UNIVERSITI PUTRA MALAYSIA

EVALUATION OF HISTOPATHOLOGICAL APPEARANCE OF COLON TISSUES IN THE DMH-INDUCED RATS FED WITH HIGH FAT DIET SUPPLEMENTED WITH ANDROGRAPHIS PANICULATA (HEMPEDU BUMI) ETHANOLIC EXTRACT

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A FINAL YEAR PROJECT SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR OF BIOMEDICAL SCIENCES WITH HONORS

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ABSTRACT

Evaluation of Histopathological Appearance of Colon Tissues in The DMH-Induced Rats Fed With High Fat Diet Supplemented With *Andrographis paniculata* (*Hempedu Bumi*) Ethanolic Extract

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Introduction: Colorectal cancer (CRC) is considered the most common malignancy occurring globally. The incidence rate has climbed dramatically during the previous several decades in Asian countries. Dietary habits have a high correlation with disease incidence. Recently, fluoropyrimidine 5-fluorouracil (5-FU) is a conventional chemotherapeutic drug of choice for the treatment of CRC. The significant effect of fluorouracil on tissues raises the possibility of substantial adverse effects. *Andrographis paniculata* (*A. paniculata*) is one of the most popular herbal plants that has been widely cultivated for its curative properties against several diseases. *A. paniculata* have recently been revealed to possess anti-carcinogenic properties that are effective against various etiological causes of cancer. Additionally, consuming medicinal herbs enhances mitochondrial bioenergetics and suppresses the production of reactive oxygen species (ROS), thereby decreasing the chance of carcinogenesis. **Objective:** This study was aimed to evaluate the chemoprotective effects of *A. paniculata* ethanolic extract (APEE) on colon tissues in the high-fat diet (HFD)-induced colorectal cancer in Sprague Dawley (SD) rats by histopathological analysis. **Methodology:** This study employed colon tissue samples from prior HFD-induced CRC SD rats. The tissue samples were subjected to a series of histological methods and stained with Hematoxylin and Eosin for histopathological evaluation. The colons of the animals were analyzed for detection and quantification of aberrant crypt foci (ACF) using a Leica compound microscope at 400x magnification. **Results:** Histologically, the reduction of abnormality towards neoplasticity in the ACF was observed in APEE-treated groups. The findings showed that ACF of the APEE-treated group at a dosage of 250mg/kg BW (16.33 ± 0.33), and 500mg/kg BW (13.83 ± 0.36) had a significant reduction as compared to the HFD-induced CRC (20.38± 0.80). **Discussion**: Antioxidant phytochemicals are prevalent in plants and comprise a diverse class that has significant antioxidant and free radical scavenging properties. A previous study illustrates that APEE contained a wide range of polyphenols or phytochemicals that possess significant free radical scavenging activity. Extracts of *A. paniculata* may possibly decrease cancer cell proliferation, trigger cell cycle arrest, and promote apoptosis. **Conclusion**: The study showed a combination of HFD and DMH-induced agent increase the number of ACF. A dosage of 500mg/kg BW of APEE was effective in lowering the number of ACF, which might be due to the phytochemicals present in the extract.

Keywords: colorectal cancer, *Andrographis paniculata*, high-fat diet, aberrant crypt foci

ABSTRAK

Penilaian Manifestasi Histopatologi Tisu Kolon pada Tikus Diaruh DMH yang Diberi Diet Tinggi Lemak Ditambah Dengan Ekstrak Etanolik *Andrographis paniculata* (Hempedu Bumi)

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Pengenalan: Kanser kolorektal (CRC) dianggap sebagai kanser yang paling biasa berlaku di seluruh dunia. Kadar insiden telah meningkat secara mendadak selama beberapa dekad sebelumnya di negara-negara Asia. Tabiat pemakanan mempunyai korelasi yang tinggi dengan kejadian penyakit. Baru-baru ini, fluoropyrimidine 5-fluorouracil (5-FU) adalah ubat pilihan kemoterapi konvensional untuk rawatan CRC. Kesan ketara fluorouracil pada tisu menimbulkan kemungkinan kesan buruk yang besar. *Andrographis paniculata* (*A. paniculata*) adalah salah satu tumbuhan herba yang paling popular dan telah ditanam secara meluas kerana sifat kuratifnya terhadap beberapa penyakit. *A. paniculata* baru-baru ini dilaporkan mempunyai sifat anti-karsinogenik yang berkesan terhadap pelbagai etiologi penyebab kanser. Tambahan pula, pengambilan herba perubatan meningkatkan bioenergetik mitokondria dan menghalang penghasilan spesies oksigen reaktif (ROS), dengan itu mengurangkan peluang karsinogenesis. **Objektif:** Kajian ini bertujuan untuk menilai kesan kemopreventif ekstrak etanolik *A. paniculata* (APEE) pada tisu kolon dalam diet diaruh diet tinggi lemak (HFD) pada kanser kolorektal tikus Sprague Dawley (SD) melalui analisis histopatologi. **Metodologi:** Kajian ini menggunakan sampel tisu kolon dari tikus CRC SD yang diaruh HFD sebelumnya. Sampel tisu digunakan untuk satu siri kaedah histologi dan diwarnakan dengan Hematoxylin dan Eosin untuk penilaian histopatologi. Kolon tikus dianalisa untuk pengesanan dan pengukuran *aberrant crypt foci* (ACF) dengan menggunakan mikroskop Leica pada pembesaran 400x. **Keputusan:** Secara histologi, pengurangan keabnormalan terhadap neoplastisiti dalam ACF diamati dalam kumpulan yang dirawat APEE. Penemuan menunjukkan bahawa ACF kumpulan yang dirawat APEE pada dos 250mg / kg BW (16.33 ± 0.33), dan 500mg / kg BW (13.83 ± 0.36) mengalami penurunan yang ketara berbanding CRC yang diaruh hanya HFD (20.38± 0.80). **Perbincangan:** Fitokimia antioksidan adalah lazim dalam tumbuh-tumbuhan dan terdiri daripada pelbagai kelas yang mempunyai sifat antioksida dan radikal bebas yang ketara. Kajian terdahulu menggambarkan bahawa APEE mengandungi pelbagai jenis polifenol atau fitokimia yang mempunyai aktiviti mengais radikal bebas yang ketara. Ekstrak *A. paniculata* mungkin dapat mengurangkan percambahan sel barah, mencetuskan penahanan kitaran sel, dan mempromosikan apoptosis. **Kesimpulan:** Kajian menunjukkan bahawa gabungan ejen yang diaruh HFD dan DMH meningkatkan bilangan ACF. Dos pada 500mg / kg BW APEE berkesan dalam menurunkan bilangan ACF, yang mungkin disebabkan oleh fitokimia yang terdapat dalam ekstrak.

