EVALUATION OF POTENTIAL ROLE OF *PHALERIA MACROCARPA* AND *GYNURA PROCUMBENS* IN THE PREVENTION OF AZOXYMETHANE-INDUCED ABERRANT CRYPT FOCI IN RAT COLON

ABDRABUH NAJI SHWTER

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

EVALUATION OF POTENTIAL ROLE OF *PHALERIA MACROCARPA* AND *GYNURA PROCUMBENS* IN THE PREVENTION OF AZOXYMETHANE-INDUCED ABERRANT CRYPT FOCI IN RAT COLON

ABDRABUH NAJI SHWTER

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Abdrabuh Naji Hussein Shwter

Passport No:

Matric No: MHA 100057

Name of Degree: PhD

Title of Thesis: Evaluation of potential role of *Phaleria macrocarpa* and *Gynura procumbens* in the prevention of azoxymethane-induced aberrant crypt foci in rat's colon.

Field of Study: Molecular Medicine

I do solemnly and sincerely declare that:
(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this work;
(4) I do not have any actual knowledge nor ought I reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature **Date**

Subscribed and solemnly declared before,

Witness's Signature **Date**

Name:

Designation:

ABSTRACT

Phaleria macrocarpa and *Gynura procumbens* extracts are traditionally used to treat different diseases in Malaysia and Indonesia. The aim of this research is to evaluate the chemopreventive effects of ethanol extracts of *P. macrocarpa* and *G. procumbens* against azoxymethane-induced (AOM) aberrant crypt foci (ACF) in male Sprague Dawley rats. Our acute toxicity studies revealed no hepatotoxic and nephrotoxic effects. *In vitro*, both plant extracts exhibited high antioxidant activities which were due to the presence of high phenol and flavonoid content. Moreover, the results from the MTT assay showed anti-proliferative activity of both plant extracts against colon cancer cell line HT29. Gross examination of rats treated with the plant extracts showed a significant reduction in the number of ACF. Histological evidence showed remarkably elongated glands with stratified nuclei, and depletion of mucin in the colon of AOM group compared to the plant extract-treated group. Immunohistochemical staining showed proliferating nuclear cell antigen (PCNA)-positive cells were significantly higher in the AOM group compared to the plant extract-treated rats. In colon tissue homogenate, glutathione S-transfarease (GST) and superoxide dismutase (SOD) activities were significantly higher whilst malondialdehyde (MDA) level was significantly lower in extract-fed rats compared to AOM groups. RT-PCR showed that *P. macrocarpa* and *G. procumbens* altered the expression of apoptosis-related genes; the anti-apoptotic protein Bcl-2 was downregulated whereas the pro-apoptotic protein Bax was upregulated. Furthermore, the active constituents isolated from both plants were identified. Based on the results of this study, *P. macrocarpa* and *G. procumbens* ethanol extracts were nontoxic after oral administration. The chemopreventive potential of these plant extracts was demonstrated by reductions in the numbers of ACFs which could be attributed to their antioxidant properties and down-regulation of PCNA-promoting proteins in cancer cells. In addition, gene expression analyses revealed that both plant extracts activated apoptosis.

ABSTRAK

Ekstrak *Phaleria macrocarpa* dan *Gynura procumbens* digunakan secara tradisional untuk merawat pelbagai penyakit di Malaysia dan Indonesia. Tujuan kajian ini adalah untuk menilai kesan chemopreventif ekstrak etanol *P. macrocarpa* dan *G. procumbens* terhadap fokus kripta aberan (ACF) yang dicetuskan oleh azoxymethane (AOM) pada tikus Sprague Dawley. Kajian toksisiti akut dengan ekstrak tumbuhan-tumbuhan tersebut mendedahkan bahawa tiada kesan ketoksikan hepar dan ginjal. Kajian *in vitro* menunjukkan kedua-dua tumbuhan ekstrak mempamerkan aktiviti antioksidan yang tinggi yang disebabkan kandungan tinggi fenol dan flavonoid. Selain itu, keputusan daripada assay MTT menunjukkan aktiviti anti-proliferatif kedua-dua ekstrak tumbuhan terhadap zuriat sel kanser usus besar HT29. Pengamatan kasar usus besar tikus AOM yang dirawat ekstrak *P. macrocarpa* dan *G. procumbens* menunjukkan pengurangan ketara bilangan ACF. Kajian histologi mempamerkan ACF dengan nukleus yang panjang dan berstrata, berserta pengurangan mucin pada kumpulan tikus AOM berbanding dengan kumpulan-kumpulan AOM yang dirawat dengan ekstrak tumbuhan-tumbuhan tersebut. Perwarnaan immunohistochemical menunjukkan bahawa sel-sel yang positif terhadap pembiakan antigen sel nuklear (PCNA) lebih tinggi dalam kumpulan AOM berbanding yang dirawat dengan ekstrak tumbuhan. Aktiviti glutathione-S-transfarease dan superoxide dismutase adalah lebih tinggi dengan signifikannya manakala paras MDA adalah lebih rendah dalam homogenat usus besar dari tikus AOM yang dirawat ekstrak tumbuhan berbanding dengan kumpulan AOM tanpa rawatan. RT-PCR menunjukkan bahawa *P. macrocarpa* dan *G. procumbens* mengubah ekspresi gen yang berkaitan apoptosis; pengawalaturan protein anti-apoptotic Bcl-2 diturunkan manakala pengawalaturan protein pro-apoptotic Bax dipertingkatkan. Di samping itu, komponen-komponen aktif daripada kedua-dua ekstrak tumbuhan tersebut telah diasingkan dan dikenal pasti. Berdasarkan hasil kajian ini, disimpulkan bahawa ekstrak etanol *P*

.*macrocarpa* dan *G. procumbens* tidak menunjukkan kesan toksisiti apabila diambil secara oral. Tambahan pula, potensi chemopreventif esktrak tumbuhan-tumbuhan tersebut telah ditunjukkan dengan pengurangan dalam bilangan ACF yang boleh dikaitkan dengan cir-ciri antioksidan ekstrak tumbuhan-tumbuhan tersebut dan penurunan pengawalaturan protein promosi PCNA dalam sel-sel kanser. Lagi, analisis ekspresi gen mendedahkan bahawa ekstrak etanol kedua-dua tumbuhan tersebut mengaktifkan apoptosis.

ACKNOWLEDGEMENTS

"In the name of Allah, the most gracious and the most merciful" Alhamdulillah, all praises to Allah (God) for the assets and His blessing in completing this thesis. Special thanks to my supervisor, Professor Dr Mahmood Ameen Abdullah, for his supervision and constant support. His invaluable help in putting it all together, and for his constructive comments and suggestions during the experimental and thesis works have contributed to the success of this research.

I am particularly grateful to my co-supervisor Professor Dr Nor Azizan Binti Abdullah, who provided me the needed support, good comments and valuable suggestions.

I wish to mention my thanks to the following: Animal Experimental Unit, CDL-UM Hospital, CARIF, Biotechnology Department and all staff in Biomedical Science Department, Faculty of Medicine, University of Malaya.

I would like to express my sincere appreciation and thanks to University of Malaya for supporting this work through the research grant PV046/2012A which gave me the opportunity to obtain the PhD degree. I am especially grateful to all my friends particularly Dr Mohammed Al-Shawesh for their kindness and moral support during the period of my study. I wish to express my thanks to the Ministry of Higher Education-Yemen for the scholarship. I wish to express my thanks to my parents who constantly pray for me, to my wife for her support and encouragement, to my kids who keep me happy. Last but not least, I wish to acknowledge with thanks all those who showed their cooperation during the course of this study, including the lab work and in the preparation of this thesis.

Abdrabuh Shwter

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Description
G. procumbens	*Gynura procumbens*
P. macrocarpa	*Phaleria macrocarpa*
ACF	Aberrant crypt foci
AOM	Azoxymethane
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ANOVA	Analysis of variance
ATCC	American type culture collection
BAX	BCL-2–associated X
BCL2	B-cell leukemia / lymphoma-2
BCAC	Beta-catenin-accumulated crypts
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DMEM medium	Dulbecco's modified eagle medium
DPPH	α, α-Diphenyl-β-picryl-hydrazyl radical scavenging assay
FRAP	Ferric reducing antioxidant power
FC	Flavonoid content
FBS	Fetal bovine serum
GST	Glutathione S-transferase

HPRT1	Hypoxanthine phosphoribosyltransferase 1.
H&E stain	Hematoxylin & eosin stain
HD	High dose
HPLC	High performance liquid chromatography
Kg	Kilogram
LC-MS	Liquid chromatography-mass spectrometry
LD	Low dose
MDA	Malondialdehyde
Mg	Milligram
Mm	Millimeter
Mmol	Mill mole
MTT	3-(4,5-Dimethylthiazol2-yl)-2,5-diphenyltetrazoliubromide assay
NCCLS	National committee for clinical laboratory standards
nm	Nanometer
PBS	Phosphate buffer saline
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Sprague dawley
SEM	Standard error of the mean

SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substance
TP	Total protein
TS	*Thymidylate synthase inhibitors*
TPC	Total phenolic content
WHO	World Health Organization
Ml	Microliter
μm	Micrometer
P value	Level of significance
RT-PCR	Real time PCR
Ct	Threshold cycle
BHT	Butylated hydroxyltoluene
FOBT	Fecal occult blood test
FdUMP	Fluoro deoxy uridine monophosphate
TGF-β	Transforming growth factor beta

LIST OF APENDEXES

CHAPTER 1: INTRODUCTION

1.1. Introduction

Cancer is a genetic disorder which includes dynamic changes in the genome leading to uncontrolled cell growth, cell division, ability to invade distant organs through blood or lymph and metastasize. In Malaysia, cancer was reported to be one of the most serious problems, and cancerous diseases became a major public health problem that is of great concern to the Malaysian population. Development and progress in cancer research in the country aim to overcome this problem. In Malaysia, lung cancer is the most common whilst colorectal cancer (CRC) is the third most common cancerous diseases in Malaysia resulting in death among malignancies patients (Shin et al., 2012).

Cancer is predicted to be progressively an important cause of morbidity and mortality all over the world. According to National Center for Health Statistics, it was estimated that a total of 1,665,540 new cancer cases and 585,720 cancer deaths would occur in the United States in 2014 if global cancer rates remain unchanged (Siegel et al., 2014). Incidence data from the National Center for Health Statistics also estimated that 65,000 women and 71,830 men were diagnosed with CRC and 24,040 women and 26,270 men would have died of the disease. Azoxymethane (AOM) and its precursor dimethylhydrazine induces colon tumours in rodents by DNA mutations in β-catenin (Ochiai et al., 2001). Aberrant crypt foci (ACF) are proposed to be pre-neoplastic lesions of colons of both animal models and humans (Bird, 1995). Aberrant crypt foci exist in the colon of carcinogen-treated rodents and humans and there is a high risk that the lesions will progress to cancer. Medicinal herbs and their phytocompound derivatives have been proven to be complementary treatment for tumours. The beneficial effects of herbal medicines on the immune modulation and survival of cancer patients were reported by clinical studies when used in combination with conventional therapeutics (Yin et al.,

2013). The utilisation of medicinal plants was shown, as indicated by research, to improve the prognosis in advanced colon cancer patients provided they were used as an adjuvant therapy (Auyeung & Ko, 2010). Over the years, plants are recognised to be good sources of disease-healing drugs. Their role in traditional healthcare systems is already established and they contributed to a significant percentage of modern drugs and allopathic medicine in many nation-states of the world (Shaw et al., 2010). A number of studies indicate the use of numerous medicinal plants for chemopreventive effects (Almagrami et al., 2014; Shwter et al., 2014).

Phaleria macrocarpa Boerl (Thymelaceae) is commonly known as mahkota dewa or crown of god. The plant originated from Indonesia and Papua Island is the habitat of this plant. *P. macrocarpa* is considered to be one of the most popular traditional drugs in and is a dense evergreen tree with a height of around 1-6 m. The tree has all the components of a mature tree (stem, leaves, flowers and fruit). The fruit of the tree is oval in shape with a diameter of around 3 cm. It is green in colour before ripening and red when maturing. Traditionally, this plant is used to prevent or treat cancer, impotency, kidney disorders, diabetes mellitus, blood diseases, acne, allergies, liver and heart disease, migraine, stroke, and various skin diseases (Zhang et al., 2006; You et al., 2010).

Gynura procumbens Merr is an annual evergreen shrub with a fleshy stem that has a purple tint. This medicinal plant is known as Sambung nyawa and is commonly used in South-East Asia countries including Thailand, Malaysia and Indonesia. Locals have used it to treat rash, eruptive fevers, migraines, constipation, kidney disease, hypertension, cancer and diabetes mellitus. *G. procumbens* is pharmacologically active and is known for having virucidal and antireplicative actions against the herpes simplex virus HSV-a and HSV-2 (Jarikasem et al., 2013), anticancer properties against the growth of a breast cancer cell line, MDA-MB-231 (Hew et al., 2013) and anti-ulcerogenic activities (Mahmood et al., 2010). Research has highlighted the advantages of the

traditional use of *G. procumbens* in light of the identification of pharmaceutically active flavonoids in the plant (Habila et al., 2010). Previous studies have described that the consumption of ethanol extract of *Gynura procumbens* inhibited tongue carcinogenesis in rodents. Additionally, the extract inhibited the carcinogenicity of mice lung tumours induced by benzo[a]pyrene (BaP) (Nisa et al., 2012). The existence of side effects of chemotherapy treatment is a major problem, which may lead to undesirable consequences in the human body. These consequences range from hair loss to multidrug resistance. Cancers such as non-small cancer, rectal cancer and lung cancer may not respond to standard chemotherapy from the beginning and this is known as primary or natural resistance (Chidambaram et al., 2011). Because of the disadvantages associated with the use of standard chemotherapy, medicinal plants with fewer side effects have been promoted for the prevention and/or treatment of cancer. The current study aims at evaluating the chemopreventive properties of *P. macrocarpa* fruits and *G. procumbens* in azoxymethane-induced aberrant crypt foci in male Sprague Dawley rats. Results from this study will pave the way for further investigation in cancer prevention.

1.2 Objectives of the Study

1.2.1 General objective

The main objective of this study is to evaluate the chemopreventive effects of *P. macrocarpa* and *G. procumbens* against AOM-induced aberrant crypt foci in male Sprague Dawley (SD) rats.

1.2.2 Specific objectives

1. To evaluate the acute toxicity of *P. macrocarpa* fruits and *G. procumbens* crude leaf extracts in male SD rats.
2. To evaluate the antioxidant properties of *P. macrocarpa* and *G. procumbens* extracts *in vitro* and in colon tissue homogenates of AOM-induced ACF in rats.
4. To evaluate the cytotoxicity of each of the plant extracts by MTT assay.
5. To assess the chemopreventive effects of both plant extracts in AOM-induced ACF in rats (gross examination, histological evaluation and biochemical tests).
6. To identify the active constituents of the plant extracts by HPLC and LC-MS.
7. To investigate the possible mechanisms of chemopreventive action of both plant extracts in AOM-induced ACF in rats.

CHAPTER 2: LITERATURE REVIEW

2.1 Colorectal cancer

Colorectal cancer is the third most common malignant cancer and one of the leading causes of cancer deaths worldwide. More than a million new cases of CRC are identified globally every year and CRC is also the second most common cause of cancer deaths in the US and other countries (Jemal et al., 2011). The rate of CRC is increasing in many nations (Béjar et al., 2012) including Asian region (Sung et al., 2015). About 60% of cases are identified worldwide. In 2012 there were about 40,290 cases of rectal cancer and an estimated 103,170 cases of colon cancer (Saslow et al., 2012). The Asia Pacific Working Group which includes Asian countries such as South Korea, China, Singapore and Japan recommended CRC screening be implemented in states where the incidence of CRC of colorectal cancer is high (Sung et al., 2015). Colorectal cancer was the most common cancer in Singapore, while in Malaysia a recent study reviewing 1, 212 CRC patients who undertook treatment between January 2001 and December 2010 (Magaji et al., 2014) showed that the majority of patients being above 40 years old (Yusoff et al., 2012; Shah et al., 2014).

Benefits of early diagnosis of colorectal cancer and occurrence of malignant neoplasm include reduction of mortality (Lieberman et al., 2012). Mortality due to CRC is proven to be reduced with screening through faecal occult blood test (FOBT), (Bretthauer, 2011). Consequently, a number of countries developed guidelines to include screening for colorectal cancer, even in their national screening programs (Power et al., 2009). Nonetheless, there is still a low rate of screening activity in a number of countries even in developed countries. Screening for colorectal cancer from the age of 50 is recommended by the Asia Pacific Consensus (Sung et al., 2008). The introduction of guidelines on screening for CRC in Malaysia took place in 2001 (Yusoff et al., 2012). Colorectal cancer screening is less established than cervical

cancer screening. So, it is expected that low levels of colorectal cancer screening exist, which explains why the majority of colorectal cancer patients in Malaysia are diagnosed when the disease is in the advanced stages. Besides, the knowledge of Malaysian patients regarding CRC and its screening activity is extremely low (Harmy et al., 2011).

2.1.1 Signs and symptoms of colorectal cancer

The signs and symptoms of CRC have been defined, with the main ones being rectal bleeding (GI bleeding), constipation or diarrhoea, worsening fever, loss of appetite and body weight, abdominal pain and vomiting in patients who are over 50. While rectal bleeding or anaemia are high-risk features in those over the age of 50, other generally described signs and symptoms including change in bowel habit and weight loss are typically only of concern if associated with haemorrhage (Adelstein et al., 2011; Astin et al., 2011). Other signs are dark or black stools, pain in the lower stomach, new onset of diarrhoea or constipation for more than a few days (Pal et al., 2012)

2.1.2 Cause of colorectal cancer

Most of the CRC cases occur as a result of lifestyle and advancing age with only a minority of causes related to an underlying common genetic disorder (Watson & Collins, 2011; Abotchie et al., 2012). It usually begins in the lining of the colon or rectum and if left untreated can develop into the muscle layers underneath, and then through the bowel wall (Fattori et al., 2011). Risk factors for CRC include a patient's age and gender, a high intake of alcohol, fat or red meat, obesity and an absence of physical exercise (Watson & Collins, 2011). The risk for alcohol appears to increase when more than one drink is consumed per day (Fedirko et al., 2011).

2.1.3 Inflammation and cancer

The role of inflammation in the development of cancer was discovered on the basis of findings that tumours frequently arise at the sites of chronic inflammation and that inflammatory cells were present in tumours. Inflammation alters host physiology to promote cancer, as seen in colitis-associated colorectal cancer. Indeed, up to 20% of cancers arise from chronic inflammation and persistent infections (Arthur et al., 2012; Chang et al., 2014). Inflammation is a critical process in the generation of neoplasms. It causes genomic variability, which allows cells to acquire the fundamental properties of a cancer cell (Hanahan & Weinberg, 2011). People with inflammatory bowel diseases, which are characterised by chronic intestinal inflammation, are at increased risk of CRC. Furthermore, risk of disease increases with severity and period of inflammation reaching as high as 40% in people diagnosed with pan colitis before 15 years of age (Kwon et al., 2011). Inflammation has been implicated in development of colorectal cancer and its role has been validated by epidemiological studies. Latest work in pre-clinical models suggests that this association is not simply attributable to production of DNA-damaging reactive oxygen species but rather that the role of inflammation in colorectal neoplasia is multi-faceted and complex (Kozicky et al., 2013).

2.1.4 Genetic of colon carcinogenesis

Statistically, the third most diagnosed cancer in both males and females is colorectal cancer. The percentage of patients with sporadic forms of the disease is 75% while those with a family history of the disease represent 25%, which signifies the roles played by shared genes and the environment. Major genes with highly inherited mutations, however, contribute to 5-6% of CRC. The interaction of less penetrant genes and environment factors is responsible for the remaining familial forms (Jasperson et al., 2010). Individuals diagnosed with CRC and with a family history in two or more first-degree relatives have a 2 to 3 fold increased risk of the disease and this group accounts

for about 20% of all cases. Once the family history contains two or more relatives with CRC, the probability of a genetic disorder is increased substantially (Ebada et al., 2008).

Tumourigenesis is a multistep process with each step reflecting genetic variations that initiate the progressive transformation of normal tissue in human to highly malignant derivatives (Hanahan & Weinberg, 2011). It is therefore fitting that The Cancer Genome Atlas (TCGA) published a comprehensive characterisation of the genetics of CRC as their third publication (Muzny et al., 2012). Because CRC is often identified at a late stage, and the early detection of cancer dramatically increases survival, the identification of genomic risk factors in CRC is of utmost importance. The most commonly recognised pathway describing CRC progression is the adenoma carcinoma sequence (Pancione et al., 2012), beginning as benign polyps/dysplastic lesions before developing to advanced adenoma, and finally to aggressive carcinoma. A variety of genetic pathways and genes are involved in the progression, which typically occurs over years or decades (Trust & Surrey, 2010).

Colorectal adenomatous polyps are demarcated lumps of epithelial tumour cell that can be categorised into the following major three histological types: villous, tubular and tubule-villous adenomatous (Takuji, 2009). These adenomatous are benign glandular neoplasms originating from intestinal mucosal epithelium characterised by incomplete cell differentiation and unrestricted cell division.

Colorectal cancer is one of the cancers that are thought to develop from adenomatous polyps, and may be due to acquired genetic mutations in tumour suppressor genes. Polyps grow at an increasing rate in people above 50 years of age. There are certain types of polyps that raise the patient's risk of developing CRC. For instance, a rare genetic condition, known as familial polyposis, has more than ninety polyps forming in the colon and rectum. Unless this condition is treated, familial polyposis will usually lead to CRC (Fearon, 2011). Most CRC is adenocarcinoma which develops from adenoma. About 5%

of adenomas become malignant (Cairns et al., 2010). Studies on colorectal cancer support the hypothesis that colon cancer develops through a multi-step process. Small benign adenomatous polyps develop differently from normal mucosa, the mucosa is hyperproliferative or exhibit abnormal tissue architecture such as aberrant crypt foci (ACF) (Lipkin, 1988; Tudek et al., 1989). Subsequent regression of a small adenoma to a large adenoma with enhanced malignant potential may occur in some cases. Finally, a fraction of the larger adenomas may progress into invasive and metastatic cancer (Vogelstein & Kinzler, 1993). These abnormal crypts can be recognised by increased size, pericryptal zone and thicker epithelial lining when visualised with methylene blue (Tudek et al., 1989). But recent reports have demonstrated that adenomas arise from other preneoplastic lesions, i.e. mucin depleted foci which are formed by dysplastic crypts devoid of mucin (Caderni et al., 2003) (Fig. 2.1).

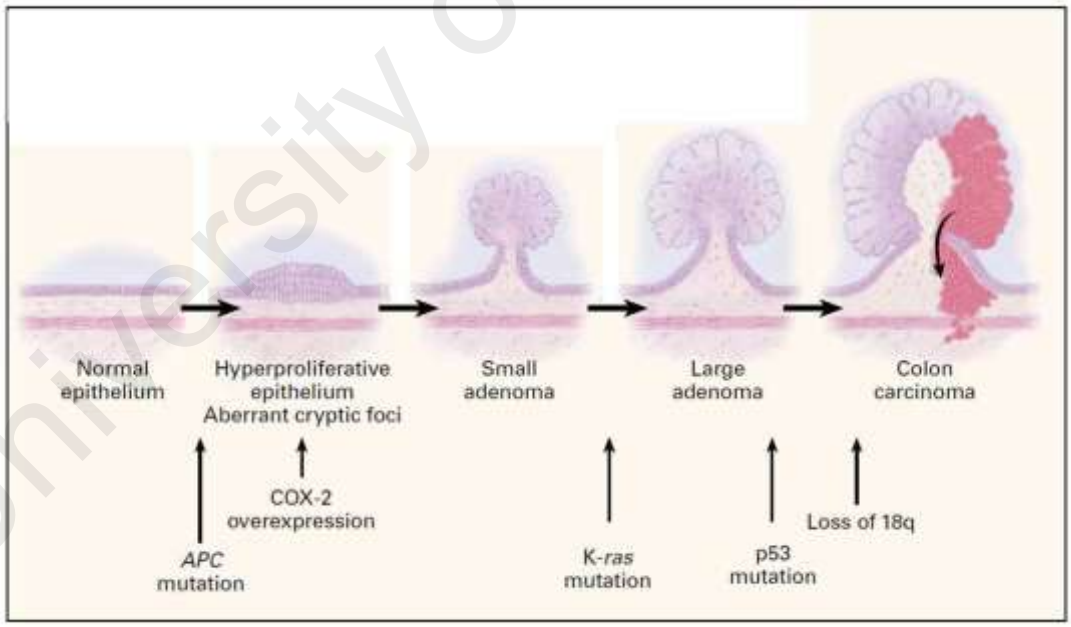

Figure 2.1: Colon Carcinogenesis (Source: Jänne & Mayer, 2000).

There exist a number of genetic disorder that are associated with higher rates of CRC. Hereditary nonpolyposis colorectal cancer or Lynch syndrome is the most common of these kinds and accounts for 3% of people with colorectal cancer. Other syndromes

strongly associated with CRC include Gardner syndrome and familial adenomatous polyposis (FAP) (Juhn & Khachemoune, 2010).

Females and males are similarly affected. Familial adenomatous polyposis (FAP) accounts for less than 1% of colorectal cancer cases, with an expected prevalence of 1/11,300–37,600 in the European Union (Half et al., 2009). Familial adenomatous polyposis may perhaps exist with some extra intestinal manifestations such as congenital hypertrophy, dental abnormalities of the extracolonic tumours, and retinal pigment epithelium (stomach tumours, desmoid, hepatoblastoma, central nervous system tumours and pancreas tumours (Half et al., 2009; Jasperson et al., 2010).

The onset of colorectal cancer takes place in two stages: cellular and molecular. It begins from a mutation within the Wnt signaling pathway. Wnt signaling happens when β-catenin binds to nuclear partners to create a transcription factor which then enters the nucleus and regulates genes involved in cellular activation. Adenomatous polyposis coli (APC) gene which encodes the APC protein degrades β-catenin and thus is a negative regulator of the Wnt pathway. Mutations in the APC gene cause the Wnt pathway to remain activated giving rise to colon cancer (Markowitz & Bertagnolli, 2009).

Environmental in addition to hereditary factors play an important role in the progression of colorectal cancer. Individuals older than 50 years with sporadic cancer, account for over 75% of the various cases of CRC. These people have no identifiable predisposing factors. Inflammatory bowel diseases and familial constitute the remaining percentage. Familial adenomatous polyposis (FAP), known as an autosomal dominant colon cancer syndrome develops upon the inheritance of a single copy of a mutant gene. Familial adenomatous polyposis is characterised by the appearance of hundreds to thousands of colonic polyps in the second and third period of life. It may be considered the least common type of colon cancer (Farinella et al., 2010).

In addition, interruption of colon cell homeostasis leading to disproportionate apoptotic and proliferative cell programs is a reason for development of colon cancer (Dupaul-Chicoine et al., 2010). Both mechanisms are supposed to contribute to the development of malignancies. The regulation of apoptosis requires Bcl2 family of proteins. The pro-apoptotic Bax and anti-apoptotic Bcl-2 are amongst the proposed proteins used as prognostic indicators of several advanced stages of colon cancer. Bcl-2 protein inhibits apoptosis while Bax is the crucial protein that stimulates cell death programming in response to cellular stress. Bcl-2 and Bax actively interact with each other to regulate cellular death program. Thus, a higher expression of Bax levels may result in apoptotic cell death, whereas a relative higher Bcl-2 levels may prevent cell death (Fearon, 2011). Furthermore, proliferating cell nuclear antigen that accelerates proliferation is used as a prognostic marker for colorectal cancer either in the evaluation of the proliferative alteration or in the determination of the cancer stages (Lumachi et al., 2012).

2.1.5 Aberrant crypt foci (ACF)

Aberrant crypt foci were first described in the colon of carcinogen treated rodents (Bird, 1987) and observed in a person with FAP sporadic CRC. APC have thicker epithelial lining, elevated crypts, increased distance from lamina to basal surfaces of cells and enlarged pericryptal zone compared to normal crypts when visualised with methylene blue (Bird, 1987). They display preneoplastic features viz., dysplasia (Paulsen et al., 2005) K-ras mutations (Pretlow et al., 1993) and hyperproliferation (Fig. 2, 2).

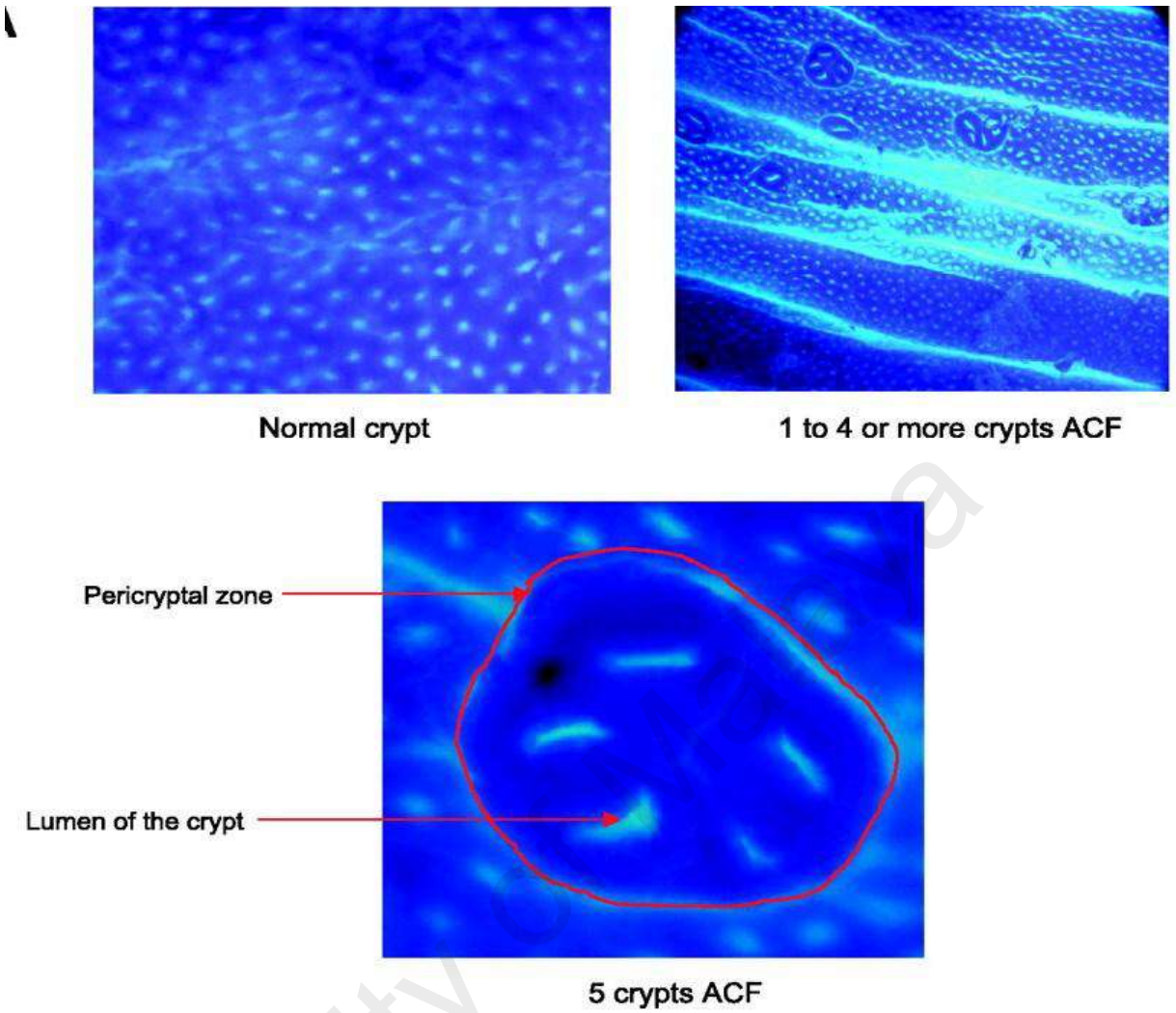

Figure 2.2: Morphological appearance of ACF (Pretlow et al., 1993)

Figure 2.3: Topographical appearance of ACF in mice, rat and human and scanning electron micrograph of ACF. (Tudek et al., 1989; Latham et al., 1999)

ACF replication process is basically identical to that of normal crypts with the replication beginning at the bottom of the crypt pushing cells upwards and outwards to form new colonic crypts and simultaneously replenishing the cells in the original crypt. This is a budding and branching process, known as crypt fission, which forms larger sized foci

over time (Fujimitsu et al., 1996). This process does, nevertheless, happen at an increased rate in several disease conditions of the bowel (Figure 2.4).

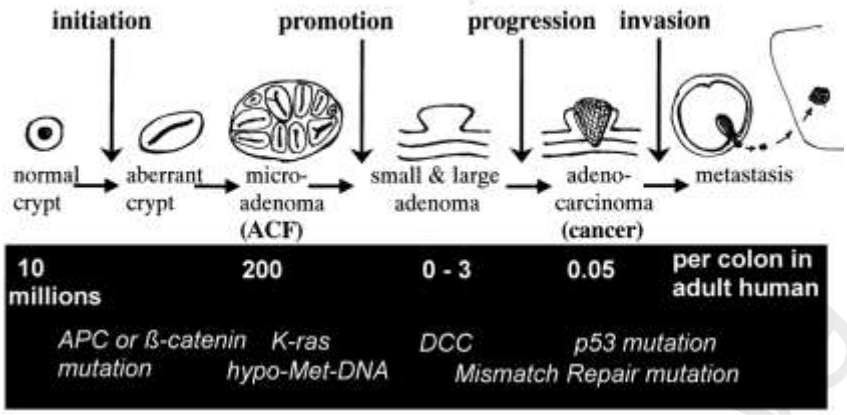

Figure 2.4: Stages of ACF formation in colon cancer (Corpet & Taché, 2002)

According to observations, there are two types of aberrant crypts foci: dysplastic and hyperplastic or non-dysplastic crypt. Hyperplastic or non-dysplastic crypt is the most common and it is a hyper-cellular crypt with normal individual cells. Hyperplastic or non-dysplastic crypt is considered unlikely to result in clinically significant lesions. The least common type is dysplastic ACFs, being the precursors of the adenomas and carcinomas (Aust & Baretton, 2010).

