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INVESTIGATING THE EFFECTS OF MALAYSIAN TUALANG HONEY ON HUMAN COLON CANCER CELLS

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A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE (BIOMEDICAL SCIENCES)

DEPARTMENT OF BIOMEDICAL SCIENCES

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ABSTRACT

INVESTIGATING THE EFFECTS OF MALAYSIAN TUALANG HONEY (MTH) ON COLON CANCER CELLS

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Introduction: Colon cancer has been announced as the third most common cancer and the second leading cause of cancer-related death worldwide. Current treatments affect normal cells that lead to varieties of side effects which affect the patients' life quality. Therefore, exploration of alternatives or adjunct therapy is much needed. The anticancer properties of MTH on cancer cells such as breast and cervical cancer have been well documented. However, gaps in knowledge still exist on the effect of MTH on colon cancer cells. **Objectives:** This study aimed to investigate the effect of MTH on the proliferation and migration of colon cancer cells, namely HCT-116. **Methodology:** To obtain the inhibitory concentration (IC_{50}), human colon carcinoma cell line HCT-116 were treated with increasing concentrations of MTH (0.625% - 10%) for 24 hours and the cell viability was assessed using MTT assay. The cells were treated with MTH inhibitory concentrations of 25%, 50% and 75% for 72 hours to determine the proliferation rate via MTT assay. The effect of MTH exposure on the migration of the cells was assessed via migration assay. **Results:** From the plotted dose-response curve, the IC_{25}, IC_{50}, and IC_{75} values of HCT-116 cells treated with MTH are 0.9884%, 1.928% and 3.761%. In the proliferation assay, the cell viability of HCT-116 cells decreased significantly as the concentration of MTH increased ($p < 0.05$). The migration assay showed significant reduction in migration rate of HCT-116 cells with exposure to higher MTH concentration ($p < 0.05$). **Discussion:** Based on the findings, MTH exerted cytotoxic effects on HCT-116 cells in dose-dependent manner, in which consistent with earlier study of MTH on lung adenocarcinoma cells. MTH also inhibited the proliferation of HCT-116 cells. Phenolic content in MTH explained the cytotoxicity and antiproliferation properties of MTH. The study of migration showed migration of HCT-116 cells were suppressed by MTH in concentration-dependent manner. This finding in line with previous study on HCT-116 cells using Manuka honey which proved metastasis of colon cancer were affected by honey. **Conclusion:** Present study proved that MTH could be a promising potential anticancer agent due its antiproliferative and antimigratory effect on colon cancer cells.

Keywords: human colon carcinoma, HCT-116, Malaysian Tualang Honey (MTH), cytotoxic, proliferation, migration

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ABSTRAK

MENYIASAT KESAN MADU TUALANG MALAYSIA (MTH) PADA SEL KANSER KOLON

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Pengenalan: Kanser kolon telah diumumkan sebagai kanser ketiga paling biasa dan penyebab kedua kematian berkaitan kanser di seluruh dunia. Rawatan semasa menjejaskan sel normal yang membawa kepada pelbagai kesan sampingan yang menjejaskan kualiti hidup pesakit. Oleh itu, penerokaan alternatif atau terapi tambahan amat diperlukan. Sifat antikanser MTH pada sel kanser seperti kanser payudara dan serviks telah didokumenkan dengan baik. Walau bagaimanapun, jurang dalam pengetahuan masih wujud mengenai kesan MTH pada sel kanser kolon. **Objektif:** Kajian ini bertujuan untuk menyiasat kesan MTH terhadap percambahan dan migrasi sel kanser kolon iaitu HCT-116. **Metodologi:** Untuk mendapatkan konsentrasi perencatan (IC_{50}), sel karsinoma kolon manusia HCT-116 dirawat dengan konsentrasi MTH yang meningkat (0.625% - 10%) selama 24 jam dan daya hidup sel dinilai menggunakan ujian MTT. Sel-sel telah dirawat dengan konsentrasi perencatan MTH sebanyak 25%, 50% dan 75% selama 72 jam untuk menentukan kadar percambahan melalui ujian MTT. Kesan pendedahan MTH pada migrasi sel telah dinilai melalui ujian migrasi. **Keputusan:** Daripada keluk tindak balas dos yang diplot, nilai IC_{25} , IC_{50} dan IC_{75} daripada sel HCT-116 yang dirawat dengan MTH ialah 0.9884%, 1.928% dan 3.761%. Dalam ujian proliferasi, daya hidup sel sel HCT-116 menurun dengan ketara apabila kepekatan MTH meningkat (p <0.05). Ujian migrasi menunjukkan pengurangan ketara dalam kadar migrasi sel HCT-116 dengan pendedahan kepada kepekatan MTH yang lebih tinggi (p <0.05). **Perbincangan:** Berdasarkan penemuan, MTH memberikan kesan sitotoksik pada sel HCT-116 dalam cara bergantung kepada dos, yang konsisten dengan kajian awal MTH pada sel adenokarsinoma paru-paru. MTH juga menghalang percambahan sel HCT-116. Kandungan fenolik dalam MTH menerangkan sifat sitotoksisiti dan antiproliferasi MTH. Kajian migrasi menunjukkan migrasi sel HCT-116 telah ditindas oleh MTH dalam cara yang bergantung kepada konsentrasi. Penemuan ini selaras dengan kajian lepas terhadap sel HCT-116 menggunakan madu Manuka yang membuktikan metastasis kanser kolon telah terjejas oleh madu. **Kesimpulan:** Kajian sekarang membuktikan bahawa MTH boleh menjadi agen antikanser yang berpotensi menjanjikan kerana kesan antiproliferatif dan antimigrasinya pada sel-sel kanser kolon.

Kata kunci : karsinoma kolon manusia, HCT-116, Madu Tualang Malaysia (MTH), sitotoksik, proliferasi, migrasi

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee On Cancer
ANOVA	One- Way Of Analysis
ATCC®	American Type Culture Collection
BSC	Biosafety Cabinet
CGA	Comprehensive Geriatric Assessment
CIMP	Cpg Island Methylator Phenotype
CIN	Chromosome Instability
COX2	Cyclooxygenase 2
DMSO	Dimethyl Sulfoxide
EMT	Epithelial-Mesenchymal Transition
FAMA	Federal Agricultural Marketing Authority
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration
H_2O_2	Peroxidase
HMF	5-(Hydroxymethyl) Furfural
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
IARC	International Agency for Research On Cancer
IBD	Inflammatory Bowel Disease
IC_{25}	25% Inhibitory Concentration
IC_{50}	50% Inhibitory Concentration
IC_{75}	75% Inhibitory Concentration
LDH	Lactate Dehydrogenase

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LOH	Loss Of Heterozygosity
MAAREC	Mid-Atlantic Apiculture Research & Extension Consortium
MMPs	Matrix Metalloproteinases
MSI	Microsatellite Instability
MTH	Malaysian Tualang Honey
MTT	3-(4, 5-Dimethylthiazolyl-2)-2, 5-Diphenyltetrazolium Bromide
PBS	Phosphate-Buffered Saline
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1. Background

Cancer is a condition caused by uncontrolled proliferation of cells that are influenced by various factors such as genetics, unhealthy diet, radiation, virus or bacterial infection, and tobacco smoke. In 2019, the World Health Organisation (WHO) has identified cancer as one of the leading causes of death worldwide (International Agency for Research on Cancer, 2020). As of 2020, WHO has announced colon cancer as the third most common cancer worldwide while in Malaysia, it is the second most common cancer after breast cancer with 13.5% cases from all cancer incidents.

Cancer cells are formed in multistep processes which are started with initiation, followed by promotion, progression and metastasis with the exposure to carcinogens. The initiation of colon cancer started with abnormal proliferation of colon epithelium cells before they progressed to small benign neoplasm which is known as adenoma and polyps (Cooper, 2000). Benign neoplasm will transform to malignant adenoma that have the ability to metastasis with constant exposure to carcinogens (Cooper, 2000). Metastasis is defined as the spread of cancer cells from primary tumour to other parts of the body and it is the major cause of mortality in cancer patients (Geiger & Peeper, 2009). About 50% of the colon cancer patients developed metastasis which increased the risk of mortality of the

colon cancer patients (van der Geest et al., 2015). Liver is the most common site of metastasis in colon cancer cases due to anatomical sites and portal circulation (Sheth & Clary, 2005).

Various treatments have been developed to suppress the progression of cancer cells. Chemotherapy, radiation therapy, hormone therapy, surgery and immunotherapy are common treatments provided to cancer patients based on the prognosis of the disease. However, each treatment also came with adverse effects to patients. For example, chemotherapy will not only affect the cancer cells, but the healthy cells will also be damaged (Raji, 2005). Other than that, hormone therapy may cause abnormal body functions (Khalid et al., 2018) and patients that are receiving immunotherapy also have a risk to develop autoimmune disease (Michot et al., 2016). Thus, people are searching for other alternatives to treat cancer with less adverse effects. These days, natural compounds are more preferable alternatives than chemical compounds to treat cancer as it has lower risk to develop harmful effects to patients. Cancer chemotherapeutic drugs such as pactlitaxel and vinblastine are examples of drugs extracted from natural compounds (Khalid et al., 2018).