Kata kunci: kanser kolorektal, *Andrographis paniculata*, diet tinggi lemak, *aberrant crypt foci*

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LIST OF ABBREVIATIONS

CRC	Colorectal cancer
5-FU	5-fluorouracil
A. paniculata	*Andrographis paniculata*
APEE	A. paniculata ethanolic extract
HFD	High-fat diet
SD	Sprague Dawley
ACF	Aberrant crypt foci
AICR	American Institute of Cancer Research
WCRF	World Cancer Research Fund
FAP	Familial adenomatous polyposis
VAT	Visceral adipose tissue
AOM	Azoxymethane
DMH	Dimethylhydrazine
WHO	World Health Organization
miRNAs	MicroRNAs
SRV-2	Simian retrovirus-2
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
ROS	Reactive oxygen species
PBS	Phosphate-buffered saline
H&E	Hematoxylin and eosin
SEM	Standard error of the mean

CHAPTER 1

INTRODUCTION

1.1 Background

Colorectal cancer (CRC) is considered the most common malignancy were occurring globally. The prevalence of CRC varies considerably between countries (Siegel et al., 2020). However, the incidence rate has climbed dramatically during the previous several decades in Asian countries (Veettil et al., 2017). According to epidemiological studies, Patients' lifestyles and eating habits have influenced their chance of acquiring colorectal cancer (Walther et al., 2009). Until recently, Fluorouracil is a prominent chemotherapeutic drug used to treat colon cancer and is often prescribed to relieve benign and malignant breast tumors in women. Unfortunately, fluorouracil has several adverse effects, including heartburn, diarrhea, and lip sores (Wolpin and Mayer, 2008; Xie et al., 2020).

Furthermore, the considerable effect of fluorouracil on body tissues increases the likelihood of prolonged adverse effects (Al-Henhena et al., 2014). Therefore, Phytochemicals are now widely recognized as natural cancer-fighting agents. Most importantly, phytochemicals can treat cancer with fewer or no negative consequences (Ranjan et al., 2019). Scientific evidence suggests that phytochemicals could possess significant anticancer activity. Between 1940 and 2014, around 50% of authorized anticancer medicines were generated from natural sources (Newman & Cragg, 2016). The demand for chemopreventive agents and alternative anticancer therapies has arisen due to the adverse effects associated with currently accessible treatment modalities such as chemotherapy, surgery, and radiotherapy (Gumay et al., 2020).

People have relied on plants for thousands of years to treat various ailments, including cancer. Flavonoids, phenols, lactones, and other plant-based compounds have been shown to possess anticancer effects and strengthen cancer patients' immune systems (Cai et al., 2004; Merfort, 2011). One of the most herbal plants is *Andrographis paniculata* (Burm.f.) Nees is commonly known as the 'King of Bitters' in Asia and belongs to the Acanthaceae family (www.theplantlist.org; Kaushik et al., 2020). Traditional medicines from around the world have relied on this herb for thousands of years. It has been used in Unani, Malay, Chinese, Thai, Japanese, etc. (Sharma and Sharma, 2018; Mishra et al., 2007). Typically, aerial portions, leaves, or roots of *A. paniculata* generally are used individually. Powder, infusions, or decoctions of these plant components have traditionally been used to treat a variety of ailments, either alone or in conjunction with other therapeutic herbs (Hossain et al., 2021).

To minimize severe side effects in long-term survival, *A. paniculata* is currently used as an alternative to chemotherapeutic therapies like fluorouracil (Al-Henhena et al., 2014). Despite the continuous advancements in *A. paniculata* clinical findings, the initial steps in suppressing colorectal cancer formation are not well understood. Numerous studies are now investigating the use of *A. paniculata* and its primary phytoconstituent andrographolide as an alternative therapy for the treatment of cancer (Malik et al., 2021).

Experimentally, Dimethylhydrazine (DMH) is a carcinogen commonly utilized in rodent colon cancer experiments. The DMH model of colon cancer in rats is an effective tool for examining the association between ACF and CRC (Rodrigues et al., 2002). This experiment investigated the anti-proliferative and anti-oxidative effects of *A. paniculata* ethanol extract on Sprague Dawley rats. The DMH was administered to stimulate the development of cryptal foci. The formation of cryptal foci is commonly considered a

precancerous lesion (Al-Henhena et al., 2014). Therefore, this study will shed light on the mechanism underlying colon cancer prevention and the development of safer alternative therapies for CRC patients, as well as anticipate the future prospects of APEE usage in treating various diseases through additional proposed experiments.

1.2 Objectives

1.2.1 General Objective

To observe the histopathological appearance of *Andrographis paniculata* ethanolic extract (APEE) on colon tissues in DMH-induced colorectal cancer in obese rats

1.2.2 Specific Objectives

The specific purpose of this study are:

i. To observe the histopathological changes of APEE on the aberrant crypt foci in the colon tissues of treated rats
ii. To determine the effect of APEE on the average of aberrant crypt foci in the colon tissues of treated rats

1.3 Hypothesis

In comparison to the untreated group, oral administration of APEE exhibit a lower number of ACF and low level of the histopathological changes.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide. Approximately 10% of all malignant tumors detected each year are colorectal cancers. It is the second most prevalent cancer in women and the third most common cancer in males, respectively (Bray et al., 2018). Rates vary geographically, with the most developed countries experiencing the highest rates. With continued advancements in emerging countries, the global prevalence of CRC is expected to rise to 25 million new cases by 2035 (Arnold et al., 2017; Bray et al., 2018). There has been some fluctuation in global estimates of CRC prevalence throughout time. In developing countries, including those in South America, Asia, and Eastern Europe, the incidence increases dramatically (Arnold et al., 2017). Approximately 1.8 million new cases and 900,000 deaths a year were estimated by the International Agency for Research on Cancer in 2018, putting CRC the third predominantly diagnosed cancer and the second largest cause of cancer mortality worldwide (Bray et al., 2018).

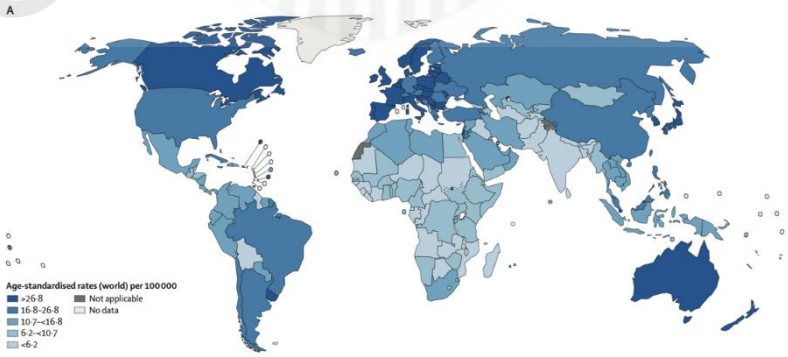

Figure 1: Geographical distribution of Colorectal cancer (Dekker et al., 2019)

However, genetics has a substantial influence on individual risk. On the other hand, CRC incidence is strongly influenced by modifiable variables such as nutrition and lifestyle (Jasperson et al., 2010). Usually, a western diet, obesity, and a sedentary lifestyle are significant risk factors for CRC, increasing prevalence due to industrialization and economic expansion (Keum & Giovannucci, 2019). Consequently, the CRC prevalence rate tends to climb as countries become more westernized (Center et al., 2009). The American Institute of Cancer Research (AICR) and the World Cancer Research Fund (WCRF) conducted a review of studies from throughout the world in 2017. They found that being overweight or obese, lacking exercise, consuming a poor diet, and alcohol consumption all increased the risk of developing CRC (Clinton et al., 2020). This convergence of factors is probably the source of escalating CRC incidence in economically transitioning populations (Keum & Giovannucci, 2019). Additionally, individuals with a family background of colorectal cancer or hereditary syndromes have a significantly higher risk of developing CRC (Jasperson et al., 2010). Accordingly, roughly 15–20% of patients with CRC have a positive family history (Kuipers et al., 2015). However, the risk varies according to the degree of relatedness and the number of affected individuals (Schoen et al., 2015). Nevertheless, the risk increases if a family member has been diagnosed with CRC before age 50 (Keum & Giovannucci, 2019).