2.1.6. Pathogenesis of CRC

A surgical or biopsy sample is normally taken from the tumourous portion for pathological investigation. A pathological report commonly contains a description of the cell type and status. The majority of the CRC cell types is adenocarcinoma which constitutes 95% of the cases. Other uncommon types include squamous carcinoma and lymphoma. Most CRC originates in polyps as non-cancerous growth in the lining of the large intestine inner surface. Generally, larger polyps present higher risk factor for cancer as compared to smaller polyps. In addition, the cellular characteristics of the polyps are important in determining risk for cancer as villous adenomas carry a higher risk than the

tubular adenomas. Eliminating these polyps is helpful in reducing the incidence of cancer since polyps that are not removed can develop over time into malignancies (Goel & Boland, 2010). For instance, familial adenomatous polyposis will likely develop into CRC in the absence of surgical intervention (Lynch et al., 2013). If the colonic epithelial cells lose the process of "epithelial homeostasis," the balance between proliferation and apoptosis will be lost, or programmed cell death that produces normal regeneration will be disturbed. The process of colorectal carcinogenesis generally follows the predictable sequence of invasion of the pericolic tissue, muscularis mucosa, lymph nodes, and finally, distant metastasis (Armaghany et al., 2012).

2.2 Azoxymethane (AOM)

Azoxymethane (methyl-methylimino-oxidoazanium) is a compound of oxide of azomethane, having molecular formula of $C_2H_6N_2O$, with chemical structure as shown in Figure 2.5. It is a carcinogenic and neurotoxic chemical, with a wide application in biological research, particularly effective in inducing colon carcinomas. Besides being soluble in water, this compound is reported to be sensitive to prolonged exposure to air and elevated temperatures.

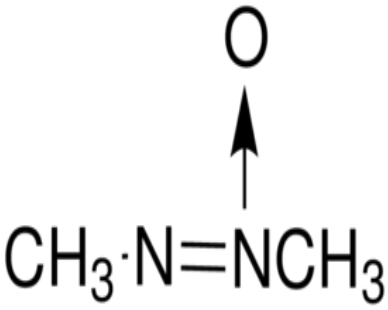

Figure 2.5: Azoxymethane (methyl-methylimino-oxidoazanium)

The use of AOM to induce foci of aberrant crypts in rats has been reported in many studies (Wargovich et al., 2010), one study investigated the chemopreventive effect of zerumbone isolated from *Zingiber zerumbet* in AOM-induced ACF in male F344 rats.

The investigators induced ACF in these rats by subcutaneous injections of azoxymethane (15 mg/kg B.W) for two weeks. They evaluated the effects of zerumbone on tissue proliferation activity by counting silver-stained nucleolus organiser regions protein in colonic cryptal cell nuclei. Other studies used AOM-induced aberrant crypt foci to investigate the effect of *Acanthus ilicifolius* and Schiff base derived copper (II) complex respectively for suppression of ACF (Almagrami et al., 2014; Hajrezaie et al., 2014).

In general, AOM is frequently used for colon cancer induction in animal model. The induction of colon cancer by AOM is probably similar to the pathogenesis of human sporadic colon cancer. Accordingly, such induction has been utilised in other studies of molecular biology, prevention and treatment of colon cancer. When administered, AOM is absorbed and converted into methyl-azoxymethanol by CYP2E1, which causes DNA mutations. Mutation of K-ras stimulates this pathway and its down-stream PI3K/Akt pathway and mitogen-stimulated protein kinase (MAPK) pathway (Chen & Huang, 2009).

The interaction of AOM with DNA is not direct. Azoxymethane needs to be metabolically activated in vivo to develop carcinogenesis. Cytochrome P450, in particular the isoform CYP2E, metabolises AOM through hydroxylation of the methyl group of the compound to form methyl-azoxymethanol. Methyl-azoxymethanol breakdowns into formaldehyde and a highly reactive alkylating compound, most likely methyl-diazonium. This compound causes alkylation of DNA, 06-methylguanine and 04-methylthymine (Chen & Huang, 2009). Of interest, O6-methylguanine formation and colon polyp numbers were reduced in response to azoxymethane treatment in CYP2E1 knockout mice (Chen & Huang, 2009).

2.2.2 Mechanisms of AOM-induced cancer

Numerous activation pathways have been shown to describe the mechanism of azoxymethane-induced cancer (Figure 2.6). These include β-catenin, K-ras and TGF-β. On the other hand, there is no unified explanation for the mechanism of this model. The K-ras is a small G-protein that controls both the PI3K/Akt and MAPK intracellular signaling pathway, which, in turn, controls cell growth, proliferation and glucose metabolism. K-ras is purported to play an important role in the carcinogenesis of colon cancer. AOM has been shown to cause a K-ras gene transversion mutation from G:C pair to A:T pair at codon 12 stemming from O6-methyl-deoxyguanine adducts and transmutation of glycine to aspartic acid. This alteration causes the activation of the K-ras protein (Chen & Huang, 2009).

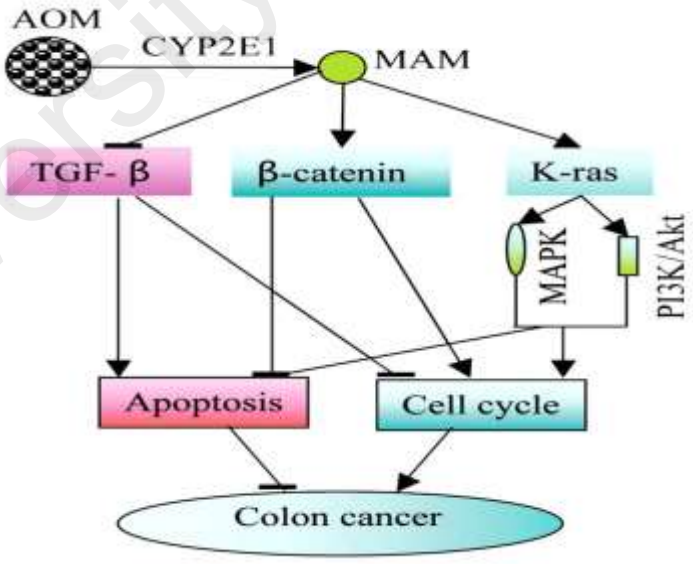

Figure 2.6: Azoxymethane (AOM) mechanism of colon cancer (Chen & Huang, 2009).

2.3 Fluorouracil (5-FU)

There is a significant treatment options for different types of malignancies including colon and breast cancer. Since 1957, 5-FU is still commonly used for anticancer treatment (Breda & Barattè, 2010). Fluorouracil has a similar structure to the pyrimidine molecules in RNA and DNA; it has a similar structure to uracil except that at the C-5 position it contains a fluorine atom instead of hydrogen (Figure 2.7). In the literature, there is only one study that reported a crystal structure for pure fluorouracil, in which 5-FU crystallises with four molecules in the asymmetric unit and the molecule adopts a hydrogen-bonded sheet structure (Hulme et al., 2005). Due to its analogous structure, 5-FU interrupts nucleoside metabolism and can lead to cytotoxicity and cell death through its integration into RNA and DNA (Noordhuis et al., 2004).

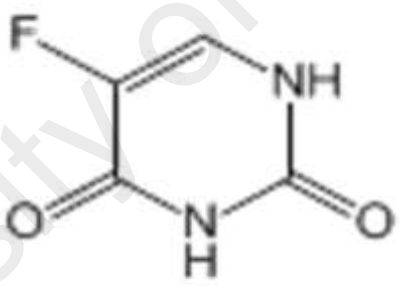

Figure 2.7: Chemical structure of 5-Fluorouracil

2.3.1 Mechanism of action

In mammalian cells, fluorouracil is converted by the enzyme thymidine phosphorylase to fluorodeoxyuridine monophosphate (FdUMP) which is an active metabolite. This metabolite inhibits thymidylate synthase (TS) by forming a stable complex. Inhibition of TS interferes with the production of deoxythymidine monophosphate (dTMP) which is required for DNA replication and repair, and its depletion leads to cytotoxicity (Parker & Cheng, 1990).

Up to 80% of the fluorouracil administered dose undergoes degradation in the liver before elimination (He et al., 2008). The rate-limiting step of fluorouracil catabolism in both normal and tumour tissues is the conversion of fluorouracil to dihydrofluorouracil by the enzyme dihydropyrimidine dehydrogenase (DPD).

2.4 Studies on medicinal plants

Traditional herbal medicine practitioners have described the therapeutic efficacies of many native plants for various ailments (Natarajan et al., 2003). Individuals turn to natural products as a source of synthetic and traditional herbal medicine which is still preferred in some parts of the world as the primary healthcare programs (Singh & Singh, 2001).

2.4.1 *Phaleria macrocarpa*

Phaleria macrocarpa (Scheff.) Boerl (Thymelaceae) is called crown of god, mahkota dewa, and pau. The natural habitat of the plant is in Papua Island, Indonesia and it grows in tropical areas. It is reputed to be one of the most popular tradtional medicinal plants in Indonesia (Figure 2.8).

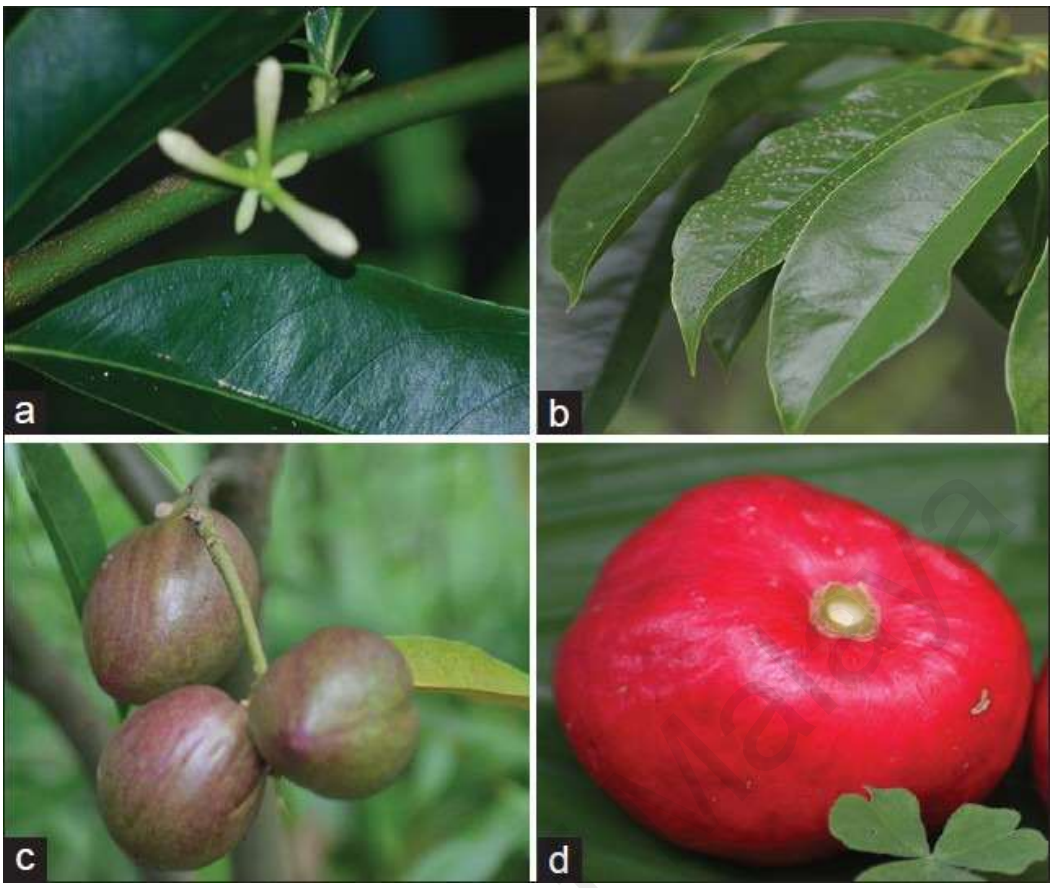

Figure 2.8: *Phaleria macrocarpa* showing a typical (a) small flower bud (b) green tapering leaves (c) un-ripened green fruit (c) and (d) fully grown red fruit (Altaf et al., 2013).

2.4.1.1 Description

P. macrocarpa is an evergreen tropical plant reaching a height of around 1-6 m. It has a stem, leaves, flowers and fruits. The leaves are green, 7 cm to 10 cm in length and 3-5 cm in width. The flowers are composed of two to four petals, and vary between green to maroon; and the the fruit is ellipse in shape with a diameter of around 3 cm. The fruit is green before ripening and red when fully mature. There are 1 to 2 seeds per fruit and they are brown, ovoid and anatropous. Although the herb may be consumed processed or unprocessed, the latter form can be poisonous and toxic. *P. macrocarpa*, of which the extract has been evaluated scientifically for pharmacological uses, can be applied as a treatment for diseases caused by lifestyle (Yosie et al., 2011).

2.4.1.2 Pharmacological properties and uses

P. macrocarpa is known to have a number of pharmacological activities, including anti-tumour, anti-inflammation, anti-hyperglycemia, anti-viral, anti-oxidant, vasodilator, anti- fungal and anti-bacterial effects. Parts from the plants, such as the fruits, leaves and stems, are used to treat bone cancer, cervix cancer, breast cancer, liver and heart diseases, lung diseases while the leaves contain constituents that can be used to treat blood disorders, diabetes, mellitus, allergies and tumours (Hendra, et al., 2011). Research has shown that *P. macrocarpa* has plant secondary metabolites that could combat not only infectious diseases or cancers , but also the so-called lifestyle diseases including hypertension, diabetes and atherosclerosis. It is also used as traditional medicine to treat disorders in animals. The methanol extract of differents parts of *P. macrocarpa* is believed to be a natural antimicrobial source due to the presence of flavonoids (Hendra, et al., 2011).

2.4.1.3 Phytochemistry

A variety of chemical constituents have been found in different parts of *P. macrocarpa* in different amount. These include dodecanoic acid, palmitic acid, mahkoside A, des-acetyl, flavicordin-A, flavicordin-D, ethyl stearate, flavicordin-A glucoside, sucrose and lignans (Hendra et al., 2009). Yang and co-reserachers isolated mahkoside A from the pit of *P. macrocarpa* along with six constituents including kaempferol-3-o-β-D-glucoside, magniferin (a C-glucosylxantone), palmitic acid, dodecanoic acid, ethyl stearate, and sucrose (Zhang et al., 2006). The bark and fruits are rich in phenols, alkaloids , polyphenolics, flavanoids, saponin, tanins and lignans (Chong et al., 2011; Hendra, et al., 2011). Isolated compounds from the fruit include magniferin, icariside C3, and gallic acid (Kim, et al., 2010; Ahmad et al., 2012).

Methanol extracts of *P. macrocarpa* fruits have phalerin content up to 9.52% (Ali et al., 2012). Another study described an extraction method using pressurised hot water

as solvent that yielded as much as 2.1% of magniferin (Kim, et al. 2010). This high yield of magniferin depended strongly on extraction temperature and less on pressure. The yield of magniferin with pressurised hot water was found not to be as much as with methanol (2.5%) but higher than that with water (1.8%). The mesocarp, pericarp and seeds have a total phenolic content around 60.5 ± 0.17, 59.2 ± 0.04 and 47.7 ± 1.04 mg gallic acid equivalent/gram of dry weight (GAE/gDW) respectively. Mesocarp has a higher total flavonoid content when compared to that of pericarp and with the seeds having the least (Hendra, et al., 2011). Fruits and leaves of *P. macrocarpa* are utilised for the treatment of different types of cancer (Hendra, et al., 2011) mainly against brain tumour (Lu et al., 2010) and breast cancer (Winarno, 2010). *P. macrocarpa* supplementation with adriamycin cyclophosphamide is known not only for its synergistic effect which reduced tumour development in breast cells via inducing apoptosis, but also for its defensive effect on kidney and liver damage due to adriamycin cyclophosphamide (Riwanto et al., 2011). Gallic acid and phalerin are two of its ingredients that largely contribute to its cytotoxic properties (Sutiono et al., 2008). The toxic effects of free radicals or ROS on human body, foodstuffs and fats are recognised. Because of this, there is a need to search for antioxidant substances from natural sources, which either prevent or reduce oxidation (Halliwell, 2012). Scientists associate the antioxidant activity of an extract with its free radical scavenging activity. Great effort has been made to develop analyses to determine the antioxidant properties of plant such as ferric reducing antioxidant power assay, thiobarbituric acid assay and ferric thiocyanate assay (Hendra, , et al., 2011; Yosie et al., 2011). The leaves and fruits of *P. macrocarpa* are found to possess phenolics and flavanoids (Yosie et al., 2011) which are known to be effective antioxidants. The constituents found in pericarp, mesocarp and seed extract of *P. macrocarpa* which are responsible for the antioxidant activity include gallic acid (Faried et al., 2007). The

ethanol extracts of *P. macrocarpa* fruits have been shown to enhance the level of superoxide dismutase (SOD).

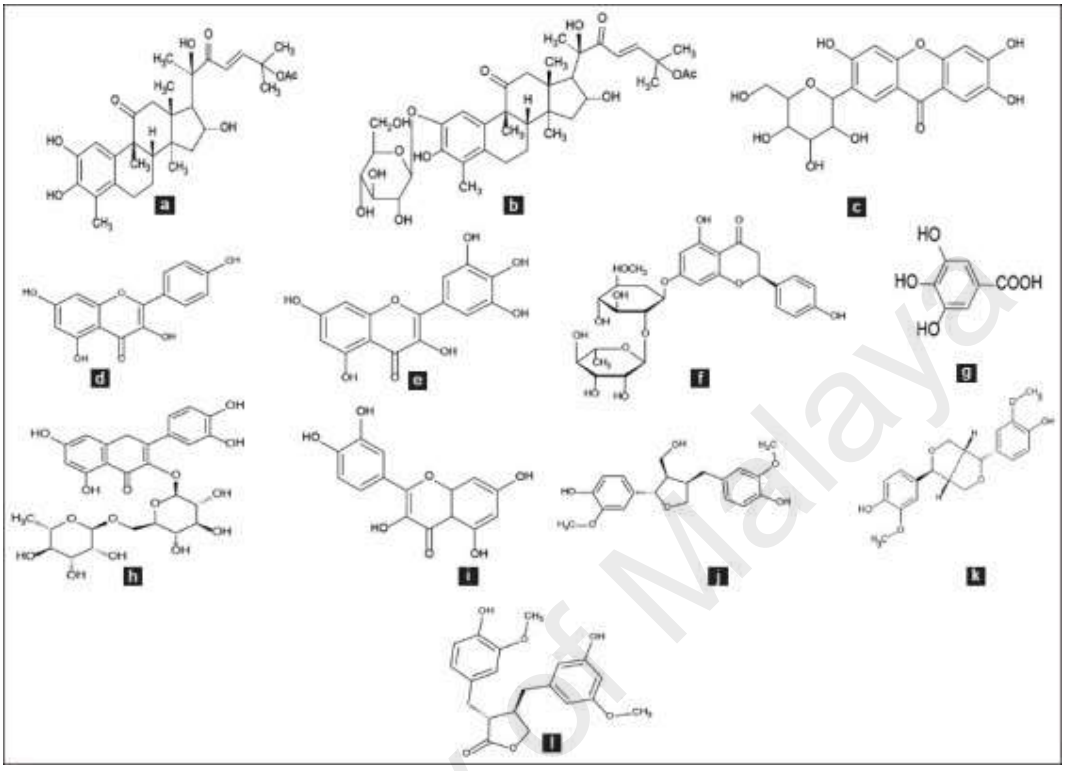

Figure 2.9: Chemical structures of constituents isolated from *P. macrocarpa* extracts (Altaf, et al, 2013).

2.4.2 *Gynura procumbens*

Gynura procumbens (Lour.) Merr. Family of Asteraceae is an important traditional medicinal plant indigenous to Malaysia, Thailand and Indonesia. It is commonly known as 'sambung nyawa' by the Malays and (bai bing ca) by the Chinese in Malaysia. In Malaysia, *G. procumbens* (Figure2.10) is mainly found in the western part of the peninsular (Yam et al., 2009).

Figure 2.10: *G. procumbens* plant (http://ashitabaplant.blogspot.com/2011/06/ashitaba-vs-*gynura-procumbens*.html)

2.4.2.1 Description

G. procumbens (Merr), a member of the Compositae family, is a herbaceous plant that is widely distributed in South East Asia countries including Borneo, the Philippines, Peninsular Malaysia and particularly Indonesia (Hoe et al., 2011). *G. procumbens* has oblance-ovate leaves, with alternate whorled phyllotaxy, acumununate base, acute apex and serrate-cilliated margin. The foliar surface is made up of simple craspedodumous viens, moderately hairy and dark green on the adaxial side while light to yellow green on its abaxial side.

2.4.2.2 Uses and pharmacological properties

G. procumbens has been long used as ethnoherbal products to treat different ailments such as, hypertension, diabetes, urinary infection and is used as anti-allergic and anti-inflammatory agents (Nurulita et al., 2012; Hew et al., 2013). Other uses of the plant

include treatment of kidney disease, eruptive fevers, rash, constipation, migraine, and cancer. Indeed, *G. procumbens* possesses pharmacological actions such as anticancer (Hew et al., 2013) and anti-ulcerogenic effects (Mahmood et al., 2010). The advantages of the traditional uses of this plant is supported by the identification of pharmaceutically active chemical components such as saponins, flavonoids and terpenoids (Kaewseejan et al., 2015). Consumption of the ethanol leaf extract of *G. procumbens* was reported to delay tongue carcinogenesis in rats. Besides, the leaf extract displayed an inhibitory effect on the carcinogenicity of lung tumour in mice induced by benzo (a) pyrene (BAP) (Nisa et al., 2012). A previous *in vitro* study reported that the acetate fraction of the leaf extract of this plant has a cytotoxic impact on breast cancer cells (Luerang et al., 2010). The lack of studies on the effects of *G. procumbens* on colon cancer is being recognised, particularly dietary chemoprevention with regards to colon carcinogenesis.

2.4.2.3 Phytochemistry

A variety of chemical components have been found in different parts of *G. procumbens*, previous studies have isolated flavonol glycosides (e.g. kaempferol 3-O-glucoside and quercetin-3- O -rhamnosyl (1-6) glucoside) as the major constituents in the n-butanol fraction of methanolic leaf extracts (Akowuah et al., 2002). In addition it has been reported that the plant leaf extracts have phenolic acids namely chorogenic, vanillic, gallic, p-rotocatechuic phydroxybenzoic caffeic, syringic p-coumaric and ferulic acids (Kaewseejan et al., 2015).

The roots of *G. procumbens* have a variety of compounds like leucine, valine, isoleucine, alanine, threonine, c-aminobutyric acid (GABA), glutamic acid, aspartic acid, succinic acid, and malic acid (Saiman et al., 2012). Moreover, other studies have discovered valuable plant defense proteins, such as peroxidase, thaumatin like miraculin and proteins from the leaves of *G. procumbens* (Hew et al., 2013).

2.5 Treatment of colon cancer by natural crude extract with anticancer properties

Cancer chemoprevention is described as the use of natural product, synthetic or biologic inducing biological mechanisms necessary to preserve genomic fidelity (Steward & Brown, 2013). Such mechanisms include protection against mutagens/ carcinogens via the inhibition of uptake, activation or through enhanced DNA repair/replication or apoptosis (Hauser & Jung, 2008). A potential chemopreventive agent has to be able to inhibit, or reverse carcinogenesis earlier in the progression of the invasive disease (Pan & Ho, 2008). A perfect agent is one that (1) kills cancer cells while leaving normal ones safe (2) has a known mechanism of action (3) is effective at multiple sites (4) can be orally administered (5) has low cost and high human acceptance (Ouyang et al., 2014).

2.6 *In vitro* and in *vivo* antioxidant properties of the plant extracts

2.6.1 Free radicals and reactive oxygen species

Free radicals are chemical composites which have unpaired electrons in their outer electron orbit. The free radicals are energetic and highly unstable; in order to achieve stability, they constantly find further electrons to pair, steal and attack electrons from other molecules such as proteins, DNA, lipids and carbohydrates. They can even damage DNA giving rise to mutation and chromosomal damage. The attacked molecule becomes devoid of its electron and becomes free radicals itself; this initiates an uncontrolled chain reaction that can damage the natural function of a living cell, resulting in several diseases (Valko et al., 2006).

There are several types of free radicals but those of utmost importance in biological systems are reactive oxygen species (ROS). ROS are highly reactive due to their unstable electron conformations that tend to attract electrons from other molecules (Matés et al., 2012). ROS are the most important free radicals in our body and it refers to any free radical involving oxygen-centred free radical containing two unpaired electrons

in the outer shell. There are two sources of free radicals, endogenous and exogenous. Endogenous sources include free radicals produced during nutrient metabolism and energy production in the mitochondria. Another endogenous source of ROS, especially in the liver, is a group of enzymes called the cytochrome P450 mixed–function oxidases. The biochemical reactions catalysed by the cytochrome P450 molecules employ molecular oxygen, and limited amounts of ROS are produced in these reactions. The production of ROS varies greatly depending on which compound is to be degraded and on the catalysing cytochrome P450 molecule. One type of cytochrome molecule that is especially active in producing ROS is known as cytochrome P450 2E1 (CYP2E1) (Abdelmegeed et al., 2012). Free radicals are involved in many physiological processes and human diseases such as cancer, toxin and liver injury (Alshawsh et al., 2011; Almagrami et al., 2014).

Examples of ROS include free radicals such as hydroxyl and superoxide radicals and also non-radicals such as hydrogen peroxide and singlet oxygen. Generally, ROS can be found in all aerobic cells and are generated by various endogenous metabolic processes such as mitochondrial respiration or exogenous sources, which includes UV light, ionizing radiation, inflammatory cytokines, smoking, alcoholic beverages, food and carbonated drinks (San Miguel et al., 2011).

Oxidative stress seems to play a pivotal role in the development of cancer as reported in many studies (Firuzi et al., 2011; Fuchs-Tarlovsky, 2013). Oxidative stress results from an imbalance between intracellular production of ROS and antioxidant defences (Kashyap & Farrugia, 2011).

2.6.1.1 Role of oxidative stress in carcinogenesis

Excessive ROS is associated with decreased levels of antioxidants in the body and this condition is known as oxidative stress. There are accumulating evidences that correlate oxidative stress and antioxidant status in several malignant cancer such as

colorectal (Al-Henhena et al., 2014; Hajrezaie et al., 2014), breast (Panis et al., 2012), and prostate cancer (Thapa & Ghosh, 2012). Biological molecules such as carbohydrates, lipids, DNA and proteins are always at risk of being oxidised by ROS that lead to chronic inflammation and cancer.

The most common effect of ROS is DNA damage, which potentially leads to carcinogenesis. ROS can directly cause DNA base changes, produce single or double-stranded DNA breaks and cross-links as well as chromosomal instability, gene mutations, altered gene expression (Figure 2:9). In general, cancer development is characterised by three steps: initiation, promotion and progression, of which ROS plays a role in all these stages (Steinberg, 2009). Several base damage products generated by ROS include 8-hydroxy-deoxyguanosine (8-OHdG), 8-hydroxyguanine (8-OH-Gua) and thymine glycol. 8-OHdG is commonly used as a biomarker of oxidative stress and is known as a potential biomarker in the early stages of cancer initiation and promotion. Furthermore, level of 8-hydroxyguanine (8-OH-Gua) determines the transformation of benign to malignant cancer (Hong et al., 2010).

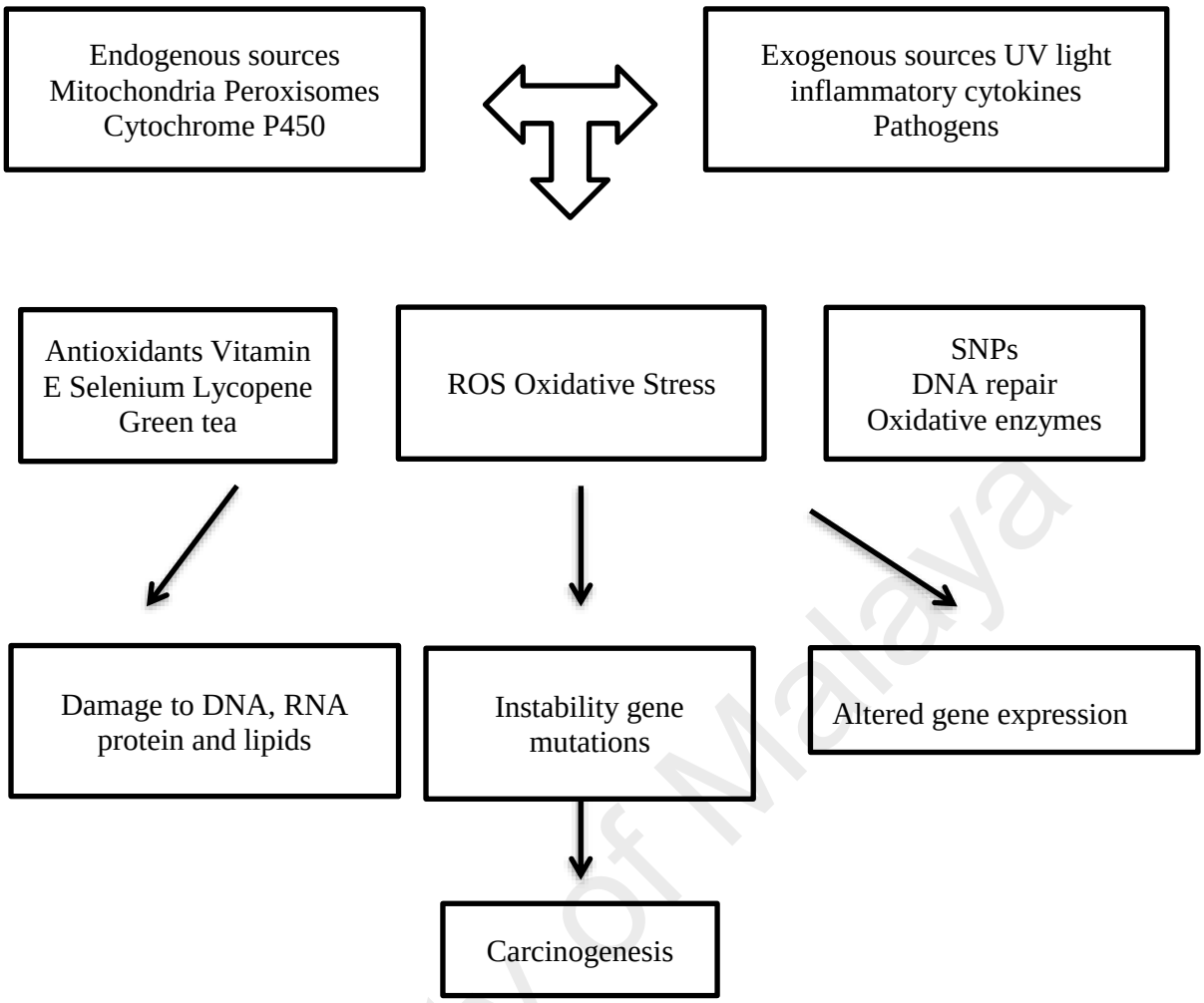

Figure 2.11: Role of reactive oxygen species (ROS) in the development of cancer (Klaunig et al., 2010).

2.6.1.2 Antioxidant defence mechanisms

Supplementation of antioxidants may protect against ROS and avoid damage to cells (Halliwell, 2012). The main property of antioxidant is the ability to trick free radicals and it plays a role as electron or H donating agents (Kilgour & Roberts, 2014). Due to its ability to react with free radicals and to act as oxygen scavenger, the antioxidants can interfere with the oxidation process.

There are two main antioxidant defence systems that can reduce ROS and protect tissues against damage by ROS, which include enzymatic and non-enzymatic antioxidants. Non- enzymatic antioxidant include vitamin E, vitamin C, carotenoids, thiol antioxidants and natural flavonoids. The enzymatic counterparts comprise superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT) (Valko et al.,

2006). Initiation of enzymatic antioxidant is important for protection against oxidative stress (Gill & Tuteja, 2010). Superoxide dismutase is the most effective intracellular antioxidant since it provides the first line of defence against elevated levels of ROS. The protective effects of antioxidants in the diet is mostly attributed to micronutrients such as vitamins C and E carotenoids and plant polyphenols (Jakesevic et al., 2011).

2.6.1.3 Evaluation of antioxidant activity

In vitro and *in vivo* antioxidant assays were used to measure the antioxidant potential of herbal extracts. A number of *in vitro* assays were developed to monitor the effectiveness of natural antioxidants as compounds and also as plant crude extracts. Electron transfer reactions are the most known *in vitro* methods, examples are trolox equivalent antioxidant capacity, 2,2 diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), ferric reducing antioxidant power (FRAP), α, α- Hydroxyl radical scavenging assay and total phenol content. These methods are popular because they are highly sensitive and not time consuming. However, it is recommended to verify the antioxidant capacity by using more than one method due to the complex nature of phytochemicals (Chanda & Dave, 2009).

In addition to *in vitro* assays, there are several *in vivo* cellular enzymes and compounds that may be measured in experimental animal tissue homogenates to assess the antioxidant capacity of plant extracts. Cells contain an effective array of antioxidants that act in a coordinated way to combat the deleterious effects of free radicals. Superoxide dismutase, GST and catalase CAT are major antioxidant enzymes because of their role in the direct elimination of ROS. GST enzymes are the main players in phase II drug-metabolism where they enhance cell protection by detoxification of foreign compounds. (Tew et al., 2011).

Malondialdehyde (MDA) was reported to be the main secondary oxidation product of polyunsaturated fatty acids. It was reported as having mutagenic and cytotoxic

effects. The levels of MDA were elevated in various diseases related to free radical damage and MDA is used as a biomarker for assessment of lipid peroxidation (Kilic et al., 2014). Measurement of MDA is a simple, sensitive and inexpensive method to assess the degree of cellular lipid peroxidation affected by oxidative stress. Thus, MDA measurement in biological samples, such as rat and human plasma, urine, other tissues/ organs such as liver and lung has been adapted to assess the extent of oxidative stress. The possibility of using it as a biomarker for oxidative stress *in vivo* has been reported (Bordoloi et al., 2013).