Besides, grape, soybean, green tea, garlic, and olive are examples of natural alternatives that have been proven to be therapeutics to colon cancer (Aiello et al., 2019). Nowadays, the studies of honey on cancer development have increased, which lead to the development of new natural treatments for cancer patients (Othman, 2012). Honey is one of the natural compounds used for therapeutic purposes as it has high nutritional value and healing properties. Honey is produced

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by bees from various types of floral nectar. Types of honey are determined and named by their floral types. Colour, aroma and taste of each type of honey differ based on their flower types. Gelam honey, Pineapple honey, Kelulut honey as well as Tualang honey are the common honey types in Malaysia. In traditional medicine, honey is commonly used to soothe sore throats and treat topical wounds or infection (Abeshu & Geleta, 2016). Honey also contains flavonoid and phenolic compounds that influence the antioxidant effect (Iurlina et al., 2009). Besides, honey exhibit antibacterial, antifungal, antihypertensive, anti-inflammatory, gastroprotective, hepatoprotective, and hypoglycemic effects (Muhammad et al., 2016). In addition, the presence of hydrogen peroxide, high sugar contents and low pH level in most honeys give rise to antibacterial activities (Mandal & Mandal, 2011).

These days, Malaysian Tualang honey (MTH), which is produced by giant bee (*Apis Dorsata*) on branches of Tualang tree, has received more recognition by the locals due to its higher antioxidant properties than other local honey. In addition, MTH also has lower pH than other Malaysian honeys which indicates MTH is more acidic than other honeys (Ghazali, 2009). Hence, MTH is able to be an antibacterial agent on a wider range of harmful bacteria (Tan et al., 2009). There have been few earlier studies that found MTH is effective against several cancer cells. The anticancer properties of MTH were observed on lung adenocarcinoma cell lines as MTH inhibits cell growth and promotes apoptosis of the cells via apoptosis signalling pathway-related proteins (Amran at al., 2020). Other than that, MTH also affects breast and cervical cancer cells by inducing apoptosis of the cells through the depolarization of mitochondrial membrane. (Fauzi at al.,

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2011). The study on oral cell carcinoma and osteosarcoma also showed that the cancer cells are affected by MTH by its antiproliferative and apoptosis effect (Ghashm et al., 2010).

1.2. Problem Statement

Cancer metastasis process involves the initial activities of cancer cells such as cell proliferation and migration (Hong et al., 2012). As many colon cancer patients developed metastasis, a study to support MTH as a new therapeutic compound needs to be conducted to suppress the colon cancer cells metastasis and improve survival rate of cancer patients. Besides, current cancer treatments also cause adverse effects that impair the life quality of the patients. Hence, people are choosing natural alternatives to treat colon cancer to avoid suffering. Therefore, present study is performed to prove that MTH could be one of the effective natural alternatives to treat colon cancer.

1.3. Justification

To date, no studies have addressed the effect of MTH in the gastrointestinal tract especially on colon cancer cells although colon cancer has been announced as the third most common cancer and the second leading cause of cancer-related death worldwide. Thus, there is a need to do this study as it will provide novel insight on the anticancer effect of MTH on human colon cancer that has not been fully elucidated.

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1.4. Objectives

1.4.1. General Objectives

To investigate the antiproliferative and antimigration effects of MTH on colon cancer cells.

1.4.2. Specific Objectives

a. To identify the cytotoxic concentrations of MTH on colon cancer cells namely HCT-116 using MTT assay.
b. To determine the cell proliferation rate of colon cancer cells (HCT-116) upon induction with cytotoxic concentrations of MTH using MTT assay.
c. To determine the migration effect of MTH on colon cancer cells (HCT-116) via migration assay.

1.5. Hypothesis

The present study aims to observe the anticancer properties of MTH on colon cancer cells by suppressing the proliferation and migration of the colon cancer cells, namely HCT-116.

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CHAPTER 2

LITERATURE REVIEW

2.1 Colon Cancer

2.1.1 Introduction

Cancer is one of the main causes of death worldwide beside cardiovascular disease with approximately 10 million deaths in 2020 (International Agency for Research on Cancer, 2020). Cancer which is also known as tumors and neoplasm is a condition that affects any part of the body caused by abnormal cell proliferation. A tumor can be either benign, which is a non-invasive tumor or malignant, which is an invasive tumor that is able to spread throughout the body via circulatory or lymphatic systems (Cooper, 2000). Cancer can be caused either by internal and external factors such as physical and chemical carcinogens. Breast, lung, prostate, rectum and colon are body parts that are commonly affected by cancer.

Colon cancer has drawn the attention of many researchers as the number of incidences keeps increasing that make it the third most common type of cancer (Sung et al., 2021). Genetic mutation is the initiation of colon cancer development. This type of cancer can be caused by inherited syndromes such as Familial Adenomatous Polyposis (FAP) and Lynch Syndrome (Recio-Boiles & Cagir, 2022). At an early stage of colon cancer, proliferation of colon epithelium cells increased before they progressed to small benign neoplasm which is also known as adenoma and polyps (Cooper, 2000). With the exposure to carcinogens, benign

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adenoma gives rise to malignant adenoma that may metastasize to the whole body through blood and lymphatic vessels (Cooper, 2000). Colon cancer can be classified to three subtypes that refer to the location of the tumor which are proximal colon, distal colon and rectum (Keum & Giovannucci, 2019). Blood in stools, change in bowel habits, abdominal pain, fatigue, weight loss and anemia-related symptoms such as pale appearance and shortness of breath are some of the common symptoms of the disease that require assessment such as colonoscopy for diagnosis (Kuipers et al., 2015).

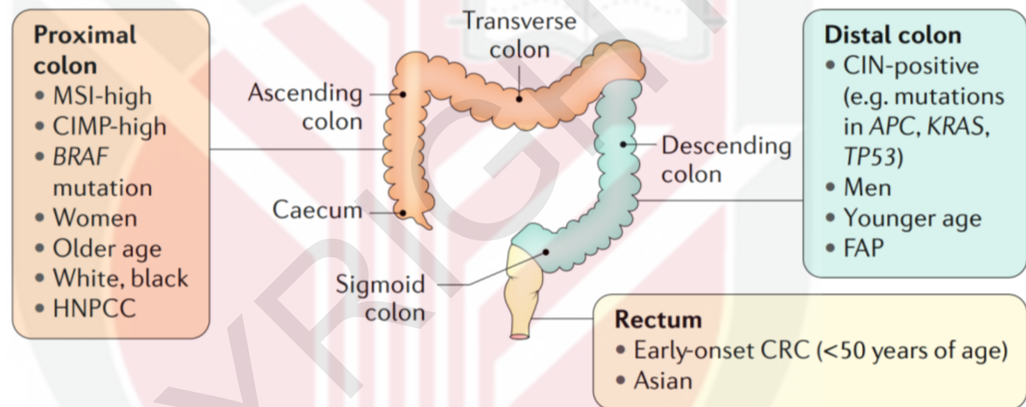

Figure 2.1 Subtypes of colon cancer and the risk factors (Keum & Giovannucci, 2019).

2.1.2 Epidemiology

Besides being known as the third most common cancer, colon cancer also comes second as the most life-threatening cancer worldwide. In 2020, there were about 1, 148, 515 new cases and 576, 858 deaths due to colon cancer (Sung et al. 2021). It is found that citizens in developed countries such as Southern Europe, Australia, New Zealand and Northern Europe have higher incidence of colon

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cancer than developing nations and men are more prone to develop colon cancer than women (Rawla et al., 2019). By age, adults aged more than 50 years old have a higher chance of developing early-onset colon cancer (Sung et al., 2021). Hence, the recommended age for cancer screening has been lowered from 50 to 45 years in 2018 by American Cancer Society (Sung et al., 2021). In addition, children and teenagers aged 20 years and below are less likely to be diagnosed with colon cancer (Ahn & Kim, 2017). In Malaysia, the highest prevalence of colon cancer cases is among people aged 60-69 years and the Chinese population has a highest rate of the cases (28.8 per 100,00) compared to Malay (11 per 100,000) and Indian population (14.3 per 100,00) (Veettil et al., 2017). As colon cancer cases are commonly diagnosed at a later stage, the risk of colon cancer death increases in Malaysia (Schliemann et al., 2020). Compared to lung cancer, hematopoietic and lymphoid malignancies, cancer mortality rate for cancers that require screening for early detection such as colon cancer decline slowly over the year since 1991 (Siegel et al., 2020).

2.1.3 Risk Factors

Many factors may contribute to colon cancer progression either they are non-modifiable factors or modifiable factors. Hereditary mutations play a big role in increasing the risk of developing cancer. About 10% of colon cancer cases are inherited syndrome which includes hereditary nonpolyposis colorectal cancer (HNPCC), adenomatous, and hamartomatous polyposis syndrome (De Rosa et al., 2015). Germline genetic mutations in any of the DNA mismatch repair genes, including MLH1, MSH2, MSH6, and PMS2, give rise to development of HNPCC,

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which is the most common hereditary colon cancer syndrome (Markowitz & Bertagnolli, 2009). Although FAP accounts for less than 1% of all colon cancer cases, it is the second-most type of hereditary colon cancer syndrome (Rawla et al., 2019). FAP is associated with the formation of hundreds to thousands of adenomas in the distal colon commencing in adolescence and caused by inherited germline APC mutations (Patel & Ahnen, 2012).

In addition, age is one of the non-modifiable factors that are associated with the disease. People over 65 years old have a higher risk of developing cancer than younger people. However, in recent years, the incidence rate of the disease for people under 50 years old has increased and that might be due to sedentary lifestyle and lack of screening (Edwards et al., 2010). Besides, having inflammatory bowel disease (IBD) will also increase the risk of colon cancer development. IBD is associated with inflammation in the colon which will lead to carcinogenesis by releasing excess growth cytokines, excess blood flow and metabolic free-radicals (Rawla et al., 2019).

