UNIVERSITI PUTRA MALAYSIA

ANTI-PROLIFERATIVE ACTIVITY OF ACMELLA ULIGINOSA FLOWER EXTRACT ON HUMAN COLORECTAL CARCINOMA CELLS (HCT-116)

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Anti-proliferative activity of *Acmella uliginosa* flower extract on Human Colorectal Carcinoma cells (HCT-116)

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ABSTRACT

Colorectal cancer (CRC) is rated as the third most common cancer among men and the second most common cancer among women. The survival rate of colorectal cancer has been greatly affected by metastasis as it may be fatal. In addition, the current cancer therapy treatments have high toxicities so alternative therapy with lesser adverse effects and greater effectiveness is urged to develop. *Acmella uliginosa* (AU), locally known as "Subang Nenek" or "Butang Baju Siti Fatimah" is a viable and flowering herb with various medicinal properties include antimicrobial, antioxidant, antinociceptive and anti-inflammatory properties. This study is conducted to determine the anti-proliferative activity of *Acmella uliginosa* flower extract (AUFE) on HCT 116 cells by assessing the cytotoxic effect and its anti-migratory potential towards HCT 116 cells. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was conducted to evaluate the cell proliferation when treated with AUFE at the range of 0.5-2 mg/mL and inhibitory concentration (IC) observed at 50% of cell population (IC_{50}) was determined with 24 hours incubation time. Next, wound scratch assay was followed to determine the anti-migratory potential of AUFE on HCT 116 cells which were tested at three different concentrations (0.21 mg/mL, 0.42 mg/mL and 0.63 mg/mL). 2 µg/mL of sunitinib was used as the positive control in wound scratch assay. From the MTT result, the IC_{50} obtained from HCT 116 cells when treated with AUFE was 1.125 ± 0.0052 mg/mL after 24 hours of incubation. AUFE treatment at concentrations ranging from 1.25 mg/mL to 2 mg/mL significantly reduced the HCT116 viability from 53.82 ± 4.45% to 15.6 ±6.43% when compared to untreated cells ($p < 0.05$). For wound scratch assay, the cells that have been treated with 0.42 mg/mL of AUFE showed the highest inhibition (78.89 ±5.75%) towards HCT116 migration. Whilst, AUFE at 0.63 mg/mL (68.82 ±9.88%) and 0.21mg/mL (25.97 ±14.01%) showed lesser cell migration when compare to untreated cells (0 mg/mL). The concentration of 0.63mg/mL and 0.42mg/mL had significantly inhibited the cell migration when compared to negative control ($p<0.05$), although the effect was not in a concentration dependent manner. From the MTT result, AUFE was able to inhibit 50% of cells proliferation when treated at the concentration of 1.125 mg/mL on HCT 116 cells which shows that AUFE exhibits anti-proliferative effect towards the colorectal cancer cell. The anti-migratory potential of AUFE also can be observed via wound scratch assay but there is no concentration dependent effect can be noticed in wound scratch assay. Treatment with AUFE can suppress the cell proliferation and inhibit the migration of HCT 116 cells *in -vitro.*

Keywords: Acmella uliginosa, colorectal cancer, wound scratch assay, MTT assay, HCT116

Kegiatan anti-proliferatif ekstrak bunga *Acmella uliginosa* pada Sel Human Kolorektal Carcinoma (HCT-116)

Khor Mei Hooi, Abdul Hamid Roslida

ABSTRAK

Kanser kolorektal dikenali sebagai penyebab kematian kanser ketiga dalam kalangan lelaki dan penyebab kematian kedua dalam kalangan wanita. Jangka hayat pesakit kanser kolorektal sangat dipEngaruhi oleh metastasis dan kemungkinan membawa maut. Di samping itu, rawatan terapi kanser zaman sekarang mempunyai ketoksikan yang tinggi sehingga terapi alternatif dengan kesan buruk yang lebih rendah dan keberkesanan yang lebih besar digesa untuk dikembangkan. *Acmella uliginosa* (AU), yang dikenali sebagai "Subang Nenek" atau "Butang Baju Siti Fatimah" adalah satu tumbuhan yang mempunyai pelbagai khasiat perubatan termasuk sifat antimikroba, antioksidan, antinociceptive dan anti-radang. Kajian ini dilakukan untuk mengetahui aktiviti anti-proliferatif ekstrak bunga *Acmella uliginosa* (AUFE) pada sel HCT-116 dengan menilai kesan sitotoksik dan potensi anti-migrasi terhadap sel HCT 116. 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) dilakukan untuk menilai proliferatif sel ketika dirawat dengan AUFE pada kepekatan 0.5-2 mg/mL dan kepekatan penghambatan (IC) diperhatikan pada 50% populasi sel (IC_{50}) ditentukan dengan masa inkubasi 24 jam. Kesan AUFE pada migrasi sel kanser ditentukan dengan menggunakan asai migrasi dengan 3 kepekatan yang berlainan (0.21 mg/mL, 0.42 mg/mL and 0.63 mg/mL). 2 µg/mL sunitinib digunakan sebagai kawalan positif. Dari hasil asai MTT, nilai IC_{50} untuk sel HCT116 yang dirawat dengan AUFE adalah 1.125 ± 0.0052 mg/mL selepas 24jam inkubasi. Rawatan AUFE pada kepekatan 1.25 mg/mL hingga 2 mg/mL secara signifikan mengurangkan daya maju HCT116. Untuk asai migrasi, sel HCT116 yang dirawat dengan 0.42mg/mL menunjukkan migrasi sel paling sedikit manakala 0.21 dan 0.63mg/mL menunjukkan kurang migrasi sel di kawasan celahan berbanding dengan celahan untuk sel yang tidak dirawat. Kepekatan 0.63mg/mL dan 0.42mg/mL telah secara signifikan menghalang migrasi sel jika dibandingkan dengan kawalan negatif (p <0.05), walaupun kesannya tidak bergantung pada kepekatan. Dari hasil MTT, AUFE dapat menghalang 50% proliferatif sel ketika dirawat pada kepekatan 1.125mg/mL pada sel HCT 116 yang menunjukkan bahawa AUFE menunjukkan kesan anti-proliferatif terhadap sel kanser kolorektal. Potensi anti-migrasi AUFE juga dapat diperhatikan melalui migrasi asai tetapi tidak ada kesan bergantung pada kepekatan yang dapat diperhatikan. Rawatan dengan AUFE dapat mempamerkan kesan sitotoksik dan menghalang migrasi sel dalam sel-sel kanser kolon.

Kata kunci: Acmella uliginosa, kanser kolorektal, migrasi asai, MTT asai, HCT116

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUFE	*Acmella uliginosa* flower extract
CADD	Computer-aided drug design
CDK	Cyclin-dependent kinases
COX	Cyclooxygenase
CRC	Colorectal cancer
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ESI-MS	Electrospray ionization ion trap mass spectrometry
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
HCT-116	Human Colorectal Carcinoma cells
HNPCC	Hereditary nonpolyposis colorectal cancer
HPLC	High-performance liquid chromatography
HTS	High throughput screening
IC_{50}	Inhibitory concentration (IC) observed at 50% of cell population
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NO	Nitric Oxide
PBS	Phosphate buffer saline
PDGFR	Platelet-derived growth factor receptor
PGE_2	Prostaglandin endoperoxide synthase-2
PIGF	Placental growth factor
ROS	Reactive Oxygen species
SEM	Standard error of mean
SPSS	Statistical Program for Social Sciences
5-FU	5-fluorouracil

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Colorectal cancer (CRC), defined as a type of cancer that can be developed in colon or rectum is the most common type of gastrointestinal cancer (American Cancer Society, 2018a; Tomislav, 2020). CRC is rated as the third most common cancer among men and the second most common among women. In addition, the mortality rate that has been caused by CRC is increasing and becomes the second leading cause of cancer death worldwide (WHO, 2018). In Malaysia, GLOBOCAN (2018) reveals that CRC is the second most common cancer with male exhibit higher incidence rate (16.2%) than women (12%). In addition, Chinese populations have been affected by CRC the most (Abu Hasan e al, 2016).

