EVALUATION OF POTENTIAL ROLE OF *CATHARANTHUS ROSEUS* AND *ACANTHUS ILICIFOLIUS* IN THE PREVENTION OF AZOXYMETHANE-INDUCED ABERRANT CRYPT FOCI IN RAT'S COLON

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To my friends and colleagues

ABSTRACT

Catharanthus roseus extract is traditionally used to treat different diseases including cancer in Southeast Asia. *Acanthus ilicifolius*, is a mangrove medicinal plant, and is widely used by the local inhabitants of the Sundarbans (India) to treat a variety of diseases. The aim of current research is to assess the chemoprotective outcomes of ethanolic extracts of *C. roseus* and *A. ilicifolius* against azoxymethane (AOM) induced colonic aberrant crypt foci (ACF) in rats. The bioactivities of the crude ethanol extracts of *C. roseus* and *A. ilicifolius* were investigated in both of their *in vitro* antioxidant and *in vivo* oral toxicity tests. Antioxidant activity was evaluated by different assays, including: 2, 2-diphenlyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), total phenolic content (TPC) and total flavonoids content (TFC) assays. Five groups of rats, normal control group were given subcutaneous injection of normal saline, once weekly for 2 weeks. The AOM control group, reference group and treatment groups were given subcutaneous injection of AOM, 15 mg/kg body weight, once weekly for 2 weeks each. Normal and AOM control groups continued on 10% Tween 20 feeding for 8 weeks. Reference group continued on 35 mg/kg of 5-Fluorouracil intraperitoneal injection once weekly for 8 weeks, and treatment groups continued on 250 and 500 mg/kg *C. roseus* and *A. ilicifolius* extracts feeding for 8 weeks respectively. After 60 days treatment, all rats were sacrificed. Colons were evaluated grossly, histopathology and immunohistochemistry staining for ACF. Also body weight, biochemical parameters, catalase (CAT), superoxide dismutase (SOD) activities and lipid peroxidation (MDA) level in the colon homogenates were tested. Gene expressions also were studied for p53, Bax and Bcl2 by real time PCR. In addition, the crude extracts of *C. roseus* and *A. ilicifolius* and their isolated fractions were investigated against HT29 and (CCD_{841}) human cell line cell line, and the

percentage of inhibition by using MTT assay was assessed. Our results revealed that, extracts of *C. roseus* and *A. ilicifolius* exhibited high free radical scavenging activity at the same time, showed high total phenolic and flavonoids contents. Acute toxicity test could not demonstrate any morbidity as well as mortality. Rats treated with *C. roseus* and *A. ilicifolius* showed significantly decreased total colonic ACF formation by 61 % to 65 % and 53 % to 65 % respectively when compared with AOM control group and they were effectively comparable to that of 5-Fluorouracil, a standard cemoprotective agent. Histopathological study confirmed the result. In immunohistochemical staining, proliferating nuclear cell antigen (PCNA) positive cells were significantly higher in AOM control group than *C. roseus* and *A. ilicifolius*-treated groups. *C .roseus* showed significant increase in total protein, albumin, hemoglobin, white blood cell, SOD and CAT, and significant decrease in MDA, LDH and urea when contrasted with AOM control group. While *A. ilicifolius* showed significant increase in total protein, albumin, Hb, SOD and CAT, and significant decreased in MDA and urea when compared with AOM control group. RT-PCR showed that *C. roseu*s and *A. ilicifolius* caused change in regulation of gene expression of p53, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax and inverting the Bcl-2/Bax ratio to <1. Moreover, the results observed from the MTT assay showed that *C. roseus* and *A. ilicifolius* plants extract stimulated the normal cell line (CCD_{841}) proliferation *in vitro* in a dose-dependent manner, at the same time both extracts have remarkable activity against cancer cell line (HT29). The active constituents of the fractions that are proved to have chemoprotective activity in *C. roseus* are vindoline, vindolidine and ajmalicine while in *A. ilicifolius* is stigmasterol. The results of the current study showed that *C. roseus* and *A. ilicifolius* may be an effective herbal and efficient remedial for chemical-induced against cancer in rats colon.

ABSTRAK

Catharanthus roseus digunakan secara tradisional untuk merawat penyakit yang berbeza termasuk kanser di Asia Tenggara. *Acanthus ilicifolius*, adalah tumbuhan perubatan bakau, dan digunakan secara meluas oleh penduduk tempatan Sundarbans (India) untuk merawat pelbagai penyakit. Tujuan kajian semasa adalah untuk menilai hasil chemoprotective ekstrak ethanolic *C. roseus* dan *A. ilicifolius* terhadap azoxymethane (AOM) disebabkan kolon menyeleweng kubur tumpuan (ACF) pada tikus. The bioactivities daripada ekstrak etanol mentah *C. roseus* dan *A. ilicifolius* disiasat dalam kedua-dua mereka antioksida in *vitro* dan dalam ujian Ketoksikan oral *vivo*. Aktiviti antioksida yang telah dinilai oleh ujian yang berbeza, termasuk: 2, 2-diphenlyl-1-picrylhydrazyl (DPPH), kuasa penurunan ferik (FRAP), jumlah kandungan fenolik (TPC) dan flavonoid jumlah kandungan (TFC) ujian. Lima kumpulan tikus, kumpulan kawalan normal diberikan suntikan subkutaneus masin biasa, sekali seminggu selama 2 minggu. Kumpulan kawalan AOM, kumpulan rujukan dan kumpulan rawatan telah diberikan suntikan subkutaneus AOM, 15 mg / kg berat badan, sekali seminggu selama 2 minggu setiap. Kumpulan kawalan normal dan AOM berterusan 10% Tween 20 makan selama 8 minggu. Kumpulan rujukan berterusan 35 mg / kg 5-Fluorouracil suntikan intraperitoneal sekali seminggu selama 8 minggu, dan kumpulan rawatan diteruskan pada 250 dan 500 mg / kg *C. roseus* dan *A. ilicifolius* ekstrak makan untuk 8 minggu masing-masing. Selepas rawatan 60 hari, semua tikus telah dikorbankan. Titik bertindih dinilai terlalu, histopatologi dan mengotorkan Immunohistokimia untuk ACF. Juga berat badan, parameter biokimia, katalase (CAT), superoksida dismutase (SOD) aktiviti dan lipid peroksidaan peringkat (MDA) dalam homogenates kolon telah diuji. Ungkapan gen juga dikaji untuk p53, Bax dan Bcl2 oleh masa PCR sebenar. Di samping itu, ekstrak mentah *C. roseus* dan *A. ilicifolius* dan pecahan terpencil mereka telah disiasat terhadap HT29 dan (CCD_{841}) sel manusia garis sel talian, dan peratusan perencatan dengan menggunakan MTT assay telah dinilai. Keputusan kami menunjukkan bahawa, ekstrak *C.*

roseus dan *A. ilicifolius* dipamerkan aktiviti percuma yang tinggi memerangkap radikal pada masa yang sama, menunjukkan jumlah fenolik yang tinggi dan flavonoid kandungan. Ujian ketoksikan akut tidak boleh menunjukkan mana-mana morbiditi serta kematian. Tikus yang dirawat dengan *C. roseus* dan *A. ilicifolius* menunjukkan ketara menurun jumlah pembentukan ACF kolon sebanyak 61% kepada 65% dan 53% kepada 65% masing-masing berbanding dengan kumpulan kawalan AOM dan mereka berkesan setanding dengan 5 Fluorouracil, standard ejen cemoprotective. Kajian histopatologi mengesahkan keputusan. Dalam mengotorkan immunohistochemical, membiak antigen sel nuklear (PCNA) sel-sel yang positif adalah lebih tinggi dalam kumpulan kawalan AOM daripada *C. roseus* dan *A. ilicifolius* dirawat. *C. roseus* menunjukkan peningkatan ketara dalam jumlah protein, albumin, hemoglobin, sel darah putih, SOD dan CAT, dan penurunan ketara dalam MDA, LDH dan urea apabila dibandingkan dengan kumpulan kawalan AOM. Walaupun *A. ilicifolius* menunjukkan peningkatan ketara dalam jumlah protein, albumin, Hb, SOD dan CAT, dan ketara menurun dalam MDA dan urea berbanding dengan kumpulan kawalan AOM. RT-PCR menunjukkan bahawa *C. roseus* dan *A. ilicifolius* disebabkan perubahan dalam peraturan gen p53, anti-apoptotic protein Bcl-2 dan pro-apoptotic protein Bax dan menyongsang nisbah Bcl-2/Bax hingga <1. Selain itu, keputusan dilihat dari assay MTT menunjukkan bahawa *C. roseus* dan A. tumbuhan ilicifolius ekstrak dirangsang garis sel normal (CCD_{481}) percambahan in vitro dengan cara yang bergantung kepada dos, pada masa yang sama kedua-dua ekstrak mempunyai aktiviti luar biasa terhadap sel kanser line (HT29). Juzuk aktif pecahan yang terbukti mempunyai aktiviti chemoprotective di *C. roseus* adalah vindoline, vindolidine dan ajmalicine manakala di *A. ilicifolius* adalah stigmasterol. Keputusan kajian semasa menunjukkan bahawa *C. roseus* dan *A. ilicifolius* mungkin merupakan pemulihan yang berkesan herba dan berkesan untuk bahan kimia disebabkan daripada kanser pada tikus kolon.

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LIST OF SYMBOLS & ABBREVIATIONS

Abbreviation	Description
%	percentage
/	Divide by
˚C	Degree Celsius
<	Less than
±	Plus minus
A. ilicifolius	*Acanthus ilicifolius*
ACF	Aberrant crypt foci
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AOM	Azoxymethane
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BCAC	Beta-Catenin-Accumulated Crypts
C. roseus	*Catharanthus roseus*
CAT	Catalase
CYP2E1	Cytochrome P450 2E1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	α, α-diphenyl-β-picryl-hydrazyl radical scavenging assay
et al.	and other people
EtOH	Ethanol
FBS	Fetal bovine serum
FC	Flavonoid content
FRAP	Ferric reducing antioxidant power
H&E stain	Haematoxylin-eosin stain
HD	High dose
HPLC	High performance liquid chromatography
I.P	Intraperitoneal
Kg	Kilogram
LC-MS	Liquid chromatography-mass spectrometry
LD	Low dose
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDF	Mucin Developed Foci
mg	Milligram
Min	Minute/s
ml	Millilitre
mM	Micromole
Mm	Millimetre
mmol	Millimole

MTT	3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide assay
NCCLS	National committee for clinical laboratory standards
nm	nanometre
PBS	Phosphate buffer saline
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S.C	Subcutaneous
SD	Sprague Dawley
SEM	Standard error of the mean
SOD	Superoxide Dismutase
TBARS	Thiobarbituric acid reactive substance
T.P	Total protein
TPC	Total phenolic content
WHO	World Health Organization
µl	Microliter
µm	Micrometre

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CHAPTER I

INTRODUCTION

1.1. Introduction

Colorectal cancer (CRC) is one of the most prevalent causes of cancer deaths in developed countries, including the United States (Hisamuddin & Yang, 2006). The etiology of colon cancer is multifactorial including familial, environmental and dietary agents. Despite several advancements in the understanding of the processes involved in carcinogenesis the currently available therapies that include surgery, radiation and chemotherapeutic drugs still only have limited success for treating the advanced stages of colon cancer (Boursi & Arber, 2007).

To that end nutritional intervention is an effective and promising complimentary strategy for controlling the incidence of colon cancer (Hoensch & Kirch, 2005). Several epidemiological and experimental studies have indicated that plant products exert a protective influence against this disease, and beneficial effects may be partly attributable to polyphenolic phytochemicals, which have a wide range of pharmacological properties (Higdon *et al.*, 2007). Moreover, the search for putative chemopreventive compounds with minimal toxicity raises particular interest in regards to phytochemicals. Colorectal carcinogenesis provides a huge screen including years approaches (Boursi & Arber, 2007). With the aid of efficient preclinical designs it is possible to assess the effect of potential chemopreventive agents on the carcinogenic process. These preclinical designs consist of the induction of colon carcinogenesis in rats by the administration of the chemical carcinogen azoxymethane (AOM) (Bird & Good, 2000).

AOM and its precursor compound dimethylhydrazine are alkylating agents that cause DNA mutation by binding methyl or alkyl groups to guanine residues, resulting in

G to A transition mutations (Dipple, 1995). Aberrant crypt foci (ACF) are putative pre-neoplastic lesions in the colons of both animals and humans (Cheng & Lai, 2003). They serve as intermediate biomarkers to rapidly evaluate the chemopreventive potential of several agents, including naturally occurring agents against colon cancer (Corpet & Taché, 2002). Aberrant crypt foci (ACF) are hyper proliferative lesions located in the human colon as well as carcinogen-treated laboratory creatures that share ordinary characteristics with colon tumours (Bird, 1995). In addition ACF are recognized as colonic carcinogenesis biomarkers as well as alleged precursors of colon cancers (Takayama *et al.*, 1998).

ACFs are monoclonal gatherings of strange crypts always formed in a dose-dependent way in reaction to carcinogen exposure (Bird, 1987). Within 6–8 hours of post exposure to AOM, crypt progenitor cells undergo apoptosis in response to DNA damage. Progenitor cells that avoid apoptosis then start a proliferative response 48–72 hours afterwards (Hirose *et al.*, 1996). These foci of aberrant crypts started as monoclonal (Ponder & Wilkinson, 1986) and arise by a process of incomplete crypt fissioning (Siu *et al.*, 1999).

Catharanthus roseus (L) G. Don. (Family: Apocynaceae) is a plant whose leaves have been traditionally used for the treatment of cancer, Hodgkin's disease, leukemia, rhrumatism, enorrhagia, indigestion, dyspepsia, dysmenorrheal, hypertension, diabetes, menstrual disorders, malaria and skin diseases (Holdsworth, 1990; Natarajan *et al.*, 2012). This plant has several therapeutic properties including wound healing (Nayak & Pinto Pereira, 2006), anticancer (Lu *et al.*, 2003), antihyperglycemic (Rasineni *et al.*, 2010a), antibacterial (Ramya, 2008) along with biochemical specialization (Murata *et al.*, 2008) such as enhancing the kidney and liver functions (Adekomi, 2010). *C. roseus* has a variety of medicinal properties, such as leukaemia treatment, the cure of Hodgkin infection (Jaleel *et al.*, 2007), hypoglycaemia (Kar *et al.*, 2003), wound healing (Nayak

& Pereira, 2006), significant reduction of protein levels, lipid peroxidation of liver (Iweala & Okeke, 2005), hypolipidaemic properties (Rasineni *et al.*, 2010b), antioxidant activity (Rasool *et al.*, 2011) and antibacterial efficacy (Raza *et al.*, 2009). Phytochemically, *C. roseus* contains serpentine, vindoline, catharanthine, tabersonine, vincristine, vinblastine, ajmalicine, tryptamine, tryptophan and secologanine (Tikhomiroff & Jolicoeur, 2002).

Acanthus ilicifolius Linn (Family: Acanthaceae) is a spiny herb of the mangrove species, distributed widely throughout Southeast Asia where it is used locally for the treatment of cancers, kidney stones, snakebites, rheumatism, paralysis, ulcers, wound healing and asthma (Wöstmann & Liebezeit, 2008a). The leaves of *A. ilicifolius* has been reported to exhibit hepatoprotective (Babu *et al.*, 2001) and tumour reducing activities (Babu *et al.*, 2002). Furthermore antioxidant, hepatoprotective, leishmanicidal, tumour reducing and anticancer activities of various extracts of *A. ilicifolius* have been reported (Babu *et al.*, 2001, 2002). Plant derived compounds such as flavonoids (Osawa *et al.*, 1990), terpenes and alkaloids (Di Carlo *et al.*, 1999) have pharmacological properties including cancer chemopreventive effects and cytotoxicity (Roja & Heble, 1994). The phytochemical literature reveals the presence of 2-benzoxazolinone, lignanglucosides, benzoxazinoideglucosides, flavone glycosides and phenylethanoid glycosides (Kanchanapoom *et al.*, 2001a; Wu *et al.*, 2003). To date, there are no studies with *in vivo* (colon) cancer models in evaluating anti-carcinogenic potential of *A. ilicifolius*. Thus, our investigation in well-established models of chemically-induced colon carcinogenesis helped to validate the chemopreventive effects of *A. ilicifolius* in AOM-induced aberrant crypt foci in rat colons as a form of animal modeling.

1.2 Objectives of the study

1.2.1 General objective

The main objective of this study is to evaluate the chemoprotective effects of *C. roseus* and *A. ilicifolius* against AOM-induced aberrant crypts foci in rats, using rats as an experimental model animal. The understanding of these affects should pave the way to providing an alternative therapy for treating colorectal cancer.

1.2.2 Research objectives

1. To determine the antioxidant properties of *C. roseus* and *A. ilicifolius* extracts *in vitro* and *in vivo.*
2. To determine the acute toxicity of the use of *C. roseus* and *A. ilicifolius* crude leaf extracts in rats.
3. To assess the preventative effects of *C. roseus* and *A. ilicifolius* extracts in AOM-induced ACF in rats.
4. To investigate the possible mechanism of action for the preventative effect of *C. roseus* and *A. ilicifolius* extracts on AOM-induced aberrant crypt foci in rats.
5. To determine the cytotoxicity of crude extracts and fractions isolated from *C. roseus* and *A. ilicifolius* extracts by MTT assay.
6. To identify the active constituents of the *C. roseus* and *A. ilicifolius* extracts by HPLC and LC-MS.

CHAPTER II

LITERATURE REVIEW

2.1 Colorectal cancer

Colorectal cancer is the most common gastrointestinal cancer and a major cause of cancer deaths worldwide. It also called large bowel cancer or colon cancer and includes cancerous growths in the colon, appendix and rectum. colorectal cancer is the fourth leading causes of cancer deaths worldwide (Yusoff *et al.*, 2012). The incidents of colorectal cancer is rising in many countries (Béjar *et al.*, 2012), especially in the Asian region (Sung *et al.*, 2005). Around 60% of cases are currently diagnosed in the developed world. In 2008, it is approximated that 1.23 million new cases of colorectal cancer globally were clinically diagnosed, and that it killed 608,000 people (Ferlay *et al.*, 2010). In Malaysia, colorectal cancer is the most common cancer in males and the third most common in females, with the majority of patients being above 50 years old. It also contributes to the highest number of hospital discharges due to neoplasm related problems (Yusoff *et al.*, 2012).

Early diagnosis can reduce mortality due to colorectal cancer and the incidence of malignant neoplasm. Screening through faecal occult blood test (FOBT), sigmoidoscopy and colonoscopy is proven to reduce mortality (Walsh & Terdiman, 2003). Therefore, many countries have produced guidelines to include screening for colorectal cancer and included colorectal cancer screening in their national screening programmes (Power *et al.*, 2009). Despite that, screening activity is still low in many countries, including the developed world. In the Asian region, The Asia Pacific Consensus recommends screening for colorectal cancer from the age of 50 years onwards (Sung *et al.*, 2008). In Malaysia, guidelines on screening for colorectal cancer were introduced in 2001 (Yusoff *et al.*, 2012), recommending annual screening to individuals at average risk using FOBT. However, information on cancer screening participation in Malaysia is lacking, except for cervical cancer screening and breast

cancer screening. Even then, the uptake of Pap smear screening is only 26% and mammography uptake is only 3.8% (Lim, 2002). Considering that cervical cancer screening is more established than colorectal cancer screening, it is expected that the uptake of colorectal cancer screening would be lower. This is probably the reason why the majority of colorectal cancer patients in Malaysia present themselves in an advanced stage of the illness (Goh *et al.*, 2005).

2.1.1 Signs and symptoms of colorectal cancer

Colorectal cancer symptoms and signs depend on the location of tumours in the bowel, and whether it has extended to elsewhere in the body (metastasis). The stellate notice signs include: blood in the stool, worsening constipation, fever, loss of appetite, nausea, weight loss and vomiting in someone over 50 years of age. While anaemia or rectal bleeding are high-risk features in those over the age of 50 (Astin *et al.*, 2011), other usually substantive symptoms including weight loss and change in bowel habits are typically only around if associated with bleeding. Cancers that are pent within the wall of the colon are predominantly curable with surgery, while cancer that has distributed vastly around the body is predominantly not curable via surgery, therefore the focus moves on to extending the person's life via chemotherapy and other treatments designed to generally improve the patients quality of life (Adelstein *et al.*, 2011; Astin *et al.*, 2011).

2.1.2 Cause of colorectal cancer

Colorectal cancer mostly occurs due to increasing age and lifestyle choices, with only a minority of cases related with implied genetic disorders. It typically begins in the lining of the bowel and if untreated, can grow into the muscle layers, and then through the bowel wall. Screening is successful at decreasing the chance of dying from colorectal cancer and is recommended beginning at the age of 50 and continuing until a person is

75 years old. Diagnosis of bowel cancer is usually by sigmoidoscopy or colonoscopy. Little or no genetic risk of colon cancer occurs in more than 75-95% of people (Watson & Collins, 2011). Other risk factors include male gender, older age, alcohol, high intake of fat or red meat, smoking and obesity, a lack of physical exercise (Watson & Collins, 2011).

2.1.3 Inflammatory bowel disease

In cases of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis there is an increased risk of contracting colon cancer (Jawad *et al.*, 2011). Prevention with aspirin and regular colonoscopies are recommended for these high risk groups (Xie & Itzkowitz, 2008). However, people with inflammatory bowel disease account for less than 2% of colon cancer cases per year. In those with Crohn's disease 2% get colorectal cancer after 10 years, 8% after 20 years, and 18% after 30 years. In those with ulcerative colitis around 16% develop either a cancer precursor or cancer of the colon over 30 years (Triantafillidis *et al.*, 2009).

2.1.4 Types and molecular genetics

Genetic analysis shows that rectal and colon tumours are basically genetically the same cancer (Muzny *et al.*, 2012). Those with a family history in two or more first-degree relatives have a two to three fold increased risk of disease, and this group accounts for about 20% of all cases. Several genetic syndromes are also related with higher rates of colorectal cancer.

Lynch syndrome or (HNPCC) is the most common type of hereditary nonpolyposis colorectal cancer and is present in about 3% of people with colorectal cancer. Other syndromes that are strongly associated include: familial adenomatous polyposis (FAP) and Gardner syndrome, in which cancer nearly always occurs and is the cause of 1% of cases (Half *et al.*, 2009). Genetics, epidemiological data, and experimental, suggest that

colorectal cancer develops from complex interactions between environmental factors and inherited susceptibility. The existing hypothesis is that adenomatous polyps are the precursors of the great majority of colorectal cancers. Thus a gauge that can detect and reduce the prevalence of these adenomatous polyps can reduce the risk of colorectal cancer. Amongst colorectal cancer, some are considered to be invasive cancers that are confined within the wall of the colon (TNM stages I and II), these are curable with surgery. If untreated, they extend to regional lymph nodes (stage III), where up to 73% are curable by surgery and chemotherapy. Cancer that metastasizes to distant sites (stage IV) is ordinarily not curable, although chemotherapy can prolong survival, and in rare cases, surgery and chemotherapy combined have seen patients through to a cure.

Hereditary and environmental factors play a role in CRC development. For example, sporadic cancer. Those cases that occur in individuals over 50 without any identifiable predisposing factors, account for greater than 75% of the various causes of CRC, while the remaining are accounted for by inflammatory bowel diseases and familial incidence.

Familial adenomatous polyposis (FAP) is an autosomal dominant colon cancer syndrome that appears upon the inheritance of a single copy of a mutant gene (Farinella *et al.,* 2010; Kucherlapatia *et al.,* 2001). FAP syndrome is described by the appearance of hundreds to thousands of colonic polyps during the second and the third decade of life and is assumed to be the least common type of colon cancer (Farinella *et al.,* 2010; Kucherlapatia *et al.*, 2001). The gene responsible for FAP was recognized in (1991) by Groden and termed the adenomatous polyposis coli (APC) gene. The *APC* gene was recognized on chromosome 5q as one of the genes generally deleted in some FAP kindred's (Groden *et al.*, 1991; Kinzler *et al.,* 1991). It encodes a huge protein (about 2850 amino acids) that forms a complex with axin and helps glycogen synthase kinase3β to phosphorylate N-terminal serine/threonine residues of β-catenin, accelerating its rapid degradation through ubiquitylation (Polakis, 1999).

Hereditary non-polyposis colon cancer (HNPCC), also known as lynch syndrome, is another autosomal dominant disorder that represents 2-4% of colon cancer cases (Jasperson *et al.,* 2010; Kucherlapatia *et al.,* 2001). Individuals with HNPCC are also at increased risk of other cancers, such as kidney, stomach, biliary, small bowel and ovarian (Jasperson *et al.,* 2010). The mutations in any mismatched repair genes mut l homolog 1 [MLH1], mut s homolog 2 [MSH2], mut s homolog 6 [MSH6], postmeiotic segregation increased 1 [PMS1], post-meiotic segregation increased 2 [PMS2], and mut s homolog 3 [MSH3] results in HNPCC (Neibergs *et al.,* 2002). Mutations in MLH1 and MSH2 are usually observed mutations in HNPCC patients (Markowitz & Bertagnolli, 2009).

2.1.5 Pathogenesis of colorectal cancer

Colorectal cancer is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract, most consistently as a result of mutations in the WNT signaling pathway that artificially increase signaling activity. The mutations can be inherited or acquired, and most probably occur in the intestinal crypt stem cell.

On the molecular and cellular level, colorectal cancer begins with a mutation to the WNT signaling pathway, when WNT binds to a receptor on the cell that sets in motion a chain of molecular proceedings that ends with β-catenin moving into the nucleus and activating a gene on DNA. In colorectal cancer, genes along this chain are damaged, usually by a gene called APC, which is a "brake" that works on the WNT pathway. Without a working APC brake, the WNT pathway is stuck in the "on" position (Markowitz & Bertagnolli, 2009). If the *APC* gene is mutated, glycogen synthase kinase3β cannot phosphorylate β-catenin and therefore stabilized β-catenin accumulates in the cytoplasm and moves to the nucleus, where it activates TCF/LEF transcription factors to transcribe WNT target genes (Korinek *et al.,* 1997). Activation of the canonical WNT pathway in the colonic epithelium seems to be one of the key events in

the polyp initiation process, as shown in Figure 2.1 (Oshima *et al.*, 1997; Taketo, 2006). While APC is mutated in most colon cancers, some cancers have increased β-catenin because of mutations in β-catenin (CTNNB1) that block its degradation, or they have mutations in other genes whose functions are analogous to APC such as AXIN1, AXIN2, Transcription factor 7-like 2 (TCF7L2), or Naked cuticle 1(NKD1) (Markowitz & Bertagnolli, 2009).

Beyond the defects in the WNT-APC-beta-catenin signalling pathway, other mutations must occur for the cell to become cancerous. The p53 protein, produced by the *TP53* gene, normally monitors cell division and kills cells if they have WNT pathway defects. Eventually, a cell line acquires a mutation in the tumour protein (*TP53)* gene and transforms the tissue from an adenoma into an invasive carcinoma (sometimes the gene encoding p53 is not mutated, but another protective protein named BAX is mutated).

Other apoptotic proteins commonly deactivated in colorectal cancers are TGF-β and DCC (Deleted in colorectal Cancer). TGF-β has a deactivating mutation in at least half of colorectal cancers. Sometimes TGF-β is not deactivated, but a downstream protein named SMAD is. DCC commonly has deletion of its chromosome segment in colorectal cancer (Mehlen & Fearon, 2004).

Some genes are oncogenes they are over expressed in colorectal cancer. For example, genes encoding the proteins KRAS, RAF, and PI3K, which normally stimulate the cell to divide in response to growth factors, can acquire mutations that result in over-activation of cell proliferation. The chronological order of mutations is sometimes important, with a primary KRAS mutation generally leading to a self-limiting hyperplastic or borderline lesion, but if occurring after a previous APC mutation it often progresses to cancer (Vogelstein & Kinzler, 2004). PTEN, a tumour suppressor,

normally inhibits PI3K, but can sometimes become mutated and deactivated (Markowitz & Bertagnolli, 2009). Comprehensive genome-scale analysis have revealed that colorectal carcinomas are clearly separable into hypermutated and non-hypermutated tumour types. In addition to the oncogenic and inactivating mutations described for the genes above, non-hypermutated samples also contain mutated CTNNB1, FAM123B, SOX9 and ARID1A. Progressing through a distinct set of genetic events, hypermutated tumours display mutated forms of ACVR2A,TGFBR2, MSH3, MSH6, SLC9A9, TCF7L2, and BRAF. The common theme among these genes, across both tumour types, is their involvement in WNT and TGF-β signalling pathways, which in turn results in increased activity of MYC, a central player in colorectal cancer (Muzny *et al.*, 2012).

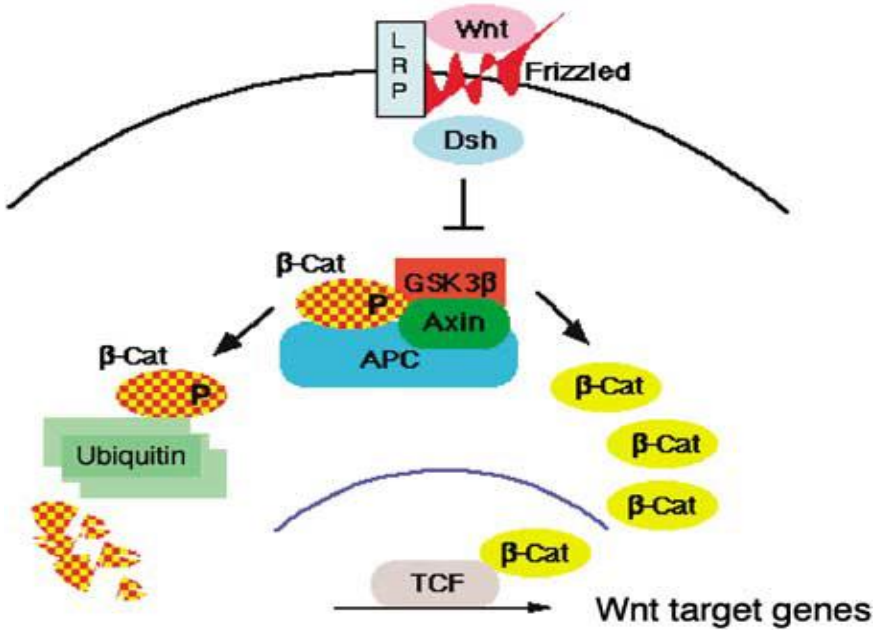


Figure 2.1: Wnt signalling activation in colon cancer cells (Taketo, 2006).

2.1.6 Pathology of colorectal cancer

The classifications of colon cancers are well-differentiated, moderately well differentiated, or poorly differentiated on the degree of preservation of normal glandular architecture and cytologic features. Gradually more poor differentiation is perhaps a histological marker of further underlying genetic mutations, but the mutations associated with poor differentiation are currently unknown. About 20% of cancers are poorly differentiated. They have a poor prognosis. About 15% of colon cancers are classified as mucinous or colloid, because of prominent intracellular accumulation of mucin. These cancers are more aggressive (Kanazawa *et al.*, 2002). About 65% of colon cancers are distal to the splenic flexure and potentially detectable by sigmoidoscopy (McCallion *et al.*, 2001). Contrariwise, about 35% of colon cancers are proximal to the sigmoid and not detectable by flexible sigmoidoscopy. Colon cancer can occur in a pedunculated polyp, sessile polyp, mass or stricture. Small polyps rarely contain cancer. Only about 1% of diminutive polyps contain cancer. Cancer in a sessile polyp may metastasize faster than cancer in a pedunculated polyp because of the closer proximity of the lymphatic drainage (Nivatvongs, 2002).

2.1.7 Aberrant crypt foci (ACF)

Aberrant crypt foci were first disclosed by Bird in (1987). Treating mice with the carcinogen azoxymethane (AOM) induced the growth of colonic crypts that were larger, darker staining and thicker than normal crypts when conceived with methylene blue. Aberrant crypts were first noticed in the surrounding normal colonic mucosa of patients with colon cancer. In human mucosa, the crypt clusters found appear raised from the normal mucosal surface of the colon (Pretlow *et al.*, 1991). Bird (1995) reported the role of aberrant crypt foci in knowing the pathogenesis of colon cancer. The Case for

Modulation as a Biomarker for Colon Cancer (Takayama, (2005) elucidate the detection, gene abnormalities and clinical usefulness of ACF.

Wargovich (2010) mentioned the use of ACF as a biomarker in their research Aberrant Crypt Foci: Due to the observed rapid conversion of intestinal and colonic cells under normal conditions, it is expected that aberrant crypts would replicate at the same rate, if not faster than normal crypts. However, in humans there is discordant evidence as to how much of an increase in replication exists, if any (Wargovich *et al.*, 2010).

This discrepancy can be due to many factors, most notably differences between the methods of analysis and sampling in various studies defining colonic epithelial cell proliferation (Jass *et al.*, 2003). Aberrant crypt replication is basically identical to that of normal crypts, with the replication process beginning at the bottom of the crypt pushing cells upward and outwards to form new colonic crypts, in addition to 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 occurs at an increased rate in various diseased states of the bowel (Figure 2.2).

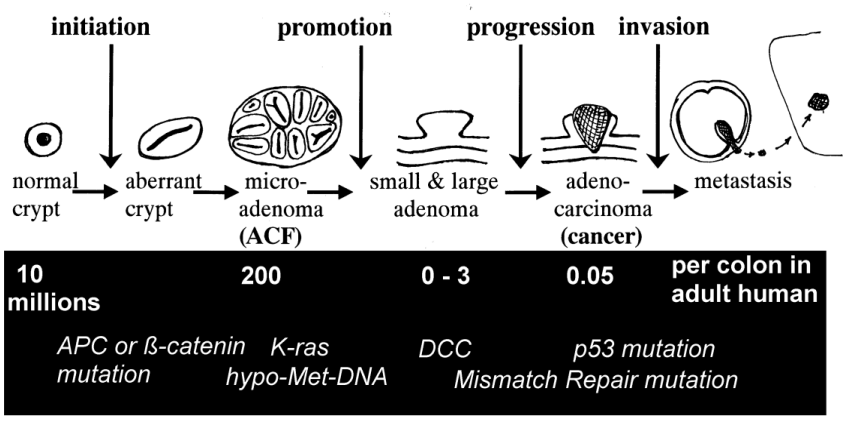


Figure 2.2: Stages of ACF formation in colon cancer (Corpet & Taché, 2002)

As mentioned, ACF was first noticed in the colon epithelium of rodents treated with chemical carcinogens. They are single to multiple crypt clusters of abnormally staining crypts after short-term staining with either indigo-carmine solutions or methylene-blue and fixation with either alcohol-based fixatives or buffered formalin (Bird, 1987).

ACF are readily visible ordinarily with the assistance of a dissection microscope at a magnification at 40x strength. There are many examples in literature describing the key histopathological signatures of ACF, though categorizing them in humans has met with considerable controversy (Gupta *et al.*, 2009; Gupta *et al.*, 2007; Khare *et al.*, 2009; Lance & Hamilton, 2008). Microscopically, a discrepancy has been made between dysplastic ACF and non-dysplastic ACF (often including serrated hyperplastic ACF) (Wargovich *et al.*, 2010). Before 1990, the gold standard endpoint for chemoprevention in rodents was the incidence of macroscopic tumours and colon cancers; colon adenomas and adenocarcinomas induced by a chemical carcinogen.

Though these endpoints are clearly related to cancer, the have three major drawbacks: (i) a tumour requires a long time to develop (usually 5-8 months); (ii) each tumour must be confirmed by histology, which is long and costly; (iii) each animal brings little information to the study (each rat has either no tumour or a tumour), thus large groups of rats are needed for statistical analysis (typically 30 rats or more per group). In lieu of the above draw backs, ACF was found to be advantageous as endpoint chemoprotective screening biomarkers because (i) ACF was induced by all colon carcinogens in a dose and species dependant manner; (ii) their number and growth were modified by the modulators of colon carcinogenesis, and they predicted the tumour outcome in several rodent studies; (iii) they correlate with colon cancer risk, and adenoma size and number in humans; (iv) the morphological and genotypic features of ACF in human colons were similar to those in animal colons, and many alterations are

similar in ACF and in tumours; (v) some ACF show dysplasia, and carcinoma were observed in rodents and humans‟ ACF (Corpet & Taché, 2002).

This is why ACF is now widely used as a preliminary endpoint in colon cancer chemoprevention studies, as it provides a simple and economical tool for preliminary screening of potential chemopreventive agents, and it allows a quantitative assessment of the mechanisms of colon carcinogenesis.

2.2 Azoxymethane AOM

Azoxymethane (methyl-methylimino-oxidoazanium) is an oxide compound of azomethane, with the molecular formula of $C_2H_6N_2O$, whose chemical structure is shown in (Figure 2.3). It is a carcinogenic and neurotoxic chemical, with wide applications in biological research, such as being particularly effective in inducing colon carcinomas. Besides been soluble in water, this compound is reported to be sensitive to prolonged exposure to air and elevated temperatures.

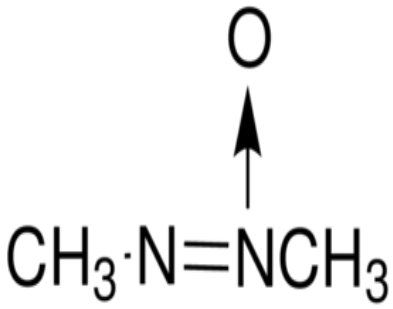


Figure 2.3: Chemical structure of Azoxymethane (methyl-methylimino-oxidoazanium)

The use of azoxymethane to induce foci of aberrant crypts in rats has been reported in many papers (Adler, 2005; Velmurugan *et al.*, 2008; Wargovich *et al.*, 2010) studied the Chemoprevention effect of zerunbone isolated from *Zingiber zerumbet* by using azoxymethane to induce aberrant crypt foci in male F344 rats. The researchers induced ACF in these rats by subcutaneous injections of AOM (15mg/kg body weight) for two weeks. They assessed the effects of zerumbone on cell proliferation activity by counting

silver-stained nucleolar organizer regions protein (AgNORs) in colonic cryptal cell nuclei.

Challa (1997) reported the induction of aberrant crypt foci using azoxymethane while studying the effect of phytic acid and green tea in interactive suppression of ACF. Magnuson (2000) published an extensive study on the increased susceptibility of adult rats to AOM-induced aberrant crypt foci. Verghese (2002) studied the suppression of AOM-induced ACF using dietary insulin.

In general, azoxymethane is commonly used as a model for colon cancer induction. It can induce colon cancer similar to the pathogenesis of human sporadic colon cancer. Thus, it has been widely used in the study of the molecular biology, prevention and treatment of colon cancer. After administration, AOM is metabolised into methylazoxymethanol by CYP2E1, which causes DNA mutations. Mutation of K-ras activates this pathway and its downstream PI3K/Akt pathway and Mitogen-activated protein kinase (MAPK) pathway. Mutation of β-catenin also prevents it from being degraded by GSK-3 and an accumulation of β-catenin leads to cell proliferation. TGFβ, a pro-apoptotic protein, is thus inhibited. These changes form the basis of AOM carcinogenesis (Chen & Huang, 2009).

2.2.1 Metabolism for AOM causing colon cancer

Azoxymethane does not react with DNA directly, it has to be activated *in vivo* to develop carcinogenesis. Azoxymethane is metabolised by cytochrome P450, specifically isoform CYP2E1. The first step is the hydroxylation of the methyl group of AOM to form methylazoxymethanol (MAM). Methylazoxymethanol then breaks down into formaldehyde and a highly reactive alkylating species, probably the methyldiazonium. This chemical actually causes alkylation of DNA guanine to O6-MEG and to O4-methylthymine. These mutations can initiate tumourigenesis through several key genes in intracellular signal pathways. The inhibition of CYP2E1 (for

example by disulfiram, an agent used for avoidance therapy in alcohol abuse) has been shown to prevent chemical carcinogenesis. In CYP2E1 knockout mice, O6-MEG formation and colon polyp numbers decrease in response to AOM treatment (Chen & Huang, 2009).

2.2.2 Mechanisms of AOM causing colon cancer

Several activation pathways have been revealed to explain the mechanism of AOM-induced colon cancer (Figure 2.4). These include K-ras, β-catenin and TGFβ. However, there is no unified explanation for the mechanism of this model.

K-ras: Is a small G-protein that regulates both MAPK and PI3K/Akt intracellular signal pathways, which in turn, regulate cell growth, proliferation and glucose metabolism. In K-ras pathways, K-ras plays an important role in the carcinogenesis of colon cancer. Azoxymethane has been shown to cause a K-ras gene transversion mutation from G:C to A:T at codon 12 deriving from O6-methyl-deoxyguanine adducts. It changes glycine to aspartic acid. This mutation causes the activation of the K-ras protein (Chen & Huang, 2009).

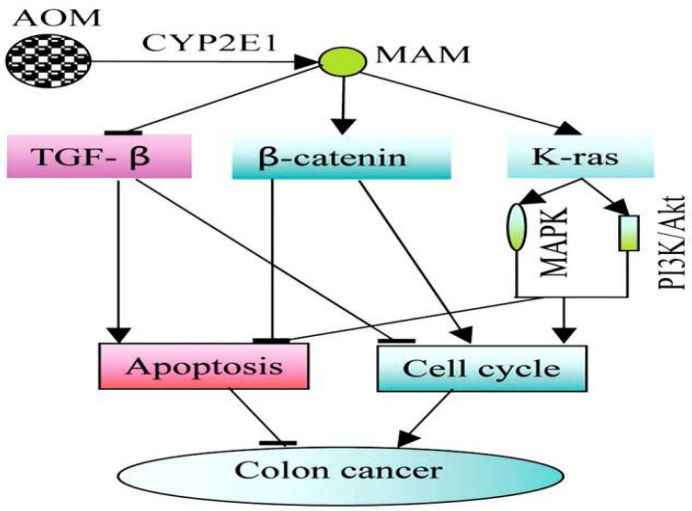


Figure 2.4: Azoxymethane (AOM) mechanism of colon cancer (Chen & Huang, 2009).

In fact, both pathways play important roles in the carcinogenesis of many types of cancers including colon cancer. This is similar to human colon cancer. A study showed that pEGFR, pAkt and pMAPK are increased in colon tumours compared to normal colon tissue.

The PI3K/Akt pathway: Is important in understanding colon cancer as 20% of patients have PIK3CA mutations. The activation of PI3K/Akt can increase cell survival pathways via phosphorylation of downstream targets, including NFκB, and Bcl-xl. PI3K/ Akt also blocks p53 and the forkhead/Fas ligand to decrease apoptosis (Chen & Huang, 2009; Whiteside, 2007).

. In the cell cycle pathway, PI3K/Akt deactivates glycogen synthase kinase 3 (GSK3) and promotes cyclin D1 and myc to increase cell proliferation (Sears *et al.*, 2000). In the cell growth pathway, PI3K/Akt activates the mammalian target of rapamycin (mTOR), a conserved Ser/Thr kinase that increase's cell size. Whether mToractivity is increased in the AOM model has not yet been elucidated. Downstream of PI3K/Akt, COX2 has also been shown to be involved in the carcinogenesis of AOM (Rao *et al.*, 2002). Activated Ras stimulates the serine/threonine-selective protein kinase: Raf kinase, which is an oncogene. The protein encoded has regulatory and kinase domains. Ras binds to CR1 in their regulatory region and phosphorylates CR2, which is rich in serine/threonine. This leads to activation of CR3 in the kinase region. It then, in turn, activates MAPK and ERK kinase (MEK), which activates mitogen-activated protein kinase (MAPK) and ERK. MAPK and ERK promote carcinogenesis via target proteins like c-myc, CREB, RSK, Mcl1, p16, Rb and cyclins. Inhibition of these pathways has been demonstrated to cause cancer cell death (Chen & Huang, 2009). Over expression of the cell cycle promoter, cyclin D1, may contribute to the

AOM model as well. Cdk4 has been detected in the early stages in the AOM cancer induced mouse colon.

β-catenin pathway: β-catenin plays an important role in cell adhesion and is also an oncogenic protein. It associates with cadherin or a-catenin to link the actin cytoskeleton. It is also a co-transcriptional activator of genes in the WNT signal pathway. In its free form, it associates with the scaffolding proteins axin and Apc, and is phosphorylated by GSK-3β resulting in degradation by the proteasome. The N-terminus of β-catenin is also mutated in some cases, so that β-catenin cannot form the complex and be degraded. Thus, free β-catenin is increased and binds with the T-cell factor/lymphoid enhancer factor TCF/LEF to form a complex, which activates gene transcription and cell proliferation. It targets *c-myc*and *cyclinD1* genes, which are well known carcinogens. Azoxymethane causes β-catenin mutations at codons 33 and 41, which are the serine and threonine residues that are targets for GSK-3β phosphorylation. This leads to the accumulation of β-catenin for the carcinogenesis. It has been shown that AOM treatment increases both β-catenin and cyclin D.