2.2 Risk Factors

Worldwide, the likelihood of developing colorectal cancer is approximately 4%–5% (Mármol et al., 2017). However, patients with a hereditary colorectal cancer syndrome are a separate segment of the population of patients, accounting for around 5–10% of all

patients. Since they carry mutations in high-penetrance genes, the chance of developing CRC is relatively higher compared to the general population at any given age (Rahner & Steinke, 2008). Interestingly, people may develop hereditary colorectal cancer syndromes even if there is no background history in their families (Mork et al., 2015). Lynch syndrome is the most frequent ailment in this category. Subsequent mutations of the DNA mismatch-repair genes: MLH1, MSH2, EPCAM, or PMS2 lead to the development of this condition. The accumulation of DNA mutations is caused by inefficient mismatch repair throughout replication, particularly in microsatellite DNA pieces with repeated nucleotide sequences (Kuipers et al., 2015). A common feature of CRC associated with Lynch syndrome is mucinous, poorly differentiated, and heavily infiltrated with lymphocytes (Jass & Edgar, 1994).

The second most common hereditary colorectal cancer syndrome is familial adenomatous polyposis (FAP). When the APC gene is mutated, the WNT signaling system becomes defective, precipitating the development of this condition (Vasen et al., 2015). A distinguishing feature of FAP is the formation of hundreds or even thousands of adenomas in the distal colon, which usually begins in adolescence (Patel & Ahnen, 2012). Additionally, specific personal characteristics and behaviors have been considered risk factors since they enhance the probability of developing polyps. Despite age being considered a significant risk factor for CRC, the likelihood of having the disease significantly increases after 50. (Mármol et al., 2017). Obesity, another major cause of colorectal cancer, is intimately associated with a sedentary lifestyle. Remarkably, the risk of developing CRC increases with food consumption and visceral adipose tissue (VAT), which is known as an active hormone component that promotes the acquisition of CRC by stimulating the secretion of proinflammatory cytokines, which results in a variety of

consequences of inflammation in the rectum and colon, as well as insulin resistance (Martinez-Useros & Garcia-Foncillas, 2016).

2.3 Pathophysiology

CRC is initiated by genetic and environmental factors that encourage epithelial cells in the colon to acquire cancer hallmarks (Hanahan & Weinberg, 2011). Oncogene activation and tumor suppressor gene inactivation are two distinctive hallmark traits acquired throughout time by accumulating epigenetic alterations and genetic mutations (Kuipers et al., 2015). One of the most underlying pathophysiological processes in developing CRC (specifically, aberrant crypt foci) is the impairment of genomic stability, which has been observed in many neoplastic lesions (Colussi et al., 2013).

The majority of cancers begin as polyps. Over the period of 10 to fifteen years, aberrant crypts grow into precancerous polyps, eventually leading to colorectal cancer. Most colorectal tumors are thought to originate from stem cells or cells that resemble stem cells (Medema, 2013). Generally, CRC is caused by two separate pathways: chromosomal instability, also known as the adenoma-carcinoma, accounting for 70–90 % of colorectal malignancies. Additionally, a serrated neoplasia route accounts for 10%–20% of cases. These pathways represent many epigenetic modifications that occur sequentially (Muzny et al., 2012). Chromosomal instability is frequently the outcome of genomic processes initiated by an APC mutation, followed by loss of TP53 function or activation of RAS. On the other hand, the serrated neoplasia pathway is associated with RAF and RAS mutations, along with epigenetic instability manifested by CpG island methylation, resulting in stable and unstable microsatellite cancer (Dekker et al., 2019).

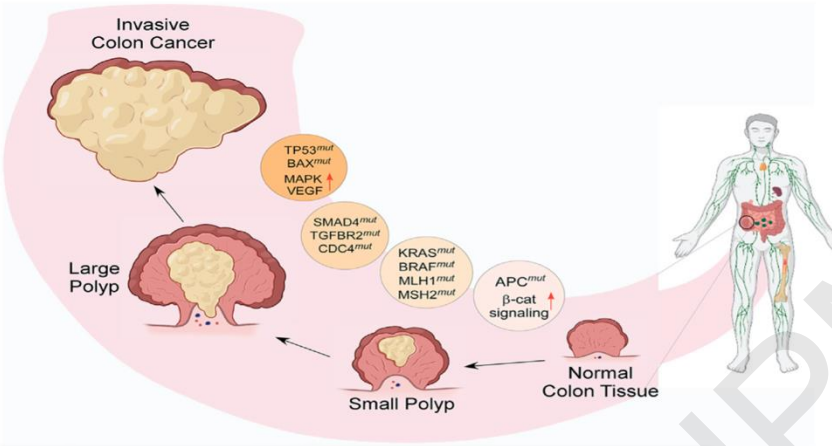

Figure 2: Formation of invasive polyps (Paul et al., 2021)

2.4 Background of aberrant crypt foci (ACF)

Due to the considerable variability of the genetic mutations associated with colorectal tumor formation, the focus of research on predictive biomarkers has primarily emphasized early histological changes occurring in the colonic mucosa. Aberrant crypt foci (ACF) are regarded as the first step in developing histologic changes that occur during the development of the colorectal neoplasm (Clapper et al., 2020). Bird described these lesions for the first time in 1987 when he discovered clusters of distinctively staining crypts with a remarkably thick epithelial lining inside the colonic mucosa of C57BL/6 mice treated with azoxymethane (AOM) (Bird, 1987). Using methylene blue to stain complete mounts of human colon tissue, researchers discovered comparable atypical crypts in 1991 and validated their clinical significance (Roncucci et al., 1991).

ACF is known as localized lesions in colonic mucosa characterized by many crypt enlargements and are triggered mainly by colon carcinogens (Bird, 1987). They can be easily detected under a stereoscopic microscope using methylene blue staining. They are frequently counted in numbers per colon or animal as early markers of colon

carcinogenesis (Bird, 1995). Dimethylhydrazine (DMH) is a commonly utilized carcinogen in rodent colon cancer experiments. In addition to being a highly carcinogenic substance, it also promotes the stages of carcinogenesis, resulting in the development of macroscopically visible neoplastic lesions. The DMH model of colon cancer in rats is an effective tool for examining the association between ACF and CRC (Rodrigues et al., 2002).

2.5 Aberrant crypt foci (ACF) as a biomarker

According to several studies, an increased risk of colon cancer is associated with dysplastic ACF; 93.6% of FAP patients exhibited dysplastic ACF, whereas only 7.0% of patients without an APC mutation had similar lesions (Takayama et al., 2001). It was found that in patients with CRC, the relative risk ratio for acquiring dysplastic ACF was substantially higher (RR, 18.14) when compared to those having nondysplastic ACF (RR, 1.29). Even though the function of dysplastic ACF in indicating the risk of CRC is still debatable, numerous researchers have discovered a correlation between the disease progression and the prevalence of ACF (Alrawi et al., 2006). Anderson and colleagues discovered that individuals with a significant number of ACF (>6) in the distal colon or rectum were found to have an increased risk of developing progressive neoplasm after the next five years (Anderson et al., 2011). Regardless of dysplasia, total ACF numbers rise with age; single ACF are more prevalent in younger individuals (under 40), but the most significant numbers are recorded in people aged 50 to 70 (Kowalczyk et al., 2018). Considering the recent findings, researchers should continue to investigate dysplastic ACF as an initial biomarker of CRC (Clapper et al., 2020).