Lifestyle and nutritional factors have huge influences on colon cancer incidence. Active lifestyle helps in gut motility, immune system, inflammation and metabolic hormones, thus, reducing the risk of colon cancer (Ruiz-Casado et al., 2017). According to the American Cancer Society, adults should practice moderate-intensity activity for at least 150 min, vigorous-intensity activity for 75 min or an equivalent of the two activities in a week (Kushi et al., 2012). Physical inactivity usually leads to many health problems especially obesity which will then disturb the gut microflora and irritate large intestine epithelium. Besides, excess adipose

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tissue due to obesity is expected to cause cancer progression as adipose tissue is the most inflammatory tissue (Rawla et al., 2019).

In addition, poor diet also increases the risk of colon cancer development. Consumption of red and processed meats may increase the risk of colon cancer. A study by Chan et al. (2011) found high relative risk (RR = 1.22) among red and processed meats consumers. Therefore, the International Agency for Research on Cancer (IARC) has classified processed meat as "carcinogenic". In contrast to meats, high intake of fruit, vegetables, calcium and fiber decrease the risk of colon cancer. Fiber, which is abundant in fruits, vegetables, and whole grains, can promote faster stool transit times, hence, reducing exposure to possible carcinogens (Song et al., 2015).

The intake of non-steroidal anti-inflammatory drug, aspirin, for a long period of time is believed to have a protective effect against colon cancer. A study by Pearson (2011) discovered that the use of aspirin on a daily basis was found to be inversely related to the development of colon cancer and death over time. In addition, after 3–4 years of treatment, aspirin also proved beneficial in avoiding adenoma recurrence (Cole et al., 2009). Aspirin's chemo preventive potential may be mediated in part by decreasing cyclooxygenase 2 (COX2), an enzyme that promotes tumor-promoting inflammation while suppressing T cell-mediated antitumor immunity (Zelenay et al., 2015).

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2.1.4 Stages

Staging is a process to describe the cancer's location in a person's body and to identify whether the cancer has spread to other body parts. Staging is an important step for physicians to determine the best treatment for the patients. TNM classification and staging system by American Joint Committee on Cancer (AJCC) is the preferred reference for most medical professionals to determine the cancer's stage. TNM classification system is based on characteristics of primary tumor (T), the involvement of nearby lymph nodes (N), and tumor metastasis to distant sites (M) (**Table 2.1**).

T Category	Definition of primary tumor (T) T Criteria
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ, intramucosal adenocarcinoma (involvement of lamina propria, no extension through the muscularis mucosae)
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the pericolonic tissue
T4a	Tumor penetrates to the surface of the visceral peritoneum (serosa)
T4b	Tumor invades and/or is adherent to other organs or structures
	Regional lymph node staging (N)
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	1 to 3 regional lymph nodes are positive (tumor in lymph nodes measuring ≥0.2mm), or any number of tumor deposits are present and all identifiable lymph nodes are negative
N1a	1 regional lymph node is positive
N1b	2–3 regional lymph nodes are positive
N1c	No regional lymph nodes are positive, but there are tumor deposits in subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastases
N2a	4 or more regional lymph nodes are positive
N2b	7 or more regional lymph nodes are positive
	Distant metastasis staging (M)
M0	No distant metastasis
M1a	Metastasis confined to 1 organ or site is identified without peritoneal metastasis
M1b	Metastasis confined to 2 or more organs or sites is identified without peritoneal metastasis
M1c	Metastasis to the peritoneal surface is identified alone or with other site or organ metastases

Stage	*T*	*N*	*M*
0	Tis	N0	M0
I	1–2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1-N1c	M0
	T1	N2a	M0
IIIB	T3-T4a	N1-N1c	M0
	T2-T3	N2a	M0
	T1-2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b
IVC	Any T	Any N	M1C

Table 2.1 TNM classification and AJCC 8th edition Staging of Colon Cancer (Vogel et al., 2017).

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2.1.5 Pathogenesis

Various pathways are involved in carcinogenesis of colon that cause normal colonic epithelium to transform into benign adenoma or polyps before turning into malignant tumor. Most colon tumors are developed from precancerous lesions or adenomatous polyps. Accumulation of genetic and epigenetic alterations over time lead to carcinogenesis of benign tumors to invasive malignancy (Kasi et al., 2020). Mutation of tumor suppressor gene such as APC gene, and oncogene such as KRAS gene are the genetic alterations that initiate the cancer progression (Nguyen et al., 2020). This process is known as adenoma-carcinoma sequence.

Besides, colon cancer that arises from heterogenous outgrowth such as hyperplastic polyps, sessile serrated adenomas or mixed hyperplastic polyps and serrated adenoma is usually associated with serrated polyp pathway (De Palma et al., 2019). The most common initial insult in this pathway is BRAF mutations, which are worsened by the epigenetic CpG island methylator phenotype (CIMP) (Kasi et al., 2020).

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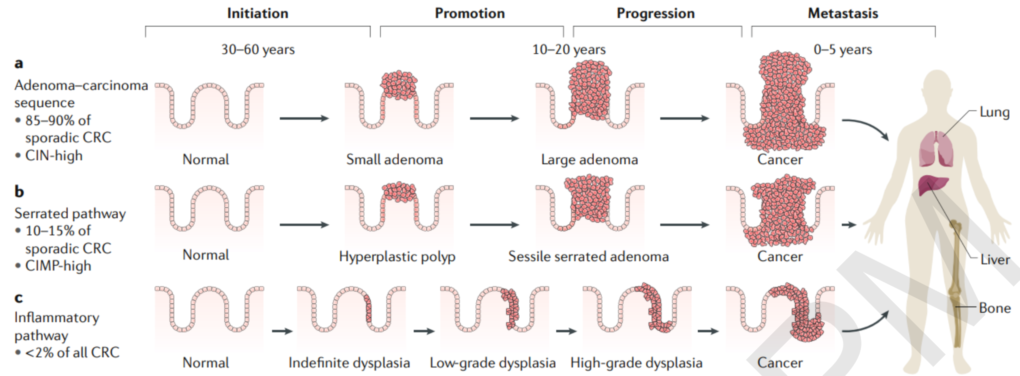

Figure 2.2 Pathogenesis of colon cancer through various pathways which are a) adenoma-carcinoma sequence, b) serrated pathway, and c) inflammatory pathway (Keum & Giovannucci, 2019).

The major molecular pathways associated in colon cancer pathogenesis are chromosome instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) hypermethylation (Currais et al., 2022). CIN is defined by chromosome modifications such as deletions, loss of aneuploidy, insertions, and amplifications, which result in somatic copy number changes. In about 70% of colon cancer cases, chromosomal instability can be seen (Walther et al., 2009). Karyotypic abnormalities such as defects in chromosomal segregation and loss of heterozygosity (LOH) at chromosome 18, deletions of tumor suppressor genes including APC and TP53 gene, and amplifications of oncogenes such as KRAS gene are associated with the chromosomal instability (Nguyen et al., 2020).

MSI, which is observed in 15% of sporadic cases of colon cancers, is defined by a high frequency of changes in genomic copy number (Currais et al., 2022). MSI occurs due to mutations of the mismatch repair genes which are MLH1, MSH2, MSH6, PMS2 or EPCAM through epigenetic silencing or constitutional mutations

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(Ryan et al., 2017). Most sporadic cases of colon cancer are associated with the silencing of MLH1 due to DNA hypermethylation (Nguyen et al., 2020).

Increased methylation of at least three loci from a panel of five gene-associated CpG islands (MINT1, MINT2, MINT31, CDKN2A, and hMLH1) is classified as CIMP hypermethylation (Grady & Markowitz, 2014). CIMP-high subtypes, which means more than 3 positive methylation markers found at the five genes, give rise to carcinoma through serrated pathway (Keum & Giovannucci, 2019). Furthermore, CIMP hypermethylation gives rise to the activation of the BRAF mutations (Jones et al., 2017).

2.1.6 Metastasis

The spread of cancerous cells from a primary tumor to other parts of the body is known as metastasis and it is the major cause of mortality in cancer patients (Geiger & Peeper, 2009). In colon cancer cases, about 50% of the patients develop distant metastasis in which 25% of them are identified in the metastatic stage at the time of diagnosis while 25% acquire metastatic spread over time (van der Geest et al., 2015). Due to anatomical sites and portal circulation, liver is the most common site of metastasis in colon cancer cases (Sheth & Clary, 2005). In a study by Holch et al. (2017), about 70% of colon cancer patients had liver metastasis and 15-25% of the patients developed pulmonary, distant lymph nodes, and peritoneal metastasis. Bones and central nervous system are the least affected organs and it usually occurred together with respiratory metastasis (Holch et al., 2017; Riihimäki et al., 2016).

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The invasion-metastasis cascade describes the process of tumor cells leaving their originating site and creating new colonies in distant tissues (Paschos, 2014). According to Cao et al., 2015, there are five steps that make up this procedure: local tumor cell invasion into surrounding matrix (Step 1), tumor cell intravasation into circulatory system (Step 2), tumor cell systemic transportation (Step 3), tumor cell extravasation into parenchyma of distant tissue sites (Step 4), colonization of distant organs, and establishment of macroscopic tumors (Step 5) (**Figure 2.3**).