TGFβ pathway: Transforming growth factor-β (TGFβ) including isoforms 1, 2 and 3 can inhibit cell growth, proliferation and the cell cycle progression and thus has an anti-tumour effect. Defects in TGFβ signalling have been found in 20–30% of colon cancer patients. The activity of the TGFβ pathway is decreased after AOM treatment, which mediates AOM-induced colon cancer. It has been demonstrated that the active form of TGFβ is decreased in AOM treated mice (Chen & Huang, 2009).

Montenegro (2009) cited that TGF-β induces apoptosis through several signal pathways. First, TGFβ forms dimers and binds to its type 2receptor. This complex then associates and phosphorylates its type 1 receptor. The type 1 receptor in turn phosphorylates receptor-regulated SMAD (R-SMAD) to cause apoptosis. Second, the activated type 2 receptor binds to death associated protein 6 to induce apoptosis. Third,

TGFβ has also been shown to inhibit the phosphorylation of the p85 subunit of PI3K/Akt induced by GM-CSF in several myeloid leukaemia cell lines, including MV4-11, TF-1 and Tf-1a (Montenegro *et al.*, 2009).

The evolution of colorectal cancer through the adenoma-carcinoma sequence can be simplified by highlighting the intervention of some of the most important markers. Normal epithelium, upon loss or mutation of the APC gene, is transformed into hyper-proliferative epithelium. DNA methylation is responsible for the transformation of the hyper-proliferative epithelium into early adenoma. Due to the mutation of the K-ras gene and loss of DCC, the early adenoma is transformed into dysplastic adenoma, upon which the loss of p53 function results in the appearance of carcinoma. Though the adenoma-carcinoma sequence is a well-established concept for the development of colorectal cancer, it does not however, imply that all adenomas will transform into carcinomas since many of them may regress (Sillars-Hardebol *et al.*, 2010).

In fact, the total accumulation of the genetic alterations, rather than their order according to a preferred sequence, is responsible for the determination of the tumour's properties (Vogelstein *et al.*, 1988).

The chance of long-term survival for colon, rectal, or other cancers improves significantly with early detection. For example, it was reported that the 5-year survival rate for people whose colorectal cancer is found and treated at an early stage is > 90%, but once the cancer has spread to nearby organs the 5-year survival rate drops to 64%, and it is < 10 % when the spread includes distant organs, such as the liver and lungs.
Because the survival rates improve with early detection, considerable effort has gone into devising screening tests that might provide an 'early warning system' for individuals with no symptoms, as well as for those who exhibit one or more symptoms. It has been cited that if intermediate biomarkers are identified, one might be able to recognize very early stages of cancer development, long before the formation of a frank

tumour. With appropriate intervention, progression of the lesion could be reversed or slowed significantly. One promising candidate for an intermediate biomarker for colon cancer is known as an Aberrant Crypt Foci, or "ACF" (Wargovich *et al.,* 2010).

On a much shorter time scale, Azoxymethane, PhIP and related heterocyclic amines generate ACF in rats when fed to them over the course of a few weeks.

The ACF assay also provides a quick method to screen compounds that might be effective at inhibiting the development of colon cancer, and several promising candidates for chemoprevention such as plant extracts. As mentioned elsewhere in this paper, the development of colorectal cancer depends on many lifestyle related factors in addition to genetic factors all of which influence the digestive tract. Hence, identifying the point at which normal colonic epithelium becomes neoplastic, hyperplastic, dysplastic, or an early indicator of disease is of interest to many research groups. There is an overwhelming idea that aberrant crypt foci (ACF) are colon cancer precursors (Wargovich *et al.,* 2010), whose size and numbers directly correlate with the risk of developing colon cancer.

2.3 5-Flourouracil

5-Fluorouracil (5-FU) is still a widely used anticancer drug. Since 1957, it has played an important role in the treatment of colon cancer and is used for patients with breast and other cancers, like those of the head and neck (Grem, 2000). 5-FU is a heterocyclic aromatic organic compound with a structure similar to that of the pyrimidine molecules of DNA and RNA. It is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen (Figure 2.5). Only one crystal structure is reported in the literature for pure 5-FU, in which the compound crystallizes with four molecules in the asymmetric unit and the molecule adopts a hydrogen-bonded sheet structure (Hulme *et al.,* 2005). Due to its structure, 5-FU interferes with nucleoside metabolism and can be

incorporated into RNA and DNA, leading to cytotoxicity and cell death (Noordhuis *et al.*, 2004).

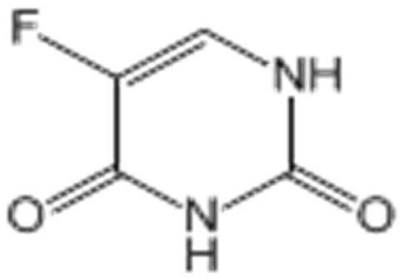


Figure 2.5: Chemical structure of 5-Fluorouracil (Zhang *et al.*, 2008).

2.3.1 Mechanism of action

In mammalian cells, 5-FU is converted to fluorodeoxyuridine monophosphate (FdUMP), which forms a stable complex with thymidylate synthase (TS), and thus inhibits deoxythymidine monophosphate (dTMP) production. dTMP is essential for DNA replication and repair, its depletion therefore causes cytotoxicity (Longley *et al.*, 2003; Parker & Cheng, 1990). Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells. Up to 80% of administered 5-FU is broken down by DPD in the liver (He *et al.*, 2008). TS *inhibition* TS, an essential enzyme for catalyzing the biosynthesis of thymidylate, is implicated in the regulation of protein synthesis and apoptotic processes (Chernyshev *et al.*, 2007). TS catalyzes the methylation of deoxyuridine monophosphate (dUMP) to dTMP, for which 5,10-methylenetetrahydrofolate (CH_2THF) is the methyl donor, and finally provides the reaction with thymidylate to maintain DNA replication and repair (Roberts *et al.*, 2006). The reaction has a seriatim binding sequence, and dUMP binds at the active site before CH_2THF does. Then the reaction is initiated by the nucleophilic addition of the active

site Cys 146 (numbering of amino acid residues used is according to the sequence of EcTS) to the pyrimidine C (6) atom of dUMP. Specifically, at the onset of catalysis, the binding position and orientation of the substrate, if correctly adopted, support an efficient binding of the cofactor, and then allows the formation of the ternary TS–dUMP–CH_2THF complex, and the subsequent reaction (Newby *et al.*, 2006). Research has indicated that 5-FU exerts its anticancer effects mainly through inhibition of TS, for which the pathways have not been fully interpreted. Santi has pointed out that the formation of the ternary TS–FdUMP–CH_2THF complex is time-dependent, and the reaction stops as the fluorine substituent fails to dissociate from the pyrimidine ring, resulting in a slowly reversible inactivation of the enzyme (Sotelo-Mundo *et al.*, 2006). Reduction of dTMP leads to downstream depletion of deoxythymidine triphosphate (dTTP), which induces perturbations in the levels of the other deoxynucleotides (dATP, dGTP and dCTP). Finally the imbalances (the ATP/dTTP ratio specifically) are thought to severely disrupt DNA synthesis and repair, resulting in lethal DNA damage (Danenberg, 1977). Accumulation of dUMP, which might subsequently lead to increased levels of deoxyuri-dine triphosphate (dUTP), can be misincorporated (Figure 2.6)

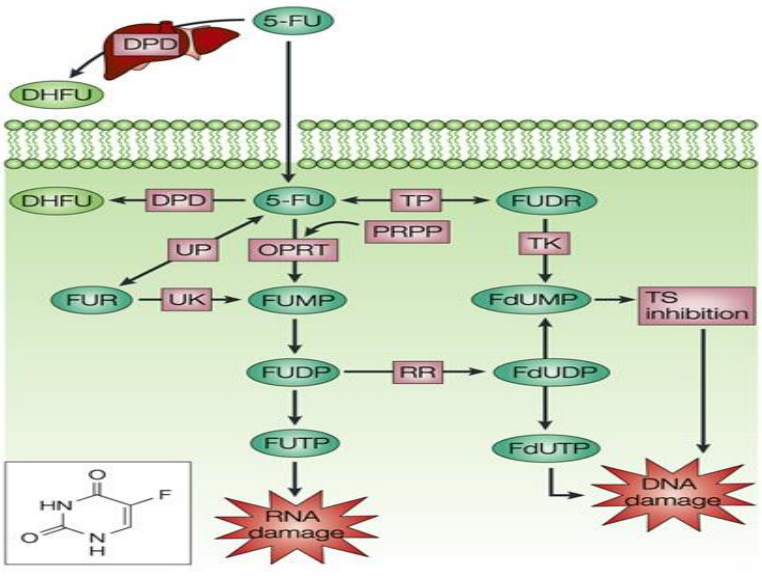


Figure 2.6: Mechanism of action of 5-Fluorouracil

2.4 Investigated medicinal plants

The therapeutic efficacies of many indigenous plants for various diseases have been described by traditional herbal medicine practitioners (Natarajan *et al.*, 2003). Natural products are a source of synthetic and traditional herbal medicine. They are still the primary health care system in some parts of the world (Singh & Singh, 2001). Fifty percent of the drugs in clinical usage around the world have been derived from natural products (Gurib-Fakim, 2006). Recent research into medicinal plants signified that new phytochemicals could be developed for many health problems. Multiple factors lend themselves to the increasing demand for herbal medicines in the last few decades, either due to the perception that herbs might treat and prevent diseases in a safer way than pharmaceutical medicines, because they are natural and therefore seen as being the harmless option, or because no unfavorable side effects could be identified, along with relatively low cost and the easy availability of herbal remedies (Stickel & Schuppan, 2007).

2.4.1 *Catharanthus roseus*

C. roseus (L) G. Don (Family: Apocynaceae) also known as *Vinca rosea*, Periwinkle, Nityakalyani, Billaganneru, Ainskati, Nayantra, Rattanjot, Sada bahar, Sadaphul, Ushamanjairi, Madagascar periwinkle, *Vinca rosea* or *Lachnera rosea* worldwide (Antia & Okokon, 2005) and in Malaysia known as Kemuning Cina. It is native to the Madagascar, Caribbean Basin, abundantly naturalized in many regions, particularly in arid coastal locations (Figure 2.7).

2.4.1.1 Description

The leaves are glossy, dark green (1-2 inch long), oblong elliptic, acute, rounded apex. Flowers are fragrant, white to pinkish purple in terminal or axillar y cymose clusters. Follicle is hairy, multi-seeded and 2-3 cm long. The seeds are oblong, minute and black. The plant is commonly grown in gardens for beddings, borders and for mass effect. It blooms throughout the year and is propagated by seeds or cuttings. The bloom of natural wild plants are pale pink with a purple eye in the center, but horticulturist have developed varieties (more than 100) with colour ranging from white to pink to purple (Aslam *et al.*, 2010).

2.4.1.2 Uses and pharmacological properties

C. roseus has historically been used to treat a wide assortment of diseases, and as such is one of the most highly exploited and studied medicinal plants. European herbalists used the plant for conditions as varied as headaches to a folk remedy for diabetes. Traditionally the leaves have been soaked in water which is then drunk to ease menstrual pain and the leaf extract is applied to insect bites. In China, it was used as an astringent, cough remedy and diuretic. It was used as a homemade cold remedy to ease lung congestion and inflammation and sore throats in Central and South America and

throughout the Caribbean. An extract from the flowers has also been used to make a solution to treat eye irritation and infections. Finally, In India and all over the world, it is used as folk remedy for diabetes mellitus and this is proven by the fact that ethanolic extracts of leaves and flowers of Madagascar periwinkle lower blood glucose level. Diabetes mellitus, a major endocrine disorder affecting nearly 10% of the population all over the world, is possibly the world's largest growing metabolic disease. Traditional plant medicines including leaves and lower parts of *C. roseus* are used throughout the world for a range of diabetic complications (Nammi *et al.*, 2003). *C. roseus* has a variety of potential medicinal chemotherapeutic properties. It is estimated that about 30% of all plants contain alkaloids, though *C. roseus* has more than 400 known alkaloids, some of which are approved as antineoplastic agents to treat leukemia, Hodgkin's disease, malignant lymphomas, neuroblastoma, rhabdomyosarcoma, Wilms' tumour, wound healing and other cancers (Nayak & Pereira, 2006). It can also be used to treat Hypoglycemia (Kar *et al.*, 2003) as it significantly reduces the levels of protein, lipid peroxidation and liver (Iweala & Okeke, 2005). Its vasodilating and memory-enhancing properties have been shown to alleviate vascular dementia and Alzheimer's disease (Hindmarch *et al.*, 1991). It also has antidiabetic and hypolipidemic properties (Rasineni *et al.*, 2010b), antioxidant activity (Rasool *et al.*, 2011) and antibacterial activity (Raza *et al.*, 2009).

2.4.1.3 Phytochemistry

Phytochemically, *C. roseus* contain catharanthine, serpentine, tabersonine, vindoline, vinblastine, and vincristine. Ajmalicine, tryptophan, tryptamine and secologanine (Tikhomiroff & Jolicoeur, 2002) (Figure 2.8).

The two classes of active compounds in *Vinca* are alkaloids and tannins. The major alkaloid is vincamine and it's closely related semi-synthetic derivative widely is

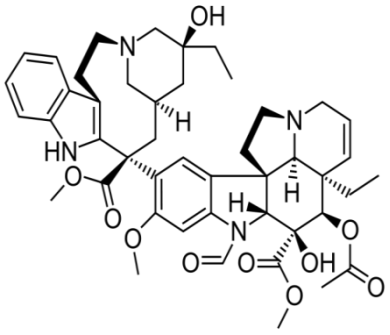
used as a medicinal agent, known as ethyl-apovincaminate or vinpocetine, has vasodilating, blood thinning, hypoglycemic and memory-enhancing properties (Chattopadhyay, 1999; Chattopadhyay *et al.*, 1991). The extracts of *Vinca* have demonstrated significant anticancer activity against numerous cell types (El-Sayed & Cordell, 1981). Currently, cancer is Malaysia's number two killer, preceded only by cardiac disease.

The leaves have been known to contain 150 useful alkaloids among other pharmacologically active compounds which are found in the roots and stem of the plant (Antia & Okokon, 2005). In particular, two dimeric indole alkaloids, vincristine and vinblastine are valuable drugs used in the treatment of cancer of the embryonic membranes and acute leukaemia. They have been used as active immunosuppressive agents due to the inhibitory activity they induce against cells of the lymphoid line (Aisenberg & Wilkes, 1964), and have been used for many years as chemotherapeutic agents in the treatment of leukemia and Hodgkin's disease. Other separated ingredients are vindolinine, vincamijine, venalstonine, tabersonine and vincubine. The crude alkaloids, if taken in high dosages, could be detrimental to the heart (Muhamad & Mustafa, 1994).

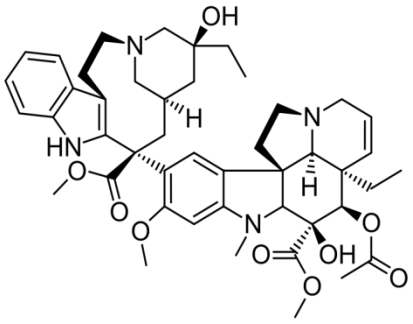
Catharanthine and vindoline are the most abundant alkaloids found in the *C. roseus*. This plant has been extensively studied because of the pharmaceutical importance of its alkaloids, vincristine and vinblastine. These two alkaloids have been used for many years as chemotherapeutic agents in the treatment of acute leukemia and hodgkin's disease (Noble, 1990). Catharanthine and vindoline are the major monomer alkaloids, as well as biosynthetic precursors for vinblastine and vincristine. Other compounds with important biological activities that can be found in *C. roseus* are ajmalicine as anti-hypertensive and serpentine with sedative properties (Pereira *et al.*, 2010).

High performance liquid chromatography (HPLC) has been used for the separation and the identification of ajmalicine, serpentine, vinblastine, vincristine, vindoline, catharanthine, tabersonine, lochnericine, secologanine, strictosidine, tryptophan and tryptamine (Noble, 1990). HPLC can be efficiently coupled to different detectors such as Photo Diode Array (PDA), fluorescence, ESI-MS, MS-MS, NMR and CD (Bringmann *et al.*, 2002), for precise identification and/or quantification of alkaloids. LC-MS methods that use time-of-flight (TOF) as the detection system are of particular interest in herbal component analysis as TOF analyzers are well-suited to perform structure elucidation or confirmation. TOF instruments also provide accurate mass measurement and full-scan spectral sensitivity. Accurate mass measurement gives elemental composition of parent and fragment ions (Zhou *et al.*, 2009) . In this study, the chemical constituents in *C. roseus* leaves and stem from three different flower colours that consists of pink, red and white were separated and identified by liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS) analysis. The total-ion-current (TIC) chromatogram was acquired in positive ion mode, and the molecular formula was inferred from accurate mass measurement.

Figure 2.7: *Catharanthus roseus*

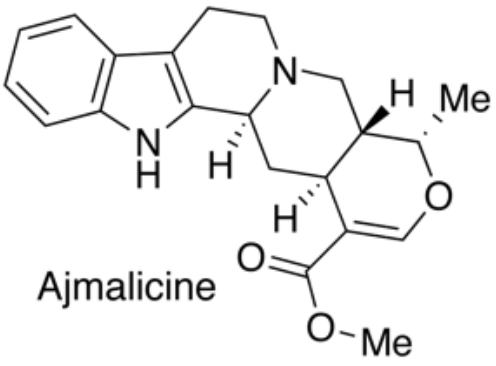


(A)



(B)

(C)



(D)

Figure 2.8: Chemical structure of (A) Vinocristine; (B) Vinblastine; (C) Vindoline; (D) Ajmalicine (O'Connor & Maresh, 2006)

2.4.2 *Acanthus ilicifolius*

A.ilicifolius Linn. (Acanthaceae) is a spiny herb of the mangrove species, distributed widely throughout southeast Asia. The plant is commonly known as Holly-Leaved mangrove and locally known as *jemuju, jeruju* or *daruju* (Figure 2.9).

2.4.2.1 Description

A. ilicifolius is a strictly mangrove plant of the *Acanthaceae* family. It is a low sprawling or somewhat vine-like herb, scarcely woody and can grow up to 2m in height. The axes initially are erect, but tend to recline with age. Branching is infrequent and commonly seen in the older parts. The aerial roots tend to appear from the lower surface of the reclining stems. The leaves are decussating and have a pair of spines at their insertion. They are glabrous, gradually tapering below, either broadly lanceolate with entire margins or more usually with a sinuous, spiny margin. The apex is broadly tridentate including an apical spine. Major spines are seen at the end of leaf lobes horizontal or erect, minor spines between major lobes erect. The inflorescences are terminal spikes measuring 10-20 cm in length that extend with age. The flowers are in four ranks, numbering up to 20 pairs, the bract below each flower is 5mm long or shorter, often caduceus, with 2 lateral bracteoles, conspicuous and persistent. The flowers are perfect calyx 4-lobes, the upper lobe conspicuous and enclosing the flower in bud, the lower lobe somewhat smaller, lateral calyx lobes are narrow and wholly enclosed by upper and lower sepal. The corolla is zygomorphic, at least 3cm long with a short tube closed by basal hairs, abaxial lip broadly 3-lobed, adaxial lobes absent. There are 4 stamens, subequal, with thick hairy connectives, anthers medifixed, each with 2 cells, aggregated around the style. The ovary bilocular, with 2 superposed ovules in each loculi style enclosed by stamens, that capitates to exposed pointed stigma. The fruit is a capsule measuring 2-3cm in length and 1cm in width, usually with 4 rugose

angular seeds measuring about 1cm in length with delicate testa, the seeds are wrinkled and whitish green in colour (MacKinnon, 1996).

2.4.2.2 Uses and pharmacological properties

In India, the plant is found in the Sundarban mangroves and is used locally for the treatment of rheumatism, snakebite, paralysis, asthma, ulcers, and wound healing (Wöstmann & Liebezeit, 2008a).

In Malaysia its leaves are used for rheumatism, neuralgia, and poison arrow wounds. In Thailand decoction of the whole plant is used for kidney stones. In Goa mucilaginous leaves are used as emollient in rheumatism and neuralgia. Methanolic extract of the leaves have been reported to exhibit hepatoprotective (Babu *et al.*, 2001) and tumour reducing activities (Babu *et al.*, 2002). Antioxidant, hepatoprotective, leishmanicidal, tumour reducing and anticancer activities of various extracts of *A. ilicifolius* have been reported (Babu *et al.*, 2001, 2002), it is also reported to be resistant to fungi (Bandaranayake, 2002; Wu *et al.*, 2003).

Anticancer activity: A study on evaluated tumour reducing and anti-carcinogenic activities of extracts of *A. ilicifolius* reported that the alcoholic extract of *A. ilicifolius* was effective in controlling tumour progression and inhibiting carcinogenisis of induced papilloma formation in mice. The extract showed cytotoxic activity against lung fibroblast cells and Ehrlich's ascites carcinoma (EAC) cells (Babu *et al.*, 2002; Chakraborty *et al.*, 2007).

Anti-inflammatory activity: It also reduced protein exudation and leucocyte migration in the peritoneal fluid effectively inhibiting peritoneal inflammation, inhibited COX (1 and 2) and 5-LOX activity, and the production of pro-inflammatory cytokines (TNF alpha and IL-6) (Mani Senthil Kumar *et al.*, 2008).

Anti-oxidant activity: It was found that the alcoholic extract of *A. illicifolius* leaves inhibited the formation of oxygen derived free radicals (ODFR) in vitro. The methanolic fraction of *A. ilicifolius* leaf extract was found to have significant free radical scavenging activity (DPPH, ABTS, superoxide and hydroxyl radical). Upon intraperitoneal administration it was found that it augmented the endogenous antioxidant status (Babu *et al.*, 2001; Mani Senthil Kumar *et al.*, 2008).

Hepatoprotective activity: Oral administration of alcoholic extract of *A. ilicifolius* leaves was seen to significantly reduce carbon tetrachloride (CCl_4) induced hepatotoxicity in rats, as judged from the serum and tissue activity of marker enzymes: glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) (Babu *et al.*, 2001).

Osteoblastic activity: The methanolic extract of leaves of *A. ilicifolius* contained acancifoliuside, acteoside, isoacteroside, acanthaminoside, (+)-lyoniresinol 3a-o-b-glucopyranoside, (-)-lyoniresinol and a-amyrin. Acteoside, isoacteoside and (+)-lyoniresinol 3a-O-β-glucopyranoside was found to increase the growth and differentiation of osteoblasts significantly (Van Kiem *et al.*, 2008).

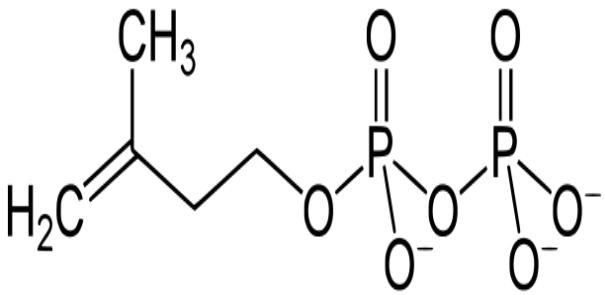
2.4.2.3 Phytochemistry

The phytochemical analysis results showed that *A. ilicifolius* contains saponin, tannins, cardiac glycosides, terpenoids, flavanoids, anthraquionones, alkaloids, total flavonoid and total phenolic (Poornaa, 2011). *A. ilicifolius* has been reported in various studies as being a rich sources of long chain alcohols, terpenes, steroids and triterpenoidal saponins (Bandaranayake, 2002). 2- benzoxazolinone and acanthicifoline (Cordell, 1999). Stigmasterol, a common plant steroid, is present in this plant in large quantity (Wöstmann & Liebezeit, 2008b). A phenylethanoid glycoside (ilicifolioside A), aliphatic alcohol glycoside (ilicifolioside B (Kanchanapoom *et al.*, 2001b). Two lignan

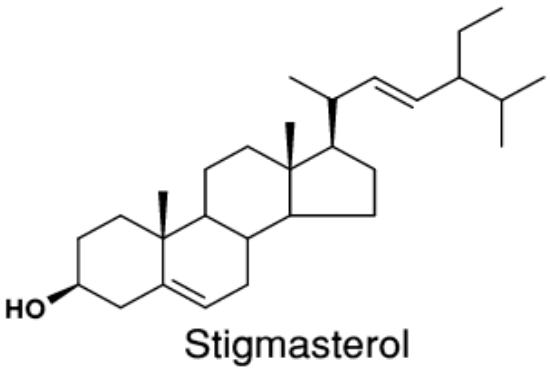
glucosides, (+)-lyoniresinol3a-[2-(3,5-dimethoxy-4-hydroxy)-benzoyl]-O-beta-glucopyranoside, and dihydroxymethyl-bis (3,5-dimethoxy-4-hydroxyphenyl) tetrahydrofuran-9 (or 9')-O-beta-glucopyranoside have been isolated from the aerial parts (Kanchanapoom *et al.*, 2001b). 11-epoxymegastigmane glucoside and megastigmane glucosides (roseoside) have been reported from a *A. ilicifolius* growing in China (Wu *et al.*, 2003). Blepharin (Singh *et al.*, 2011). A new coumaric acid derivative acancifoliuside, acteoside, isoacteoside, acanthaminoside, (+)-lyoniresinol 3a-O-beta-glucopyranoside, (-)-lyoniresinol, and alpha-amyrin (Huo et al., 2008). Protein, Amino acids (Kumar *et al.*, 2009). Triterpenoids, alkaloids, phenolic compounds, lignan, flavonoid, steroids, and terpenoids (Wöstmann & Liebezeit, 2008b). Phytosterols (Law, 2000). Sitosterol, stigmasterol (Figure 2.10) and campesterol (Jones & Raeini-Sarjaz, 2001).

Therefore based on the above mentioned papers, this study was undertaken in rats to evaluate any possible chemoprotective properties of ethanolic extracts of *C. roseus* and *A. ilicifolius* against foci of aberrant crypts.

Figure 2.9: *Acanthus ilicifolius*



(A)



(B)

Figure 2.10: Chemical structure of (A) terpenoid isopentenyl pyrophosphate; (B) Stigmasterol (Kamboj & Saluja, 2011)

2.5 Treatment of colon cancer by natural anticancer

Cancer chemoprevention is defined as the use of a natural crude extract capable of inducing biological mechanisms necessary to preserve genomic fidelity (Hauser & Jung, 2008; Pan & Ho, 2008; Umar *et al.*, 2002). This mechanisms comprise protection against mutagens/carcinogens through the inhibition of uptake, activation or via enhanced DNA repair/replication or apoptosis (Hauser & Jung, 2008). A potential chemopreventive agent has to be able to prevent, inhibit, or reverse carcinogenesis prior to the development of the invasive disease (Krzystyniak, 2002; Pan & Ho, 2008). A model agent is one that has a defined mechanism of action, kills cancer cells while sparing normal ones, is effective in multiple sites, can be given orally, and has low cost and high human acceptance. These agents belong to four different classes; as alkaloids, the epipodophyllotoxins, the taxanes, and the camptothecin derivatives (Darwiche *et al.*, 2007). In 1942 the anti-cancer properties of podophyllotoxin, from the plant extract of *Podophyllum peltatum,* were discovered, but due to its high toxicity it has been ignored (You, 2005). In the mid-1960, semi-synthetic analogues of podophyllotoxin, etoposide and teniposide, were discovered and are used for the treatment of lymphomas, Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute leukemia, lung, prostate, bronchial, testicular and cancers (Bhutani *et al.*, 2006; Kelland, 2005; You, 2005). *Taxus brevifolia*, Paclitaxel, and its semi-synthetic analogue docetaxel are widely used taxanes, a class of alkaloids that possess potent anticancer activities. While paclitaxel is used for the treatment of ovarian, breast, prostate, urological and bladder cancers, docetaxel is used for the treatment of metastatic breast, ovarian, urothelial, lung, head and neck, gastric and prostate cancers (Kelland, 2005; Lyseng-Williamson & Fenton, 2005; Yusuf *et al.*, 2003). Camptothecin (CPT), isolated in 1966 from the Chinese tree *Camptotheca acuminate,* was ignored in the 1970s due to its severe toxicity, despite its strong anti-tumour properties against gastrointestinal and urinary bladder tumours (Li *et*

al., 2006; Wall *et al.*, 1966). Other plant derived compounds such as combretastatin, roscovitine and flavopiridol (among others) are still under clinical trials and show promising anticancer results (Cragg & Newman, 2005; Darwiche *et al.*, 2007; Desai *et al.*, 2008).

For instance, vinblastine and vincristine (vinca alkaloids) isolated from the *C. roseus* plant have been used for the treatment of hematologic cancers (Advani *et al.*, 2006; Cragg & Newman, 2005; Kumar *et al.*, 2006). Vinorelbine and vindesine are semi-synthetic analogues of vinca alkaloids that show less toxicity and a wider range of anti-tumour properties when compared to vinblastine and vincristine (Darwiche *et al.*, 2007). Due to the fact that plants are host to many secondary metabolites that may provide chemoprevention against cancer, they potentially represent an almost inexhaustible source for the discovery of new drugs.

2.6 Antioxidant properties of extracts *in vitro* and *in vivo*

2.6.1 Free radicals and reactive oxygen species

Free radicals are chemical compounds which contain unpaired electrons in their outer electron orbit. Free radicals are energetic and highly unstable. In order to gain stability they always seek other electrons to pair with, attacking and stealing electrons from other molecules such as lipids, proteins, DNA and carbohydrates. They can even damage DNA which leads to mutation and chromosomal damage. The attacked molecule loses its electron and becomes a free radical itself, this initiates an uncontrolled chain reaction that can damage the natural function of the living cell, resulting in various diseases (Valko *et al.*, 2006).

An important incident associated with cancer and its progression is oxidative stress, which has been proven in many studies (Valko *et al.*, 2006; Wiseman &

Halliwell, 1996).Oxidative stress results from an imbalanced intracellular production of ROS and antioxidant defences (Kashyap & Farrugia, 2011).

ROS are highly reactive due to their unstable electron configurations that contribute to the attraction of electrons from other molecules (Matés & Sánchez-Jiménez, 2000). ROS includes free radicals, such as hydroxyl and superoxide radicals, as well as non-radicals including hydrogen peroxide and singlet oxygen (Table 2.1) Generally, ROS can be found in all aerobic cells and are generated by various endogenous metabolic processes such as mitochondrial respiration or exogenous source, which includes UV light, ionizing radiation, inflammatory cytokines, smoking, alcoholic beverages, food and carbonated drinks (San Miguel *et al.*, 2011; Waris & Ahsan, 2006).

Table 2.1: Reactive Oxygen Species (ROS) and their reactivity

ROS	Symbol	Half-life (in seconds)	Reactivity
Superoxide	$O^{2\bullet}$	10^{-6}	Generated in mitochondria, in cardiovascular system and others
Hydroxyl radical	·OH	10^{-9}	Very highly reactive, generated during iron overload and such conditions in our body
Hydrogen peroxide	H_2O_2	stable	Formed in our body by large number of reactions and yields potent species like ·OH
Peroxyl radical	ROO·	second	Reactive and formed from lipids proteins, DNA, surges ect. During oxidative damage.
Organic hydroperoxide	ROOH	stable	Reacts with transient metal ions to yield reactive species.
Singlet oxygen	$^1O_2$	10^{-6}	Highly reactive, formed during photosensitization and chemical reaction.

(Devasagayam *et al.*, 2004).

2.6.1.1 Role of oxidative stress in carcinogenesis

When there is an overload of ROS concomitant with the decreased levels of antioxidants in the body the phenomenon is called oxidative stress. There is amble

evidence that correlates oxidative stress and antioxidant status with various malignant cancers, such as breast (Ray *et al.*, 2000), prostate (Sikka, 2003), colorectal (Özdemirler *et al.*, 1998) and many more (Valko *et al.*, 2004; Waris & Ahsan, 2006). Biological molecules such as lipids, carbohydrates, DNA and proteins are always at risk of being oxidized by ROS, which leads to chronic inflammation and cancer.

The most striking result caused by ROS is DNA damage, which potentially leads to carcinogenesis. In general, cancer development is characterized by three stages: initiation, promotion and progression, of which ROS plays a role in all these stages (Steinberg, 2009). Among these three stages the promotion stage is the main line of ROS-related cancer promotion (Valko *et al.*, 2006). ROS can directly produce single or double-stranded DNA breaks and cross-links, as well as chromosomal instability, gene mutations and altered gene expression (Figure 2.11).

The free radicals can be classified as reactive oxygen species (ROS) or reactive nitrogen species. ROS are the most important free radicals in our body, referring in this case to any free radical involving oxygen-centred free radicals containing two unpaired electrons in their outer shell. There are two sources of free radicals, namely endogenous and exogenous sources. 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 oxidase. The biochemical reactions catalyzed by the cytochrome P450 molecules use molecular oxygen, and during these reactions small amounts of ROS are generated. The extent of ROS generation may vary considerably depending on the compound being degraded and on the cytochrome P450 molecule involved. One type of cytochrome molecule that is especially active in producing ROS is known as cytochrome P450 2E1 (CYP2E1) (Lieber, 1997).

The exogenous sources come from environmental contaminants such as smoking, toxic chemicals, radiation, air pollution, organic solvents and pesticides (Büyükokuroğlua *et al.,* 2001). Free radicals are involved in many physiological processes and human diseases such as cancer, arteriosclerosis, aging, arthritis, Parkinson syndrome, ischaemia, toxin induced reaction, alcoholism and liver injury (Willcox *et al.*, 2004).

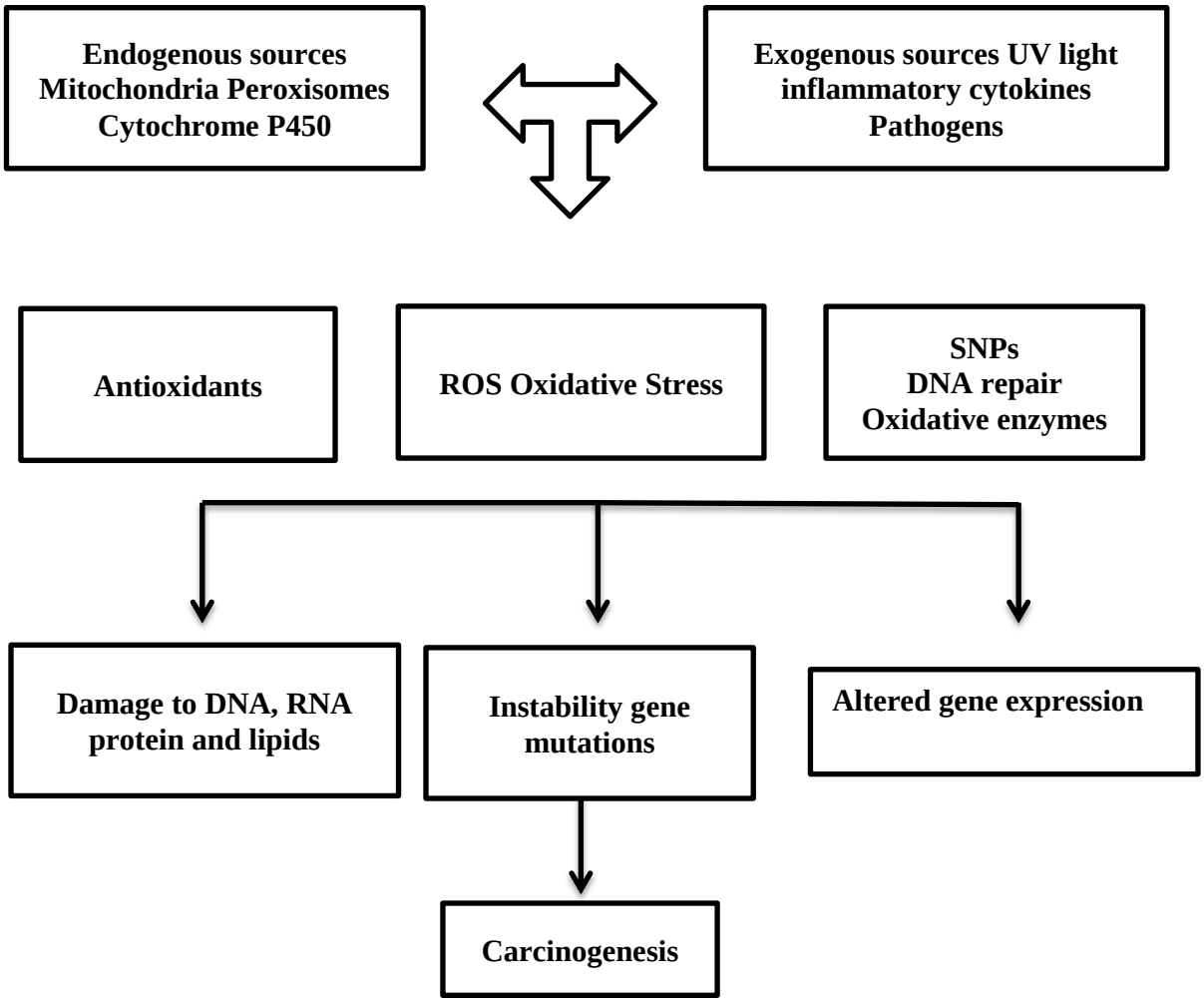


Figure 2.11: Role of reactive oxygen species (ROS) in the development of cancer (Klaunig *et al.*, 2010)

2.6.1.2 Antioxidant defence mechanisms

Since cancer may enhance oxidative stress through various pathways, supplementation of antioxidants may protect against ROS and prevent damage to cells (Halliwell, 2001). The main characteristic of an antioxidant is its ability to trap free radicals and play a

role as an electron or H^+ donating agent (Prakash, 2001). In most cases antioxidants function either in preventing ROS from being formed, or else remove them before they can damage vital cell components (Sies, 1997; Vertuani *et al.*, 2004). The antioxidants can interfere with the oxidation process by reacting with free radicals chelating catalytic metals and by acting as an oxygen scavenger.

There are two major antioxidant defence systems that can be used in order to reduce ROS and protect against tissue damage caused by ROS, which includes enzymatic and non-enzymatic antioxidants. Non-enzymatic antioxidant include vitamin C, vitamin E, carotenoids, thiol antioxidants (glutathione, thiol antioxidants (glutathione, thioredoxin, lipoic acid)) natural flavonoids, a hormonal product of the pineal gland and melatonin (Valko *et al.*, 2006). Their enzymatic counterparts comprise of superoxide dismutase (SOD) and catalase (CAT) (Valko *et al.*, 2006). Induction of enzymatic antioxidants is important for protecting against oxidative stress (Gill & Tuteja, 2010) as shown in (Figure 2.12). SOD is the most effective intracellular antioxidant, since it provides the first line of defence against elevated levels of ROS. SOD catalyzes the dismutation of $O_2^{\cdot}$ to O_2 and to the less reactive species H_2O_2 which is then converted to in to H_2O and O_2 by the CAT that is located in peroxisome. However, the innate defence system may not be effective enough for handling severe oxidative stress. It is believed that additional antioxidants obtained through nutrition may help counterbalance this problem. Mostly, the protective effects of antioxidants in the diet have been attributed to micronutrients such as vitamins C and E, carotenoids and plant polyphenols (Jakesevic *et al.*, 2011).

Superoxide dismutase: $O_2^{\cdot} + O_2\cdot + 2H^+ \longrightarrow 2H_2O_2 + O_2$

Catalase: $2H_2O_2 \longrightarrow 2H_2O + O_2$

Figure 2.12: Reaction catalysed by ROS scavenging antioxidant enzymes (Valko *et al.*, 2006)

2.6.1.3 Role of nutrition in preventing cancer

In the last decade, research explored a crucial role of nutrition in the prevention of chronic diseases especially cancer (Lobo *et al.*, 2010). Reduced cancer risk by diet is reported through cancer specific mechanisms such as antioxidant activity, modulation of detoxification enzymes, stimulation of immunologic response, modulation of hormonal level and antiproliferative activities (Boffetta *et al.*, 2010).

To date, dietary intervention has been extensively explored as a way to reduce the risks of developing colon cancer (Liong, 2008), as diet is one of the modifiable risk factors which have been found to influence colonic microflora and their enzymes in colon carcinogenesis (Gourineni *et al.*, 2011). Waladkhani and Clemens (2001) reported that consumption of fruits ,vegetables, whole grains, beans, nuts and seeds contain fibre and biologically active phytochemicals that the human body needs to fight diseases, to the extent that such a diet can help to reduce the chances of colorectal cancer from reoccurring.

2.6.1.4 Evaluation of antioxidant activity

In vitro and *in vivo* antioxidant models were used to assess the antioxidant potential of the herbal extract. A great number of *in vitro* methods have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. Electron transfer reactions are the most famous *In vitro* methods, like trolox equivalent

antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), α, α-diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), Hydroxyl radical scavenging assay, nitric oxide radical scavenging assay and total phenol content. These methods are popular due to their high speed and sensitivity. However, it is essential to use more than one method to evaluate the antioxidant capacity of plant materials because of the complex nature of phytochemicals (Chanda & Dave, 2009).

In addition to the *in vitro* assay that can be carried out on plant extracts, there are many *in vivo* cellular enzymes and compounds that may be leveraged on experimental animal tissue homogenate. Cells have developed a comprehensive array of antioxidants that act co-operatively *in vivo* to combat the deleterious effects of free radicals. Superoxide dismutase (SOD) and catalase (CAT) are considered primary antioxidant enzymes since they are involved in the direct elimination of ROS. SOD scavenges the superoxide radical (O_2•-) by converting it to hydrogen peroxide (H_2O_2), hence reducing the toxic effects due to this radical or other free radicals derived from secondary reactions. CAT subsequently reacts with H_2O_2 and decomposes it into water and molecular oxygen (Halliwell & Gutteridge, 1986; Vattem *et al.*, 2005).

A delicate balance between the generation of free radicals, endogenous and exogenous antioxidants is of critical importance for the physiological functioning of the cell. It has been proposed that a net pro-oxidant state, which occurs when there is a loss of homeostasis between ROS generation and detoxification, i.e. oxidative stress, results in lipid peroxidation and DNA damage leading to carcinogenesis (Khanzode *et al.*, 2003). Several reports have demonstrated that increases in the oxidant/antioxidant ratio are directly correlated with tumour progression, angiogenesis and migration/invasion (Ho *et al.*, 2001; Kumaraguruparan *et al.*, 2002; Nishikawa, 2008; Ścibior *et al.*, 2008).