ACF has been utilized as an alternative outcome in various preclinical studies that evaluated a chemopreventive agent's efficacy. In most studies, ACF dysplasia is not validated regarding the degree of dysplasia by histology or mucin-specific staining, but the overall number of ACF is generally reported. Since most ACF do not proceed to colorectal adenomas, comprehensive assessments are essential for a complete analysis of the results (Clapper et al., 2020). Several studies have shown that different experimental diets effectively prevent the development of dysplastic ACF. Compared to rats fed a normal AIN93M diet, rats treated with AOM were more likely to develop dysplastic ACF by 53% when administered a 20% high-fat diet (Baijal et al., 1998). Based on these findings, dysplastic ACF can be used as a potential biomarker for dietary therapies in vivo intended to either prevent or promote cancer in humans (Clapper et al., 2020).

2.6 Current therapies of CRC

The introduction of therapeutic approaches such as radical colostomy and chemotherapy, which are recognized for their curative effects in localized curable situations, has significantly raised the five-year survival rate (Norwati et al., 2014). However, one of the most commonly used chemotherapeutic agents is fluoropyrimidine 5-fluorouracil (5-FU), a widely utilized anti-tumor agent in treating tumors, including CRC (Pinedo & Peters, 1988). It is considered the most effective agent which could be used exclusively for treating CRC in its advanced stages (Raderer & Scheithauer, 1995). Unfortunately, like other chemotherapeutics drugs, fluorouracil can cause side effects such as indigestion, diarrhea, and sores on lips. Additionally, body tissues can be

influenced by the significant impact of fluorouracil, increasing the chance of prolonged negative consequences (Al-Henhena et al., 2014).

Numerous natural products have recently been revealed to possess anti-carcinogenic properties that are effective against various etiological causes of cancer (Khalifa et al., 2014). Consumption of medicinal herbs has been shown to enhance mitochondrial bioenergetics and suppress the production of numerous sources of reactive oxygen species (ROS), thereby decreasing the chance of carcinogenesis (El-Seedi et al., 2013). Natural compounds that inhibit or stimulate specific transcriptional machinery essential for mitochondrial biosynthesis and other processes involved in the formation of pro-inflammatory mediators may contribute significantly toward the treatment and prevention of different cancers (Al-Henhena et al., 2014). The demand for chemopreventive agents and alternative anticancer therapies has arisen due to the adverse effects associated with currently accessible treatment modalities such as chemotherapy, surgery, and radiotherapy. Nowadays, Colorectal cancer treatment is increasingly reliant on chemopreventive agents (Gumay et al., 2018).

2.7 Plant medicine as a potential treatment

Since antiquity, humans have relied on plants for their therapeutic properties (Schaal, 2018). According to the World Health Organization (WHO), the reliance on herbal remedies continues to grow constantly to treat medical needs by more than 80% of the global population (Haque et al., 2018). Currently, herbal medicine appears to replace chemoprevention and adjunctive treatment, which have a few adverse effects (Bi et al., 2017). Phytochemicals are now universally acknowledged as potential cancer treatments.

The real benefit of phytochemicals is that they can be utilized to treat cancer with few or no adverse effects (Ranjan et al., 2019). Scientific evidence suggests that phytochemicals could possess significant anticancer activity. Between 1940 and 2014, around 50% of authorized anticancer medicines were generated from natural sources (Newman & Cragg, 2016). The anti-cancer activity of these phytochemicals has been evaluated in vitro and in vivo. They scavenge free radicals (Lee et al., 2013), inhibiting malignant cell survival and proliferation (Yan et al., 2018), as well as reducing tumor angiogenesis and invasiveness (Lu et al., 2018). These phytochemicals have a complex and varied set of actions on a variety of biological targets and signaling pathways, such as membrane receptors (Deng et al., 2017), kinases (Dou et al., 2018), microRNAs (miRNAs) (Cojocneanu et al., 2015), transcriptional factors (Zhang et al., 2017), cyclins, and caspases (Yan et al., 2018).

However, plant-derived compounds, especially those with antioxidant properties to counteract free radicals and oxidative stress, are promising solutions in cancer chemoprevention. Moreover, these phytochemicals are proven to possess none-to-minimal toxicity toward normal cells, making them preferred agents in chemoprevention (George et al., 2021). Given the critical role of oxidative stress in the etiology of many malignancies, the antioxidant action of dietary phenolic substances may provide a promising technique for cancer prevention. Antioxidant phytochemicals are prevalent in plants and comprise a diverse class of molecules with various modes of action against malignancies. Thus, chemoprevention through plant-based antioxidants has the potential to significantly reduce the risk factors linked with cancer development (George et al., 2021).

2.8 *Andrographis paniculata*: Background and traditional uses

Andrographis paniculata (Burm.f.) Nees. It is a common plant that belongs to the Acanthaceae family and is commonly referred to as the "King of Bitters" in Asia and as "Kalmegh" in India (Kaushik et al., 2021). This plant is indigenous to Sri Lanka and India, but it is also found in China, the Caribbean, Indonesia, Malaysia, and Thailand (Jiao et al., 2019). Various phytoconstituents, including diterpenoids, xanthones flavonoids, quinic acids, diterpenoids, and noriridoids, have been identified in the leaves and stem of *A. paniculata* (Joseph & Joseph, 2016).

A. *paniculata* crude extracts were found to possess various pharmacological properties. For instance, anticancer, analgesic, antimalarial, antifertility, antidiabetic, anti-inflammatory, antimicrobial, antioxidant, antivenom, antipyretic, hepatoprotective, hepatoprotective, and immunomodulatory properties (Singha et al., 2003; Dai et al., 2019; Kaushik et al., 2020). Typically, aerial portions, leaves, or roots of *A. paniculata* generally are used individually. Powder, infusions, or decoctions of these plant components have traditionally been used to treat a variety of ailments, either alone or in conjunction with other therapeutic herbs (Hossain et al., 2021). For instance, *A. paniculata* leaves juice is extensively used in Malaysia, Japan, and the Philippines to treat hypertension, rheumatic fever, cough, diabetes, and the common cold. Additionally, the leaves have been used in Thai medicine since ancient times to cure various diseases, including gastrointestinal disorders, colic, liver toxicity, parasite infections, gallbladder inflammation, etc. (Churiyah et al., 2015).

Figure 3: The leaf of *Andrographis paniculata* (Mussard et al., 2019)

2.9 Pharmacological properties of *Andrographis paniculata*: Anticancer properties

According to several studies, extracts of *A. paniculata* and their main diterpenoid component effectively decrease cancer cell proliferation, trigger cell cycle arrest, and promote apoptosis (Banerjee et al. 2016, Cheung et al. 2005, Kumar et al. 2012, Mi et al. 2016). Besides that, it suppresses the phosphorylation of AKT and mTOR in colon cancer cells, resulting in ER stress-induced apoptosis (Banerjee et al., 2017). Andrographolide, either alone or in conjunction with cisplatin, caused apoptotic cell death via increased Bcl-2, Bax expression, and Fas/FasL interaction in human colorectal cancer cells, leading to increased production of cytochrome c and caspase activation (Lin et al., 2014).