According to a study done by Zhang et al. (2018), the most effective and extensive plans for cancer treatment are chemotherapy and radiotherapy. Fluorouracil (5-FU) is a chemotherapy drug which has been used for the past 50 years and it is known as the most effective anti-cancer drugs. With the aid of the drug, 21% of CRC recurrence has been decreased. However, the continuous consumption of the drug may lead to many serious side effects such as mucositis, myelosuppression, peripheral sensory neuropathy, myocardial infarction, pulmonary embolus and the most seriously can cause death (Westley et al., 2010).

Hence, effective ways of management for cancer treatment-induced adverse effects are necessary to develop (Zhang et al., 2018).

Natural products are derived from the natural resources which include plants, animals and microorganism. These natural products are believed to possess therapeutic properties such as wound healing and pain relieving. Traditional medicine is the knowledge of medicine based on generations of experience that is documented or passed down to the descendants which largely relies on plants for their medicinal properties (Ji et al., 2009; Wachtel-Galor & Benzie, 2011). Betti et al (2013) declare that the uses of natural products such as plants are very essential as daily health care as plants are cheaper than modern medicine and they can be more effective. Not only this, documentation, conservation and sustainability of use of flora component from any country is very essential.

According to the World Health Organization (2002), around 80% of the population in developing countries relies on medicinal plants and drugs based on these plants. The increased awareness in the limited ability of synthetic drugs used in modern medicine and the harmful side effects have to lead to the society turning to complementary and alternative medicine that utilises plant-based medicine that is used traditionally for several diseases (Hussain et al., 2011a). Hussein et al. (2011b) also state that many plants are used in pharmacological research in search of new medicine.

In addition, Riahi-Chebbi et al. (2016) claim that polyphenols are the secondary metabolites which believed to have essential role in cancer prevention and therapy. Meanwhile, these secondary metabolites are normally presented in plant kingdom so the plants are known as the important source for anti-cancer drugs. According to the researches that had been conducted by using cancer cell lines and

carcinogenesis in animal models, polyphenols have been proven to have anti-cancer effects because polyphenols are able to initiate apoptosis via cell death pathways; able to inhibit proliferation of cancer cells and inhibit metastasis by inhibiting anti-apoptotic molecules and cell cycle arrest (Riahi-Chebbi et al., 2016). Meanwhile, there is one plant scientifically named *Acmella uliginosa* (AU) which is enriched in polyphenols such as flavonoids and phenolic which are essential for inhibiting the proliferation of cancer cells. Not only that, spilanthol, the major bioactive constituent in AU was also found to possess significant anti-proliferative effect on human breast cancer MCF-7 cell line (Nelofar & Mukta, 2016; Siti & Intan, 2017).

Acmella uliginosa (Sw.) Cass is an herbal plant belongs to Asteraceae family. It is a viable and flowering herb with various medicinal properties including anti-cancer, antimicrobial, antioxidant, anti-nociceptive and anti-inflammatory properties. This plant can be widely found in the tropics and sub-tropics especially in West Indies, Brazil, Africa, Indonesia as well as Malaysia. (Ong et al., 2011). In Malaysia, it is locally known as "Subang Nenek" or "Butang Baju Siti Fatimah". Traditionally, this plant is usually used to relieve pain which include mouth ulcers, toothache, sore throat and stomach ache by Malay population (Ong et al., 2011; Lagnika et al., 2016). This plant has been reported to exhibit significant anti-proliferative effect against breast cancer cell with the presence of important active metabolites, therefore this current study was conducted in order to evaluate the anti-proliferative effect of AU in suppressing the growth of colorectal cells.

1.2 Objective of Study

1.2.1 General Objectives

To investigate the effect of *Acmella uliginosa* flower ethanolic extract (AUFE) on Human colorectal carcinoma cell line (HCT-116).

1.2.2 Specific Objectives

- To evaluate the cell proliferation activity and cytotoxic effect of AUFE on HCT 116 via MTT assay.
- To determine the anti-migratory potential AUFE on HCT-116 via scratch assay.

1.3 Hypothesis

AUFE possesses anti-proliferative activity by exhibiting cytotoxic effects and inhibiting migration of HCT-116 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Colorectal Cancer

Colorectal cancer had caused 861,000 deaths in 2018 and the addition of 1.8million new diagnosed cases. In addition, CRC is the third most commonly diagnosed cancer among males and the second among females (Finlay et al., 2020). Based on Global Cancer Statistics 2018, CRC ranks as the third most commonly diagnosed cancer and ranks as second the most mortal cancer (Bray et al., 2018). Therefore, CRC is considered as one of the top leading cause of death globally (Abu Hassan et al., 2016).

In 2018, there are 576,000 cases have been diagnosed among male while 521,000 cases had been detected among female. In addition, men who age 0-74 years have comprised of 1.51% cumulative CRC risk and 1.12% risk among women. The developed countries like North America Southern Europe, Australia, Eastern Asia and Northern Europe are having the highest risk CRC incidence (Rawla et al., 2019). Gandomani et al. (2017) also declare that there are more than 90% of diagnosed patients exceed 50 years and the average CRC diagnosed age is 64 years old. Hence, the older population are more likely to develop CRC.

Apart than its global statistic, Intentional Agency for Research on Cancer revealed that CRC is the second most common cancer in Malaysia in 2018.

Singapore has the highest incidence of CRC in South East Asia while Malaysia ranks as the third highest incidence after Brunei. In term of mortality, Brunei has the highest mortality rate followed by Singapore and Malaysia exhibited as the third-highest mortality rate. The people more than 50 years old are the susceptible group for colorectal cancer as there are about 80% of CRC in Malaysia diagnosed in the population older than 50 years. Recently, Malaysia does not have any structured national CRC screening program so majority of the patients present were already at the late stage with poor diagnosis. This leads to a surge of health burden as the cost of treatment is expensive and quality of life at the late stage is poor (Veettil et al., 2017).

In comparison with the incidence rate among genders, males undeniably possess a higher incidence rate in Malaysia where there are 2,518 (55.9%) among males and 1,933 (42.9%) colorectal cases among females (Abu Hassan et al., 2016). In terms of ethnicity, Chinese demonstrated the highest rate of CRC where there are 28.8 per 100,000 annually followed by Indian which are 14.3 per 100,000 and the lowest is among Malay population which are11 per 100,000 (Veettil et al., 2017).The similar result among ethnic has further supported by the study from Abu Hassan and his teammate (2016) which again illustrated that Chinese ethnicity has the highest incidence rate and followed by Malay ethnicity while the least is Indian ethnicity. Chinese population also shows the highest mortality rate for CRC (11.85), then Malay population (9.56) and lastly 7.08 in Indian population (Abu Hassan et al., 2016). Both studies from Veettil et al. (2017) and Abu Hassan et al. (2016) suggest that the highest occurrence of CRC among Chinese population are due to genetic factors.

2.2 Pathogenesis of Colorectal Cancer

Cancer is the uncontrolled growth and spread of abnormal cells (American Cancer Society, 2019a). A metastasis process will cause invasion of cancerous cells to other parts of body through bloodstream or lymphatic system (Cancer.Net, 2019a). According to National Cancer Institute (NIH), there are two ways to classify the cancers which are by the type of tissue where cancer originates (histological type) or by the location of the body where cancer first developed. There are hundreds of variable cancers in the world while the cancers can be categorized into six major groups such as carcinoma, sarcoma, myeloma, leukaemia, lymphoma and mixed typed from the histological standpoint.

American Cancer Society (2018a) defines colorectal cancer (CRC) as a type of cancer which develops in colon or rectum (large intestine). CRC is also commonly known as colon cancer or rectal cancer while the name of the cancer relies on where the cancer starts. In other words, colon cancer is the most common type of gastrointestinal cancer (Tomislav, 2020). Colon is a muscular tube and the longest part of large intestine, 5 feet long. The colon starts with ascending colon which begins with a pouch known as cecum for receiving undigested food from small intestine. Then, the colon continues with transverse colon, descending colon and the last section known as sigmoid colon with "S" shape (Hamza et al., 2017).