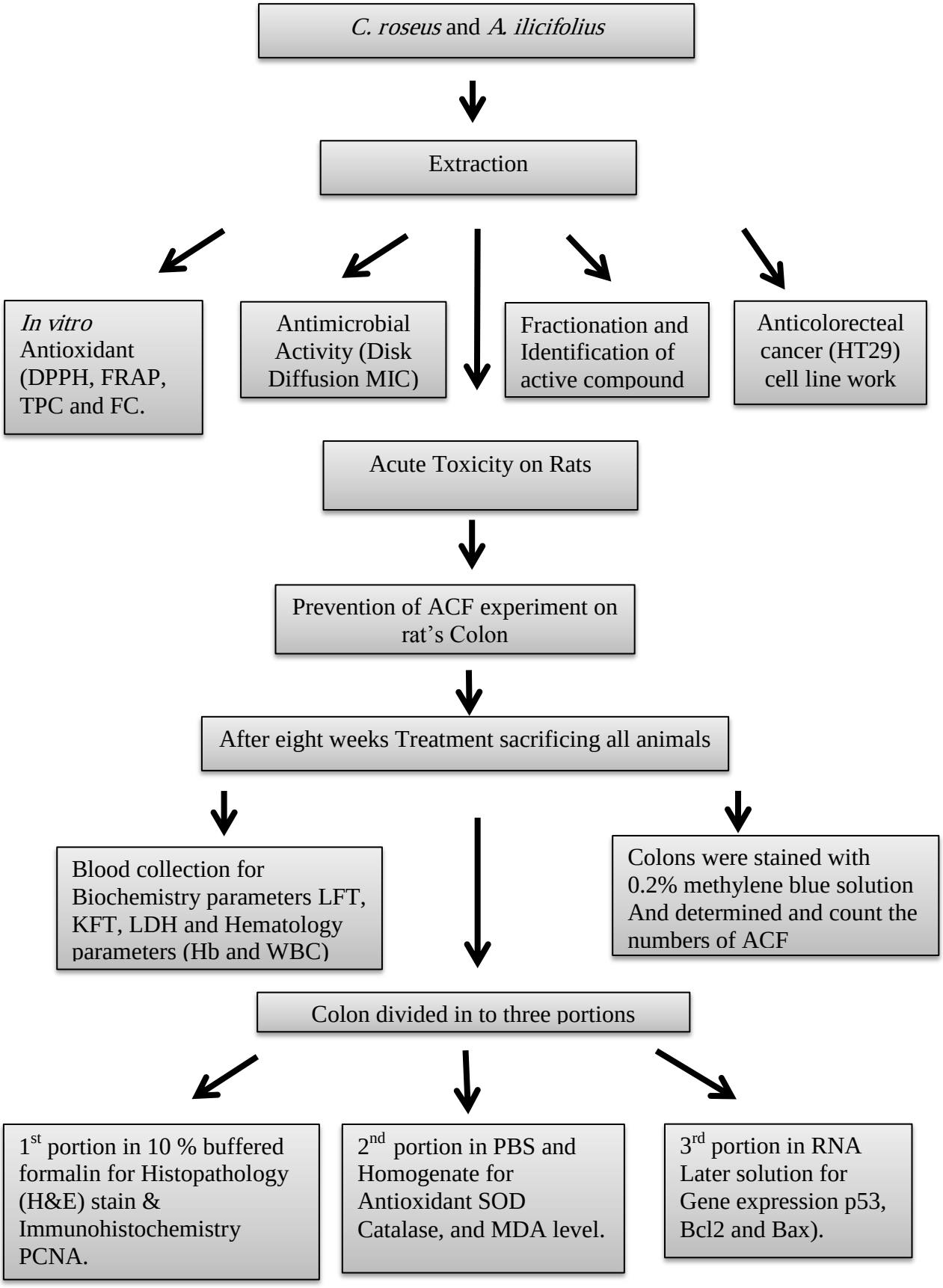
Malondialdehyde (MDA) was reported as being one of the major secondary oxidation products of peroxidized polyunsaturated fatty acids. It has been reported to

have mutagenic and cytotoxic effects (Mateos *et al.*, 2005). MDA has been found elevated in various diseases that relate to free radical damage. Hence, it has been spectrophometrically used as a biomarker for the assessment of lipid peroxidation in biological and medical sciences (Mateos *et al.*, 2005). It is a simple, sensitive and inexpensive method to observe the effect of oxidative stress on the levels of lipid peroxidation in cells; it has been adapted in biological samples, such as rat plasma, human plasma and urine, as well as other tissues organs such as the liver and lungs. Its feasibility as a biomarker for oxidative stress *in vivo* in a rat model has been deemed a success (Mateos *et al.*, 2005).

CHAPTER III

METHODOLOGY

3.1 Experimental design



3.2 Plants material

3.2.1 Preparation of plant extracts

Fresh leaves of *C. roseus* and *A. ilicifolius* were obtained from Ethno Resources Sdn Bhd, Selangor Malaysia, and identified by comparison with the voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur. The leaves were tap washed and oven dried for 7 days before finely ground using an electrical blender to produce 100g of fine powder from each plant, these were then soaked separately in 900ml of absolute ethanol in conical flasks for 3 days at room temperature. The mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No1) and distilled under reduced pressure in an Buchi-type R-215 (Rotavapor, Switerland), then dried in a freeze-drying machine (LabConco, Kansas City, MO, USA) to give the crude-dried extract. The dried mass yielded 15.7% and 13.5% (w/w) dry weights, referring to *C. roseus* and *A. ilicifolius* respectively. The dry extracts of each plant were then dissolved in Tween-20 (10 % w/v) and administered orally to rats at a dosage of 250 and 500 mg/kg body weight.

3.2.2 Chemicals and reagents

Azoxymethane (AOM) was purchased from Sigma-Aldrich St. Louis, MO, USA and was used to induce ACF in the rat colon. All other chemicals used were of analytical grade and purchased mostly from Sigma-Aldrich and Fisher. AOM was dissolved in normal saline and injected subcutinuse to the rats at a dosage of 15mg/kg body weight (Marotta *et al.*, 2003). 5-Fluorouracil (purchased from Calbiochem, USA) as a standerd drug was dissolved in normal salin and injected intraperitoneally to the rats at a dosage of 35mg/kg body weight (Srimuangwong *et al.*, 2012).

3.3 Determination of antioxidant activity *in vitro*

The *in vitro* antioxidant activity of the *C.roseus* and *A.ilicifolius* ethanolic extract was measured using the ferric reducing antioxidant power (FRAP), DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity assays, total phenolic content (TPC) and flavonoid content (FC).

3.3.1 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing activity of the plant extracts were estimated using the method with slight modification developed by (Benzie & Strain, 1996).

3.3.1.1 The principle

At low pH, in the presence of antioxidants, a ferric- tripyridyltriazine (Fe III TPTZ) complex will be reduced to the ferrous Fe (II) and yielded an intense blue colour that absorbed at 593nm.

3.3.1.2 Materials

All chemicals were supplied by Sigma-Aldrich (Steinhem,Germany). Comprising of sodium acetate trihydrate, glacial acetic acid, 40mmol/L HCL, 2,4,6-tripyridly-s-triazine (TPTZ), iron III chloride ($FeCl_3$), ferrous iron II sulphate ($FeSO_4$), ascorbic acid (Vitamin C), quercetin, butylated hydroxytoluene 99% (BHT) and DMSO.

3.3.1.3 Preparation of FRAP reagent

A –Preparing the Acetate buffer (300 mM, pH 3.6):

Glacial acid (0.4ml) added to 0.0775g of sodium acetate trihydrate, the solution was then made up to 25ml using distilled water. The pH of the solution was checked by using a pH meter.

B – Preparing the TPTZ solution:

TPTZ 0.00781 g (M.W. 312.34) 10 mM was added to 0.1ml of 40 mmol/L HCL (M.W.36.46) and was made up to 2.5ml with double distilled water.

C – Preparing the Ferric chloride Reagent:

Ferric chloride 0.0135 g ($FeCl_3H_2O$) (M.W. 270.30) 20 mM was dissolved in 2.5ml of double distilled water.

D- Preparing the FRAP Reagents:

2.5 ml of reagent B and 2.5 ml of reagent C were added to 25 ml of reagent A to make 30 ml of FRAP reagent.

E-Preparing the standard solution:

Dissolving 0.00278g of $FeSO_4.7H_2O$ in 10ml DMSO (stock solution).

F-Preparing the standard serial dilutions:

The stock solution dilutes as follows to make a serial of standard solution:

Standard concentration (µM)	**$FeSO_4.7H_2O$ solution (µl)**	**Distilled water (µl)**	**Total volume (µl)**
0	0	1000	1000
200	200	800	1000
400	400	600	1000
600	600	400	1000
800	800	200	1000
1000	1000	0	1000

Which the first dilution was reagent blank

G-Preparation of control:

Vitamin C 0.001g, BHT, Quercetin, Gallic acid were dissolved in 1ml DMSO.

3.3.1.4 Preparation of crude extracts (*C. roseus* and *A. ilicifolius*)

C. roseus and *A. ilicifolius* 0.001g were dissolved in 1ml DMSO separately.

3.3.1.5 The procedure of FRAP assay

The reaction mixture involved 300 mmol/L acetate protect, 10 mmol/L TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol/L of HCl level of acidity and 20 mmol/L of $FeCl_3.6H_2O$. The essential FRAP reagent was prepared clean by mixing 25ml of acetate protect, 2.5ml of TPTZ solution and 2.5ml of $FeCl_3.6H_2O$. The clean prepared mixture was incubated at 37°C in the water bath for five minutes and then a blank reading was taken spectrophotometrically at 593nm. After that, 10µl of extract or standard was introduced to 300µl of the working FRAP reagent. Absorbance was measured immediately upon addition of the working FRAP reagent after mixing. Thereafter, absorbance measurements were taken four minutes later. The standard curve was plotted using $FeSO_4$ solution absorbance as standard, and the results were indicated as µmol Fe (II)/g dry body weight of crude extract (Appendix A1).

3.3.2 Free radical scavenging activity (DPPH) assay

The antioxidant activities of extracts were determined using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The assay was carried out as described by (Brand-Williams *et al.,* 1995) with minor modification.

3.3.2.1 Materials

All chemicals were supplied by Sigma-Aldrich (Steinhem, Germany),1,1-diphenyl-2-picryl-hydrazyl (DPPH). The chemicals comprised of acid, ascorbic acid (Vitamin C), quercetin, BHT, and trolex.

3.3.2.2 The principle

DPPH is a molecule containing a stable free radical. In the use of an anti-oxidant which can offer an electron to DPPH, the purple colour which is typical for a 100 % free DPPH excessive decays and modifies its absorbency at 515nm which can then be studied. This assay measures the free radical scavenging capacity of the investigated extracts.

3.3.2.3 Preparation of reagent

A- Preparing the DPPH reagents

0.002g of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) dissolved in 50ml of 95% ethanol solution.

B- Preparing the standard solution

0.001g of (Vitamin C) was dissolved in 1ml DMSO, 900µl of distilled water was add to 100µl of vitamin C, the solution was than mad up to 1000µl.

C- Preparing the standard serial dilutions

The stock solutions dilute as follows to make a serial of standard solution:

Standard concentration (µg/ml)	DPPH solution (µl)	Distilled water (µl)	Total volume (µl)
0	0	100	0
3.90	3.90	996.1	1000
7.81	7.81	992.19	1000
15.62	15.62	925	1000
31.25	31.25	984.38	1000
62.5	62.5	937.5	1000

D-Preparation of control

0.001g of each BHT, quercetin, and trolex were dissolved in 1 ml DMSO.

3.3.2.4 Preparation of crude extract (*C. roseus* and *A. ilicifolius*)

0.001 g of each *C. roseus* and *A. ilicifolius* dissolved in 1 ml DMSO.

3.3.2.5 The procedure of DPPH assay

Briefly 0.001g from ethanolic extracts dissolved in 1ml DMSO and then serial diluted to get different concentrations (50, 25, 12.5, 6.25 and 3.125 µg/ml). Qurecrtine, BHT, and trolox were used as standard antioxidants. A quantity of (5µl) from each plant extract and standard were mixed with 195µl of DPPH in triplicate. The decrease in absorbance value was measured at 515nm after 20min intervals. The radical scavenging activity was calculated from the following equation and the results were expressed as:

Mean ± Standard Error (S.E.M.)

% of radical scavenging activity = [(Abs Blank – Abs Sample)/Abs Blank) x100].

3.3.3 Determination of total phenolic and flavonoid content

3.3.3.1 Total phenolic content assay

3.3.3.1.1 The principle

The *C. roseus* and *A. ilicifolius* extracts were evaluated for their total phenolic content by using the Folin-Ciocalteu reagent and was calculated as gallic acid equivalents in mg (GAE)/g of extract according to the Folin-Denis colourimetric method (AOAC, 1995).

The reagent oxidizes phenolates (ionized phenolic) present in the sample reduces the acids to form a blue complex .The colour develops in a slightly alkaline environment which is provided by the sodium carbonate. The blue chromagen is quantified at 765 nm absorbance.

3.3.3.1.2 Materials

All chemicals were supplied by Sigma-Aldrich and Merck (Germany), commercial Folin-ciocalten reagent, Sodium carbonate and Gallic acid.

3.4.3.1.3 Preparation of reagent

A- Preparing the 10 % Folin reagent

10 ml of commercial Folin reagent diluted with 90ml H_2O the solution was than made up to (1:10).

B- Preparing the 10% sodium carbonate solution

5.75g of sodium carbonate dissolved in 50ml distilled water.

C- Preparing the standard solution

Gallic acid was used as standard

Dissolving 0.002g of Gallic acid in 1 ml DMSO (stock solution)

D- Preparing the standard serial dilutions

The stock solution dilutes as follows to make a serial of standard solution:

Standard concentration (mg/ml)	Gallic acid (µl)	Distilled water (µl)	Total volume (100 µl)
0	0	100	100
10	5	95	100
50	25	75	100
100	50	50	100
150	75	25	100
200	100	0	100

3.3.3.1.4 Preparation of crude extract (*A. ilicifolius* and *C. roseus*)

0.001g of each *A. ilicifolius* and *C .roseus* dissolved in 1ml DMSO.

3.3.3.1.5 The procedure of TPC assay

Briefly, 10µl of each standard and extract was mixed with 500 µl of Folin–Ciocalteu reagent. After 5 minutes, 0.35ml of sodium carbonate (Na_2Co_3) were added and the mixture was incubated at 30°C for 2 hours with intermittent shaking. The absorbance of the resulting blue-coloured solution was measured at 765nm and the total phenolic content was expressed as gallic acid equivalents mg (GAE)/g of dry weight.

Each test sample dilution was tested in triplicate to allow a mean absorbance to be calculated. TPC values of the plant crude extract were determined using the standard curve of absorbance versus of the concentration gallic acid equivalents (mg GAE/g).

3.3.3.2. Determination of total flavonoids content

The total flavonoids was determined by using the aluminium chloride colourimetric method and expressed as quercetin equivalents in mg (QE)/g of extract as described by (Dowd, 1959). Both assays were carried out in triplicate.

3.3.3.2.1 Materials

All chemicals were supplied by (Fisher chemicals, UK). They comprised of aluminium trichloride, potassium acetate (CH_3COOH), quercetin and DMSO.

3.3.3.2.2 Preparation of Flavonoid Reagent

A- Aluminium trichloride ($AlCl_3$) solution:

0.5g of aluminium trichloride ($AlCl_3$) dissolved in 5ml of distilled water.

B- Potassium acetate solution:

0.491g of potassium acetate dissolved in 5ml of distilled water.

C- 80 % Ethanol alcohol:

84.21ml of ethanol (95%) was added to 15.79ml of distilled water to make an 8.0% ethanol alcohol solution.

D- Preparing the standard solution

Dissolving 0.001g of quercetin in 1ml DMSO (stock solution)

E- Preparing the standard serial dilutions

200µl of stock solution was adding to 800µl of distilled water.

The stock solution dilutes as follows to make a serial of standard solution:

Standard concentration (mg/ml)	Quercetin solution (µl)	Distilled water (µl)	Total volume (1000µl))
0	0	1000	1000
25	25	975	1000
50	50	950	1000
100	100	900	1000

3.3.3.2.3 Preparation of crude extract (*A. ilicifolius* and *C. roseus*)

0.001g of each *A. ilicifolius* and *C. roseus* dissolved in 1ml DMSO.

3.3.3.2.4 The procedure of total flavonoid content assay

Briefly, 500 µl of extract was mixed with 1.5ml of 80 % ethanol, 100µl of 10% aluminum ($AlCl_3$) 100µl of 1 M potassium acetate (CH_3COOH), and 2.8ml of deionized water. 250µl of mixture was incubated at room temperature for 30 minutes and the absorbance was measured at 415nm. The data was expressed as milligram quercetin equivalents (QE)/g of extract. The total flavonoid content was expressed as quercetin equivalents mg (QE)/g dry weight.

3.4 Acute toxicity test

Adult male and female *Sprague Dawley* rats (6 - 8 weeks old) were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur. The rats weighed between 120g–150g. The animals were given standard rat pellets and tap water. The acute toxic study was used to determine a safe dose for *C. roseus* and *A. ilicifolius* extracts. The test extracts should be administered to animals to identify doses causing no adverse effect and doses causing major (life-threatening) toxicity. Thirty six *Sprague Dawley* rats (18 males and 18 females) were assigned equally into 3 groups labelled as vehicle (10% Tween-20), low dose (2 g/kg) and high dose (5 g/kg) of *C. roseus* and *A. ilicifolius* extracts. The animals fasted overnight (no food, but allowed water) prior to dosing. Food was withheld for a further 3 to 4 hours after dosing. The animals were observed for 30 minutes and 2, 4, 8, 24 and 48 hours after the administration for the onset of clinical or toxicological symptoms. Mortality (if any) was observed over a period of 2 weeks. The animals were sacrificed on the 15^{th} day after overnight fasting. Histology and serum biochemical parameters were determined using standard methods (OECD, 2001).

3.5 Animals and treatments

Sprague Dawley healthy adult male rats were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya (Ethic No. PM/07/05/2012/MMA (b) (R). The rats were divided randomly into seven groups of 6 rats each. Each rat that weighed between 120g-150g was placed individually in separate wire-bottomed cages. The animals were maintained on a standard pellet diet and tap water. Throughout the experiments, all animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institutional Animal Care and Use Committee, University of Malaya (UM IACUA).

Group 1 (normal control) received subcutaneous injection normal saline, once weekly for 2 weeks. Normal control rats were continued on 10 % Tween-20 feeding for 8 weeks.

In Group 2-5 received subcutaneous injection of AOM, 15mg/kg body weight, once weekly for 2 weeks.

Group 2 (AOM group) were continued on 10 % Tween-20 feeding for 8 weeks.

Group 3 (reference group) were continued on 35 mg/kg 5-Fluorouracil drug by intraperitoneal injection once weekly for 8 weeks.

Groups 4 and 5 (treatment groups) continued on 250 and 500 mg/kg for each plant for 8 weeks respectively.

The body weight of all animals were recorded once weekly for 10 weeks, and were sacrificed after overnight fasting by intramuscular injection of ketamine and xylazine anesthesia. Blood samples were collected and serum was separated for assay of colon biomarker. The colons were washed with cold normal saline.

3.6 Quantification of ACF on colon mucosa grossly

Colons were split open longitudinally cut into proximal, middle and distal portions of the same length. Topographic examination of the colonic mucosa with slight modification as Bird suggested (1995) was completed following 5 minutes fixation in 10% phosphate buffered formalin. Colons were stained with a 0.2% methylene blue solution for 10 minutes, placed mucosal side up on a microscopic slide, and observed under a light microscope (10x). The sum of ACF in the whole colon was counted in each 2cm part of the colon, beginning from the distal to the proximal end of the colons. Aberrant crypts were differentiated from the surrounding regular crypts by their enlarged size, improved distance from lamina to basal surfaces of cells, as well as easily perceptible pericryptal zone. The variables utilized to evaluate the aberrant crypts were occurrence and multiplicity. Aberrant crypt multiplicity was considered as the number

of crypts in every focus and classified as containing up to four or more aberrant crypts/focus. Under light microscopy, a microfeather scalpel blade was used to excise the ACF of interest and the surrounding normal crypts.

3.7 Histopathological examination

The Colon was fixed 24 hours in 10% buffered formalin solution for histological study. The fixed tissues were processed by an automated tissue processing machine (Leica Microsystm, Nussloch Germany). Tissues were embedded in paraffin wax by conventional methods. Serial Sections of 5µm thickness were cut parallel to the muscular mucosa using a microtome (Leica Microsystm, Nussloch Germany) and stained with haematoxylin and eosin (H & E) stain according to standard protocol (Appendix A). The histology of each ACF was assessed for crypt architecture and nuclear features by comparison with the normal surrounding crypts which were absorbed under a light microscope (Colympus, Tokyo, Japan) for histopathologyical, and their photomicrographs were captured with a Nokion, Tokyo, Japan).

3.8 Biochemical and haematological analysis

The gathered blood samples were centrifuged at 2500 rpm for 15 minutes subsequent to being completely clotted. Serum for assay of biochemical parameters such as albumin, total protein, urea and lactate dehydrogenase and EDTA blood samples for assay of haematological parameters such as Hb and WBC were examined spectrophotometrically by normal standard automated techniques according to the procedures described by the manufacturer in Central Diagnostic Laboratory (CDL), University of Malaya Medical Centre.

3.9 Estimation of antioxidant in colon tissue

Colon samples were rinsed instantly with ice cold saline to take away as much blood as possible. Colon homogenates (10% w/v) were arranged in a cold 50 mM PBS (pH 7.4) using homogenizer in ice (Wise mix HG-15,Daihan scientific, seoul, Korea). The cell particles were eliminated by centrifugation in a refrigerated centrifuge at 4500rpm for 15 minutes at 4°C using a refrigerated centrifuge (Rotofix32 Hettich Zentrifuge, Germany). The supernatant was used for the estimation of the following *in vivo* antioxidant, using commercially available kits from (Cayman Chemical Company, USA): Malondialdehyde (MDA) or thiobarbituric acid reactive substance (TBARS) (cat # 10009055), Superoxide Dismutase (SOD) (cat # 706002) and catalase (CAT) (cat # 707002) Appendix B. All assays were performed as directed by the producer's manual. Protein concentration was determined by the Bardford method using bovine serum albumin as a standard.

3.9.1 Malondialdehyde (MDA)

3.9.1.1 Principle

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.

3.9.1.2 General procedure

The assay was performed by add 100 µl of the sample ,100 µl stand SDs solution and 4 ml of the colour Reagent .The reaction were initiated by boiling 1hour. To stop the reaction incubate on ice for 10 min, than centrifuge 10 min and adding 150µl to all the wells being used. The absorbance reading was taken at 530-540nm.

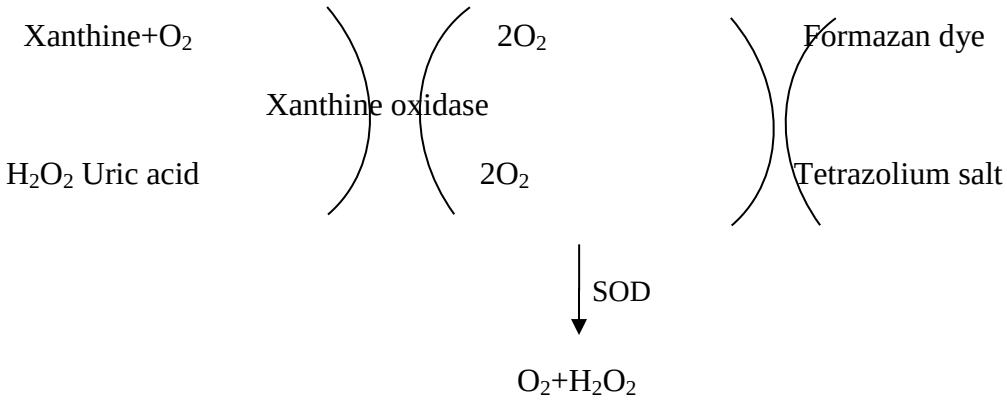
The MDA activity was determined by using the following formula:

$$\text{MDA } (\mu M) = \frac{(\text{Corrected absorbance})-(\text{y-intercept})}{\text{Slop}}$$

3.9.2 Superoxide dismutase (SOD) assay

3.9.2.1 Principle

One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical. Its measures all three types of SOD (Cu/Zn, Mn and FeSOD).



3.9.2.2 General procedure

The assay was performed by adding 200 µl of the diluted radical detector and 10 µl of sample to wells .The reactions were initiated by adding 20 µl of diluted Xanthine Oxidase to all the wells being used. The plate was incubated for 20 minutes at room temperature and the absorbance reading at 440 nm. The SOD activity was determined by using the following formula:

$$\text{SOD (U/ml)} = \frac{(\text{sample LR-y-intercept} \times \text{sample dilution}}{\text{Slope}}$$

3.9.3 Catalase assay

3.9.3.1 Principle

Catalyses conversion of two molecules of H_2O_2 and two molecules of water (catalytic activity).it also demonstrates peroxidatic activity, in which low molecular weight alcohols can sever as electron donors.

(Catalytic Activity) $2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$

(Peroxidatic Activity) $H_2O_2 + AH_2 \xrightarrow{\text{Catalase}} A + 2H_2O$

3.9.3.2 General procedure

The assay was performed by add 100 µl of the Assay buffer, 30 µl of methanol, and 20 µl of the sample. The reaction was initiated by adding 20 µl of Hydrogen Peroxide. To terminate the reactions adding 30 µl of potassium Hydroxide and 30 µl of purpled (chromagen), than incubate for 10 minutes at room temperature, finally adding 10 µl of Potassium Periodate .The absorbance reading was taken at 540 nm.

The formaldehyde concentration of sample was determined by using the following formula:

$$\text{Formaldehyde } (\mu M) = \frac{(\text{Sample absorbance}) - (\text{y-intercept})}{0.02 \text{ ml}} \times \frac{0.17 \text{ ml}}{\text{Slop}}$$

The Catalase activity was determined by using the following formula:

$$\text{CAT Activity} = \frac{\mu M \text{ of sample}}{20 \text{ min}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

3.10 Immunohistochemistry

Colon biopsies were fixed in buffered formalin and were processed by a tissue processing automated machine. Colon specimens were embedded with paraffin. Sections (3-5 µm) used poly-L-lysine-coated slides, colon sections were prepared and heated in an oven (Venticell, MMM, Einrichtungen, Germany) for 25 minutes at 60^{o}C. After heating, colon sections were deparaffinized in xylene and rehydrated in graded alcohol. Concentration of 10mM sodium citrate buffer was boiled in a microwave for antigen retrieval.

Immunohistochemistry staining steps were performed following the manual's instructions (DakoCytomation, USA). In brief, 0.03% hydrogen peroxide sodium azide was used to block the endogenous peroxidase for 5 minutes, followed by washing the tissue sections gently with a wash buffer and then incubated with Proliferating Cell Nuclear Antigen (PCNA) (1:200) (Santa Cruz Biotechnology Inc, California, USA) biotinylated primary antibodies for 15 minutes. After incubation, sections were gently re-washed with a wash buffer and kept in the buffer bath in a humid chamber. Streptavidin-HRP was then added and incubated for 15 minutes followed by washing. Diaminobenzidine-substrate chromagen was added to the sections and incubated for over 7 minutes followed by washing and counterstaining with hematoxylin for 5 seconds. The sections were then dipped 10 times in weak ammonia (0.037 mol/L), washed and then cover slipped. Light microscopy was used to examine the brown-stained positive antigens. The PCNA labeling index (PI) was computed as the [(number of positive cells) / (total number of epithelial cells)] X 100 for each field.

3.11 Real-time quantitative polymerase chain reaction analysis

3.11.1 Reagents

RNA*later*, RNeasy plus Mini Kits, QIA shredder homogenizer, Mortar and pestle, 50%-100% ethanol.

3.11.2 General procedure

From the tissue colon mucosa of normal saline injected rats, AOM-injected rats, 5-Flurouracil-treated AOM-injected rats and of *A. ilicifolius* and *C. roseus*-treated AOM-injected rats colon samples were placed immediately into RNAlater solution (Ambion, USA), incubated over night at 4°C and stored at -80^{o}C. Total RNA was isolated according the manufacturer's instructions using RNeasy plus Mini Kits (catalogue No74134, Qiagen, Germantown, Maryland, USA).

RNA was extracted using the RNeasy-Plus Mini kit according to the manufacturer's protocol, at a weight of 30mg rat tissue and disruption of the tissue being done via a mortar. To hemogenize tissue 600µl of Buffer RLT was added directly to each mortar. The tissues then became lysate. The lysate was collected into a microcentrifuge (QIA shredder) tube and centrifuged twice for 2 minutes and 3 minutes respectively at maximum speed. The resultant lysate was then filtered passed a DNA eliminator spin column and centrifuged for 30 seconds at ≥ 8000 x g ≥ (10,000 rpm). 50% ethanol was added to the homogenized lysate in a1:1 ratio and mixed using pipetting. 600µl of the sample, including any precipitate that may have formed, was transferred to an RNeasy spin column placed in a collection tube and centrifuged for 15 seconds at ≥ 8000 x g. Successive aliquots of any excess of the sample were centrifuged in the same RNeasy spin column. Flow-through was discarded after each centrifugation. After all the samples had been loaded, 600µl of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at ≥ 8000 x g to wash the spin column

membrane. Flow-through was discarded and 500µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at ≥ 8000 x g. Flow-through was discarded and this step was repeated for a longer centrifugation time of 2 minutes. RNeasy spin column was then placed in a new 1.5ml collection tube and 25µl of RNase free water was added directly to the membrane. The column was centrifuged for 1 minute at ≥ 8000 x g to elute the RNA. This step was repeated using another 25µl of RNase free water. All steps were performed at room temperature from the worn out mucosa of the treated and untreated rats. RNA concentrations were measured by a Nano Drop ND-2000 spectrophotometer (Thermo Fisher, USA). RNA quality and integrity were concluded through the A260/A280 ratio and agarose gels electrophoresis respectively.

3.11.3 RNA agarose gel electrophoresis

3.11.3.1 Reagents

Ethidium Bromid (BASE), agarose gel, bromophenol blue loading dye, Tris-Borate EDTA buffer.

3.11.3.2 Principle

The integrity and size distribution of total RNA purified with QIAamp RNA pulse Mini Kits was checked by denaturing agarose gel electrophoresis and ethidium bromide staining through the use of agarose gel electrophoresis apparatus (Bio-Rad, Richmond, CA, USA). The apparent ratio of 28S RNA to 18S RNA should be approximately 2:1. The extracted RNA was stored at −80ºC until used.

3.11.3.3 General procedure

Agarose (0.5g) was dissolved in a Tris-Borate EDTA buffer (50ml; TBE) by heating in a microwave until boiled, this solution was then allowed to cool before ethidium bromide was added (0.5µl). Gels (0.25%) were then cast in a 10 x 20 cm gel tank, any

bubbles were removed with a pipette tip and then gel comb was added before allowing the gel to set. Gel electrophoresis was carried out in a horizontal tank first containing then loading the Gel by Mix reaction volumes onto a piece of parafilm, at the appropriate volumes of 3µl loading dye onto parafilm with 2µl of each sample. Once the appropriate volume of sample and mix are prepared to make up the 5µl sample, they were slowly and carefully placed into the well. TBE buffer was run at 95V for 45 minutes. Migration of the bands was monitored by visualizing bromophenol blue. Gels were imaged using the Typhoon 9410 Variable Mode Imager. The gel was then carefully exposed to UV trans-illuminator. The bands are analysed and pictures of the results are taken.

3.11.4 Reverse transcription and cDNA synthesis

3.11.4.1 Principle

One microgram of total RNA was converted to cDNA using High capacity RNA-to-cDNA reagent from Applied Biosystems, USA.

3.11.4.2 Reagents

High Capacity RNA-to-cDNA Master Mix, nuclease-free water.

3.11.4.3 General procedure

The High Capacity RNA-to-cDNA Master Mix kit (Applied Biosystems, USA) was used for cDNA synthesis. The kit uses the random primer scheme for initiating cDNA synthesis. Random primers ensure that the first strand synthesis occurs efficiently with all species of RNA molecules present, including mRNA and rRNA. Reverse transcription of RNA was carried out in a 20µl final volume using 1000ng total RNA. The measured RNA concentration values were calculated and 1X RT Master mixture was prepared as shown in (Table 3.1) below.

Table 3.1: making the 1X Reverse Transcription (RT) Master Mix for cDNA synthesis

Reagent	Volume per Reaction	Final conc
Master Mix	4_µl	1x
RNA	--µl (Variable (up to 16 µl), calculated according to RNA concentration in order to obtain 1µg cDNA per reaction	10pg- 1µg-1000 ng
Deionized water	--µl	-
Total volume	20µl	-

Referring to the table, the volume of master mix needed to prepare one sample is indicated to the left, from which the required numbers of reactions, for instance 20µl total, were calculated. This calculation also included additional reactions to provide excess volume for the loss that occurred during reagent transfers.

4µl of 1X RT master mix was added into each tube containing 1000ng of RNA sample before adding deionized water (total volume of RNA with deionized water 16µl). The content was mixed by pipetting up and down before the tubes were centrifuged for a short period (1 minute) in order to spin down the contents and to eliminate any air bubbles. The tubes were placed on ice until loading on the thermal cycler conditioner (PTC-100 Programmable Thermal Controller, MJ, USA) as shown in (Table 3.2).

Table 3.2: Thermal cycler condition steps

	Step1	Step2	Step3	Step 4
Temperature	25 °C	42°C	85°C	4°C
Time	5 min	30	5	Hold to 4°C
Event	Random primers to RNA	cDNA synthesis	Inactivation of enzymes	

The cDNA product was stored at -20°C. All the above steps were performed on ice and a nuclease free bench.

3.11.5 Real- time quantitative reverse-transcriptase polymerase chain reaction analysis

3.11.5.1 Procedure

The cDNA synthesis was used as suggested by the manufacturer. RT-PCR was carried out by ABI TaqMan gene expression assays for Bcl-2, Bax and p53.

3.11.5.2 Reagents

ABI TaqMan gene expression assays Tbp (assay ID: Rn01455646_ml), HPRT1 (assay ID: Rn01527840_ml), Bcl-2 (assay ID: Rn99999125), Bax (assay ID: Rn02532082), and P53 (assay ID: Rn00755717), TaqMan Fast Advanced Master Mix reactions, nuclease-free water. All reagents and genes applied from Applied Biosystems, USA.

3.11.5.3 General procedure

To make a standard curve, 20µl of each sample with RT was used to make a cDNA pool. From this cDNA pool, 10µL was taken out and diluted 1:2 as shown in (Table 3.3) below:

Table 3.3: Standard curve dilution for real time PCR

Dilution	
1:2	10µl of pooled sample+10µl dH_2O
1:5	5µl (of 1)+ 25µl d H_2O
1:5	5µl (of 2)+ 25µl d H_2O
1:5	5µl (of 3)+ 25µl d H_2O
1:5	5µl (of 4)+ 25µl d H_2O

The samples are dilution same as standard dilution

3.11.5.4 Prepare the PCR reaction mix

Table 3.4: Components volume and concentration used in the RT-PCR reaction mix (total volume 10 µl)

Component	96-well plate Volume (µl) for 1 reaction	Final concentration
Master Mix	5.0	1x
Gene Expression	0.5	1x
cDNA	1.0	100 ng to 1 pg
Nuclease free water	3.5	-
Total volume per reaction	10	-

Both standard and samples were placed on ice.

According to the protocol guidelines, all samples were run in triplicate in total reaction volume 30µl: 15µl master mix, 1.5µl TaqMan gene expression assay 3µl cDNA and 10.5 µl nuclease-free water. Quantitative real-time RT-PCR was done using TaqMan fast advanced master mix (Applied Biosystems, USA) and the step one plus Real-Time PCR system (Applied Biosystems, USA) in triplicate wells. Cycle parameters were as follows: activation at 95°C for 20 s, 40 cycles of denaturation at 95°C for 1s, and then annealing and extension at 60°C for 20s. The data was analysed by utilising a comparative threshold cycle (Ct) technique. Gene expressions were calculated using the Gene Ex software (MultiD Analyses AB, Sweden). The corresponding mRNA level from colonic mucosa of the normal group (calibrator) was used as an external reference. The levels of Hprt1 (assay ID: Rn01527840) and Tbp (assay ID: Rn01455646) mRNA were used as an internal reference to standardize the data. The fold changes of each mRNA (mRNA relative expression) were expressed relatively to the mean value of the related mRNA located in the mucosa of the normal control rats, and was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Statistical analysis of differences

was performed by a two-tailed unpaired Student's t test. A *p* value of <0.05 was considered indicative of a statistically significant difference.

3.12 Chromatography materials

Silica gel 60 powder (0.063 - 0.200 mm) and 70 - 230 mesh (Merck, Darmstadt, Germany), HPLC grade hexane, HPLC grade ethyl acetate, HPLC grade methanol, HPLC grade acetonitrile were purchased from Merck, Germany. 3.0 x 50 cm Kontes Scientific Glassware (Vineland, NJ, USA) connected with an EYEL-4 pump (Tokyo Rikakikai, Tokyo, Japan).

3.12.1 Profiling and fractionation of crude extracts

The ethanol crude extract of both plants (1g) were subjected to column chromatography fractionation using 3.0 x 50 cm glass columns packed with silica gel G60 powder (0.063-0.200) and (70–230) then connected with an EYEL-4 type pump. The crude extracts were eluted stepwise with gradients (25ml each time at five different concentrations; 20, 40, 60, 80 and 100 %) of different solvents in the order of increasing polarity, starting with the least polarity towards the higher polarity. The solvents used were hexane, ethyl acetate, methanol, acetone, acetonitrile and water. All fractions were collected in clean tubes and all eluents were then pooled to give six major fractions (Fraction 1–8). The solvents were then evaporated under reduced pressure in a centrifuge evaporator and freeze dryer (Awaad *et al*., 2008). All fractions and crude extracts were then subjected to an *in vitro* chemoprotective cell line assay, and then given positive result identification through the use of high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) to know the active constituents.

3.13 Thin layer chromatography

3.13.1 Principle

Thin layer Chromatography (TLC) is a solid-liquid technique consisting of two phases, those being the solid phase (stationary phase) and liquid phase (mobile phase). The most commonly used solid in TLC are silica gel. In this case silica gel 60 F254 plate, 20x20 cm, 0.2 mm (Merck, Darmstadt, Germany) was used.

TLC is a sensitive, fast, simple and inexpensive analytical technique. It's used to determine the number of components in a mixture and to identity the RF values of substances

3.13.2. General procedure

All collected fractions from column chromatography banded on TLC aluminum sheets coated with silica gel 60. The sheets were developed in a TLC tank using five various compositions of solvent (hexane, isopropanol, methanol, acetonitrile, acetic acid) the sheets were than visualized under ultraviolet lamps (254nm short wave and 366nm long wave). The isolation of the compounds was monitored by TLC using mixtures of ethyl acetate and methanol. The bands were circled using a pencil and respective RF values were calculated as follows:

$$Rf = x/y$$ where x= distance travelled by sample in cm

Y= solvant front in cm

3.14 Cytotoxicity and anticancer activity

3.14.1 Cells and chemical

Human colorectal carcinoma cell lines HT-29 and normal colon epithelial cell line (CCD841) were purchased from ATCC, USA. RPMI 1640 cell culture media, Fetal Bovine Serum (FBS) and penicillin/streptomycin solution were purchased from (Sigma-Aldrich, Gillingham, UK). Trypsin EDTA solution, trypan blue, MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide), sterile tissue culture flasks and analytical grade ethanol were purchased from (Merck, Germany). Sterile cell culture plates, sterile centrifuge tubes, phosphate buffer saline and dimethyl sulfoxide reagent were purchased from (Sigma Aldrich, Germany). Cell culture flasks and plates were purchased from (Jet Biofil). Hemocytometer (Weber, Teddington, UK). Cell culture work was done under sterile conditions in class II bio-safetycabinat (ESCO, USA).

3.14.2 Cytotoxicity procedure

For the estimation of the cytotoxicity and potential anticancer activity of both plant fractions, an established MTT assay as described by (Mosmann, 1983) was used with two cell lines. Human colorectal cancer cell line (HT-29) and normal colon human cell line (CCD_{841}) were grown in RPMI medium supplemented with 10% FBS (Sigma-Aldrich, USA) and 1% penicillin-streptomycin solution. Both cell lines were cultured at 37°C in a humidified 5% CO_2 incubator, and sub-cultured twice weekly using trypsin/EDTA. The MTT assay was carried out for eight fractions for *C. roseus* and two fractions for *A. ilicifolius*, with the assay being performed in triplicates in three independent experiments.

The effect of the extracts on human cell HT 29 and (CCD_{841}) were determined by using MTT assay as described by Mosmann and Scudiero using MTT reagent (5mg/ml) (Mosmann, 1983; Scudiero *et al.*, 1988). Briefly, 100µl of the isolated HT 29

suspension were cultured in 96-well flat bottom microtiter plates at 5×10^4 cells/ml in RPMI medium containing 10% (v/v) FBS and incubated at 37°C, 5% CO_2 and 90% humidity incubator for 48 hours. On the second day, the cells were treated with 20µl of two fold serial dilution with final concentration (166.6, 83.3, 41.7, 20.8 µg/ml) of fractions and re-incubated at 37°C in a humidified 5% CO_2 incubator for 24 hours. After 48 hours, 10µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) was added into each well in the plates and again re-incubated for four hours at 37°C in a humidified 5% CO_2 incubator. The yellow MTT is reduced to purple formazan in the mitochondria of living cells (Mosmann, 1983). Approximately 80µl of medium with MTT was removed from all wells and 100µl of Dimethyl sulfoxide (DMSO) was added to each well to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution measured at 595nm using power wave x340 ELISA Reader (Bio-Tek Instruments, USA). The assay was performed in triplicates in three independent experiments.

The identification and characterization of active compounds were performed by LCMS.

3.14.3 Identification of active constituents

In this study liquid chromatography-mass spectrometry (LC-MS) was used for identification of phenolics, alkaloids and other active constituents in the fractions that give positive result identified by the *in vitro* anticolon cancer activity assay i.e., *C. roseus* fractions 5 (F 5) and *A. ilicifolius* fraction 3 (F 3). The identification and characterization of isolated compounds from active fractions were performed by comparing ultraviolet (UV) and mass spectrometry (MS) as described by (Lin *et al.*, 2008). The components of fractions such as phenolics showed positive mass spectra containing intense [M+H] ions as well as fragmented ions created after the cleavage of

compound bonds. The method development and optimization were done by the technician at BST consultancy and services Sdn Bhd.

3.14.3.1 Principle

3.14.3.2 Sample preparation

The sample was prepared by dissolving 2mg in 2ml methanol inside a volumetric flask. The solution was then filtered by using SRP-4 membrane 0.45 mm. Stock solution 1 mg/ml was kept in a fridge at 4°C.

3.14.3.3 Instrumentation and parameter used equipment

Acquity ultra performance liquid chromatography (UPLC)-PDA system coupled to a Synapt High Definition Mass Spectrometry (HDMS) quadrupole-orthogonal acceleration time-of-flight (TOF) detector (Waters Corporation, USA) equipped with an ESI source (Table 3.5).

Column: Acquity BEH C18, 2.1 x 50 mm, 1.7 µm UPLC column

Injection volume: 3 l

Mode: Positive ESI mode

Solvents: A: Water + 0.1% Formic acid **B:** Methanol+ 0.1% Formic acid

Flow rate: 0.5ml/min

Gradient:

Table 3.5: UPLC and LC-MS gradient conditions

Time	% H_2O+ Formic Acid	% MeOH
0.00	100	0
0.83	60	40
2.50	55	45
3.33	50	50
3.75	50	50
4.17	20	80
4.25	100	0

3.15 Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post Hoc test. All values were expressed as mean ± S.E.M. and a value of $P < 0.05$ was considered significant as compared to the respective control group using SPSS programme for windows version 17.0 (SPSS Inc. Chicago, IL, USA).

Real-time RT-PCR data exported form "Step One Plus" software to Ms. Excel where all results arranged in one study and finally analysed by using GenEx program version 5.4 (GenEx software, www.multid.se), fold changes were calculated and T-test was used to examine the difference between groups for all genes and $P < 0.05$ was considered as the level of significance.

CHAPTER IV

RESULTS

4.1 Antioxidant activity of *C. roseus* extract *in vitro*

4.1.1 Ferric-reducing antioxidant power (FRAP)

The FRAP value of the *C. roseus* extract was determined to be 471.9 ± 0.274 µmol FeII/g. (standard curve equation: $y = 0.0006x + 0.0013$, $R^2 = 1$) as shown in appendix A1. The FRAP result of *C. roseus* is comparable to that of BHT. Ascorbic acid, quercetin and gallic acid were used as reference compounds and their FRAP values were calculated to be 521.4 ± 0.82, 1181.9 ± 0.274, 2267.1 ± 0.55 and 2640 ± 0.277 µmol Fe II/g respectively, which was almost the same as that of the standard BHT, but significantly lower than that of ascorbic acid, quercetin and gallic acid. There are statistical differences between *C. roseus* and these standards, with the results shown in (Figure 4.1).

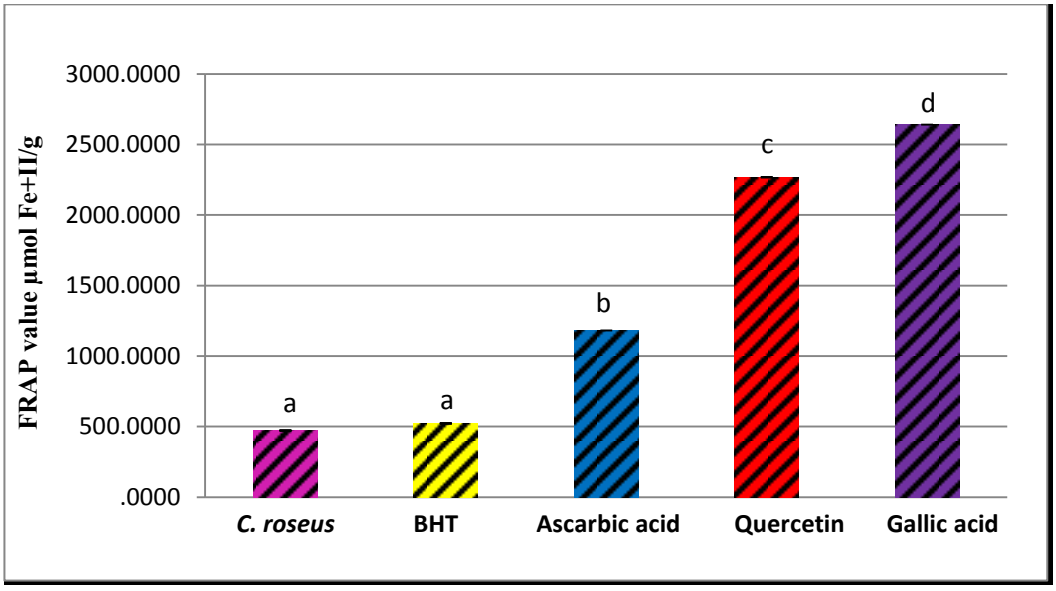


Figure 4.1: Ferric-reducing antioxidant power (FRAP) of *C. roseus*. All values are expressed as mean ± SEM. Mean with different letters indicate significantly different values $P < 0.05$.BHT; Butylatedhydroxyl toluene.