CHAPTER 3: MATERIALS AND METHODS

3.1. Preparation of plant extracts

Fresh leaves of *G. procumbens* and fruits of *P. macrocarpa* were taken from Ethno Resources Sdn Bhd, Selangor Malaysia, and recognized by comparison with the voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of biological science, University of Malaya, Kuala Lumpur. Both the leaves and fruits were rinsed with tap water followed by drying and grounding into powder by an electrical blender. A hundred grams of the powder of each was soaked in 900 ml of 95% ethanol for 48h. Next, the ethanol extract was filtered using filter paper (Whatman No. 1) and then dried using rotary evaporator (Buchi) to produce the crude-dried extract. The percentage yield of ethanol extracts was 4.0% (w/w) for the leaves of *G. procumbens* and 12.0% (w/w) for the fruits of *P. macrocarpa*. The dry extract was then dissolved in 10% Tween 20 and administered orally to the rats in concentrations of 250 and 500 mg/kg body weight, respectively.

3.2 Chemicals and reagents

AOM is a colon specific carcinogen and was used to induce ACF in the rat colon. The chemical was purchased from Sigma (Sigma-Aldrich, Switzerland) in 100 mg vial and stored at -20ºC before use. After dilution with normal saline, it was administered subcutaneously at 15 mg/kg body weight once a week for two weeks. 5-FU (Sigma Chemical Co., St. Louis, MO, USA) was used as standard drug, dissolved in 0.9% normal saline and administered intraperitoneally to the rats at a dose of 35 mg/kg body weight (Tanaka et al., 2001).

3.2.1 Preparation of AOM

100 mg azoxymethane (the content in one vial) was diluted in 10 ml normal saline. The solution was used to induce ACF in rat colon.

3.3 Determination of *in vitro* antioxidant activity

The *in vitro* antioxidant activity of the *P. macrocarpa* and *G. procumbens* ethanolic extracts were measured using the FRAP, DPPH, TPC and FC assays.

3.3.1 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing activity of the plant extracts was estimated using the method of Benzie and Strain (Benzie & Strain, 1996). This method is based on the reduction of colourless ferric-tripyridyltriazine (Fe^{3+} TPTZ); the complex is reduced to the ferrous ion (Fe^{2+}) which yield an intense blue colour by the action of electron-donating antioxidants at low pH that is absorbent at 593 nm. The reaction mixture contained 300 mmol/liter acetate buffer, 10 mmol/liter TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol/liter of HCl acid and 20 mmol/L of $FeCl_3$. $6H_2O$. The FRAP reagent was freshly prepared by mixing the ingredients; 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3$. 6H2O. Subsequently the mixture was incubated at 37°C in water bath for five minutes and then a blank reading was obtained with spectrophotometer at 593 nm. After that, 10µl of extract or standard was added to 300µl of the working FRAP reagent. Absorbance is measured at 0 minutes upon the addition of the FRAP reagent immediately after mixing. The second absorbance reading was measured four minutes later. The standard curve was plotted using $FeSO_4$ solution absorbance as standard, and the results were expressed as µmol Fe (II)/g dry weight of crude extract (Appendix A).

3.3.2 Free radical scavenging activity (DPPH) assay

The antioxidant activities of the plant extracts were determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The assay was carried out as described by Brand et al. (Brand-Williams et al., 1995). All chemicals were supplied by Sigma-Aldrich (Steinhem, Germany), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), glacial acetic acid, ascorbic acid (vitamin C), quercetin, rutin, butylated

hydroxytoluene (BHT), DMSO and trolex. DPPH is a stable free radical that can donate hydrogen atoms and can be scavenged by any antioxidant compound. When it is dissolved in a solvent, it gives a purple colour. The colour changes to yellow when a single electron of DPPH radical is doubled with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH form. The yellow colour becomes less intense or colourless as an increasing number of electrons are captured (Generalić et al., 2011).

Briefly, 1 mg of the ethanolic extract of each plant was dissolved in 1 ml solvent and the solution was then diluted to obtain different concentrations. Ascorbic acid and BHT are used as antioxidant control. Five µl of each plant extract solution or standard were mixed with 195 µl of DPPH in triplicate. The reduction in absorbance reading was measured at 515 nm for 2 hours with 20 min intervals (Appendix A). The radical scavenging activity was obtained using the following equation. The percentage of radical scavenging activity = {(Abs Blank – Abs Sample)/Abs Blank} X100 and the readings were expressed as mean ± standard error. A graph was plotted representing the sample concentration versus the percentage of DPPH scavenging activity to evaluate the IC_{50} (the concentration needed to inhibit 50% of DPPH radical scavenging activity).

3.4. Determination of total phenolic and flavonoid content

3.4. 1 Total phenolic content assay (TPC)

The phenolic content of *P. macrocarpa and G. procumbens* extracts were evaluated using Folin-ciocalten reagent and was calculated, based on Folin-Denis colorimetric method, as gallic acid equivalents in mg (GAE)/g of extract (AOAC, 1995). The test was based on the oxidation-reduction reaction that occurs between the Folin-Ciocalteu reagent solution (phosphomolybdic and phosphotungstic acid) and the phenolic compounds in the sample. Briefly, for measurement of TPC, a mixture of equal volumes of crude extract (1mg/ml DMSO) and 10% Folin-Ciocalteu reagent was placed in a 96-well plate, incubated for 5 minutes followed by the addition of 10% sodium carbonate

solution. The mixture was further incubated for 90 minutes after which the total phenolic content was measured at wavelength 750 nm. The readings were compared to a standard curve drawn based on gallic acid solution readings (Appendix A). The readings of different dilutions of test samples were performed in triplicate and the mean absorbance was calculated. TPC values of the plant crude extract were determined using the standard curve of absorbance versus the concentration gallic acid equivalents (mg GAE/g). (AOAC., 1995.)

3.4.2 Determination of total flavonoid content (TFC)

The total flavonoid contents were obtained using the aluminium chloride colorimetric assay and were expressed as quercetin equivalents in mg (QE)/g of plant extract as described by Dowd (1959). The assay was carried out in triplicates. All chemicals, aluminium trichloride, potassium acetate, quercetin and DMSO were purchased from Fisher chemicals, UK.

Briefly, 500 µl of the 1:20 diluted and filtered extract (at an original concentration of 100 mg/ml in ethanol) was mixed with 1.5 ml of 95% ethanol, 100 µl of 10% aluminum trichloride ($AlCl_3$), 100 µl of 1 M potassium acetate, and 2.8 ml of deionized water. A total of 250 µl of mixture was incubated at room temperature for 40 minutes and the absorbance was measured at 415 nm. The data were expressed as milligram quercetin equivalents (QE)/g of extract (Appendix A). The total flavonoid content was expressed as quercetin equivalents mg (QE)/g dry weight.

3.5 Cytotoxicity and anticancer activity

3.5.1 Cells and chemical

The normal colon human cell line CCD841 and colorectal carcinoma cell line HT29; catalogue number (HTB-38) were obtained from American Type Culture Collection. RPMI 1640 cell culture media, fetal bovine serum (FBS), penicillin/streptomycin solution, trypsin EDTA solution, trypan blue, MTT (3-(4,5-

dimethyl thiazol-2-yl-2,5-diphenyl tetrazolium bromide), sterile tissue culture flasks, ethanol analytical grade (Merck, Germany). Sterile centrifuge tubes, sterile cell culture plates, phosphate buffer saline, dimethyl sulfoxide, and reagents were provided by Sigma Aldrich, Germany. Cell culture flasks and plates were provided by (Jet Biofil). Cell culture studies were performed in class II, biosafety cabinets (ESCO, USA) under sterile conditions.

3.5.2 Cytotoxicity procedure

Cytotoxicity and potential anticancer activity of the crude extracts were evaluated by an established MTT assay (Mosmann, 1983). Two cells line, human colorectal cancer cell (HT29) and normal colon human cell (CCD841) were grown in RPMI medium (Sigma-Aldrich, USA) supplemented with 10% FBS (Sigma-Aldrich, USA) and 1% penicillin-streptomycin solution (Sigma-Aldrich, USA). Both cell lines were cultured in humidified 5% CO_2 incubator at 37°C, and sub-cultured twice weekly using trypsin/EDTA (Sigma-Aldrich, USA). The MTT colorimetric assay were carried out on the crude extracts and the assay were performed in triplicates and repeated in three independent experiments.

The MTT assay of the plant crude extracts were performed on HT29 and CCD841 cell line according to the methods established by Mosmann and Scudiero (Mosmann, 1983; Scudiero et al., 1988) as mentioned in the following. Briefly, 100 µl of the HT29 cell line suspension were seeded in 96-well flat bottom micro titer plates at 5×10^4 cells/ml in RPMI 1640 medium containing FBS 10% (v/v) and allowed to grow in CO_2 incubator at 37°C, 5% CO_2 and 90% humidity for 48 hr. On the second day, the cells were treated with 20 µl of 1000, 500, 250, 125 and 65 µg/ml (final concentrations) crude extracts respectively followed by re-incubation (37°C in humidified 5% CO_2 incubator) for 24 hr. After 48 hr., 10 µl of 5mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide obtained from Merck, USA) was added into each well of a

micro-titre plate and re-incubated for 4 hr. at 37°C in humidified 5% CO_2 incubator. The yellow tetrazole MTT solution is reduced to purple formazan in living cells (Mosmann, 1983). After incubation, the MTT was removed carefully and re-suspended in 100 µl of dimethyl sulfoxide (DMSO, Fisher Scientific, UK) to solubilize purple formazan product into a coloured solution whose absorbance was obtained using power wave x340 ELISA Reader (Bio-Tek Instruments, USA) at 595nm. The assay was performed in triplicate from three independent experiments. The percentage of cell viability was calculated by the following formula and the results were expressed as mean ± S.E.M.:

% Cell viability = {(Abs sample – Abs blank) / Abs blank} X 100

The dose response curves were obtained by plotting the percent inhibition versus the concentrations. Data is expressed as mean ± S.E.M. and the inhibition concentration (IC_{50}) calculated by linear regression equation.

3.6 Acute toxicity test

A total of 36 healthy, adult Sprague Dawley rats, of which 18 are males and 18 are females (6-8 weeks old) were obtained from the animal experimental unit, Faculty of Medicine, University of Malaya, Kuala Lumpur. The body weight of the rats ranged between 120 – 150 g. The animals were given standard rat pellets and tap water. The acute toxic study was carried out to identify a safe dose for *P. macrocarpa* and *G. procumbens* extracts. The rats were randomly divided into three groups and each group either received Tween20 (vehicle control group), 2 g/kg or 5 g/kg of *P. macrocarpa* or *G. procumbens* preparation respectively. Following overnight fasting, appropriate doses of the plant extracts were administered, and the animals were monitored for 30 min, 2hrs, 4hrs, 8hrs, 24hrs and 48 hrs., for any toxicological or clinical symptoms. No mortality was recorded when the animals were administered all doses of each of the plant extracts over a period of two weeks. The rats were sacrificed on the 15th day. Biochemical and

histological (liver and kidney) parameters were assessed under light microscopy according to standard protocol (OECD, 2001).

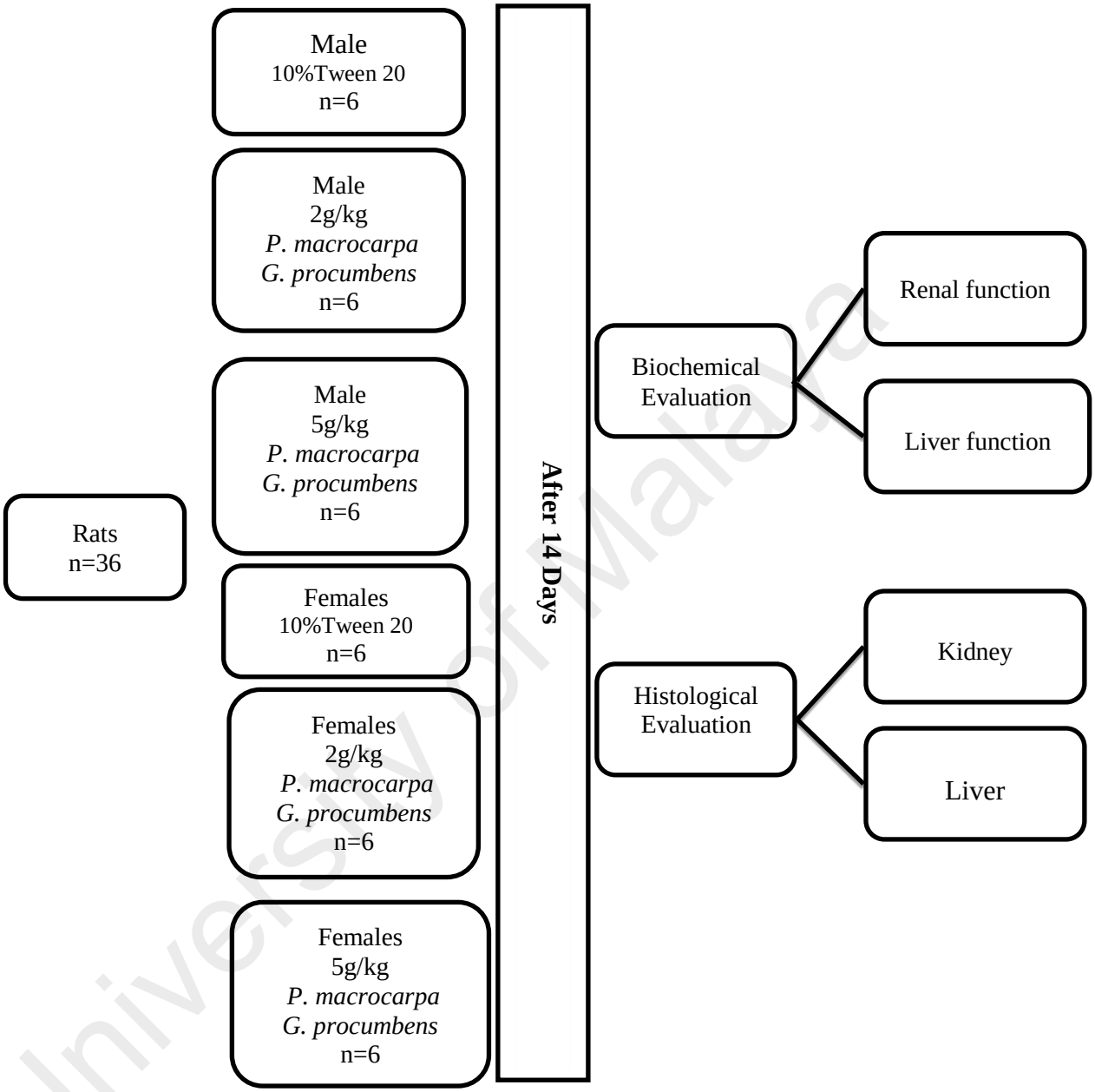

Figure 3.1: Design of acute toxicity

3.7 Experimental animals for chemoprevention study

Healthy male adult Sprague-Dawley rats weighing 120-150 g were obtained from the Animal Experimental Unit, Faculty of Medicine, University of Malaya, Kuala Lumpur. For each plant extract, thirty rats (six-weeks old) were sorted randomly into five groups of six animals in each group. The rats were placed individually in separate cages and were fed standard pellet diet and tap water. The protocol of this study was accepted by the ethics committee for animal experimentation, Faculty of Medicine, University of Malaya, Malaysia (Ethic No. PM/07/05/2012/MMA (b) (R). Throughout the experiments, all the animals received care according to the standards outlined in the "Guide for the care and use of laboratory animals" published by the National Institute of Health. Animal groupings are as follow:

Group 1 (control/normal group): received subcutaneous injection of normal saline (5 ml/kg) and oral administration of 5 ml/kg 10% Tween 20 daily for 10 weeks

Group 2 (AOM group): received subcutaneous injections of 15 mg/kg AOM once per week, consecutively for two weeks (Robles et al., 2010), oral administration of 5 ml/kg 10% Tween 20 daily for 10 weeks

Group 3 (5-FU group): received subcutaneous injections of 15mg/kg AOM once per week, consecutively for two weeks and intraperitoneal injections of 35 mg/body weight 5-FU daily for five successive days (Tanaka et al., 2001). Body weight of all rats was recorded weekly throughout the experiment.

Groups 4 and 5 (extract treatment groups): received subcutaneous injections of 15 mg/kg AOM once per week, for two consecutive weeks and oral administration of 250 mg/kg or 500 mg/kg body weight of ethanol plant extract, respectively, daily for 10 weeks.

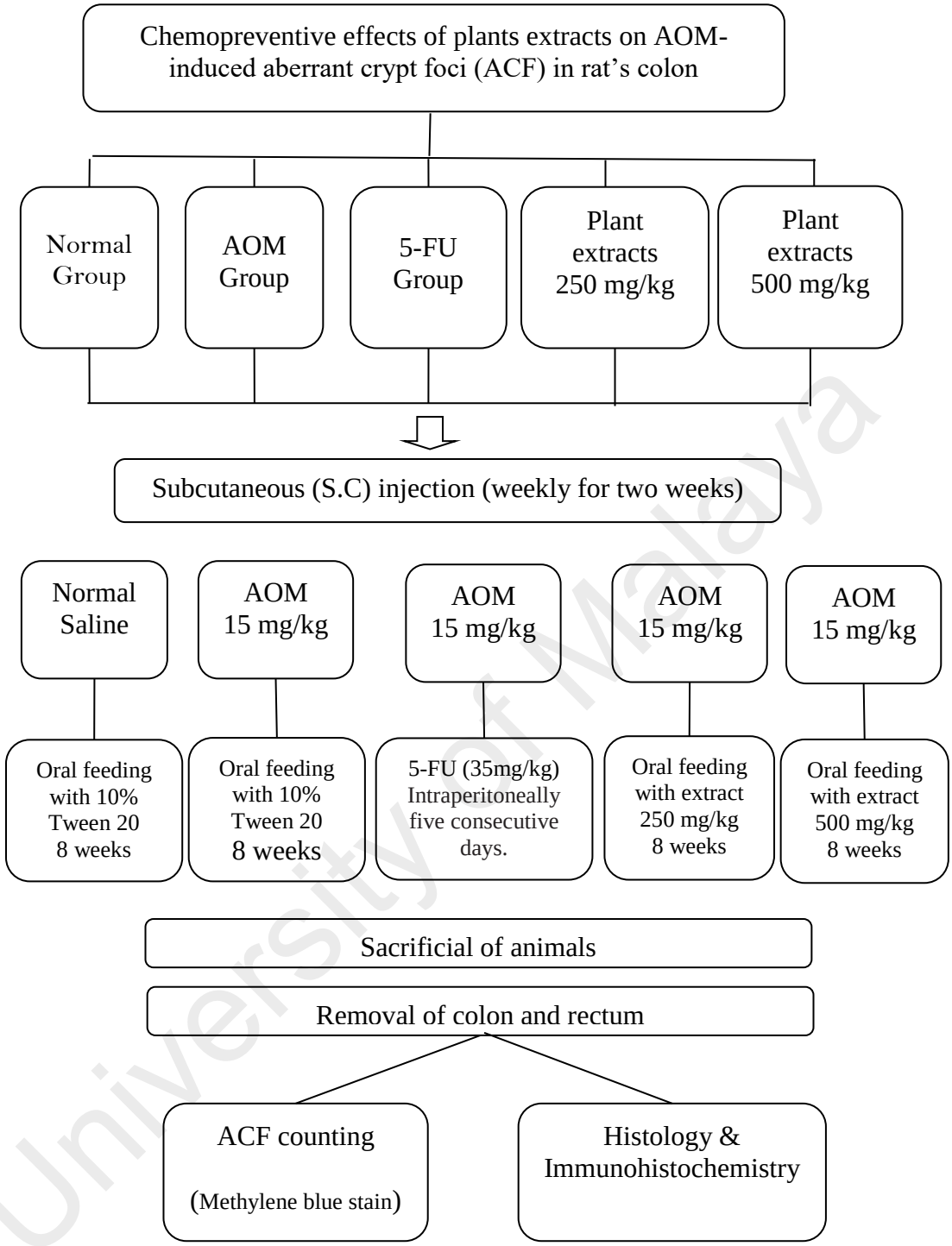

Figure 3.2: Flowchart showing induction of ACF and chemopreventive study

3.8 Gross evaluation of clone mucosa

The animals were sacrificed under anesthesia using 30 mg/kg of ketamine and 3 mg/kg xylazine. The colon from caecum to rectum were collected, gently flushed with PBS, followed by longitudinal opening of the colon. Subsequently, the colon was fixed in formalin and stained using 0.2% methylene blue/PBS for 20 min at room temperature. The stained tissues were washed twice with PBS, and then viewed under a light microscope for scoring based on the number of ACF (foci containing more than two aberrant crypts). The number of ACF per colon and the number of aberrant crypts in each focus were recorded. Due to the swelling nature and noticeable pericryptal zone, ACF were readily identified from normal crypts (Kawamori et al., 1994).

3.9 Histopathological examination

Colon tissues were dissected, and soaked in 10% buffered formalin for 24h and treated using a programmed tissue processing machine. The tissues were immersed in paraffin wax using standard protocol, and the tissue blocks were cut into sections at 5 μm thickness and dyed with hematoxylin and eosin (H & E) to evaluate tumour histopathology. The ACFs were investigated for crypt architecture and nuclear features in comparison to the normal crypts.

3.10 Immunohistochemistry

Immunohistochemistry was carried out using streptavidin-biotin and peroxidase method according to the manufacturer's protocal (Dako ARK™ USA) to identify proliferating cell nuclear antigen (PCNA). Tissues were immersed in 10 % buffered formalin, dehydrated in graded series of ethanol, and sectioned at 5 μm. The sections were thent de-paraffinised and re-hydrated in graded series of ethanol followed by incubation in a microwave for antigen retrieval using (10 mM sodium citrate buffer). The processed samples were then cleansed in PBS and endogenous peroxidase, and were blocked using

0.3% H_2O_2 for 20-30 min. Tissue sections were washed gently with wash buffer and incubated with biotinylated PCNA (1:100) for 15 min followed by another wash with PBS. Subsequently, the sections were incubated in buffer bath in a humidified chamber. The sections were incubated for 15 min in a sufficient amount of streptavidin-peroxidase. The substrate diaminobenzidine (DAB) was added, followed by incubation for over 5 min and then washed and soaked in hematoxylin for 5 sec. The slides were rinsed and dipped 10 times in 0.037 m/l of ammonia. Negative control samples were prepared following the same protocol without the primary antibodies. Subsequently the slides were washed in a bath of de-ionized water for 2–5 min. Using light microscope, brown staining of antigens was considered positive The following formula was used to calculate the PCNA labeling index (PI) which is [(number of positive cells) / (total number of cells)] X 100 for each field. The average PI for each section was calculated (Yamashita et al., 1994).

3.11 Biochemical analysis

The animals' blood samples were collected in gel activating tubes followed by centrifugation at 3400 rpm for 10 min. The serum was isolated and kept for the determination of glucose, alkaline phosphatase (ALP), aspartate aminotransferase (AST), albumin, alanine aminotransferase (ALT), urea and creatinine levels. These enzymes were measured, at the Central Diagnostic Laboratory, University of Malaya Medical Centre, using standard automated techniques according to the provider's instructions.

3.12 Colon tissue homogenates

Colon samples were rinsed immediately with ice cold saline to get rid of blood. The tissue samples were then homogenised in cold 50 mM PBS (pH 7.4) on ice using a homogeniser to prepare colon homogenates (10% w/v). The cell particles were eliminated by centrifugation in a refrigerated centrifuge Rotofix 32A (Hettich Centrifuge, Germany) at 4500 rpm for 15 min at 4°C. The supernatant was used for the estimation of the following *in vivo* antioxidants using ELISA commercial kits (Cayman Chemical

Company, USA): malondialdehyde (MDA) or thiobarbituric acid reactive substance (TBARS), glutathione S-transfer (GST) and superoxide dismutase (SOD). All assays were performed according to the manufacturer's manual.

3.12.1 Superoxide dismutase (SOD) assay

3.12.1.1 Principle

Superoxide dismutase is a metalloenzyme that catalyse the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defence mechanism.

$$2O_2 + 2H^+ + SOD \longrightarrow H_2O_2 + O_2$$

Superoxide dismutase assay kits were used to measure the SOD activity in the samples (Appendix B).

3.12.1.2 General procedure

The assay was performed according to the instruction manual of the manufacturer (appendix B).

3.12.2 Glutathione S-transferase (GST)

3.12.2.1 Principle

Glutathione S-transferases (GSTs) are ubiquitous multifunctional enzymes which play a key role in cellular detoxification. The enzymes protect cell against toxicants by conjugating them to glutathione, herby neutralizing their electrophilic sites, and rendering the products more water soluble.

3.12.2.2 General procedure

The assay was performed by adding 150 µl of the diluted radical detector, 20 µl of glutathione and 20 µl of sample to 96 wells plate. The reactions were initiated by adding 10 µl of 1-chloro-2, 4-dinitrobenzen (CDNB) to all the wells being used. The plate

was shaken for a few seconds at room temperature and the absorbance reading was taken at 340 nm (Appendix B).

3.13. Estimation of malondialdehyde (MDA)

3.13.1 Principle

Malondialdehyde (MDA) is produced as a result of lipid peroxidation which is a cellular injury mechanism in animals and plants that is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable derivatives of polyunsaturated fatty acids that decompose to form a complex series of compounds including reactive carbonyl compound, such as MDA. Cayman's TBARS Assay Kit is a simple, reproducible, and standardised tool to monitor lipid peroxidation in tissue homogenates. The MDA-TBA adducts formed by the reaction of MDA and TBA under high temperature (90-100˚C) and acidic conditions is measured colorimetrically at 530-540 nm and an emission wavelength of 550 nm.

3.13.2 General procedure

The assay was performed by adding 100 µl sodium dodecyl sulphate (SDS) solution and 4 ml of the colour reagent to the sample. The reactions were initiated by boiling for 1hour. To stop the reaction, the mixture was incubated on ice for 10 min, then centrifuged for 10 min and adding 150 µl of the mixture (100 µl SDS +4 ml colour reagent) to all the wells being used. The absorbance reading was taken at 530-540 nm (Appendix B). The MDA activity was determined by using the following formula: MDA (µM) = {(Corrected absorbance)-(y-intercept)}/slope.

3.14 Real-time quantitative polymerase chain reaction analysis

3.14.1 Reagents and equipment

RNA *later*® (Sigma), RNeasy plus Mini Kits, QIA shredder homogenizer (catalog No.79654), mortar and pestle, 70-100% ethanol.

3.14.2 General procedure

Rats were sacrificed and colon samples were taken and kept immediately in RNA *late*r (Ambion, USA), incubated overnight at 4°C and stored at -80ºC until use. Total RNA was isolated using RNeasy- plus Mini Kits (catalog No.74134, Qiagen, Germantown, Maryland, USA & Germany) according to the manufacturer's protocol as follows: 30 mg colon tissue was homogenised using a homogeniser. 600 µl of RLT buffer was added to the homogenised tissue in the mortar. Then the tissue lysate was collected into micro centrifuge tubes (QIA shredder) and centrifuged twice; for 2 min and 3 min respectively. The lysate was then filtered and passed through DNA eliminator spin column and centrifuged for 30 sec at ≥ 8000 x g ≥ (10,000 rpm). Ethanol (50%) was added to the homogenised lysate in a 1:1 ratio and mixed by pipetting. A total of 600 µl of the sample was transferred to an RNeasy spin column placed in a collection tube and centrifuged for 15 s at ≥ 8000 x g.

Total RNA concentration and quality was evaluated by determining 260/280 absorbance ratio using a Nano Drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The A260/A280 ratio should be within the range of 1.8 to 2.0. The RNA was of high integrity and purity, being detected by agarose gel electrophoresis using 0.5% agarose gels and ethidium bromide staining in a buffer system of Tris-borate- (TBA) buffer and agarose gel instrument (Bio-Rad, Richmond, CA, USA). The ratio of 28S RNA to 18S RNA appeared to be 2:1 and this is the required ratio. The RNA was stored after extraction at -80 until used.

3.14.3 RNA agarose gel electrophoresis

3.14.3.1 Reagents

Ethidium bromide (BASE), agarose gel, bromophenol blue loading dye, TrisBorate EDTA buffer.

3.14.3.2 Principle

Agarose gel electrophoresis shows the integrity and size distribution of total RNA (Bio-Rad, Richmond, CA, USA). The apparent ratio of 28S RNA to 18S RNA should be approximately 2:1 (Figure 3.3).

3.14.3.3 General procedure

Agarose (0.5 g) was dissolved in Tris/Borate EDTA buffer (50 ml; TBE) using a microwave until boiling, and ethidium bromide was added (0.5 µl) before casting the gel. Gel (0.25%) was then casted in a 10 x 20 cm gel tray, any bubbles on the gel were removed by pipette tip, a gel comb was then inserted and the gel was allowed to set. Gel electrophoresis was carried out in a horizontal tank.

The integrity and size distribution of total RNA purified was checked by denaturing agarose gel electrophoresis and ethidium bromide staining using agarose gel electrophoresis apparatus (Bio-Rad, Richmond, CA, USA). The apparent ratio of 28S RNA to 18S RNA should be approximately 2:1 (figure 3.3). The extracted RNA was stored at −80º C until used.

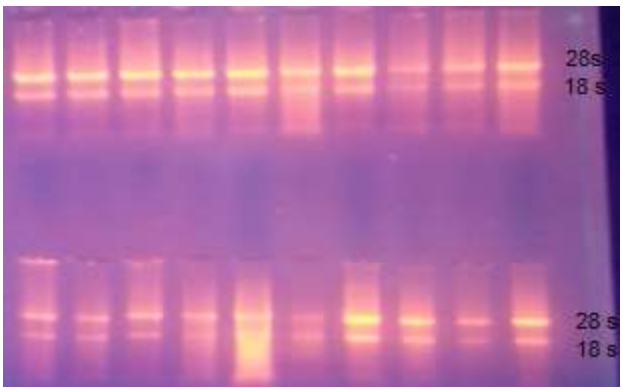

Figure 3.3: RNA profile using ethidium bromide-stained agarose gel

3.14.4 Reverse transcription and cDNA synthesis

3.14.4.1 Principle

One µg of total RNA was reverse transcribed to cDNA with high capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA).

3.14.4.2 Reagents

High capacity RNA-to-cDNA, master mix and nuclease free water.

3.14.4.3 General procedure

Complementary DNAs (cDNAs) were synthesized using master mix kit (Applied Biosystems, USA). cDNAs were produced from 1 µg RNA of each sample using the High Capacity RNA to- cDNA Master Mix protocol (PN 4375575, Applied Biosystem, Foster City, CA, USA). RNA was reverse transcribed to cDNA according to manufacturer's protocol in which the RNA was adjusted to one microgram/20 µl with nuclease-free water and 4 µl RT master mix to obtain the required number of reverse transcription reactions. The tubes were sealed and mixed well before it was loaded in a thermal cycler (Major Science, CA, USA). The run was done under the conditions of 5 minutes at 25°C in the first step, then 30 minutes at 42°C followed by the third step that was 3.5 minutes at 85°C, and finally held at 4°C. The cDNA was stored at -80°C until used.

3.14.5 Real- time quantitative reverse-transcriptase PCR analysis

3.14.5.1 Procedure

RT-PCR of cDNA was carried out using ABI TaqMan gene expression assays for Bcl-2 (assay ID: Rn99999125) and Bax (assay ID: Rn02532082_gl).

3.14.5.2 Reagents

ABI TaqMan gene expression assays (assay ID: Rn 00690933_m1 Ppia) (assay ID: Rn01527840_mlHPRT1), Bcl-2 (assay ID: Rn99999125), Bax (assay ID: Rn02532082_gl) and TaqMan® Fast Advanced Master Mix reactions, nuclease-free water. All reagents and genes were obtained from Applied Biosystems, USA.

3.14.5.3 General Procedure

A standard curve was prepared as follows: 20 µl of each sample with RT were used to make a cDNA pool. From this cDNA pool, 10 µl was taken out and diluted 1:2 as shown in (appendix C).

3.14.5.4 Preparation of the PCR reaction mix

The volume and concentration of each component in the RT-PCR reaction mix was done as in Appendix C. Both standard and samples were placed on ice. Based on the NormFinder algorithm, the genes with the lowest variability were HPRT-1, while geNorm showed that the HPRT-1 and Ppia are the best combination endogenous reference genes. The transcriptional levels of the target genes were normalized using both HPRT-1 and Ppia endogenous reference genes. These two genes were consistently expressed in rat colon tissue and experiments showed a stable expression of both endogenous reference genes in all groups. The cDNA was diluted with nuclease-free water (Applied Biosystems, Foster City, CA, USA) to a concentration of 20 ng/µl after that 1 µl was applied for each RT-PC reaction. The PCR reaction was carried out in TaqMan fast advanced Master Mix (Applied Biosystems, Foster City, CA, USA).

All components were added to 1.5 ml optical tube and capped (Applied Biosystems, Foster City, CA, USA). The amplification reaction was performed using the step one plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to TaqMan gene expression protocol with the following cycling conditions: 2 min at 50°C, 20 sec at 95°C, followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. The reactions were performed in triplicate, and threshold cycle (Ct) average values were used for further calculations according to the comparative Ct method. The values of gene expression was calculated with the 2-ΔΔCt method (Livak & Schmittgen, 2001). The ΔCt value was calculated by subtracting the average Ct value of the endogenous reference gene from the average Ct value of the target gene. The ΔΔCt value was then calculated by subtracting the ΔCt value of the treated sample from the control (untreated) ΔCt value. The gene expression level was then calculated as 2-ΔΔCt giving the final value that is normalised to the reference genes and relative to the control sample values of the studied genes. GenEx Enterprise software for real-time quantitative PCR (qPCR) expression profiling (MultiD Analyses AB, Göteborg, Sweden) was used to analyse and normalise the RT-qPCR data (Kubista et al., 2006). This software allows the correction of PCR efficiencies and the normalisation with more than one endogenous reference genes. Statistical analysis of differences was performed by a two-tailed unpaired Student's t test. A $p<0.05$ is considered indicative of a statistically significant difference."