Furthermore, the ability of andrographolide to reverse 5-FU resistance in colorectal cancer cells was also investigated. In vitro and in vivo studies show that andrographolide re-sensitizes 5-FU-resistant cells to the toxic effects of 5-FU. Recently, Wang and colleagues discovered that andrographolide overcomes 5-FU resistance in human colorectal cancer via enhancing BAX expression. It targets BAX explicitly, preventing its

breakdown and increasing apoptosis mediated by mitochondria. They also found that andrographolide reduces 5-FU resistance in colorectal cancer patients (Wang et al., 2016).

2.10 Antiretroviral properties

Recent studies have examined the antiretroviral activity of 96 % ethanolic extract of *A. paniculata* leaves against Simian retrovirus-2 (SRV-2). Numerous studies have demonstrated that *A. paniculata* and its derivatives have a potent inhibitory effect on the primary protease of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). It has been shown to be a potential candidate for treating COVID-19 through silico studies (Enmozhi et al., 2020). Several computational techniques research has discovered that *A. paniculata* and its derivatives are responsible for suppressing COVID-19 proteins. However, clinical trials must be followed by more in vivo and in vitro studies (Park et al., 2016; Solnier & Fladerer, 2020).

2.11 Antioxidant properties

A condition known as oxidative stress arises when excessive reactive oxygen species (ROS) levels accumulate within cells and overcome the body's natural defenses. Because of their toxicity is implicated in a wide range of disorders, including type 2 diabetes and cancer (Mussard et al., 2019). Several studies have discovered that phytochemicals such as glycosides, alkaloids, phenols, terpenoids, and steroids have significant antioxidant and free radical scavenging properties (Farhan et al., 2012). *A. paniculata* ethanolic extract contained a wide range of polyphenols or phytochemicals, including flavonoids, terpenoids, phenolics, steroids, and alkaloids. Significant free

radical scavenging activity was observed by the ethanolic extract, which contains essential phytochemicals. These findings indicate that *A. paniculata* could be a promising therapeutic agent in the oxidative stress prevention (Rajendrakumar T, Suguna Rao, Satyanarayana, M.L., Narayanaswamy, H.D. and Byregowda, 2020).

Table 1: Traditional uses of *Andrographis paniculata* (Liaqat, 2021)

Countries/traditional medicine system (TMS)	Medicinal uses	References
Traditional Chinese medicine	Inflammation, burn, common cold, eczema, fever, respiratory infections, pelvic inflammation, snake bite, etc.	(Akbar, 2011)
Traditional Indian medicine	Enteritis, dysentery, diabetes, herpes, skin infections, peptic ulcer	(Jarukamjorn & Nemoto, 2008)
Ayurvedic	Fever, vitiligo, liver disease, torpid liver	(Boopathi, 2000)
Malaysia	Diabetes, hypertension	(Borhanuddin et al., 1994)
Japan	Fever, common cold	(Borhanuddin et al., 1994)

CHAPTER 3

METHODOLOGY

3.1 Animal model and experimental design

This experiment used 42 Sprague Dawley rats aged 4-22 weeks and obtained from the Animal Colony Unit, Faculty of Veterinary Medicines, UPM, Serdang, Selangor. After simple random selection, rats were grouped into seven groups with sex rats each (six rats/ group). The rats were acclimatized for one week on receiving a standard commercial feed. After acclimatization, the animals were fed either a normal diet or a high-fat diet (HFD) with energy (414 kcal/100g) made of 43% carbohydrate, 17% proteins, and 40% fats. HFD was prepared and mixed well before a bake in the oven at 60 °C for 2 hours. Then, the HFD was cut into small pieces and stored in the fridge (Perumal et al., 2019; Balan et al., 2019). Fresh diets were provided daily to allow the rats free access to food. Daily food intake was monitored throughout the study.

After acclimatization, the rats intended for carcinogen treatment were injected subcutaneously with DMH at a dose level of 40 mg/kg body weight once weekly for ten weeks. *A. paniculata* ethanol extract were administered via oral gavage throughout the experimental period (19 weeks) for group HCAP125, HCAP250 and HCAP500. The Sprague Dawley rats are distributed into the following groups:

I. Normal (N): fed with standard chow diet and subcutaneously injected with normal saline.

II. High Fat Diet (H): fed with high fat diet and s.c. injected with normal saline.

III. High Fat Diet (HC): fed with high fat diet with s.c. injection 40mg/kg BW of DMH.

IV. HCF Group: fed with high-fat diet with s.c. 40mg/kg BW DMH and 35mg/kg BW of Fluorouracil (5FU) injection.

V. HCAP125: fed with high-fat diet with s.c.40mg/kg BW DMH injection and 125mg/kg BW *A. paniculata* ethanol extract.

VI. HCAP250: fed with high fat diet with s.c.40mg/kg BW DMH injection and 250mg/kg BW *A. paniculata* ethanol extract.

VII. HCAP500: fed with high fat diet with s.c.40mg/kg BW DMH injection and 500mg/kg BW *A. paniculata* ethanol extract.

3.2 Termination of experiment

Before euthanasia, gross measurements had been taken weekly for the last seven weeks of treatment. The rats were fasted for 12 hours before receiving an intraperitoneal dose of 125/10 mg/kg BW of ketamine/xylazine. After that, blood samples were obtained through cardiac puncture before collecting liver, colon, kidney, and adipose tissue samples. Colon samples obtained in a previous study will be utilized to evaluate the histopathological appearance and quantify the ACF percentage.

3.3 Colon sample for histopathological evaluation

Euthanasia was followed by immediate gentle rinsing of the colon with phosphate-buffered saline (PBS). Colon tissue was then dissected from the anal side and embedded in paraffin blocks after being fixed in 10% formalin (v/v) to prevent autolysis and stabilize

tissue structures. Then, colons were sectioned to 5um and stained with hematoxylin and eosin (H&E) for histological evaluation.

3.4 Histological classification of ACF

The stained specimens are examined histopathologically using a Leica Compound Microscope at 400x magnification. Norazalina et al. (2010) method was utilized to identify ACF. However, ACF was recognized as rounded and well-defined clusters of crypts with polymorphism and relatively more significantin size than normal. The average number of ACF from each group was calculated by comparing the overall number of crypts to the number of ACF. Additionally, the cluster number of crypts were counted and categorized into 1 crypt, 2 crypts, 3 crypts, 4 crypts, and more than 5 crypts.

3.5 Statical analysis

The data are presented as mean ± standard error of the mean (SEM). One-way ANOVA was performed to analyze the mean difference between multiple samples. When the ANOVA result was significant, the Tukey test was used to compare the variations between the control group and the treatment groups using IBM SPSS Statistics Campus Edition V26.0. A value of P less than 0.05 was considered significant, and the experiment was carried out in triplicate.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Effect of APEE on the histological appearance of aberrant crypt foci (ACF)

Homeostasis of the colorectal epithelial layer is attributed to be caused by a balance between cell division and cell death. However, cancer of the colon develops when cells grow in an irregular and excessive manner. The underlying mechanisms of CRC formation and progression are heavily influenced by oxidative stress (Dekker et al., 2019). According to the findings of numerous epidemiological studies, a diet high in fat contributes to the development of 80 % of CRC. Most studies conducted on animals have reported that the consumption of HFD leads to an increase in the number of ACF. These lesions are regarded as preneoplastic in the progression of CRC (Campos FG et al., 2005; Nor JE et al., 2001; Lin S et al., 2000).