4.1.2 Scavenging activity of *C. roseus* extract (DPPH)

Antioxidant activity is measured as the capacity of the tested compound to reduce the stable DPPH radical. The scavenging capability increases with the increase in concentration of the *C. roseus* as shown in (Figure 4.2). The IC_{50} value represents the concentration needed to cause 50% inhibition of DPPH. Different concentrations of the extracts, as well as BHT, quercetin and trolox were used and the IC_{50} values were determined to be 47.20µg/ml for *C. roseus*, 17.40µg/ml for BHT, 7.40µg/ml for quercetin and 6.20µg/ml for trolox (standard curve equation: $y = 1.2997x + 0.1834$, $R^2 = 0.997$) as shown in appendix A2.

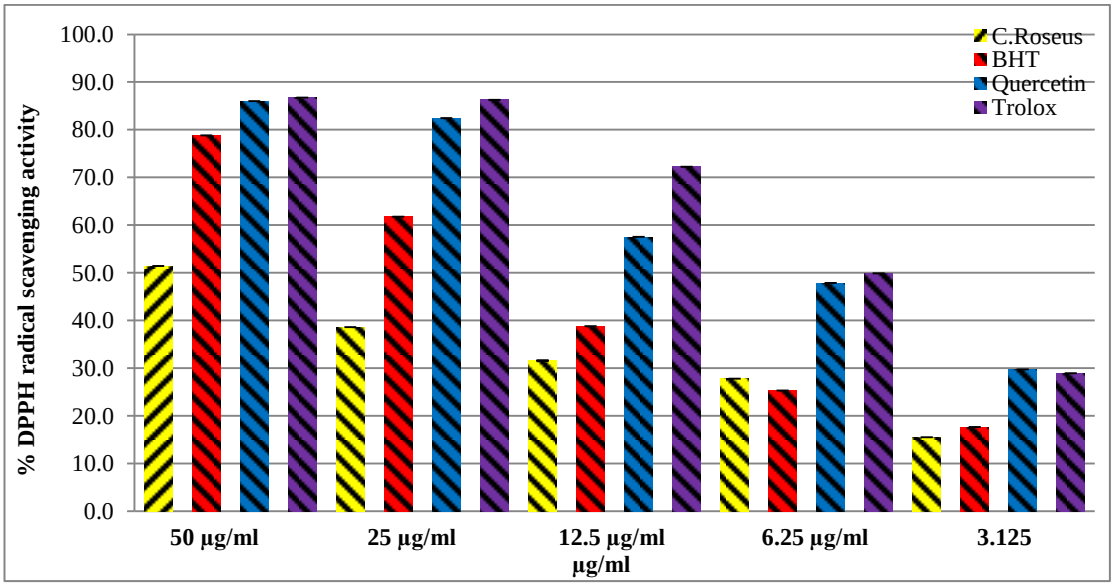


Figure 4.2: Scavenging Activity of *C. roseus* extract (DPPH). All values are expressed as mean ± SEM, $P < 0.05$.

4.1.3 Total phenolic and flavonoids content

Total phenolic content of the ethanolic extract of *C. roseus* was determined to be appreciable amounts of phenolic compound to 113.81 ± 0.027 mg (Gallic acid equivalent) per g of extracts, with standard curve equation: $y = 0.0007x + 0.0015$, $R^2 = 0.989$ as shown in Appendix A3.

The total Flavonoids value was 56.97 ± 0.008 mg (Quercetin equivalents) per g of extract (standard curve equation: $y = 0.0054x + 0.005$, $R^2 = 0.999$) as shown in appendix A4 with a ratio flavonoids/phenolic of 0.50. Thus, phenolic compounds were the predominant antioxidant components in *C. roseus* extract, which leads to more potent radical scavenging effect.

4.2 Acute toxicity study

Acute toxicity study is an experimental study in which selected rats are treated with the *C. roseus* extract at a dose of 2g/kg and 5g/kg. The rats were closely observed for a period of 14 days. At the end, it was found that all the rats were alive and did not show any noticeable significant toxicity at these doses. Both biochemical and histological indicators showed no hepatic or renal toxicity. The finding from the applications of the *C. roseus* extract to the control rats provided sufficient evidence to conclude that the orally administered extract was safe and presented no extract-related toxicity even at the highest dose of 5g/kg as shown in (Table 4.1, Table 4.2 and Figure 4.3).

Table 4.1: Effects of *C. roseus* extract on kidney biochemical parameters in acute toxicity

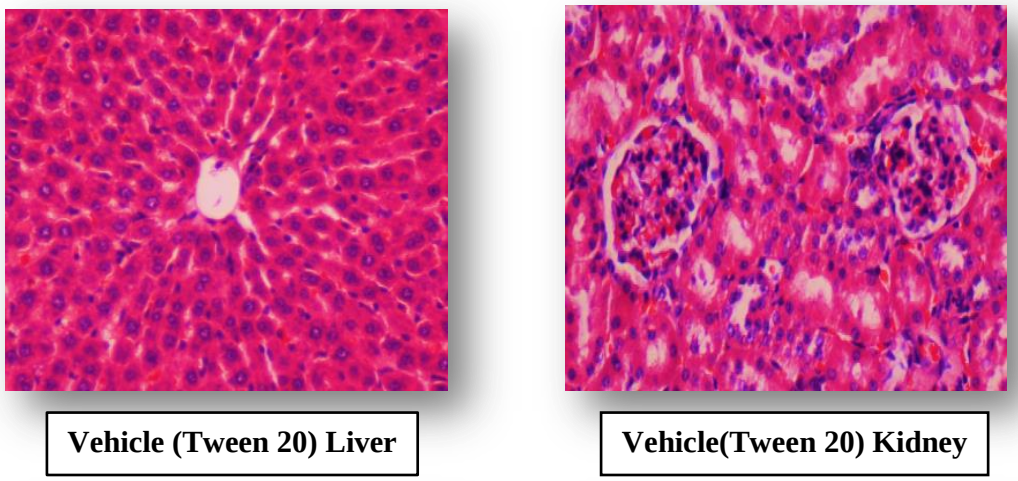
Dose	Sodium (mmol/L)	Pottasium (mmol/L)	Chloride (mmol/L)	CO_2 (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Vehicle (10% Tween-20)	142.05 ± 0.58	4.89 ± 0.07	104.01 ± 0.87	24.85 ± 0.55	18.11 ± 0.58	5.50 ± 0.38	34.88 ± 2.38
LD *C. roseus* (2 g/kg)	142.03 ± 1.54	5.00 ± 0.06	106.13 ± 1.03	22.60 ± 1.19	18.17 ± 0.61	6.20 ± 0.50	33.67 ± 2.65
HD *C. roseus* (5 g/kg)	142.50 ± 0.86	4.95 ± 0.05	105.17 ± 0.58	23.96 ± 1.05	18.52 ± 0.45	5.63 ± 0.494	34.51 ± 4.90

Values expressed as mean ± SEM. There are no significant differences between groups $P< 0.05$.

Table 4.2: Effects of *C. roseus* extract on liver biochemical parameters in acute toxicity

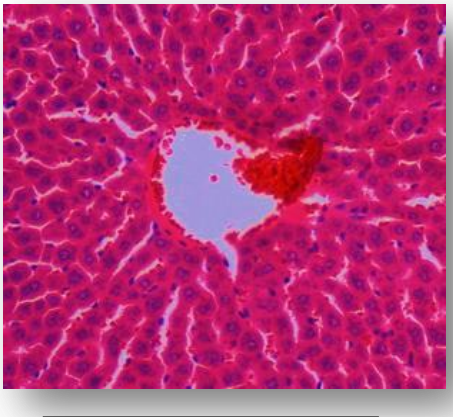
Dose	T.protein (g/L)	Albumin (g/L)	Globulin (g/L)	T.b (µmol/L)	Cb (µmol/L)	Alp (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)
Vehicle (10% Tween-20)	61.13 ± 1.08	9.16 ± 0.53	51.39 ± 1.33	2.13 ± 0.17	1.00 ± 0.00	153.07 ± 15.01	50.81 ± 1.71	173.53 ± 7.17	3.17 ± 0.16
LD *C. roseus* (2 g/kg)	58.97 ± 0.48	8.67 ± 0.33	49.73 ± 0.69	2.11 ± 0.15	1.00 ± 0.00	156.33 ± 15.24	48.02 ± 0.88	178.18 ± 5.84	3.67 ± 0.42
HD *C. roseus* (5 g/kg)	60.04 ± 1.03	9.70 ± 0.47	50.08 ± 1.05	2.08 ± 0.10	1.00 ± 0.00	155.17 ± 9.09	46.52 ± 1.76	175.87 ± 8.55	3.17 ± 0.17

Values expressed as mean ± SEM There are no significant differences between groups $P< 0.05$. Tb: Total bilirubin; Cb: Conjugated bilirubin; Alp: Alkaline phosphatase; ALT: Alanine aminotransferase: AST: Aspartate aminotransferase: GGT: G-Glutamyl Transferase.

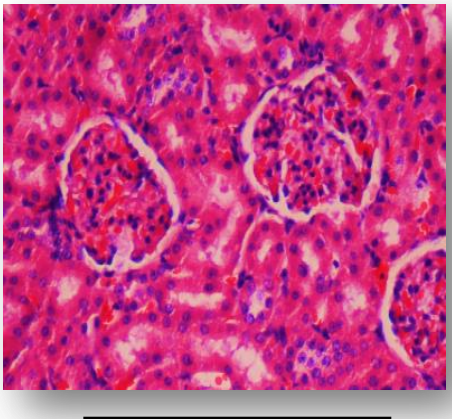


Vehicle (Tween 20) Liver

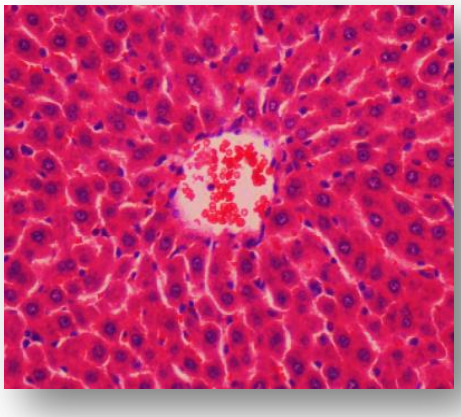
Vehicle(Tween 20) Kidney



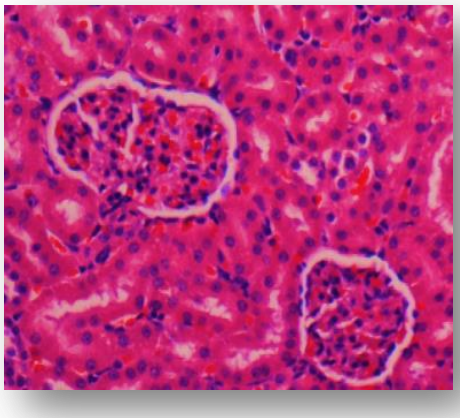
LD *C. roseus* Liver



LD *C. roseus* Kidney



HD *C. roseus* Liver



HD *C. roseus* Kidney

Figure 4.3: Histological sections. Effect of *C. roseus* on liver and kidney on acute toxicity test (H & E stain; 20x).

4.3 Analysis of body weight

The body weight of all rats were taken at the beginning (week 1) of the experiment, then weekly up to 10 weeks (Figure 4.4). Although there is a difference between groups, statistically there were no significant differences in the body weight among the groups.

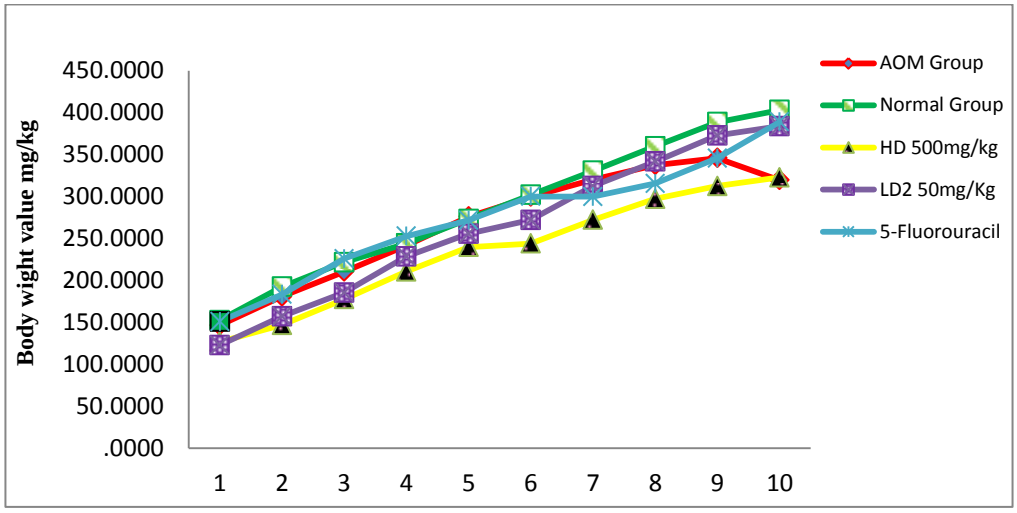


Figure 4.4: Effect of *C. roseus* on body weight of rat's in different experimental groups**.** All values expressed as mean ± SEM. No significant changes in body weights were observed in all rat's group $P < 0.05$.

4.4 Efficacy of *C. roseus* on ACF formation

ACF were mostly observed in the middle colon. ACF present in the colon were counted and the average of the total number of ACF and the number of crypts per focus was obtained. ACF were observed in the colon as well as in multicrypt clusters (more than four crypts/focus) of aberrant crypts (Table 4.3 and Figure 4.5). Rats treated with AOM and fed with the low and high dose of *C. roseus* extract showed a significant reduction of total ACF/colon compared with the AOM-control rats 65% and 61% inhibition respectively, $P<0.0001$. The incidence of multiple aberrant crypts/focus was also significantly inhibited in rats fed with *C. roseus* when contrasted with the AOM-induced rats.

Table 4.3: Effect of *C. roseus* on AOM-induced colonic ACF containing four or more aberrant crypts in rats

Groups	1 crypt	2 crypts	3 crypts	4 crypts	5&more crypts	Total ACF	inhibition %
AOM control	19.50 ± 3.62	29.79 ± 4.89	23.04 ± 2.70	24.67 ± 2.22	34.33 ± 4.94	131.33 ± 15.09	0
5-Fluorouracil	10.16 ± 1.04	9.50 ± 2.04*	7.91 ± 0.69**	6.25 ± 0.81**	7 ± 0.64**	40.83 ± 3.06**	69%
LD 250 mg/kg *C .roseus*	15.66 ± 3.27	9.50 ± 2.33*	5.58 ± 0.43**	3.58 ± 0.41**	12 ± 1.13**	46.33 ± 3.95**	65%
HD 500 mg/kg *C .roseus*	10.25 ± 1.63	15.16 ± 1.60	8.58 ± 0.95**	6.41 ± 0.86**	10.83 ± 1.27**	51.25 ± 4.83**	61%

Values are expressed as mean ± S.E.M., Values with different symbol are significantly differences. $^{*}P < 0.001$ versus AOM control group and $^{**}P < 0.0001$ versus AOM control group.

4.4.1 Macroscopical Findings

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, were significantly higher in the AOM-control group compared to the *C. roseus* treated groups. At the same time the multiplicity of ACF (number of crypt per ACF), which is considered as a marker for tumour promotion, was significantly higher in AOM group.

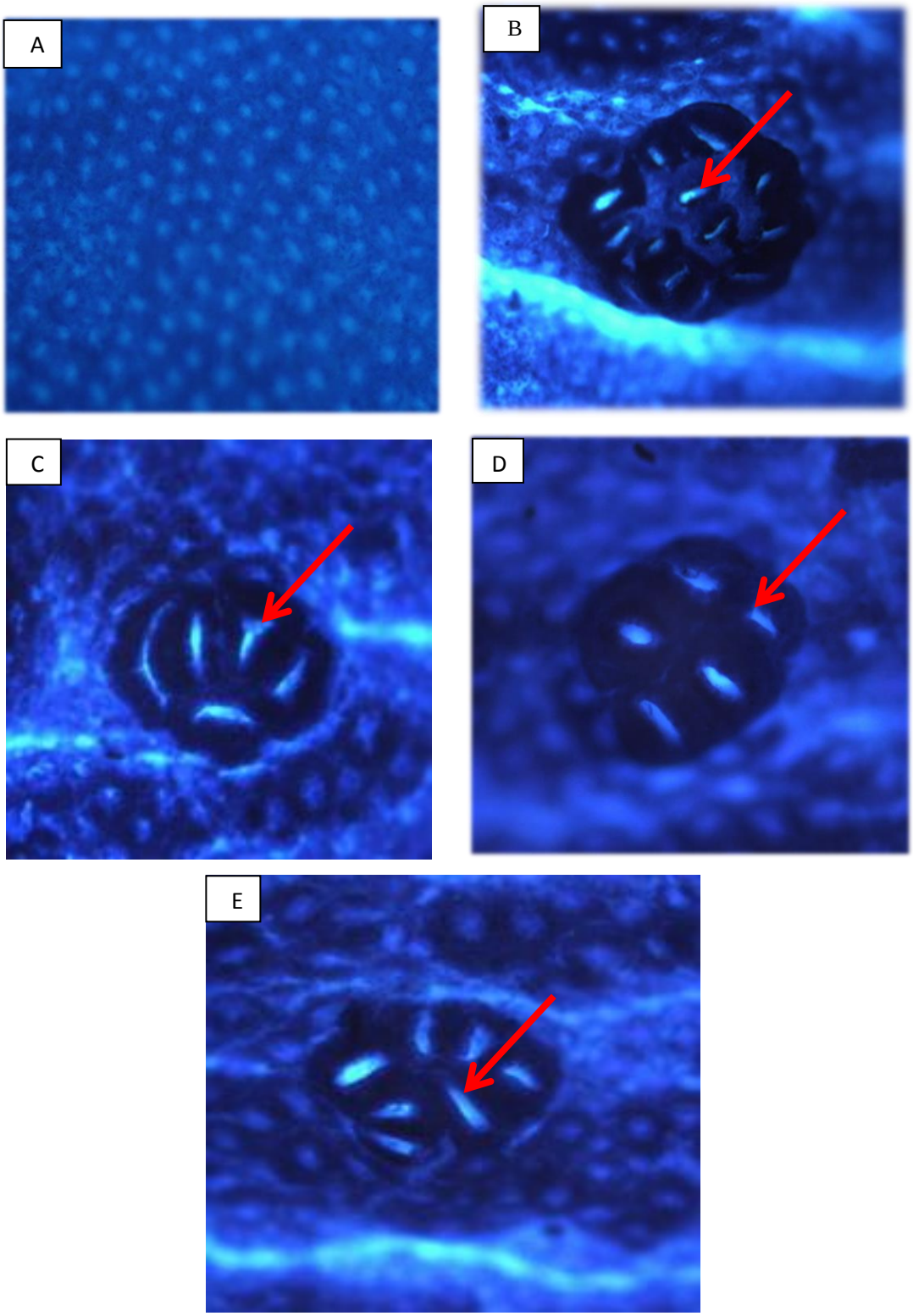


Figure 4.5: Topographic views shows effect of *C. roseus* on ACF in methylene blue staining on rat colonic tissue; (A) Normal crypts from rats treated with 10% Tween 20, normal control; (B) AOM control group. (C) Reference group (5-Fluorouracil) + AOM (D) 250 mg/kg *C. roseus* + AOM; (E) 500 mg/kg *C. roseus* + AOM (10x).

4.4.2 Histopathology examination

All the grossly visible colon lesions were evaluated histologically. Hematoxylin and Eosin stains in the AOM-control group contained comparatively more ACF, exhibiting marked nuclear nuclear atypia, with the crypts being longer and larger than normal. In higher magnification view the mildly dysplastic areas show elongation and slight stratification of nuclei. The epithelial cells in ACF have distinctive elongated nuclei, loss of cell polarity, increase in mitoses, lack of goblet cells and narrow lumen compared to the surrounding normal crypts (Figure 4.6).

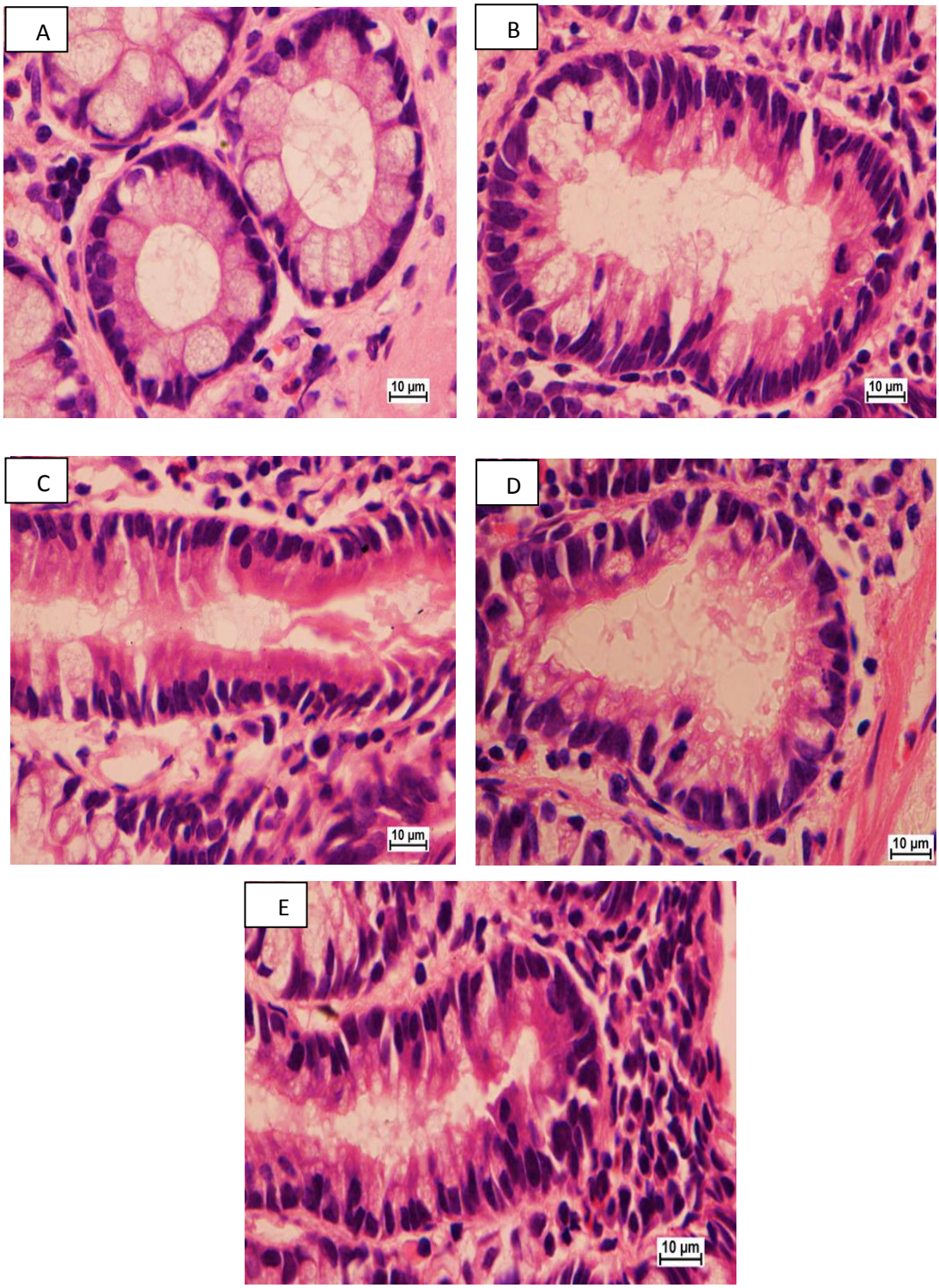


Figure 4.6: Histological patterns of aberrant crypt foci. An effect of *C. roseus* on AOM-induced ACF in rat's colon; (A) Normal control group; (B) AOM control group; (C) 5-Fluorouracil, Standard drug + AOM; (D) 250 mg/kg *C. roseus* + AOM; (E) 500 mg/kg *C. roseus* + AOM (H& E stain 100x).

4.5 Effects of *C. roseus* on biochemical parameters

The total protein albumin was significantly increased in the treated *C. roseus* group compared to the AOM group. However, LDH and urea were decreased in treated groups compared to the AOM group. While there were no significant differences in gloucose, createnine, AST, ALT, ALP, and T. cholestrol in the *C. roseus* treated group compared to the AOM group. *C. roseus* treated groups showed restored levels of total protein, albumin, urea and LDH corresponding to normal values (Table 4.4, 4.5).

Table 4.4: Effects of *C. roseus* on glucose, urea and createnine tests

Animal Group	**Glucose (mmol/L)**	**Urea (mmol/L)**	**Createnine µmol/L**
Normal	10.38 ± 0.56	5.44 ± 0.10	56.4 ± 5.52
AOM	9 ± 0.51	7.13 ± 0.65$^{\#}$	40 ± 3.67
5-flourouracil	7.2 ± 0.45	5.56 ± 0.25	46 ± 5.52
LD *C. roseus*	7.7 ± 0.63	5.03 ± 0.29 **	42 ± 2.06
HD *C. roseus*	7.52 ± 0.18	4.86 ± 0.24 **	43 ± 4.11

All values expressed as mean ± SEM. Values with different symbol are significantly differences. $^{\#}P < 0.05$ versus normal control group, $^{*}P < 0.05$ versus AOM control group, $^{**}P < 0.01$ versus AOM control group

Table 4.5: Effects of *C. roseus* on biochemical parameters

Animal Group	T. protein G/L)	Albumin (G/L)	AST IU/L	ALT IU/L	ALP IU/L	LDH U/L	T.chol mmol/L
Normal	64.37 ± 0.33	12.33 ± 0.21	203.2±18.48	60 ± 3.43	98.4 ± 3.02	1080.2 ± 24.88	1.3 ± 0.00
AOM	58.20 ± 1.13^{t}	11.00 ± 0.44^{t}	181.83 ± 15.04	60.83 ± 5.36	86.66 ± 8.97	1414.33 ± 105.38^{t}	1.42 ± 0.11
5-flourouracil	65.66 ± 1.54**	12.16 ± 0.16	164.83 ± 10.8	52.33 ± 3.43	133.16 ± 7.24	796.33 ± 55.18$^{\#}$	1.35 ± 0.50
LD C. roseus	64.83 ± 1.44*	12.33 ± 0.49*	164.66 ± 5.38	46 ± 4.31	120.66 ± 13.95	904.83 ± 77.85$^{\#}$	1.43 ± 0.04
HD C. roseus	64.40 ± 1.80*	12.7 ± 0.19*	206.6 ± 44.1	92.2 ± 35.84	131 ± 12.54	816.60 ± 73.61$^{\#}$	1.96 ± 0.04

All values expressed as mean ± SEM. Values with different symbol are significantly differences $^{t}P< 0.05$ versus normal control group, $^{*}P< 0.05$ versus AOM control group, $^{**}P< 0.01$ versus AOM control group and $^{\#}P< 0.001$ versus AOM control group. T. protein: Total protein, AST; Aspartate aminotransferase, ALT; Alanine Aminotransferase, ALP; Alkaline Phosphatase, LDH: Lactate dehydrogenase, and T. cho; Total cholesterol.

4.6 Effects of *C. roseus* on hematological parameters

The Hb, WBC, HCT, RBC, and Plt were significantly increased in the *C. roseus* treated group compared to the AOM group. While there were no significant differences in MCV, MCH, MCHC, RDW and WBC differential count in the *C. roseus* treated group compare to the AOM group. AOM induced oxidative damage in erythrocytes, leading to reduction of RBC counts and Hb, WBC, HCT, RBC and plt concentration. *C. roseus* treatment leads to an increase in concentration to reach normal values (Table 4.6, 4.7).

Table 4.6: Effects of *C. roseus* on hematological parameters

Animal Group	Hb G/L	HCT (L/L)	RBC (10^{12}/L)	MCV (fL)	MCH (pg)	MCHC (G/L)	RDW (%)
Normal	154.00 ± 1.12	0.464 ± 0.01	8.33 ± 0.13	55.8 ± 0.65	18.26 ± 0.20	328.4 ± 0.76	14.2 ± 0.46
AOM	142.17 ± 2.35^{t}	0.455 ± 0.01	8.195 ± 0.13	55.16 ± 0.96	17.55 ± 0.22	317.5 ± 2.46	14.76 ± 0.65
5-flourouracil	154.83 ± 2.18**	0.488 ± 0.00*	8.435 ± 0.11	57.83 ± 0.79	18.36 ± 0.30	317.66 ± 2.72	15.08 ± 0.25
LD *C. roseus*	162.33 ± 1.68$^{\#}$	0.498 ± 0.01**	8.861 ± 0.10*	56.66 ± 0.92	18.3 ± 0.14	325.16 ± 4.47	13.8 ± 0.20
HD *C. roseus*	156.40 ± 3.21**	0.486 ± 0.01	8.522 ± 0.22	57.4 ± 0.66	18.38 ± 0.13	321 ± 1.59	15.72 ± 0.55

All values expressed as mean ± SEM. Values with different symbol are significantly differences $^{t}P< 0.05$ versus normal control group, $^{*}P< 0.05$ versus AOM control group $^{**}P< 0.01$ versus AOM control group and $^{\#}P< 0.001$ versus AOM control group. Hb: Hemoglobin; HCT: Hematocrit RBC: Red blood cell, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular hemoglobin, MCHC: Mean Corpuscular hemoglobin Concentration, RDW: Red Cell Distribution.

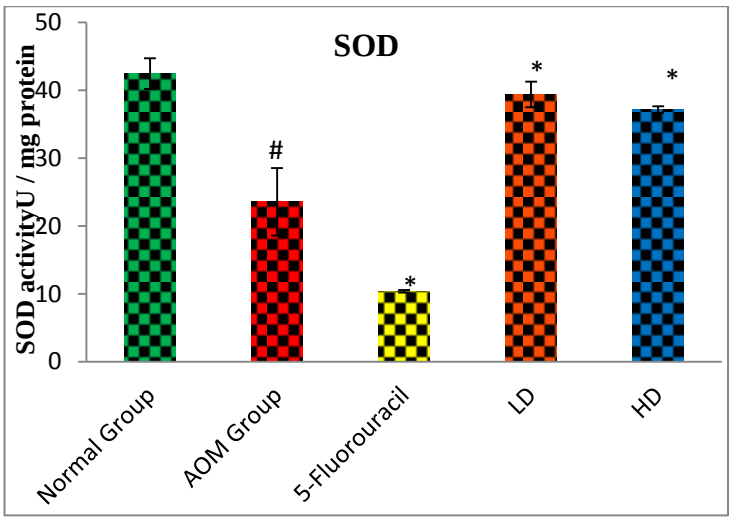
Table 4.7: Effects of *C. roseus* on hematological parameters

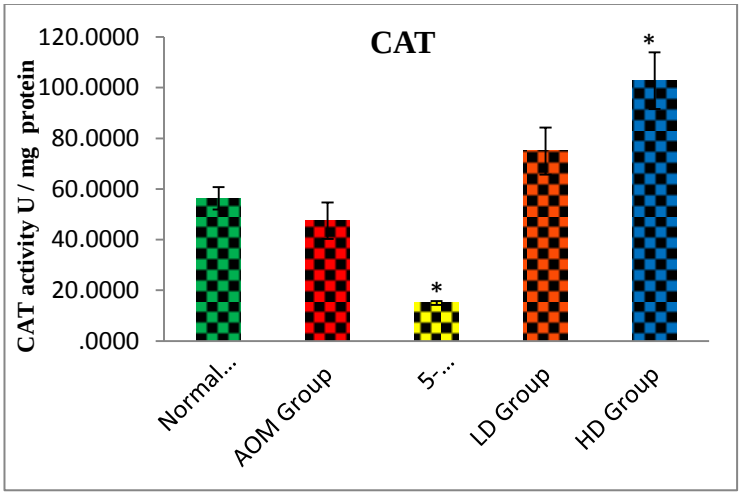
Animal Group	WBC 10^9/L	Platelet 10^9/L	Neutrophil %	Lymphocyte %	Monocyte %	Eosinophil %	Basophil %
Normal	12.98 ± 1.50	732.6 ± 26.33	18.36 ± 1.94	74.62 ± 2.45	3.96 ± 0.69	1.44 ± 0.43	0 ± 0.00
AOM	8.08 ± 0.66 ^{t}	542.66 ± 94.38	17.93 ± 4.54	59.45 ± 12.37	5.35 ± 1.06	1.61 ± 0.54	0.1 ± 0.03
5-flourouracil	7.28 ± 0.52	683 ± 35.33	28.46 ± 2.07	64.15 ± 3.21	5.86 ± 0.94	1.43 ± 1.19	0.08 ± 0.03
LD *C. roseus*	12.35 ± 1.06*	924.66 ± 68.04**	29.75 ± 6.74	66.78 ± 6.97	3.2 ± 0.47	0.083 ± 0.07	0.016 ± 0.02
HD *C. roseus*	10.10 ± 0.73	836.4 ± 48.97*	24.5 ± 2.04	72.42 ± 1.85	2.8 ± 0.65	0.94 ± 0.63	0.24 ± 0.16

All values expressed as mean ± SEM. Values with different symbol are significantly differences. $^{t}P < 0.05$ versus normal control group, $^{*}P< 0.05$ versus AOM control group, WBC: white blood cell.

4.7 Effects of *C. roseus* on antioxidant and lipid peroxidation (TBRAS) in AOM induced ACF colonic aberrants

Figure 4.7 shows the *in vivo* activities of oxidative enzymes involved in the colon antioxidant defense system. SOD enzyme level LD and HD were significantly increased in treatment groups compared with the AOM control group, whereas CAT enzyme HD levels were significantly increased in the treatment group compared to the AOM control group. In contrast, MDA values in colon rats were significantly decreased in the treatment groups when compared to the AOM control group. However, the 5-Flourouracil values were lower than those in the low dose and high dose groups.





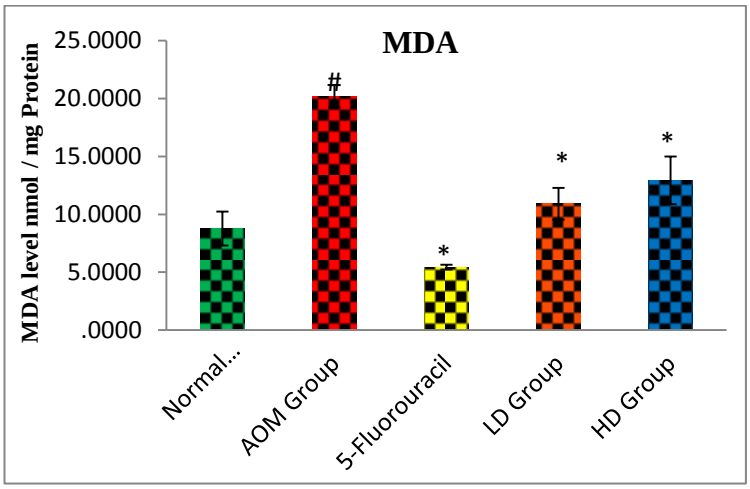


Figure 4.7: Effect of *C. roseus* extract *in vivo* antioxidant activities parameters, in AOM -induced colon cancer rats. All values are expressed as the mean ± SEM. * = significantly different means compared to the AOM group, # = significantly different means compared to the normal group, $p < 0.05$.

4.8 Immunohistochemistry analysis

Photomicrographs of ACF exhibiting grades of nuclear morphology from colons of rats receiving AOM were captured. All sections were cut parallel to the muscle layer. The presence of elongated and stratified nuclei were noted throughout the crypt.

4.8.1 PCNA staining of colons and cell counting

The PCNA was evaluated as a marker for cell proliferation in the colon specimens. Sections of colon samples from the control group and the *C. roseus*-treated group are shown (Figure 4.8). The PCNA positive staining cells in the nucleus (brown red) of mucosa of the colon tissue were much stronger in azoxymethane-treated rats than in the *C. roseus*-fed group. PCNA-negative cells (blue) were stained with hematoxylin. The PCNA labelling index is also shown in (Figure 4.9). The colon sections from the AOM control group showed a higher number of positive cells than those from the AOM + *C. roseus*-treated group.

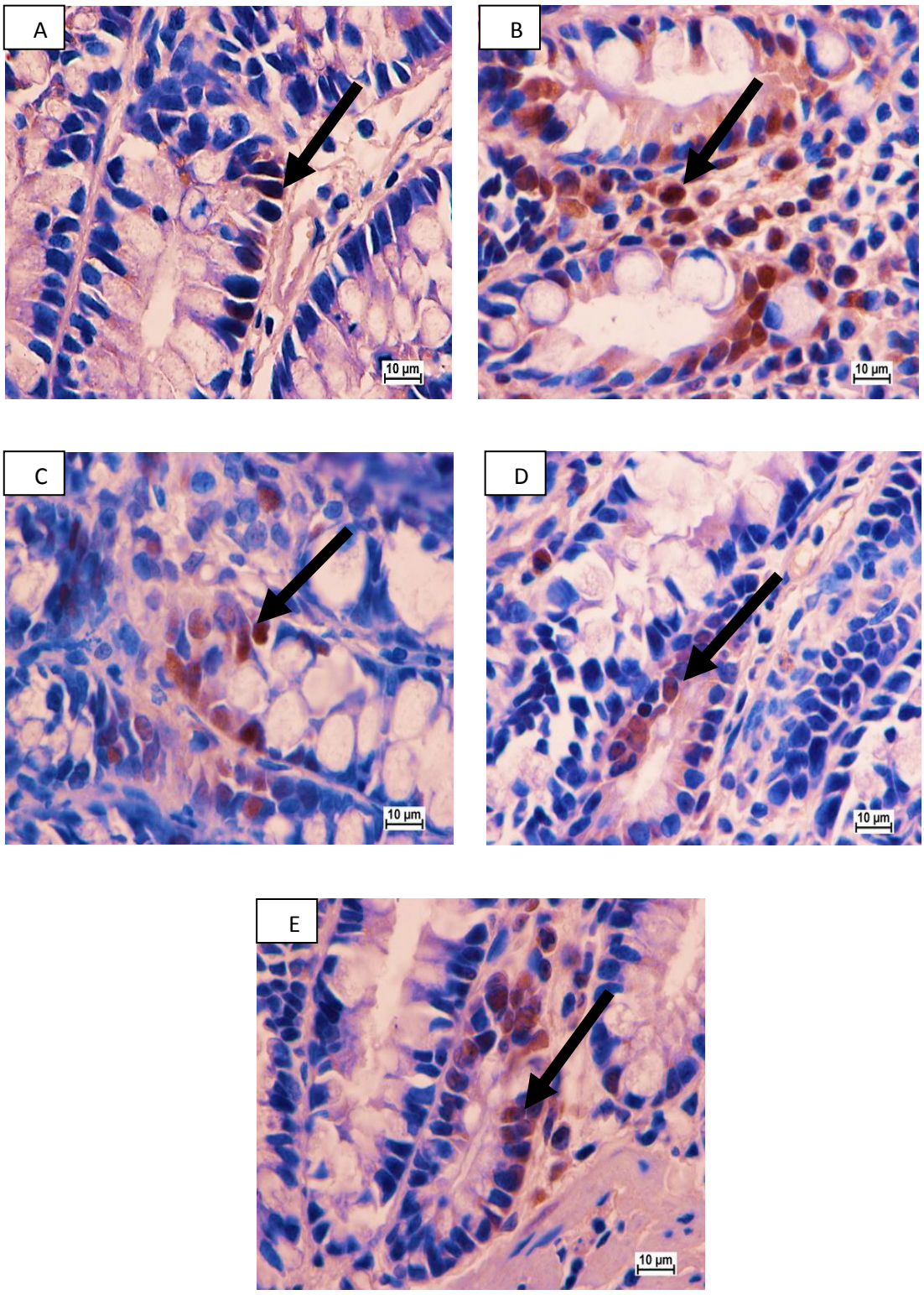


Figure 4.8: Immunohistochemical staining: Effect of *C. rosues* on PCNA staining of colon mucosa; (A) Normal control group; (B) AOM-control group; (C) 5-Fluorouracil drug treated group + AOM; (D) 250 mg/kg *C. roseus* + AOM. (E) 500 mg/kg *C. roseus* + AOM (IH staining 100x).

The PCNA-positive cells (%) of the colon tissue in the control group were 100%, whereas PCNA-positive cells (%) from the *C. roseus*-treated group were 41.8, 57.6 and 67.5 % respectively. The two groups were significantly different at ($P < 0.01$) (Figure 4.9).

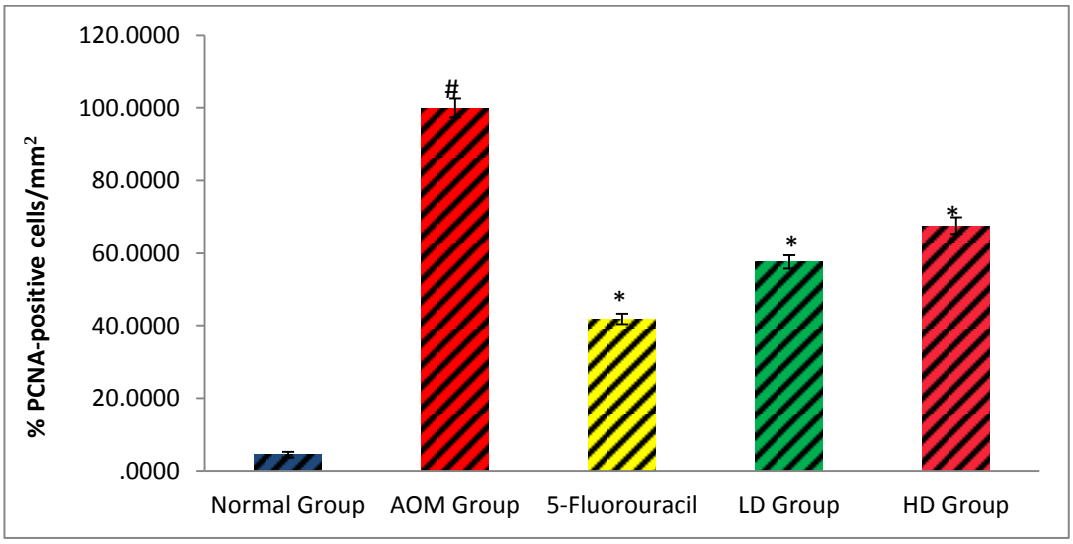


Figure 4.9: Effect of *C. rosues* on PCNA counting in AOM-induced ACF in rats. All values expressed as mean ± SEM. Values with symbol are significantly different *versus* control group, $P < 0.01$.

4.9 Gene expression profile

4.9.1 Integrity of RNA

The ratio of absorbance readings at 260nm and 280nm were used to indicate the quality of the RNA. The 260/280 ratio for our RNA preparation always ranged from 1.6-2.1 (Table 4.8), which suggested good quality RNA.