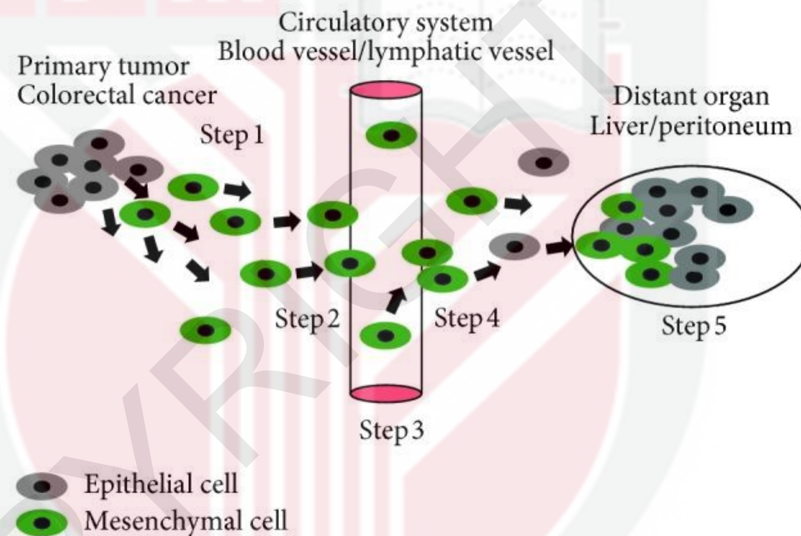

Figure 2.3 Invasion-metastasis cascade processes (Pretzsch et al., 2019).

Pretzsch et al. (2019) highlighted that the epithelial-mesenchymal transition (EMT) is the critical procedure that allows stationary epithelial cells to loss cell-cell adhesion and acquire mesenchymal features that are required for invasion and metastasis. Several pathways are involved in the activation of EMT through downregulation of E-cadherin which are abnormal activation of WNT pathway,

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PI3K/AKT pathway, and RAS/RAF/MEK/ERK/MAPK pathway and downregulation of the TGF-β/Smad pathway (Pretzsch et al., 2019).

2.1.7 Treatment

Many alternatives to colon cancer treatment have been discovered by medical professionals such as polypectomy and surgery, radiation therapy, chemotherapy, targeted therapy and immunotherapy. Methods of cancer treatment are chosen based on several parameters, including the type of tumor, the stage of the cancer, the patient's age, health, and his view on life (Mishra et al., 2013). According to Mishra et al. (2013), polypectomy and surgery are the conventional methods which are suitable to treat solid tumors that are resistant to radiation and chemotherapy. It is least favorable for older patients that have higher risk of serious post-surgical complications that require comprehensive geriatric assessment (CGA) (Kristjansson et al., 2010). Stage I disease can be cured by surgery while other stages need additional treatments to completely cure the disease (Recio-Boiles & Cagir, 2022).

Radiation therapy is used to treat a wide range of cancers and it involves the use of ionizing radiation to suppress the multiplication of malignant cells. Despite the effectiveness of this therapy to treat cancer, it also provides risk to normal tissue as it promotes cancer formation due to radiation exposure.

Other than that, chemotherapy is the main alternative used for cancer treatment and is effective to improve survival for high-risk conditions of stage II and all patients of stage III with the involvement of lymph nodes (Stintzing, 2014).

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Chemotherapy involves the use of a drug or combination of drugs to suppress the tumor cells development. Capecitabine (xeloda), fluorouracil (5-FU), irinotecan (camptosar) and oxaliplatin (eloxatin) are some drugs approved by U.S. Food and Drug Administration (FDA) and used to treat colon cancer. The combination of drugs such as 5-FU with leucovorin (folinic acid) will enhance the efficacy of the drugs to cure the cancer cells. The anticancer drugs can be administered to the body either through mouth, vein, muscle, cerebrospinal fluid, organ or body cavity and the administration method is determined based on the type and stage of the cancer (PDQ Adult Treatment Editorial Board, 2022). However, the drug's activity is not specific, hence, it will also harm normal cells by interrupting the cell division processes (Mishra et al., 2013).

The conventional alternatives may be ineffective and harmful to some patients due to high toxicity, treatment resistance or comorbidities. Therefore, scientists are discovering new approaches to improve survival rates of cancer patients. Immunotherapy is one of the new discoveries of treatment methods that use human's immune system to destroy cancer cells. This biological therapy helps strengthen the body's immune system by enhancing their capability to recognize and destroy tumors (PDQ Adult Treatment Editorial Board, 2022). For example, T-cells are induced in T-cell enhancement therapy to responses against tumor-associated antigens in the mechanism of cancer vaccine (Mishra et al., 2013).

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2.1.8 HCT-116 Human Colon Cancer Cell Line

HCT-116 cell line is a product of isolation from the colon of an adult male, colon cancer patient. The cells have epithelial morphology and growth in adherent mode. The cell lines have been used for 3D cell culture, therapeutic research, tumorigenicity studies, and high-throughput screening. HCT- 116 also has been used in various studies of colon cancer proliferation and corresponding inhibitors. For example, there is a study by Qu et al. (2018) using HCT-116 cell lines to investigate the proliferation effect of Bisphenol A on colon cancer.

2.2 Malaysian Tualang Honey

2.2.1 Introduction

Honey is a natural product that has been introduced to the human population since ancient times. Honey is produced by bees through collection of flower nectar and stored in the honeycomb. The evaporation process, caused by the design of the honeycomb and fanning of the bee's wings, turns the nectar into liquid honey. The nectar collected by bees will determine the color and flavor of the honey. Hence, there are about 300 various types of honey including clover honey, acacia honey, manuka honey and buckwheat honey. In Malaysia, kelulut honey, acacia honey, and tualang honey are the common honey consumed by the population.

Malaysian Tualang honey (MTH) is Malaysian multifloral jungle honey that is produced by the giant bee (*Apis dorsata*). The bees build up their hives hanging on the branches of the Tualang tree (*Kompassia excelsa*) which is commonly found

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in tropical rainforests (Erejuwa et al., 2010). The trees are abundant in Malaysia's north-eastern state of Kedah. Besides, the tree also can be found in Sumatra, Borneo, and South Thailand (Othman et al., 2015). A tree of Tualang honey can have about 100 nests with 30 000 bees and can produce up to 450 kg of honey (Ahmed & Othman, 2017).

Honey is often consumed as a natural sweetener and energy food. It is also a good ingredient in breads, cakes, cookies, and candies as the moisture absorbing quality of honey helps the food to stay fresh longer (Mid-Atlantic Apiculture Research & Extension Consortium (MAAREC), 2004). Besides, honey has been used as traditional medicine for decades as it can treat various conditions such as infertility, respiratory and gastrointestinal symptoms (Hassan & Abdul Karim, 2019). Honey is also used to soothe sore throats and treat topical wounds or infection (Abeshu & Geleta, 2016). In addition, various studies have been conducted to prove that MTH can be an alternative to treat cancer such as breast cancer, lung cancer, and oral cancer (Ahmed & Othman, 2017; Amran et al., 2020; Ghashm et al., 2010).

2.2.2 Chemical Constituents

MTH appears in dark brown and has a pH of 3.55-4.00 which is more acidic than other Malaysian honeys such as Gelam, Kelulut Hitam and Kelulut Putih (Ghazali, 2009). Because of this property, MTH is effective against a wide range of harmful bacteria (Tan et al., 2009). The primary contents of honey are fructose,

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glucose and water which are about 38%, 31% and 17% (Abeshu & Geleta, 2016). **Table 2.2** shows the summary of physical properties of MTH.

Physiochemical properties	Tualang Honey
Appearance	Dark brown
Specific gravity	1.335
pH	3.55–4.00
Moisture content	23.30%
Total Reducing sugars	67.50%
Fructose	29.60%
Glucose	30.00%
Sucrose	0.60%
Maltose	7.90%
Potassium	0.51%
Calcium	0.18%
Magnesium	0.11%
Sodium	0.26%
Carbon	41.58%
Oxygen	57.67%

Table 2.2 Physicochemical properties of MTH (Ahmed & Othman, 2017).

Besides, honey is made up of around 180 different compounds, including amino acids, vitamins, minerals, and enzymes (Pérez et al., 2002). In MTH, contents of phenolic acids and flavonoids are higher than other local Malaysian honeys (Kishore et al., 2011). The phenolic acids found in MTH are gallic, syringic, benzoic, transcinnamic, p-coumaric, and caffeic acids while the flavonoid compounds of MTH are catechin, kaempferol, naringenin, luteolin and apigenin (Mohamed & Hamad Alfarisi, 2017). Phenolic contents and color intensity are associated with the antioxidant capacity of honey (Mohd Kamal et al., 2021).

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Hence, MTH is concluded to have higher antioxidant activity than other local Malaysian honey as it has a darker-colored appearance and higher phenolic content. Other than that, stearic acids, 2-cyclopentene-1, 4,-dione, 2[3H]-furanone or dihydro-butyrolactone, gamma-crotonolactone or 2[5H]- furanone, 2-hydroxy-2-cyclopenten-1-one, hyacinthin, 2, 4- dihydroxy-2, 5-dimethyl-3[2H]-furan-3-one, and phenylethanol are some compounds present specifically in MTH (Ahmed & Othman, 2017). **Table 2.3** shows the summary of chemical constituents of MTH.