The development of colorectal cancer is slow which may need to take a period of 10 to 20 years as it is a progressive and multistep genetic disease while the environmental factors also may contribute towards the development of CRC (Hamza, Hadeer & Hanaa, 2017; Arnold et al., 2005). Furthermore, there will be increasingly loss of genomic and progressively colonocyte replication that caused by the

accumulation of genetic mutations while these may result in the changes from normal mucosa become benign adenoma to severe dysplasia and frank carcinoma (Cappell, 2005). Specifically, the epigenomic instability accelerates the malignant transformation of colon cells as tumour suppressor genes and oncogenes are undergoing epigenetic alterations (Kuipers et al., 2015).

Usually, a polyp, a noncancerous growth, will firstly develop on the inner lining of colon or rectum while there are two most common types of histology that have malignant potential which are hyperplastic and adenomatous (Cappell, 2005; Hamza, Hadeer & Hanaa, 2017). Meanwhile, the adenomas or adenomatous possess a higher probability to progress into invasive cancer when it grows larger. However, Fleming et al. (2012) declare that more than 90% of CRC are adenocarcinomas which arise from colorectal mucosa epithelia cells.

The people suffering from colorectal cancer in the earlier stage normally do not show any significant sign and symptoms. However, the sign and symptoms will show when the tumour grows larger. The most common symptoms are like abdominal pain, bowel habit alteration and rectal bleeding. Meanwhile, there is approximately 10% of patients may develop iron deficiency anaemia (Ballinger et al, 2007). Bright red or dark blood appears in the stool; narrow or thinner shape stool when compare to normal stool; unexplained weight loss and fatigue are also known as the symptoms for CRC. The people that experienced these sign and symptoms no matter in younger or older than the recommended screening age are encouraged to visit a medical doctor and have a colonoscopy (Cancer.Net, 2019c).

Table 2.1: Cancer stage classifications (MarryAnne, 2019; Cancer.Net, 2019b)

Stage	Description
0	The earliest stage of cancer and it is called cancer in situ. The cancer cells only grows in mucosa or the inner lining of colon or rectum.
I	The cancer is limited to mucosa and muscular layer of the colon or rectum but not yet invasion in lymph node.
IIA	The cancer is limited to the wall or outermost layer of colon or rectum but did not spread to nearby tissue or organs. No invasion in lymph node.
IIB	The cancer is limited to muscle lining of abdomen (visceral peritoneum. No invasion in lymph node.
IIC	The cancer has grown through the wall of colon or rectum and spread into nearby tissue or organs. No invasion in lymph node.
IIIA	The cancer has grown through the muscular layers and inner lining but do not exceed the wall of bowel. 1 to 3 lymph nodes are involved.
IIIB	The cancer has already exceeded the bowel and starts penetrating the visceral peritoneum or metastasising to other organs. Involvement of lymph node.
IIIC	The cancer has grown beyond the muscular layers and more than 4 lymph nodes are involved.
IVA	The cancer has metastasized to single distant part such as liver or lungs.
IVB	The most advanced stage of colorectal cancer where the cancer cells has metastasized to two or more distant sites.

2.3 Risk Factors of CRC

There are many risk factors related to the occurrence of CRC where the risk factors can be categorized into 2 groups which are non-modifiable and environmental risk factors. The non-modifiable risk factors include age, hereditary or genetic factors, ethnicity and gender; meanwhile environmental factors include smoking, alcohol intake, sedentary lifestyle and dietary (Wong et al., 2019; Marley& Nan, 2016; Hagger & Boushey, 2009).

First and foremost, age is the most significant risk factor that increases the risk of developing CRC. According to the study done by Hagger et al. (2019), the risk of CRC increases progressively from the age of 40 while the risk urges dramatically after the age of 50. This statement has been further supported by the studies done by Wong et al. (2019) and Hamza et al. (2017) who deduce that more than 90% of CRC diagnosed cases are found for patients aged more than 50 years old while the incidence rate is 50 times fold for the populations aged between 60-79 years old when compared to the population aged 40 years old and below However, they also remind that there is increasing incidence rate among younger populations (Hamza, Hadeer & Hanaa, 2017; Hagger & Boushey, 2009).

Moreover, the ethnicity risk factor is more applicable in the Asia countries such as Indonesia, Japan, Korea, Singapore and Malaysia as the ethic like Chinese, Japanese and Korean are more likely to have CRC. In Malaysia, the Chinese population had shown the highest age-standardized incidence and highest mortality rate when compared to Malay and Indian. The similar result from Singapore also demonstrated that Chinese Singaporean are more susceptible to CRC (Wong et al., 2019). For gender specificity, most of the incidence rate from Asian countries illustrate higher incidence

was associated in male but the valid reason for this data is remained uncertain. However, Newcomb et al. (2007) claim that estrogens and progestins may play a role as protective ingredients against CRC in therapy for hormone replacement among postmenopausal women. Other than hormone, the reason that there is higher incidence among male is due to varying lifestyle and awareness of healthcare between male and female (Wong et al., 2019).

The statistic deduces that about 5 to 10% of CRC is due to genetic conditions while the commonest inherited associated CRC are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), also named as Lynch syndrome. Not only this, the lifetime risk for the person associated with HNPCC-related mutations will have 70 to 80% of risk higher than normal people and normally will be diagnosed in their mid-40s. Meanwhile, a family history of colorectal cancer or adenomatous polyps again increase 20% of risk to associate with CRC. The findings demonstrate that the risk elevates when the persons with a CRC history in one or more first-degree relatives (Hamza, Hadeer & Hanaa, 2017; Hagger & Boushey, 2009).

The populations with unhealthy habits like alcohol consumption and smoking are also the risk factors for CRC (Marley & Nan, 2016). Alcohol intake is the reason why there is an onset of CRC among the younger population. The statistic establishes that the people who consume more than 4 drinks per day are 52% more likely to develop CRC. This is because the reactive metabolites in alcohol such as acetaldehyde can be carcinogenic while the alcohol itself can also acts as the solvent in order to enhance the penetration of other carcinogenic into mucosal cells (Marley & Nan, 2016; Hagger & Boushey, 2009). Furthermore, smoking has contributed 12% of mortality rate in colorectal cancer because the tobacco in a cigarette is able to induce mutation

in DNA and increase the proliferation of cancer cells in the colon or rectum. Long term smoking habit can develop larger polyps in colon and rectum (Hagger & Boushey, 2009).

The sedentary lifestyle like physical inactivity in daily routines can be attributed to a higher incidence rate of obesity which is believed to link with CRC. In contrast, regular physical activity can lower the risk of developing CRC by 25% as exercises can raise the metabolic rate and elevates maximal oxygen uptake as well as increases the motility of gut (Marley & Nan, 2016; Hagger & Boushey, 2009). On the contrary, adequate fibre dietary can be protective factor against CRC as Marley and Nan (2016) reported that the diagnosed CRC in populations with high fibrous diet are extremely rare but more commonly observed in low-fibre diet's developed countries. Not only for fibre, nutritional practices like consuming vitamin D and calcium can also be the additional protection factors against CRC. 50% of CRC risk can be reduced when 1000 IU/day of vitamin D and 1250 mg/day of calcium is taken. This statement can be explained mechanistically as calcium can reduce the colonic epithelium proliferation and neutralize bile acids (Marley & Nan, 2016).

2.4 Colorectal Cancer Cell Line

There are various type of human colorectal cancer cell lines which are used to study about tumour in colon or rectum's biology, experimental therapy and biomarkers (Mouradov et al., 2014). Meanwhile, the colorectal cancer cell lines that are widely used for investigating colorectal carcinogenesis are HCT-116, LoVo, SW-480, LS174T, Colo205 and WiDr (Oh et al., 1999).

HCT-116 is a human colorectal carcinoma cell line which is initiated from an adult male and the cells are adherent with an epithelial morphology. This cancer cell line is an orthotopic model of colon cancer which is widely used for studying cancer biology. HCT-116 also can be applied in both in-vivo and in-vitro experiments. Due to the invasive and highly motile growth factor-independent cell line, HCT-116 is suitable to be used in *in-vitro* investigation with a doubling time of approximately 18 hours. In contrast, the reporter cell lines of HCT-116 are able to be tracked in *in-vivo* models which making them excellent tools to study tumour growth and metastasis mechanism as well as identify the effect of new drugs and therapies (Imanis Life Science, 2015-2020; Rajput et al., 2008).