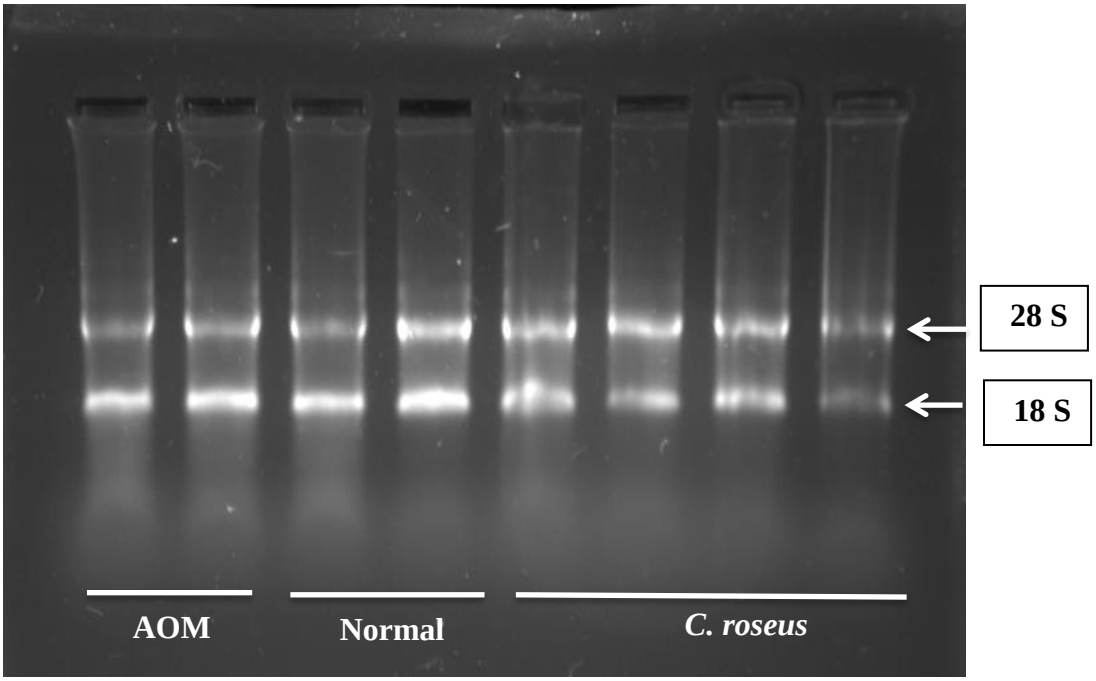
The integrity of RNA was checked by agarose gel electrophoresis. Discrete 28S and 18S ribosomal RNA bands were obtained in each case, with the 28S band about twice the 18S band, indicating that the RNA extracted was intact and could be applied for RT-PCR. (Figure 4.10) shows a typical ethidium bromide stained-agarose RNA gel.

Table 4.8: RNA concentration and purity measured by NanoDrop Spectrophotometer at 230, 260, 280, 320, 260/320 nm absorbance

Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230
Calibrator 1	1082.8	ng/µl	27.07	12.677	2.14	1.97
Calibrator 2	634.6	ng/µl	15.865	7.546	2.1	2.04
Calibrator 3	632.2	ng/µl	15.805	7.561	2.09	2.08
Calibrator 4	464.1	ng/µl	11.603	5.549	2.09	1.45
Calibrator 5	410.5	ng/µl	10.264	4.912	2.09	1.91
Calibrator 6	502.5	ng/µl	12.561	6.076	2.07	2
AOM1	271.4	ng/µl	6.785	3.259	2.08	1.94
AOM2	320	ng/µl	8	3.806	2.1	0.84
AOM3	569.3	ng/µl	14.233	6.771	2.1	1.76
AOM4	1089	ng/µl	27.224	12.911	2.11	2.04
AOM5	879.6	ng/µl	21.991	10.421	2.11	1.83
AOM6	671	ng/µl	16.774	8.018	2.09	2.15
C. roseus1	460.6	ng/µl	11.515	5.572	2.07	2.02
C. roseus2	826.8	ng/µl	20.669	9.93	2.08	2.13
C. roseus3	740	ng/µl	18.5	8.942	2.07	2.16
C. roseus4	1014.9	ng/µl	25.374	12.064	2.1	2.1
C. roseus5	905.1	ng/µl	22.628	10.902	2.08	2.11
C. roseus6	829.3	ng/µl	20.733	9.872	2.1	2.1

N: Normal control group, AOM: Azoxymethan treated group.

(A)



(B)

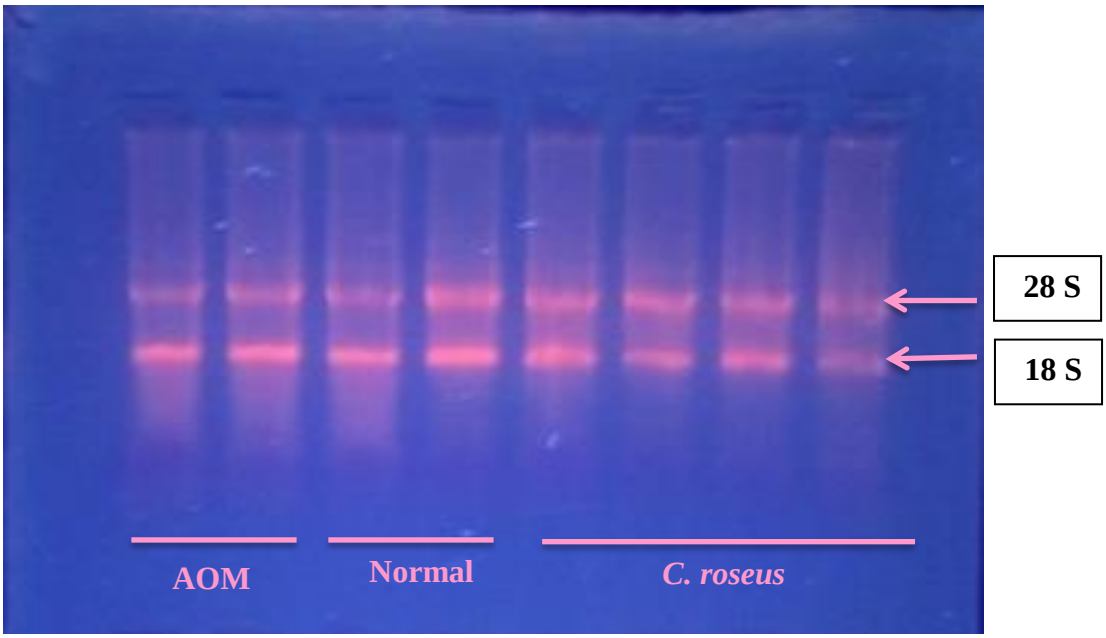


Figure 4.10: A typical ethidium bromide-stained agarose gel showing the integrity of the extracted RNA; (A) Visualized under Vilber Lourmat gel documentation system; (B) Visualized under UV light.

4.10 *C. roseus* activates apoptosis cell death

4.10.1 Effect of *C. roseus* on apoptosis gene expression

The gene expression levels of the target genes in animal colon tissue samples were normalized using both HPRT-1 and Tbp endogenous reference genes. The expression levels of Bax, Bcl2, p53 and the two endogenous reference genes were subsequently validated by RT-PCR measurements. According to a standard curve (appendix A9) all genes showed that efficiency was between 90-110 % and the slope between (-3.1) and (-3.5), which is within the reference criteria necessary to run the quantitative RT-PCR (Table 4.9). After data analysis of Ct values by Gene EX software and normalized to the reference genes HPRT-1 and Tbp, all measured mRNAs showed different expressions between calibrator group (normal control tissue), AOM group (cancer group) and *C. roseus* treatment rats group. The expression of Bax and Bcl2 genes in AOM group were significantly decreased, being (1.197 ± 0.003), $P < 0.003$, (0.24 ± 0.20) $P < 0.003$ respectively, with the fold being lower when compared with the normal rat group (Calibrator group). However, the difference was insignificant for the p53 gene (0.76 ± 0.23) $P < 0.23$ in terms of the fold being lower when compared with normal rats.

*C. roseu*s induced the expression of apoptosis-related genes that caused significant up-regulation of pro-apoptotic protein Bax (1.24 ± 0.34), $P < 0.01$ and p53 1.30 ± 0.01, $P < 0.01$. Although *C. roseus* treatment did not significantly modify the expressed levels of anti-apoptotic protein Bcl-2 (0.28 ± 0.13), $P < 0.003$ in the mucosa compared of the AOM –injection rats. Bcl2 gene expression did not change with *C. roseus* treatment in contrast to Bax and p53 transcript, which was significantly up-regulated. In addition *C. roseus* treatment caused a switch in the Bcl-2/Bax ratio, which was elevated in the mucosa of AOM-injected rats (Bcl-2/Bax>1), and was reversed after *C. roseus* treatment (Bcl-2/Bax <1) as shown in (Figure 4.11 and Table 4.10).

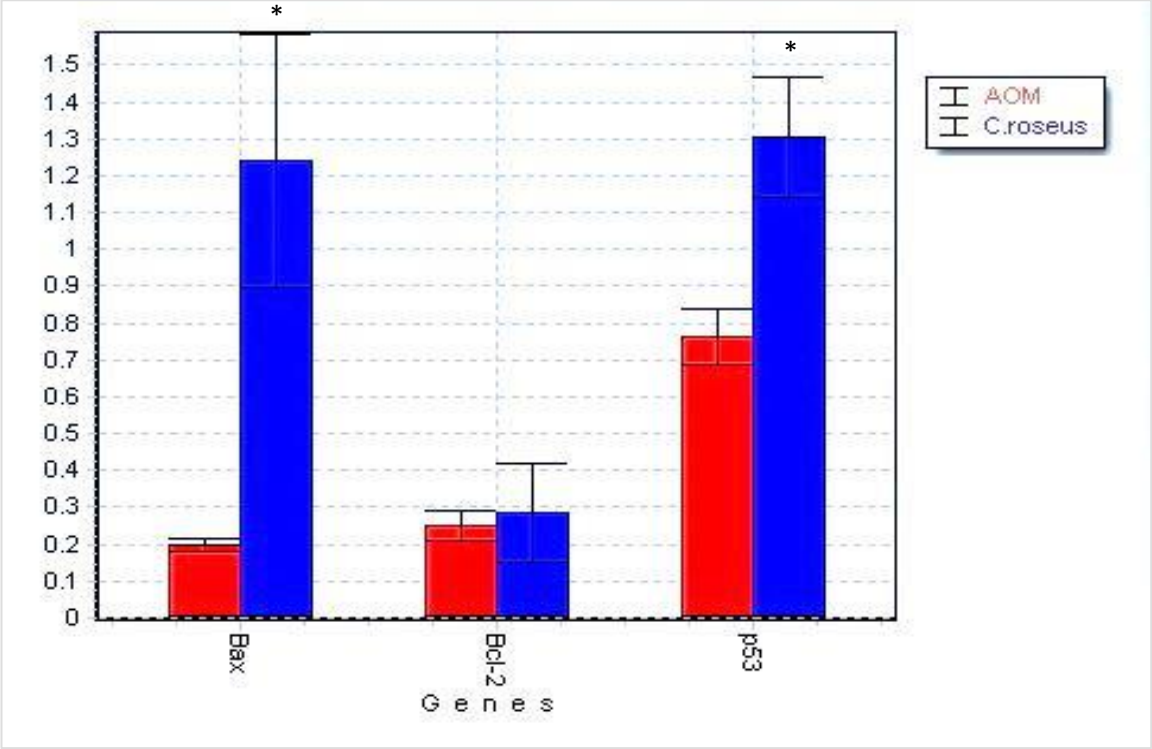


Figure 4.11: Changes in the mRNA expression levels of p53, Bcl-2 and Bax in the colonic mucosa of AOM-injected rats and *C. roseus* treated AOM-injected rats. Real-time PCR was done in triplicate. The mRNA level from colonic mucosa of normal control rats was used as an external reference. The levels of Hprt1 and Tbp mRNA were used as an internal reference to normalize the data. Data are presented as the mean ± SEM, $P < 0.05$.

Table 4.9: Measured efficiency slope and R^2 of target and endogenous reference genes using the Ct slope method with 5 concentrations points

Genes	Hprt1	Tbp	Act-b	P53	Bax	Bcl_2	Reference criteria
Efficiency	109.4	108.4	96.0	110	109.4	108.9	90-110
Slope	-3.1	-3.1	-3.4	-3.1	-3.1	-3.1	(-3.1) - (-3.5)
y-inter	30.3	23.3	32.1	33.4	30.3	30.2	--
R^2	0.99	0.99	0.98	0.99	0.99	0.98	Not less than 0.98

Table 4.10: Effect of AOM, *C. roseus* extract intake on mRNA gene expression levels in AOM-induced rat colon tissue

Group	Bax	Bcl2	p53
Calibrator	1	1	1
AOM	1.197 ± 0.003*	0.24 ± 0.200*	0.76 ± 0.23
C. roseus	1.24 ± 0.34*	0.28 ± 0.13	1.30 ± 0.01*

All values are expressed as mean fold changes of mRNA from 6 rats ± S.E.M. Statistical analysis of differences was performed by a two-tailed unpaired student's t test. Means with different superscripts are significantly different. $P < 0.05$

4.11 *In vitro* chemo-protective activity of *C. roseus* crude extracts and isolated fractions

The inhibitory effect of the crude extract and fractions of *C. roseus* has been tested *in vitro* against the human colon cancer HT-29 cell line and normal human colon cells CCD_{841} using MTT assay. The crude extract inhibited colon cancer cells showing maximum inhibition on HT-29 cells (13.84 %) at the concentration 166.66µg/ml, with no variations in the cell viability of CCD_{841} cells having been observed. At the same time the fractions of the crude extract of *C. roseus* were also tested for their antiproliferative effect on both HT-29 and CDD_{841} cell lines. Amongst the 8 fractions tested, fraction 5 (F5) showed maximum inhibition on HT-29 cells (86.27 %) at the concentration 166.66µg/ml. F4 and F7 also showed strong inhibition activities against HT-29 cells of 57.83% and 62.53 % respectively. The remaining fractions showed moderate inhibition activities. On the other hand, there were almost no variations in the cell viability of CCD_{841} cells observed. While the increase in the percentage of inhibition was seen with F4, F5 and F7 of *C. roseus*, which also showed no cytotoxicity against normal colon cells (Figure.4.12 and Figure 4.13), photomicrographs showed the

effects of *C. roseus* fractions treatment on CCD_{841} and the HT-29 cells in comparison to the untreated cells.

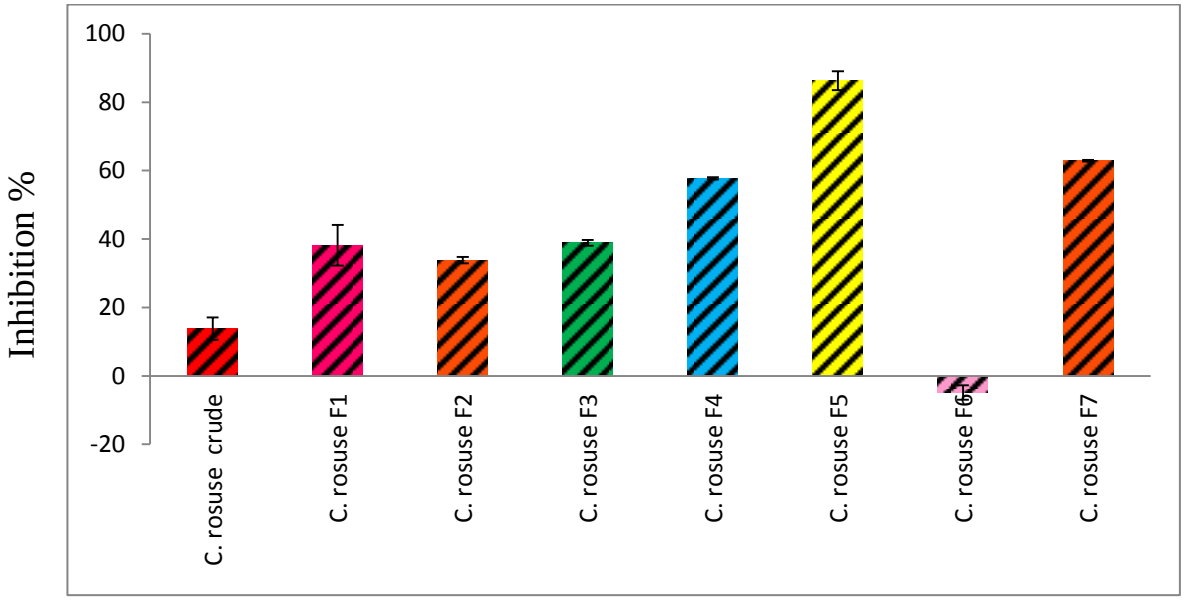


Figure.4.12: The effect of the crude extract of *C. roseus* and its fractions on the growth of human HT-29 cell line. Cells were seeded in 96-well plate and treated with different concentrations of both *C. roseus* crude extract and fractions (166.66 µg/ml) for 48h. The inhibition of HT-29 growth was assessed by MTT assay. The results represent the mean of triplicate measurement.

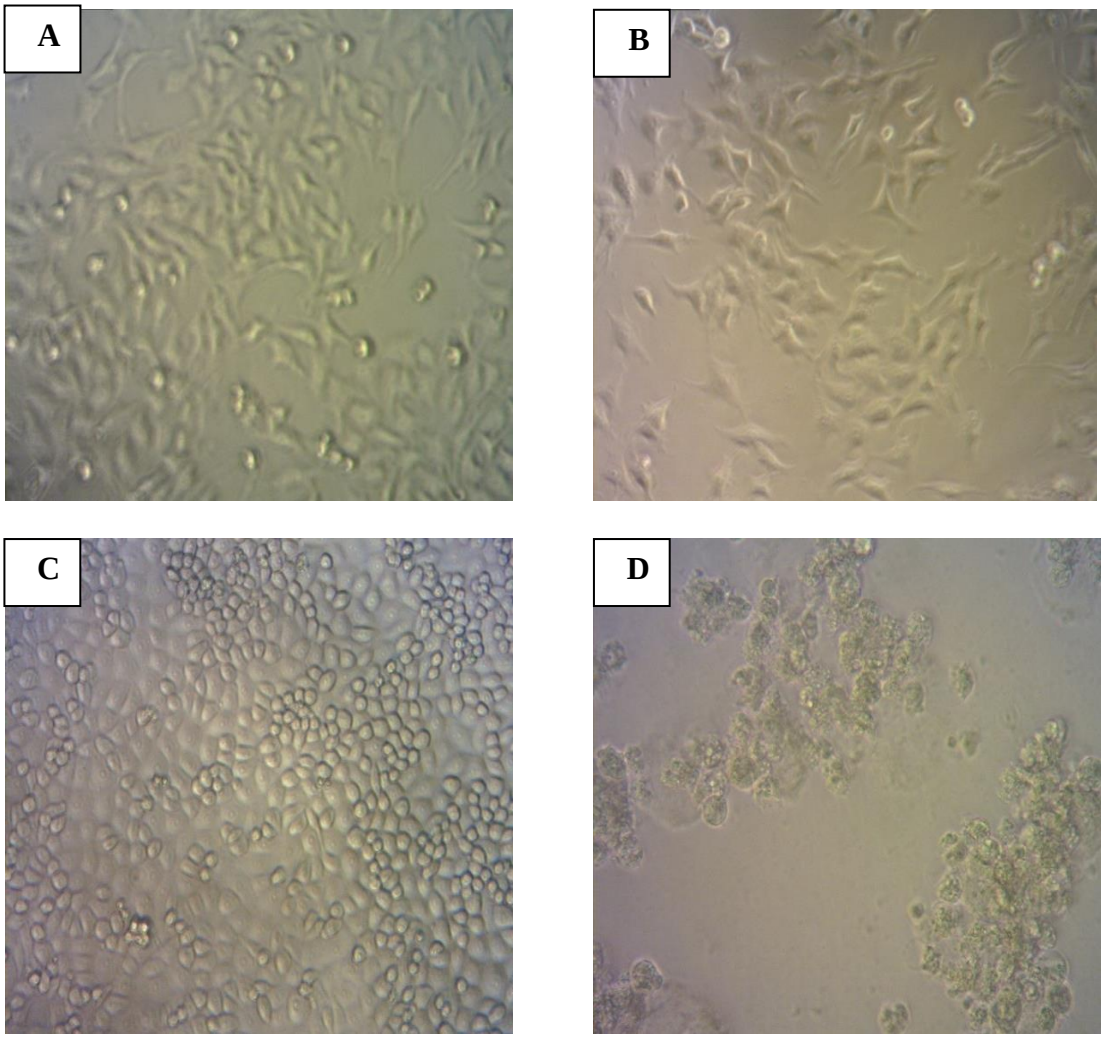


Figure 4.13: Photomicrographs of HT29 and CCD_{841} cells showing the effects *C. roseus* fractions treatment; (A) CCD_{841} cells without treatment; (B) cells treated with 166.66µg/ml F5; (C) HT29 cells without treatment; (D) HT29 cells treated with 166.66µg/ml F5 (magnification 10x).

4.11.1 Identification of active constituents of the active fractions

We applied UPLC-PDA and LC-MS using positive and negative ionization mode in order to know the chemical structural information and to identify the alkaloid or phenolic constituents and other active compounds of *C. roseus* Approximately three compounds were detected in F5 from them were characterized on the basis of the UV spectra and MS fragmentation patterns in comparison with literature or by searching the dictionary of natural products on DVD, Version 20:2 (2011) (CRC Press, Taylor &

Francis Group, London, UK). In addition three active compounds were detected in *C. roseus* F5 and three of them were identified Typical HPLC-TOF/MS peaks and UV diode array chromatograms of the *C. roseus* F5 fractions are shown in (Figure 4.14).Tables 4.11 showed the identification of all the peaks detected with their retention time, UV max, observed *m/z* and the *m/z* of fragment ions. The *C. roseus* F5 compounds include ajmalicine at *m/z* 351.1722 (Figure 4.15), vindoline at *m/z* 457.2222 (Figure 4.16) and vindolidine at *m/z* 427.2140 (Figure 4.17)

Table 4.11: Identification of alkaloid compounds in *C. roseus* F3 by UPLC-PDA and LC-MS data using positive ionization mode

Sample	Rt time(min)	m/z of fragment ions observed	Suggested formula	Observed m/z	Tentative identification	M.W. (g/mol)
C. roseus F5	1.188	280, 350, 191, 300, 254	$C_{26}H_{22}O$	351.1722	Ajmalicine	350.17
	2.231	280, 350, 191, 300, 254	$C_{21}H_{32}N_2O_9$	457.2222	Vindoline	456.22
	2.411	280, 350, 191, 300, 254	$C_{25}H_{30}O_6$	427.214	Vindolidine	426.21

RT: Retention Time, M.W.: Mol

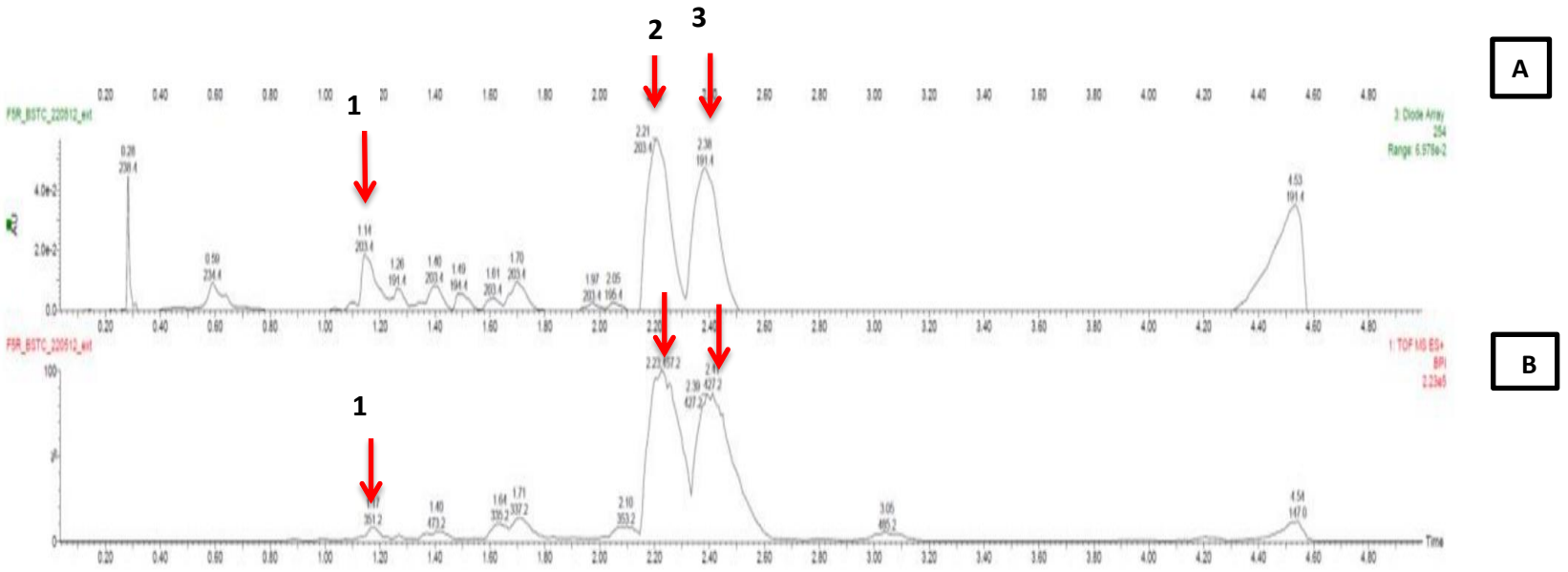
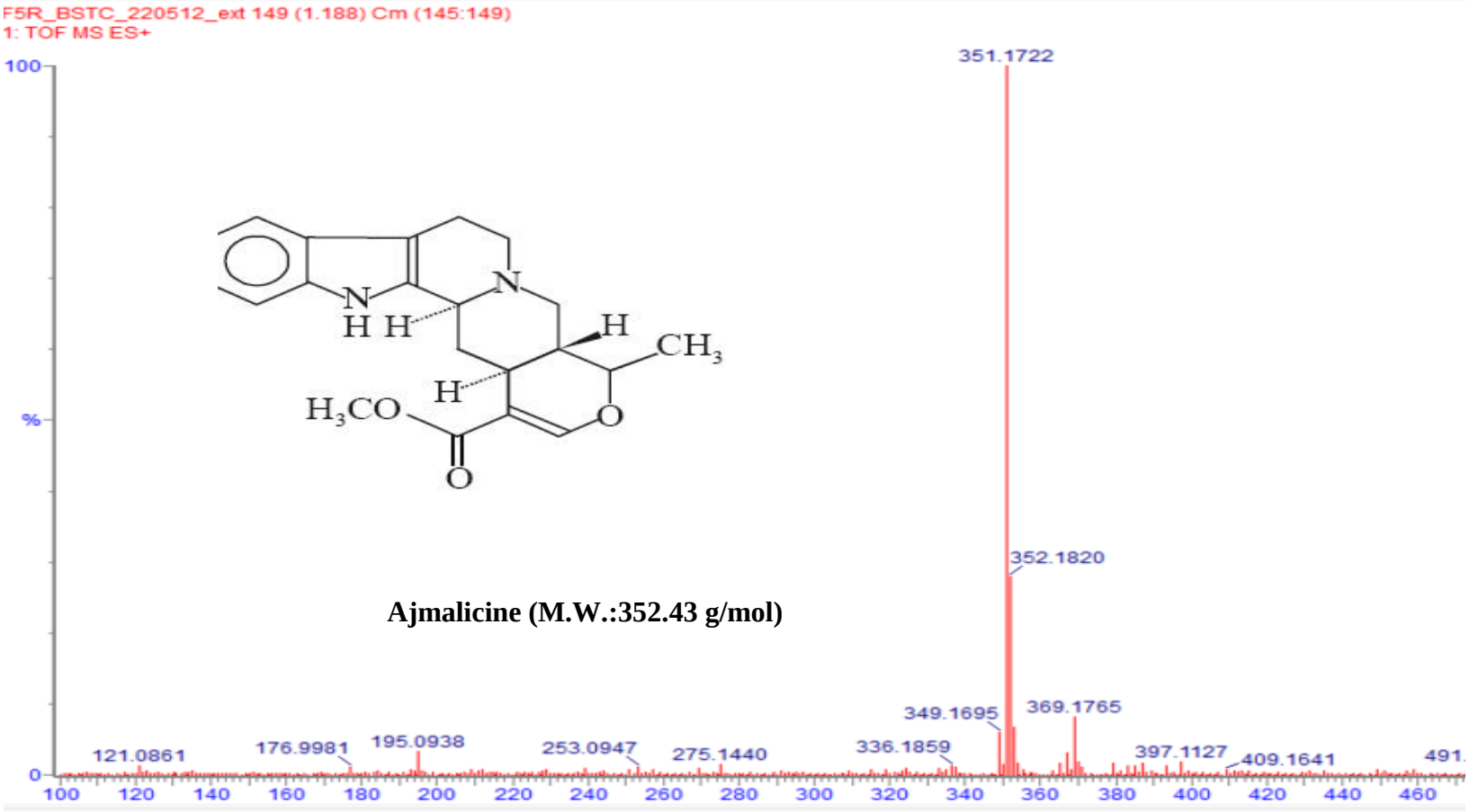


Figure 4.14: HPLC-TOF/MS and UV diode array chromatograms of *C. roseus* fraction 5 (F5); (A) Diode array detection UV spectra at 351 nm; (B) TOF MS peaks in positive mode ionization



Ajmalicine (M.W.:352.43 g/mol)

Figure 4.15: Mass Spectrum (TOF MS ES+), chemical structure and UV max spectra of ajmalicine (peak No. 1) identified in *C. roseus* F5.

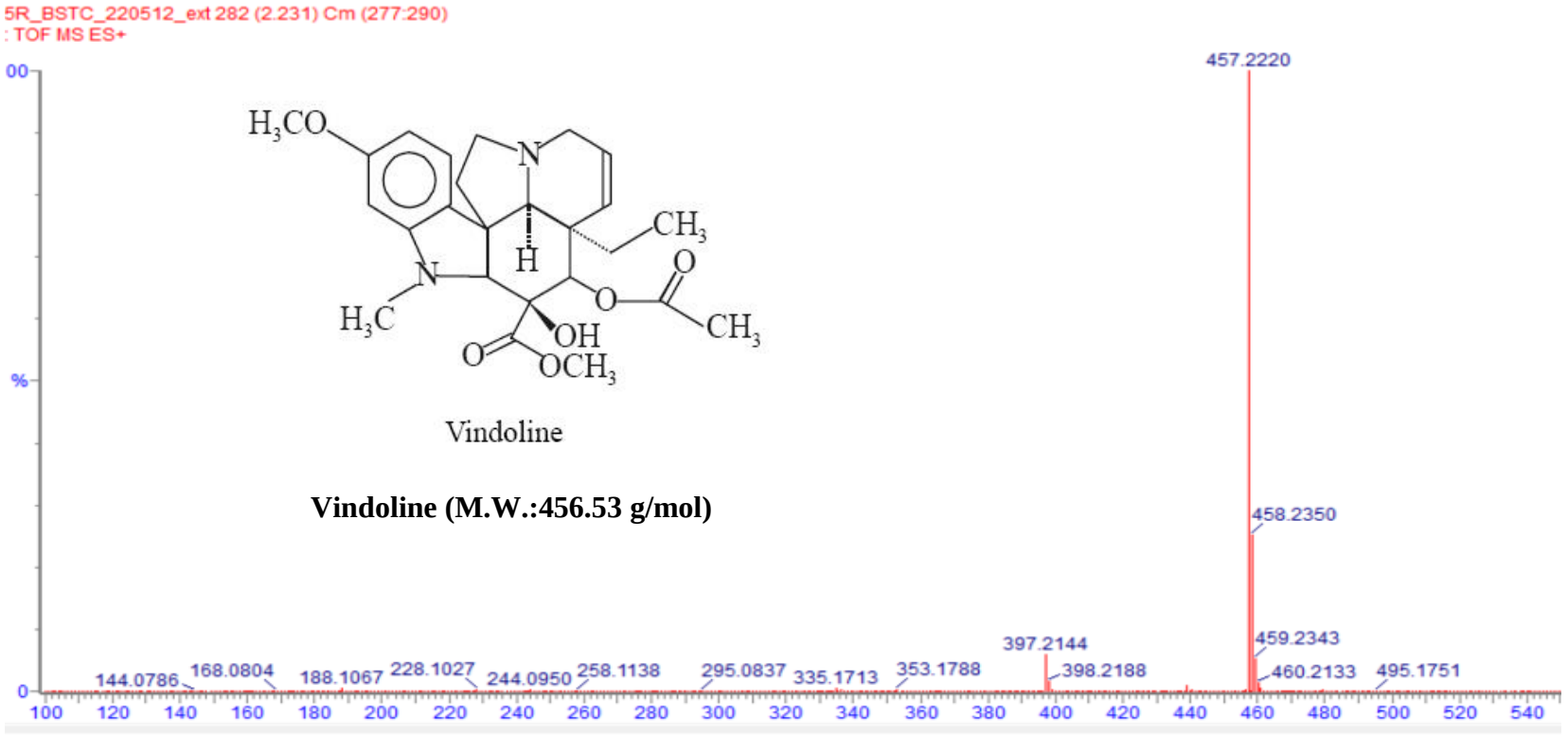


Figure 4.16: Mass Spectrum (TOF MS ES+), chemical structure and UV max spectra of vindoline (peak No. 2) identified in *C. roseus* F5.

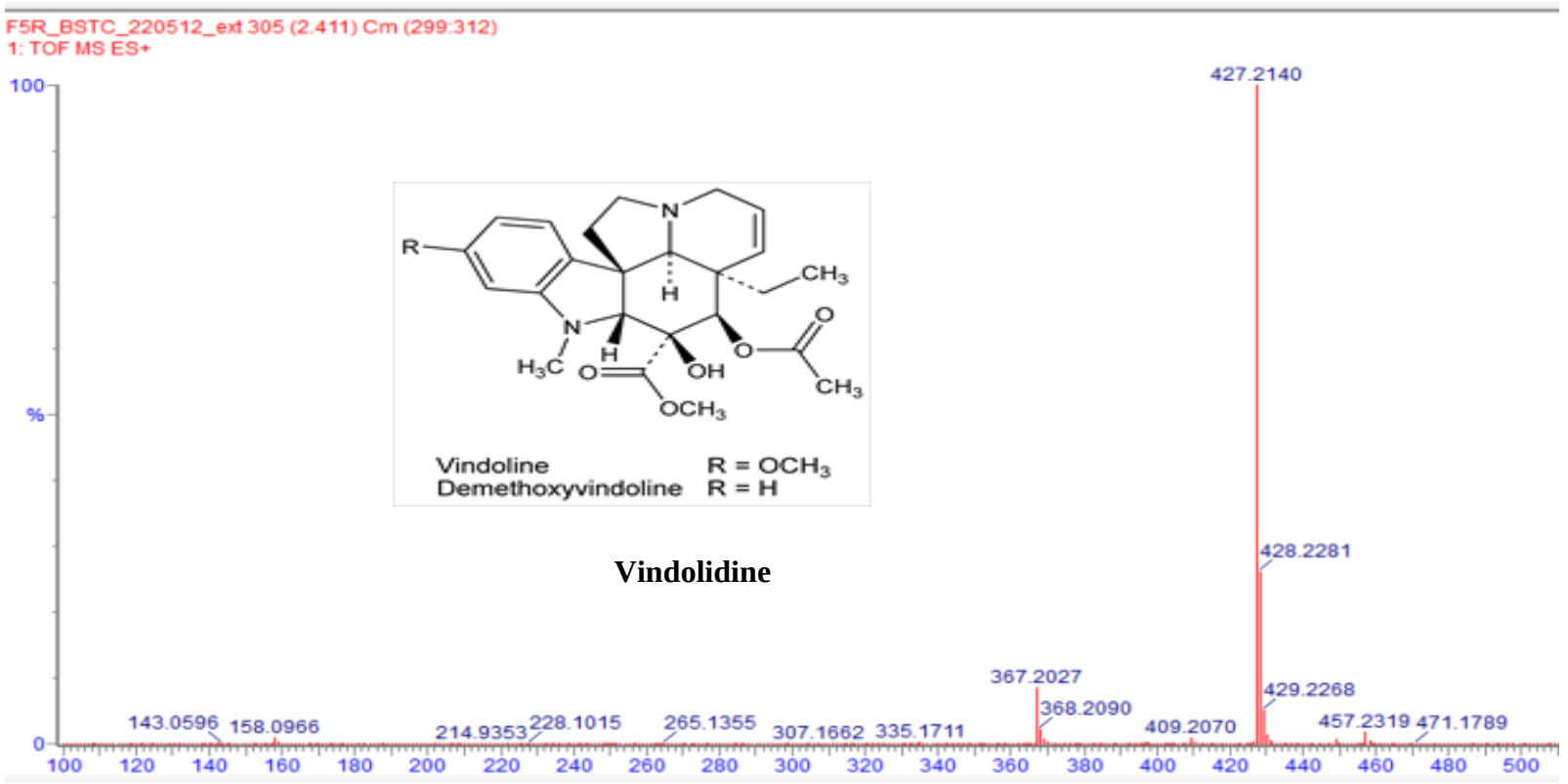


Figure 4.17: Mass Spectrum (TOF MS ES+), chemical structure and UV max spectra of vindolidine (peak No. 4) identified *C. roseus* F5.

4.12 Antioxidant activity of *A. ilicifolius* extract *in vitro*

4.12.1 Ferric-reducing antioxidant power (FRAP) assay results

Figure 4.18 shows that *A. ilicifolius* had a strong reducing power as it attained significantly $p<0.05$ with higher FRAP value 1670 ± 0.277 µmol Fe II/g as compared to BHT and Ascorbic acid 521.4 ± 0.82 and 1181.9 ± 0.274µmol Fe II/g respectively. Furthermore, the FRAP value for the synthetic reference antioxidants qurcetin and gallic acid were reported to be 2267.1 ± 0.55 and 2640 ± 0.277 µmol Fe II/g respectively with regression coefficient of (standard curve equation: $y = 0.0006x + 0.0013$, $R^2 = 1$) as shown in appendix A1.

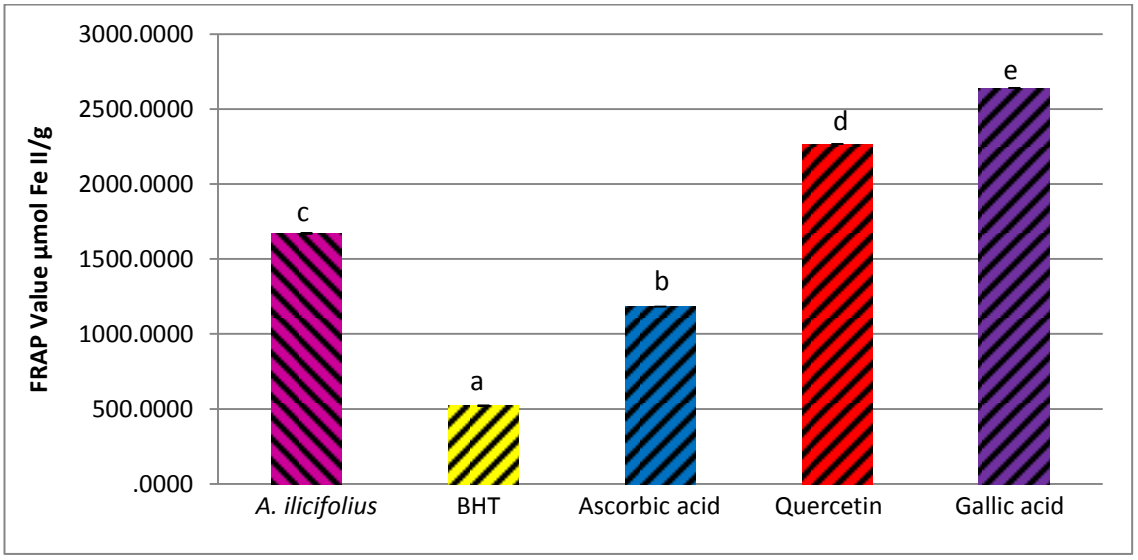


Figure 4.18: Ferric reducing activity of *A. ilicifolius* extract. All values are expressed as mean ± SEM. Mean with different letters indicate significantly different values $P< 0.05$. BHT; Butylatedhydroxyl toluene

4.12.2 DPPH free radical scavenging activity of *A. ilicifolius* extract *in vitro*

Antioxidant activity is measured as the capacity of the tested compound to reduce the stable DPPH radical. The scavenging capability of *A. ilicifolius* increases with the increase in concentration of the *A. ilicifolius* and is same as the BHT. Figure 4.19 The IC_{50} value represents the concentration needed to cause 50 % inhibition of DPPH Different concentrations of the extracts, as well as BHT, quercetin and trolox, were used and the IC_{50} values were determined to be 12.20 µg/ml for *A. iIlicifolius* 17.40 µg/ml for BHT, 7.40 µg/ml for quercetin and 6.20 µg/ml for trolox with standard curve equation: $y = 1.2997x + 0.1834$, $R^2 = 0.997$) as shown in Appendix A2.

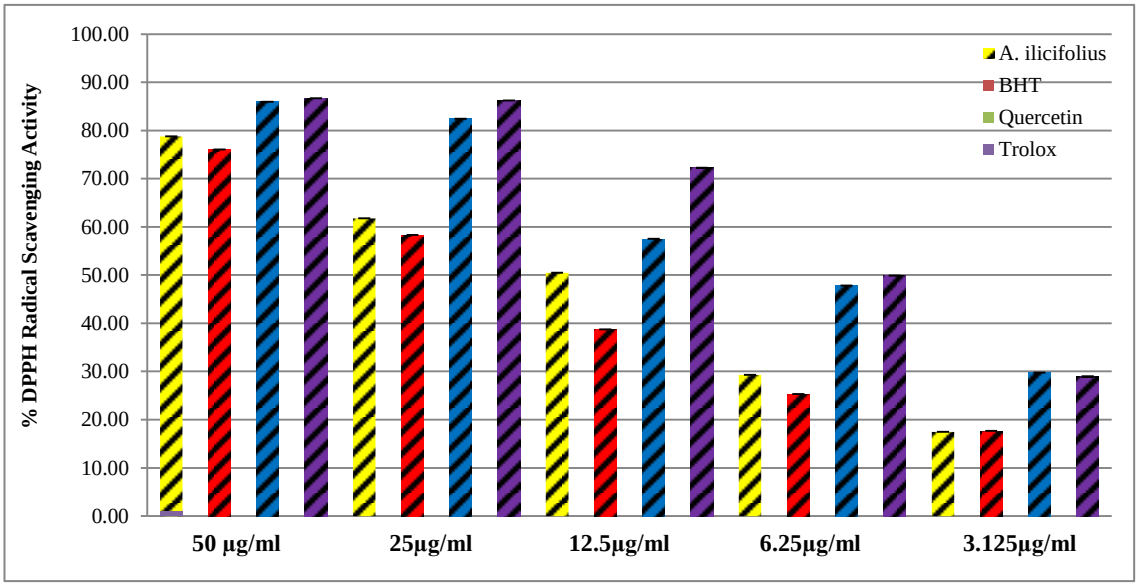


Figure 4.19: Scavenging Activity of *A. ilicifolius* extract (DPPH). All values are expressed as mean ± SEM, $P< 0.05$.

4.12.3 Total phenolic and flavonoids content assay

Total phenolic content of the ethanolic extract of *A. ilicifolius* was determined to 281.90 ± 0.27 mg (Gallic acid equivalents) per g of extracts with standard curve equation: $y = 0.0007x + 0.0015$, $R^2 = 0.989$ as shown in Appendix A3.

The total Flavonoids were 46.79 ± 0.005 mg (Quercetin equivalents) per g of extracts (standard curve equation: $y = 0.0054x + 0.005$, $R^2 = 0.999$) as shown in appendix A4 with ratio flavonoids/phenolic of 0.2. Thus, phenolic compounds were the predominant antioxidant components in *A. ilicifolius* extracts, which leads to more potent radical scavenging effect.

4.13 Acute toxicity study

Acute toxicity study is an experimental study in which selected rats are treated with the *A. ilicifolius* extract at a dose of 2 g/kg and 5 g/kg and were kept under observation for 14 days. All the animals remained alive and did not manifest any significant visible of toxicity at these doses. By chemical and histologic indicators, no hepatic or renal toxicity was seen. The finding from the applications of the *A. ilicifolius* extract to the control rats provided sufficient evidence to conclude that the orally administered extract was safe and presented no extract-related toxicity even at the highest dose of 5 g/kg as shown in (Table 4.12, 4.13 and Figure 4.20).

Table 4.12: Effects of *A. ilicifolius* extract on kidney biochemical parameters in acute toxicity

Dose	Sodium (mmol/L)	Pottasium(mmol/L)	Chloride (mmol/L)	CO_2 (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Vehicle (10% Tween-20)	138.68 ± 0.49	5.25 ± 0.19	104.43 ± 1.95	22.55 ± 0.88	18.25 ± 0.97	5.86 ± 0.38	51.05 ± 1.51
LD *A. ilicifolius* (2g/kg)	138.35 ± 0.52	5.33 ± 0.18	103.07 ± 1.52	23.61 ± 0.79	17.13 ± 1.12	5.76 ± 0.41	48.94 ± 1.28
HD *A. ilicifolius* (5g/kg)	137.42 ± 0.58	5.09 ± 0.17	101.95 ± 1.21	23.38 ± 0.95	17.41 ± 0.91	6.02 ± 0.45	49.36 ± 1.73

Values expressed as mean ± SEM. There are no significant differences between groups, $P < 0.05$.

Table 4.13: Effects of *A. ilicifolius* extract on liver biochemical parameters in acute toxicity.