Biochemical compounds	
5-(hydroxymethyl)-furfural (HMF)	2-hydroxy-1-[hydroxymethyl] ethyl ester
Furfural	Dihydro-butyrolactone
2-furyl methyl ketone	2[5H]-furanone
5-methyl furfural	Benzoic Acid
Acetic acid	Gallic Acid
Phenylethane	Syringic Acid
2-hydroxy-2-cyclopenten-1-one	P-Coumaric Acid
2 furan carboxaldehyde	Hyacinthine
Furfural alcohol	Trans-cinnamic acid
2-cyclopentene-1,4, -dione	Caffeic acid
2[3H]-furanone	Kaempferol
Gamma-croton lactone	Naringenin
Palmitic acid	Luteolin
Ethyl oleate	Linoleic acid
2,4-dihydroxy-2,5-dimethyl-3[2H]-furan-3 one	Octadecanoic acid
Oleic acid	

Table 2.3 Chemical compounds in MTH (Ahmed & Othman, 2017).

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2.2.3 Properties of Malaysia Tualang Honey

Since recently, various studies have proved that MTH is a beneficial natural compound in providing therapeutic effects to humans. Hence, in this section, we will discuss more on the medicinal properties of MTH. Anti-bacterial, anti-oxidant, wound healing, anti-neoplastic and anti-proliferative, and anti-inflammatory are some of the medicinal properties commonly discussed in the articles.

Due to the high osmolarity, acidity, and peroxidase (H_2O_2) and non-peroxidase components, honey becomes one of the natural compounds that exhibit antibacterial activity (Albaridi, 2019). MTH is claimed to have bacteriostatic and bactericidal effect on wide range of bacteria strains either gram positive such as *Staphylococcus aureus and Staphylococcus epidermidis*, or negative gram such as *Escherichia coli, Pseudomonas aeruginosa and S. enterica Serovar Typhimurium* (Shehu et al., 2015). Compare to other Malaysian honeys such as Manuka honey, MTH is more effective against certain gram-negative bacteria strains as MTH has higher contents of non- peroxidase contents which are phenolics, flavonoids, and 5-(hydroxymethyl) furfural (HMF) (Ahmed & Othman, 2017).

Besides, honey is commonly associated with antioxidant effects. By scavenging free radicals from body cells and minimizing the damage produced by oxidative stress, antioxidants assist to lessen the risk of numerous chronic diseases such as heart disease and cancer. The presence of flavonoids, phenolic acids, amino acids, and protein contribute to antioxidant properties in honey (Kishore et al., 2011). As

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MTH appears darker than other Malaysian honey, has high ferric reducing power values and high concentration of phenolic acids and flavonoids, it can be concluded that MTH has the highest antioxidant properties among the other honeys (Moniruzzaman et al., 2013).

Nowadays, MTH has been widely known to have anticancer potential. There have been several *in vitro* studies that investigate the chemo preventive capabilities of MTH on various cancer cells including cervical cancer cell lines, and breast cancer cell lines (Fauzi et al., 2011). The study by Fauzi et al. (2011) shows that antitumor properties of MTH is caused by the depolarisation of mitochondrial membrane that leads to the apoptosis of cancer cells. Other than that, the presence of specific compounds such as flavonoids also contribute to the antiproliferative effect of MTH by inhibiting the COX2 (Ahmed & Othman, 2017). In addition, in an animal study by Al-Koshab et al. (2020) found MTH inhibit the progression of oral squamous carcinoma by suppressing the expression of TWIST1 and RAC1 genes that control EMT, and overexpressing β-catenin and E-cadherin.

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CHAPTER 3

METHODOLOGY

3.1. Materials

These materials were provided by Anatomy and Histology Laboratory 1 in the Faculty of Medicine and Health Science, UPM.

No.	Materials	Supplier
1	HCT-116 (ATCC CCL-247)	American Type Culture Collection (ATCC®) Manassas, Virginia, USA
2	McCoy's 5A Medium	Sigma-Aldrich® St Louis, Missouri, USA
3	Fetal Bovine Serum (FBS)	Capricorn Scientific GmbH Ebsdorfergrund, Germany
4	Trypsin-EDTA 10X	Biosera Nuaille, France
5	Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich® St Louis, Missouri, USA
6	Phosphate Buffered Saline Tablet	Beijing Solarbio Science & Technology Co., Ltd.

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		Beijing, China
7	Trypan Blue Solution	Sigma-Aldrich® St Louis, Missouri, USA
8	Thiazolyl Blue Tetrazolium Blue (MTT reagent)	EMD Millipore Corporation Billerica, Massachusetts, USA
9	Malaysian Tualang Honey	Federal Agricultural Marketing Authority (FAMA)
10	Tissue Culture Flask	SPL Life Sciences Co., Ltd. Gyeonggi-do, Korea
11	Multi-well Plates	SPL Life Sciences Co., Ltd. Gyeonggi-do, Korea
12	Serological Pipette 5 mL & 10 mL	Kirgen® Shanghai
13	Galaxy® 170 R CO_2 Incubator	Eppendorf® AG Hamburg, Germany
14	Benchtop Centrifuge	Andreas Hettich GmbH & Co. KG Tuttlingen, Germany

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15	Class II Biological Safety Cabinet	BioAir^{TM} Italy
16	CKX41 Inverted Microscope	Olympus Corporation Tokyo, Japan
17	Water bath	Memmert GmbH + Co. KG Schwabach, Germany
18	VersaMax Microplate Reader	Molecular Devices California, USA

Table 3.1 Materials, reagents, and equipment used for the study and their sources.

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3.2 Methods

3.2.1 Preparation of culture media

HCT-116 cells were cultured in Dulbecoo's modified Eagle's medium (DMEM) which was completed with 10% FBS. The preparation of the complete media was performed in biosafety cabinet (BSC) class II. The complete media was stored at 4°C.

3.2.2 Preparation of trypsinizing solution

The 10× stock solution of trypsin-EDTA was diluted to 2.5× with PBS to prepare the optimum trypsin solution for HCT-116 cells detachment.

3.2.3 Preparation of phosphate-buffered saline (PBS)

PBS was prepared by dissolving 1 tablet of PBS in 100 mL of distilled water. The solution was autoclaved, filtered and kept at 4°C to keep sterility. PBS solution was used as washing solution, preparation of MTT reagent and preparation of trypsinizing solution.

3.2.4 Cell thawing

To start culturing the cells, cryopreserved cells in cryovial need to be defrosted in a process called cell thawing. The process requires fast handling as DMSO used to cryopreserve the cells is toxic to the cells in room temperature and can cause cell death. 5 mL pre-warmed complete medium was prepared in a T25 culture flask before taking out the cryovial from -80°C freezer. After taking out the cryovial, thawed the vial in 37°C water bath for about 2 minutes or until the

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cell suspension turned into liquid form. To avoid any contamination, cap was kept out of the water. As soon as the vial contents turned into liquid form, cells were transferred to T25 culture flask that contain complete medium in BSC class II. Cells were observed under microscope and incubated the culture in incubator with temperature of 37ºC and air atmosphere at 5% CO_2.

3.2.5 Cell culture

HCT-116 human colon cancer cells were cultured in a T25 culture flask that contained medium completed with 10% FBS. Cells were maintained in the incubator that provided a humidified atmosphere with 5% CO_2 at 37ºC. Cell's morphology and confluency was observed under inverted microscope daily until the cells reached the 80% confluency. Medium was changed for every two days or when the colour of the medium changed to orange or yellow. All cell culture works were performed in BSC class II to provide a sterile environment for the cells.

3.2.6 Cell subculture

Once the cells in the T25 culture flask reach 70% -80% confluency, passaging cells or also known as subculturing need to be done to maintain the growth of cells. In this process, some or all previous culture from the flask were transferred to fresh complete medium. Complete medium, PBS, and 2.5x trypsin-EDTA were pre-warmed in the water bath at 37ºC. Old culture medium was removed and discarded carefully. The cells were washed with 1 mL of PBS to ensure all traces of serum that act as trypsin inhibitor were removed. After swirling the flask for a while, PBS was discarded. 1 mL of trypsin-EDTA solution was

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added to flask and cells were observed under inverted microscope until cell layer is detached. If the cells were difficult to detach, flask was placed in the 37ºC incubator for a few minutes. 2 mL of complete medium was added and flushed to the flask wall to ensure cells were detached from the flask. The cell suspension was aspirated by gentle pipetting and transferred to a 15 mL centrifuge tube before centrifuged at 2000 RPM for 10 minutes under 4ºC. After removing the supernatant, 2 mL complete medium was added and resuspended to ensure the cells become a single cell. 5 mL of complete medium was added to a T25 culture flask and 500 µL of cell suspension was added to the flask. Flask was swirled to ensure the cell distribution was uniform. Cells were observed under microscope before incubating the cells in the 37ºC incubator with 5% CO_2.

3.2.7 Cryopreservation of cells

Cell cryopreservation is a process of preserving the cell in a very low temperature. 2.5x trypsin-EDTA solution and FBS were pre-warmed prior to the process. Cells were detached from the flask and centrifuged with the same procedure mentioned in section 3.2.6. After discarding the supernatant, 900 µL of FBS was added into the pellet and resuspended. Cell suspension was transferred into cryovial that was labelled with the type of cell line, passage number, and date. 100 µL of DMSO was added slowly around the wall of the vial. Vial was sealed with parafilm and immediately stored in a -20ºC freezer for 4 hours. After that, cryovial was transferred to -80ºC freezer.