A recent study concluded that consuming HFD may increase the risk of developing CRC by disrupting the normal ratio of bile acids found in the intestines and leading to an imbalance in hormones that allows possibly malignant cells to proliferate (Arnold et al., 2017; Torre et al., 2016; Wolf et al., 2018). According to the findings of a study that was conducted in 2014, intake of an HFD may contribute to the development of CRC via inflammation, changes in metabolic function, and an increase in the progression of the cell cycle in rats that have been induced with DMH (Zhu et al., 2014). These findings might help to explain why there has been a spike in the number of young adults diagnosed with the disease. (Araghi et al., 2019; Haggar & Boushey, 2009; Siegel et al., 2019).

In our study, an attempt was made to examine the number of ACF and the histological changes in the dynamics of the disease, as well as to investigate whether a diet high in fat influenced the progress and number of ACF. ACF was counted after being administered subcutaneously with 40mg/kg of 1,2-Dimethylhydrazine (DMH) once a week for 10 consecutive weeks. From the qualitative analysis, the histopathological appearance of the colon from the microscopic result is shown in Figure 4 which represents different groups. The crypts showed various forms of abnormalities. There was an enlarged and elongated appearance of the nucleus with depleted mucin in the NC and H groups. In the HC group, we can see crowded glands with complex architecture while in the HCF group, the nucleus still has dysplastic features with some atypical changes. However, in the HCAP125 treatment group, there are still some enlarged crypts with a slight elongation appearance of the nucleus, while there is an absence of mucin depletion or stratification of the nucleus in both groups HCAP250 and HCAP500. The treated rats show a significant reduction of histopathological changes at a dose of 250, and 500 mg/kg BW *A. paniculata* ethanolic extract.

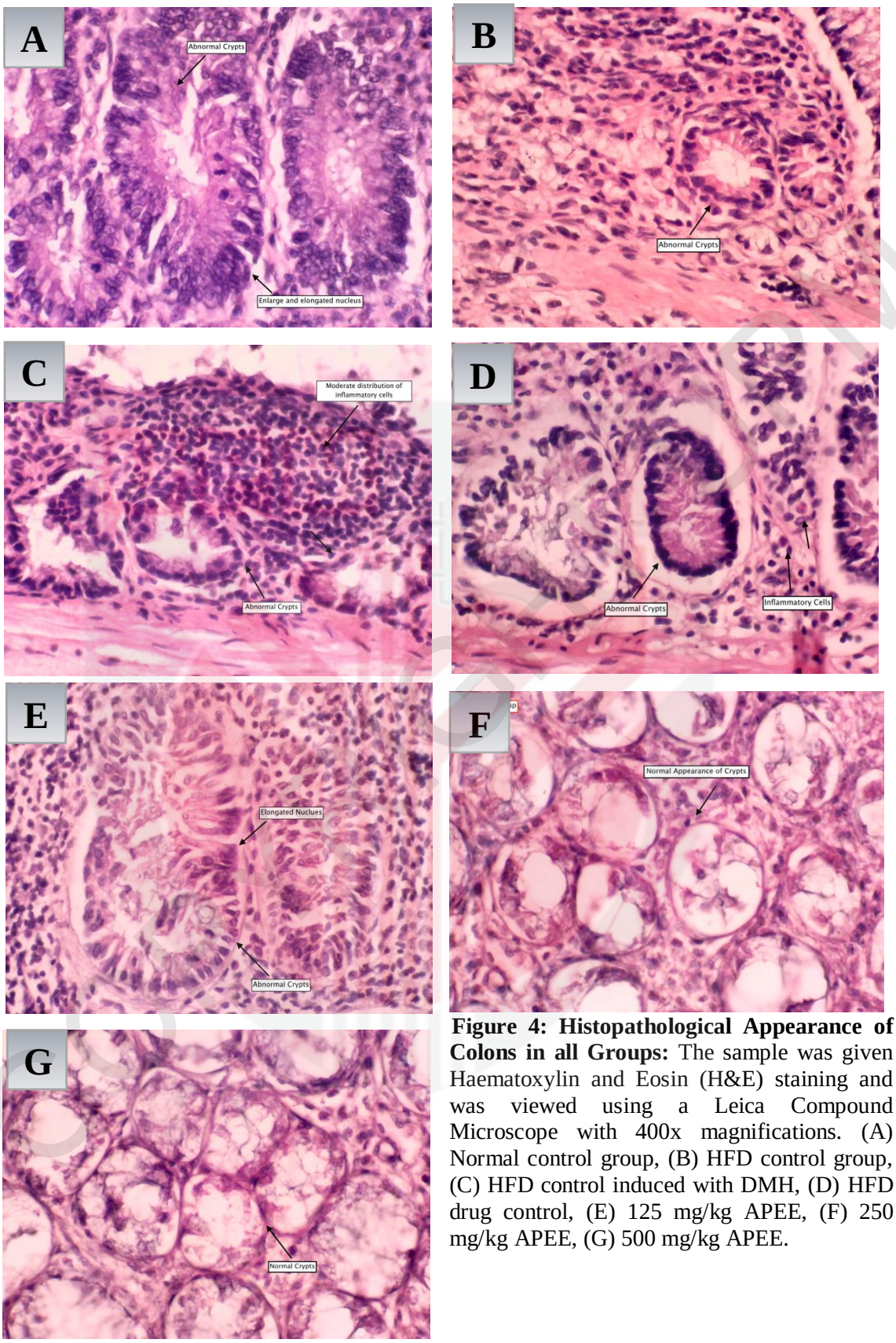

Figure 4: Histopathological Appearance of Colons in all Groups: The sample was given Haematoxylin and Eosin (H&E) staining and was viewed using a Leica Compound Microscope with 400x magnifications. (A) Normal control group, (B) HFD control group, (C) HFD control induced with DMH, (D) HFD drug control, (E) 125 mg/kg APEE, (F) 250 mg/kg APEE, (G) 500 mg/kg APEE.

4.2 Five categories of aberrant crypt foci (ACF)

The characterization of ACF and the number of crypts per focus were illustrated in Figure 5. The observation was seen using a Leica Compound Microscope at 400x magnification. The figures illustrate the histological appearance of crypts of various sizes.