3.15. Profiling and fractionation of crude extracts

3.15.1. Column chromatography

One gram of both ethanol extracts plant were subjected to column chromatography fractionation using a 3.0 x 50 cm glass columns (Kontes Scientific Glassware, Vineland, NJ, USA) packed with silica gel G60, 70 - 230 mesh (Merck,

Darmstadt, Germany) and connected with an EYEL-L1 type pump (Tokyo Rikakikai, Tokyo, Japan). The crude extracts were eluted stepwise with different solvents in the order of increasing polarity, the first solvent has less polarity than the later solvent with the following gradient (25 ml each time of five different concentrations; 20, 40, 60, 80 and 100 %). The solvents used were hexane, ethyl acetate, methanol, acetone, acetonitrile and water; all fractions collected in clean tubes and all eluents were then pooled to give ten major fractions (Fraction 1–10) The solvents were evaporated in centrifuge evaporator and freeze dryer under reduced pressure (Ebada et al., 2008). The fractions and crude extracts were tested for cytotoxicity and anticancer activities in cancer cell line and those with positive result will be analysed by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) to identify the active components.

3.15.2. Determination of chemopreventive effects of plant fractions

Human epithelial colon cell line CCD841 (ATCC® CRL-1790™) and colorectal adenocarcinoma cell line HT29 (ATCC® HTB-38™) were gifts from Department of Molecular Medicine, Faculty of Medicine, University of Malaya. . Similar to the procedure described in section 3.5., the determination of active fractions was performed through investigation of their chemopreventive effect in *in vitro* studies.The human epithelial colon cell line CCD841 and colorectal adenocarcinoma cell line HT29 were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) FBS (J R Scientific, Inc, USA) and 1% antibiotic solution (penicillin and streptomycin) (Sigma Aldrih, UK), and incubated in a humidified incubator at 37 ^{o}C, 5% CO_2. The cells were sub-cultured periodically to keep them in an exponential growth phase. The CCD841 and HT29 cells were seeded in 96-well plates at a density of 5 x 10^4 viable cells/well and incubated for 24-48 hours to allow cell adherence. Both cell types were treated with the ten fractions from the plant extracts in a similar manner to the crude

extracts. Subsequently, 10 µl MTT (5gm/ml PBS) was added to each well and the cells were re-incubated for four hours. The medium containing MTT was removed and 100 µl DMSO was added. Cell viability was determined spectrophotometrically at 595nm. The fractions which showed positive results were further tested and identified by HPLC and LC/MS to determine the active compounds that were responsible for the cytotoxicity effects.

3.15.3 High performance liquid chromatography (HPLC)

High-performance liquid chromatography is a procedure through which the components of a compound mixture can be separated and the individual components can be identified, quantified or purified. In this analysis, a reversed phase HPLC is done using a non-polar stationary phase and an aqueous, moderately polar mobile phase. RP-HPLC operates on the principle of hydrophobic interactions.

3.15.3.1 Sample preparation

A total of 5mg of each sample of *G. procumbens* or *P. macrocarpa* was dissolved in 2 ml of methanol and the gradient used was methanol being substituted with acetonitrile, isocratic HPLC grade (Scharlau). The solution was filtered through 0.22 µm cellulose acetate syringe into HPLC vial for analysis.

3.15.3.2 Instrumentation and parameter used

Equipment: HPLC Agilent 1100 series equip with auto sampler and fraction collector.

Supported by HPChem System.

Column: Agilent Zorbax ODS C-18 (4.6 X 250 mm, 5.0 µm, 70 A)

Volume of injection 15 µl

Solvents:

A: Methanol, acetonitrile, isocratic HPLC grade (Scharlau)

B: Deionized water

Flow rate: 1.0 ml/ min

3.15.4 Identification of active constituents

In this study liquid chromatography-mass spectrometry (LC-MS) was used for identification of phenolics and other active constituents in the fractions that produced positive result with *in vitro* chemoprotective studies i.e., fraction 10 (F10) of *G. procumbens* and fraction 6 (F6) of *P. macrocarpa*. The identification and characterisation of isolated compounds from active fractions were achieved by comparing ultraviolet (UV) and mass spectrometry (MS). The components of fractions such as phenolics give positive or negative ion mass spectra containing intense [M+H]+,[M-H]- ions as well as fragment ions created after the cleavage of bonds within the compound. Development of the method and procedure were done by a technical staff at Cancer Research Initiatives Foundation (CARIF), Malaysia.

3.15.2.1 Sample preparation

A 2mg sample was dissolved in 2 ml methanol and the solution was filtered using 0.45 µm SRP-4 membrane. The stock solution (1 mg/ml) was stored at 4°C.

3.15.2.2 Instrumentation and parameter used equipment

Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESIsource

Column: Agilent Zorbax SB-C18 Narrow-Bore (2.1 x 150 0mm, 3.5micronm) Part no: 830990-902

Flow rate: 0.21mmin

Solvents: 0.1% formic acid in water (A); 100% acetonitrile with 0.1% formic acid (B)

Injection volume: 0.5µl of methanol fraction 10 and 1µl of ethyl acetate.

Sample analysis

Method: U-ESI-MS-28(33)-Pos & U-ESI-MS-28(33)-Neg

Post runs time: 5 minutes.

Total run time: 33 minutes.

MS Parameters.

Ion polarity: positive & negative.

Vcap: 4000V.

Fragmentor voltage: 125V.

Skimmer: 65V.

OCT 1 RF Vpp: 750V.

Drying gas: 10L/min.

Gas Temperature: 300 °C

Nebulizer: 45psig Data acquisition: positive polarity - range 105-2000 m/z

Negative polarity – range 115-2000 m/z

Acquisition rate/time

For MS only analysis: Rate 1.03 spectra/s; Time 973ms/spectrum; Transient/spectrum: 9632

Reference masses: Positive Polarity – 121.050873, 922.009798

Negative Polarity – 119.03632, 966.000725

Data Analysis: Data was processed with

1) Agilent MasHunter Qualitative Analysis B.05.00 (Method 1: Metabolomics) (Method 2: Metabolomics 2)

For Method 1: Metabolomics

Molecular Feature Extraction (MFE): Raw data was processed with MFE, with below settings

1) Peak filters: Use peaks with height >=100 counts

2) Compound filter: Only look for compound with absolute height >=5000 counts and relative height >=2.5%

3) Ion species: Allow positive ions: +H, +Na, +K, +NH4 Allow negative ions: -H, +Cl

4) Input data range:

5) Charge State: Limit assigned charge states to a maximum of 2 (default value for metabolomics analysis)

Identify Compound:

Search Database: METLIN_AM_PCDL-N-130328.cdb.

3.16 Statistical analysis

The analysis of the values was conducted with SPSS One-way ANOVA and then Tukeys post-hoc tests were used to compare the measurements between groups. The data were studied for normality and shown as the mean ± S.E.M. All values at $p<0.05$ were considered significant.

CHAPTER 4: RESULTS

4.1 *In vitro* antioxidant activity of *P. macrocarpa* and *G. procumbens* extracts

4.1.1 Ferric-reducing antioxidant power (FRAP)

The FRAP value of the *P. macrocarpa* fruit extract and *G. procumbens* leaf extract were calculated using ferrous sulphate hepta hydrate ($FeSO_4.7H_2O$) to generate a standard curve and were determined to be 2122.81 ± 56.12 µmol Fe^{2+}/g. for *P. macrocarpa* and 226.72 ± 8.66 µmol Fe^{2+}/g for *G. procumbens* (standard curve equation: $y = 0.0006x + 0.0013$, $R^2 = 0.9931$). The FRAP result indicated that *P. macrocarpa* fruit extract exhibited high antioxidant activity. Table 4.1 show that the FRAP result of *P. macrocarpa* fruit extract was comparable to that of standard gallic acid as there was no statistically significant difference between them, while *G. procumbens* leaf extract has a relatively lower FRAP activity.

Table 4.1: *In vitro* antioxidant activities, total phenolic and flavonoid contents of *P. macrocarpa* and *G. procumbens*

Plant extracts and standards	FRAP (µmol Fe (II)/g)	DPPH IC_{50} (µg/ml)	TPC value mg Gallic acid/g of extracts	Flavonoids mg Quercetin/g of extracts	Flavonoids/Phenolic Ratio
P. macrocarpa ethanol extract	2122.81 ± 56.12^{a}	21.18 ± 0.10^{b}	124.66 ± 5.02^{b}	84.63 ± 7.81^{b}	0.67
G. procumbens ethanol extract	226.72 ± 8.66^{b}	>25	64.72 ± 0.01^{b}	40.72 ± 1.94^{b}	0.62
Gallic acid	1987.49 ± 34.98^{a}	-	237.62 ± 8.02^{a}	-	-
Quercetin	-		-	245.33 ± 4.52^{a}	-
BHT	-	5.32 ± 0.03^{a}	-	-	-
Ascorbic acid	-	11.69 ± 0.02^{b}	-	-	-

Data are expressed as mean ± S.E.M for triplicate; [a-b] Means followed by the same letters are not significantly different. Difference is significant at $p<0.05$.

4.1.2 Scavenging activity of plant extracts (DPPH)

P. macrocarpa exhibited high free radical scavenging activity towards DPPH as shown in Table 4.1; there was no significant difference between the IC_{50} of the ethanolic fruit extract of *P. macrocarpa* (21.18 ± 0.10 µg/ml), and the IC_{50} of the synthetic antioxidant BHT (11.69 ± 0.03 µg/ml). On the other hand, ethanolic leaf extract of *G. procumbens* had very low free radical scavenging activity with IC_{50} (>25 µg/ml) (Table 4.1). (Standard curve equation: $y = 1.989x$ $R^2 = 0.9697$) (Figure 4.1)

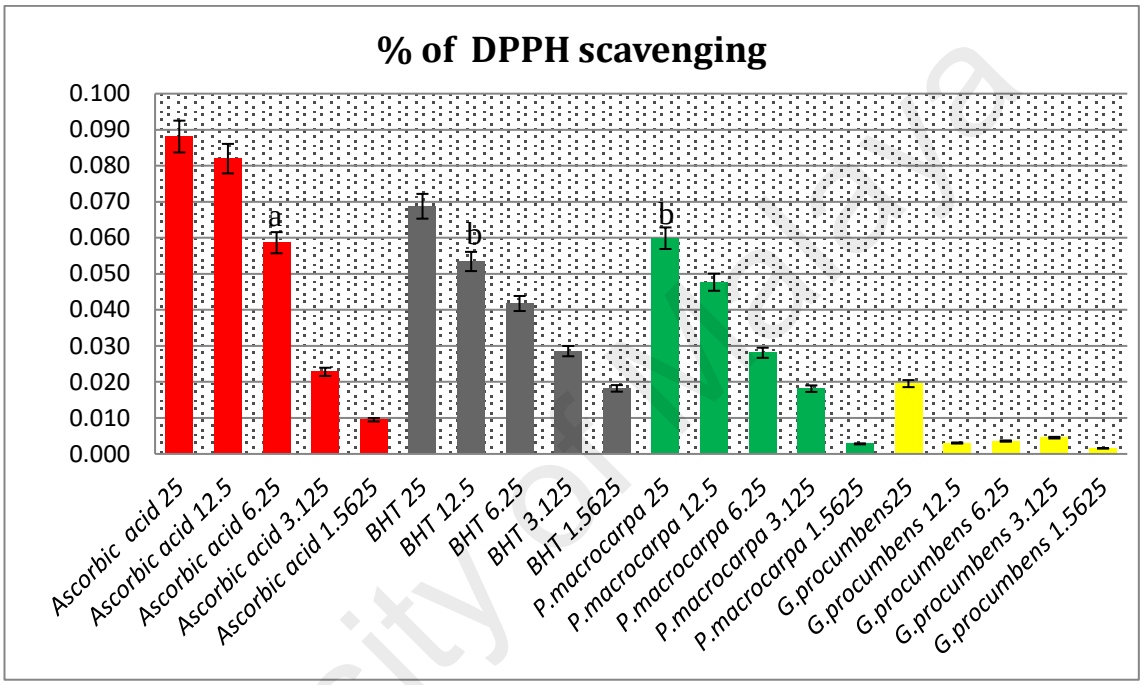

Figure 4.1: DPPH scavenging activity of different concentrations (µg/ml) of crude extracts of *P. macrocarpa* and *G. procumbens.* Data were expressed as mean ± S.E.M for triplicate. Significant difference at a level of $p<0.05$

4.1.3 Total phenolic and flavonoid content

Total phenolic content of the ethanolic extracts of P. macrocarpa and G. procumbens were estimated by the Folin–Ciocalteu colorimetric method using gallic acid to generate the standard curve and was determined to be 124.66 ± 5.02 mg (gallic acid equivalents) per g of extracts of P. macrocarpa and 64.72 ± 0.01 mg (gallic acid equivalents) per g in extract of G. procumbens (standard curve equation: y = 0.0077x + 0.0036, R2= 0.9942). The total flavonoids were 84.63 ± 7.81 mg (quercetin equivalents) per g of ethanol extract of P. macrocarpa and 40.72 ± 1.94 mg (quercetin equivalents) per g of ethanol extract of G. procumbens (standard curve equation: y = 0.0054x + 0.005, R2= 0.999). The flavonoid/phenolic ratio of P. macrocarpa and G. procumbens were 0.67 and 0.62 respectively. Thus, phenolic compounds were the predominant antioxidant components in both extracts, which lead to more potent radical scavenging effect.

4.2 Chemopreventive activity of plants extracts and their isolated fractions *in vitro*

The crude extract and ten fractions of *P. macrocarpa* and *G. procumbens* have been investigated *in vitro* against human colon cancer HT29 cell line and normal human colon cells CCD884 using MTT assay. The crude extract inhibited colon cancer cells HT29 in a dose-dependent manner (Figure 4.2) with maximum inhibition at the highest concentration used i.e. 200µg/ml. At the same time, *P. macrocarpa* and *G. procumbens* extract showed no toxicity against normal cell line CCD884. The fractions from the crude extracts of both plants were also tested for their anti-proliferative effect on both HT29 and CDD884 cell lines. Amongst the 10 fractions tested, F10 of *G. procumbens* showed maximum inhibition of 89.50% on HT29 cells at the concentration of 200µg/ml and F6 of *P. macrocarpa* showed strong inhibition activities 76.70% against HT29 cells. The remaining fractions showed moderate inhibition activities. On the other hand, almost no changes were observed in the CCD884 cell viability. The result clearly showed that the

crude extracts exhibited significant anti-proliferative activity against HT29 and hardly any effects on the growth of the normal colon cells CCD884. While a significant increase in the percentage of inhibition was also seen with F10 of *G. procumbens* and F6 of *P. macrocarpa* and these fractions showed no cytotoxicity against normal colon cells.

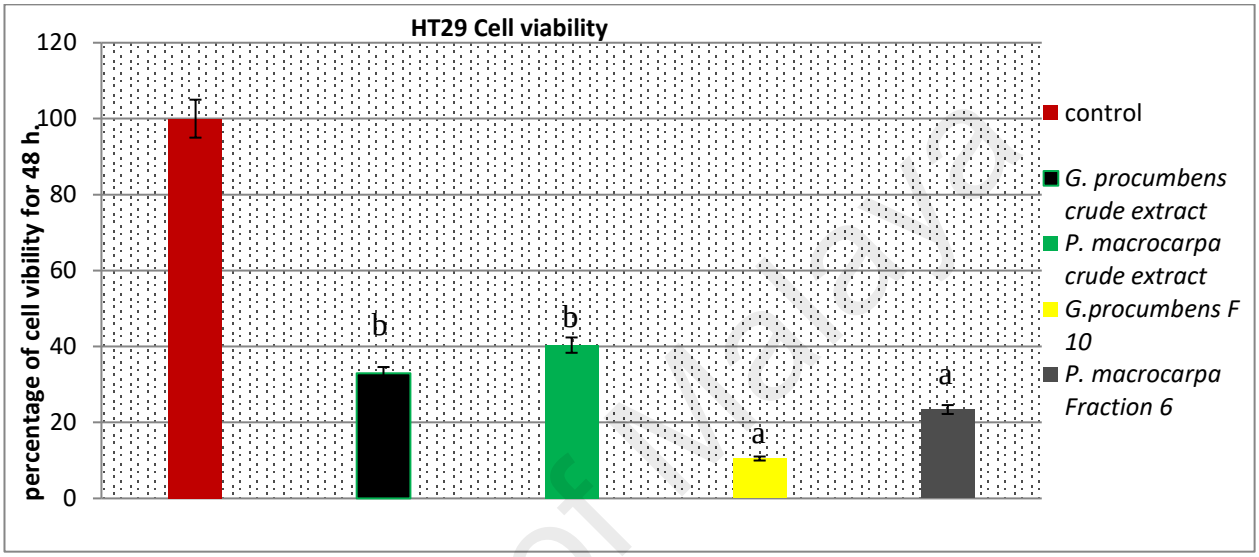

Figure 4.2: Effect of the crude extracts of *P. macrocarpa* and *G. procumbens* and their fractions on the growth of HT29 cell line. Cells were seeded in 96-well plate and treated with 200µg/ml of both plant crude extracts and fractions for 48h. The inhibition of HT29 growth was assessed by MTT assay. The results represent the mean of triplicate measurement. Symbols are significantly different versus control group, $p < 0.05$.

4.3 Acute toxicity study

All animals showed no mortality with orally administered *P. macrocarpa* and *G. procumbens* extracts at doses 2 g/kg and 5 g/kg. In addition, there were no visible manifestations of hepatotoxic and nephrotoxic effects, based on outward abnormal behaviour at these doses. Biochemical and histopathology data did not show any noticeable significant differences between the vehicle (Tween 20) group and treated groups. These findings provided sufficient evidence that the orally administered *P .macrocarpa* and *G. procumbens* extracts were deduced to be safe and that no drug-related toxicity was detected even at the highest dose investigated (Figure 4.3A & 4.3B, Table 4.2A & 3A and Table 4.2B & 3B).

T able 4.2A: Acute toxicity study: Effects of *P. macrocarpa* and *G. procumbens* extracts on renal function in male Sprague Dawley rats

Dose	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	CO2 (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Vehicle (Tween 20) (5 ml/kg)	141.81 ± 4.82	4.81 ± 0.14	101.01 ± 1.48	24.85 ± 0.55	18.16 ± 0.60	6.08 ± 0.43	30.40 ± 7.32
P. macrocarpa (2 g/kg)	139.82 ± 4.01	4.93 ± 0.16	102.42± 1.46	22.60 ± 1.19	17.88 ± 0.29	5.72 ± 0.93	33.60 ± 7.74
P. macrocarpa (5 g/kg)	139.23 ± 4.71	4.81 ± 0.15	100.03 ± 1.40	23.96 ± 1.05	17.90 ± 0.70	5.10 ± 0.68	31.08 ± 8.03
G. procumbens (2 g/kg)	140.24 ± 3.13	5.14 ± 0.20	101.01± 0.86	21.80 ± 0.50	18.10 ± 0.37	5.90 ± 0.63	33.00 ± 7.71
G. procumbens (5 g/kg)	142.01 ± 1.62	4.93 ± 0.16	99.00 ± 087	21.60 ± 0.61	18.20 ± 0.54	6.00 ± 0.57	31.20 ± 5.52

Data are expressed as mean ± S.E.M; there are no significant differences between the vehicle control and treated groups.

Table 4.3A: Acute toxicity study: Effects of *P. macrocarpa* and *G. procumbens* extracts on liver function in male Sprague Dawley rats

Dose	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	TB (µmol/L)	CB (µmol/L)	AP (IU/L)	AST (IU/L)	GGT (IU/L)
Vehicle Tween 20 (5 ml/kg)	61.42 ± 1.51	11.02 ± 0.71	55.04 ± 2.44	2.62± 0.12	0.98 ± 0.01	116.81 ±14.31	177.01 ±5.63	4.92 ± 0.19
P. macrocarpa (2 g/kg)	61.01 ± 3.00	11.01 ± 0.72	52.03 ± 2.40	2.43± 0.24	0.98 ± 0.01	153.01 ± 5.47	153.43 ± 6.37	4.80 ± 0.16
P. macrocarpa (5 g/kg)	66.20 ± 1.21	11.43 ± 0.44	54.83 ± 3.00	2.42 ± 0.24	0.97 ± 0.02	129.42 ± 5.63	163.23 ± 6.33	4.52 ± 0.20
G. procumbens (2 g/kg)	61.02 ± 3.33	11.24 ± 0.71	54.82 ± 2.94	2.83 ± 0.20	0.98 ± 0.01	153.00 ± 4.60	169.44 ± 5.20	4.91 ± 0.24
G. procumbens (5 g/kg)	62.01 ± 3.14	12.01 ± 0.33	59.61 ± 3.44	2.82 ± 0.20	0.96 ± 0.03	139.02 ± 5.02	157.40 ± 5.40	4.60 ± 0.22

Data are expressed as mean ± S.E.M; there are no significant differences between the vehicle control and treated groups. (TB: total bilirubin; CB: conjugated bilirubin; AP: alkaline phosphatase; AST: aspartate aminotransferase: GGT: G-glutamyl transferase)

Table 4.2B: Acute toxicity study: Effects of *P. macrocarpa* and *G. procumbens* extracts on renal function in female Sprague Dawley rats

Dose	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	CO2 (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Vehicle (Tween 20) (5 ml/kg)	140.62 ± 4.41	4.61 ± 0.13	102.13 ± 1.50	23.75 ± 0.75	18.12 ± 0.72	5.94 ± 0.67	31.41 ± 6.33
P. macrocarpa (2 g/kg)	139.11 ± 4.32	5.11 ± 0.18	101.00 ± 1.46	22.90 ± 1.20	18.18 ± 0.31	5.55 ± 0.83	33.04 ± 6.73
P. macrocarpa (5 g/kg)	139.01 ± 4.61	4.91 ± 0.14	102.01 ± 1.10	22.96 ± 1.02	17.60 ± 0.74	5.32 ± 0.72	31.00 ± 8.02
G. procumbens (2 g/kg)	140.50 ± 3.90	5.02 ± 0.19	101.02 ± 0.84	21.61 ± 0.54	18.32 ± 0.34	6.11 ± 0.53	32.81 ± 7.03
G. procumbens (5 g/kg)	141.00 ± 4.62	4.91 ± 0.16	100.12 ± 0.92	21.33 ± 0.51	18.01 ± 0.54	5.91 ± 0.59	30.90 ± 5.54

Data are expressed as mean ± S.E.M; there are no significant differences between the vehicle control and treated groups.

Table 4.3B: Acute toxicity study: Effect of *P. macrocarpa* and *G. procumbens* extracts on liver function in female Sprague Dawley rats

Dose	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	TB (µmol/L)	CB (µmol/L)	AP (IU/L)	AST (IU/L)	GGT (IU/L)
Vehicle Tween 20 (5 ml/kg)	60.11 ± 2.52	11.21 ± 0.72	53.02 ± 3.42	2.73 ± 0.11	0.96 ± 0.03	116.82 ±14.31	175.01 ±5.53	4.43 ± 0.19
P. macrocarpa (2 g/kg)	61.42 ± 2.60	11.32 ± 0.71	52.00 ± 3.51	2.53 ± 0.24	0.97 ± 0.02	143.02 ± 5.47	152.40 ± 6.77	4.62 ± 0.21
P. macrocarpa (5 g/kg)	63.20 ± 2.21	11.24 ± 0.44	54.8 ± 3.03	2.42 ± 0.24	0.98 ± 0.01	139.42 ± 5.63	164.22 ± 5.33	4.73 ± 0.20
G. procumbens (2 g/kg)	62.01 ± 3.14	11.74 ± 0.73	55.84 ± 2.52	2.50 ± 0.23	0.98 ± 0.01	143.00 ± 4.61	167.40 ± 5.33	4.93 ± 0.24
G. procumbens (5 g/kg)	62.10 ± 3.13	11.60 ± 0.42	58.62 ± 3.43	2.62 ± 0.21	0.97 ± 0.02	149.02 ± 5.02	156.42 ± 5.92	4.60 ± 0.22

Data are expressed as mean ± S.E.M; there are no significant differences between the vehicle and treated groups.
(TB: Total bilirubin; CB: Conjugated bilirubin; AP: Alkaline phosphatase; AST: Aspartate aminotransferase: GGT: G-Glutamyl Transferase)

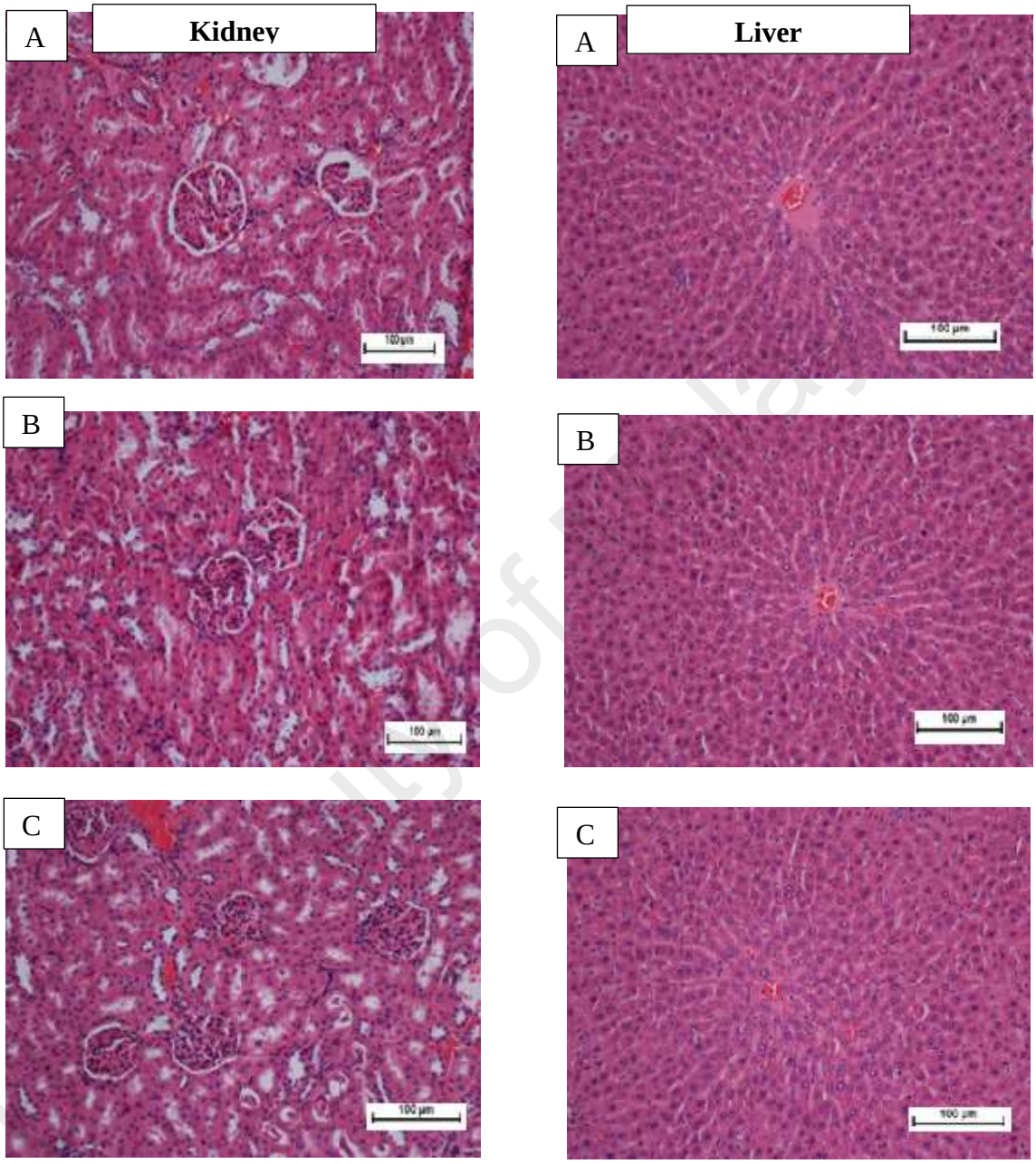

Figure 4.3A: Histological sections of kidney and liver stained with haematoxylin and eosin in acute toxicity study. (A) Normal control (B) *P. macrocarpa* 2 g/kg (C) *P. macrocarpa* 5g/kg. Bar =100µm.

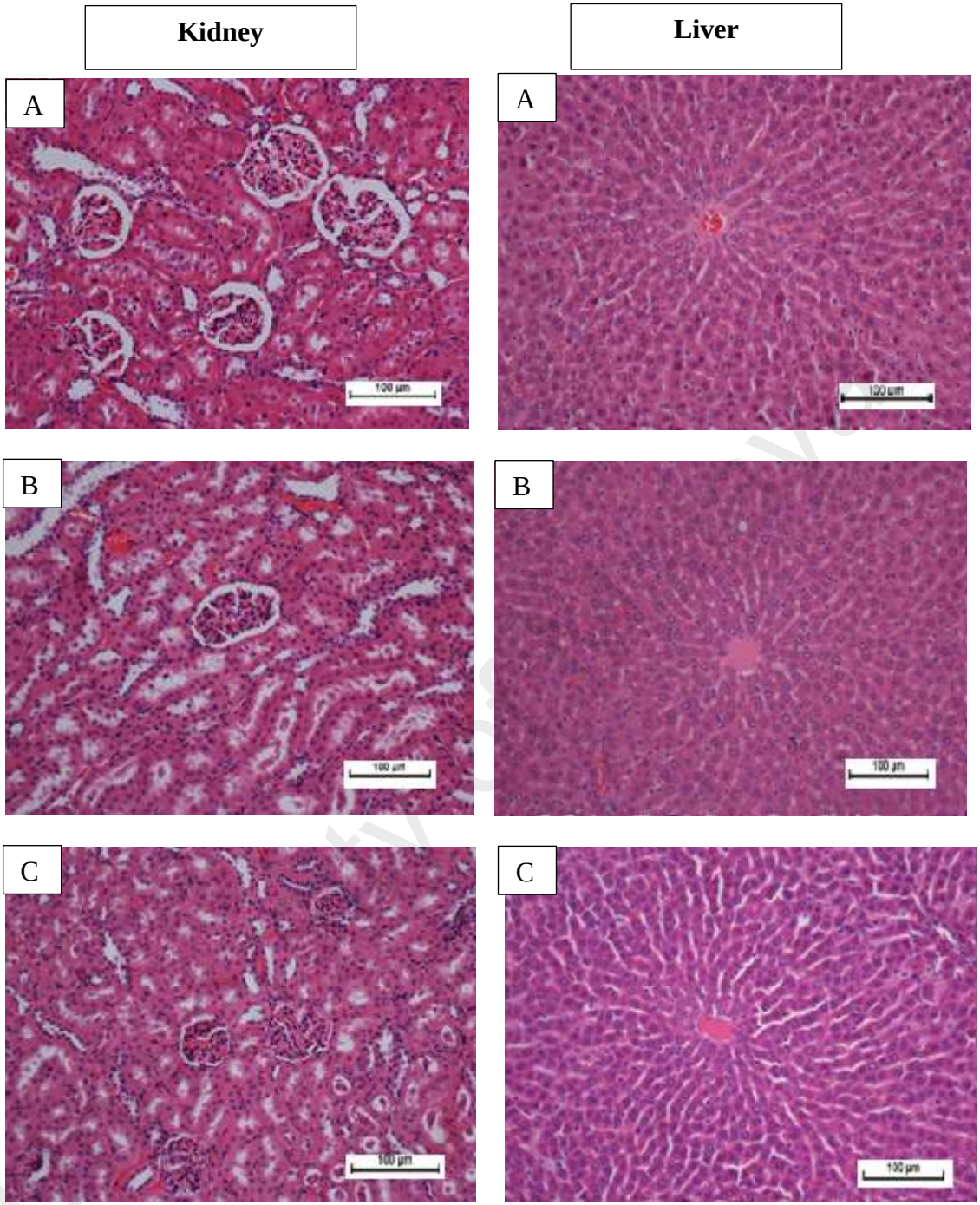

Figure 4.3B: Histological sections of kidney and liver stained with haematoxylin and eosin in acute toxicity study. (A) Normal control. (B) *G. procumbens* 2 g/kg. (C) *G. procumbens* 5 g/kg. Bar = 100µm.

4.4 Analysis of body weights

Body weights of all rats were taken at the beginning (0 week) of the experiment then weekly up to 10 weeks (Figure 4:4). Colon, liver and kidney were weighed after the rats were sacrificed (Table 4.4). Although there were differences in body and organ weights between all the experimental groups, these differences were not statistically significant (Figure 4.4, Table 4.4).