2.5 Angiogenesis

Angiogenesis refers to the formation of new blood vessels which involves the processes such as migration, growth, and differentiation of endothelial cells (National Cancer Institute, 2018). In normal development, angiogenesis is carried out for the whole life as it is important for some developmental processes such as bone formation, embryonic development and others (Thomas & Jean-Pierre, 2010). However, angiogenesis also can contribute to pathologic conditions such as metastatic spreading

of cancer tissue as growth of the vascular network is important for the tumour cells to obtain sufficient oxygen and nutrient as well as to remove waste products (Nishida et al., 2006). Rmali et al. (2007) also deduce that once the tumour has reached a volume of 2 to 3mm, the proliferation and the growth of tumour needs to be dependent on angiogenesis. Due to this angiogenesis, the tumour can enlarge and invade to adjacent tissue and form new colonies of cancer cells, this process pathologically known as metastasis (National Cancer Institute, 2018).

Normally, angiogenesis plays a critical role in solid tumours including colorectal cancer. Angiogenesis is controlled by chemical signals that can be generated from the body such as vascular endothelial growth factor (VEGF) while in normal development, the stimulation of angiogenesis is balanced with the angiogenesis inhibitors stimulation. However, when imbalance equilibrium occurs, this will lead to increasing of new blood vessels formation and cause metastasis to occur (National Cancer Institute, 2018).

In addition, previous study discovers that the patients that have lower VEGF expression will have a significantly better survival rate when compare to the patients that have a higher expression of VEGF (Rmali et al., 2007). It is applicable in lung cancer, breast cancer including CRC as they are reported to have significant correlation between the expression of VEGF and prognosis (Nishida et al., 2006).

2.6 Cell migration

Cell migration is a biological fundamental process which the cells are translocated from one location to another location in order to establish and maintain the proper organization of multicellular organism (Trepat, Chen & Jacobson, 2012). In multicellular organism, cell migration is essential for wound healing, embryo formation, tissue homeostasis as well as immune system functions. However, aberrant cell migration can be found in various pathologies which normally can be seen in cancer where the tumour cells detach from primary site and attached to neighbouring cells to form secondary tumour (Nikolaou & Machesky, 2020).

The cancer cells can be migrated to another location either by individually or collectively. Cytoskeleton activity without the present of cell-cell interactions are individual cell migration while collectively migration is a group of cohesive cells that retains cell junctions and coordinates cytoskeletal activities with nearby cells which both the mobile and immobile cells will be carried and migrate together. Hence, cell migration can be known as metastasis in pathological perspective (Pijuan et al., 2019).

There are some different assays that can be carried out to investigate the invasion–metastatic cascade. The information regarding to each cell migration assays are stated in the Table 2.2.

Table 2.2: Cell Migration Assays

Type of assays	Principle	Advantages	Limitations	Reference
Wound Healing Assay (Scratch assay)	• A sterile pipette tip is used to create a scratch manually. • The effect of treatment can be observed by comparing the width of the gap before and after treatment.	• Easy to conduct • Inexpensive	• Time consuming • Large number of cells needed • Scratching may cause physical damage onto the cells	Ediriweera et al. (2018)
Transwell Assay (Boyden chamber assay)	• A physical barrier is included which can help to obtain a thorough analysis of the ability of cells to sense chemoattractant and undergo migration. • Cells are seeded in the upper chamber migrate vertically through the pore membrane into the lower chamber containing medium rich in attractant or high serum.	• Can measure the migratory response of both adherent and non-adherent cells	• Difficult to obtain a time-lapse data of cell invasion by using conventional microscopy • Time consuming	Justus et al. (2014)
Microfluidic Assays	• The cells are seeded in a chamber that has internal channel to bridge to a second chamber. • Able to measure chemokinetic by knowing linear chemotactic signals.	• Low sample volume which can save on reagents • Multiple chemoattractants analysis are available	• Imaging quantitation is more technically challenging • Expensive • Need to change media frequently to maintain cell viability	Pouliot et al. (2013)

Spheroid migration assay	• An *in-vitro* model which can mimic the micrometastasis as well as mimic *in-vivo* system such as gradients of oxygen and proliferation.	• Least time consuming and reproducible • Automated imaging is available	• Unable to control size and uniformity • Expensive	Katt et al. (2016); Vinci et al. (2013)
Tumor-Microvessel assay	• Endothelial cells are seeded onto Predefined ECM Scaffold or self-assembled through matrix remodelling in order to study the interactions of tumour cells and tumour vasculature. • Able to evaluate several key events in the metastatic such as invasion, intravasation, and extravasation.	• Vessel endothelium and shear stress are well-defined	• Limited vessel diameter ranges which need to be smaller than 50μm	Katt et al. (2016)
Real time monitoring of cell migration	• A sensing electrodes are placed on the underside of porous member which is used to monitor the migration of cells and it is monitored in real-time. • The migration of cells towards the chemoattractant that has been placed on the bottom chamber also will be monitored in real time.	• Less number of labour needed in performing experiment	• Need a skilful people in determining the incubation time to obtain linear relationship between the absorbance reading and cell number.	Xi et al. (2008); Ke et al. (2011)

2.7 Metastasis

Metastasis is defined as a process where the cancer cells are spread to tissue and organs so a secondary or even the tertiary tumours formed (Martin et al., 2000-2013). According to the explanation from Cancer Treatment Centers of America (2020) in terms of colorectal cancer, when the cancer cells are spread to other part of the body from colon or rectal through the bloodstream or lymphatic system, it is known as metastatic colorectal cancer but it is different from recurrent colorectal cancer. This is because recurrent of CRC is where the cancer cells reoccur at the same part of colon or rectal after receiving treatment but not spreading to other parts of the tissue or organs. Furthermore, the colorectal cancer cells can be spread to lungs, bones, brain or spinal cord but liver is known as the most common part for metastatic colorectal cancer (Cancer Treatment Centers of America, 2020).

There are some important processes that have been involved in metastasis which include proliferation of primary tumours; local infiltration (invasion through nearby tissue and basement membranes); intravasation (invasions through blood vessels or lymphatic system); extravasation and proliferation at the secondary tumour site. Not only this, metastasis can be successfully performed when the survived apoptotic signals and host immune responses are prevented by tumour cells (Hunter, Crawford & Alsarraj, 2008; Zijl, Krupitza & Mikulits, 2011).

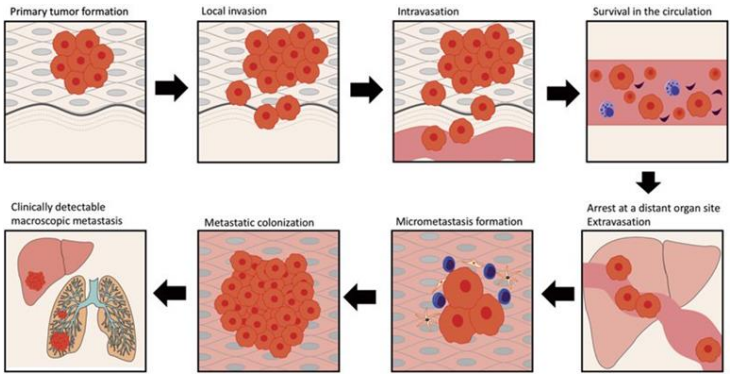

Figure 2.1: Flow chart of invasion-metastasis cascade. (Adapted from Pachmayr, Treese & Stein, 2017)

2.8 Current therapy for CRC

The factors such as the grade of cancer, the location of tumour, the patient's age, the overall health of patient and the potential side effects of the treatment will influence the treatment choices (MarryAnne, 2019; Cancer.Net, 2019d). Meanwhile, surgical resection is the most common treatment for colorectal cancer at stage I or II (early stage) but chemotherapy or combination of chemotherapy and radiation therapy are used for later stage cancer before or after surgery (Akhtar et al., 2014).