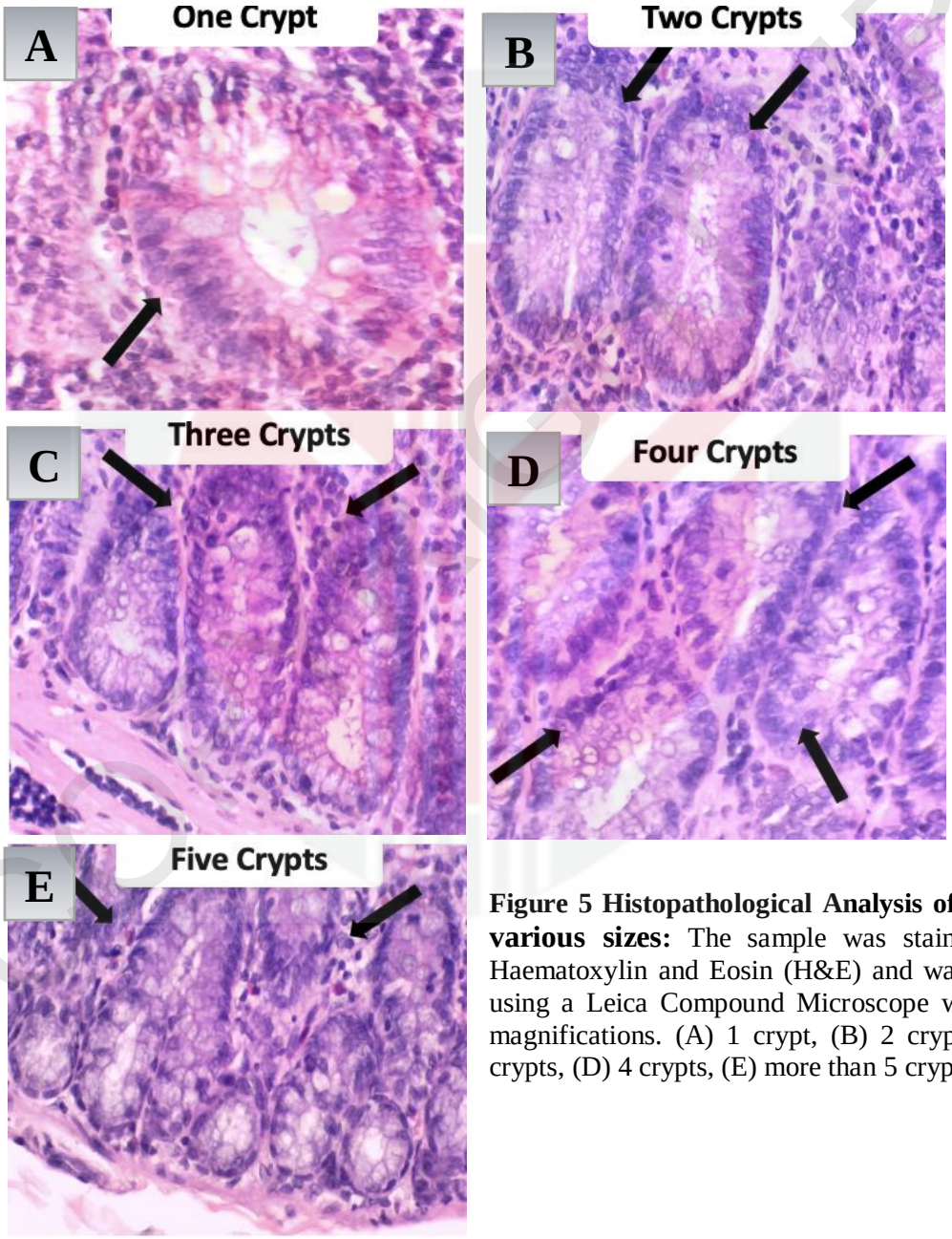

Figure 5 Histopathological Analysis of ACF of various sizes: The sample was stained using Haematoxylin and Eosin (H&E) and was viewed using a Leica Compound Microscope with 400x magnifications. (A) 1 crypt, (B) 2 crypts, (C) 3 crypts, (D) 4 crypts, (E) more than 5 crypts.

4.3 Effect of APEE on the average score of ACF for control and treatment groups

The effect of APEE on DMH-induced rats fed with HFD is illustrated in Figure 5. The presence of ACF was identified in the colon and the overall number of ACF were counted. The result showed that the colonic ACF appeared in all rats induced with DMH. However, there is no significant difference in the average ACF between the NC (15.38 ± 0.263) group fed a normal diet and the H (15.13 ± 0.133) group given HFD. Noticeably, the HC group fed an HFD and induced with DMH had a conspicuous rise in the number of ACF, suggesting that the combination of an HFD and DMH agent leads to the progression of CRC and increases the quantity of ACF in the colon. These findings have been matched with previous studies that were conducted in 2014, intake of an HFD may contribute to the development of CRC via inflammation, changes in metabolic function, and an increase in the progression of the cell cycle in rats that have been induced with DMH (Zhu et al., 2014).

Furthermore, there was a significant decrease in ACF formation in the HCF and treatment groups at two doses of 250 and 500 mg/kg BW, indicating that the treatment is effective at reducing the quantity of ACF. However, at a dose of 125 mg/kg BW, no significant reduction of ACF was detected, indicating that this dose may not be adequate to reduce the quantity of ACF. Overall, the ACF of HCAP250 (16.33 ± 0.333) and HCAP500 (13.83 ± 0.367) had a significant reduction compared to HC (20.38± 0.800). Among the extract treatment, the lowest quantity of ACF was in HCAP500 (13.83 ± 0.367). The findings of this study indicate that *A. paniculata* exhibits antioxidant and antiproliferative properties, and hence could be a promising candidate for the development of new CRC therapeutic agents.

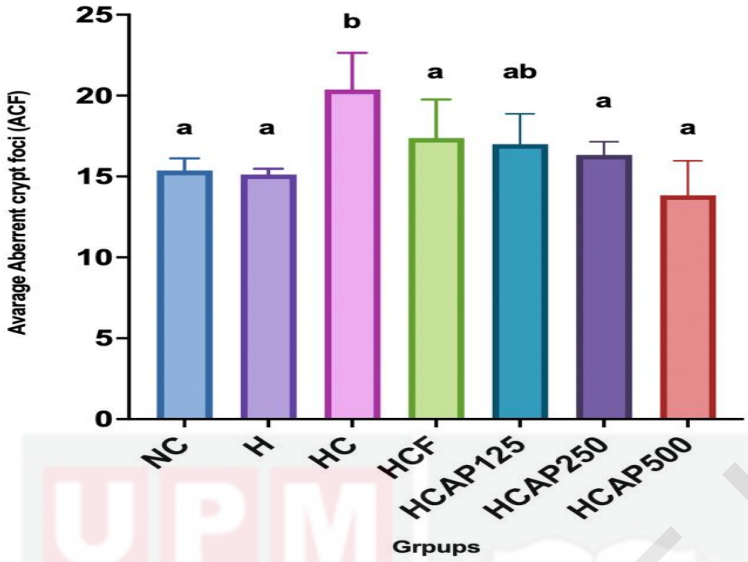

Figure 6: Comparative Analysis of the average number of ACF Between Groups The comparative analysis of the average number of ACF between groups. Results are expressed as mean ± SEM (n=7) Results were analysed by using one-way ANOVA followed by a post hoc Tukey test. Different letters indicated a significant difference (p<0.05). Groups. NC: Normal control group, H: HFD control group, HC: HFD control induced with DMH, HCF: HFD drug control, HCAP: 125 mg/kg APEE, 250 mg/kg APEE, 500 mg/kg APEE.

Mutations and genetic alterations are more prevalent in the gastrointestinal tract since it is more susceptible to oxidative stress. Specifically, the segments of the colorectal due to their biological role and distant location. Consequently, CRC accounts for 13% of all cancer globally and is among the most prevalent malignancies (Al-Henhena et al., 2015). There has been a significant amount of research on the antioxidant properties of natural products, specifically looking at their pro-apoptotic and anti-proliferative capabilities, with the goal of presenting them in the future as potential preventative or therapeutic agents. Approximately, 74% of cancer treatments are derived from herbal extracts which exhibited an antioxidant and anti-inflammatory activities (Al-Asmari et al., 2015; Pucci et al., 2019).