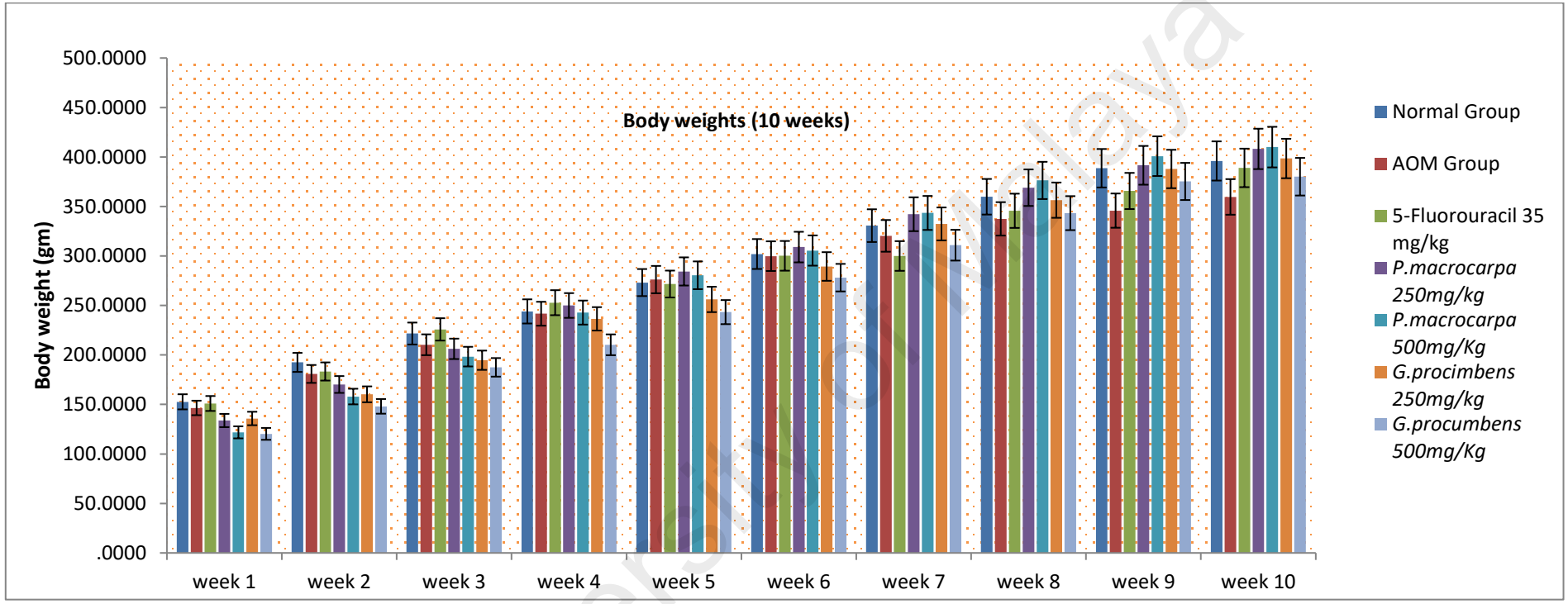

Figure 4.4: Effects of *P. macrocarpa* and *G. procumbens* on body weights of rats with AOM- induced ACF. AOM: azoxymethane, 5-FU: fluorouracil.

Table 4.4: Effects of *P. macrocarpa* and *G. procumbens* extracts on body, liver, colon and kidney weights of rats with AOM-induced ACF

Experimental groups	Body weight (g)	Colon weight (g)	Liver weight (g)	Kidney weight (g)
Vehicle (Tween 20), (5ml/kg)	396.11 ± 22.00	2.07 ± 0.21	10.12 ± 0.06	2.89 ± 0.07
AOM (15 mg/kg)	359.62 ± 18.10	1.92 ± 0.37	10.40 ± 0.38	2.93 ± 0.09
AOM(15mg/kg) + 5-FU (35mg/kg)	389.02 ±17.38	2.18 ± 0.16	10.09 ± 0.05	2.93 ± 0.07
AOM (15mg/kg) + *P. macrocarpa* (250 mg/kg)	408.22 ± 46.84	2.47 ± 0.33	10.60 ± 2.21	3.02 ± 0.19
AOM (15mg/kg) + P. macrocarpa (500 mg/kg)	410.34 ± 15.27	2.23 ± 0.11	9.45 ± 1.33	2.40 ± 0.37
AOM (15mg/kg) + G. procumbens (250 mg/kg)	398.53 ± 25.26	2.40 ± 0.19	9.97 ± 0.20	2.90 ± 0.10
AOM (15mg/kg) + G. procumbens (500 mg/kg)	380.14 ± 19.97	2.20 ± 0.14	9.82 ± 0.67	2.44 ± 0.22

Data are expressed as mean ± SER. No significant differences was observed between any of the experimental groups.

4.5 Effects of *P. macrocarpa* and *G. procumbens* extracts on the incidence of AOM-induced ACF in male Sprague Dawley rats

The majority of ACF were observed in the proximal and distal colon and were stereoscopically distinguished from normal crypts by their darker staining, elliptical shape, larger size, thicker epithelial lining, and larger pericryptal zone. The incidence of ACF is shown in Tables 4.5 and 4.6. Rats fed with ethanol extracts of *P. macrocarpa* and *G. procumbens* showed significantly lower ACF numbers compared to untreated AOM group ($p<0.05$). In all experimental groups, ACF incidence in the distal colon was significantly higher compared with proximal colon ($p<0.05$). However, the number of ACF in both the distal and proximal colon in AOM group was significantly higher compared to that of the AOM groups that received 250 mg/kg or 500 mg/kg of either plant extracts or 5-FU. Treatments with high or low doses of the plant extracts led to a 78.71, 78.2%, 82.76% and 83.6% decrease in the total crypt number in the groups fed with 250 mg/kg or 500 mg/kg of *P. macrocarpa* or *G. procumbens* respectively as compared to the AOM group.

Table 4.5 Effects of *P. macrocarpa* and *G. procumbens* on AOM-induced ACF number in colons of male Sprague Dawley rats.

Experimental groups	Incidence of Aberrant Crypt Foci (ACF)			
	Distal colon	Proximal colon	Total ACF	% Inhibition
AOM (15mg/kg)	111.00 ± 8.80	19.60 ± 2.60	130.60 ± 9.81	0.00
AOM 15mg/kg + 5-FU (35 mg/kg)	26.80 ± 2.10[a]	5.20 ± 1.78[a]	32.00 ± 2.20[a]	75.49
AOM 15mg/kg + *G. procumbens* (250 mg/kg)	18.40 ± 2.80[a]	3.00 ± 0.60[a]	21.4 ± 3.17[a]	83.61
AOM 15mg/kg + *G. procumbens* (500 mg/kg)	18.60 ± 1.20[a]	3.80 ± 0.37[a]	22.40 ± 2.10[a]	82.76
AOM 15mg/kg + *P. macrocarpa* (250 mg/kg)	24.40 ± 2.01[a]	3.80 ± 0.37[a]	28.20 ± 1.70a	78.40
AOM 15mg/kg + *P. macrocarpa* (500 mg/kg)	23.60 ± 3.10[a]	4.20 ± 1.09[a]	27.80 ± 4.09[a]	78.71

Data are expressed as mean ± S.E.M. [a] *p<0.05 vs* untreated AOM group. 5-FU: 5-Fluorouracil; ACF: aberrant crypt foci; AOM: azoxymethane

Table 4.6: Effects of *P. macrocarpa* and *G. procumbens* extracts on crypt number per focus in AOM-induced ACF in colons of male Sprague Dawley rats

Experimental groups	Foci with the following crypt number:				
	1 crypt	2 crypts	3 crypts	4 crypts	5 & more crypts
AOM (15 mg/kg)	13.01 ± 3.30	31.01 ± 6.08	22.82 ± 3.80	25.40 ± 2.60	38.02 ± 1.40
AOM 15 mg/kg + 5-FU (35 mg/kg)	8.00 ± 1.90[a]	7.22 ± 1.35[a]	8.30 ± 1.10[a]	3.22 ± 0.80[a]	5.20 ± 0.80a
AOM (15 mg/kg) + *P. macrocarp* (250 mg/kg)	3.21 ± 0.58[a]	6.64 ± 1.20[a]	5.20 ± 0.58[a]	4.84 ± 0.74[a]	5.42 ± 0.83[a]
AOM (15 mg/kg) + *P. macrocarp* (500 mg/kg)	2.84 ± 1.10[a]	5.20 ± 0.86[a]	5.80 ± 0.73[a]	8.00 ± 4.08[a]	6.00 ± 0.80[a]
AOM (15 mg/kg) + *G. Procumbens* (250 mg/kg)	3.02 ± 0.70[a]	6.82 ± 1.31[a]	5.80 ± 0.86[a]	2.20 ± 0.58[a]	3.62 ± 1.02[a]
AOM(15mg/k+ *G. Procumbens* (500 mg/kg)	2.42 ± 0.74[a]	4.23 ± 0.91[a]	6.21 ± 0.58[a]	5.02 ± 0.94[a]	5.40 ± 0.81[a]

Data are expressed as mean ± S.E.M. [a] *p<0.05 vs* untreated AOM group. 5-FU: 5-Fluorouracil; ACF: aberrant crypt foci; AOM: azoxymethane.

.

4.6 Macroscopical appearance of colon tissue

The aberrant crypt foci were visualised and counted using 0.2% methylene blue. The ACF was easily seen as they were slightly elevated above the surrounding mucosa and demonstrated a characteristic oval or slit-like orifices. Frequently, some ACF were detected as almost normal-sized lesions but with more densely staining by methylene blue. The results are presented in Figure 4.5. AOM-control rats developed grossly identifiable ACF in the colon. No aberrant crypts were identified with methylene blue in the intact colons of normal control rats. The number of ACF per colon, which is considered as a marker for tumour initiation, was significantly higher in AOM group compared to treated groups.

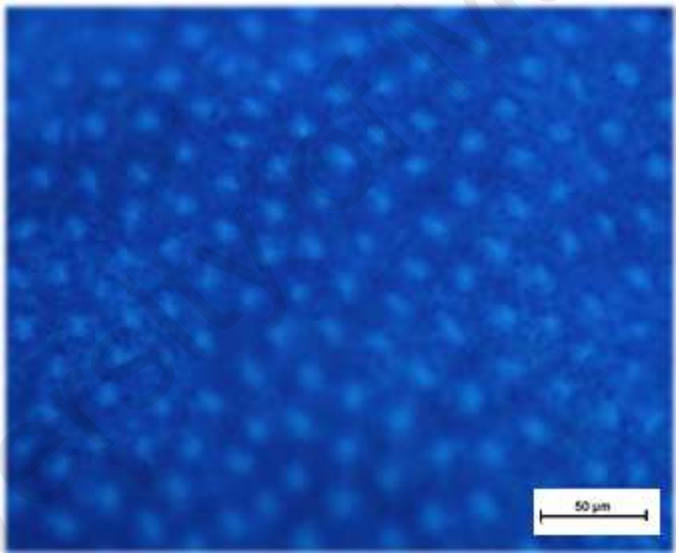

Figure 4.5: Topographical appearance of colon mucosa stained with methylene blue of rats treated with 10% Tween 20 (normal control group) showing normal crypts. Bar = 50µm.

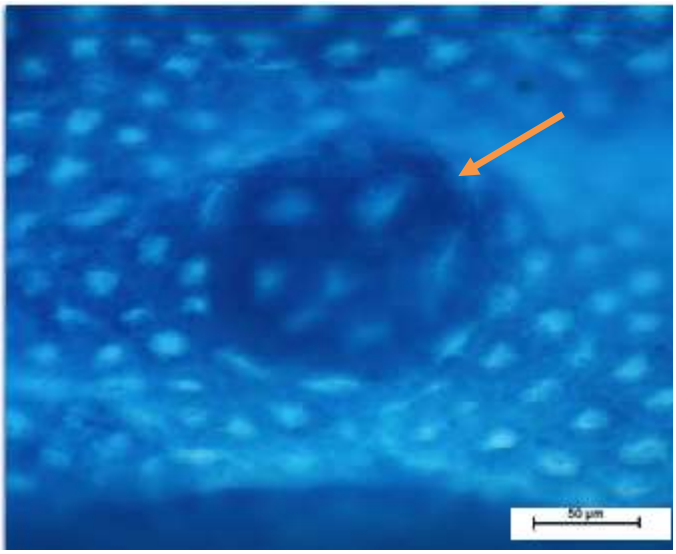

Figure 4.6: Macroscopical appearance of ACF stained with methylene blue in colon of rat with AOM-induced ACF. Arrow indicates ACF with more than 5 crypts. Bar = 50µm.

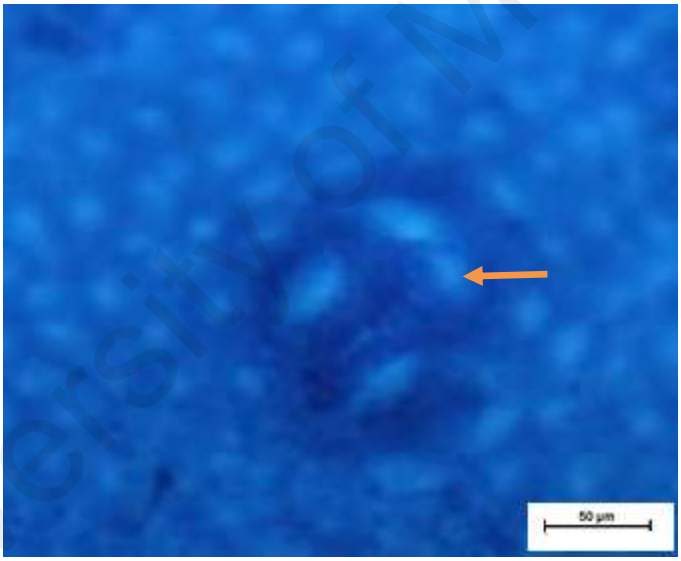

Figure 4.7: Macroscopical appearance of ACF stained with methylene blue in colon of rat with AOM-induced ACF treated with 35 mg/kg 5-FU. Arrow indicates ACF with less than 5 crypts. Bar = 50µm.

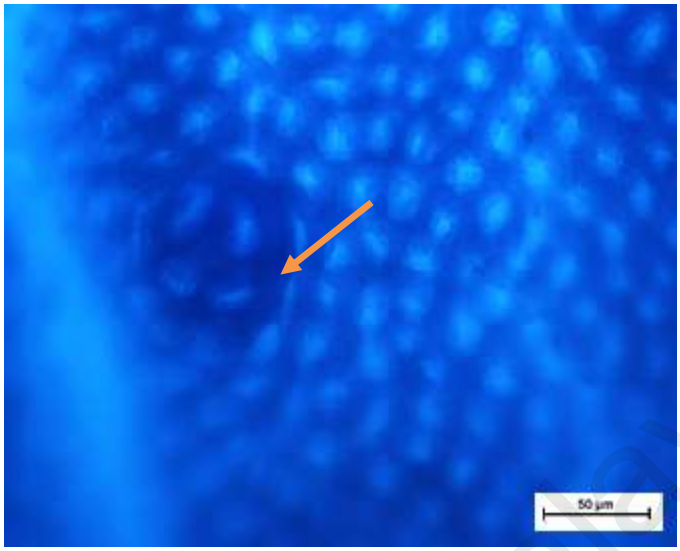

Figure 4.8: Macroscopical appearance of ACF stained with methylene blue in colon of rat with AOM-induced ACF treated with 250 mg/kg *P. macrocarpa* extract. Arrow indicates ACF with less than 5 crypts. Bar = 50µm.

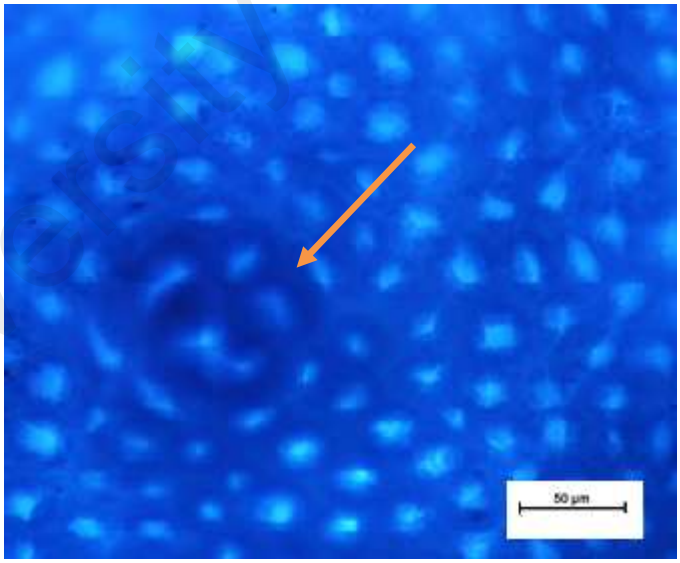

Figure 4.9: Macroscopical appearance of ACF stained with methylene blue in colon of rat with AOM-induced ACF treated with 500 mg/kg of *P. macrocarpa* extract. Arrow indicates ACF with less than 5 crypts. Bar = 50µm.

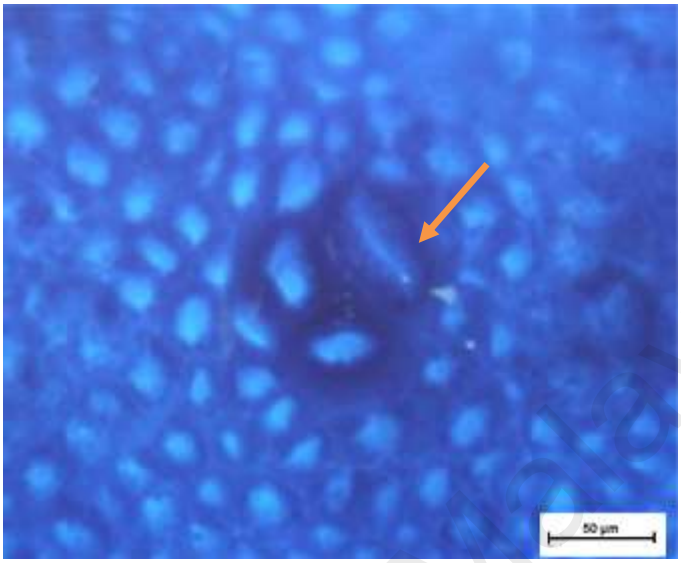

Figure 4.10: Macroscopical appearance of ACF stained with methylene blue in colon of rat with AOM-induced ACF treated with 250 mg/kg *G. procumbens* extract. Arrow indicates ACF with less than 5 crypts. Bar = 50µm.

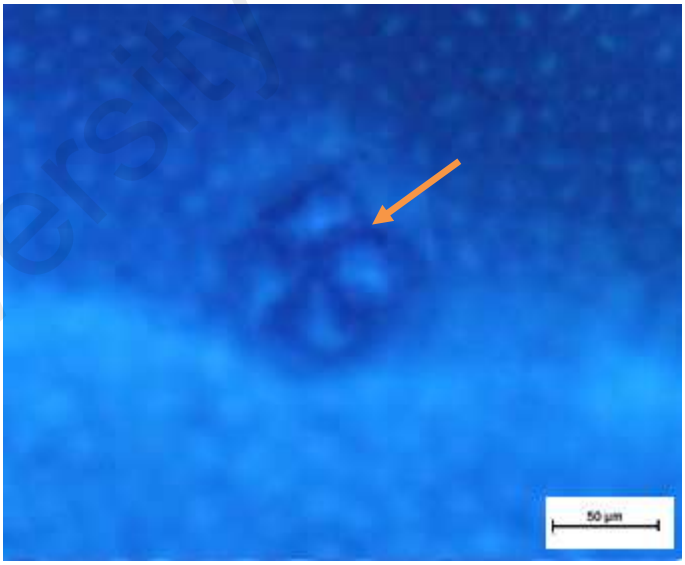

Figure 4.11: Macroscopical appearance of ACF stained with methylene blue in colon of rat with AOM-induced ACF treated with 500 mg/kg *G. procumbens* extract. Arrow indicates ACF with less than 5 crypts. Bar = 50µm.

4.7 Histopathological examination of colon tissue

Histological features of dysplastic ACF and normal colon cells were illustrated by H&E staining. Histological examination of colon sections from normal and treated groups showed normal architecture of the mucosal and submucosal layers. Similar observations were made with colon of AOM administered rats that are treated with 5FU or the plant extracts. Proliferating mucosal glands with severe dysplastic changes that signify possible transformation to carcinoma existed in the colon of AOM administered rats. The epithelial cells in ACF show distinctive elongated nuclei, deficit of cell polarity, shortage of goblet cells, increase in mitoses and narrow lumen as compared to the surrounding normal crypts (Figure 4.12-4.18).

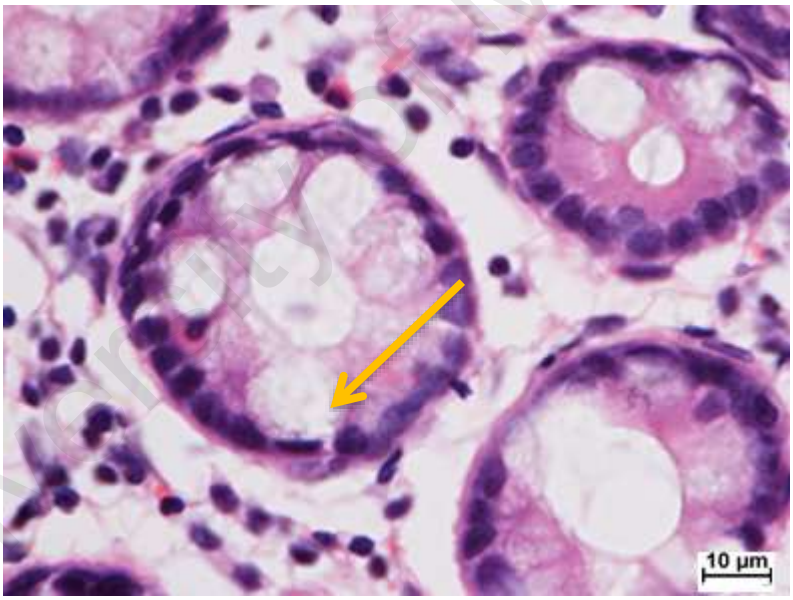

Figure 4.12: H&E stained rat colonic tissue showing crypt with normal mucosa. The section was cut parallel to the muscle layer. Arrow indicates normal colon cells with round shaped nuclei located at the base. Bar = 10µm.

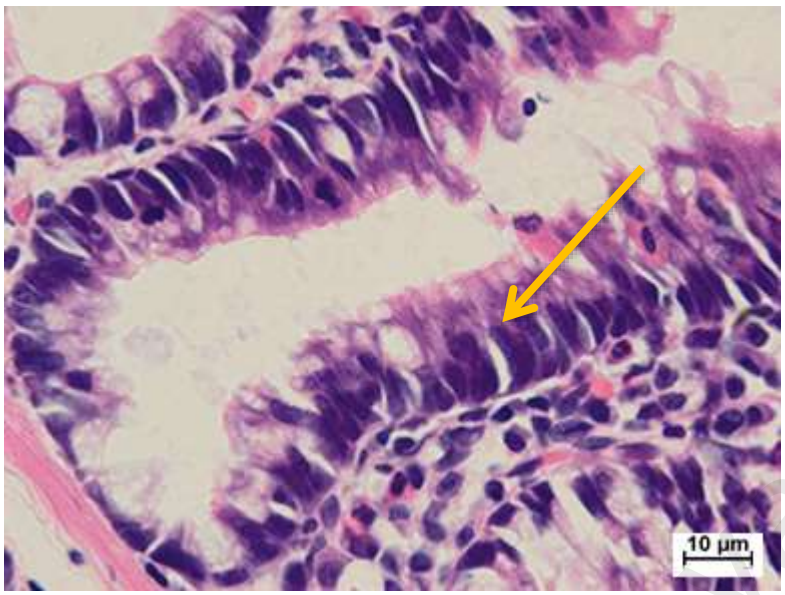

Figure 4.13: H&E stained AOM-induced ACF in rat colon. The section was cut parallel to the muscle layer. Arrow indicates ACF cell with elongated and stratified nuclei, and depletion of mucus. Bar = 10µm.

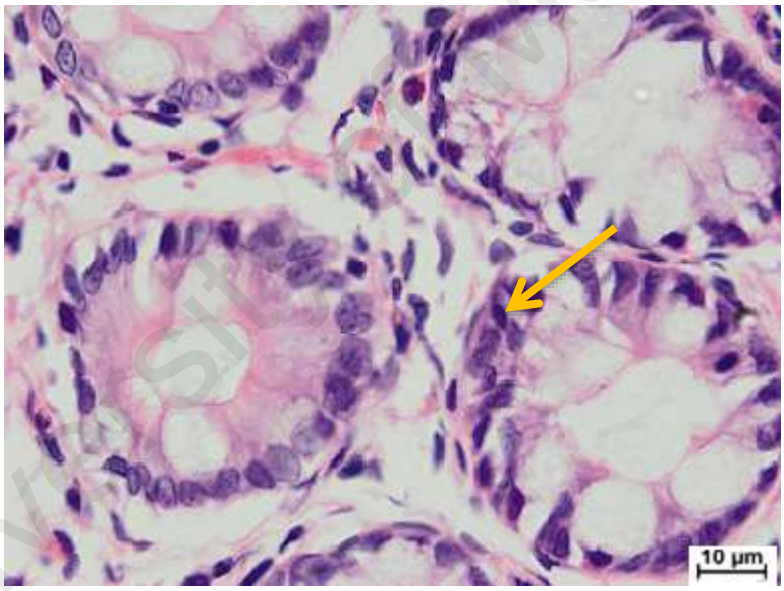

Figure 4.14: H&E stained colonic tissue of rat with AOM-induced ACF and treated with 35mg/kg 5FU. The section was cut parallel to the muscle layer and showed mucosal and submucosal layer with minimal changes and normal architecture. Arrow indicates normal colon cells with round nuclei. Bar = 10µm.

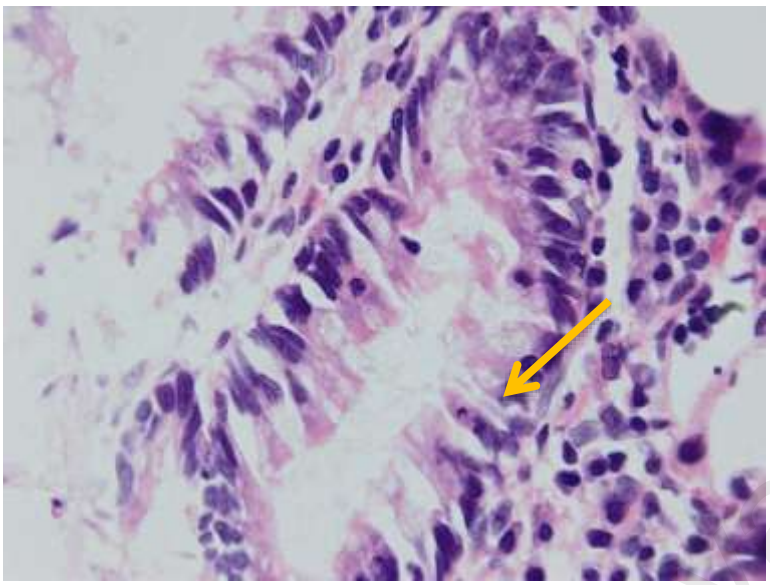

Figure 4.15: H&E stained colonic tissue of rat with AOM-induced ACF and treated with 250mg/kg *P. macrocarpa* extract. The section was cut parallel to the muscle layer and showed mucosal and submucosal layers with minimal changes and normal architecture. Arrow indicates normal colon cells with round nuclei. Bar = 10µm.

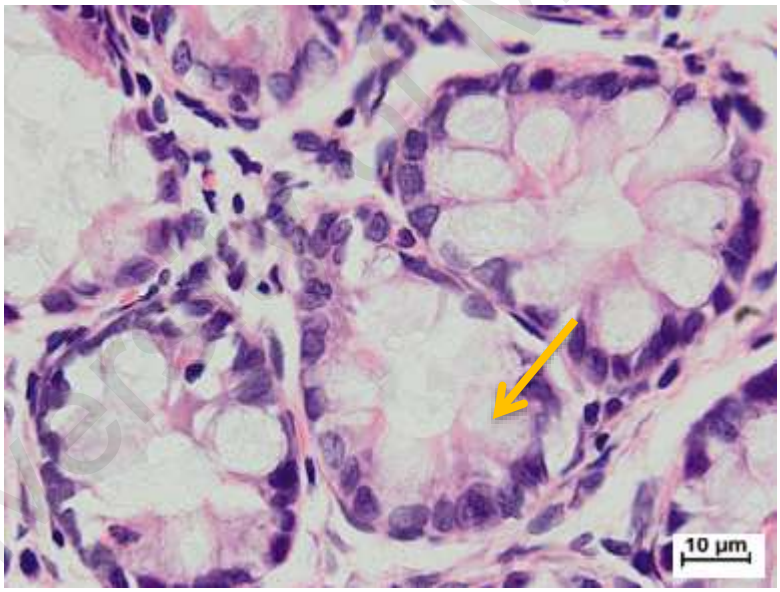

Figure 4.16: H&E stained colonic tissue of rat with AOM-induced ACF and treated with 500mg/kg *P. macrocarpa* extract. The section was cut parallel to the muscle layer and showed mucosal and submucosal layers with minimal changes and normal architecture. Arrow indicates normal colon cells with round nuclei Bar = 10µm.

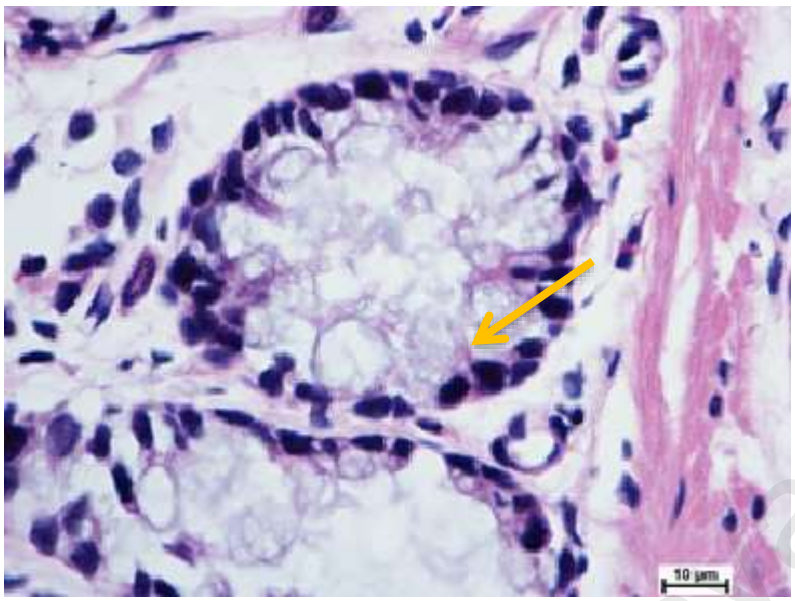

Figure 4.17: H&E stained colonic tissue of rat with AOM-induced ACF and treated with 250mg/kg *G.procumbens* extract. The section was cut parallel to the muscle layer and showed mucosal and submucosal layers with minimal changes and normal architecture. Arrow indicates normal colon cells with round nuclei. Bar = 10µm.

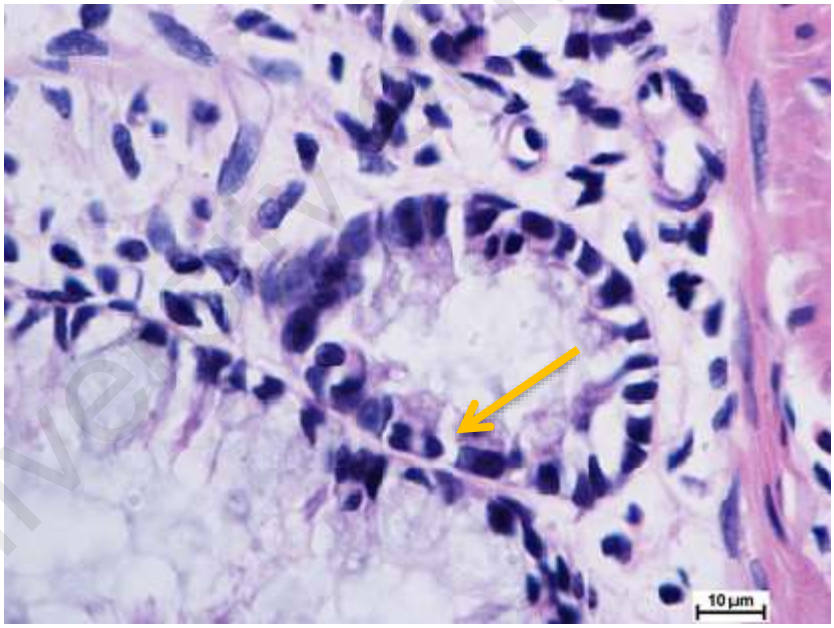

Figure 4.18: H&E stained colonic tissue of rat with AOM-induced ACF and treated with 500mg/kg *G.procumbens* extract. The section was cut parallel to the muscle layer and showed mucosal and submucosal layers with minimal changes and normal architecture. Arrow indicates normal colon cells with round nuclei. Bar = 10µm.

4.8 Biochemical parameters

The plasma albumin and protein were significantly increased in treated groups except with low dose of *P. macrocarpa* extract compared to the untreated AOM group. However, LDH was decreased in treated groups compared to the untreated AOM group. While there were no significant differences in urea, creatinine, Na^+, K^+, AST, ALT, and ALP in treated groups compared to the untreated AOM groups (Tables 4.7 & 4.8).

Table 4.7: Effects of *P. macrocarpa* and *G. procumbens* on plasma Na^+, K^+, urea and creatinine in rat with AOM-induced ACF.

Experimental groups	Sodium (mmol/L)	Potassium (mmol/L)	Urea (mmol/L)	Creatinine (μmol/L)
AOM (15 mg/kg)	140.62 ± 4.01	4.20 ± 0.18	5.06 ± 0.51	28.81 ± 2.03
AOM (15mg/kg) + 5FU (35 mg/kg)	144.92 ± 1.00	4.70 ± 0.17	6.07 ± 1.23	30.10 ± 7.80
AOM (15mg/kg) + *P. macrocarpa* (250 mg/kg)	141.01 ± 1.00	4.50 ± 0.15	6.04 ± 0.90	29.60 ± 7.09
AOM 15 mg/kg + *P. macrocarpa* (500 mg/kg)	140.43 ± 4.03	4.56 ± 0.15	6.14 ± 1.33	27.84 ± 5.70
AOM 15 mg/kg + *G. procumbens* (250 mg/kg)	140.44 ± 1.83	4.46 ± 0.01	6.04 ± 0.40	29.64 ± 3.17
AOM 15 mg/kg + *G. procumbens* (500 mg/kg)	141.02 ± 0.40	4.85 ± 0.15	5.98 ± 0.52	28.81 ± 2.60

Data are expressed as mean ± S.E.M. No significant difference was observed between any of the experimental groups. (5-FU): 5-Fluorouracil; AOM: azoxymethane.

Table 4.8: Effects of *P. macrocarpa* and *G. procumbens* on liver function in rat with AOM-induced ACF.