Dose	T. protein (g/L)	Albumin (g/L)	Globulin (g/L)	TB (µmol/L)	CB (µmol/L)	AP (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)
Vehicle (10% Tween-20)	72.29 ± 1.62	11.47 ± 0.65	59.76 ± 1.69	1.94 ± 0.19	1.00 ± 0.00	132.58 ± 9.03	52.35 ± 3.68	1513.45 ± 8.55	4.96 ± 0.91
LD *A. ilicifolius* (2 g/kg)	71.68 ± 1.23	11.31 ± 0.49	58.81 ± 1.73	2.06 ± 0.17	1.00 ± 0.00	133.45 ± 8.27	52.47 ± 2.13	154.26 ± 6.59	5.02 ± 1.13
HD *A. ilicifolius* (5 g/kg)	71.83 ± 1.08	10.84 ± 0.41	60.13 ± 1.88	1.87 ± 0.20	1.00 ± 0.00	134.15 ± 8.62	53.24 ± 3.28	158.02 ± 5.91	5.28 ± 1.02

Values expressed as mean ± SEM There are no significant differences between groups, $P < 0.05$. Tb: Total bilirubin; Cb: Conjugated bilirubin; Alp: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: G-Glutamyl Transferase.

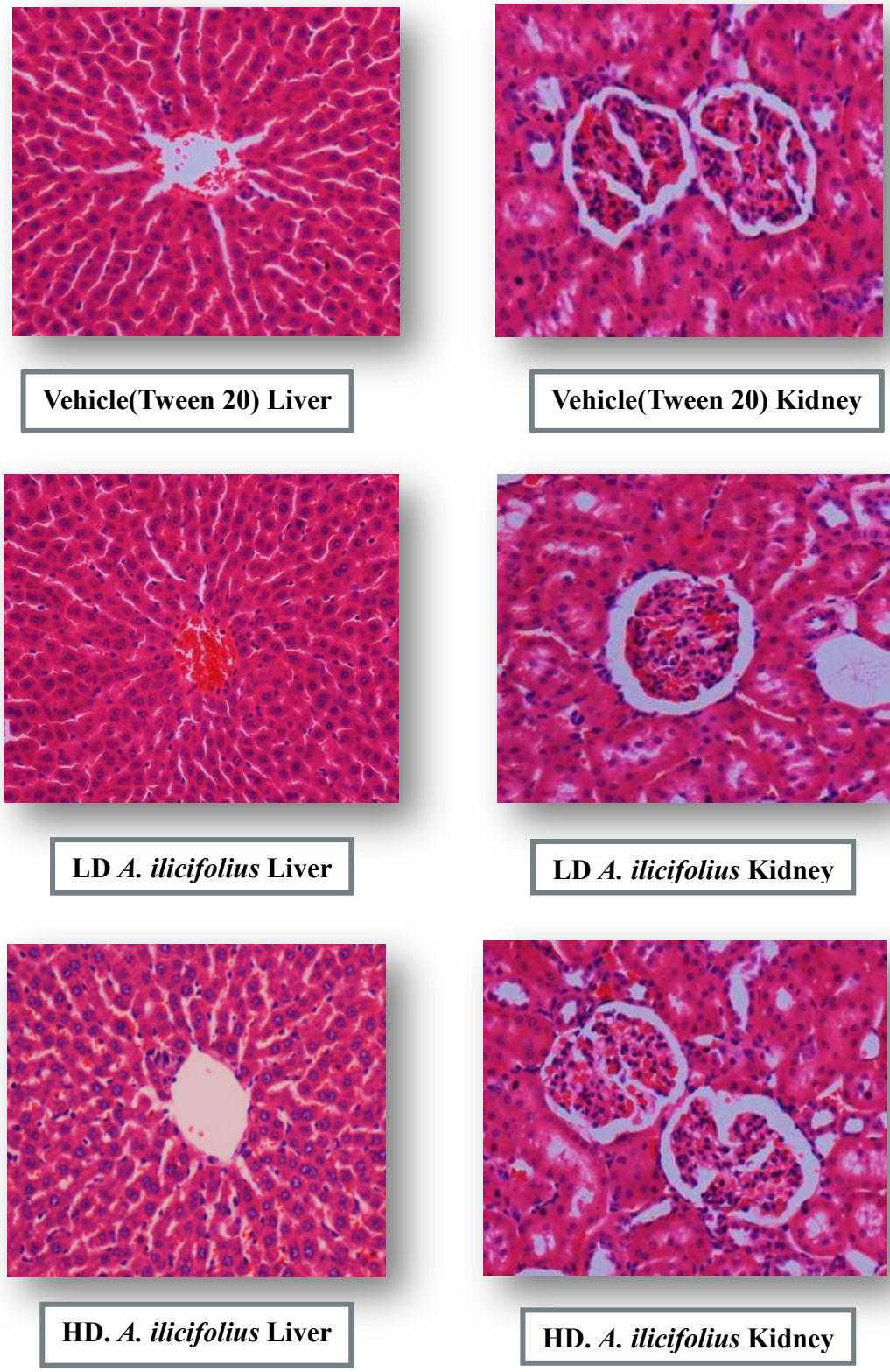


Figure 4.20: Histological sections. Effect of *A. ilicifolius* on liver and kidney on acute toxicity test (H & E stain; 20x).

4.14 Analysis of body weight

The body weight of all rats were taken at the beginning (1 week) of the experiment then weekly up to 10 weeks (Figure 4.21). Although there is differences between groups, statistically there were no significant differences in the body weight among the groups.

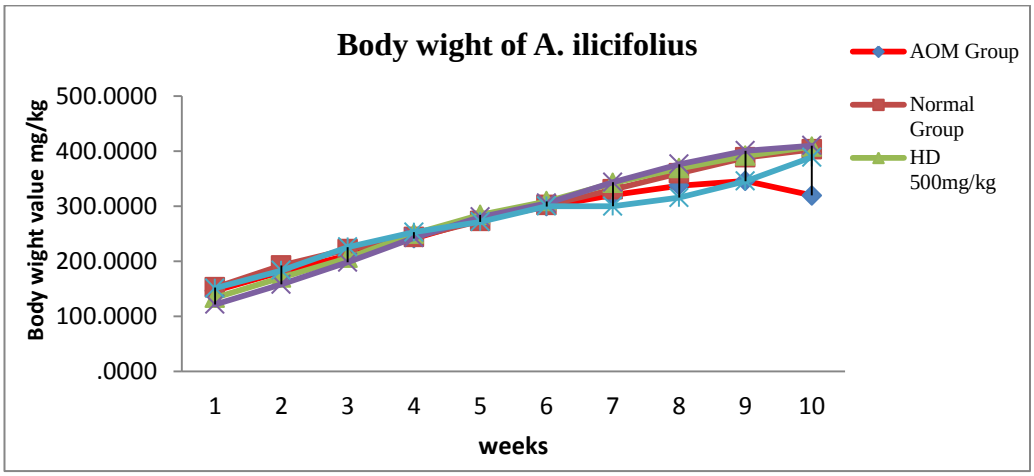


Figure 4.21: Effect of *A. ilicifolius* on body weight of rat's in different experimental groups**.** All values expressed as mean ± SEM. There were significant changes in body weights were observed in all rat's group, $P< 0.05$.

4.15 Effect of *A. ilicifolius* on colonic ACF

ACF were predominantly observed in the middle colon. ACF present in the colon were counted and the average of total number of ACF as well as the number of crypts per focus was obtained. ACF were observed in the colon as well as multicrypt clusters (more than four crypts/focus) of aberrant crypts (Table 4.14) Rats treated with AOM and fed with the *A. ilicifolius* extract showed a significant lower number of total ACF/colon compared with the AOM-control rats (65 % and 53 % respectively inhibition, $P< 0.0001$). The incidence of multiple aberrant crypts/focus was also significantly inhibited in rats fed with *A. ilicifolius* as compared with the AOM-control rats.

Table 4.14: Effect of *A. ilicifolius* on AOM-induced colonic ACF containing four or more aberrant crypts in rats

Groups	1 crypt	2 crypts	3 crypts	4 crypts	5&more crypts	Total ACF	inhibition %
AOM control	19.50 ± 3.62	29.79 ± 4.89	23.04 ± 2.70	24.67 ± 2.22	34.33 ± 4.94	131.33 ± 15.09	0
5-Fluorouracil	10.16 ± 1.04	9.50 ± 2.04*	7.91 ± 0.69**	6.25 ± 0.81**	7 ± 0.64**	40.83 ± 3.06**	69%
LD 250 mg/kg *A. ilicifolius*	18.36 ± 3.14	10.05 ± 1.63*	4.30 ±.0.16**	4.47 ±.0.57**	8.13 ± 0.89**	45.33 ± 4.43**	65%
HD 500 mg/kg *A. ilicifolius*	17.08 ± 2.37	13.08 ± 2.55	11.91 ±.0.95**	8.75 ±.0.86**	9.08 ± 1.55**	62 ± 3.72**	53%

Values are expressed as mean ± S.E.M., Values with different symbol are significantly differences. $^{*}P< 0.001$ versus AOM control group and $^{**}P< 0.0001$ versus AOM control group.

4.16 Macroscopical Findings

The results are presented in (Figure 4.22) 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 were significantly higher in the AOM-control group compared to the *A. ilicifolius* treated groups. The multiplicity of ACF (number of crypt per ACF), which is considered as a marker for tumour promotion, was significantly higher in AOM group.

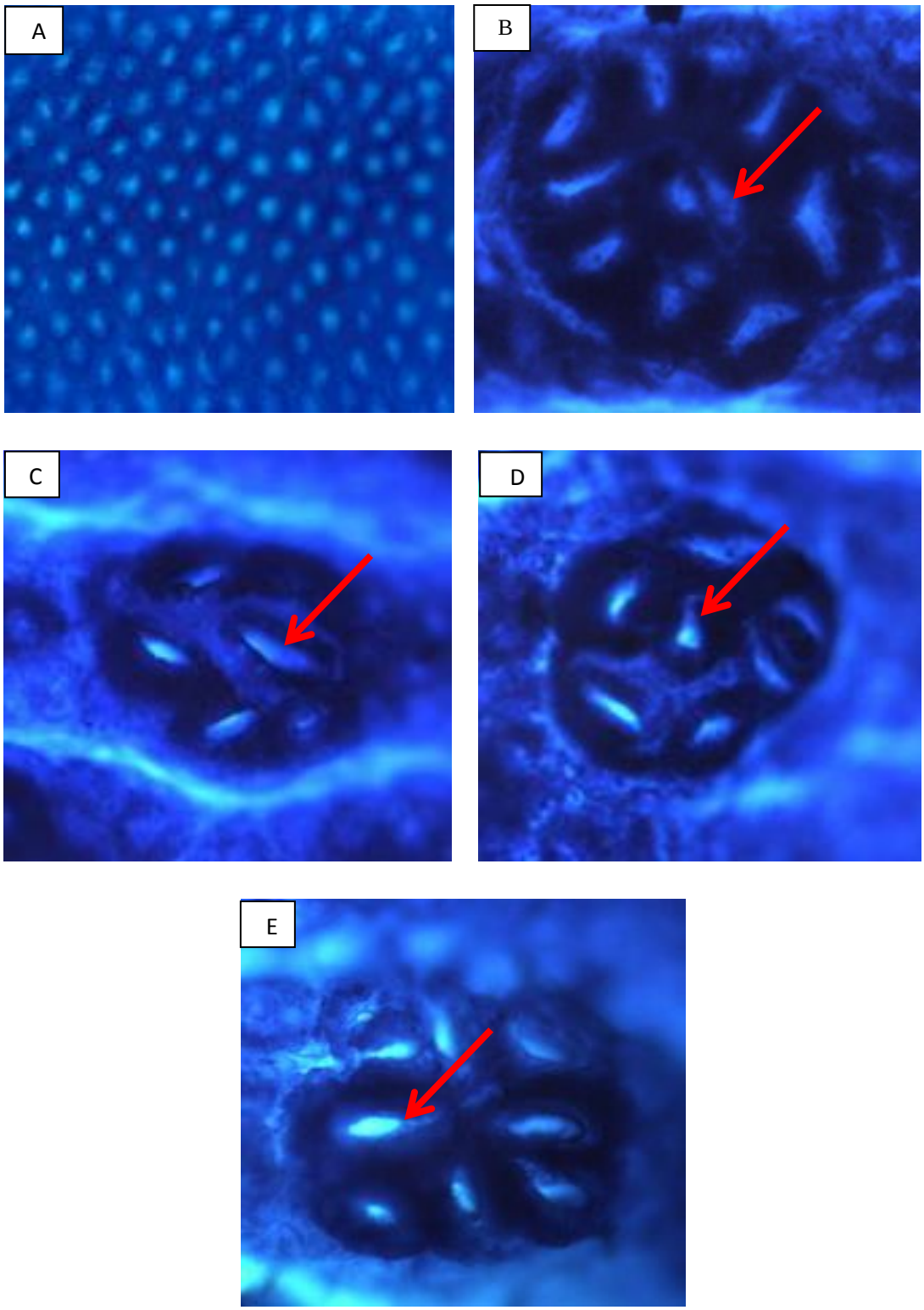


Figure 4.22: Topographic views show effect of *A. ilicifolius* on ACF in methylene blue staining on rat colonic tissue; (A) Normal crypts from rats treated with 10% Tween 20, normal control; (B) AOM control group; (C) Reference group (5-Fluorouracil) + AOM (D) 250 mg/kg *A. ilicifolius* + AOM; (E) 500 mg/kg *A. ilicifolius* + AOM (10x).

4.17 Histopathology examination

All the grossly visible colon lesions were evaluated histologically (Haematoxylin and Eosin stains). Normal control group showed circular shape and basal location of the nuclei. AOM control group contain comparatively more ACF exhibiting marked nuclear atypia, significantly decreased goblet cells, narrow lumen, ACF are longer and larger than normal, intense staining appearance of cell's cytoplasm. The epithelial cells in ACF have distinctive elongated and stratified nuclei, loss of cell polarity, and increase in mitoses compared to the surrounding regular crypts (Figure 4.23). Plant extract treated groups showed increases the apoptosis, reduced mitosis and cellular proliferation, and reduced the induction of ACF in rat colon with mild morphological changes of ACF compared with AOM-treated group (cancer control).

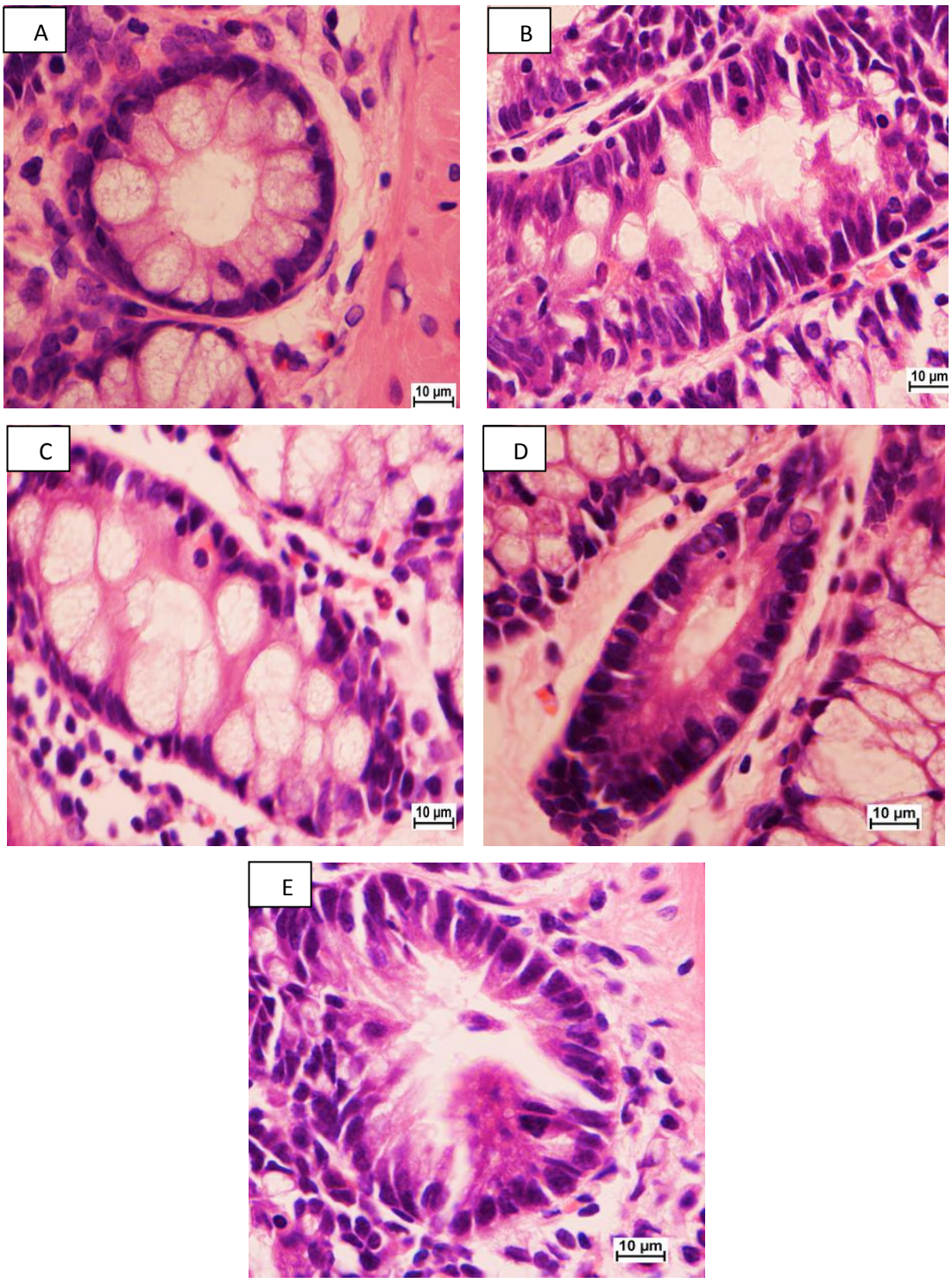


Figure4.23: Histological patterns of aberrant crypt foci. Effect of *A. ilicifolius* on AOM-induced ACF in rat's colon; (A) Normal control group; (B) AOM control group; (C) Standard drug group, 5-Fluorouracil + AOM; (D) 250 mg/kg *A. ilicifolius* + AOM; (E) 500 mg/kg *A. ilicifolius* + AOM (H& E stain 100x).

4.18 Effects of *A. ilicifolius* on biochemical parameters

The Total protein, and Albumin significantly increased in treated *A. ilicifolius* group compared to the AOM However, urea was significantly decreased in the treated *A. ilicifolius* groups compared to the AOM group, While there were no significant differences in glucose, createnine, AST, ALT, ALP, LDH and T. cholesterol in the *A. ilicifolius* treated group compare to the AOM group. A. *ilicifolius* treated group restored the level of T. protein, Alb and urea to reach normal values (Table 4.15, 4.16).

Table 4.15: Effects of *A. ilicifolius* on glucose, urea and createnine

Animal Group	**Glucose (mmol/L)**	**Urea (mmol/L)**	**Createnine µmol/L**
Normal	10.38 ± 0.56	5.44 ± 0.10	56.4 ± 5.52
AOM	9 ± 0.51	7.13 ± 0.65^{t}	40 ± 3.67
5-flourouracil	7.2 ± 0.45	5.56 ± 0.25*	46 ± 5.52
LD *A. ilicifolius*	8.51 ± 0.26	4.76 ± 0.20*	39 ± 4.48
HD *A. ilicifollius*	7.66 ± 0.32	5.31 ± 0.19*	38.6 ± 3.12

All values expressed as mean ± SEM. $^{t}P< 0.05$ versus normal control group, $^{*}P< 0.01$ versus AOM control group.

Table 4.16: Effects of *A. ilicifolius* on biochemical parameters

Animal Group	T. protein G/L)	Albumin (G/L)	AST IU/L	ALT IU/L	ALP IU/L	LDH U/L	T. chol mmol/L
Normal	64.37 ± 0.33	12.33 ± 0.21	203.2± 18.48	60 ± 3.43	98.4 ± 3.02	1080.2 ± 24.88	1.3 ± 0.00
AOM	58.20 ± 1.13^{t}	11.00 ± 0.44	181.83± 15.04	60.83 ± 5.36	86.66 ± 8.97	1414.33 ± 105.38	1.42 ± 0.11
5-flourouracil	65.66 ± 1.54**	12.16 ± 0.16	164.83± 10.8	52.33 ± 3.43	133.16 ± 7.24	796.33 ± 55.18 $^{\#}$	1.35 ± 0.50
LD *A. ilicifolius*	61.5 ± 0.84	13.16 ± 0.16$^{\#}$	157.5± 5.22	44.33 ± 4.08	107.5 ± 8.17	1218.1 ± 140.08	1.38 ± 0.03
HD *A. ilicifollius*	63.2 ± 0.97*	13.4 ± 0.41$^{\#}$	168.8 ± 9.54	39.6 ± 0.99	111.6 ± 6.14	1233.4 ± 156.45	1.4 ± 0.04

All values expressed as mean ± SEM. Values with different symbol are significantly differences. $^{t}P < 0.05$ versus normal control group, $^{*}P< 0.05$ versus AOM control group, $^{**}P< 0.01$ versus AOM control group and $^{\#}P< 0.001$ versus AOM control group. T. protein: Total protein, AST; Aspartate aminotransferase, ALT; Alanine Aminotransferase, ALP; Alkaline Phosphatase, LDH: Lactate dehydrogenase, and T. cho; Total cholesterol

4.18.1 Effects of *A. ilicifolius* on hematologic parameters

The Hb and Plt were significantly increased in the treated *A. ilicifolius* group compared to the AOM group, while there were no significant differences in HCT, RBC, MCV, MCH, MCHC, RDW and total WBC count in the *A. ilicifolius* treated group compared to the AOM group. AOM induced oxidative damages in erythrocytes, leading to reduction Hb and plt concentration. *A. ilicifolius* treatment leads to increase the concentration to reach normal values. (Table 4.17, 4.18)

Table 4.17: Effects of *A .ilicifolius* on hematological parameters

Animal Group	Hb G/L	HCT (L/L)	RBC (10^{12}/L)	MCV (fL)	MCH (pg)	MCHC (G/L)	RDW (%)
Normal	154.00 ± 1.12	0.464 ± 0.01	8.33 ± 0.13	55.8 ± 0.65	18.26 ± 0.20	328.4 ± 0.76	14.2 ± 0.46
AOM	142.17 ± 2.35 ^{t}	0.455 ± 0.01	8.195 ± 0.13	55.16 ± 0.96	17.55 ± 0.22	317.5 ± 2.46	14.76 ± 0.65
5-flourouracil	154.83 ± 2.18**	0.488 ± 0.00	8.435 ± 0.11	57.83 ± 0.79	18.36 ± 0.30	317.66 ± 2.72	15.08 ± 0.25
LD *A. ilicifolius*	151 ± 1.15*	0.45 ± 0.01	8.29 ± 0.08	55 ± 0.73	18.2 ± 0.17	330 ± 5.24	14.26 ± 0.48
HD *A ilicifollius*	153 ± 1.57**	0.46 ± 0.01	8.46 ± 0.06	55 ± 0.73	18.08 ± 0.18	328.2 ± 3.03	14.78 ± 0.33

All values expressed as mean ± SEM. Values with different symbol are significantly differences. ^{t}P < 0.05 versus normal control group, $^{*}P$< 0.05 versus AOM control group,$^{**}P$< 0.01 versus AOM control Hb: Hemoglobin; HCT: Hematocrit RBC: Red blood cell, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular hemoglobin, MCHC: Mean Corpuscular hemoglobin Concentration, RDW: Red Cell Distribution.

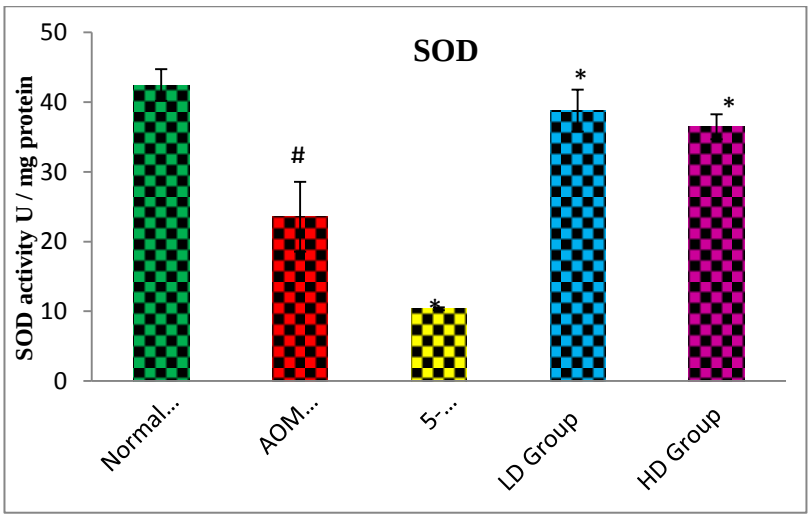
Table 4.18: Effects of *A. ilicifolius* on hematological parameters

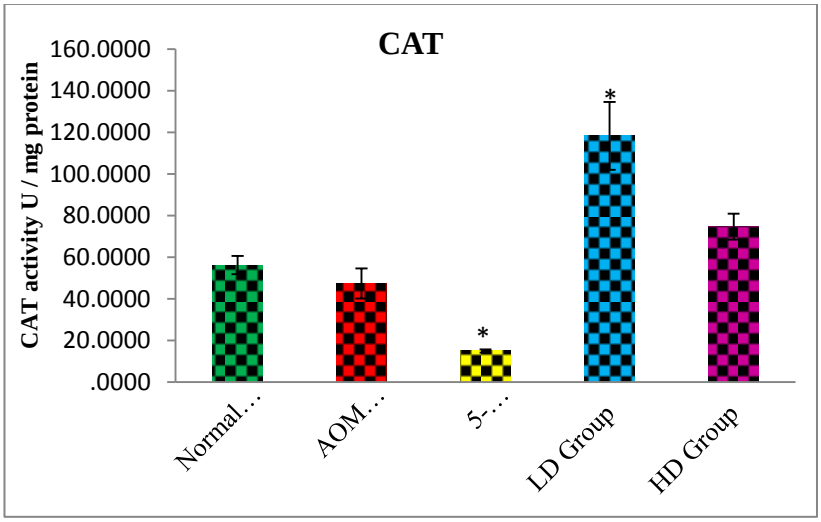
Animal Group	WBC 10^9/L	Platelet 10^9/L	Neutrophil %	Lymphocyte %	Monocyte %	Eosinophil %	Basophil %
Normal	12.98 ± 1.50	732.6±26.33	18.36±1.94	74.62±2.45	3.96±0.69	1.44±0.43	0±0.00
AOM	8.08 ± 0.66 ^{t}	542.66±94.38	17.93±4.54	59.45±12.37	5.35±1.06	1.61±0.54	0.1±0.03
5-flourouracil	7.28 ± 0.52	683±35.33	28.46±2.07	64.15±3.21	5.86±0.94	1.43±1.19	0.08±0.03
LD *A. ilicifolius*	8.76 ±1.15	771±29.04	22.8±2.51	70.6±2.98	5.56±1.17	0.86±0.19	0.05±0.02
HD *A. ilicifollius*	11.1 ± 0.54	834±53.49**	25.66±2.59	69.04±2.93	4.72±0.48	0.46±0.13	0.06±0.02

All values expressed as mean ± SEM. Values with different symbol are significantly differences, $^{t}P < 0.05$ versus normal control group, $^{*}P< 0.05$ versus AOM control group, and $^{**}P< 0.01$ versus AOM control WBC: white blood cell.

4.19 Effects of *A. ilicifolius* on antioxidant and lipid peroxidation (TBRAS) in AOM induced ACF colonic aberrant

Figure 4.24 shows the *in vivo* activities of oxidative enzymes involved in the colon antioxidant defense system. SOD and CAT enzyme levels were decreased in the AOM groups when compared with the normal control group and similarly their levels were lower in the high dose plant group when compared with the low dose group. Furthermore, MDA values in colon of the low dose group rats were lower than those in the high dose groups However, the 5- Fluorouracil values was lower than those in the low dose and high dose groups.





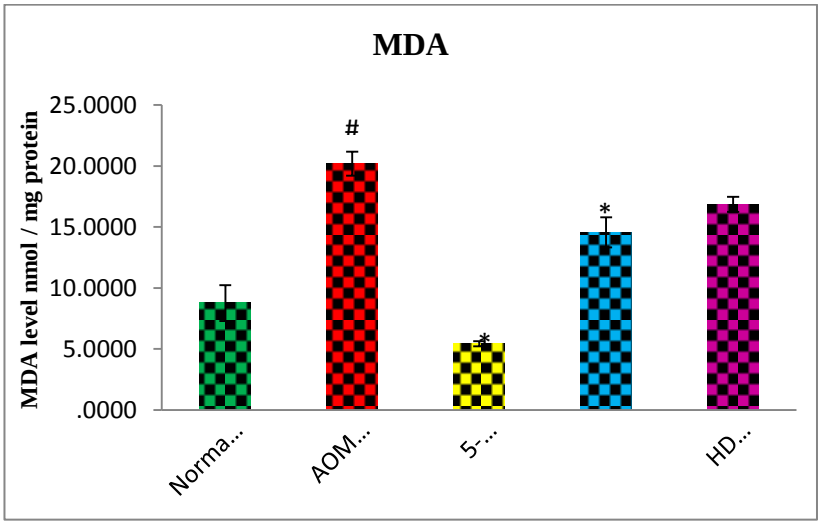


Figure 4.24 Effect of*A.ilicifolius* extracts *in vivo* antioxidant activities parameters, in AOM -induced colon cancer rats. All values are expressed as the mean ± SEM. * =

significantly different means compared to the AOM group, # = significantly different means compared to the normal group, $p < 0.05$.

4.20 ImmunohistochemistryAnalysis

Photomicrographs of ACF exhibiting grades of nuclear morphology from colons of rats receiving AOM were captured. All sections were cut parallel to the muscle layer. Note the presence of elongated and stratified nuclei were noted throughout the crypt.

4.20.1 PCNA staining of colons and cell counting

The PCNA was evaluated as a marker for cell proliferation in the colon specimens. Sections of colon samples from the control group and the or *A.ilicifolius*-treated group are shown (Figure 4.25). The PCNA positive staining cells in the nucleus (brown red) of mucosa of the colon tissue were much stronger in azoxymethane-treated rats than in the *C. roseus*-fed group. PCNA-negative cells (blue) were stained with hematoxylin.

The PCNA labelling index is also shown in (Figure 4.26). The colon sections from the AOM control group showed a higher number of positive cells than those from the AOM + *A. ilicifolius* -treated group

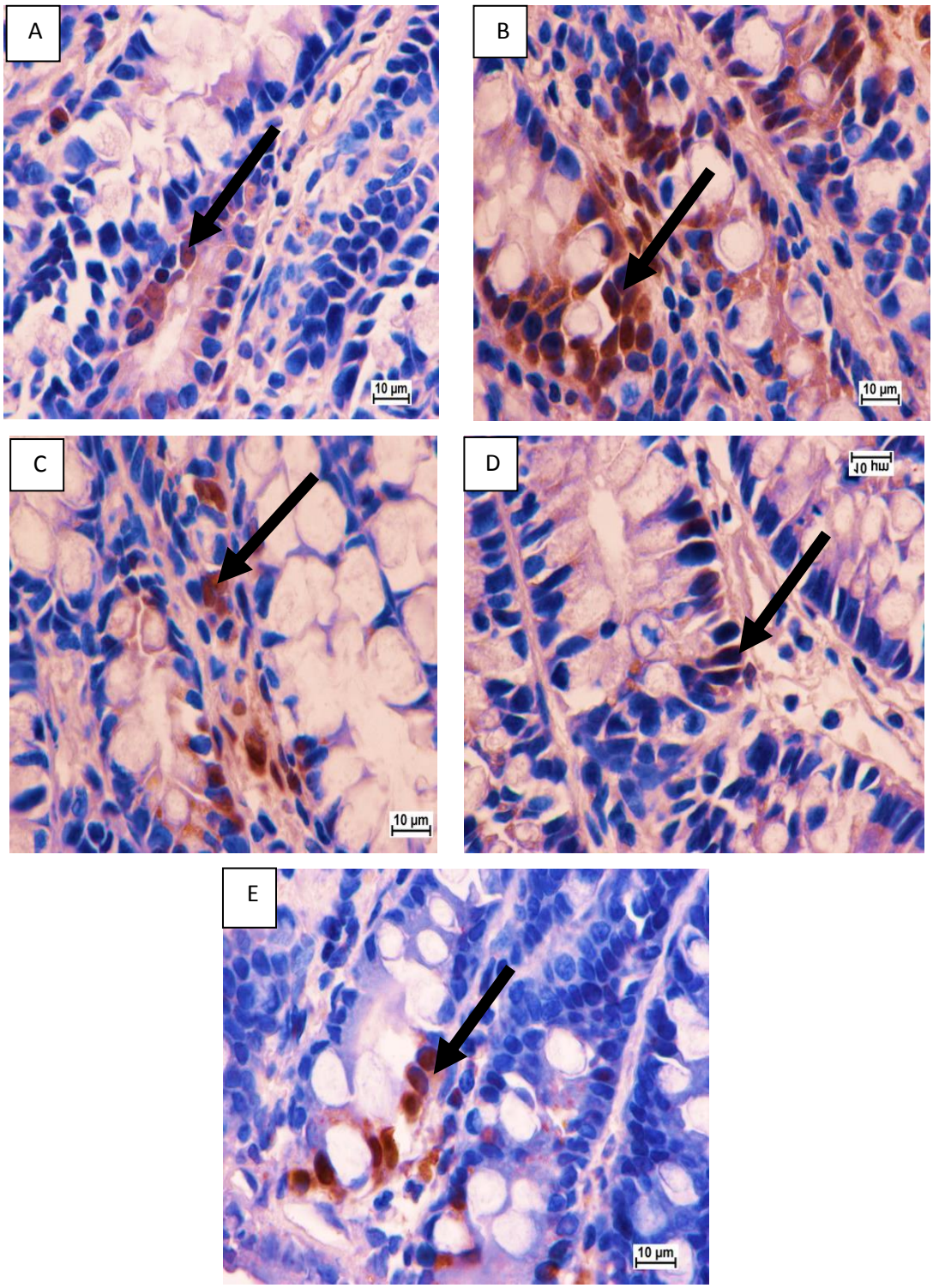


Figure 4.25: Immunohistochemical staining: Effect of *A. ilicifolius* on PCNA staining of colon mucosa in AOM-induced ACF in rats. (A) Normal control group. (B) AOM-control group. (C) 5-Fluorouracil drug treated group + AOM. (D) 250 mg/kg *A. ilicifolius* + AOM; (E) 500 mg/kg *A. ilicifolius* + AOM (PCNA staining 100x).

The percentage of PCNA-positive cells of the colon tissue in the AOM control group were 100 %, whereas the percentage of PCNA-positive cells from the 5-Fluorouracil and *A. ilicifolius* -low and high dose treated group were 25.47, 35.47 and 47.30 % (Figure 4.31).

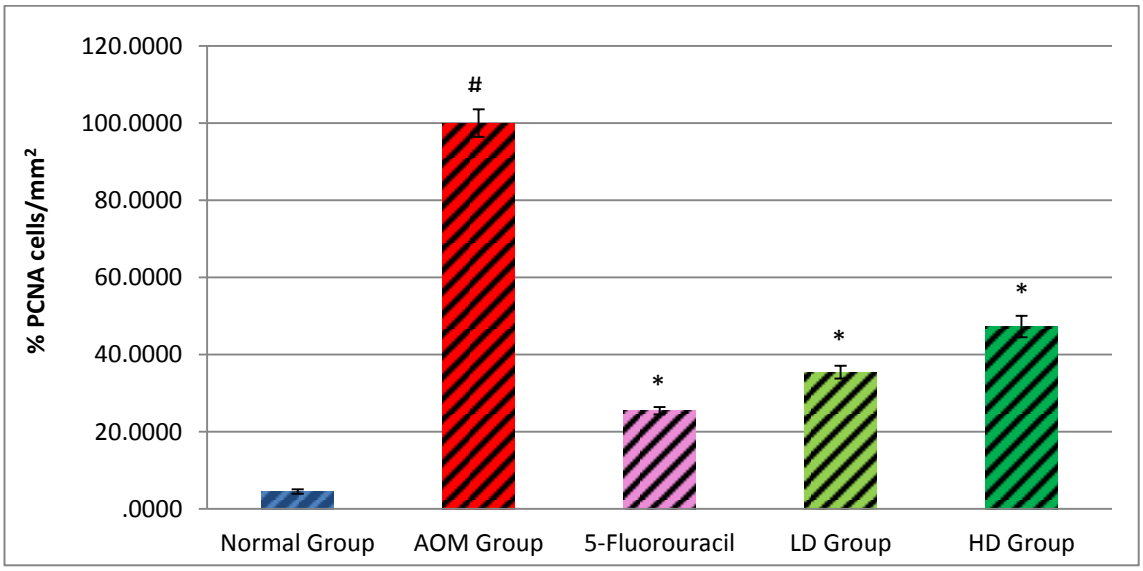


Figure 4.26: Effects of *A. ilicifolius* on PCNA counting in AOM-induced ACF in rats. All values are expressed as the mean ± SEM. * = significantly different means compared to the AOM group, # = significantly different means compared to the normal group, $P< 0.01$.

4.21 Gene expression profile

4.21.1 Integrity of RNA

The ratio of absorbance reading at 260 nm and 280 nm were used to indicate the quality of the RNA .The 260/280 ratio for our RNA preparation always ranged from 1.6-2.1 (Table4.19), which suggested good quality RNA.

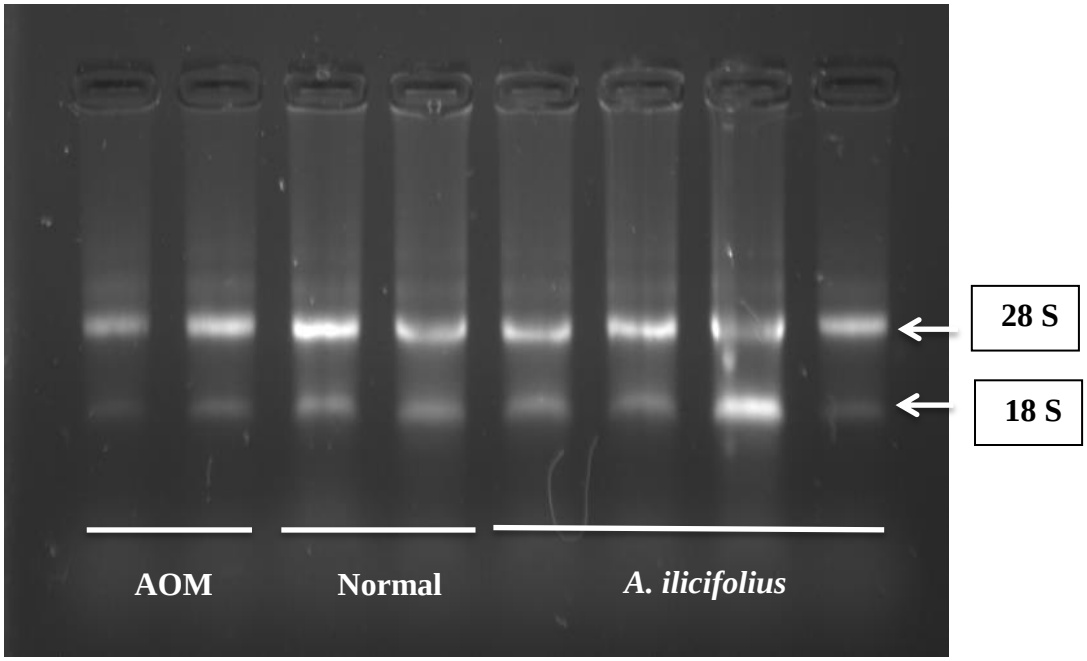
The integrity of RNA was checked by agarose gel electrophoresis. Discrete 28S and 18S ribosomal RNA bands were obtained in each case, with the 28S band about twice the 18S band, indicating that the RNA extracted was intact and could be applied for RT-PCR (Figure 4.27) shows a typical ethidium bromide stained-agarose RNA gel.

Table 4.19: RNA concentration and purity measured by NanoDrop Spectrophotometer at 230, 260, 280, 320, 260/320 nm absorbance

Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230
Calibrator 1	1082.8	ng/µl	27.07	12.677	2.14	1.97
Calibrator 2	634.6	ng/µl	15.865	7.546	2.1	2.04
Calibrator 3	632.2	ng/µl	15.805	7.561	2.09	2.08
Calibrator 4	464.1	ng/µl	11.603	5.549	2.09	1.45
Calibrator 5	410.5	ng/µl	10.264	4.912	2.09	1.91
Calibrator 6	502.5	ng/µl	12.561	6.076	2.07	2
AOM1	271.4	ng/µl	6.785	3.259	2.08	1.94
AOM2	320	ng/µl	8	3.806	2.1	0.84
AOM3	569.3	ng/µl	14.233	6.771	2.1	1.76
AOM4	1089	ng/µl	27.224	12.911	2.11	2.04
AOM5	879.6	ng/µl	21.991	10.421	2.11	1.83
AOM6	671	ng/µl	16.774	8.018	2.09	2.15
A. ilicifolius1	690.9	ng/µl	17.273	8.136	2.12	2.15
A. ilicifolius2	1144.1	ng/µl	28.603	13.362	2.14	2.17
A. ilicifolius3	1007.2	ng/µl	25.18	11.782	2.14	1.84
A. ilicifolius4	1317.7	ng/µl	32.943	15.552	2.12	2.16
A. ilicifolius5	1024.2	ng/µl	25.605	12.17	2.1	2.21
A. ilicifolius6	617.5	ng/µl	15.438	7.625	2.02	1.9

N: Normal control group, AOM: Azoxymethan treated group.

(A)



(B)

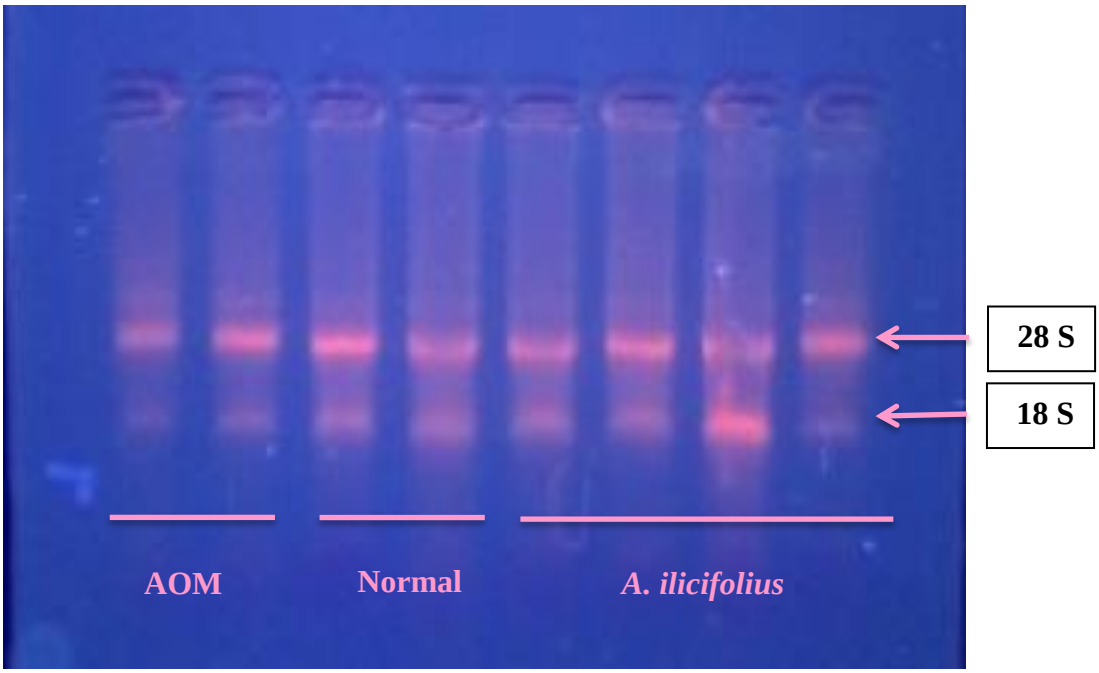


Figure 4.27: A typical ethidium bromide-stained agarose gel showing the integrity of the extracted RNA; (A) Visualized under Vilber Lourmat gel documentation system; (B) Visualized under UV light.