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3.2.8 Cell counting

Cell counting is a process of measuring the total number of live cells in the cell population. Hemocytometer is one of the equipment used to count the live cells accurately. Glass hemocytometer and coverslip were cleaned with 70% alcohol before use. Coverslip was moistened with water and affixed to the hemocytometer. Cells were harvested from the flask and centrifuged to get the pellet under the same procedure mentioned in section 3.2.6. Supernatant was discarded before 1 mL of media was added into the pellet and resuspended to break up clumps. Trypan blue exclusion test was performed to calculate the cell viability. Serial dilution was done by mixing 10 µL of o.4% trypan blue and 10 µL of cell suspension. The cell mixture was loaded between the cleaned hemocytometer and coverslip. Hemocytometer was viewed under an inverted microscope to count the number of viable cells (bright cells) and non-viable cells (stained blue). The total of cell viability was calculated by using the formula as below:

$$Total\ of\ cell\ viability\ = \left(\frac{Number\ of\ viable\ cells}{4}\right) \times\ 10^4 \times Dilution\ factor$$

Where,

Dilution factor = 2

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3.2.9 Experimental Design

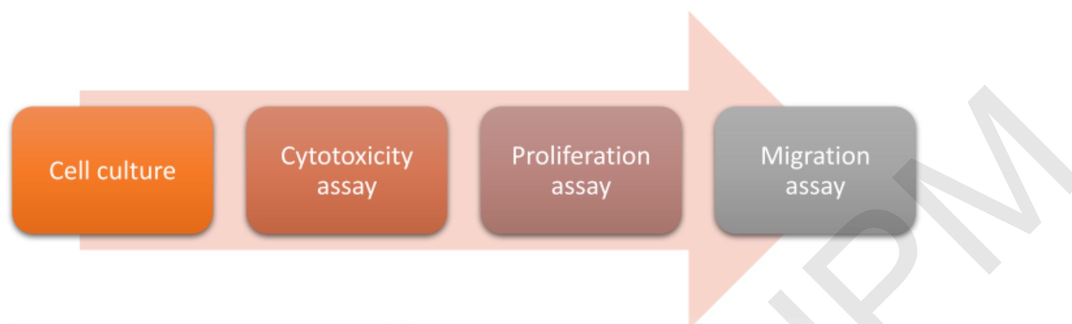

Figure 3.1 Experimental design of present study.

3.2.10 Cytotoxicity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine the cytotoxic concentration of MTH on HCT-116 (Riss et al., 2016). Number of viable cells were observed as the cells will convert the MTT substrate into purple coloured formazan product (Riss et al., 2016). HCT-116 was seeded in 96-well microplates at a density of 1 x10^5 cells/ well and incubated overnight at 37°C in 5% CO_2 condition. The medium from each well was discarded after overnight incubation. Then, the cells were treated with MTH that is diluted with complete medium at various concentrations (0%, 0.625%, 1.25%, 2.5%, 5% & 10%) for 24 hours (Amran et al., 2020). After 24 hours of incubation, 50 µL of 2 mg/mL MTT solution was added in each well. MTT solution was dissolved in PBS and filtered to keep the solution sterile. The solution needed to be protected from light and stored at 4°C (Mazloum-Ardakani et al., 2019). After 4 hours of incubation, the media containing MTT solution was removed and replaced with 100 µL of DMSO in each well to dissolve the purple

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formazan crystals. The absorbance of each well was read at a wavelength of 570 nm using microplate reader. The absorbance readings were normalised and the cell viability percentage was calculated using formula:

$$Percentage\ of\ cell\ viability\ = \frac{The\ mean\ of\ sample\ absorbance}{The\ mean\ of\ control\ absorbance} \times 100\%$$

The 50% inhibitory concentration (IC_{50}), 25% inhibitory concentration (IC_{25}) and 75% inhibitory concentration (IC_{75}) of MTH on HCT-116 was measured using GraphPad Prism 9. The IC_{25}, IC_{50}, and IC_{75} were used for subsequent experiments. Five replications of three independent experiments of MTT assay were performed.

3.2.10 Proliferation assay

To determine antiproliferative effects of MTH on HCT-116, MTT assay was performed. The cells were seeded in a 96-well plate at 1 x10^5 cells/ well and incubated overnight at 37°C in 5% CO_2 condition. After overnight incubation, medium was discarded from each well before being treated with IC_{25}, IC_{50} and IC_{75} of MTH for 72 hours. There is also a negative control group in which the cells remain untreated. After 72 hours of incubation, 50 µL of 2 mg/mL MTT solution was added in each well. After 4 hours of incubation, the solution of media and MTT were discarded and 100µL of DMSO was added in each well to dissolve the purple formazan crystals. For proliferation assay, the baseline control group was prepared to justify the proliferation activity of the cells. The cells were seeded and incubated for 4 hours before MTT was added. Then, the cells were incubated for

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another 4 hours. Similar to the previous protocol, the solution of media and MTT were discarded and DMSO was added. The absorbance was read using microplate reader at a wavelength of 570 nm.

3.2.11 Migration assay

Migration assay also known as wound healing assay is a common method to study cell migration. To study the anti-migratory effect of MTH, artificial gap or scratch was made on confluent cell monolayer and the distance between the edges of cells was observed (Liang et al., 2007). HCT-116 cells were seeded in 24 well plates at the concentration of 5×10^5 cells/ well and incubated until 80-90% of confluency was achieved. To reduce the proliferation effect of HCT-116, serum-free medium was used. When the cell was confluence, the medium was removed and 500 µL of PBS was added in each well. The cell monolayer was scratched using 200 µL pipette tips vertically (Liang et al., 2007). The plate was swirled a few times before PBS was removed to completely discard the detached cells and any debris. The cells were treated with suitable concentrations of MTH gained from previous assay (1%, 2%, 4%) and a group control that remained untreated. The scratched cells were observed under an inverted microscope with a digital camera and the distance between the edges of cells were measured by using Motic Live Imaging Module. The wound closure was observed at different time intervals which is 0 hour up to 48 hours. Experiment was carried out in triplicates (n=3).

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3.2.12 Statistical Analysis

GraphPad Prism 9 was used to analyse the data statistically. Data of three independent experiments were presented in means ± standard deviation. One- Way of Analysis (ANOVA) and post-hoc comparison, specifically Dunnett's test were used to determine significant data ($p < 0.05$).

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CHAPTER 4

RESULTS

4.1. Cytotoxic Effect of Malaysian Tualang Honey on HCT-116 Cells

Cytotoxicity assay was done to evaluate and identify the non-cytotoxic concentration of Malaysia Tualang Honey (MTH) on HCT-116 cells. HCT-116 cells were treated with various concentrations (0.625%, 1.25%, 2.5%, 5%, and 10%) of MTH for 24 hours. **Figure 4.1** shows the percentage of cell viability of HCT-116 cells treated with various concentrations of MTH. The dose-response curve concludes that viability of HCT-116 cells decreases as the concentration of MTH increases. These results demonstrate that induction of MTH on HCT-116 cells showed cytotoxic effects in a dose-dependent manner. By using the dose-response curve, three inhibitory concentrations of MTH on HCT-116 cells were measured. As shown in **Figure 4.1** and **Table 4.1**, the IC_{25}, IC_{50} and IC_{75} for MTH on HCT-116 cells were 0.9884%, 1.928% and 3.761%, respectively.

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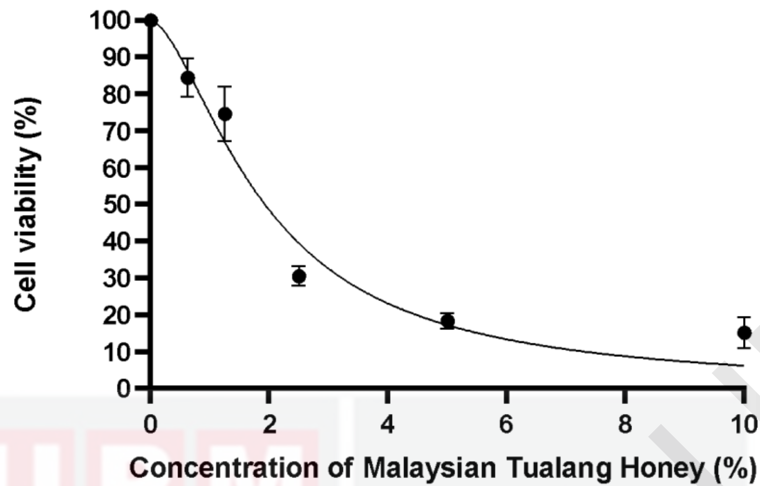

Figure 4.1 The dose response curve on the percentage of cell viability of HCT-116 cells after being treated with 0%, 0.625%, 1.25%, 2.5%, 5%, and 10% of MTH for 24 hours.

Cell line	**Inhibitory concentration of MTH (%)**		
	IC_{25}	**IC_{50}**	**IC_{75}**
HCT-116	0.9884	1.928	3.761

Table 4.1 Inhibitory concentration of MTH on human colon cancer cell line (HCT-116).

4.2. Anti-proliferative Effect of Malaysian Tualang Honey on HCT-116 Cells

MTT assay was performed to evaluate the anti-proliferative effect of MTH against HCT-116 cells. HCT-116 cells were treated with IC_{25}, IC_{50} and IC_{75} of MTH for 72 hours. It was observed that MTH had significantly inhibits the proliferation of HCT-116 cells ($p < 0.05$) in a dose-dependent manner. Baseline control group was included in the

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experimental design to prove the proliferation of HCT-116 cells after 72 hours of incubation period.