It has been hypothesized that the presence of flavonoid and diterpenoid compounds in *A. paniculata* is contributed to its anti-cancer and antioxidant bioactivates (Al-Henhena et al., 2014). The findings of our study on lowering the ACF number could be attributed to the fact that APEE contained a wide range of phytochemicals such as flavonoids, terpenoids, and alkaloids that show various pharmacological properties (Rajendrakumar T, Suguna Rao, Satyanarayana, M.L., Narayanaswamy, H.D. and Byregowda, 2020). The anti-cancer activity of these phytochemicals has been evaluated in vitro and in vivo. They scavenge free radicals (Lee et al., 2013), inhibiting malignant cell survival and proliferation (Yan et al., 2018), as well as reducing tumor angiogenesis and invasiveness (Lu et al., 2018). Furthermore, DPPH radical scavenging assay revealed considerable levels of antioxidant activity due to flavonoid content when phytochemical extracts of *A. paniculata* were investigated (Liaqat, 2021). According to these findings, *A. paniculata* has the potential to be an effective therapeutic agent in the reduction of oxidative stress (Mussard et al., 2019).

The possible anticancer mechanisms could be due to the presence of diterpenoids, specifically, andrographolide, which was shown to be responsible for the decrease in cancer cell proliferation, as well as the induction of cell cycle arrest and the enhancement of apoptosis (Banerjee et al. 2016, Cheung et al. 2005, Kumar et al. 2012, Mi et al. 2016). Andrographolide was able to induce apoptosis in human colorectal cancer (CRC) Lovo cells by increasing the expression of Bax and Bcl-2 as well as the interaction between Fas and FasL, which led to an increase in cytochrome c release and caspase activation (Islam et al., 2018).

The type of solvent that was used for the process and the concentration of the solvent both play an important role in determining the maximum amount of the bioactive substance that can be extracted from the plant. Additionally, phytochemicals generated from medicinal plants require tight quality control to maintain consistent biological activity, which is directly related to their composition. The extract made with 50% ethanol contained the highest quantities of andrographolide, followed by the extracts that were made with 100%, 70%, and 30% ethanol, respectively. However, the water extract contained the lowest levels of andrographolide. According to these findings, the polarity of the solvent used for extraction had a direct impact on the quantity of andrographolide extracted (Rafi et al., 2020).

4.4 Number of crypts/ACF quantified in control and treatments groups

The number of crypts foci per focus was illustrated in Figure 7. From the figure, multiple clusters of crypts (4 crypts and more than 5) were reported. DMH-induced rats fed with HFD and given APEE with a dose of 250, and 500 mg/kg showed a significant reduction of multiple crypts per focus. However, a dose of 500 mg/kg of APEE significantly lower the number of clusters compared to the drug control.

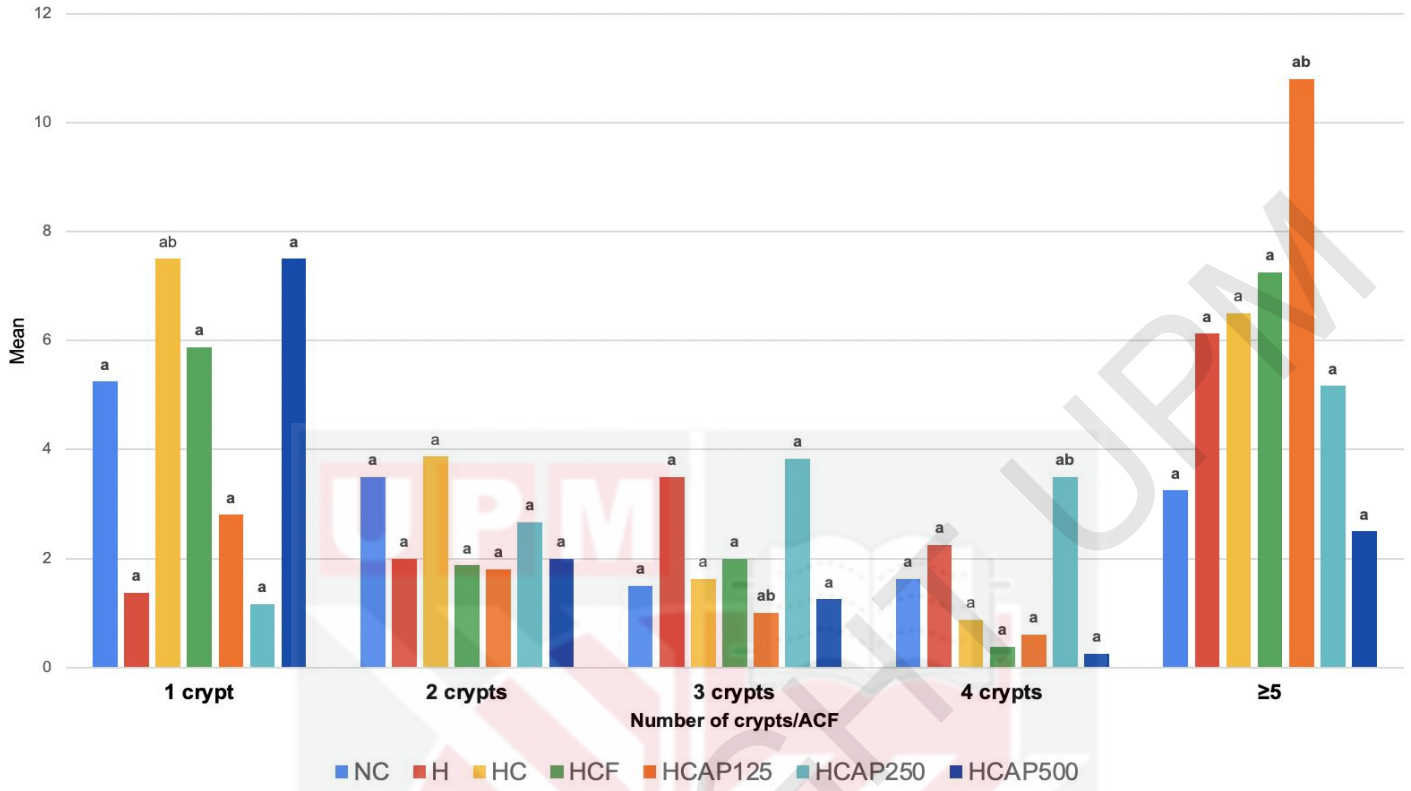

Figure 7: Comparative Analysis of the number of crypts/ACF Between Groups The comparative analysis of crypts/ACF between groups. Results are expressed as mean ± SEM (n=7) Results were analysed by using one-way ANOVA followed by a post hoc Tukey test. Different letters indicated a significant difference (p<0.05). Groups. NC: Normal control group, H: HFD control group, HC: HFD control induced with DMH, HCF: HFD drug control, HCAP: 125 mg/kg APEE, 250 mg/kg APEE, 500 mg/kg APEE.

CHAPTER 5

CONCLUSION AND RECOMENDATIONS

In conclusion, the findings revealed that a combination of HFD and DMH-induced agent increase the number of ACF . A dosage of 500mg/kg BW of APEE was effective in lowering the numbers of ACF and in exhibiting low level of the histopathological changes. The findings of this study indicate that *A. paniculata* exhibits antioxidant and antiproliferative properties, and hence could be a potential candidate for the development of new CRC therapeutic agents.

Further studies on cytotoxic effect of the plants need to be carried out to understand the underlying mechanism in colon cancer. Moreover, other stains such as methylene blue its recommended to be performed for more validation of result.

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