Surgery is known as the most effective treatment option for the localized colorectal tumours. The tumours and abnormal growth of cells will be removed by surgical resection (WebMD, 2015-2020; Onco Life Centre, 2020). However, Westley et al. (2010) reported that approximately 24% of surgically resected patients suffer recurrent disease while 85% of them died due to metastatic disease.

For chemotherapy, the drugs will be used to destroy the cancer cells when the tumour size is large or had metastasized to lymph nodes. Chemotherapy drugs can be administered systemically or regionally. Systemic chemotherapy is when the drug is either injected into a blood vein or taken it orally so the drug can reach every part in the body through bloodstream in order to reduce the risk of spreading. On the other hand, regional chemotherapy is injected into an artery that only has access to the area near to tumour. The aim of using regional chemotherapy is to reduce the side effects of the drug (American Cancer Society, 2018b).

For radiation therapy, the high-energy of X-rays is used to destroy the cancer cells. The combination of chemotherapy and radiation therapy before surgery is to help in shrinking the size of tumours and easier to remove; meanwhile, the radiation therapy is given after the surgery is to kill the leftover cancer cells that are unable to be

removed during the surgery, for example, the cancer cells which attach to internal organ or the lining of abdomen. In addition, radiation therapy is also given together with chemotherapy when the overall health status of patients are not suitable to undergo resection (American Cancer Society, 2018c). Table 2.3 below summarizes the detail of some chemotherapeutic drugs used for CRC.

Table 2.3: Current chemotherapeutic drugs for colorectal cancer treatment

Drug name	Mechanism of Action	Limitations/ Side effects	Reference
5-fluorouracil (5-FU)	• Reduce DNA synthesis and DNA repair (anti-proliferative effects) by inhibiting the synthesis of thymidylate synthase.	• poor oral absorption and intra-patient variability	Chintala et al. (2011); Pardini et al. (2011)
Irinotecan (Camptosar)	• Inhibit topoisomerase I so the DNA replication is interrupted as topoisomerase I is important for catalysing breakage and re-joining of DNA strands. • Stimulation of SN-38 to undergo DNA fragmentation and programmed cell death.	• Diarrhea, myelosuppression, alopecia	Wolpin, & Mayer (2008)
Capecitabine (Xeloda)	• Known as a crystalline substances which will be absorbed through gastrointestinal tract and converted into 5-FU via 3 steps enzymatic reactions. • Thymidine phosphorylase, the final enzyme that will be presented in the pathway is presented in higher concentration at tumour site.	• Diarrhea, neutropenia, neuropathy, thrombocytopenia, nausea, neuropathy	Hirsch & Zafar (2011)
Oxaliplatin (Eloxatin)	• Induce the inhibition of DNA synthesis and cytotoxic lesion by forming intra- and inter-strand platinum DNA adducts.	• Acute neuropathy such as muscle spasms, breathing or swallowing difficulties) • Chronic neurotoxicity	Comella et al. (2009)

Trifluridine and tipiracil (Lonsurf)	• Trifluridine is a thymidine-based nucleoside analogue while tipiracil is a thymidine phosphorylase inhibitor. • Trifluridine can interfere with DNA synthesis when has phosphorylated into active form and demonstrate the anti-proliferative effects while tipiracil reduces the degradation in order to boost the effect of trifluridine.	• Neutropenia, leukopenia, anaemia, thrombocytopenia • Nausea, decreased appetite, fatigue, diarrhoea and respiratory tract infections.	Trifluridine/tipiracil for colorectal cancer (2018)
Bevacizumab (Avastin)	• It is a monoclonal IgG_1 antibody which able to inhibit vascular endothelial growth factor (VEGF)and stimulate the anti-angiogenesis effect	• Hypertension, bleeding, venous and arterial thromboembolic events, proteinuria, wound healing complications, gastrointestinal perforation • Not encourage to use for elder patients	Strickler & Hurwitz, (2012)
Ramucirumab (Cyramza)	• A fully humanized monoclonal IgG1 antibody which having higher binding affinity to a specific epitope on the extracellular domain of VEGFR-2 and inhibit tumour growth	• Hypertension and abdominal pain • Fatigue, headache, peripheral oedema, diarrhoea, nausea, respiratory infections	Verdaguer, Tabernero & Macarulla (2016)
Ziv-Aflibercept (Zaltrap)	• A soluble recombinant fusion protein which can binds to VEGF-A, VEGF-B and placental growth factor (PIGF) with high affinity to inhibit the angiogenesis process so no more blood able to supply to tumour	• Hemorrhage, gastrointestinal perforation, fistula formation, compromised wound healing,	Rodriguez (2013)

	• Apply for patients with metastatic tumours which have resistant to the chemotherapy that consist of oxaliplatin regimen	hypertension, arterial thromboembolic events, and proteinuria	
Cisplatin	• Able to bind with DNA at position N7 and induce apoptosis	• Kidney damage, loss of hearing, nephrotoxicity, ototoxicity	(Mármol et al. (2017)
FOLFIRI	• Combination of chemotherapy drugs which consist of leucovorin, 5-FU and irinotecan.	• Gastrointestinal toxicities such as nausea, diarrhea • Neutropenia	Neugut et al. (2019)
FOLFOXIRI	• Combination of chemotherapy drugs which consist of oxaliplatin, irinotecan 5-FU and leucovorin	• Only applicable for very young patients with overall good health and no other medical problems • More severe adverse effect than FOLFOX and FOLFIRI	Axel et al. (2020)
FOLFOX	• Combination of chemotherapy drugs which consist of oxaliplatin, 5-FU and leucovorin	• Neuropathy	Neugut et al. (2019)

2.9 Uses of Natural Product

Globally, there are more than 35,000 species of plants been reported for their medical purposes in various human cultures but the species of plants may be much higher as this indigenous knowledge normally taught and passed orally from one generation to another generation while mostly were remained undocumented. Researchers also found out that plants are the outstanding reservoir of medicines and the active phytochemicals in the plants can be designed and synthesized for producing new drugs. Statistically, there is about 25% of modern medicines are derived from tropical forests' plants (Ibrahim, 2004). The marketed drugs that derived from natural products such as Paclitaxel from *Taxus brevifolia,* Artemisinin from traditional Chinese plant *Artemisia annua,* Silymarin extracted from the seeds of *Silybum marianum* etc (Veeresham, 2012).

Due to the burden of affording drugs, World Health Organization (WHO) claims that there are about 80% of the populations in the developing countries rely on traditional remedies while most of them are plant-based natural products (Lagnika et. al., 2016). Plant-derived products have elevated across the world and more than 85% of populations in the Middle East, Latin America, Africa and Asia are depending on traditional medicine for health care necessaries (Fateme, Zahra & Hossein, 2018).

In fact, Malaysia has great potential in order to develop herbal products because Malaysia consists of abundant natural resources. This statement is supported by the finding that had been reported in 1935 which declared that Malaysia has 550 genera of tropical plants and more than 1300 species demonstrating therapeutic effects (Jamal, 2006; Ibrahim, 2004). Because of the increasing awareness on the importance of medicinal plants, specific research funding is finally given and supported the research

and development (R&D). There are several laboratory-based scientific research in Malaysia which conduct phytochemical screening, pure compounds isolation and structure elucidation, bioassay-guided isolation, biological determination, pharmacological and toxicological effects. Not only this, the experiment is conducted in-vivo and in-vitro for herbal product development while some of the studies can enter clinical trials (Jamal, 2006).

Furthermore, the diverse population of Malaysia has given the advantages towards traditional medicine systems in Malaysia because there are varieties and diversities of traditional medicine practices which include Malay, Chinese, Indian as well as indigenous heritage. Hence, Ayurveda, Siddha, Unani, traditional Chinese medicine are known as some of the major traditional medicine systems which are practising in Malaysia (Jayaraj, 2010).

In short, due to their potent pharmacological potential as well as therapeutic value, there is an increasing demand on herbal medicines and the increasing acceptance of traditional medicine in the international market. No matter whether it is traditional medicine or plant-derived compounds, the uses of natural products as medicines contain a long history in curing various diseases. In the other words, the natural product plays a vital role in improving human health and exploring new drugs. However, the resurgence of natural products also bringing newer challenges to the scientist and researchers at the same time because of its quality control, standardization and cost-effectiveness (Veeresham, 2012). Therefore, it is very important to continue exploring, standardizing and also validating the potency, safety and efficacy of traditional medicines (Dubey et. al., 2013).