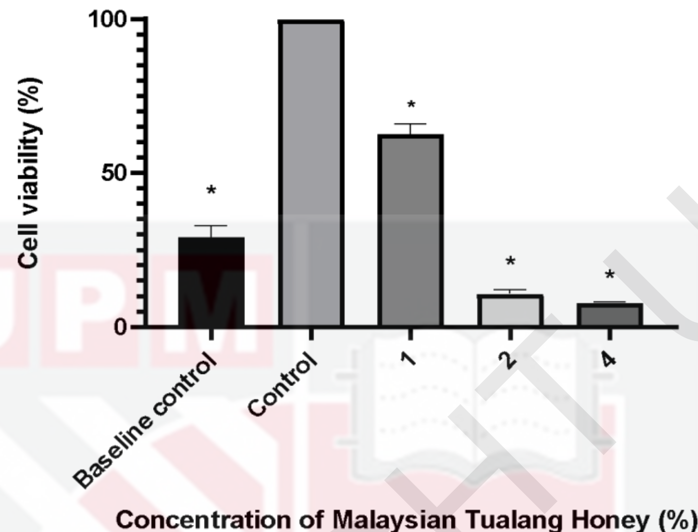

Figure 4.2 Percentage of cell viability of HCT-116 cells after treatment with IC_{25}, IC_{50}, and IC_{75} (1%, 2% and 4%) of MTH for 72 hours. The data expressed as mean values ± standard deviation at three independent experiments performed in triplicates. Results were analysed using one-way ANOVA and followed by Dunnett's multiple comparisons test. The significant mean difference of p-value less than 0.05 is expressed as *, compared to the control group.

4.3. Anti-migratory Effect of Malaysian Tualang Honey on HCT-116 Cells

Scratch assay was performed to evaluate and assess the migration rate of HCT-116 cells towards Malaysian Tualang Honey (MTH). Confluent HCT-116 cells monolayer was scratched and exposed to various concentrations of MTH (1%, 2%, and 4%) and observed for 0, 24 and 48 hours.

The migration activity was observed through a microscope and the scratch width were measured using an image analyzer. Based on microscopic observation on **Figure**

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4.3, the scratch wounds were almost the same size in each experimental group at 0 hour; however, the cell migration rate was higher in the control group MTH-treated group after 24 and 72 hours. Compared with the control group, the migration rate was slowest in 2% MTH group followed by 1% MTH group after 24 hours. However, the cells started to detach from the surface after 48 hours of exposure to 2% MTH group. No migratory activity can be observed in 4% MTH group as the cells are detaching from the surface and die after 24 hours of exposure to MTH.

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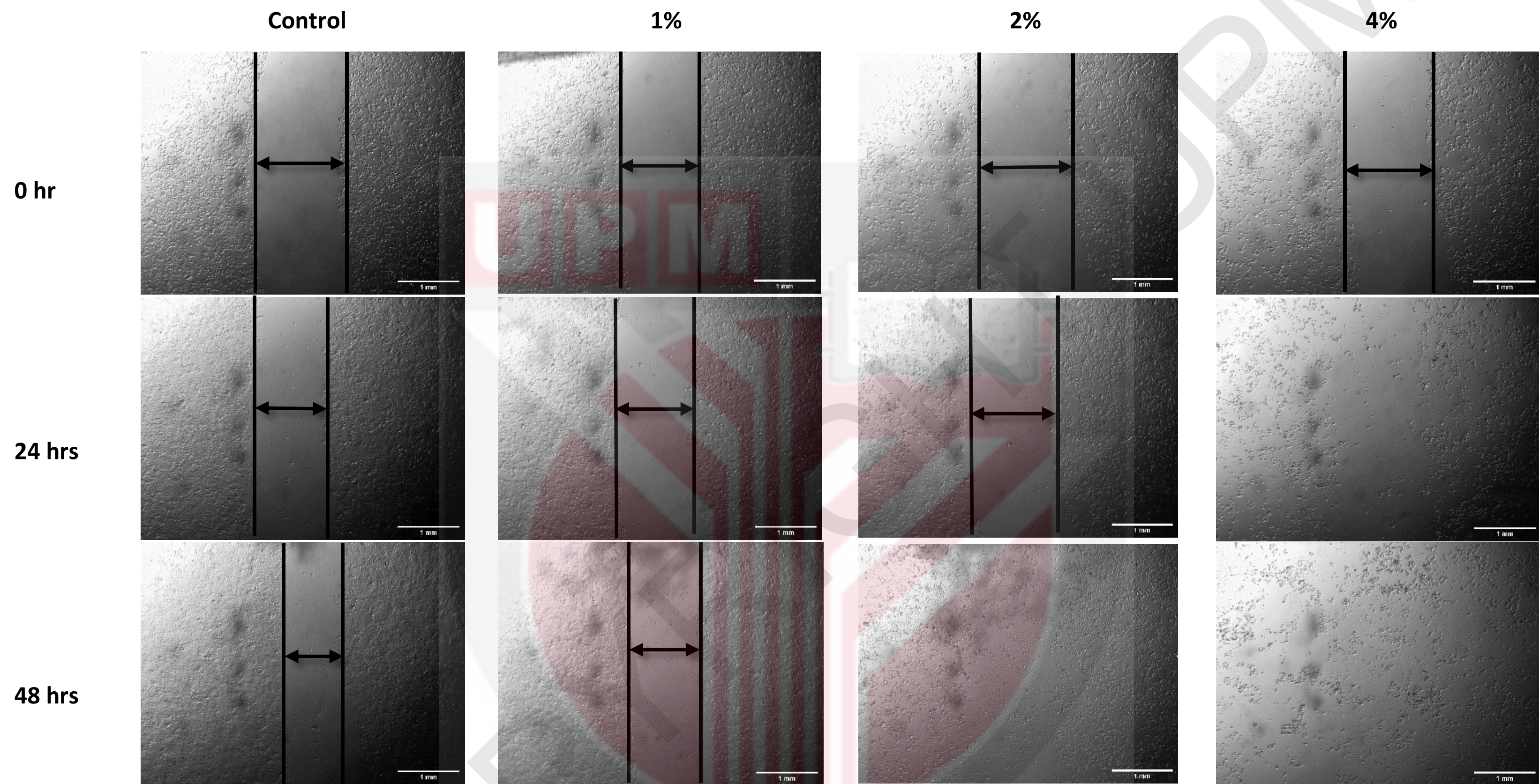

Figure 4.3 Microscopic view of migratory activity of HCT-116 cells treated with MTH for 0, 24 and 48 hours (40X magnification).

The scratch width was transformed to the percentage of initial distance between two edges to evaluate the migration rate. **Figure 4.4** shows the percentage of migration rate of HCT-116 cells treated with 1%, 2% and 4% of MTH for 24 and 48 hours. Compared to the control group, migration rate of MTH-treated HCT-116 cells significantly decreased ($p<0.05$) in a concentration-dependent manner in both 24-hour and 48-hour post treatment of MTH. Although there is a reduction in percentage of migration rate towards the MTH exposure, the migration rate cannot be measured for 48-hour post treatment of MTH (4% and 2%) and 24-hour post treatment of MTH (2%) as the cell started to detach from the surface and died (**Table 4.2**).

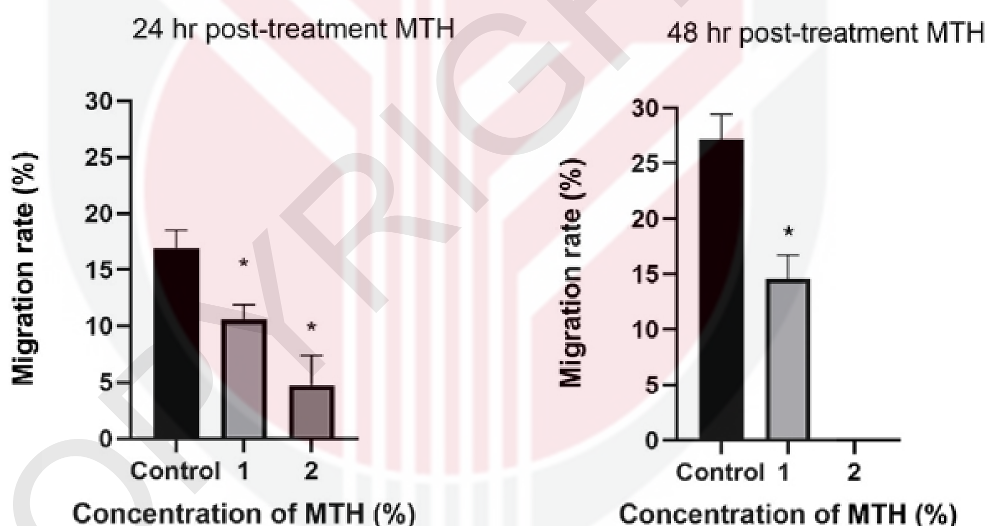

Figure 4.4 Percentage of migration rate of HCT-116 cells after exposure to various concentrations of MTH (1%, 2% and 4%) for 24 and 48 hours. The data expressed as mean values ± standard deviation at three independent experiments performed in triplicates. Results were analysed using one-way ANOVA and Dunnett's multiple comparisons test. The significant mean difference of p-value less than 0.05 is expressed as *, compared to the control group.

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MTH Concentration	Percentage of migration rate of HCT-116 cells	
	24 hours	**48 hours**
Control	16.92%	27.17%
1%	10.62%	14.6%
2%	4.76%	Cell death
4%	Cell death	Cell death

Table 4.2 Percentage of migration rate of HCT-116 cells treated with MTH for 24 and 48 hours.

