerl (PMSB)

There are many plants indigenous to Malaysia many of which have medicinal potentials. These plants have been used in traditional medicine by local people in drinking water and eaten on daily basis. One such plant is *Phaleria macrocarpa* (Scheff.) Boerl. (PMSB). This plant is originated from tropical areas of Papua island, Indonesia (Anggraini and Lewandowsky, 2015). It is also abundantly cultivated in Malaysia and has been grown in plantation for commercial usage (Nurmaryam Aini Hashim et al., 2017).

2.6.1 Botanical description and habitat of PMSB

Phaleria macrocarpa (Scheff.) Boerl (PMSB) belongs to the family Thymelaceae. This plant, prominently known as God's crown, Mahkota Dewa or Pau, grows in tropical areas. The plant is a complete tree made up of stem, leaves, flowers

and fruits (Altaf et al., 2013). Physically, the height of the tree ranges from 1 m to 18 m with 1 m long straight root exuding sap, brownish green bark and white wood (Altaf et al., 2013). The productive age of the tree spans from 10 to 20 years.

The length and width of PMSB leaves ranging from 7 cm to 10 cm and 3 cm to 5 cm respectively. The colour is green and narrowing in shape. The flowers' colours ranging from green to maroon and make a compound of 2-4. The fruit is of eclipse shape with a diameter of 3 cm, usually green when un-ripened and red when ripened (Hendra, Ahmad, Oskoueian, et al., 2011). Each fruit has 1-2 seeds that is brown in colour, ovoid in shape and has anatropous ovule (Hendra, Ahmad, Oskoueian, et al., 2011). In Figure 2.5, the botanical description of PMSB is highlighted showing the various anatomical parts while in Figure 2.6 is the seed of *Phaleria macrocarpa* (Scheff.) Boerl.

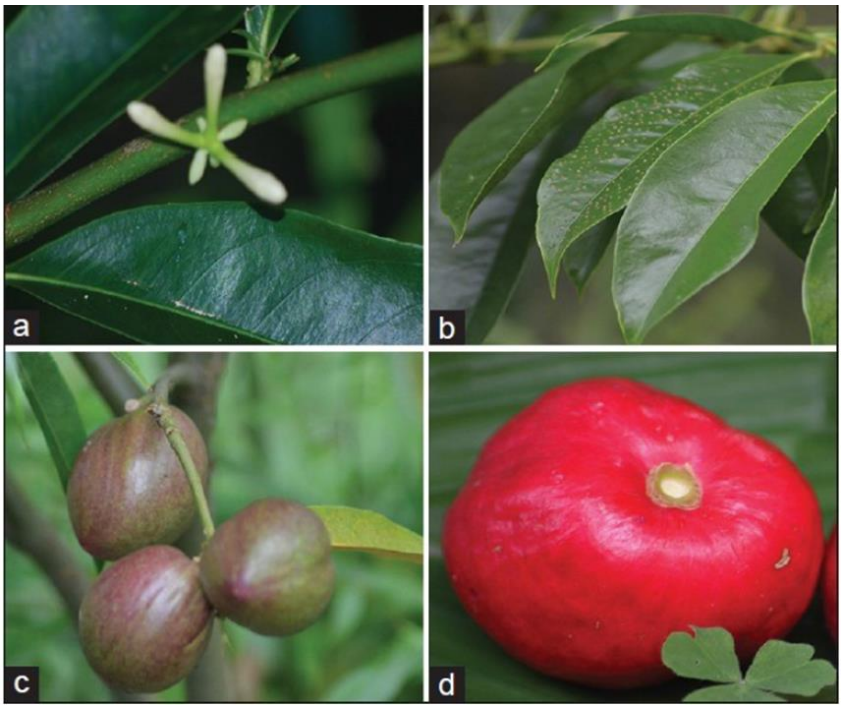

Figure 2.5 Botanical description of *Phaleria macrocarpa* (Scheff.) Boerl showing a typical (a) small flower bud, (b) green tapering leaves, (c) un-ripened green fruit (c), and (d) fully grown red fruit. Extracted from Altaf *et al*., (2013).