4.21.2 *A. ilicifolius* activates apoptosis cell death

4.21.2.1 Effect of *A. ilicifolius* on apoptosis gene expression

The gene expression levels of the target genes in animal colon tissue samples were normalized using both HPRT-1 and Tbp endogenous reference genes. The expression levels of Bax, Bcl2, p53 and the two endogenous reference genes were subsequently validated by RT-PCR measurements. According to a standard curve (Appendix A9) all genes showed efficiency was between 90-110 % and the slope between (-3.1) and (-3.5) which is within the reference criteria necessary to run the quantitative RT-PCR (Table 4.20). After data analysis of Ct values by Gene EX software and normalized to the reference genes HPRT-1 and Tbp, all measured mRNAs showed different expression between calibrator group (normal control tissue), AOM group (cancer group) and *A. ilicifolius* treatment rats group. The expression of Bax and Bcl2 genes in AOM group were significantly decreased, being (1.197 ± 0.003), $P < 0.003$, (0.24 ± 0.20) $P < 0.003$ respectively, with the fold being lower when compared with the normal rats group (Calibrator group). However, the difference was non-significant for p53 gene (0.76 ± 0.23) $P < 0.23$ fold lower compared with normal rats Table 4.21.

A. ilicifolius induced the expression of apoptosis-related genes that caused significantly up-regulation of pro-apoptotic protein Bax (0.92 ± 0.26), $P < 0.024$ and p53 1.54 ± 0.28, $P < 0.02$ while *A .ilicifolius* treatment did not significantly modify the expression level of anti-apoptotic protein Bcl-2 (0.656 ± 0.18), $P < 0.057$ in the mucosa compared of the AOM –injection rats. Bcl2 gene expression did not significantly change with *A. ilicifolius* treatment in contrast to Bax and p53 transcript which was significantly up-regulated. In addition *A. ilicifolius* treatment caused a switch in the Bcl-2/Bax ratio, which was elevated in the mucosa of AOM-injected rats (Bcl-2/Bax>1) and was reversed after *A. ilicifolius* treatment (Bcl-2/Bax <1), (Figure 4.28 and Table 4.21).

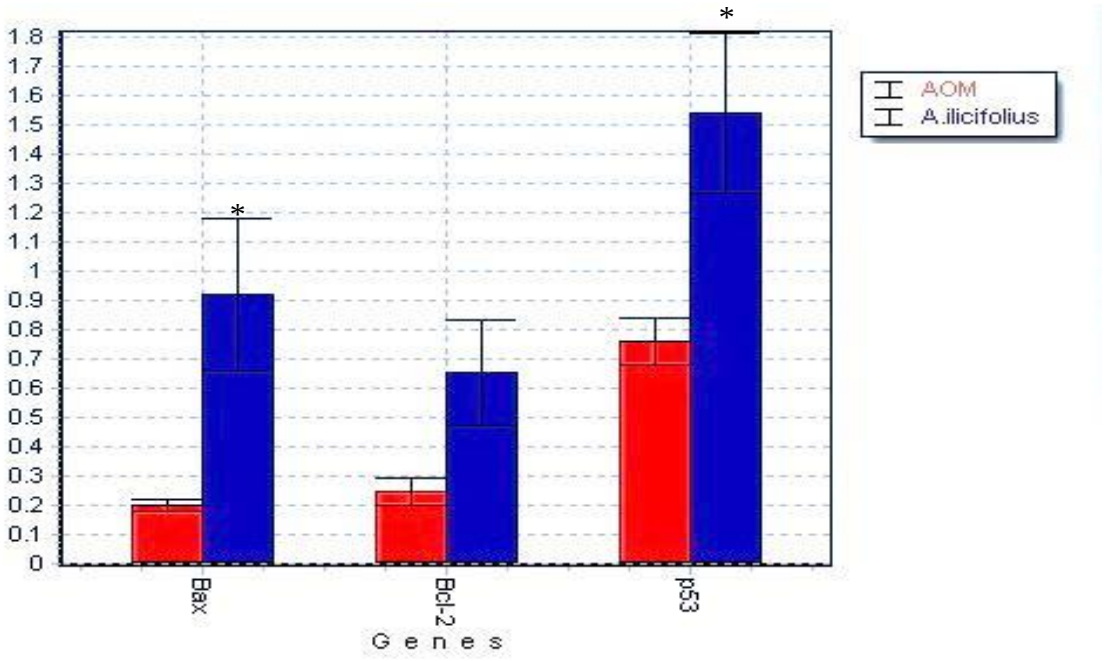


Figure 4.28: Changes in the mRNA expression levels of Bax, Bcl-2 and P53 in the colonic mucosa of AOM-injected rats and *A. illicifolius* treated AOM-injected rats. Real-time PCR was done in triplicate. The mRNA level from colonic mucosa of normal control rats was used as an external reference. The level of Hprt1 and Tbp mRNA were used as an internal reference to normalize the data. Data are presented as the mean ± SEM, $P < 0.05$.

Table 4.20: Measured efficiency slope and R^2 of target and endogenous reference genes using the Ct slope method with 5 concentrations points

Genes	Hprt1	Tbp	Act-b	p53	Bax	Bcl_2	Reference criteria
Efficiency	109.4	108.4	96.0	110	109.4	108.9	90-110
Slope	-3.1	-3.1	-3.4	-3.1	-3.1	-3.1	(-3.1)- (-3.5)
y-inter	30.3	23.3	32.1	33.4	30.3	30.2	--
R^2	0.99	0.99	0.98	0.99	0.99	0.98	Not less than 0.98

Table 4.21: Effect of AOM, *A. ilicifolius* extract intake on mRNA gene expression levels in AOM-induced rat colon tissue

Group	Bax	Bcl2	p53
Calibrator	1	1	1
AOM	0.197 ± 0.003*	0.24 ± 0.20*	0.76 ± 0.23
A. illicifolius	0.92 ± 0.26*	0.65 ± 0.18	1.54 ± 0.28*

All values are expressed as mean fold changes of mRNA from 6 rats ± S.E.M. Statistical analysis of differences was performed by a two-tailed unpaired student's t test. Means with different superscripts are significantly different, $P < 0.05$.

4.22 *In vitro* chemo-protective activity of *A. ilicifolius* crude extracts and isolated fractions

The inhibitory effect of the crude extract and fractions of *A. ilicifolius* has been tested *in vitro* against human colon cancer HT-29 cell line and normal human colon cells CCD_{841} using MTT assay. The crude extract inhibited colon cancer cells showed maximum inhibition on HT-29 cells (13.77 %) at the concentration 166.66 µg/ml and no variations in the cell viability of CCD_{841} cells observed, at the same time the fractions of the crude extract of *A. ilicifolius* were also tested for their antiproliferative effect on both HT-29 and CDD_{841} cell lines. Amongst the 2 fractions tested, fraction 3 (F3) showed maximum inhibition on HT-29 cells (51.29 %) at the concentration 166.66 µg/ml also showed no cytotoxicity against normal colon cells (Figure.4.29 and Figure 4.30) Photomicrographs showing the effects *A. ilicifolius* fractions treatment on CCD_{841} and (HT-29) cell in comparison to the untreated cells.

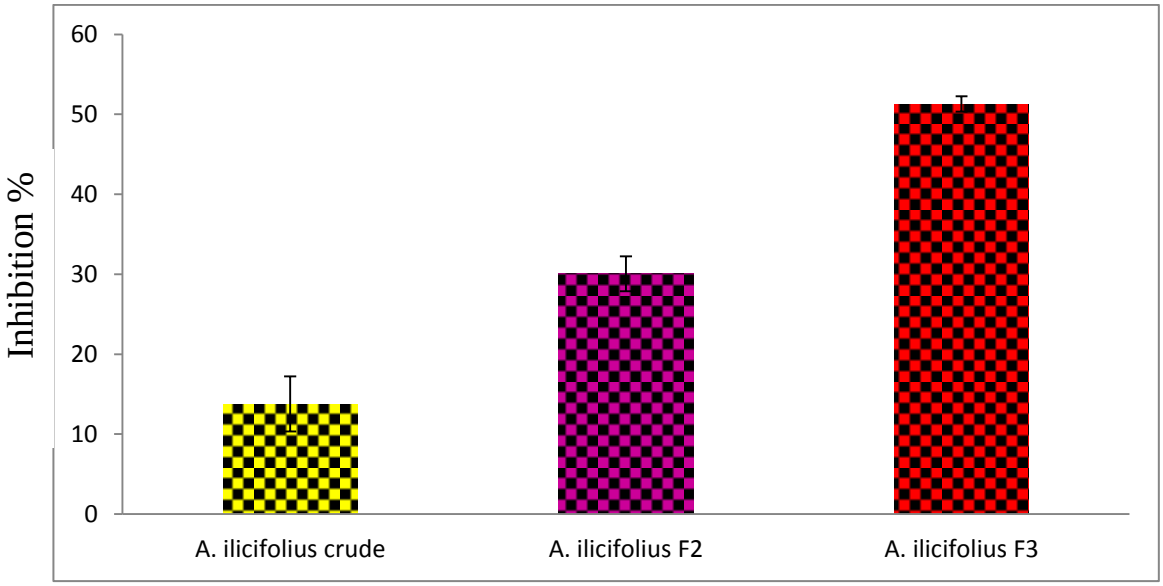


Figure.4.29: The effect of the crude extract of *A. ilicifolius* and its fractions on the growth of HT-29 cell line. Cells were seeded in 96-well plate and treated with different concentrations of both *A. ilicifolius* crude extract and fractions (166µg/ml) for 48h. The inhibition of HT-29 growth was assessed by MTT assay. The results represent the mean of triplicate measurement.

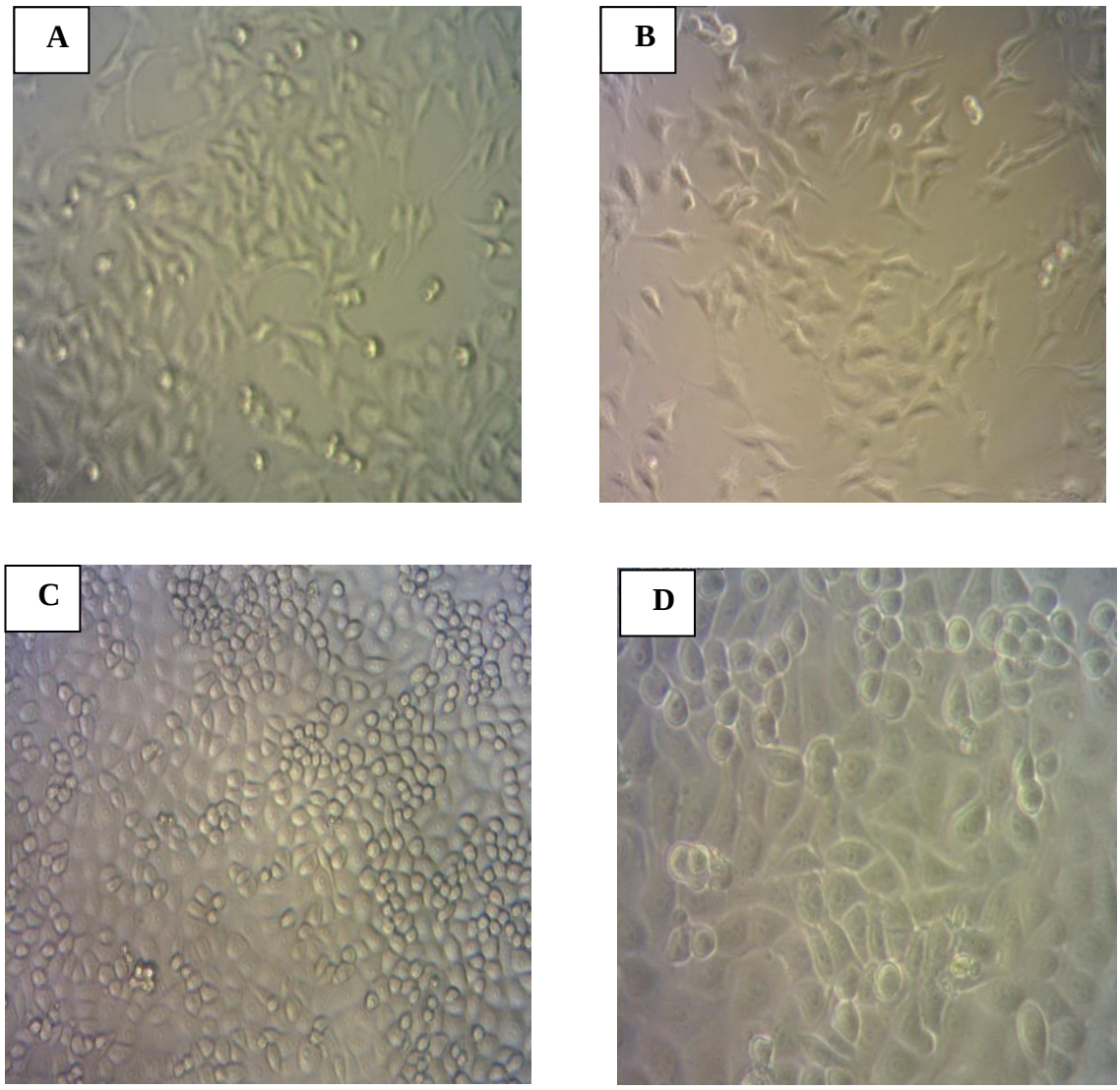


Figure 4.30: Photomicrographs of HT29 and CCD_{841} cells showing the effects *A.ilicifolius* fractions treatment; (A) CCD_{841} cells without treatment; (B) cells treated with 166.66 µg/ml F3; (C) HT29 cells without treatment; (D) HT29 cells treated with 166.66 µg/ml F3 10x. F3; Fraction number 3.

4.22.1 Identification of active constituents of the active fractions

We applied UPLC-PDA and LC-MS using positive mode in order to know the chemical structural information and to identify the phenolic constituents and other active compounds of *A. ilicifolius* F3. Approximately four compounds was detected in *A. ilicifolius* from them were characterized on the basis of the UV spectra and MS fragmentation patterns in comparison with literature or by searching the dictionary of natural products on DVD, Version 20:2 (2011) (CRC Press, Taylor & Francis Group, London, UK). In addition only one active compound was detected in *A. ilicifolius* and three of them were not identified. Typical HPLC-TOF/MS peaks and UV diode array chromatograms of the *A. ilicifolius* F3 fractions are shown in (Figure 4.31, 4.32). Table 4.22 showed the identification of all the peaks detected with their retention time, UV max, observed *m/z* and the *m/z* of fragment ions. The *A. ilicifolius* F3 compounds include Stigmasterol at *m/z* 409.173

Table 4.22: Identification of phenolic compounds in *A. ilicifolius* F3 by UPLC-DAD and LC-MS data using positive ionization mode

Sample	Rt time(min)	m/z of fragment ions observed	Suggested formula	Observed m/z	Tentative identification	M.W. (g/mol)
A. ilicifolius F3	0.994	280, 350, 191, 300, 254	$C_{14}H_{16}N_2O_3$	261.127	Unknown	260.12
	1.159	280, 350, 191, 300, 254	$C_{19}H_{20}N_6O$	349.176	Unknown	348.17
	1.2761	280, 350, 191, 300, 254	$C_{17}H_{27}O_{11}$	409.173	Stigmasterol	408.173
	1.475	280, 350, 191, 300, 254	$C_{26}H_{34}N_2O_{17}$	647.194	Unknown	646.19

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

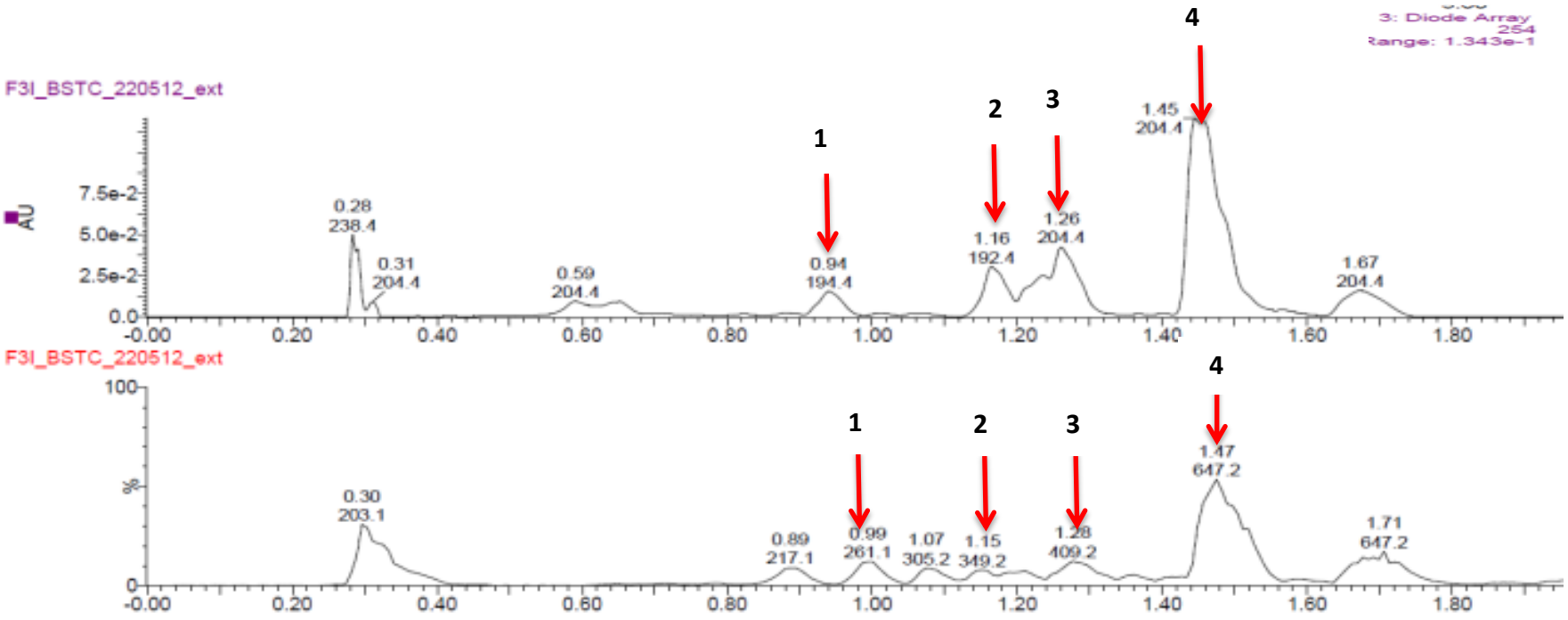


Figure 4.31: HPLC-TOF/MS and UV diode array chromatograms of *A. ilicifolius* fraction 3 (F3); (A) Diode array detection UV spectra at 351 nm; (B) TOF MS peaks in positive mode ionization

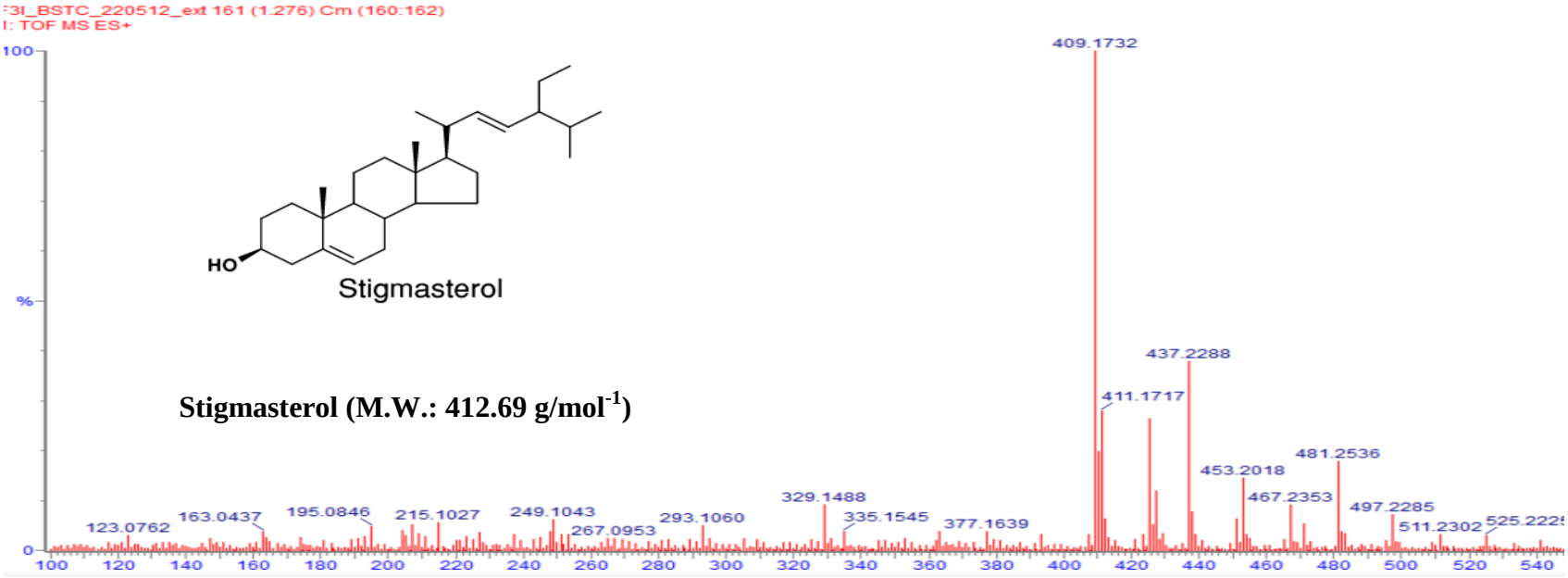


Figure 4.32: Mass Spectrum (TOF MS ES+), chemical structure and UV max spectra of Stigmasterol (peak No. 4) identified *A. ilicifolius* F3

CHAPTER V

5.1Discussion

5.1.1 Antioxidant properties of the *C. roseus* and *A. ilicifolius*.

Over the last two decades there has been an extreme interest in the role of oxygen-free radicals, usually known as reactive oxygen species (ROS), and of reactive nitrogen species (RNS) in experimental and clinical medicine (Halliwell & Gutteridge, 1999). In biological systems ROS/RNS are known to play a binary role in living systems, they can be either beneficial or harmful (Valko *et al.*, 2004). Advantageous effects of ROS involve physiological roles in cellular responses to anoxia, as for instance in the function of a number of cellular signalling systems and in defence against infectious agents. In addition, at low concentrations ROS is the induction of a mitogenic response. In contrast, ROS at high concentrations can be an important broker of damage to cell structures, including membranes, lipids, nucleic acids and proteins (oxidative stress). The harmful effects of ROS are balanced by the antioxidant action of antioxidant enzymes in addition to non-enzymatic antioxidants. In spite of the presence of the cell's antioxidant defence system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle, and radical-related damage to DNA, to proteins and to lipids has been proposed to play a key role in the development of age-dependent diseases such as arteriosclerosis, cancer, neurodegenerative, arthritis disorders and other situations (Valko *et al.*, 2006).

This study evaluated the available evidence for the involvement of cellular oxidants in the incidence of colon cancer, in damage to cellular structures and in the role of signalling cascades by ROS. A discussion on the various protective pathways that may be provided by the antioxidant network against the deleterious action of free radicals, as well as the antioxidant interactions with various regulatory factors are also addressed. Medicinal plants have been presumed to be a rich resource of natural antioxidants that

counter balance's these ROS, by removing the free radicals and minimizing the damage done too tissues.

The data of the present study showed that *A. ilicifolious* extract exhibited high antioxidant activity, as proven by FRAP results and its scavenging activity towards DPPH radicals. While extract of *C. roseus* were found to have possess antioxidant properties and a similar activity to that of BHT. Therefore the high antioxidant activity of *A. ilicifolious*, which led to more potent radical scavenging effects, is almost certainly associated with the high content of phenolic and flavonoids components. The total phenolic contents of *A. ilicifolious* and *C. roseus* are in accordance with the DPPH and FRAP results.

The phenolic compounds of antioxidant are important for helping the human body to destroy the free radicals that exist in our bodies. Many polyphenols, such as flavonoids, have been identified as powerful antioxidants. In addition they play a significant role in the treatment of many diseases. However, the antioxidant of the *A. ilicifolius* as observed in this study was not only from its phenolic contents, but could also be due to the presence of some other phytochemicals such as alkaloids and phenols. Different types of phenolic compounds have different antioxidant activities, which is dependent on their structure. The phenolics, especially flavonoids, were shown to protect mangroves (Agati *et al.*, 2007). Moreover, the leaves of *C. roseus* are reported to have the extremely high levels of antioxidant properties (Rasool *et al.*, 2011).

5.1.2 *In vivo* Antioxidant properties and biochemical parameters

Anti-oxidant activity was found in experimental animal models; azoxymethane (AOM) increases oxidative injury to colon cells and it is believed that this process play's a role in the etiology of colon cancer in rats (Al-Numair *et al.*, 2011; Rodríguez-Ramiro *et al.*, 2011). Our study showed that treatment of animals with *C. roseus* and *A. ilicifolius* extracts reduced the level of (MDA) and increases the activity of SOD. The

Catalase and SOD are responsible for the dissemination of free radicals, such as superoxide radicals.

During oxidative stress the body uses its defence mechanism to minimize the process of lipid peroxidation by using these antioxidant enzymes, thus, the activity of these enzymes become higher in early stages of AOM induction. However, when the insult continues for a long period of time, these enzymes become depleted and unable to fight against free radicals. Which means that in advanced stages of peroxidation due to AOM the activity of catalase and SOD, declined. While the activity of these enzymes continue to be high in treatment groups, the antioxidant properties of plant extracts against AOM induced free radicals. On the other hand, long term exposure to AOM led to significant increase of MDA level compared to the normal control group, indicating acute colon cell damage. Treatment of animals with *C. roseus* extracts and 5- Flourouracil significantly reduced the level of MDA and lipid peroxidation. There are numerous mechanisms of antioxidant substances having anticancer properties, such as by scavenging free radical (Jia *et al.*, 2012). However, the treatment groups of both plants showed a significant increase in the activities of these antioxidant enzymes. Similarly, previous studies have established that by enhancing the activities of antioxidant enzymes (SOD and CAT) against the AOM-induced colon cancer in animals, that the reduction of oxidative stress may well play a role in the mechanism of its chemopreventive effects (Waly *et al.*, 2012).

In the current study reduced lipid peroxidation was accompanied by a significant decrease in MDA level's in groups treated with ethanol extracts from both plants. In addition, the extent of oxidative stress was demonstrated to directly correlate with the amount of total protein (T.P) and albumin (Alb) detectable in the blood which when induced by AOM inevitably leads to oxidative damage in erythrocytes. The increased secretion of T.P and Alb from the kidneys leads to an increase in the concentration of

urea in the bloodstream, moreover the Hb is then decreased in the blood due to anemia, but *C. roseus* treatment led to T.P, Alb, urea and LDH returning to normal levels. *C. rosesus* also led to a reduction of Hb, RBC, WBC and plt concentration.

Furthermore *A.ilicifolius* also directly correlates with the amount of T.P, Alb, urea and Hb detectable in the blood, along with restoring the level of T.P , Alb, urea and Hb to their normal values. The chemopreventive effects of these extracts may be due to the presence of the large amount of phenolic and flavonoids compounds and their antioxidant effects, as well as the free radical scavenging property of the plant. Likewise, the chemopreventive activity of the extract could be due to neutralization of the toxic compounds, produced by converting AOM to a highly toxic metabolite during WNT pathway.

The results of this current study are in agreement with the results published by Shahidi & Chandrasekara (2010), that states chemopreventive effects could be attributed to their ability to reduce free radical formation and to scavenge free radicals. Several reports have shown the relationship between carcinogenesis and oxidative stress. An inflammatory response is typically accompanied by a generation of free radicals, stimulation of cytokines, chemokines, growth and angiogenic factors. Free radicals, capable of both directly damaging DNA and affecting the DNA repair machinery, enhance genetic instability of affected cells, thus contributing to the first stage of neoplastic transformation, also known as initiation (Dobrovolskaia & Kozlov, 2005).

5.1.3 Acute oral toxicity tests of *C. roseus* and *A. ilicifolius*

Many clinical and toxicological studies have been carried out for medicinal plant extracts to ensure the secure use and safeties of these plants. In Global Harmonized System of Classification and Labelling Of Chemical and Environmental Protection Agency: Health Effects Test Guidelines, the LD 50 value higher than 5000mg/Kg

showed by plants extracts are practically safe (Winder *et al.*, 2005). Therefore, the investigated plants extracts are toxicologically safe by oral administration, since there were no significant differences observed when examining the *C. roseus* and *A. ilicifolius* treated groups in comparison to the control group regarding liver and kidney functions tests. Thus, our findings support the logical usage of these plants in Malaysian folk medicine.

5.1.4 Colon cancer mechanism and chemopreventive activity of medicinal plants

Nutritional factors play an important role in human health as well as in the development of a number of chronic diseases, including cancer (Rogers *et al.*, 1993). While genetics along with environmental issues are identified as contributors in the etiology of colonic carcinoma, nutritional factors also play a major role (Vogelstein *et al.*, 1988). Plant resources, namely vegetables, fruits and spices have attracted extensive study because of their chemoprotective properties. Many are known to possess anti-inflammatory, antioxidant and antitumour or cancer chemoprevention activities (Gupta *et al.*, 2010). Aberrant crypt foci are generally considered as early markers of primary neoplastic change in the human large intestine, as seen in the experimental colon carcinogenesis AOM rat model (Park *et al.*, 1997). Colon carcinogenesis is a multistage process, quick cell proliferation as well as ablation of apoptosis are initial incidences for its progression (Patel *et al.*, 2009). The rodent AOM model of colon carcinogenesis represents a valuable approach for the development of strategies for chemoprevention. This model recapitulates many of the clinical, pathologic and molecular features occurring in sporadic human colorectal cancer (80% of cases), including crypt cell hyper proliferation and ACF formation that are considered as premalignant precursors (Bird & Good, 2000).

The preneoplastic lesions, such as ACF, which are detected 30-45 days after AOM administration, have been extensively used as an endpoint in short-term chemopre-

ventive studies (Bird & Good, 2000; Pereira *et al.*, 1994). In fact, ACFs are considered to be the 'gold standard' of colon carcinogenesis biomarkers (Pretlow *et al.*, 2004).

In the present study, both plants showed a reduction in the number of preneoplastic lesions (multicrypt aberrant and hyper proliferative crypts) at the surface of the colon. Importantly, our results showed that rats fed with *C. roseus* and *A. ilicifolius* inhibited the foci that containing multicrypt, as well as the total number of aberrant crypts. These plants considerably decreased AOM-induced total colonic ACF formation and multicrypt aberrant crypt growth. A majority of ACF exhibited morphological atypia. Using a grading system to enumerate atypia based on nuclear morphology (elongation and stratification), it became evident that some ACF exhibited a grade 4 nuclear atypia. Nuclear elongation and stratifications are the 2 main characteristics of dysplastic epithelium. Because dysplasia is generally considered to be a significant finding in the carcinogenic process, our observation that some ACF exhibit dysplasia presents strong evidence that ACF represent precursor lesions. Consistent with our results, Janakiram *et al.* (2008) showed that administration of dietary β-ionone significantly suppressed total colonic ACF formation when compared with AOM control group. In contrast to the results of the present study, pectin increased chemically induced colon cancer (Jacobs & Lupton, 1986). Soy concentrate does not protect against AOM-induced colon cancer (Bennink & Om, 1998).

5.1.5 Immunohistochemistry

Several research works involving human and animal models have shown that one of the earliest indications of preneoplasia is abnormal epithelial cell proliferation, maturation and differentiation. Increased cell proliferation has long been shown to play a crucial role in the initiation phase, as well as the promotion/progression stage of carcinogenesis. In this regard, PCNA is suggested as a factor in DNA replication by developing a moving foundation that could mediate the interaction of numerous proteins

with DNA, and hence, PCNA is reconsidered as an efficient biomarker for cell proliferation (Maga & Hübscher, 2003).

In the present study, *C. roseus* and *A. ilicifolius* at all dosage levels significantly reduced the increase in proliferative index caused by AOM treatment in rats fed with the control diet, suggesting that *C. roseus* and *A. ilicifolius*, through their ability to restrain oxidative stress could also inhibit the consequent activation of the signalling pathways involved in the proliferation and thereby the progression of preneoplastic lesions in the colonic epithelial cells.

5.1.6 Gene expression

Progressive inhibition of apoptosis has been described as taking place during the transformation of colorectal epithelium to carcinoma (Anti *et al.*, 2001). Expression of the p53 gene and those of the bcl-2 family (the best known of the genes involved in the regulation of epithelial cell apoptosis) also change progressively as the tissue passes through the consecutive stages of normal mucosa, adenoma and carcinoma (Bousserouel *et al.*, 2010b). A good number of antitumour agents obtained from plants exercise their effect through apoptosis induction in cancer cells (Kundu *et al.*, 2005). Cell proliferation and apoptosis biomarkers are generally utilized to examine the efficacy of potential chemopreventive agents (Kohno *et al.*, 2006).

The carcinogenic process entails alterations in the balance between cell death and cell renewal that regulate the normal cellular homeostasis in the colonic mucosa. The tumour suppressor protein p53 is known to play an important role in apoptosis and cell cycle arrest (Deuk Kim *et al.*, 2006). Bcl-2 protein expression in colorectal tumours (Furihata *et al.*, 1999). Bcl-2 was the first gene to be implicated in the regulation (inhibition) of apoptosis. Accumulating data shows that p53 protein is also associated in the apoptosis pathway acting in opposition to bcl-2, these studies have shown that p53 can down-regulate bcl-2 gene expression (Miyashita *et al.*, 1994). However, conflicting

reports have shown that p53 can up-regulate bcl-2 gene expression (Bousserouel *et al.*, 2011). Moreover, Bax was identified as a p53 early-response gene (Selvakumaran *et al.*, 1994), and a unique p53-regulated gene which induced apoptosis (Zhan *et al.*, 1994), and affects Bcl-2 as an apoptosis antagonist (Oltval *et al.*, 1993; Reed, 1994).

Indeed, this study showed that the AOM-triggered crypt cell hyper proliferation in the colonic mucosa was associated with the upsurge and development of preneoplastic lesions (ACF), but also with an elevated Bcl-2/Bax ratio (ratio >1). Our data showed that *C. roseus* and *A. ilicifolius* treatment induced the up-regulation of p53 gene expression, bcl-2 and pro-apoptotic Bax protein and the (Bcl-2/Bax ratio <1).

C. roseus triggered apoptosis may be at least in part responsible for its overall efficacy in inhibiting AOM-induced ACF formation in rat colons. These effects were associated with *C. roseus and A. ilicifolius* as they induced changes in the expression of apoptosis-related genes such as the subsequent Bcl-2/Bax ratio (anti-apoptotic versus pro-apoptotic gene and protein levels), which can be used as an indicator of chemopreventive efficacy, as previously reported for other drugs (Bousserouel *et al.*, 2010a; Bousserouel *et al.*, 2011) and as shown here for *C. roseus and A. ilicifolius.*

5.1.7 Phytochemicals investigation of the active fractions

By isolating and identifying extracts of *C. roseus* and *A. ilicifolius* this study has shown the presence of bioactive compounds in *C. roseus,* vindolin, ajmalicin, vindolidine, whilst stigmasterol was found in *A. ilicifolius*. As part of our investigation we have isolated and identified many alkaloid compounds including vindolin, ajmalicin and vindolidine in *C. roseus* (Tikhomiroff & Jolicoeur, 2002)**.** On the other hand, a number of studies have identified many steroids compounds in the *A. ilicifolius* plant including stigmasterol (Wöstmann & Liebezeit, 2008b). The leaves are reported to have the highest antioxidant properties, amongst which the alkaloids fraction is the most active principle among the phytochemicals studied (Rasool *et al.*, 2011). Phytosterols

are considered to be an anti-carcinogenic (Jones & Raeini-Sarjaz, 2001). In agreement with our study, Tanaka (1997) found that the hesperdin phytochemical found in orange juice might inhibit the “initiation phase” of colon carcinogenesis, which led him to suggest that drinking orange juice would inhibit the “initiation stage” of colon carcinogenesis.

5.2 Conclusion

In conclusion, this study has shown that:

1. *C. roseus* and *A .ilicifolius* ethanol extracts had relevant amount of phenolic, and flavonoid content and exhibited potent antioxidant activity in *vitro* by virtue of DPPH radical scavenging activities and ferric ion reducing power (FRAP).
2. These ethanol extracts were toxicologically safe *in vivo* by oral administration.
3. Our results indicated that *C. roseus* and *A. ilicifolius* exhibited potent chemo-preventive effects that were proven by macroscopical, biochemical, hematological, histopathological, cell line and gene expression work. Accordingly, the plant extracts could be an effective herbal remedy against AOM-induced colon cancer.
4. The mechanism underlining the chemoprotective effects of *C. roseus* and *A. ilicifolius* were suggested to be maintaining the endogenous antioxidant system of the colon and monitoring the expression of Bax, Bcl-2 and p53 genes.
5. The antioxidant and chemoprotective activities of colon cancer were probably due to the isolated chemical compounds vindoline, vindolidine and ajmalicine from *C. roseus* and stegmasteroid from *A. ilicifolius*.
6. High dose 500mg/kg extracts of *C. roseus* and *A. ilicifolius* exhibited chemoprotective activities, but less than the lower dose of 250mg/kg this activity may be attributed to the pro-oxidant of these plants.

7. From this study, promising potentials appear for the development of new therapy, which must focus on gene expression for the treatment of colon cancer.

5.3 Future work

1. Purification, identification and structure elucidation using 1D-and 2D-NMR spectroscopic processes fractions should be done for the unidentified chemical components.
2. The fractionations of the plants extracts should be repeated several times to further isolate sufficient quantities of the active fractions in order to test their bioactivities on animals.

CHAPTER VI

REFFERENCES

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CHAPTER VII

APPENNDIX A: Analytical Techniques and Preparations

A1 Ferric reducing antioxidant power (FRAP) assay

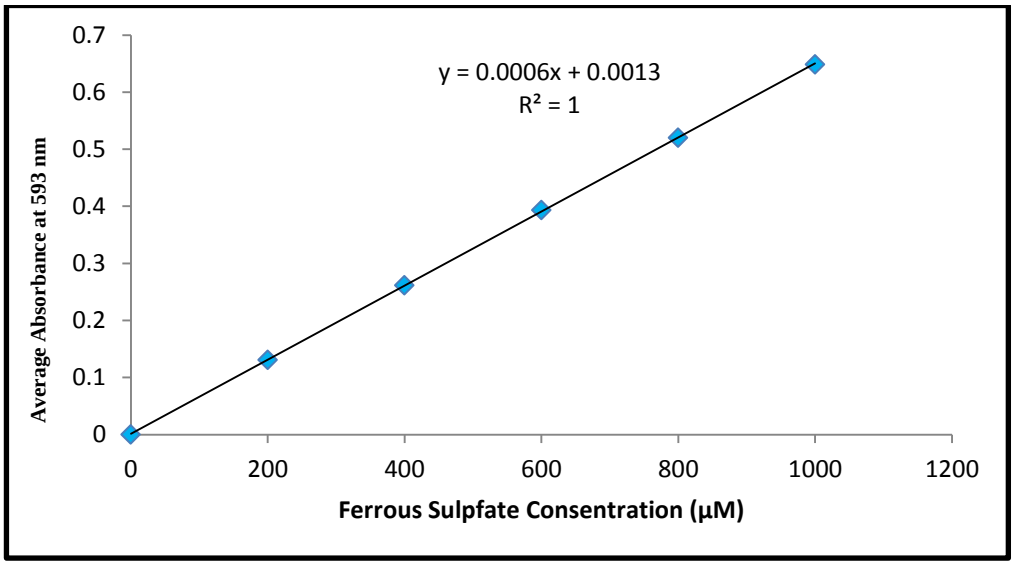


Figure A1. FRAP standard curve

A2. DPPH Free radical scavenging assay

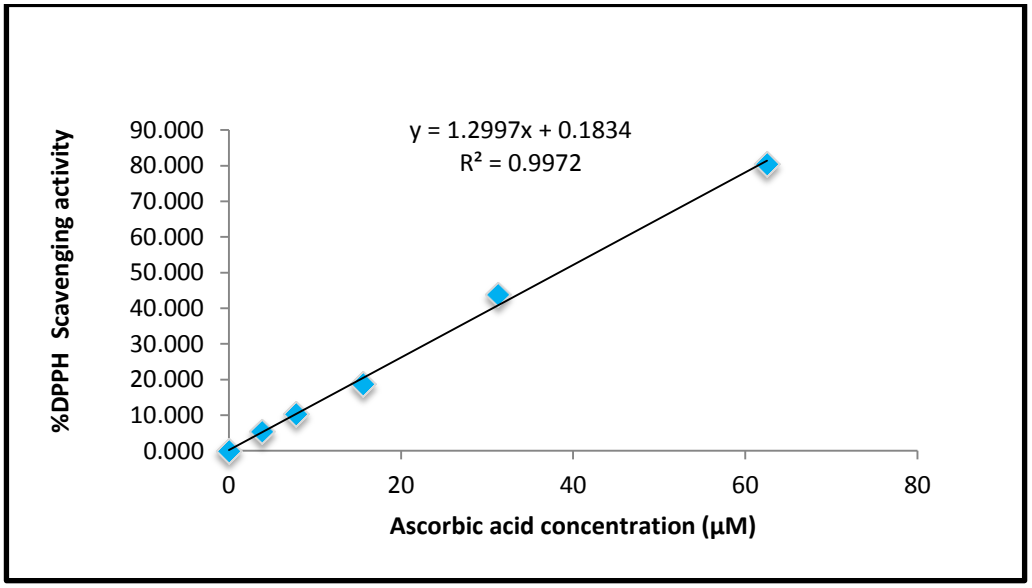


Figure A2. DPPH standard curve

A3. Total Phenolic Content (TPC)

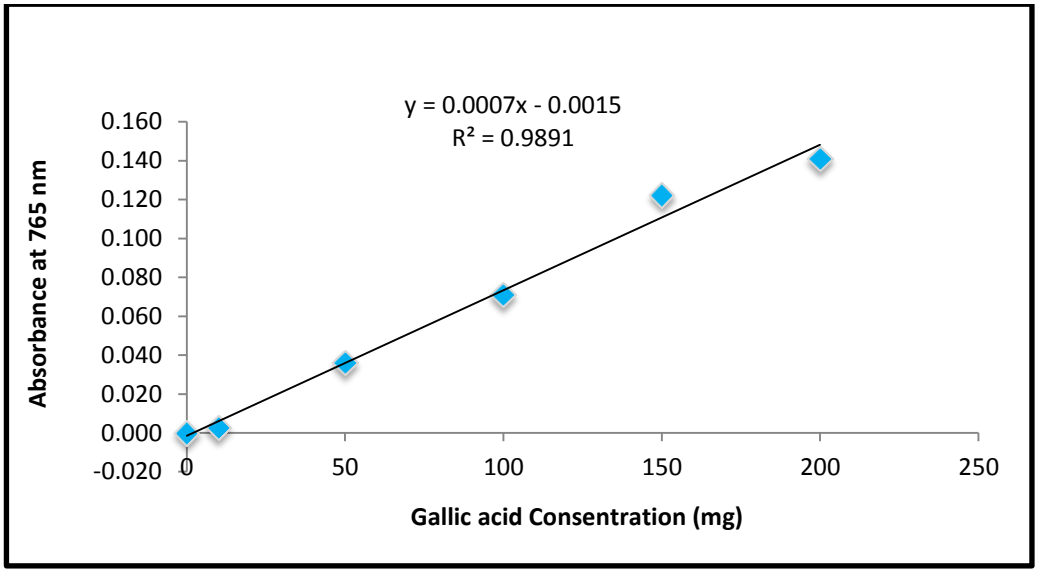


Figure A3. Total Phenolic Content (TPC) standard curve

A4. Total flavonoid Content (TFC)