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CHAPTER 5

DISCUSSION

The present study was conducted to determine the anti-cancer properties of Malaysian Tualang Honey (MTH) on HCT-116 human colon cancer cell line. Numerous studies on the anti-cancer effect of cancer cells upon treatment with MTH have been demonstrated due to its higher antioxidant properties than other local honeys which suggests the use of MTH as an alternative for cancer treatment (Mohd Kamal et al., 2021). However, the anti-carcinogenic properties of MTH on colon cancer cell lines has not been fully elucidated. To achieve the objective of the study, the cytotoxic, proliferation, and migration effects of human colon cancer cells (HCT-116) with induction of various concentrations of MTH ranging from 0.625% to 10% were evaluated. HCT-116 cell line was selected for this study over other colon cancer cell lines is because colon cancer studies commonly use the HCT-116 cell line (Zhou et al., 2017). Other than that, HCT-116 cell line is also a suitable *in vitro* model that mimics colon cancer.

Cytotoxic study was conducted as a preliminary study to identify the non-cytotoxic concentration of MTH towards HCT-116 cells before proceeding to proliferation and migration assay. To evaluate the cytotoxic effects of MTH on HCT-116 cells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed. MTT assay allows the quantification of cell viability by measuring the purple-coloured formazan product that is converted from MTT by active viable cells (Riss et al., 2016). Concentration of MTT, length of incubation period, number of viable cells, and their metabolic activity are some of the

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factors that might influence the cell viability quantification. Present study used MTT assay instead of other cell enumeration assays such as trypan blue and lactate dehydrogenase (LDH) assay because MTT assay, which is developed by Mosmann in 1980, is known as gold standard for assessing the cell viability and proliferation (van Tonder et al., 2015).

To investigate the cytotoxicity of MTH against HCT-116 cells, the cells were treated with five various concentrations (0.625%, 1.25%, 2.5%, 5% & 10%) of MTH and observed for 24 hours. This study was focused on one time point due to time restriction to observe and complete all time points. The concentrations of MTH used in this study were obtained from previous study of MTH cytotoxicity on H23 and A549 human lung adenocarcinoma cells (Amran et al., 2020). The current finding shows that MTH exerted cytotoxic effects on human colon cancer cells (HCT-116) when the treatment concentrations increased. The half inhibitory concentration (IC_{50}) is the concentration of an inhibitor where the response is reduced by half. In MTT assay, the dose of cytotoxic compound at which 50% viability was achieved is called IC_{50}. The IC_{50} values of MTH were determined in this study by interpolation from dose-response curves. This study showed that IC_{50} for MTH on HCT-116 cells were 1.928%. The result of this study is consistent to the finding of few studies on MTH, in which, half of the cell growth of H23 and A549 lung adenocarcinoma cell were inhibited upon treatment of MTH at concentration less than 3.6% in both cell line (Amran et al., 2020). Furthermore, previous study by Fauzi et al., (2011) also shows low IC_{50} value of MTH on MCF-7 cells which is 2.4%. However, the cytotoxic effect of MTH on normal colon cancer cells is not demonstrated in the present study. It is predicted that MTH will not affect normal colon cancer cells as honey is non-cytotoxic to normal cells (Erejuwa et al., 2014).

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Jaganathan and Mandal (2009a) explained the cytotoxic effect of honey on colon cancer was influenced by the level of phenolic content. As MTH has higher phenolic and antioxidant levels than other local honeys, it proves that it gives a higher cytotoxic effect on cells (Zae et al., 2020). Thus, it may be the reason of MTH at even a low concentration could exhibit cytotoxic effect HCT-116 cells in this study. The finding of this study was also supported by a study by Chang et al., (2008) that reported one of the compositions of honey, flavonoids which is also high in MTH could promote cell death on various cancer cell lines including colon cancer cells.

The antiproliferative effect of MTH on HCT-116 was assessed by also performing MTT assay. The assay was performed to investigate the effect of longer exposure of treatment on HCT-116 proliferation. Based on **Figure 4.2**, we can conclude MTH exerted anti-proliferative effects on HCT-116 in a dose-dependent manner. There is a previous study of MTH on leukemia cell lines (K562 and MV4-11) that produced similar results as the present study, in which the cell proliferation was inhibited by MTH (Nik Man et al., 2015). In addition, previous study revealed that phenolic content in MTH has been proved to cause the anti-proliferative effects after excluding the effects of hydrogen peroxide and sugars on oral squamous and osteosarcoma cell lines (Ghashm et al., 2010). Other than that, the anti-proliferative effect of MTH on HCT-116 can also be further explained by Jaganathan and Mandal (2009b) that concluded honey can cause reduction of intracellular non-protein thiols that leads to the promotion of apoptosis in cells.

Further investigation was performed to assess the migration effect of MTH on HCT-116 cells. Migration assay which is also known as scratch assay or wound closure assay was the chosen method to study the cell migration as it is the simplest method

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and has ability to evaluate the migratory capacity of whole cell masses (Justus et al., 2014). Cell migration is important in tumor cell invasion and metastasis. Hence, it is important to evaluate the effect of MTH on cancer cell migration as it could be a potential therapeutic target for anti-cancer therapy. Interestingly, the finding of this investigation revealed that MTH could inhibit the cell migration of HCT-116 cells in a concentration-dependent manner. The graph in **Figure 4.4** was plotted based on the percentage of migration rate cells that was calculated by comparing the initial wound width and the wound width on the observation time. The graph shows the percentage of migration rate was higher at lower concentration of MTH in both 12- and 24-post treatment as compared to the higher concentration of MTH. The control group showed the highest migration rate compared to the MTH- treated cells which suggests MTH could inhibit the cell migration. In addition, the result of the present study is consistent with previous migration study on HCT-116 cells using Manuka honey, in which the migratory activity of HCT-116 cells is inhibited by Manuka honey (Afrin et al., 2018).

Despite the overwhelming findings of MTH on the inhibition of HCT-116 cell proliferation and migration, further understanding on the mechanism of actions of anti-metastatic effect of MTH on colon cancer cells needs to be addressed in the future study. Cancer invasion and metastasis involved the increased production of matrix-degrading enzymes such matrix metalloproteinases (MMPs) as part of the angiogenic event (Lv et al., 2018). Thus, we predict the MMPs, one of the proteases expressed at the cell's leading edge where metastasizing cells proliferate may be inhibited by MTH and thus it may be a potential mode of action since they make the extracellular matrix to be more easily degraded (Friedl & Wolf, 2003). Furthermore, previous study by Abdul Khalid et al. (2022) proved the presence of MTH could cause a clear inhibition of endothelial cell-derived MMP-2. Therefore, deeper understanding on the underlying

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mechanism of anti-metastatic effect of MTH could be investigated in future study by quantifying the MMPs production in human colon cancer cells.

CHAPTER 6

SUMMARY, CONCLUSION & FUTURE RECOMMENDATIONS

6.1. Summary

Mortality in cancer patients is mainly caused by metastasis activity of cancer cells. Metastasis involved the proliferation and migration of cancer cells from primary tumor to another organs. In colon cancer cases, most of the patients develop metastasis in liver due to its anatomical location and portal circulation. Most of the current treatments are highly effective to kill the cancer cells and prevent relapse. However, the treatments also affect healthy normal cells which cause side effects to the patients and impairs patients' life quality. Hence, patients are searching for natural alternatives such as Malaysian Tualang honey (MTH) to treat their condition with least adverse effect. Despite the effectiveness of MTH on other cancer cells such as breast and cervical cancer, the effect of MTH on gastrointestinal tract especially colon cancer is still unknown. Thus, this study was carried out to determine the antiproliferative and antimigration effect of MTH on HCT-116 cells. MTH was found to be cytotoxic to HCT-116 cells as it is able to kill 50% population of the cells at low concentration (1.928%). Other than that, the cells proliferation was inhibited by 1%, 2% and 4% of MTH with significant difference compared to control group. Additionally, it has been demonstrated that MTH significantly suppressed the migratory activity of HCT-116 cells.

6.2. Conclusion

In short, we found that MTH can be use as anticancer agents as it is capable to inhibit the proliferation and migration of colon cancer cells, mainly HCT-116 cells. Based on previous study, the antiproliferative effect of MTH involved the apoptosis of

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the cells. However, the mechanism of antimigration effect of MTH on cancer cells were not fully explored. Hence, further investigations are needed to determine the mechanism involved in the antimigration effect of MTH.

6.3. Future Recommendations

This study has shown that MTH is cytotoxic to HCT-116 human colon carcinoma cells. However, we only conducted the cytotoxicity assay in one time point, which 24 hours due to time constraint to complete the study. Hence, cytotoxicity assay needs to be done in 24, 48 and 72 hours in future study to produce more consistency in the results with less deviation. Besides, present study also generally found that MTH can inhibit the proliferation of HCT-116 cells. Nonetheless, the underlying mechanism of antiproliferation effect of MTH on colon cancer cells are not explored. Thus, further study is needed to identify the active compounds in MTH that contributed to the antiproliferation properties by investigating the chemical compounds of MTH.

This study evaluated the effect of MTH on cancer cell migration as migration is part of the processes of cell invasion and metastasis and discovered that MTH plays a role as anticancer agent by exhibit antimigration effect on HCT-116 cells. Nevertheless, the mechanism of antimetastatic effect of MTH on colon cancer cells are not well explained. Previous study by Friedl & Wolf (2003) found that cancer invasion and metastasis involved the increased production of matrix degrading enzymes such as matrix metalloproteinases (MMPs). As this study can only cover the superficial surface of the issue, further study on underlying mechanism of actions of antimetastatic effect of MTH on colon cancer cells can be performed by quantifying the MMPs production in human colon cancer cells.

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