2.6.2 Biochemical and chemical constituents of PMSB

Several chemical constituents have been identified from various parts of PMSB in varying concentrations. The identified constituents comprise of mahkoside A, fevicordin-A, fevicordin-D, palmitic acid, des-acetyl fevicordin-A, dodecanoic acid, lignans, fevicordin-A glucoside, sucrose and ethyl stearate (Hendra, Ahmad, Sukari, et al., 2011). The bark and fruits of PMSB are rich in flavonoids, alkaloids, saponins, lignans, tannins, phenols and polyphenolics (Chong et al., 2011; Susanto et al., 2011). Chemical constituents such as magniferin, gallic acid and icariside C3 have been isolated from the PMSB fruit (Ahmad et al., 2012; Oshimi et al., 2008). Phalerin, a benzophenone glycoside (3,4,5, trihydroxy-4-methoxybenzophenone-3-O-β-D-glucoside) have been isolated from leaves of PMSB (Mae Sri Hartati et al., 2005). The same compound has also been recently isolated from the fruits (Oshimi et al., 2008). However, the proposed structure (2,4′,6, trihydroxy-4-methoxybenzophenone-3-O-β-D-glucoside) identified from the fruits was slightly different from that from the leaves (Oshimi et al., 2008). The pericarp of fruit has been reported to contain kaempferol, myricetin, naringin and rutin (Hendra, Ahmad, Sukari, et al., 2011) (Hendra, Ahmad, Sukari, et al., 2011). Quercitin and naringin are present in the mesocarp and seeds while the seeds contain fevicordin-A, phorboesters, and 29-norcucurbitacin derivatives (Diantini et al., 2012; Hendra, Ahmad, Sukari, et al., 2011; Kurnia et al., 2008) . Furthermore, some PMSB extracts have been reported to contain alkaloids and saponins (Altaf et al., 2013).

Figure 2.6 The seed of *Phaleria macrocarpa* (Scheff.) Boerl. Extracted from Faried *et al.*, (2016).

2.6.3 Pharmacological effect of PMSB

In traditional medicine, PMSB extracts have been reported to exhibit several pharmacological activities, including anti-tumour, anti-hyperglycaemia, anti-inflammation, anti-viral, anti-diarrheal, vasodilator, anti-oxidant, anti-bacterial and anti-fungal effect; however, many of these activities lack scientific understanding. In traditional medicine, the stem of PMSB is utilized for bone cancer treatment (Altaf et al., 2013). The egg shells of the seeds are also used for treatment of various cancers such as cervix cancer and breast cancer, heart diseases, liver and lung diseases (Altaf et al., 2013). The leaves are macerated and made into juice for the treatment of cancers, impotence, diabetes mellitus, allergies and blood diseases (Altaf et al., 2013; Winarno and Katrin W, 2009).

Different parts of PMSB have been described to have anti-hyperglycaemia potential (Altaf et al., 2013). Extracts isolated from the fruits of PMSB have been reported to lower the post-prandial hyperglycaemia. The highest activity of anti-hyperglycaemia was exhibited by n-butanol extract of buds and ripened fruits followed by ethyl acetate extract and then methanol extract (Sugiwati et al., 2006). Furthermore,

PMSB fruits competitively inhibits pancreatic α-amylase and membrane bound intestinal hydrolase as isomaltase, maltase and sucrose by inhibiting α-glucosidase (Triastuti et al., 2009). In the short-term, this activity delays glucose absorption and lowers post prandial, while it reduces HbA1c (glycated haemoglobin) in the long-term effect (Nagasawa et al., 2002). The anti-diabetic potential of PMSB might be due to the presence of rutin in the mesocarp and pericarp of the fruits. Rutin has been reported to cause a major anti-diabetic effect in rats (Ali et al., 2012) but currently no research that has linked the anti-diabetic potential of PMSB fruits with the presence of rutin.

The PMSB fruits contain many active compounds such as alkaloids, saponins and polyphenols (Altaf et al., 2013). One of such compounds is gallic acid which have been reported to regulate cholesterol haemostasis. Gallic acid isolated from PMSB fruits have been reported to reduce level of cholesterol by up-regulating LDL-R (low density lipoprotein receptors) and pro-protein convertase subtilisin/kexin type-9 (PCSK9) via sterol regulatory agent binding protein transcription factor (SREBP-2-TF (Altaf et al., 2013; Wong et al., 2006; Yu et al., 2004).

The leaves and seeds of PMSB have been shown to have profound antibacterial activity (Altaf et al., 2013). The extract uses various pathways to exhibit its antimicrobial behaviour, such as inhibiting nucleic acid synthesis, energy metabolism and cytoplasmic membrane function (Tri Winarni et al., 2012; Yosie et al., 2011). The methanol extract of PMSB fruit was reported to be effective against *Pseudomonas aeruginosa* and strong activity against *Escherichia coli*. The ethyl-acetate extract of the fruit also displays efficient activity against *E. coli*, *Klebsiella pneumoniae* and *Streptococcus ubellis* (Poeloengan and Komala, 2009). Phorbolesters isolated from the PMSB seeds have also been reported to inhibit growth of certain fungi such as