2.10 Natural products for CRC treatment

The treatment for CRC like chemotherapy, radiation therapy, immunotherapy and others are the common and standard treatment choices for CRC patients but these type of treatments can lead to severe adverse effects such as neutropenia, thrombocytopenia, gastrointestinal toxicities, neuropathy, nausea and many others. Not only for the side effects from the current treatment, the results shown from the treatments provided are barely effective due to the metastasis disease and responding only transiently to conventional treatment. Therefore, alternative treatments by using plants or traditional herbs are important to reduce the effects from cancer therapy and increase the effectiveness of drugs (Ramos-Silva et al., 2017). Several plants which have been reported to be therapeutic for CRC are listed in Table 2.4.

Table 2.4: Plant extracts that have significant effects on CRC

Plants	***Origin***	***Part Used***	***In-vitro/In-vivo***	***Reference***
Chamaecyparis obtusa	Korea	Leaves	*In-vitro*	Kim et al. (2015)
Opuntia ficus-indica	USA	Fruits	*In-vitro*	Flores et al. (2014)
Hedyotis diffusa Willd	China	a	*In-vitro*	Lin et al. (2012)
Borojoa patinoi Cuatrec	Columbia	Fruits	*In-vitro*	Chaves-López et al. (2018)
Imperata cylindrica	China	Aerial	*In-vitro*	Wang et al. (2018)
Diplotaxis simplex	North African	Flowers	*In-vivo* and *in-vitro*	Hamida Jdir et al. (2016)
Podocarpus elatus	Australian	Fuits	*In-vitro*	Symonds, Konczak & Fenech (2012)
Scutellaria baicalensis	China	Roots	*In-vitro*	Wang et al. (2013)
Cydonia oblonga	North African	a	*In-vitro*	Riahi-Chebbi et al. (2016)

Plants	*Origin*	*Part Used*	*In-vitro/In-vivo*	*Reference*
Apricot kernel	China	Whole plant	*In-vitro*	Cassiem & de Kock (2019)
Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf	Korea	Leaves	*In-vitro*	Son et al. (2017)
Melicope ptelefolia	Malaysia	Leaves	*In-vitro*	Kabir et al. (2017)
Cocoa tea (Camellia ptilophylla)	China	Leaves	*In-vitro*	Gao et al. (2019)
Thevetia peruviana	Mexico	Fruits	*In-vitro*	Ramos-Silva et al. (2017)
Cocoplum (Chrysobalanus icaco L.)	USA	Fruits	*In-vitro*	Venancio et al. (2017)
Sanguisorba officinalis	China	Root	*In-vitro*	Liu et al. (2016)
Origanum majorana	Emirates	Leaves	*In-vitro*	Benhalilou et al. (2019)
Xanthium strumarium L.	Cuba	Leaves and stems	*In-vitro*	Piloto-Ferrer et al. (2018)
Panax ginseng and Veratrum nigrum	China	Roots and rhizomes	*In-vitro* and *in-vivo*	Kee et al. (2018)
Ferula pseudalliacea	Iran	Roots	*In-vitro*	Bamehr et al. (2018)

a, information is not provided from the paper

2.11 Morphology of *Acmella uliginosa*

Acmella uliginosa or Spilanthes uliginosa is an annual herb where the plant completes the life cycle from germination until death within a year. Its height can grow in between 10 to 100cm. The stems can be erected, scrambling or decumbent, green or reddish in colour and can be pubescent (hairy) or glabrous (hairless). Besides, it have opposite leaves which in narrowly ovate shaped, 1-8.5cm long while 0.3-3cm wide. The flowers are in yellow colour and they are collinear, capitula solitary where 4-6mm in diameter, 6-8mm long and 1-6cm long on stalks (Beentje, Jeffrey & Hind, 2005).

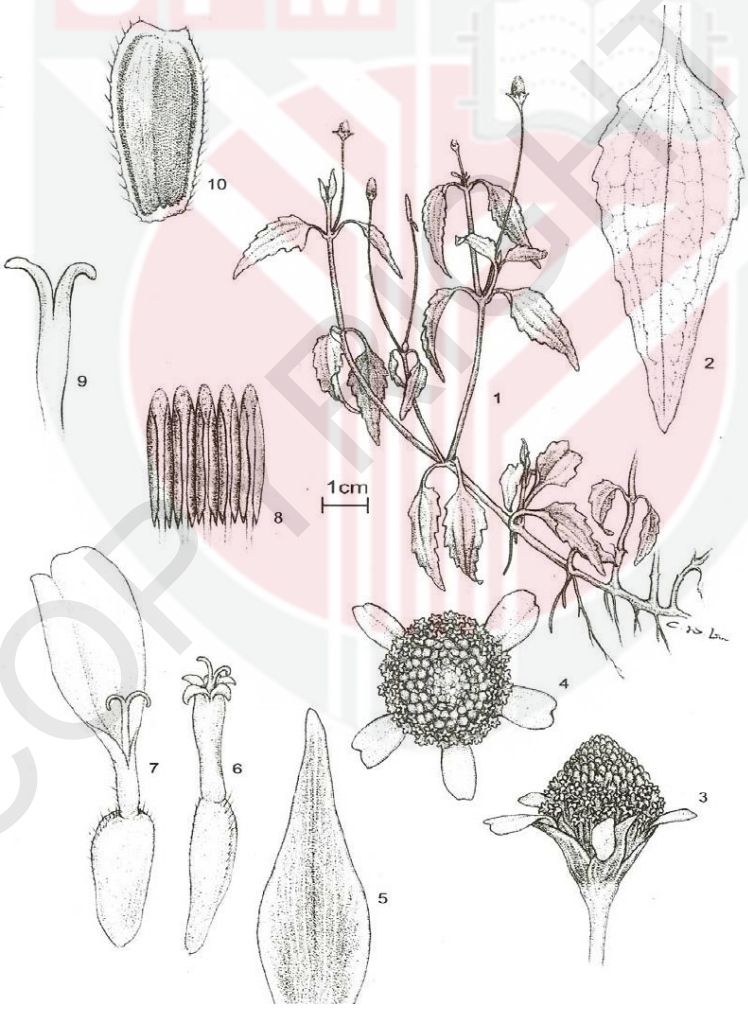

Figure 2.2: Morphology of *Acmella uliginosa*

1) Habit. 2) Leaf. 3) Lateral view of Head. 4) Dorsal view of head. 5) Involucral bract. 6) Disc floret. (Shih-Wen, Tian-Chuan & Yi-Han, 2007)

Figure 2.3: ***Acmella uliginosa (Swartz) Cassini.***

(Paul et. al., 2019)

2.12 Taxonomy and Nomenclature

The taxonomic tree of *A.uliginosa* had been reported by Julissa and Petro (2014) as below:

Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyta
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Asterales
Family	Asteraceae
Genus	Acmella
Species	Acmella uliginosa

Acmella uliginosa having several synonyms which are recognized by Porcher Michel et al. (1995 – 2020) which include *Spilanthes uliginosa, Spilanthes acmella, Acmella oleracea, and Spilanthes oleracea.* AU belongs to the family of Asteraceae or known as Compositae is one of the largest flowering plants' families as this family consists of approximately 1620 genera and 23,600 species (Jullisa & Pedro, 2014). Meanwhile, there are some important species that under the genus of Acmella or also named as Spilanthes in some of the studies such as *Spilanthes Americana, Spilanthes ocymifolia, Spilanthes alba, Spilanthes nervosa, Spilanthes urens, Spilanthes paraguyensis* while *Spilanthes acmella* also included in the important species under this genus which these plants are distributed in different locations in the world. In addition, Uraku (2016) claims this kind of genus can be widely found in damp pastures, swamp margins, on the rocks that near the sea, weed of road-sides as well as cultivations. The active ingredient in the genus of Spilanthes is spilanthol which able to produce local

astringency and anaesthetic as well as having a strong pungent taste (Kishan, Shailesh & Veenu, 2011). Therefore, Jayarai, Govindarajan and Palpu (2013) also report that the species under this genus possess various activities such as anti-inflammatory, liver protection, anti-toothache, pain relieving, anti-infective, anti-parasitic and others.