Groups	Total protein (g/L)	Albumin (g/L)	AST IU/L	ALT IU/L	ALP IU/L	LDH U/L
AOM 15 mg/kg	64.21 ± 0.60	10.01 ± 0.54	203.02 ± 10.42	47.61 ± 2.53	106.83 ± 11.00	1909.42 ± 131.00
AOM + 5-FU 35 mg/kg	68.40 ± 0.90	12.22 ± 0.16[a]	164.81 ± 10.82	64.41 ± 4.02	133.13 ± 7.20	957.02 ± 125.00a
AOM + *P.macrocarpa* 250 mg/kg	55.42 ± 1.40[a]	10.82 ± 0.48	157.42 ± 14.42	58.62 ± 3.64	167.02 ± 24.90	700.23 ± 193.00[a]
AOM + *P.macrocarpa* 500 mg/kg	70.01 ± 0.83[a]	11.22 ± 0.37[a]	181.85 ± 12.41	64.43 ± 4.01	127.42 ± 6.10	1065.03 ± 131.00[a]
AOM + *G.procumbens* 250 mg/kg	67.62 ± 1.02[a]	11.81 ± 0.44[a]	189.42 ± 13.73	73.64 ± 14.40	149.21 ± 10.20	1444.02 ± 137.60[a]
AOM + *G.procumbens* 500 mg/kg	70.03 ± 1.00[a]	11.62 ± 1.10[a]	165.82 ± 27.84	58.83 ± 5.60	166.00 ± 13.70	1065.04 ± 295.00[a]

Data are expressed as mean ± S.E.M. [a] *p<0.05 vs* AOM group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase and LDH: Lactate dehydrogenase

4.9 Effects of *P. macrocarpa* and *G. procumbens* extracts on endogenous antioxidant and lipid peroxidation (TBRAS) in AOM-induced ACF

The antioxidant SOD and GST parameters were evaluated in colon homogenate among the different groups. The results showed that colon GST activity and SOD were significantly higher in the rats fed with *P. macrocarpa*, *G. procumbens* group and 5-FU groups respectively compared with the AOM group. Treatments of animals with *P. macrocarpa* and *G. procumbens* ethanol extracts significantly reduced the level of MDA compared to AOM group (Table 4.9).

Table 4.9 : Effects of *P. macrocarpa* and *G. procumbens* extracts on endogenous antioxidant parameters in colon homogenates of rat with AOM-induced ACF

Experimental groups	Antioxidant parameters in colon homogenate		
	SOD (U/ml)	GST (nmol/min/ml)	MDA (µM)
AOM (15 mg/kg)	5.87 ± 0.21	16.65 ± 2.04	11.16 ± 0.78
AOM (15mg/kg) + 5-FU (35 mg/kg)	9.99 ± 1.11[a]	19.64 ± 3.14	3.94 ± 0.11[a]
AOM (15 mg/kg) + *P.macrocarpa* (250 mg/kg)	10.61 ± 0.37[a]	29.54 ± 4.02[a]	1.14 ± 0.15[a]
AOM (15mg/kg) + *P. macrocarpa* (500 mg/kg)	8.41 ± 0.94[a]	26.39± 0.92[a]	1.70 ± 0.11[a]
AOM (15 mg/kg) + *G. Procumbens* (250 mg/kg)	11.55 ± 0.33[a]	34.00 ±5.80[a]	3.20 ± 0.07[a]
AOM (15 mg/kg) + *G. Procumbens* (500 mg/kg)	8.11 ± 1.70[a]	17.87 ± 0.17	1.42 ± 0.11a

Data are expressed as mean ± S.E.M. [a] *p<0.05 vs* AOM group

.

4.10 Immunohistochemistryanalysis

The proliferating cell nuclear antigen (PCNA) was evaluated as a marker for cell proliferation in the colon specimens. The PCNA positive staining cells in the nucleus of the colon mucosa were more prominent in rats with AOM-induced ACF than in the treatment groups. PCNA-negative cells were stained with hematoxylin (Figures 4.19-4.25).

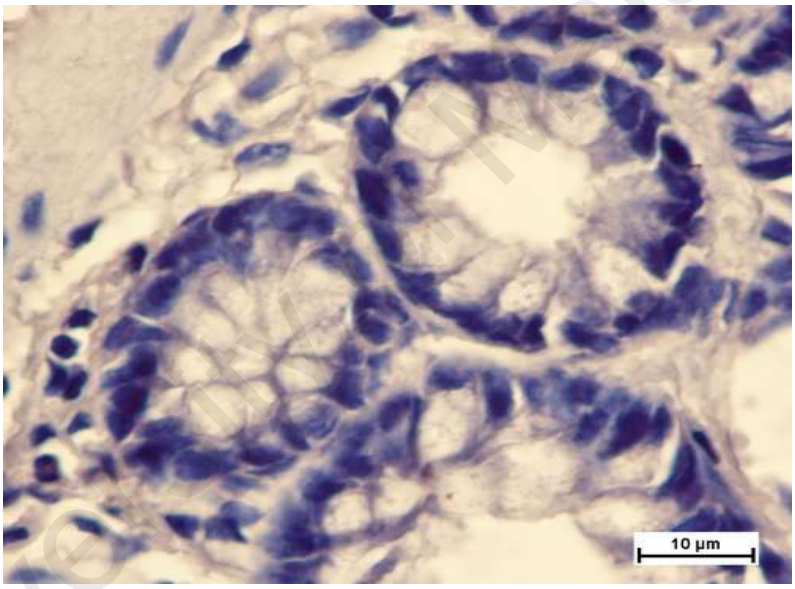

Figure 4.19: Hematoxylin stained normal colon with normal crypts. Immunohistochemical staining showed no positive PCNA cells. Bar = 10µm.

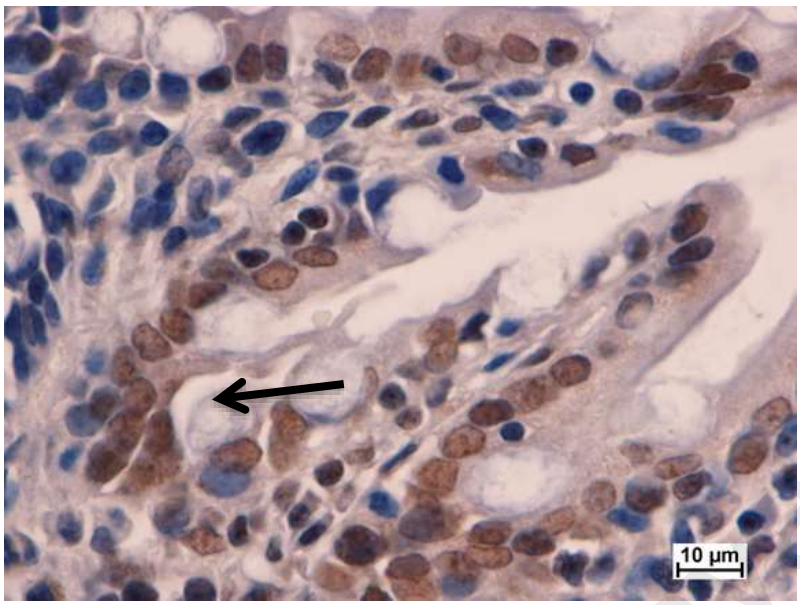

Figure 4.20: Immunohistochemical staining of colon section showing localisation of PCNA-positive cells in rat with AOM-induced ACF. The brownish stain indicates PCNA-positive cells. Bar = 10µm.

.

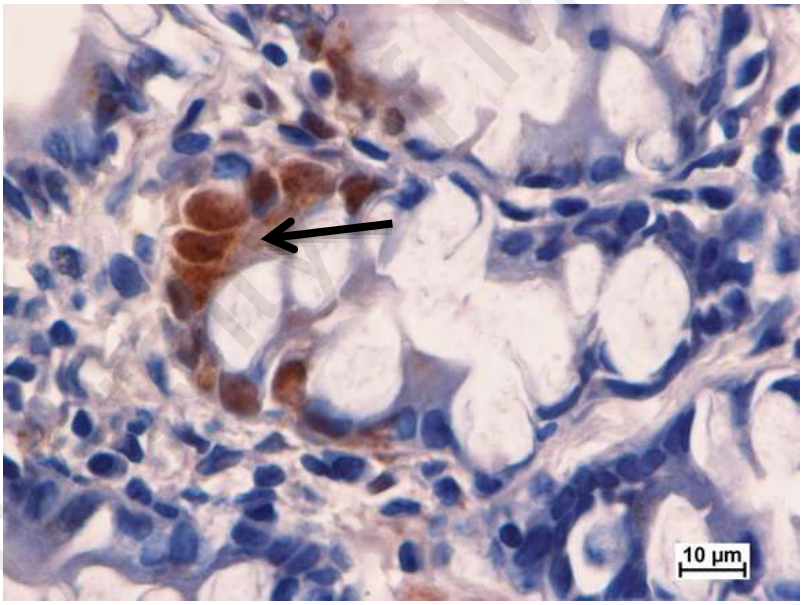

Figure 4.21: Immunohistochemical staining of colon section showing the effect of 5-FU treatment on PCNA-positive cells in rat with AOM- induced ACF. The number of PCNA-positive cells are reduced. Bar = 10µm.

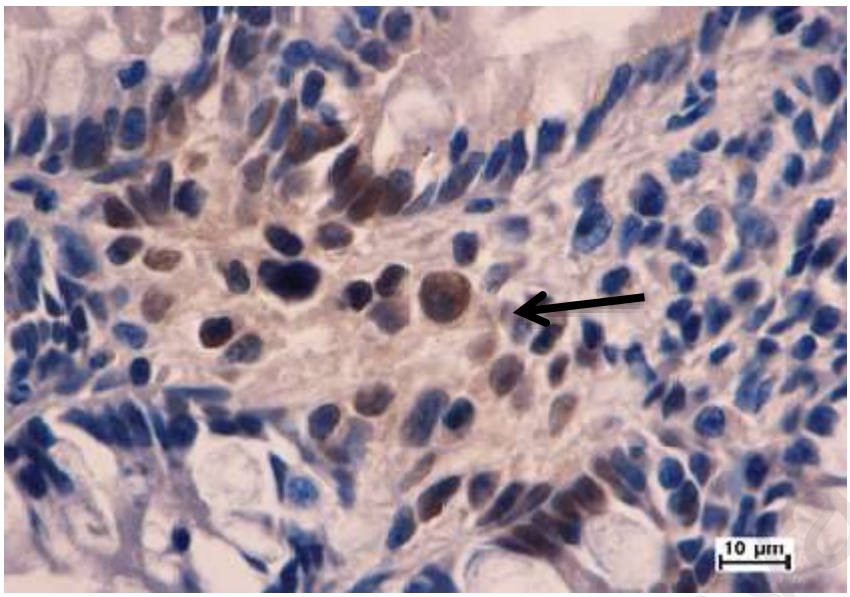

Figure 4.22: Immunohistochemical staining of colon section showing the effect of 250 mg/kg *P. macrocarpa* extract on PCNA-positive cells in rat with AOM-induced ACF. The number of PCNA-positive cells are reduced. Bar = 10µm.

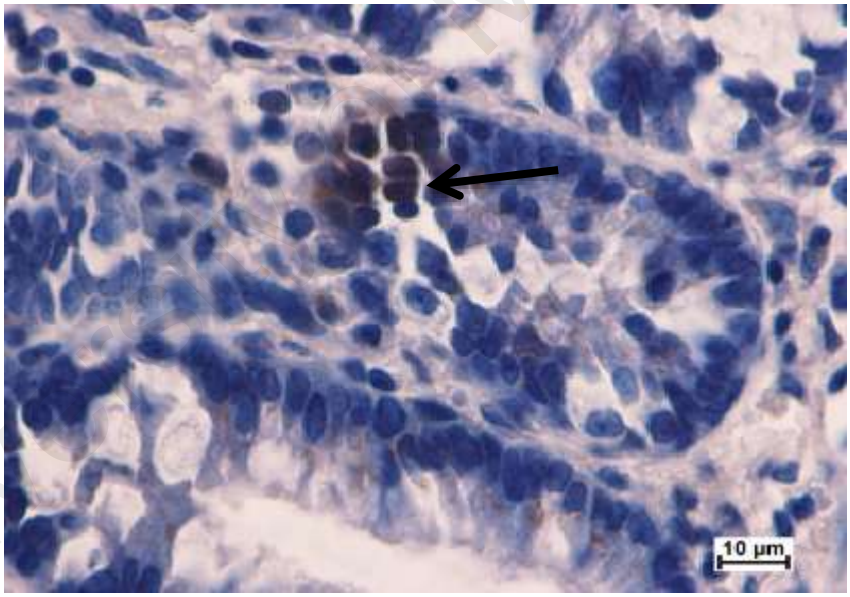

Figure 4.23: Immmunohistochemical staining of colon section showing the effect of 500 mg/kg *P. macrocarpa* extract on PCNA-positive cells in rat with AOM-induced ACF. The number of PCNA-positive cells are greatly reduced. Bar = 10µm.

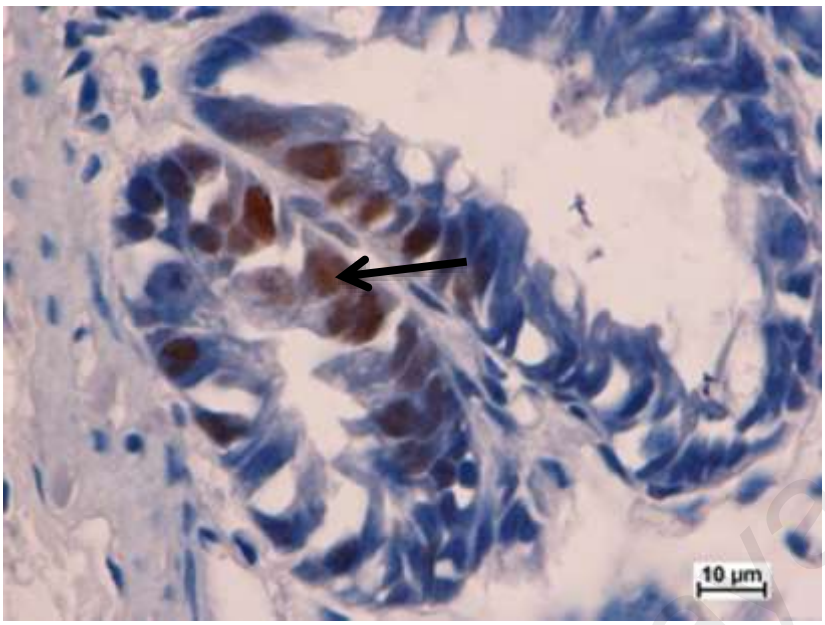

Figure 4.24: Immunohistochemical staining of colon section showing the effect of 250 mg/kg *G. procumbens* extract on PCNA-positive cells in rat with AOM-induced ACF. The number of PCNA-positive cells are reduced. Bar = 10µm.

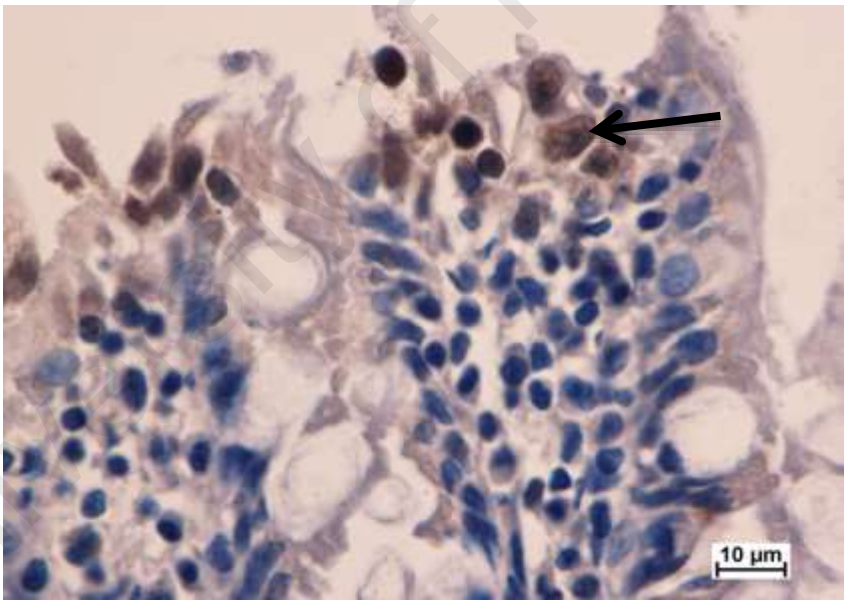

Figure 4.25: Immunohistochemical staining of colon section showing the effect of 500 mg/kg *G. procumbens* extract on PCNA-positive cells in rat with AOM-induced ACF. The number of PCNA-positive cells are reduced. Bar = 10µm.

The PCNA labelling index showed that the percentage of PCNA-positive cells in the colon tissue of the AOM group was 48.83 %, whereas the percentage of PCNA-positive cells in the colon tissue of AOM groups treated with the lower dose of *P. macrocarpa*

extract, *G. procumbens* extract and the reference drug (5-FU) were 24 %, 20 % and 10 %, respectively (Figure 4.26).

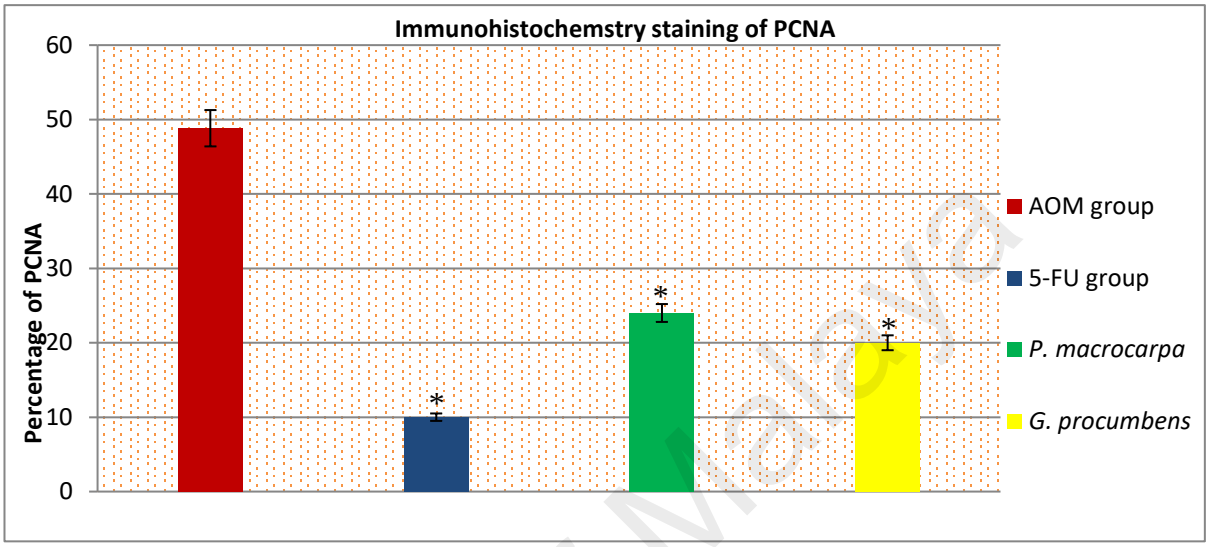

Figure 4.26: Effect of *P. macrocarpa* extract, *G. procumbens* extract and 5-FU on the percentage of PCNA in colon of rat with AOM-induced ACF. Data are expressed as mean ± S.E.M. *$p<0.05$ *vs* AOM group.

4.11. Effect of *P. macrocarpa* and *G. procumbens* on gene expression

Based on NormFinder and geNorm algorithm, HPRT-1 and Ppia showed the most stability and thus were used endogenous reference genes. The gene expression levels of the target genes in rat colon tissue samples were normalised using both HPRT-1 and Ppia endogenous reference genes. The expression levels of Bax, Bcl2 and the two endogenous reference genes were subsequently validated by RT-PCR measurements. According to the standard curve (Appendix C) all genes showed efficiency in the range of 90-110 % and the slope was between (-3.0) and (-3.5) which are within the reference criteria to run the quantitative RT-PCR (Appendix C). After data analysis of Ct values by Gene EX software and normalised to the reference genes HPRT-1 and Ppia, all measured mRNAs showed different expression from the calibrator group (normal control tissue). AOM

group, *P. macrocarpa* and *G. procumbens* altered the expression of apoptosis-related genes, that pro-apoptotic Bax gene was up-regulated while anti-apoptotic Bcl-2 gene was down-regulated (Figure 4.26 & Table 4.10).

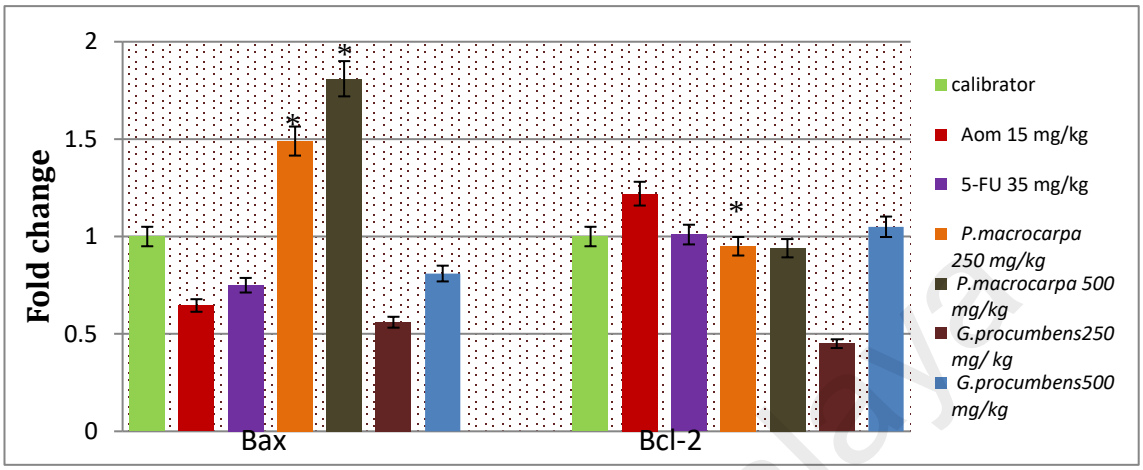

Figure 4.27: Relative gene expression levels of Bax and Bcl2 genes in rats with AOM-induced ACF treated with 250 and 500 mg/kg of *P. macrocarpa*, G. *procumbens* plant extracts, and 35mg/kg 5-FU. Data are expressed as the mean ± S.E.M. *$p \leq 0.05$ *vs*. AOM group.

Table 4.10: Effects *P. macrocarpa* extract, *G. procumbens* extract and 5-FU on mRNA expression levels of Bax and Bcl2 in colon of rat with AOM-induced ACF.

Experimental groups	Bax	Bcl2
Calibration	1	1
AOM (15 mg/kg)	0.64 ± 0.30	1.22 ± 0.51
5-FU (35 mg/kg)	0.75±0.25	1.01±0.38
P. macrocarpa (250 mg/kg)	1.49 ± 0.41*	0.95 ± 0.51
P. macrocarpa (500 mg/kg)	1.81 ± 0.61*	0.94 ± 0.52
G. procumbens (250 mg/kg)	0.56 ± 0.17	0.45 ± 0.21*
G. procumbens (500 mg/kg)	0.81 ± 0.25	1.05 ± 0.34

Data are expressed as mRNA mean fold changes ± S.E.M of 6 rats. Statistical analysis of differences was performed using a two-tailed unpaired student's, t test. *$p<0.05$ vs AOM group.

4.12. Identification of active constituents of the active fractions

The LC-MS analysis is to identify and provide information on the chemical structure of the phenolic constituents and other active compounds provides found in the *P. macrocarpa* F6 and *G. procumbens* F10. Two compounds were detected in F6 of *P. macrocarpa* and three compounds were detected in F10 of *G. procumbens.* Tables 4.11 and Table 4.12 showed the retention time and the observed m/z of fragment ions of the active compounds. The *P. macrocarpa* contain catechin-7-O-apiofuranoside at m/z 843.23 (Figure 4.28) and didesmethyl tocotrienol at m/z 367.26 (Figure 4.29) while *G. procumbens* contain scutellarein6-glucoside at m/z 447.09 (Figure 4.31), didesmethyl tocotrienol at m/z 367.26 (Figure 4.32) and Thr Tyr at m/z 300.15 (Figure 4.33).

Table 4.11: *P. macrocarpa* F6 compound identification by LC-MS using ionization mode

Sample	Rt time(min)	Observed m/z	Tentative identification	Suggested formula	M.W. (g/mol)
P. macrocarpa F6	7.07	843.23	catechin-7-O- apiofuranoside	$C_{20}H_{22}O_{10}$	422.12
	25.02	367.26	didesmethyl tocotrienol	$C_{25}H_{35}O_2$	368.27
	7.57	608.38	unknown	$C_{26}H_{54}O_{14}$	590.35

RT: Retention time, M.W.: Molecular weight

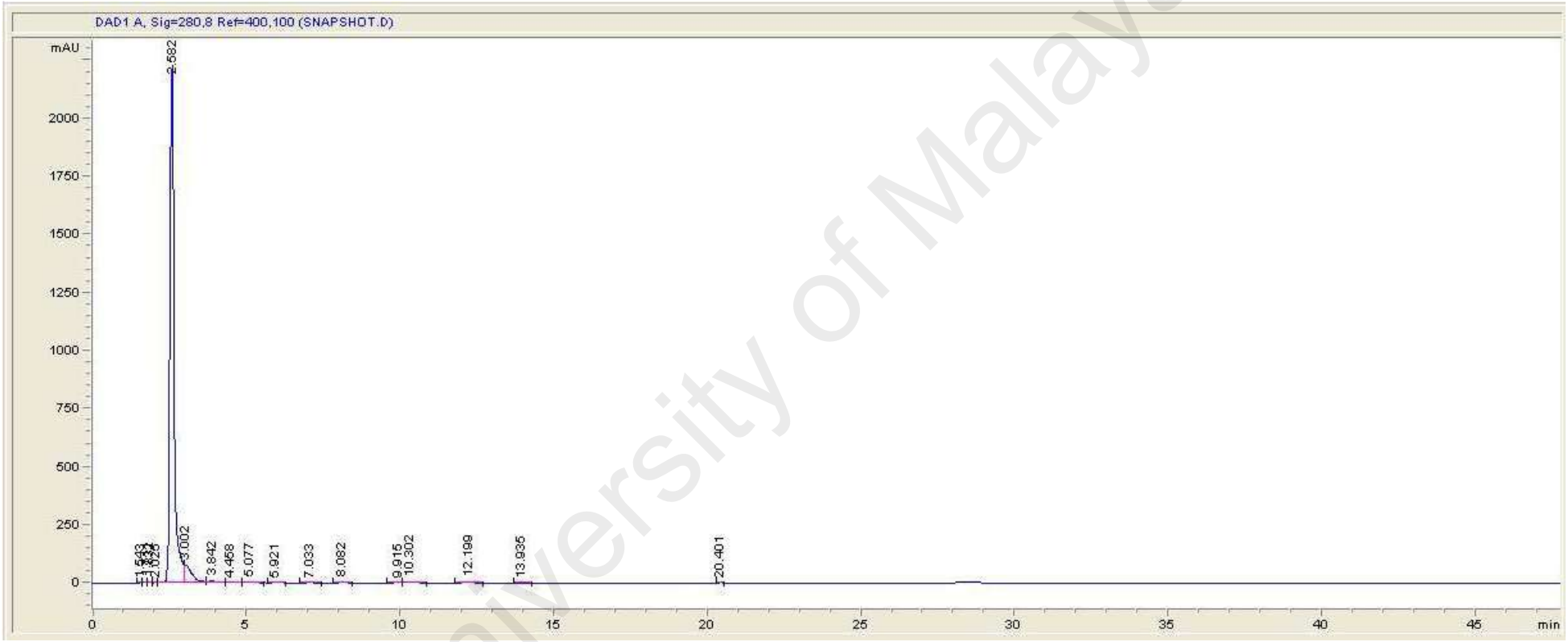

Figure 4.28: HPLC chromatogram of *P. macrocarpa* Fraction 6 (F6) showing its absorbance at 280 nm (mAU) against time (min)

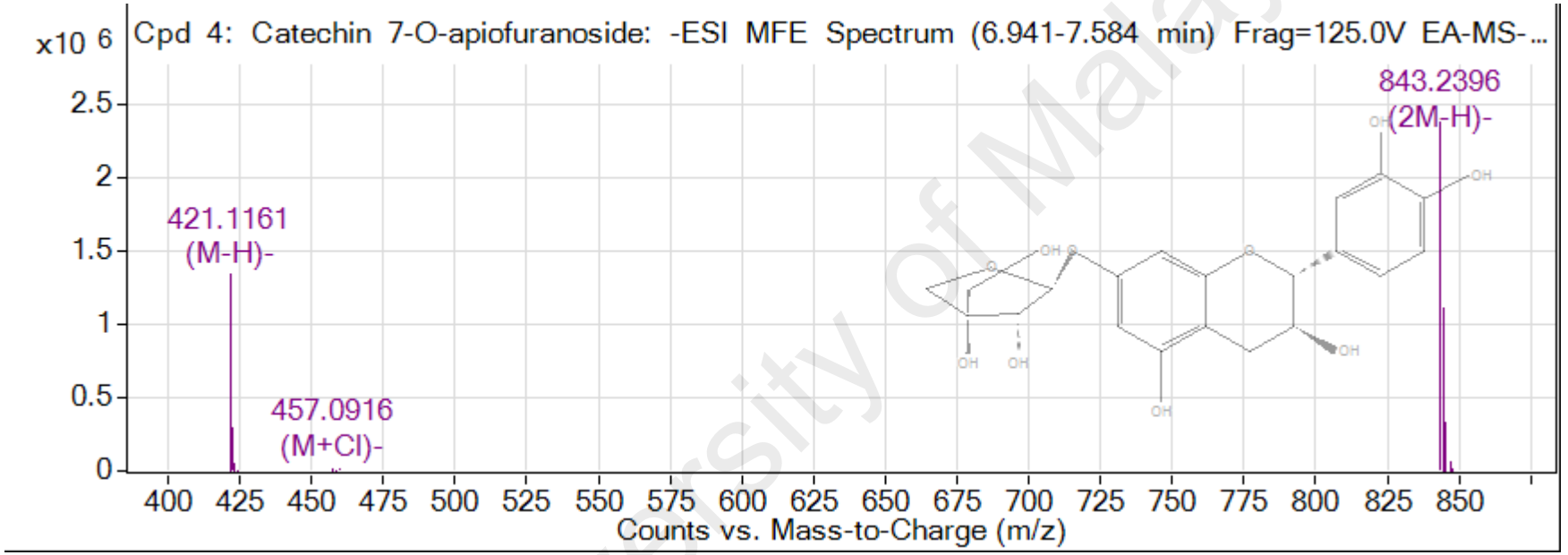

Figure 4.29: Mass spectrum and chemical structure of catechin 7-O-apiofuranoside identified in *P. macrocarpa* F6

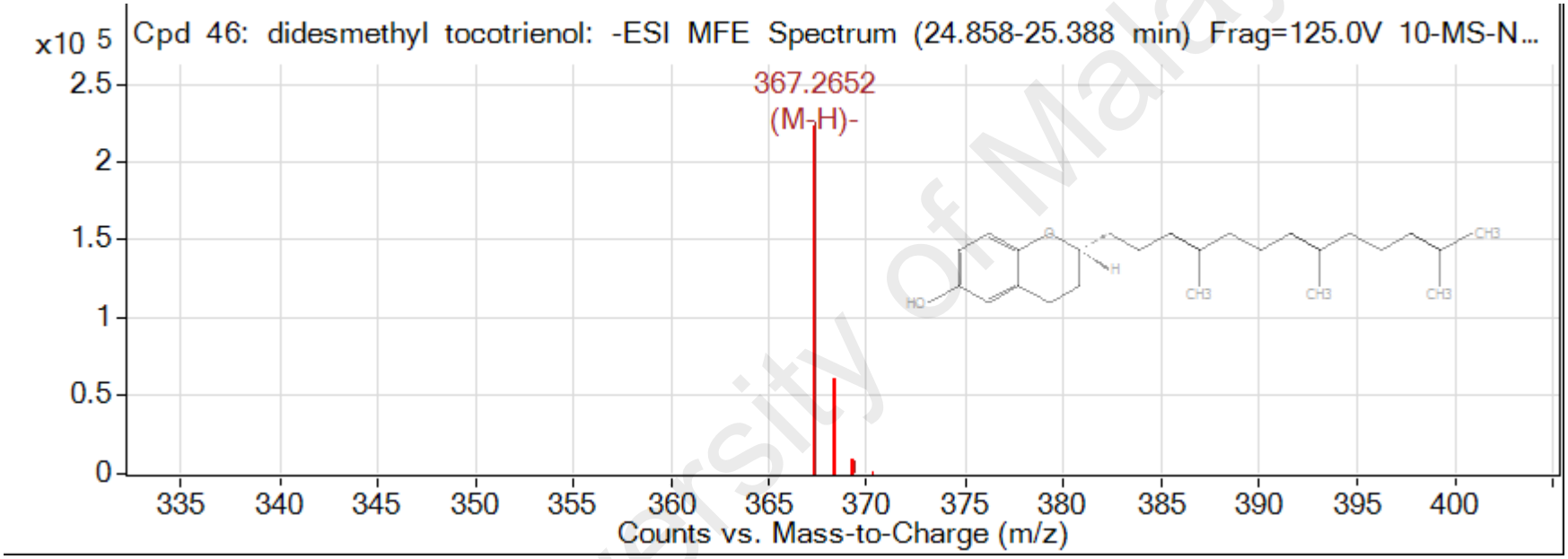

Figure 4.30: Mass spectrum and chemical structure of didesmethyl tocotrienol identified in *P. macrocarpa* F6

Table 4.12: *G. procumbens* F10 compound identification by LC-MS using ionization mode

Sample	Rt time(min)	Observed m/z	Tentative identification	Suggested formula	M.W. (g/mol)
G.procumbens F10	8.66	447.10	scutellarein 6-glucoside	C_{21} H_{19} O_{11}	448.10
	26.25	610.19	unknown	C_{37} H_{37} S_4	609.18
	25.02	367.27	didesmethyl tocotrienol	$C_{25}H_{35}O_2$	368.27
	14.54	300.15	Thr Tyr	C_{13} H_{22} N3 O_5	282.13