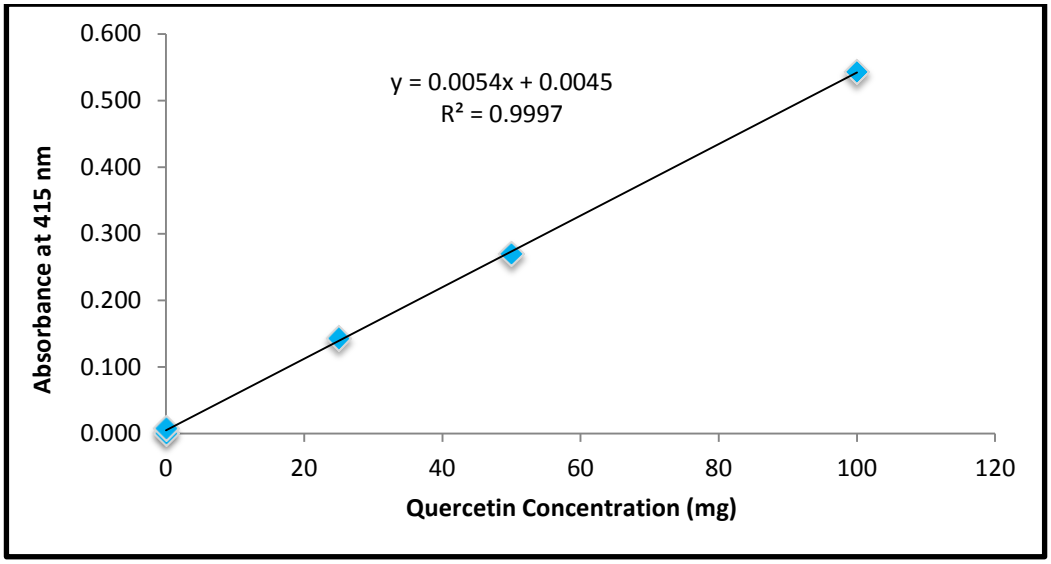


Figure A4. Flavonoid standard curve

A5. Superoxide Dismutase (SOD) assay

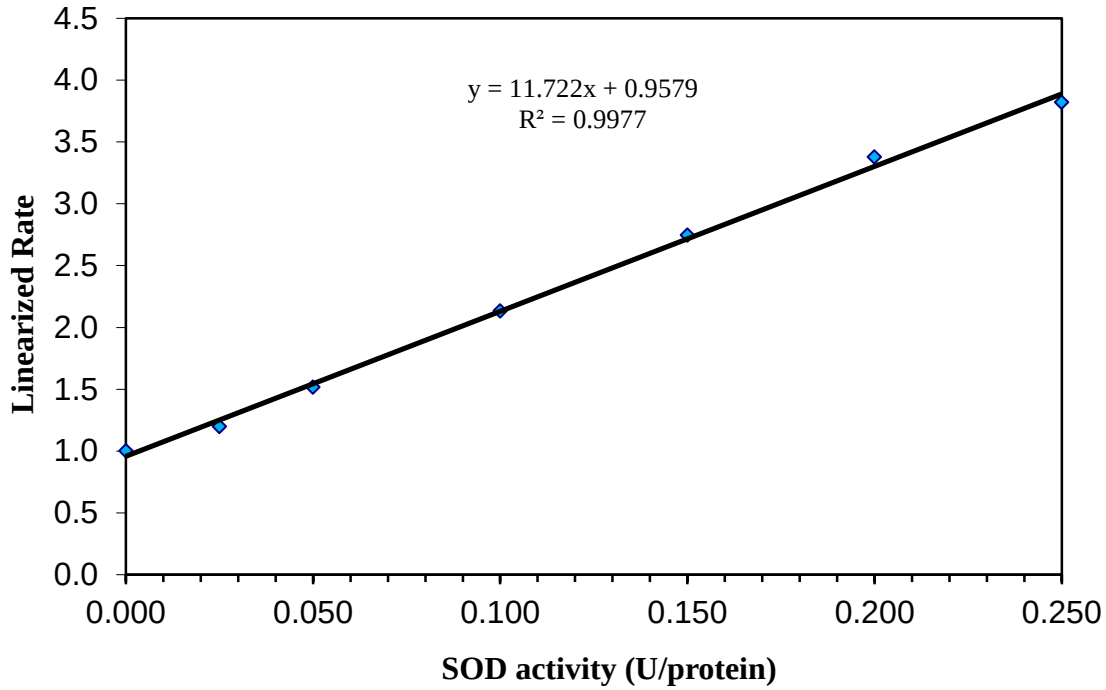


Figure A5: SOD standard curve (µM)

A6. Catalase (CAT) assay

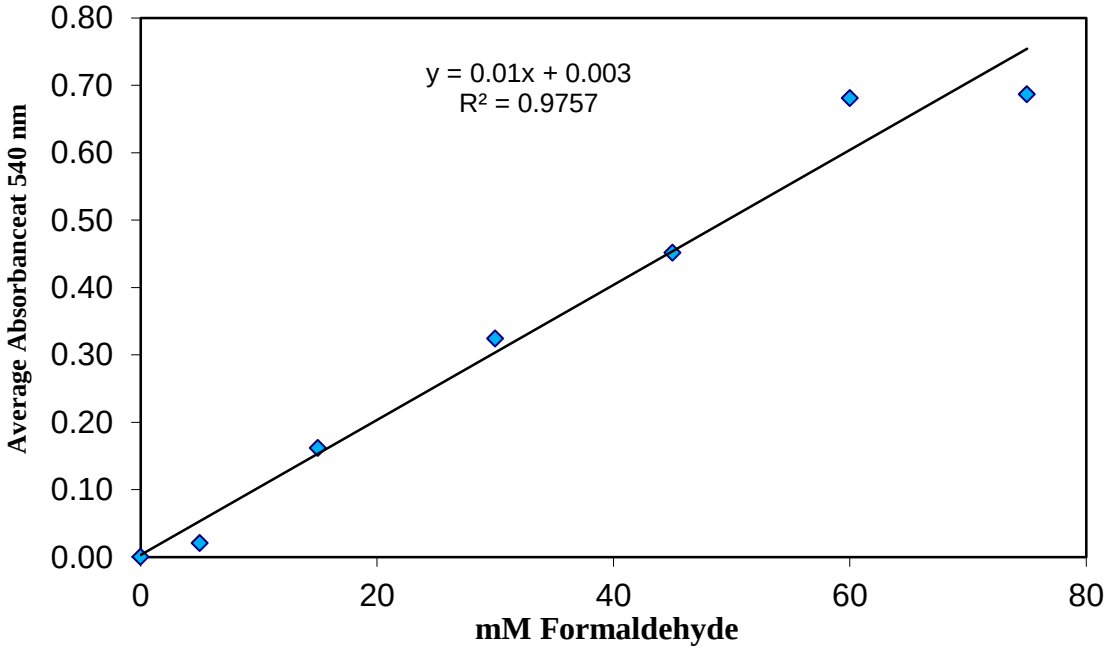


Figure A6: Formaldehyde standard curve (µM)

A7. TBARS assay

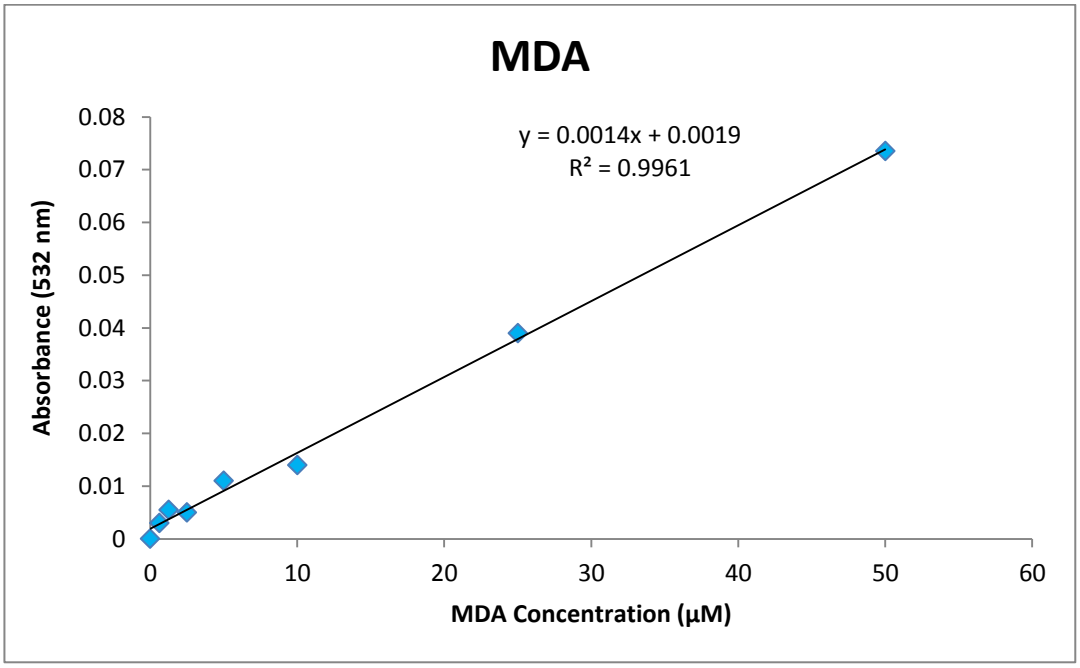


Figure A7: MDA standard curve

A8. Histopathology Techniques

1- Routine slide preparation

a- Tissue trimming and fixation

The tissue processing started with trimming and excising the tissues in to small pieces of about 1 cm in size and then put in cassettes containing fresh 1-% buffered formalin of 10:1 ratio of fixative to tissue for 48 hr. The purpose of fixation is to preserve tissues permanently in life-like sate as possible after removal of the tissues.

b- Processing

The technique of getting fixed tissue in to paraffin is called tissue processing. This process have been done by using automatic tissue processor. The main steps in this process were dehydration, clearing and infiltration in a programmed sequence.

c- Embedding

After the above processes, the tissues were manually transferred from the cassettes and put in ti the blocks with molten paraffin over them, with proper orientation of tissue in the block of paraffin.

d- Sectioning

Once the melted paraffin was cooled and hardened the blocks were trimmed into an appropriately sized block and put into freezer under -4ºC for 1 hr before sectioning. Each block was than mounted in a specially designed slicing machine, a microtome. They were cut with steel knife into sections of 5 μm thickness. These sections were floated in a 40ºC warm water bath to avoid wrinkling, then they were picked up on a labeled glass microscopic slides. All these slides were then dried less than 50ºC temperature

e- Staining

Before staining, all the slides were deparaffinized by running them through xylenes I, II for 5 min each, in order to remove the paraffin wax out of the tissues and allow water soluble dyes to penetrate the section. The stains which were used in our experiment were H and E stains. Thick paraffin sections (5μm) of colon were de-waxed in xylene, dehydrated in series of alcohol to water then immersed in hematoxylin for 15 min. Sections were then differentiated with 1% acid alcohol and washed in tap water, followed by staining with eosin or 5 min.

f- Mounting

Finaly, to protect the stained sections from damage, the stained sections were dehydrated in series of alcohol, cleared in xylene and mounted with mounting media DPX and the use of coverslip in 45º angle then bubbles were removed carefully and left to dry overnight at room temperature.

2- Haematoxylin and Eosin (H&E) stain

Reagents required

1- Harris haematoxylin working solution

2-Eosin working solution

3-0.5% acid alcohol

4-2% sodium acetate

5-80% alcohol

6-95% alcohol

7-Absolute alcohol

Procedure:

1- Bring section to water

2- Stain in Harris haematoxylin 10 min

3- Wash in running water until excess blue color gose off

4- Differentiation: Dip 2 to 3X in 0.5% acid alcohol and wash in running tab water

5- wash well in running tab water 2-3 min

6- Blue section with 2% sodium acetate 2 sec

7-Wash again in running tab water 2-3 min

8- Rinse in 80% alcohol

9- Stain in eosin solution 5 min

10- Dehydration: 95% alcohol I 5 sec

95% alcohol II 2 min

Absolute alcohol I 2 min

Absolute alcohol II 2 min

11- Clear in xylene 2 min x3

12- Mount with DPX

13- Wipe slide to remove excess xyline

14- Label slide appropriately

Results:

Nuclei blue

Cytoplasm various shades of pink and red

A9. Standard Curve and efficiency for target and endogenous reference genes

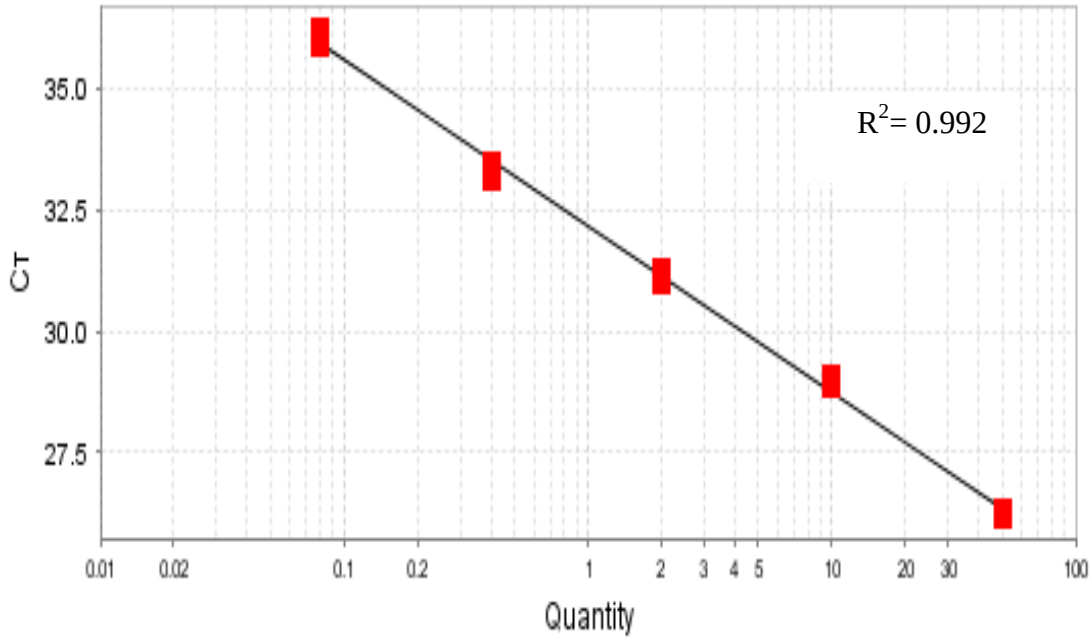


Figure Tbp standard curve

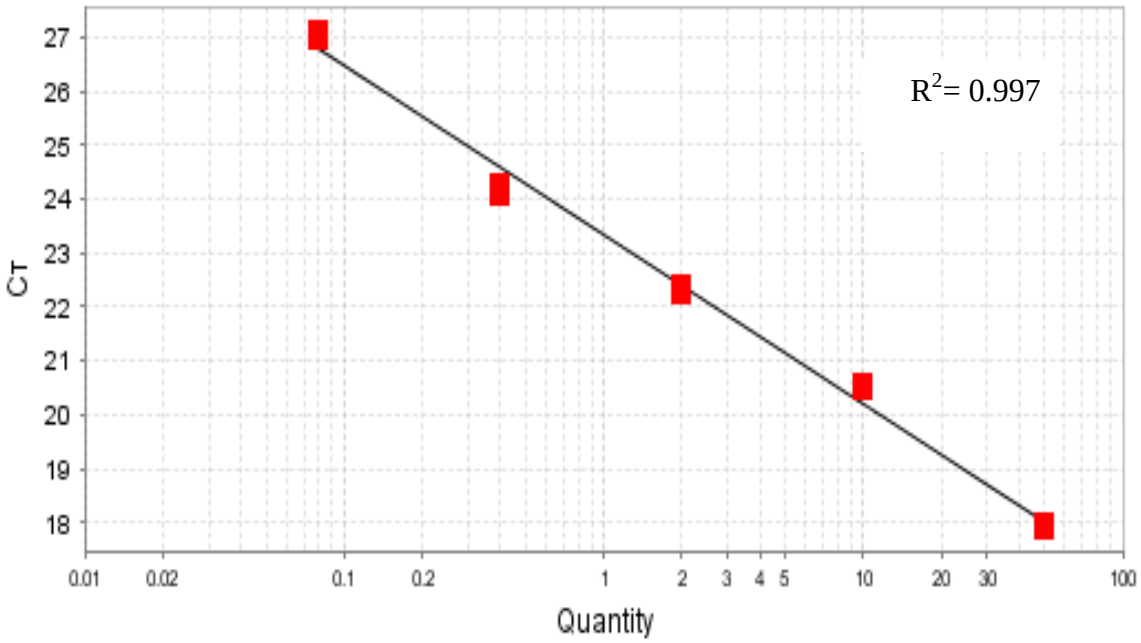


Figure actba standard curve

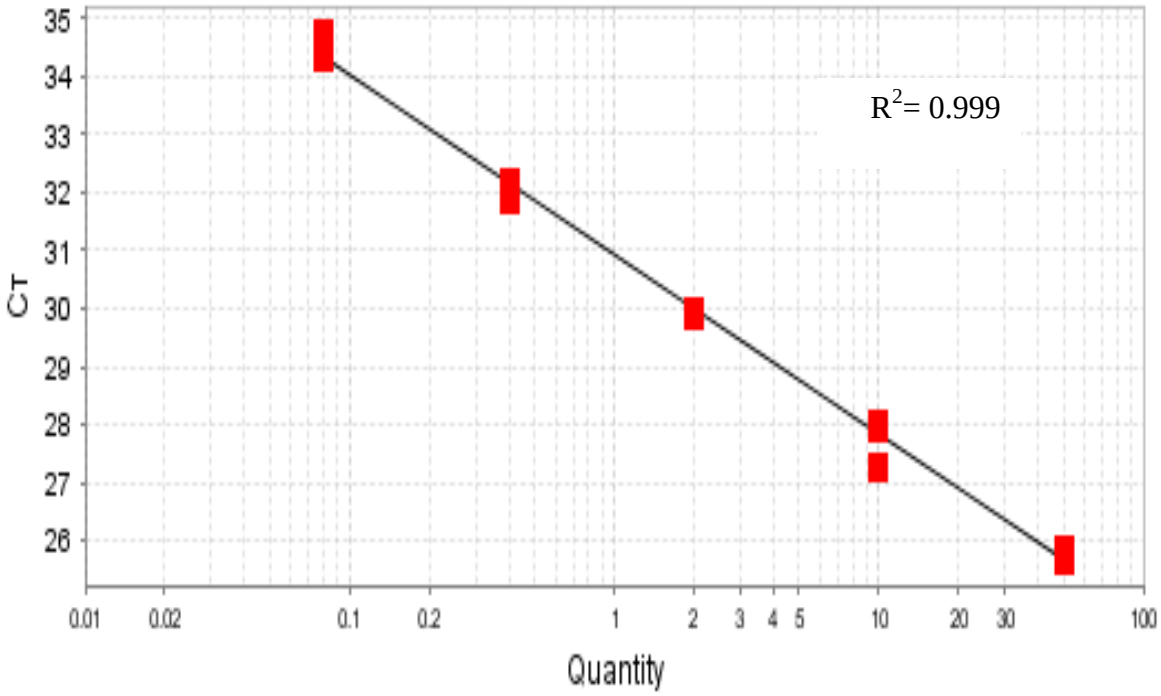


Figure Hprt1 standard curve

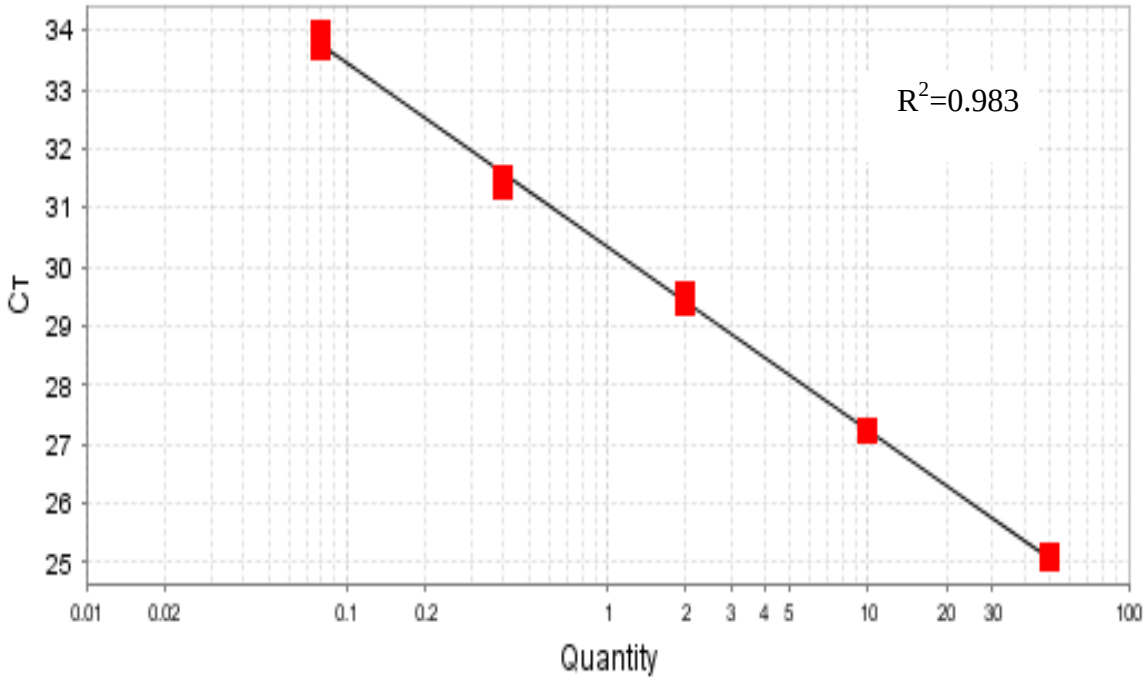


Figure p53 standard curve

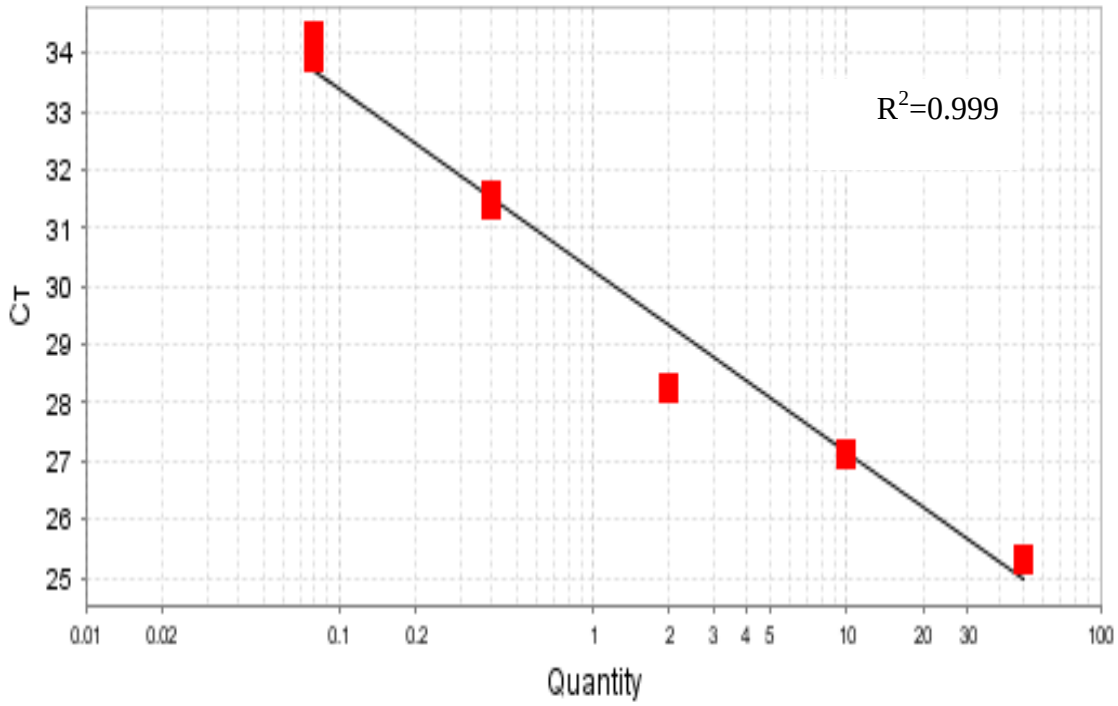


Figure bax standard curve

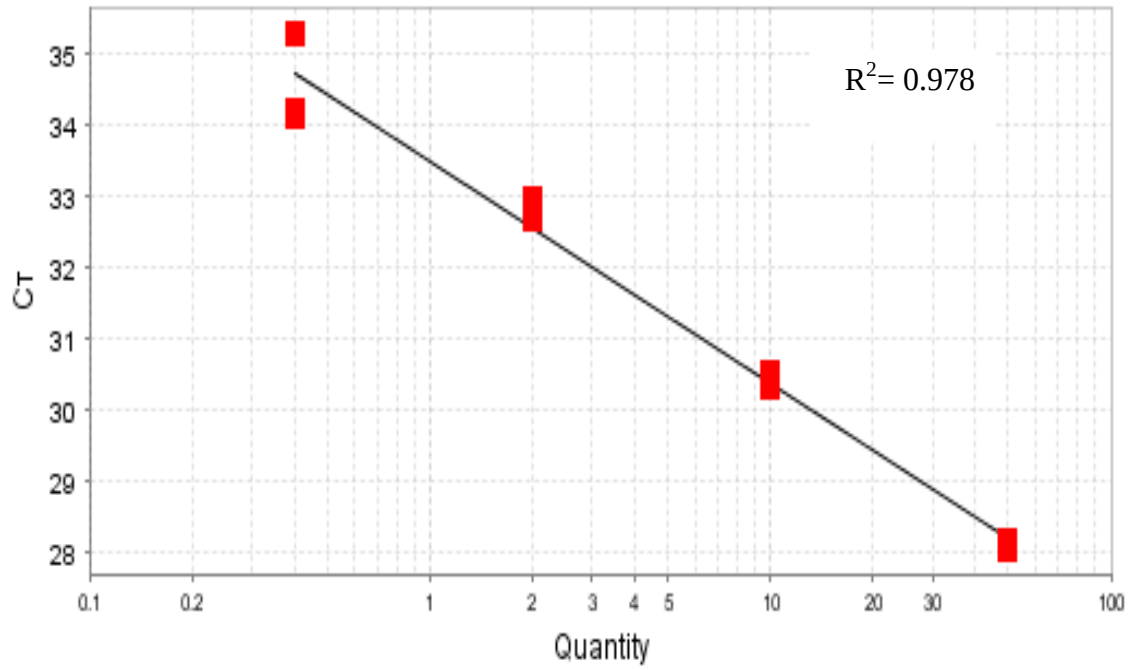


Figure Bcl2 standard curve

APENDIX B: Manufacturer kits instruction and procedures

1. Catalase assay kit (Item No. 707002 Cayman)

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that there be at least two wells designated as positive controls.

A typical layout of formaldehyde standards and samples to be measured in duplicate is shown in Figure 1. We suggest you record the contents of each well on the template sheet provided on page 23.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	+	+	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-G = Standards
+ = Positive Controls
S1-S40 = Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 240 µl in all the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the expected CAT activity of the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.
- It is recommended that the samples and formaldehyde standards be assayed at least in duplicate.
- Use the Assay Buffer (dilute) in the assay.
- Monitor the absorbance at 540 nm using a plate reader.

Standard Preparation

1. Preparation of the Formaldehyde Standards - Dilute 10 µl of formaldehyde standard (vial #3) with 9.99 ml of Sample Buffer (dilute) to obtain a 4.25 mM formaldehyde stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of formaldehyde stock and Sample Buffer (dilute) to each tube as described in Table 1 (below).

Tube	Formaldehyde (µl)	Sample Buffer (µl)	Final Concentration (µM formaldehyde)*
A	0	1,000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

Table 1

*Final formaldehyde concentration in the 170 µl reaction.

Performing the Assay

1. **Formaldehyde Standard Wells** - Add 100 µl of Assay Buffer (dilute), 30 µl of methanol, and 20 µl of standard (tubes A-G) per well in the designated wells on the plate (see sample plate format, Figure 1, page 12).
2. **Positive Control Wells (bovine liver CAT)** - Add 100 µl of Assay Buffer (dilute), 30 µl of methanol, and 20 µl of diluted CAT (control) to two wells.
3. **Sample Wells** - Add 100 µl of Assay Buffer (dilute), 30 µl of methanol, and 20 µl of sample to two wells. To obtain reproducible results, the amount of CAT added to the well should result in an activity between 0.25-4 nmol/min/ml. When necessary, samples should be diluted with Sample Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 100,000 to bring the enzymatic activity to this level.
4. Initiate the reactions by adding 20 µl of hydrogen peroxide (dilute) to all the wells being used. Make sure to note the precise time the reaction is initiated and add the hydrogen peroxide as quickly as possible.
5. Cover the plate with the plate cover and incubate on a shaker for 20 minutes at room temperature.
6. Add 30 µl of potassium hydroxide to each well to terminate the reaction and then add 30 µl of Purpald (chromogen) to each well.
7. Cover the plate with the plate cover and incubate for 10 minutes at room temperature on the shaker.
8. Add 10 µl of potassium periodate to each well. Cover with plate cover and incubate five minutes at room temperature on a shaker.
9. Read the absorbance at 540 nm using a plate reader.

2. Superoxide dismutase assay kit (Item No. 706002 Cayman)

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of SOD standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 19).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
B	B	B	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C	C	C	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
D	D	D	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
E	E	E	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
F	F	F	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
G	G	G	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
H	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41

A-G = Standards
S1-S41 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 230 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- All reagents except samples and xanthine oxidase must be equilibrated to room temperature before beginning the assay.
- It is recommended that the samples and SOD standards be assayed at least in duplicate.
- Monitor the absorbance at 440-460 nm using a plate reader.

Standard Preparation

Dilute 20 µl of the SOD Standard (Catalog No. 706005) with 1.98 ml of Sample Buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of SOD stock and Sample Buffer (dilute) to each tube as described in Table 1 on page 12.

Tube	SOD Stock (µl)	Sample Buffer (µl)	Final SOD Activity (U/ml)
A	0	1,000	0
B	20	980	0.025
C	40	960	0.05
D	80	920	0.1
E	120	880	0.15
F	160	840	0.2
G	200	800	0.25

Table 1. Superoxide Dismutase standards

Performing the Assay

1. **SOD Standard Wells** - add 200 µl of the diluted radical detector and 10 µl of standard (tubes A-G) per well in the designated wells on the plate (see sample plate format, Figure 2, page 10).
2. **Sample Wells** - add 200 µl of the diluted radical detector and 10 µl of sample to the wells. *NOTE: If using an inhibitor, add 190 µl of the diluted radical detector, 10 µl of inhibitor, and 10 µl of sample to the wells. The amount of sample added to the well should always be 10 µl. Samples should be diluted with Sample Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to fall within the standard curve range.*
3. Initiate the reactions by adding 20 µl of diluted xanthine oxidase to all the wells you are using. Make sure to note the precise time you started and add the xanthine oxidase as quickly as possible. *NOTE: If assaying sample backgrounds, add 20 µl of sample buffer instead of xanthine oxidase.*
4. Carefully shake the 96-well plate for a few seconds to mix. Cover with the plate cover.
5. Incubate the plate on a shaker for 20 minutes at room temperature. Read the absorbance at 440-460 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample. If assayed, subtract sample background absorbance from the sample.
2. Divide standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR) (*i.e.*, LR for Std A = Abs Std A/Abs Std A; LR for Std B = Abs Std A/Abs Std B).
3. Plot the linearized SOD standard rate (LR) (from step 2 above) as a function of final SOD Activity (U/ml) from Table 1. See Figure 3 (on page 14) for a typical standard curve.
4. Calculate the SOD activity of the samples using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR - y-intercept}}{\text{slope}}\right) \times \frac{0.23\ \text{ml}}{0.01\ \text{ml}}\right] \times \text{sample dilution}$$

Performance Characteristics

Precision:

When a series of 60 SOD standard measurements were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of 60 SOD standard measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.7%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0.025-0.25 units/ml SOD.

3. TBARS (MDA) assay kit (Item No. 10009055 Cayman)

Tissue Homogenates

1. Weigh out approximately 25 mg of tissue into a 1.5 ml centrifuge tube.
2. Add 250 µl of RIPA buffer with protease inhibitors of choice (see **Interferences** section on page 19).
3. Sonicate for 15 seconds at 40V over ice.
4. Centrifuge the tube at 1,600 x g for 10 minutes at 4°C. Use the supernatant for analysis. Store supernatant on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for one month.
5. Tissue homogenates do not need to be diluted before assaying.

Cell Lysates

1. Collect 2 x 10^7 cells in 1 ml of cell culture medium or buffer of choice, such as PBS.
2. Sonicate 3X for five second intervals at 40V setting over ice.
3. Use the whole homogenate in the assay, being sure to use the culture medium as a sample blank.
4. Cell lysates do not need to be diluted before assaying.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in duplicate is shown below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 23).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	H	H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-H = Standards
S1-S40 = Sample Wells

Figure 2. Sample plate format

Fluorometric Standard Preparation

Dilute 25 µl of the MDA standard (Catalog No. 10009202) with 975 µl of water to obtain a stock solution of 12.5 µM. Take eight clean glass test tubes and label them A-H. Add the amount of 12.5 µM MDA stock solution and water to each tube as described in Table 2.

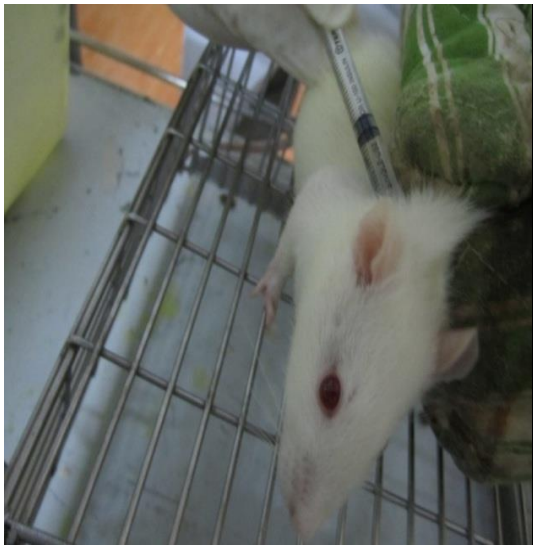
Tube	MDA (µl)	Water (µl)	MDA Concentration (µM)
A	0	1,000	0
B	5	995	0.0625
C	10	990	0.125
D	20	980	0.25
E	40	960	0.5
F	80	920	1
G	200	800	2.5
H	400	600	5

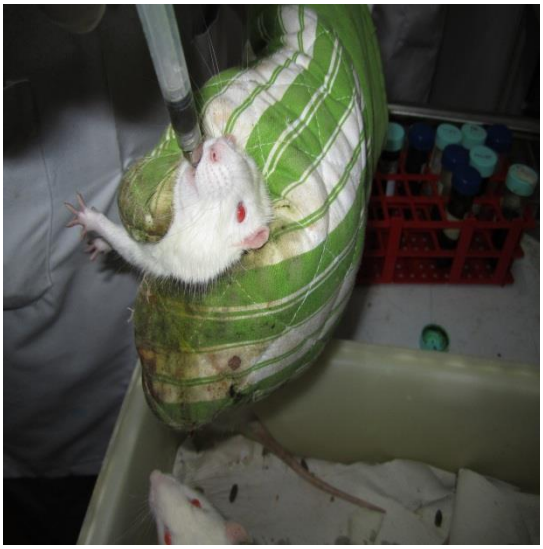
Table 2. MDA fluorometric standards

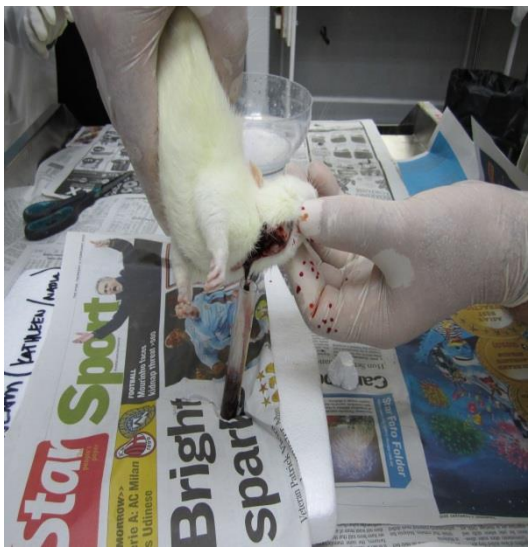
Performing the Assay

1. Label vial caps with standard number or sample identification number.
2. Add 100 µl of sample or standard to appropriately labeled 5 ml vial.
3. Add 100 µl of SDS solution to vial and swirl to mix.
4. Add 4 ml of the color reagent forcefully down side of each vial.
5. Cap vials and place vials in foam or some other holder to keep the tubes upright during boiling.
6. Add vials to vigorously boiling water. Boil vials for one hour.
7. After one hour, immediately remove the vials and place in ice bath to stop reaction. Incubate on ice for 10 minutes.
8. After 10 minutes, centrifuge the vials for 10 minutes at 1,600 x g at 4°C. Vials may appear clear or cloudy. Cloudiness will clear upon warming to room temperature.
9. Vials are stable at room temperature for 30 minutes.
10. Load 150 µl (in duplicate) from each vial to either the clear plate (colorimetric version) or to the black plate (fluorometric version).
11. Read the absorbance at 530-540 nm or read fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

APPENDIX C: PhD Project selected photographs







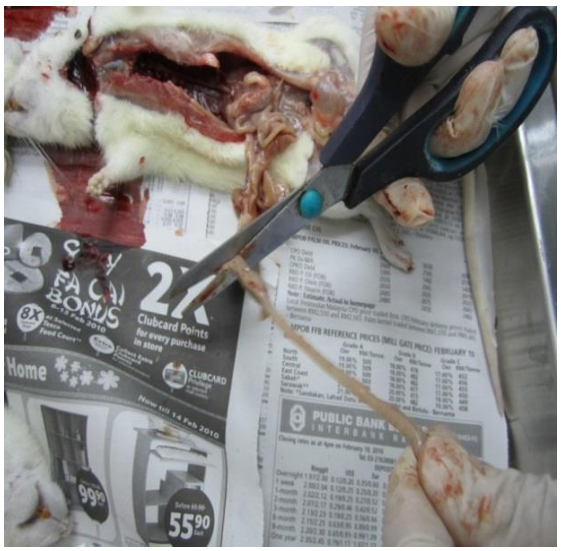


Figure B1: Animal house experiment

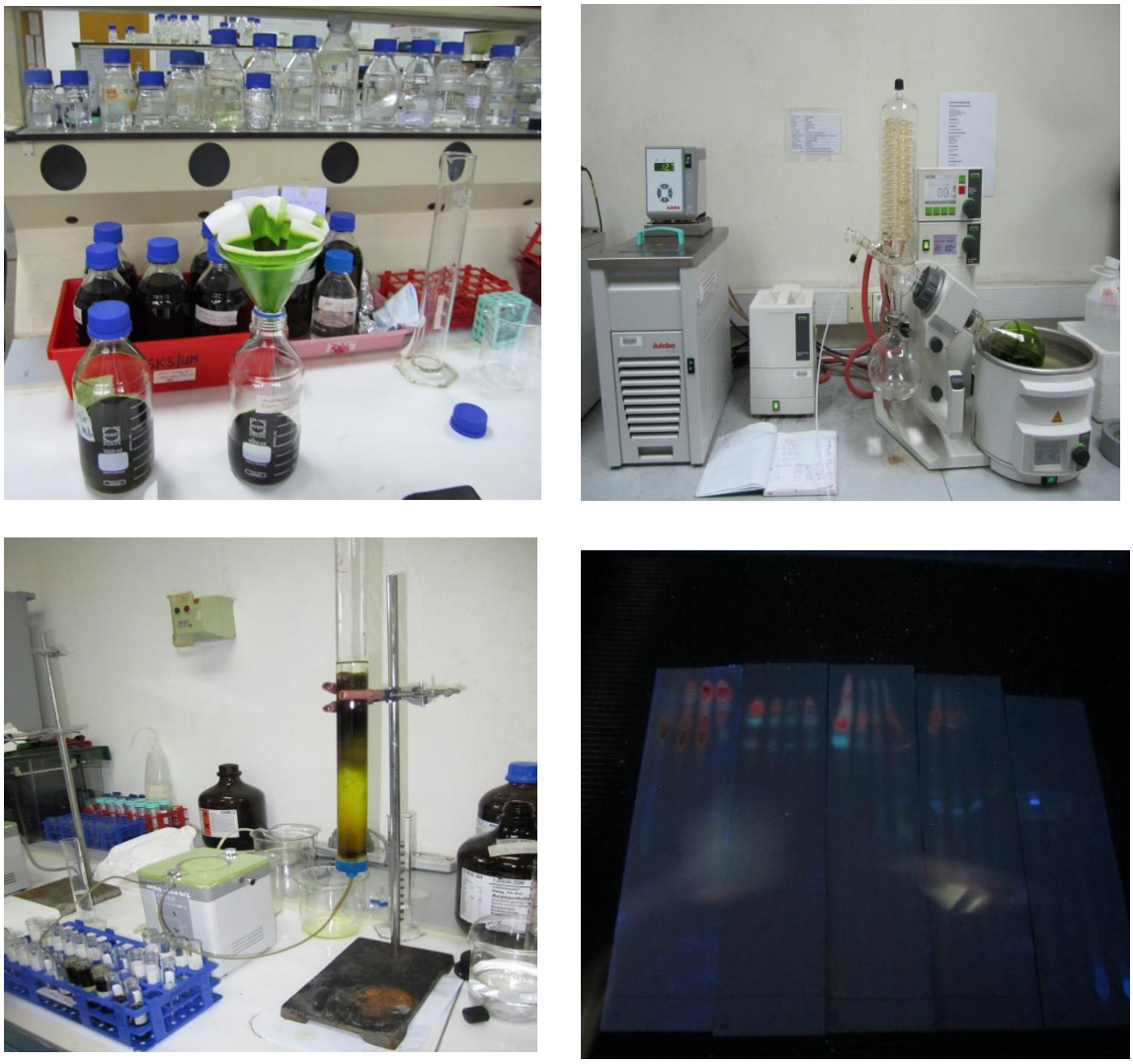
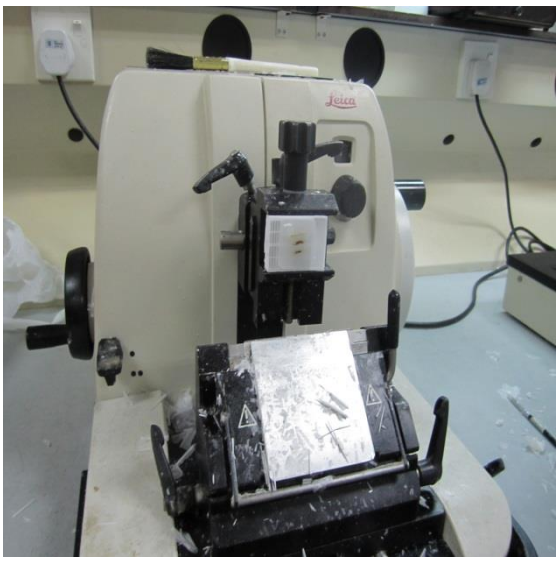


Figure B2: Column Chromatography and Thin layer Chromatography plate



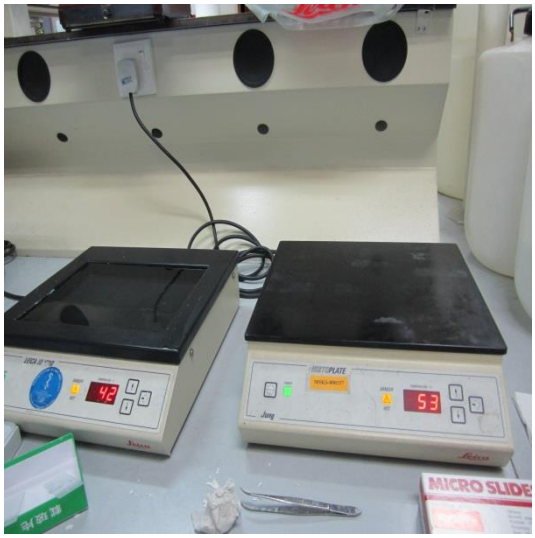


Figure B3: Histopathology slide preparation

APPENDIX D: Additional Outputs of The PhD Progect

1- List of grants, conferences and Submitted manuscripts

This work was supported by two research Grants from University of Malaya, Malaysia and (PV042-2011A).

Amel A. Almagrami[a*], Mohammed A. Alshawsh[b], Salmah Ismail[c], Suzy M. Salama[a], Shahram Golbabapour [a,c], Mahmood Ameen Abdulla[a*]. Chemopreventive Effect of Catharanthus roseus in Azoxymethane-Induced Aberrant Crypt Foci in Rat Colon" International Journal of Medical Sciences (7154v)(Q1).

Amel A. Almagrami, Mohammed A. Alshawsh, Salmah Ismail, Mahmood Ameen Abdulla*. Evaluation of Chemopreventive Effects of *Acanthus ilicifolius* Against Azoxymethane-Induced Aberrant Crypt Foci in the Rat Colon PLOS ONE (PONE-D-13-19881) (Q1).

2- List of conferences and medals

Poster presentation in the 21st International Invention, Innovation & Technology Exhibition ITEX 2010 held from 14th - 16th May 2010 Kuala Lumpur, Malaysia.

Silver Medal, INVENTION & INNOVATION Award, 21st International Invention, Innovation & Technology Exhibition ITEX 2010 held from 14th - 16th May 2010 Kuala Lumpur, Malaysia.