2.13 Phytochemistry of *A.uliginosa*

The bioactive compounds that contain in the plants show the therapeutic value of natural products while plants are also known as the oldest form of herbal medicine for human. It is essential to recognize the bioactive compound and the phytochemical constituents of the plants in order to understand the therapeutic effects of the particular plants. Plants source are always having the greater relevance with the worldwide transformation in order to manufacture a new drugs as the therapeutic effects of herbal remedies are normally safer, more effective and cheaper (Uraku, 2016). Several studies revealed that AU possess secondary metabolites such as alkaloids, glycosides, polyphenols, saponins, flavonoids and tannins (Lagnika et al., 2016).

Spilanthol, is categorized as lipophilic alkylamides or alkamides, is the major bioactive constituent that can be isolated from this plant while spilanthol is usually being used as anaesthetic and anti-inflammatory activities (Nomura et al., 2013). Spilanthol or also known as affinin has been reported to give pharmacological effects such as antimalarial, immunostimulant, antifungal and vasorelaxation (Prachayasittikul et al., 2013). The International Union of Pure and Applied Chemistry (IUPAC) name of spilanthol is (2E, 6Z, 8E)-N-Isobutyl-2, 6, 8-decatrienamide which is an unsaturated alkamide and having both hydrophilic and hydrophobic (amphiphilic nature). Therefore, the spilanthol can be easily extracted out by the extraction methods either ethanol, water,

methanol, hexane, pet ether or supercritical carbon dioxide, etc (Uthpala & Navaratne, 2020).

According to the study accomplished by Dubey et al. (2013), they discover that the concentration of spilanthol obtained can be higher when extracted by using ethanol while there is 9.04% of total N-alkylamides yet 88.84% spilanthol. Other than spilanthol, the second most abundant alkamides is 2-methylbutylamide or also known as homospilanthol (Boonen et al., 2010).

In the other perspective, flavonoids are also believed to be presented in AU which are responsible for antipyretic activity. In addition, flavonoids and saponins are known as the main active metabolites that contribute in diuretic activity (Paulraj et al, 2013). The phytochemical such as amino acids, triterpenoids, α and β amyrin esters, stigmasterol, myricyl alcohol and alkaloids being particularly rich in alkylamides and N-isobutylamides are also discovered in AU which is reported by Dias et al. (2013). Other than this, flavonoids is also important in antioxidant activity (Nelofar & Mukta, 2016).

In term of anticancer, AU consists of some biologically essential compounds that may be attributed towards anticancer and antioxidant activities such as alkylamides, α- and β-amyrin ester, isobutylamides, saponins and triterpenes. It is believed that alkylamides are the major compound in *A.uliginosa* flower to give significant effects on human breast cancer MCF-7 cell line. This is because the GC/MS analyses that has been conducted by Siti and her teammate (2017) found out that alkylamides not only can help in inhibiting the Cox-1, Cox-2 and 5lipooxygenase (anti-inflammatory activity), but also contribute in inhibiting the development of cancer and metastasis (Siti & Intan, 2017).

The Table 2.5 below summarize the various extraction methods with the respective alkamides in AU which are extracted from the study of Veenu et al. (2020).

Table 2.5: Various extraction methods with the respective alkamides in AU (Veenu et al., 2020)

Extraction Technique	Alkamides
Gradient reversed phase high performance liquid chromatography/electrospray ionization ion trap mass spectrometry (HPLC/ESI-MS)	• Spilanthol
HPLC-MS	• Undeca–2E,7Z,9E–trienoic acid isobutylamide • Undeca– 2E–en-8,10-diyonic acid isobutylamide • 2E–N-(2- methylbutyl) –2–undecene–8,10–diynamide • 2E,7Z–Nisobutyl–2,7-tridecadiene–10,12–diynamide • 7Z-Nisobutyl-7-tridecene-10,12-diynamide
HPLC-UV, ESI-MS-MS	• (2E,4E,8Z,10E)-N-isobutyl-2,4,8,10-dodecatetraenamide • (2E,7Z)-N-isobutyl-2,7-decadienamide • Homospilanthol • N-phenethyl-2,3-epoxy-6,8-nondiynamide • (2Z)-Nisobutyl-2-nonene-6,8-diynamide • (2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide

2.14 Ethnobotanical uses of AU

Traditionally, AU is used as the remedy for toothache where the flowers' head are crushed and applied at the site of toothache by the tribal people of Western Madhya Pradesh of India. (Revathi & Parimelazhagan, 2010). Besides applying the crushed of plants at the site of toothache, Ratnasooriya et al. (2004) suggested that by chewing the flower's head also can help in relieving toothache as well as stimulating the flow of saliva. In the district of Chhindwara and Betul of Madhya Pradesh state of India, Vaidhya is a traditional herbal healer which they mash the root of AU into paste and use to treat throat problem (Vijendra & Khatri, 2010). Meanwhile, the traditional Indian Systems of Medicine also uses this plant to improve male sexual performance where Sharma et al. (2011) claim that AU can be used for those having sexual feebleness while help them in achieving successful copulation as well as increase in sexual pleasure for healthy reproduction.

On the other hand, the leaves and flowers part of AU are also used to treat urinary tract infection such as leucorrhoea among the women in Bangladesh (Hossan et al., 2010). The whole plant paste of AU is used as treatment for poisonous sting in Chittagong hill tracts of Bangladesh where AU is known as Jhummosak locally (Biswas et al., 2010). This plant is also further recommended in enhancing immune system as well as curing dysentery and rheumatism. Meanwhile, the leaves of AU can be used to treat skin diseases that have been caused by bacterial or fungal (Kuldeep & Narender, 2010). AU is not only used as traditional medicine, but also commonly used as spice and food additives in Madagascar where it provides weak tingling and numbing effect on the tongue and contributes small extends of Jambu flavour (Ley et al., 2006). In Sri Lanka, potent diuretic activity of AU and urinary calculi can be dissolve when applying cold infusion of AU's flowers (Ratnasooriya et al., 2004).

In Malaysia, the plant is locally known as “Subang nenek “or “butang baju siti Fatimah”. The usage of flowers part are more common when compare to other parts of plant where the flower of AU is usually used for analgesic and antispasmodic properties. It is usually used for pain relieving such as mouth ulcers, toothache, sore throat and stomach ache. The flowers and leaves will be crushed become paste and administered topically at the site of insects bite for reducing itch, redness and swelling (Ong et al., 2011).

2.15 Pharmacological activities of *A.uliginosa*

AU is one of the important traditional herbs which has high therapeutic effects and widely distributed in tropical as well as subtropical regions worldwide. There are several pharmacological activities that had been proven by studies which include anti-inflammatory, anti-nociceptive, antibacterial, antifungal, antioxidant, anti-arthritic, antimicrobial, local anesthetic and many others (Nomura et al., 2013; Ong et al., 2011; Lagnika et al., 2016; Sathyaprasad et al., 2015; Paul et al., 2016; Chakraborty et al., 2010). Table 2.6 below provides the listing of pharmacology activities that have been evaluated on AU.