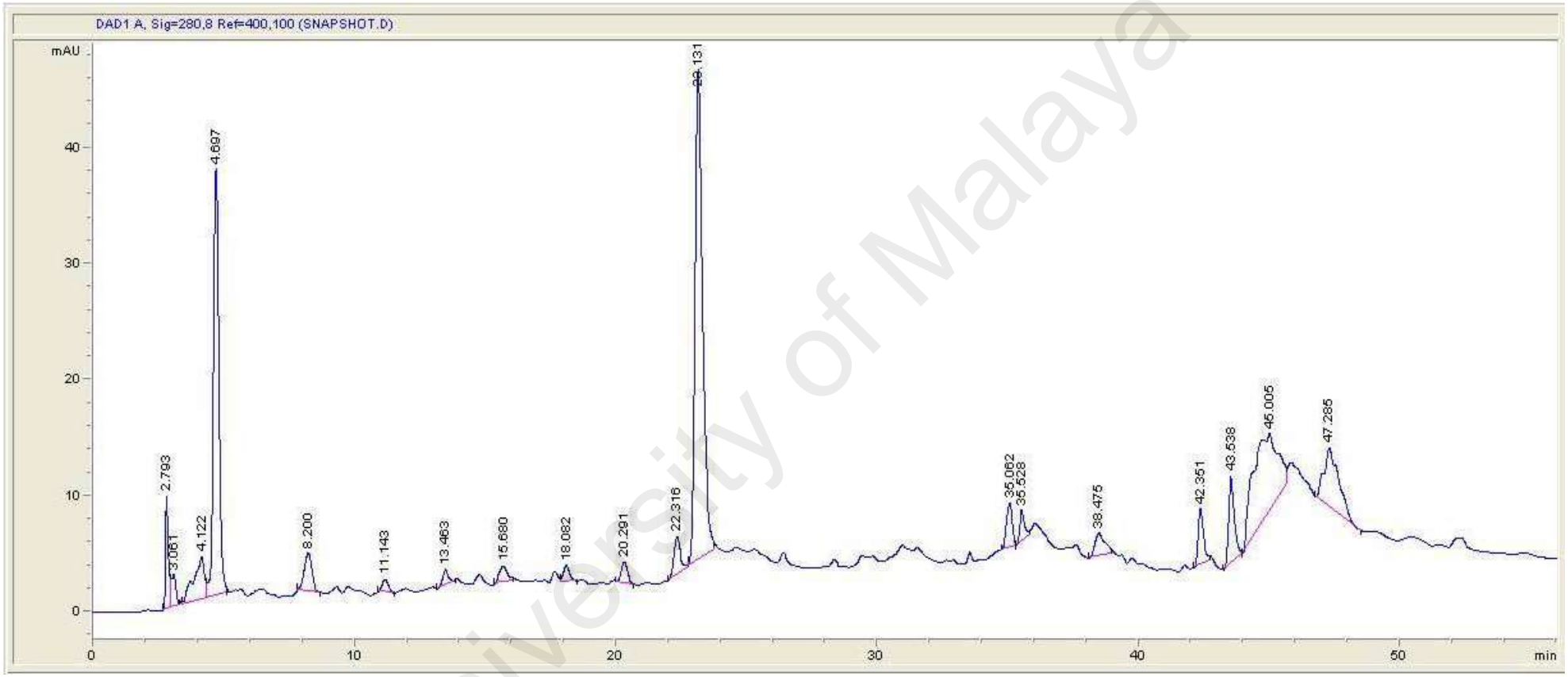

Figure 4.31: HPLC chromatogram of *G. procumbens* Fraction 10 (F10) showing its absorbance at 280 nm (mAU) against time (min)

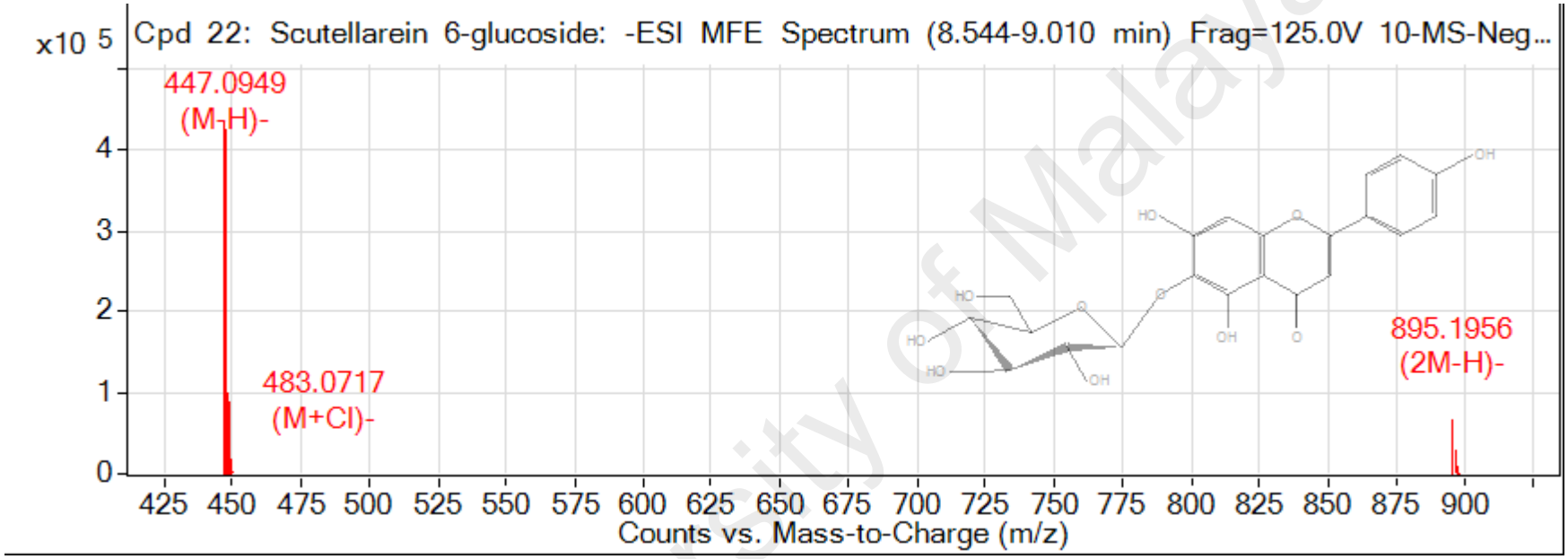

Figure 4.32: Mass spectrum and chemical structure of scutellarein 6-glucoside identified in *G. procumbens* F10

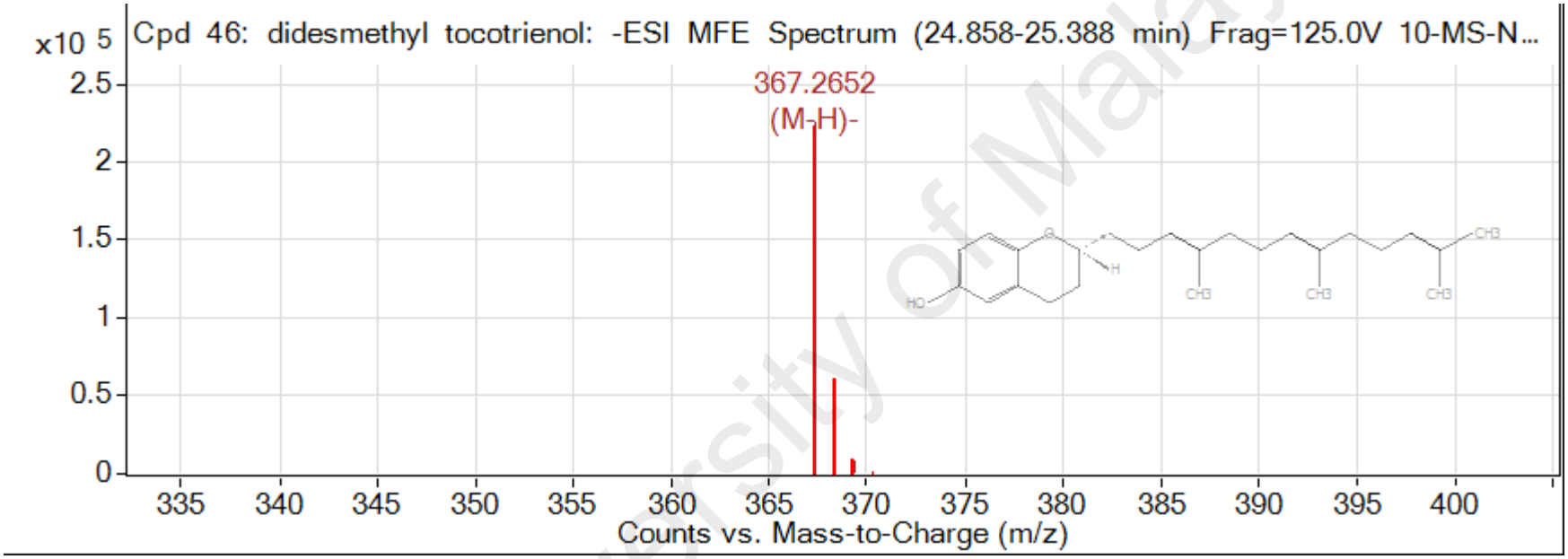

Figure 4.33: Mass spectrum and chemical structure of didesmethyl tocotrienol identified in *G. procumbens* F10

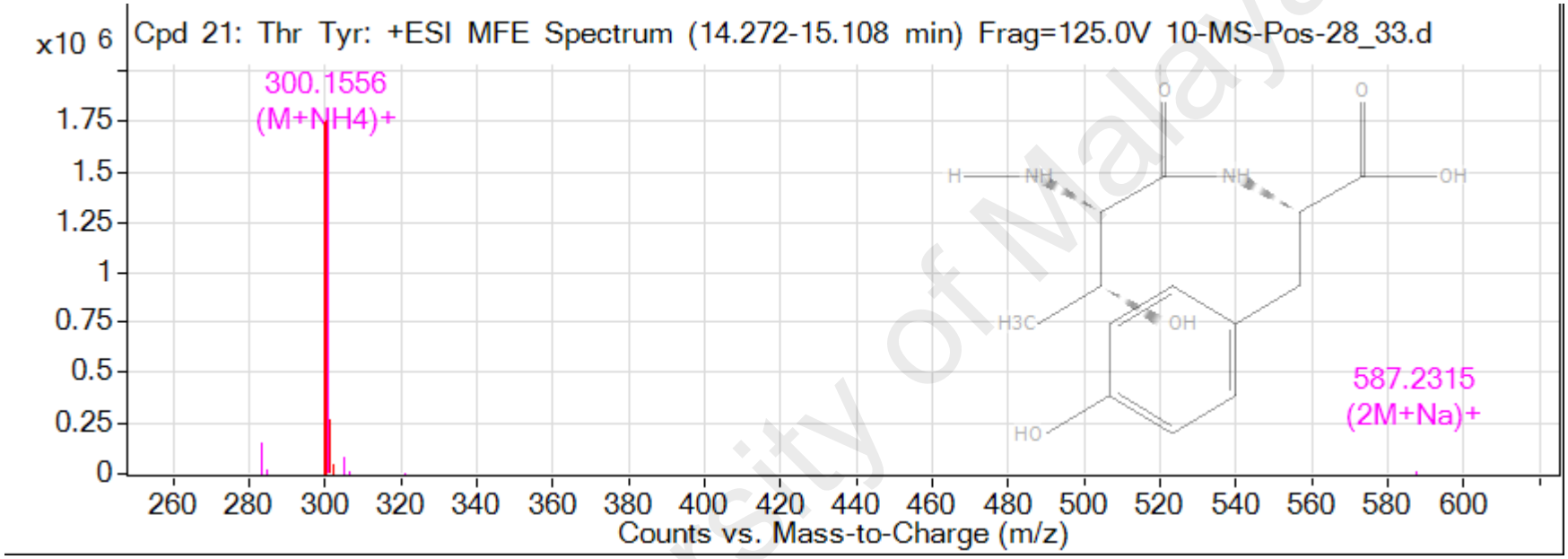

Figure 4.34: Mass spectrum and chemical structure of Thr Tyr identified in *G. procumbens* F10

CHAPTER 5: DISCUSSION AND CONCLUSION

The uncontrolled and often rapid proliferation of cells can lead to benign tumours, some types of which may turn into malignant tumours such as colon cancer and resulting in the formation of polyps over time. The disease process is associated with several other environmental, genetic factors and oxidative stress. A chemopreventive approach might potentially either suppress the appearance of the cancer phenotype or prevent further DNA damage that might develop carcinogenesis (Sporn, 1996). Plants have been used for medicinal purposes for centuries. Herbal medicine is based on the fact that plants contain natural products that can improve health (Kim et al., 2013). Plant resources namely fruits, vegetables as well as spices have attracted extensive study due to their chemoprotective properties. Many are known to possess antioxidant, anti-inflammatory, antitumour or cancer chemoprevention activities (Gupta et al., 2010). More than 180 nutritional interventions in addition to some pharmacological agents have been shown to decrease Aberrant crypt foci in chemically induced colon carcinogenesis models (Corpet & Taché, 2002). Current research interest has focused on *P. macrocarpa* and *G. procumbens* plants that may possess antitumour properties that may be useful as adjuncts to reduce the risk of cancer.

In the present study, *P. macrocarpa* and *G. procumbens* extracts showed high antioxidant activity as proven by the result obtained with FRAP assay that expressed the ferric ion reducing antioxidant power of the plant extracts. The plant extracts also exhibited high scavenging activity towards DPPH free radicals which may be due to the high phenolic content of the extracts. In agreement with previous studies, *P. macrocarpa* fruit extract contained phenols and flavonoids and exhibited high antioxidant activity (Hendra, et al., 2011; Lay et al., 2014). Similarly, *G. procumbens* leaf extract possessed high levels of antioxidant scavenging powers (Luerang et al., 2010). Many studies have

revealed positive relationships between phenolic compounds and antioxidant activity because of their ability to scavenge free radicals through their hydroxyl groups (Tosun et al., 2009; Alshawsh et al., 2011). The chemopreventive effect of both plants extracts could be due to their antioxidant and anti-proliferative activities and hence, may have the potential of being new anti-cancer therapeutic agents (Ismail et al., 2012).

Additionally, several studies have indicated a relationship between carcinogenesis and oxidative stress. Free radicals are found to be harmful to DNA. More than a few phytochemicals have been reported to activate metabolic enzymes or inactivate them. If chemical carcinogens are activated, the activation might take place via phase I metabolism or detoxified by phase II enzymes. Thus, the nature of the chemical carcinogen and the type of modulation found in the phytochemicals can be protective or detrimental.

Moreover, our results demonstrated that the exposure of HT29 to both plant extracts decreased cell viability as determined by the MTT assay. This is in consistence with the results of previous studies on *P. macrocarpa* (Hendra, et al., 2011) and *G. procumbens* (Nurulita et al., 2012).

In acute toxicity study, no mortality was observed in the animals; in addition, there were no visible manifestations of hepatotoxic and nephrotoxic effects and the serum biochemical parameters were within the normal range. Similarly, several other studies showed that the ethanol extracts of *P. macrocarpa* and *G. procumbens* were safe and no drug related toxicity was detected (Mahmood et al., 2010; Shwter et al., 2014). These results were confirmed by our *in vitro* finding, which showed that the ethanol extract of the fruit of *P. macrocarpa* and leaves of *G. procumbens* were not toxic to human colon normal cells line CCD841.

Gross examination of colon sections showed significant reduction of AFC in colon mucosa of AOM rats which were treated with plant extracts and this reduction was

comparable to 5-FU treated rats. This is in agreement with the results of other previous studies (Almagrami et al., 2014;Al-Henhena et al., 2014). Indeed, ACFs are considered to be the 'gold standard' of colon carcinogenesis biomarkers (Pretlow et al., 2004). Histological appearance of colon mucosa of the AOM group exhibited ACF with elongated stratified nuclei and depletion of mucine glands. This is in line with the results of previous studies as reported by other researchers (Hajrezaie et al., 2014; Al-Henhena et al., 2014)

Immunohistochemical analysis demonstrated that AOM treated groups, with either plant extracts or 5-FU, produced less positive staining of proliferating cell nuclear antigen (PCNA) as compared to the AOM untreated groups. Similar results were observed by Al-Henhena et al. (2014). PCNA index can be used as a marker to assess the effectiveness of chemopreventive agents to suppress cell proliferation in chemically induced colon carcinogenesis (Müller et al., 2013). The proliferation cell nuclear antigen is an important prognostic marker for colon cancer (Al-Henhena et al., 2014). Our results showed that ethanol fruit extract of *P. macrocarpa* and leaf extract of *G. procumbens* suppressed the expression of PCNA in colon mucosa. The down-regulation of cell proliferation due to both plant extracts could be considered as one of the possible mechanisms behind the inhibitory effect against ACF development and colon cancer prevention. (Al-Henhena et al., 2014; Almagrami et al., 2014)

Antioxidants act through various mechanisms such as by scavenging free radicals or by inducing antioxidant enzymes (Jia et al., 2012). SOD and GST are components of the intrinsic antioxidant defense system and responsible for dissemination of superoxide radicals and other free radicals; during oxidative stress the body uses its defense mechanism to minimise the process of lipid peroxidation. Our study indicated that both plant extracts showed a significant increase in the activities of these antioxidant enzymes compared with the AOM group. Similarly, previous studies demonstrated a negative

correlation between GST enzyme activity and tumour incidence in gastrointestinal tract, suggestive of the role of GSTs in cancer prevention (Yadav et al., 2011). In addition, the plants extracts may also act through free radical scavenging and quenching of the formation of singlet oxygen, thereby protecting the colon against oxidative stress and stimulating colon repair mechanisms (Auyeung & Ko, 2010).

Oxidative stress plays an essential role in the mechanism of cancer development and progression (Burton & Jauniaux, 2011). The current study showed that the colon homogenate of *P. macrocarpa* and *G. procumbens* treated animals had lower levels of MDA than the AOM group. Results from the current investigation are consistent with previous studies which showed reduced levels of lipid peroxidation in colonic tissues of rats treated with the plant extract (Aranganathan et al., 2009; Al-Henhena et al., 2014). It has been demonstrated that colon damage is associated with significant increases of tissue lipid peroxidation expressed as MDA level, which is a product of oxidative stress. (Jia et al., 2012). In the current study, ethanol extract of *P. macrocarpa* and *G. procumbens* treated groups exhibited a decrease in MDA levels as compared with the AOM group. It clearly suggests that the *P. macrocarpa* and *G. procumbens* reduce lipid peroxidation and protected colon cells similar to 5-FU.

Chemoprevention is a unique and promising strategy for reducing cancer risk through the administration of synthetic or dietary compounds. There are many anti-tumour agents obtained from medicinal plants which exert their effect through apoptosis induction in cancer cell (Kundu et al., 2005). Apoptotic pathway in mitochondrial has been described as an important indication of apoptotic cell death for mammalian cells (Wong, 2011). Release of cytochrome c from the inter-membrane of the mitochondria into the cytosol is a key occurrence in mitochondrial-dependent apoptotic pathway. The release of cytochrome c from mitochondria is firmly regulated by a diversity of factors. Among these factors, Bcl-2 family proteins, including anti-apoptotic members (Bcl-2)

prevent the release of cytochrome c from mitochondria. On the other hand, pro-apoptotic members such as Bax increase the release of cytochrome c from mitochondria (Wong, 2011). RT-PCR analysis was subsequently used to determine the expression of Bcl-2 and Bax mRNA. Treatments with *P. macrocarpa* and *G. procumbens* extracts induced the up-regulation of pro-apoptotic protein Bax while down-regulating anti-apoptotic protein Bcl-2. Anti-apoptotic proteins encoded by Bcl-2 genes act as check points in apoptosis regulation, while Bax are pro-apoptotic proteins of the Bcl-2 gene family. Our result is an agreement with previous studies that showed anti-apoptotic Bcl-2 protein was down-regulated, while pro-apoptotic Bax was up-regulated (Baeshen et al., 2012). However, the apparent up-regulation in colon may be in response to the dietary intake of *P. macrocarpa* and *G. procumbens.* Further, we conducted studies to elucidate other molecular mechanism underlying the apoptotic activity of *P. macrocarpa* and *G. Procumbens.* LDH is considered as a prognostic marker in malignancies and in particular for CRC and the findings of the present study demonstrated an increase in the level of serum LDH in the AOM group compared to treatment groups rather than other biochemical markers -- this was in line with the results of a previous report (Koukourakis et al., 2009).

The bioactivity and bioavailability of *P. macrocarpa* F6 against cancer was suggested to be due to phytochemicals contents of catechin-7-O-apiofuranoside and didesmethyl tocotrienol [3,4-dihydro-2-(4,8,12-trimethyltrideca-3'(E),7'(E),11'-trienyl)-2 H1benzopyran-6-ol]; and that of *G. procumbens* F10 was suggested to be which presented scutellarein 6-glucoside and didesmethyl tocotrienol.

Catechin-7-O-glucoside has been isolated in the current study. Previous studies have discussed the relationship between catechin-7-O-glucoside and cancer prevention (Kim, et al., 2010). This compound has an antioxidant activity leading to a cytoprotective effect (Kim, et al., 2010). The antioxidant activity of the extracts was suggested to be due to the presence of flavonoids.

Didesmethyl tocotrienol is a minor component of vitamin E which plays an important role in the prevention of cancer (Birt, 1986; Qureshi et al., 2000). In accordance with previous studies, it has also been isolated and identified in rice bran oil (Minhajuddin et al., 2005). Dietary supplementation of a tocopherol has been described to reduce the incidence of various cancers, such as colon, prostate, and esophageal cancers. The suggested mechanism for this anti-carcinogenic effect is thought to include at least two concepts. One of these concepts is the inhibition of lipid peroxidation and its formation of reactive products. Based on this concept, vitamin E supplementation may reduce lipid peroxidation and the binding of its reactive products to DNA, hence, preventing oxidative damage to DNA (Ames et al., 1995). Additionally, vitamin E possesses anti-proliferative properties that interfere in signal transduction and in inducing cell cycle arrest (Valadez et al., 2013).

Tocotrienol displays different types of antioxidant activities in *in vitro* and *in vivo* systems (Adachi & Ishii, 2000; Qureshi et al., 2000). The compound can quench lipid radicals and in the process become radicals themselves as a part of an antioxidant process (Rimbach et al., 2002). In addition, tocotrienol mediated activation of the intrinsic pathway involved in up-regulation of Bax, cleavage of Bid, release of cytochrome C, and activation of caspase-9. The tocotrienol form of vitamin E has clearly been identified to have other unique biological functions independent of its antioxidant activity.

Scutellarin is an active flavonoid, a type of phenolic component that is present in the medicinal plant *Erigeron breviscapus* (Vant), and is used to improve microcirculation for the treatment of cardio-cerebrovascular diseases. Scutellarin has also been suggested to have anti-cancer properties; it has been shown to induce apoptosis of tumour cells such as ovarian and breast tumour cells (Ibibia, 2013).

Phytochemical analysis of other study showed that *G. procumbens* and *P. macrocarpa* contain flavonoids, that effectively suppressed the proliferation of a human

colon carcinoma cell line, COLO 201, through apoptosis induction while phenolics showed anticancer activity against cancer colon cell by arresting the cell cycle (Imai et al., 2009)

Our promising result with *P. macrocarpa* and *G. procumbens* derived compounds are supported by other recent findings that showed the flavonoid contents have antioxidant, free radical scavenging, anti-proliferative and anti-cancer properties. Therefore, based on the above mentioned literature, this study was undertaken in rats to evaluate for any chemoprotective properties of ethanolic extracts of *G. procumbens* and *P. macrocarpa* against foci of aberrant crypts.

Conclusion

The results of this study hypothesize that *G. procumbens* and *P. macrocarpa* extracts are nontoxic after oral administration and have potential chemopreventive effects against AOM-induced colon cancer in rats via suppression of ACF formation in the distal and proximal colon mucosal sections. This hypothesis is supported by histological findings which showed significant chemopreventive activity in colon tissues. Furthermore, down-regulation of proliferating cancer cells (which was demonstrated by PCNA immunohistochemistry); activation of apoptosis via the mitochondrial pathway by down-regulating Bcl-2 and up-regulating of Bax; reduction of lipid peroxidation (MDA) and elevation of antioxidant enzymes levels (SOD & GST) which protect colon cells from oxidative injuries caused by the injection of azoxymethane by these plants extract; serves to strengthen the hypothesis that they have chemoprevention activity

Future work

Further studies are needed to confirm the active constituents which were isolated and purified from these plant extracts and also to evaluate their bioavailability, pharmacodynamics, pharmacokinetics and other pharmacological properties. In addition, it is equally important to establish the amount of the active constituents which are biologically available in the plasma after administration of the plant extract. Moreover, a study on the mechanism of action of the active compounds from the extracts on cell line using other methods such as flow cytometry is warranted.

REFERENCES

Abdelmegeed, M. A., Banerjee, A., Yoo, S.-H., Jang, S., Gonzalez, F. J. & Song, B.-J. (2012). Critical role of cytochrome P450 2E1 (CYP2E1) in the development of high fat-induced non-alcoholic steatohepatitis. *Journal of Hepatology, 57*(4), 860-866.

Abotchie, P. N., Vernon, S. W. & Du, X. L. (2012). Gender differences in colorectal cancer incidence in the United States, 1975–2006. *Journal of Women's Health, 21*(4), 393-400.

Adachi, H. & Ishii, N. (2000). Effects of tocotrienols on life span and protein carbonylation in *Caenorhabditis elegans*. *The Journals of Gerontology, 55*(6), B280-B285.

Adelstein, B. A., Macaskill, P., Chan, S. F., Katelaris, P. H. & Irwig, L. (2011). Most bowel cancer symptoms do not indicate colorectal cancer and polyps: A systematic review. *BMC Gastroenterology, 11*(1), 65.

Ahmad , A., Padhye, S. & Sarkar, F. H. (2012). Role of novel nutraceuticals garcinol, plumbagin and mangiferin in the prevention and therapy of human malignancies: mechanisms of anticancer activity. Sarkar, Fazlul H. (Ed.). *Nutraceuticals and Cancer* (pp. 179-199): Springer, Netherlands. Doi:10.1007/978-94-007-2630-7_10.

Akowuah, G, Sadikun, A. & Mariam, A. (2002). Flavonoid identification and hypoglycaemic studies of the butanol fraction from *G. procumbens*. *Pharmaceutical Biology, 40*(6), 405-410.

Al-Henhena, N., Ying, R. P. Y., Ismail, S., Najm, W., Khalifa, S. A., El-Seedi, H ,.et al. (2014). Chemopreventive efficacy of *Andrographis paniculata* on azoxymethane-induced aberrant colon crypt foci *in vivo*. *PloS One, 9*(11), e111118.

Ali, R. B., Atangwho, I. J., Kaur, N., Abraika, O. S., Ahmad, M., Mahmud, R., et al. (2012). Bioassay-guided antidiabetic study of *P. macrocarpa* fruit extract. *Molecules, 17*(5), 4986-5002.

Almagrami, A. A., Alshawsh, M. A., Saif-Ali, R., Shwter, A., Salem, S. D. & Abdulla, M. A. (2014). Evaluation of chemopreventive effects of *Acanthus ilicifolius* against azoxymethane-induced aberrant crypt foci in the rat colon. *PloS One, 9*(5), e0096004.

Alshawsh, M. A., Abdulla, M. A., Ismail, S. & Amin, Z. A. (2011). Hepatoprotective effects of *Orthosiphon stamineus* extract on thioacetamide-induced liver cirrhosis in rats. *Evidence-Based Complementary & Alternative Medicine*, Doi:10.1155/2011/103039.

Altaf, R., Asmawi, M. Z. B., Dewa, A., Sadikun, A. & Umar, M. I. (2013). Phytochemistry and medicinal properties of *P. macrocarpa* (Scheff.) Boerl. extracts. *Pharmacognosy Reviews*, *7*(13), 73.

Ames, B. N., Gold, L. S. & Willett, W. C. (1995). The causes and prevention of cancer. *Proceedings of the National Academy of Sciences*, *92*(12), 5258-5265.

Aranganathan, S., Selvam, J. P. & Nalini, N. (2009). Hesperetin exerts dose dependent chemopreventive effect against 1, 2-dimethyl hydrazine induced rat colon carcinogenesis. *Investigational New Drugs, 27*(3), 203-213.

Armaghany, T., Wilson, J. D., Chu. Q. & Mills, G. (2012). Genetic alterations in colorectal cancer. *Gastrointestinal Cancer Research*, 5(1), 19.

Arthur, J. C., Perez-Chanona, E., Mühlbauer, M., Tomkovich, S., Uronis, J .M., Fan, T.-J., et al. (2012). Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science, 338*(6103), 120-123.

Astin, M., Griffin, T., Neal, R. D., Rose, P. & Hamilton, W. (2011). The diagnostic value of symptoms for colorectal cancer in primary care: A systematic review. *British Journal of General Practice, 61*(586), e231-e243.

Aust, D. E., & Baretton, G. B. (2010). Serrated polyps of the colon and rectum (hyperplastic polyps, sessile serrated adenomas, traditional serrated adenomas, and mixed polyps)-proposal for diagnostic criteria. *Virchows Archiv, 457*(3), 291-297.

Auyeung, K. K.-W. & Ko, J. K.-S. (2010). Novel herbal flavonoids promote apoptosis but differentially induce cell cycle arrest in human colon cancer cell. *Investigational New Drugs, 28*(1), 1-13.

Baeshen, N. A., Elkady, A. I., Abuzinadah, O. A. & Mutwakil, M. H. (2012). Potential anticancer activity of the medicinal herb, *Rhazya stricta*, against human breast cancer. *African Journal of Biotechnology, 11*(37), 8960-8972.

Béjar, L. M., Gili, M., Infantes, B. & Marcott, P. F. (2012). Incidence of colorectal cancer and influence of dietary habits in fifteen European countries from 1971 to 2002. *Gaceta Sanitaria, 26*(1), 69-73.

Benzie, I. F. F. & Strain, J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*(1), 70-76.

Bird, R. P. (1987). Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: Preliminary findings. *Cancer Letters, 37*(2), 147-151.

Bird, R. P. (1995). Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Letters, 93*(1), 55-71.

Birt, D. F .(1986) .Update on the effects of vitamins A, C, and E and selenium on carcinogenesis. *Experimental Biology & Medicine, 183*(3), 311-320.

Block, K. I. & Gyllenhaal, C. (2002). Clinical corner: Herb-drug interactions in cancer chemotherapy: Theoretical concerns regarding drug metabolizing enzymes. *Integrative Cancer Therapies, 1*(1), 83-89.

Bordoloi, P., Baruah, M., Nath, C., Das, D. & Devi, R. (2013). A study of oxidative stress in cancer patient before and after supplementation of vitamin C and vitamin E. *International Journal of Health Sciences & Research, 3*(1), 14-18.

Brand-Williams, W., Cuvelier, M. & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science & Technology, 28*(1), 25-30.

Breda, M & Barattè, S. (2010). A review of analytical methods for the determination of 5-FU in biological matrices. *Analytical & Bioanalytical Chemistry, 397*(3), 1191-1201.

Bretthauer, M. (2011). Colorectal cancer screening. *Journal of Internal Medicine, 270*(2), 87-98.

Burton, G. J. & Jauniaux, E. (2011) Oxidative stress. *Best Practice & Research Clinical Obstetrics & Gynecology, 25*(3), 287-299.

Caderni, G., Femia, A. P., Giannini, A., Favuzza, A., Luceri, C., Salvadori, M., et al. (2003). Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats correlation with carcinogenesis. *Cancer Research, 63*(10), 2388-2392.

Cairns, S. R., Scholefield, J. H., Steele, R. J., Dunlop, M. G., Thomas, H. J., Evans, G. D., *et al.* (2010). Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002). *Gut, 59*(5), 666-689.

Chanda, S. & Dave, R. (2009). *In vitro* models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. *African Journal of Microbiology Research, 3*(13), 981-996.

Chang, L. C., Fan, C. W., Tseng, W. K., Chein, H. P., Hsieh, T. Y., Chen, J. R. & Hwang, C. C. (2014). IFNAR1 is a predictor for overall survival in colorectal cancer and its m RNA expression correlated with irf7 but not tlr9, *Medicine*,.93(29), e349.

Chen, J. & Huang, X. F. (2009). The signal pathways in azoxymethane-induced colon cancer and preventive implications. *Cancer Biology & Therapy, 8*(14), 1313-1317.

Chernyshev, A., Fleischmann, T. & Kohen, A. (2007). Thymidyl biosynthesis enzymes as antibiotic targets .*Applied Microbiology & Biotechnology, 74*(2), 282-289.

Chidambaram, M., Manavalan, R. & Kathiresan, K. (2011). Nanotherapeutics to overcome conventional cancer chemotherapy limitations. *Journal of Pharmacy & Pharmaceutical Sciences, 14*(1), 67-77.

Chong, S. C., Dollah, M. A., Chong, P. P. & Maha, A. (2011). *P. macrocarpa* (Scheff.) Boerl fruit aqueous extract enhances LDL receptor and PCSK9 expression *in vivo* and *in vitro*. *Journal of Ethnopharmacology*, *137*(1), 817-827.

Corpet, D. E. & Taché, S. (2002). Most effective colon cancer chemopreventive agents in rats: A systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutrition & Cancer*, *43*(1), 1-21.

Danenberg, P. V. (1977). Thymidylate synthetase-a target enzyme in cancer chemotherapy. *Biochimica et Biophysica-Reviews on Cancer, 473*(2), 73-92.

Das, U. N. (2002). A radical approach to cancer. *Medical Science Monitor, 8*(4), RA 79-92.

Dharmender, R., Madhavi, T., Reena, A. & Sheetal, A. (2010). Simultaneous quantification of bergenin,(+)-catechin, gallicin and gallic acid; and quantification of β-sitosterol using HPTLC from *bergenia ciliata* (haw.) sternb, *Pharmaceutics Analaytica Acta, 1*(1), 104.

Dupaul-Chicoine, J., Yeretssian, G., Doiron, K., Bergstrom, K. S., McIntire, C. R., LeBlanc, P. M., et al. (2010). Control of intestinal homeostasis, colitis, and colitis associated colorectal cancer by the inflammatory caspases. *Immunity, 32*(3), 367-378.

Dowd, L. (1959). Spectrophotometric determination of quercetin. *Analytical Chemistry, 31*(7), 1184-1187.

Ebada, S. S., Edrada, R. A., Lin, W. & Proksch, P. (2008). Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. *Nature Protocols, 3*(12), 1820-1831.

Elkady, A. I. (2012). Crude extract of *Nigella sativa* inhibits proliferation and induces apoptosis in human cervical carcinoma HeLa cells. *African Journal of Biotechnology, 11*(64), 12710-12720.

Elkady, A. I. (2013). Crude alkaloid extract of *Rhazya stricta* inhibits cell growth and sensitizes human lung cancer cells to cisplatin through induction of apoptosis. *Genetics & Molecular Biology, 36*(1), 12-21.

Faried, A., Kurnia, D., Faried, L., Usman, N., Miyazaki, T., Kato, H., et al. (2007). Anticancer effects of gallic acid isolated from Indonesian herbal medicine, *P macrocarpa* (Scheff.) Boerl, on human cancer cell lines. *International Journal of Oncology, 30*(3), 605-613.

Farinella, E., Soobrah, R., Phillips, R. K. S. & Clark, S. K. (2010). Familial adenomatous polyposis (FAP) and gender. Does gender influence the genetic transmission of FAP. *Familial Cancer, 9*(3), 405-406.

Fattori, R., Mineo, G. & Di Eusanio, M. (2011). Acute type B aortic dissection: Current management strategies. *Current Opinion in Cardiology, 26*(6), 488-493.

Fearon, E. R. (2011). Molecular genetics of colorectal cancer. *Annual Review of Pathology: Mechanisms of Disease, 6*, 479-507.

Fedirko, V., Tramacere, I., Bagnardi, V., Rota, M., Scotti, L., Islami, F., et al. (2011). Alcohol drinking and colorectal cancer risk: An overall and dose–response meta-analysis of published studies. *Annals of Oncology, 22*(9), 1958-1972.

Firuzi, O., Miri, R., Tavakkoli, M. & Saso, L. (2011). Antioxidant therapy: Current status and future prospects. *Current Medicinal Chemistry, 18*(25), 3871-3888.

Fuchs-Tarlovsky, V. (2013). Role of antioxidants in cancer therapy. *Nutrition, 29*(1), 15-21.

Fujimitsu, Y., Nakanishi, H., Inada, K., Yamachika, T., Ichinose, M., Fukami, H., et al. (1996). Development of aberrant crypt foci involves a fission mechanism as revealed by isolation of aberrant crypts. *Cancer Science, 87*(12), 1199-1203.

Generalić, I., Skroza, D., Ljubenkov, I., Katalinić, A., Burčul, F. & Katalinić, V. (2011). Influence of the phenophase on the phenolic profile and antioxidant properties of dalmatian sage. *Food Chemistry, 127*(2), 427-433.

Gill, S. S. & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants *Plant Physiology & Biochemistry, 48*(12), 909-930.

Goel, A. & Boland, C. R. (2010). Recent insights into the pathogenesis of colorectal cancer. *Current Opinion in Gastroenterology, 26*(1), 47.

Gupta, S. C., Kim, J. H., Prasad, S. & Aggarwal, B. B. (2010). Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer & Metastasis Reviews, 29*(3), 405-434.

Habila, J., Bello, I., Dzikwi, A., Musa, H. & Abubakar, N. (2010). Total phenolics and antioxidant activity of *Tridax procumbens* Linn. *African Journal of Pharmacy & Pharmacology, 4*(3), 123-126.

Hajrezaie, M., Hassandarvish, P., Moghadamtousi, S. Z., Gwaram, N. S., Golbabapour, S., Naji Hussien, A., et al. (2014). Chemopreventive evaluation of a Schiff base derived copper (II) complex against azoxymethane-induced colorectal cancer in rats. *PloS One, 9*(3), e0091246.

Half, E., Bercovich, D. & Rozen, P. (2009). Familial adenomatous polyposis. *Orphanet Journal of Rare Diseases, 4*(1), 22.

Halliwell, B. (2012). Free radicals and antioxidants: Updating a personal view. *Nutrition Reviews, 70*(5), 257-265.

Hanahan, D. & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646-674.

Harmy, M., Norwati, D., Noor, N. M. & Amry, A. (2011). Knowledge and attitude of colorectal cancer screening among moderate risk patients in west Malaysia. *Asian Pacific Journal of Cancer Prevention, 12*(8), 1957-1960.

Hauser, A. T. & Jung, M. (2008). Targeting epigenetic mechanisms: Potential of natural products in cancer chemoprevention. *Planta Medica, 74*(13), 1593-1601.

He, Y. F., Wei, W., Zhang, X., Li, Y. H., Li, S., Wang, F. H., et al. (2008). Analysis of the DPYD gene implicated in 5-fluorouracil catabolism in Chinese cancer patients. *Journal of Clinical Pharmacy & Therapeutics, 33*(3), 307-314.

Hendra, P., Fukushi, Y. & Hashidoko, Y. (2009). Synthesis of benzophenone glucopyranosides from *P. macrocarpa* and related benzophenone glucopyranosides. *Bioscience, Biotechnology, & Biochemistry, 73*(10), 2172-2182.

Hendra, R., Ahmad, S., Oskoueian, E., Sukari, A. & Shukor, M. Y. (2011). Antioxidant, anti-inflammatory and cytotoxicity of *P. macrocarpa* (Boerl.) Scheff Fruit. *BMC Complementary & Alternative Medicine, 11*(1), 110.

Hendra, R., Ahmad, S., Oskoueian, E., Sukari, A. & Shukor, M. Y. (2011). Flavonoid analyses and antimicrobial activity of various parts of *P. macrocarpa* (Scheff.) boerl fruit. *International Journal of Molecular Sciences, 12*(6), 3422-3431.

Hew, C.-S., Khoo, B.-Y. & Gam, L.-H. (2013). The anti-cancer property of proteins extracted from *G. procumbens* (Lour.) Merr. *PloS One, 8*(7), e0068524.

Hoe, S.-.Z., Lee, C.-N., Mok, S.-L., Kamaruddin, M. Y. & Lam, S.-K. (2011). *G procumbens* Merr. decreases blood pressure in rats by vasodilatation via inhibition of calcium channels. *Clinics, 66*(1), 143-150.

Hong, M., Xu, A., Zhou, H., Wu, L., Randers-Pehrson, G., Santella, R., et al. (2010). Mechanism of genotoxicity induced by targeted cytoplasmic irradiation. *British Journal of Cancer, 103*(8), 1263-1268.

Hulme, A. T., Price, S. L. & Tocher, D. A. (2005). A new polymorph of 5-fluorouracil found following computational crystal structure predictions. *Journal of the American Chemical Society, 127*(4), 1116-1117.

Ibibia, T. (2013). A review on antimicrobial and other beneficial effects of flavonoids. *International Journal of Pharmaceutical Sciences Review & Research, 21*(1).20-33.

Imai, M., Kikuchi, H., Denda, T., Ohyama, K., Hirobe, C. & Toyoda, H. (2009). Cytotoxic effects of flavonoids against a human colon cancer derived cell line, COLO 201: A potential natural anti-cancer substance. *Cancer Letters, 276*(1), 76-80.

Ismail, T., Sestili, P. & Akhtar, S. (2012). Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects. *Journal of Ethnopharmacology, 143*(2), 397-405.

Jakesevic, M., Aaby, K., Borge, G. I. A., Jeppsson, B., Ahrné, S. & Molin, G. (2011). Antioxidative protection of dietary bilberry, chokeberry and *Lactobacillus plantarum* HEAL19 in mice subjected to intestinal oxidative stress by ischemia-reperfusion. *BMC Complementary & Alternative Medicine, 11*(1), 8.

Jänne, P. A. & Mayer, R. J. (2000). Chemoprevention of colorectal cancer. *New England Journal of Medicine, 342*(26), 1960-1968.

Jarikasem, S., Charuwichitratana, S., Siritantikorn, S., Chantratita, W., Iskander, M., Frahm, A. W., et al. (2013). Antiherpetic effects of *G. procumbens*. *Evidence-Based Complementary & Alternative Medicine,* Doi:10.1155 / 2013/394865.

Jasperson, K. W., Tuohy, T. M., Neklason, D. W. & Burt, R. W. (2010). Hereditary and familial colon cancer. *Gastroenterology, 138*(6), 2044-2058.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians, 61*(2), 69-90.

Jia, N., Xiong, Y. L., Kong, B., Liu, Q. & Xia, X. (2012). Radical scavenging activity of black currant *Ribes nigrum* extract and its inhibitory effect on gastric cancer cell proliferation via induction of apoptosis. *Journal of Functional Foods, 4*(1), 382-390.

Jin, H., Lee, W. S., Yun, J. W., Jung, J. H., Yi, S. M., Kim, H. J., et al. (2013). Flavonoids from *Citrus unshiu* Marc. inhibit cancer cell adhesion to endothelial cells by selective inhibition of VCAM-1. *Oncology Reports, 30*(5), 2336-2342.

Juhn, E. & Khachemoune, A. (2010). Gardner Syndrome. *American Journal of Clinical Dermatology, 11*(2), 117-122.

Kaewseejan, N., Sutthikhum, V. & Siriamornpun, S. (2015). Potential of *G. procumbe*ns leaves as source of flavonoid-enriched fractions with enhanced antioxidant capacity. *Journal of Functional Foods, 12*, 120-128.

Kashyap, P. & Farrugia, G. (2011). Oxidative stress: Key player in gastrointestinal complications of diabetes. *Neurogastroenterology & Motility, 23*(2), 111-114.

Kawamori, T., Tanaka, T., Kojima, T., Suzui, M., Ohnishi, M. & Mori, H. (1994). Suppression of azoxymethane-induced rat colon aberrant crypt foci by dietary protocatechuic acid. *Cancer Science, 85*(7), 686-691.

Kilgour, J. & Roberts, R. (2014). Pathophysiological roles of reactive oxygen and nitrogen species. Laher, Ismail (Ed.), *Systems Biology of Free Radicals and Antioxidants*, (171-206), Springer-Verlag Berlin Heidelberg.

Kilic, N., Taslipinar, M. Y ,.Guney, Y., Tekin, E. & Onuk, E. (2014). An investigation into the serum thioredoxin, superoxide dismutase, malondialdehyde, and advanced oxidation protein products in patients with breast cancer. *Annals of Surgical Oncology, 21*(13), 4139-4143.

Kim, D. H ,.Hossain, M. A., Kang, Y. J., Jang, J. Y., Lee, Y. J., Im, E., et al. (2013). Baicalein, an active component of *scutellaria baicalensis* georgi, induces apoptosis in human colon cancer cells and prevents AOM/DSS-induced colon cancer in mice. *International Journal of Oncology, 43*(5), 1652-1658.

Kim, K. C., Kim, J. S., Ah Kang, K., Kim, J. M. & Won, H. J. (2010). Cytoprotective effects of catechin 7-O-β-D glucopyranoside against mitochondrial dysfunction damaged by streptozotocin in RINm5F cells. *Cell Biochemistry & Function, 28*(8), 651-660.

Kim, W.-J., Veriansyah, B., Lee, Y.-W., Kim, J. & Kim, J.-D. (2010). Extraction of mangiferin from Mahkota Dewa (*Phaleria macrocarp*a) using subcritical water. *Journal of Industrial & Engineering Chemistry, 16*(3), 425-430.

Klaunig, J. E., Kamendulis, L. M. & Hocevar, B. A. (2010). Oxidative stress and oxidative damage in carcinogenesis. *Toxicologic Pathology, 38*(1), 96-109.

Koukourakis, M, Giatromanolaki, A & Sivridis, E. (2009). Colorectal cancer: Lactate dehydrogenase (LDH) activity as a prognostic marker. In hayat, M. A. (Ed.). *Colorectal Cancer* (241-253). Springer Netherlands, 2009.

Kozicky, L., Jen, R. & Sly, L. (2013). Inflammation plays multiple roles in colorectal cancer. *Journal of Gastrointestinal & Digestive System, 3*(127), 2.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., et al. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine, 27*(2-3), 95-125.

Kundu, T., Dey, S., Roy, M., Siddiqi, M. & Bhattacharya, R. (2005). Induction of apoptosis in human leukemia cells by black tea and its polyphenol theaflavin. *Cancer Letters, 230*(1), 111-121.

Kwon, J.-H., Kim, S.-B., Park, K.-H. & Lee, M.-W. (2011). Antioxidative and anti-inflammatory effects of phenolic compounds from the roots of *Ulmus macrocarpa*. *Archives of Pharmacal Research, 34*(9), 1459-1466.

Latham, P., Lund, E. K. & Johnson, I. T. (1999). Dietary n-3 PUFA increases the apoptotic response to 1, 2-dimethylhydrazine, reduces mitosis and suppresses the induction of carcinogenesis in the rat colon. *Carcinogenesis, 20*(4), 645-650.

Lay, M. M., Karsani, S. A., Mohajer, S. & Malek, S. N. A. (2014). Phytochemical constituents, nutritional values, phenolics, flavonols, flavonoids, antioxidant and cytotoxicity studies on *P. macrocarpa* (Scheff.) Boerl fruits. *BMC Complementary & Alternative Medicine, 14*(1), 152.

Lieberman, D. A., Rex, D. K., Winawer ,S. J., Giardiello, F. M., Johnson, D. A. & Levin, T. R. (2012). Guidelines for colonoscopy surveillance after screening and polypectomy: A consensus update by the US multi-society task force on colorectal cancer. *Gastroenterology, 143*(3), 844-857

.

Lipkin, M. (1988). Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Research, 48*(2), 235-245.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT ct method, *Methods, 25*(4), 402-408.

Lu, Y., Jiang, F., Jiang, H., Wu, K., Zheng, X., Cai, Y., et al. (2010). Gallic acid suppresses cell viability, proliferation, invasion and angiogenesis in human glioma cells. *European Journal of Pharmacology, 641*(2), 102-107.

Luerang, A., Thammasarn, K., Sittiwet, C., Naowaratwattana, W., Chaichanadee, S., Puangpronpitag, D., et al. (2010). Evaluation of nutritional value and antioxidative properties of the medicinal plant *G. procumbens* extract. *Asian Journal of Plant Sciences, 9*(3), 146-151.

Lumachi, F., Marino, F., Chiara, G. B. & Basso, S. M. M. (2012). 346. Usefulness of Cathepsin D, Ki-67, and PCNA in patients with colorectal cancer and lymph node metastasis. *European Journal of Surgical Oncology, 38*(9), 838.

Lynch, P. M., Morris, J. S., Ross, W. A., Rodriguez-Bigas, M. A., Posadas, J., Khalaf, R. & Shureqi, I. (2013). Global quantitative assessment of the colorectal polyp burden in familial adenomatous polyposis by using a web-based tool. *Gastrointestinal Endoscopy, 77*(3), 455-463.

Magaji, B. A., Moy, F. M., Roslani, A. C. & Law, C. W. (2014). Descriptive epidemiology of colorectal cancer in University Malaya Medical Centre, 2001 to 2010. *Asian Pacific Journal of Cancer Prevention, 15*(15), 6059-6064.

Mahmood, A., Mariod, A. A., Al-Bayaty, F. & Abdel-Wahab, S. I. (2010). Anti-ulcerogenic activity of *G. procumbens* leaf extract against experimentally-induced gastric lesions in rats. *Journal of Medicinal Plant Research, 4*(8), 685-691.

Markowitz, S. D. & Bertagnolli, M. M., (2009) .Molecular origins of cancer: Molecular basis of colorectal cancer. *New England Journal of Medicine, 361*(25), 2449-2460.

Matés, J. M., Segura, J. A., Alonso, F. J. & Márquez, J. (2012). Oxidative stress in apoptosis and cancer: An update. *Archives of Toxicology, 86*(11), 1649-1665.

McCubrey, J. A., Abrams, S., Steelman, L. S. & Chappell, W. H. (2008). B7. Molecular mechanisms in cancer. *Journal of Clinical Oncology, 26*, 542-548.

McLellan, E. A., Medline, A. & Bird, R. P. (1991). Does response and proliferative characteristics of aberrant crypt foci: Putative preneoplastic lesions in rat colon. *Carcinogenesis, 12*(11), 2093-2098.

Minhajuddin, M., Beg, Z. H. & Iqbal, J. (2005). Hypolipidemic and antioxidant properties of tocotrienol rich fraction isolated from rice bran oil in experimentally-induced hyperlipidemic rats. *Food & Chemical Toxicology, 43*(5), 747-753.

Miyazawa, M., Ishii, T., Yasuda, K., Noda, S., Onouchi, H., Hartman, P. S., et al. (2009). The role of mitochondrial superoxide anion (O_2-) on physiological aging in C57BL/6J mice. *Journal of Radiation Research, 50*(1), 73-82.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1-2), 55-63.

Müller, M. F., Florian, S., Pommer, S., Osterhoff, M., Esworthy, R. S., Chu, F.-F., et al. (2013). Deletion of glutathione peroxidase-2 inhibits azoxymethane-induced colon cancer development. *PloS One, 8*(8), e0072055.

Muzny, D. M., Bainbridge, M. N., Chang, K., Dinh, H. H., Jennifer, A., Fowler, D. G., et al, (2012). Comprehensive molecular characterization of human colon and rectal cancer. *Nature, 487*(7407), 330-337.

Natarajan, V., Venugopal, P. & Menon, T. (2003). Effect of *Azadirachta indica* (neem) on the growth pattern of dermatophytes. *Indian Journal of Medical Microbiology, 21*(2), 98-101.

Newby, Z., Lee, T. T., Morse, R. J., Liu, Y., Liu, L., Venkatraman, P., et al. (2006) The role of protein dynamics in thymidylate synthase catalysis: Variants of conserved 2'-deoxyuridine 5'-monophosphate (dUMP)-binding Tyr-261. *Biochemistry, 45*(24), 7415-7428.

Nisa, F., Hermawan, A., Murwanti, R. & Meiyanto, E. (2012). Antiproliferative effect of *G. procumbens* (lour.) Merr. Leaves etanolic extract on 7, 12-dimethylbenz (a) antracene induced male rat liver. *Advanced Pharmaceutical Bulletin, 1*(2), 99-106.

Noordhuis, P., Holwerda, U., Van der Wilt, C., Van Groeningen, C., Smid, K ,.Meijer, S., et al. (2004). 5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibition of human colorectal cancers. *Annals of Oncology, 15*(7), 1025-1032.

Nurulita, N. A., Meiyanto, E., Sugiyanto, S., Matsuda, E. & Kawaichi, M. (2012). The ethyl acetate fraction of *G. procumbens* sensitizes widr colon cancer cell line against 5-fluorouracil but shows antagonism with cisplatin. *International Journal of Phytomedicine, 3*(3), 392-405.

Ochiai, M., Ubagai, T., Kawamori, T ,.Imai, H., Sugimura, T. & Nakagama, H. (2001). High susceptibility of Scid mice to colon carcinogenesis induced by azoxymethane indicates a possible caretaker role for DNA-dependent protein kinase. *Carcinogenesis, 22*(9), 1551-1555.

OECD Guideline for testing of chemicals (2001). Acute oral toxicity-acute toxic class method, guideline no. 423. *adopted December 2001. Organisation for Economic Cooperatio and Development, Rome, Italy*. pp. 1–24. Doi:10.1787/ 9789264078413-en

Ouyang, L., Luo, Y., Tian, M., Zhang, S. Y., Lu, R., Wang, J. H., et al. (2014). Plant natural products: From traditional compounds to new emerging drugs in cancer therapy. *Cell Proliferation, 47*(6), 506-515.

Pal, D., Saha, S. & Singh, S. (2012). Importance of pyrazole moiety in the field of cancer. *International Journal of Pharmacy & Pharmaceutical Sciences, 4*(2), 98-104.

Pan, M. H. & Ho, C. T. (2008). Chemopreventive effects of natural dietary compounds on cancer development. *Chemical Society Reviews, 37*(11), 2558-2574.

Pancione, M., Remo, A. & Colantuoni, V. (2012). Genetic and epigenetic events generate multiple pathways in colorectal cancer progression. *Pathology Research International, 201*2(509348),.8.

Panis, C., Herrera, A., Victorino, V., Campos, F., Freitas, L., De Rossi, T., et al. (2012). Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. *Breast Cancer Research & Treatment, 133*(1), 89-97.

Parker, W. B. & Cheng, Y. C. (1990). Metabolism and mechanism of action of 5-fluorouracil. *Pharmacology & Therapeutics, 48*(3), 381-395.

Paulsen, J. E., Lberg, E. M., lstrn, H. B., Knutsen, H., Steffensen, I.-L. & Alexander, J. (2005). Flat dysplastic aberrant crypt foci are related to tumorigenesis in the colon of azoxymethane-treated rat. *Cancer Research, 65*(1), 121-129.

Power, E., Miles, A ,. Wagner, C.V., Robb, K. & Wardle, J. (2009). Uptake of colorectal cancer screening: System, provider and individual factors and strategies to improve participation. *Future Oncology, 5*(9), 1371-1388.

Pretlow, T. P., Ann O'Riordan, M., Pretlow, T. G. & Stellato, T. A. (2004). Aberrant crypts in human colonic mucosa: Putative preneoplastic lesions. *Journal of Cellular Biochemistry, 50*(S16G), 55-62.

Pretlow, T. P., Brasitus, T. A., Fulton, N .C., Cheyer, C. & Kaplan, E. L. (1993). K-ras mutations in putative preneoplastic lesions in human colon. *Journal of the National Cancer Institute, 85*(24), 2004-2007.

Prochaska, H. J., Santamaria, A. B. & Talalay, P. (1992). Rapid detection of inducers of enzymes that protect against carcinogens. *Proceedings of the National Academy of Sciences, 89*(6), 2394-2398.

Qureshi, A. A., Mo, H., Packer, L. & Peterson, D. M. (2000). Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties. *Journal of Agricultural & Food Chemistry, 48*(8), 3130-3140.

Rimbach, G., Minihane, A. M., Majewicz, J., Fischer, A., Pallauf, J., Virgli, F., et al. (2002). Regulation of cell signalling by vitamin E .*Proceedings of the Nutrition Society, 61*(04), 415-425

.

Riwanto, I., Budijitno, S., Dharmana, E., Handojo, D., Prasetyo, S. A., Eko, A., et al. (2011). Effect of *P. macrocarpa* supplementation on apoptosis and tumor growth of C3H mice with breast cancer under treatment with adriamycin-cyclophosphamide. *International Surgery, 96*(2), 164-170.

Roberts, S. A., Hyatt, D. C., Honts, J. E., Changchien, L., Maley, G. F., Maley, F., et al. (2006). Structure of the Y94F mutant of *Escherichia coli* thymidylate synthase. *Acta Crystallographica Section F: Structural Biology & Crystallization Communications, 62*(9), 840-843.

Robles, F. E., Zhu, Y., Lee, J., Sharma, S. & Wax, A. (2010). Detection of early colorectal cancer development in the azoxymethane rat carcinogenesis model with fourier domain low coherence interferometry. *Biomedical Optics Express, 1*(2), 736-745.

Saiman, M. Z., Mustafa, N. R., Schulte, A. E., Verpoorte, R. & Choi, Y. H. (2012). Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of *G. procumbens*. *Plant Cell, Tissue & Organ Culture, 109*(3), 465-475.

San Miguel, S. M., Opperman, L. A. & Allen, E. P. (2011). Reactive Oxygen Species and antioxidant defense mechanisms in the oral cavity: A literature review. *Compendium of Continuing Education in Dentistry, 32*(1),e10.

Saslow, D., Solomon, D., Lawson, H. W., Killackey, M., Kulasingam, S. L., Cain, J., et al. (2012). American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer. *CA: A Cancer Journal for Clinicians, 62*(3), 147-172.

Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., et al. (1988). Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Research, 48*(17), 4827.

Sesink, A. L., Termont, D. S., Kleibeuker, J. H. & Van der Meer, R. (2001). Red meat and colon cancer: Dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium. *Carcinogenesis, 22*(10), 1653-1659.

Shah, S. A., Neoh, H., Rahim, S., Azhar, Z. I., Hassan, M. R., Safian, N., et al. (2014). Spatial analysis of colorectal cancer cases in Kuala Lumpur. *Asian Pacific Journal of Cancer Prevention, 15*(3), 1149-1154.

Shaw, J., Sicree, R. & Zimmet, P. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research & Clinical Practice, 87*(1), 4-14.

Shin, H.-R., Carlos, M. C. & Varghese, C. (2012). Cancer control in the asia pacific region: Current status and concerns. *Japanese Journal of Clinical Oncology*, Doi:10.1093/jjco/hys077.

Shwter, A. N., Abdullah, N .A., Alshawsh, M. A., Alsalahi, A., Hajrezaei, M., Almaqrami, A. A., et al. (2014). Chemoprevention of colonic aberrant crypt foci by *G. procumbens* in rats. *Journal of Ethnopharmacology, 3*(151), 1194-1201.

Siegel, R., Ma, J., Zou, Z. & Jemal, A (2014). Cancer statistics, 2014. CA*: A Cancer Journal for Clinicians, 64*(1), 9-29.

Singh, A. & Singh, D. (2001). Molluscicidal activity of *Lawsonia inermis* and its binary and tertiary combinations with other plant derived molluscicides. *Indian Journal of Experimental Biology, 39*(3), 263.

Sotelo-Mundo, R. R., Changchien, L., Maley, F. & Montfort, W. R. (2006). Crystal structures of thymidylate synthase mutant R166Q: Structural basis for the nearly complete loss of catalytic activity. *Journal of Biochemical & Molecular Toxicology, 20*(2), 88-92.

Sporn, M. B. (1996). The war on cancer. *The Lancet, 347*(9012), 1377-1381.

Sporn, M. B. & Roberts, A. B. (1984). Role of retinoids in differentiation and carcinogenesis. *Journal of the National Cancer Institute, 73*(6), 1381-1387.

Steinberg, D. (2009). The LDL modification hypothesis of atherogenesis: An update. *Journal of Lipid Research, 50*, S376-S381.

Steward, W. & Brown, K. (2013). Cancer chemoprevention: A rapidly evolving field. *British Journal of Cancer, 109*(1), 1-7.

Sung, J., Lau, J., Young, G. P., Sano, Y., Chiu, H., Byeon, J., et al. (2008). Asia pacific consensus recommendations for colorectal cancer screening. *Gut, 57*(8), 1166-1176.

Sung, J., Ng, S., Chan, F., Chiu, H., Kim, H., Matsuda, T., et al. (2015). An updated Asia pacific consensus recommendations on colorectal cancer screening. *Gut, 64*(1), 121-132.

Sutiono, A. B., Faried, A., Arifin, M., Wiriadisastra, K. & Ohta, T. (2008). Herbal medicine isolation, *P. macrocarpa* for primary glioblastoma multiforme cells. *Annals of Epidemiology, 18*(9), 718.

Takuji, T. (2009). Colorectal carcinogenesis: Review of human and experimental animal studies. *Journal of Carcinogenesis, 8*(1), 5.

Tanaka, T., Kataoka, M., Tsuboi, N. & Kouno, I. (2000). New monoterpene glycoside esters and phenolic constituents of *Paeoniae Radix*, and increase of water solubility of proanthocyanidins in the presence of paeoniflorin. *Chemical & Pharmaceutical Bulletin-Tokyo-, 48*(2), 201-207.

Tanaka, T., Kohno, H. & Mori, H. (2001). Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. *Asian Pacific Journal of Cancer Prevention, 2*(3), 165-177.

Teiten, M.-H., Gaascht, F., Dicato, M. & Diederich, M. (2013). Anticancer bioactivity of compounds from medicinal plants used in European medieval traditions. *Biochemical Pharmacology, 86*(9), 1239-1247.

Tew, K. D., Manevich, Y., Grek, C., Xiong, Y., Uys, J. & Townsend, D. M. (2011). The role of glutathione S-transferase P in signaling pathways and S-glutathionylation in cancer. *Free Radical Biology & Medicine, 51*(2), 299-313.

Thapa, D. & Ghosh, R. (2012). Antioxidants for prostate cancer chemoprevention: Challenges and opportunities. *Biochemical Pharmacology, 83*(10), 1319-1330.

Tosun, M., Ercisli, S., Sengul, M., Ozer, H., Polat, T. & Ozturk, E. (2009). Antioxidant properties and total phenolic content of eight Salvia species from Turkey. *Biological Research, 42*(2), 175-181.

Trust, L. & Surrey, U. (2010). Colorectal cancer. *The Lancet, 375*, 1030-1047.

Tsao, A. S., Kim, E. S. & Hong, W. K. (2004). Chemoprevention of cancer. *CA: A Cancer Journal for Clinicians, 54*(3), 150-180.

Tudek, B., Bird ,R. P. & Bruce, W. R. (1989). Foci of aberrant crypts in the colons of mice and rats exposed to carcinogens associated with foods. *Cancer Research, 49*(5), 1236-1240.

Valadez-Vega, C., Delgado-Olivares, L., González, J. A. M., García, E. A., Ibarra, J. R. V., Moreno, E. R., et al. (2013). The role of natural antioxidants in cancer disease. Oxidative stress and chronic degenerative diseases-a role for antioxidants, JA Morales-Gonzalez (Ed.)*.–Rijeka, Croatia: InTech*, 391-418.

Valko, M., Rhodes, C., Moncol, J., Izakovic, M. & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), 1-40.

Van der Meer-van Kraaij, C., Kramer, E., Jonker-Termont, D., Katan, M., Van der Meer, R. & Keijer, J. (2005). Differential gene expression in rat colon by dietary heme and calcium. *Carcinogenesis, 26*(1), 73-79

.

Veerakumar, K., Govindarajan, M. & Rajeswary, M .(2013) .Green synthesis of silver nanoparticles using *Sida acuta* (Malvaceae) leaf extract against *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Diptera: Culicidae). *Parasitology Research, 112*(12), 4073-4085.

Vihakas, M., Tähtinen, P., Ossipov, V. & Salminen, J.-P. (2012). Flavonoid metabolites in the hemolymph of european pine sawfly (*Neodiprion sertifer*) larvae. *Journal of Chemical Ecology, 38*(5), 538-546.

Vincent, J., Mignot, G., Chalmin, F., Ladoire, S., Bruchard, M., Chevriaux, A., *et al.* (2010). 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell–dependent antitumor immunity. *Cancer Research, 70*(8), 3052-3061.

Vogelstein, B. & Kinzler, K. W. (1993). The multistep nature of cancer. *Trends in Gentatics,9(4),* 138-141.

Wargovich, M. J., Brown, V. R. & Morris, J. (2010). Aberrant crypt foci: The case for inclusion as a biomarker for colon cancer. *Cancers, 2(3),* 1705-1716.

Watson, A. J. M. & Collins, P. D. (2011). Colon cancer: A civilization disorder. *Digestive Diseases, 29*(2), 222-228.

Winarno, H. (2010). Benzophenone glucoside isolated from the ethyl acetate extract of the bark of mahkota dewa [*Phaleria macrocarpa* (Scheff.) Boerl.] and its inhibitory activity on leukemia L1210 cell line. *Indonesian Journal of Chemistry, 9*(1), 142-145.

Wong, R. (2011). Apoptosis in cancer: From pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research, 30*(1), 1-14.

Yadav, D., Chandra, R., Saxena, R., Agarwal, D., Agarwal, M., Ghosh, T., et al. (2011). Glutathione-S-transferase M1 and T1 genes and gastric cancer: A case control study in North Indian population. *Gene, 487*(2), 166-169.

Yam, M. F., Sadikun, A., Ahmad, M., Akowuah, G. A. & Asmawi, M. Z. (2009). Toxicology evaluation of standardized methanol extract of *G. procumbens*. *Journal of Ethnopharmacology, 123*(2), 244-249.

Yamashita, N., Minamoto, T., Onda, M. & Esumi, H. (1994). Increased cell proliferation of azoxymethane-induced aberrant crypt foci of rat colon. *Cancer Science, 85*(7), 692-698.

Yin, S-Y., Wei, W.-C., Jian, F.-Y. & Yang.N-S. (2013). Therapeutic applications of herbal medicines for cancer patients. *Evidence-Based Complementary & Alternative Medicine,* Doi:10.1155/2013/302426.

Yosie, A., Effendy, M., Sifzizul, T. & Habsah, M. (2011). Antibacterial, radical-scavenging activities and cytotoxicity properties of *P. macrocarpa* (Scheff.) Boerl leaves in HEPG2 cell lines. *Journal of Pharmaceutical Sciences & Research, 2*(7), 1700-1706.

You, B. R., Moon, H. J., Han, Y. H. & Park, W. H. (2010). Gallic acid inhibits the growth of HeLa cervical cancer cells via apoptosis and/or necrosis. *Food & Chemical Toxicology, 48*(5), 1334-1340.

Yusoff, H. M., Daud, N., Noor, N. M. & Rahim, A. A. (2012). Participation and barriers to colorectal cancer screening in Malaysia .*Asian Pacific Journal of Cancer Prevention, 13*(8), 3983-3987.

Zhang, Y.-B., Xu, X.-J. & Liu, H.-M. (2006). Chemical constituents from Mahkota dewa. *Journal of Asian Natural Products Research, 8*(1-2), 119-123.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

1- Shwter, A. N., Abdullah, N .A., Alshawsh, M. A., Alsalahi, A., Hajrezaei, M., Almaqrami, A. A., et al. (2014). Chemoprevention of colonic aberrant crypt foci by *G. procumbens* in rats. *Journal of Ethnopharmacology, 3*(151), 1194-1201.

2- Shwter, A. N, Abdullah, N .A, Nawal., M A Abdulla, Shaden AM Khalifa, and Mahmood A. Abdulla. (2016). Chemopreventive effect of *Phaleria macrocarpa* on colorectal cancer aberrant crypt foci in *vivo*. *Journal of Ethnopharmacology,* (193), 195-206.

3-Chemopreventive potential of Mahkota Dewa (*Phaleria macrocarpa*) against cancer lesions in colon cancer of rats(NATPRO4 Conference in Chiang Mai, Thailand during November 28-30, 2012).

4- Chemopreventive potential of *Gynura proc*umbens against cancer lesions in colon cancer of rats. International Conference on Life Science & Biological Engineering 15-17, March, 2013 Tokyo, Japan.