Table 2.6: Listing of pharmacology activities that have been evaluated on AU

Activity	*Origin*	*Part used*	*Reference*
Toothache and throat complaints	India	Flowers and leaves	Nakatani & Nagashima (1992)
Anti-nociceptive	Brazil	Flowers	Nomura et al. (2013)
	Malaysia	Flowers	Ong et al. (2011)
Pain amelioration	Bengal	Flowers	Paul et al. (2019)
Vasorelaxant	Thailand	Aerial	Wongsawatkul et al. (2008)
Diuretic Activity	Sri Lanka	Flowers	Ratnasooriya et al. (2004)
Antibacterial	Benin	Leaves	Lagnika et al. (2016)
Antifungal	Benin	Leaves	Lagnika et al. (2016)
	India	Flowers	Sathyaprasad et al. (2015)
Antioxidant	Benin	Leaves	Lagnika et al. (2016)
	Thailand	Aerial	Wongsawatkul et al. (2008)
Acaricidal	India	Leaves	Veeramani et al. (2014)
Hepatoprotective, hepatocurative	Pakistan	Aerial	Shah et al. (2018)
	Korea	Whole plant	Kim et al. (2018)
Anti-inflammatory	Taiwan	Flowers	Wu et al. (2008)
	India	Flowers	Paul et al. (2016)
Anti-arthritic	India	Flowers	Paul et al. (2016)
Antimicrobial	India	Flowers	Sathyaprasad et al. (2015)
Aphrodisiac	India	Flowers	Sharma et al. (2011)

Bio-insecticide	Malaysia	Axillary buds	Ang. & Chan (2003)
Local anesthetic	India	Aerial	Chakraborty et al. (2010)
Antipyretic	India	Aerial	Chakraborty et al. (2010)
Antiplasmodial	USA	Flowers	Mbeunkui et al. (2011)
Antimalarial	USA	Whole plant	Spelman et al. (2011)
Anti-cancer	India	Flowers	Lalthanpuii & Lalchhandama (2019)
	Malaysia	Flowers	Siti & Intan (2017)

2.16 Sunitinib

Sunitinib is an approval treatment for renal cell carcinoma and imatinib-refractory gastrointestinal stromal tumors which sunitinib can become the inhibitor for receptor protein-tyrosine kinase (Chemocare, 2020). By inhibiting the protein-tyrosine kinase, the actions of VEGF also can be inhibited which also can act as angiogenesis inhibitor. This is because this drug possesses potent antiangiogenic activity owing to its strong activity against VEGF and PDGF signalling (Lahti et al., 2017).

Furthermore, sunitinib also has been reported in preclinical and clinical trials to illustrate a direct anti-tumour effect by targeting growth proliferation receptor such as the platelet-derived growth factor receptor (PDGFR), VEGF receptor and others against different malignancies such as thyroid carcinoma, acute myeloid leukemia, lung, breast as well as colorectal cancer (Zhang et al., 2009).

Since sunitinib is able to inhibit actions of VEGF and angiogenesis in general, therefore sunitinib can be used as the positive control in the wound scratch assay. This is because colorectal cancer is known as one of the cancer that exhibits angiogenic phenotype and it is shown to exhibit anti-tumour effect on CRC too.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents and Chemicals

The media used for HCT-116 is Dulbecco's modified Eagle's medium (DMEM) was purchased from ScienCell™ Research Laboratories (Carlsbad, USA); fetal bovine serum (FBS) was purchased from Sigma Aldrich® (St. Louis, USA); both 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Phosphate Buffer Saline (PBS) were purchased from TOCRIS Bioscience (Bristol, UK.). Not only this, 0.5% Trypsin-EDTA (10x) and Penicillin-Streptomycin Solution (100x) were purchased from Gibco® (Loughborough, USA).

3.2 Equipment and Apparatus

Biolog MicroStation™ (Hayward, CA, U.S.A.) was used to read the absorbance and calculate the viable cells for cell viability assay. Meanwhile, inverted Microscope LEICA (Weitzlar, Germay) was used to observe the cell migration under 4x magnification. In addition, Dinocapture 2.0 was used to capture the gaps produced before and after the treatment.

3.3 Plant Extracts

The flowers of *Acmella uliginosa* were collected from Felda Selancar, Pahang which had been deposited at herbarium of Instituite of Bioscience, Universiti Putra Malaysia with a voucher specimen (No.: SK225/13). Firstly, the fresh flowers were washed in order to remove the dirt and impurities. Then, the samples were blended and soaked in 95% ethanol for 48 hours. After that, the extracts were filtered and 200ml of 95% ethanol was continuously added to the extract until the solvent became colourless. The extracts were filtered and the solvent were evaporated by using a rotary evaporator. After evaporating, the extract was dried at room temperature for removing all solvent residues. Then, the ethanolic extract of AU was weighed and dissolved in 1ml of 100% DMSO for the subsequent experiment.

3.4 Cell Culture

The human colorectal carcinoma cell line (HCT 116) was purchased from American Type Culture Collection (ATCC®) (ATCC accession no. HCT116 (CCL-247™). HCT-116 cells were cultured in DMEM that being supplemented with of 10% FBS and 1% of Penicillin-Streptomycin solution. The cells were grown in T75 tissue culture flask and incubated at 37°C and 5% CO_2 incubator.

3.5 Cell Viability Assay

The cell viability assay that was conducted to quantify the cell viability was 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). MTT assay is a tetrazolium salt reduction assay to assess cell metabolic activity. HCT 116 cells (2×10^4

cells/well) were seeded in 96-well plates and cultured in 100 µL per well of complete media for 24 hours.

After 24 hours, the old media was discarded and 100 µL of new media was added into each well. Then, the cells were treated with various concentrations of extract which included 0.5, 0.75, 1.0, 1.25, 1.75 and 2.0mg/mL and incubated for another 24 hours at the atmosphere containing 37°C and 5% CO_2. Control group (untreated) was also included. After 24 hours incubation of treatment, 20 µL of MTT solution (5mg/mL) was added into each well and incubated again for another 4 hours. The untreated cells (0 mg/mL treatment) were served as negative control while 100% of DMSO were known as blank. Then, the medium containing MTT solution was carefully removed while 100 µL of DMSO was added into each well. This DMSO is important to dissolve the dark formazan crystals that had formed in intact cells. After that, the mixture in the plate was mixed carefully for 10 minutes. Then, the absorbance was measured in a 96-well plate reader at a wavelength of 570nm. The MTT assay was performed in triplicates of two independent experiments. The data obtained were tabulated and the percentage of cell viability for each concentration was calculated by using formula below:

$$\textbf{Percentage of cell viability (\%)} = \frac{Treatment - Blank}{Negative\ control - Blank} \times 100\%$$

3.6 Scratch Assay

The anti-migratory effect of AUFE was tested with wound scratch assay. 5×10^6 cells/well of HCT-116 were seeded in 6 well-plate and incubated in 37°C with 5% CO_2 until the cell confluency reached 90%. After the cells had attached and reached a confluent monolayer, a scrapper was used to do a scratch onto each concentration (0.21, 0.42 and 0.63mg/mL) including negative (0mg/mL) as well as positive control (2µg/mL of sunitinib). Then, 1mL of DMEM was used to remove the cell debris formed from the scratch. In addition, 1mL PBS was used to wash and removed the cells debris again twice. Next, the gaps of wound were photographed at 0 hour and after 24 hours incubation period by using Dinocapture under 4x magnification. The gap difference for each treatment was measured and analysed through ImageJ software. The wound scratch assay was conducted in two sets of independent experiments. The gap areas before and after treatment were recorded and the percentage of cell inhibition was obtained via the calculation below while the data were normalised to 0% cell inhibition in negative control:

$$\textbf{Percentage of Wound Closure (\%)} = \frac{A_{0hr} - A_{24hr}}{A_{0hr}} \times 100\%$$

- A_{0hr} is the area of wound measured immediately after scratching (t = 0hr)
- A_{24hr} is the area of wound measured 24 hours after scratch is performed (t = 24hr)

↓ Normalized

$$\textbf{Percentage of cell inhibition (\%)} = \frac{P_0 - P_T}{P_0} \times 100\%$$

- P_0 is the percentage of wound closure in negative control
- P_T is the percentage of wound closure in treatment

3.7 Statistical Analysis

IBM Statistical Package for the Social Sciences (SPSS) Statistics 24 software (New York, USA) was used to perform the statistical analysis. The results were presented as mean ± SEM (Standard Error of Mean) of the mean of two independent experiments. Meanwhile, One-Way Analysis of Variance (ANOVA) was used to analyse the results which followed by post hoc test Tukey HSD. The value is significance when a p-value is less than 0.05 ($p < 0.05$).

CHAPTER 4

RESULT

4.1 Cell Viability Analysis of Human Colon Carcinoma Cell (HCT-116) Treated with AUFE

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was performed in order to determine the cell viability of HCT-116 when treated with *Acmella uliginosa* flower ethanolic extract (AUFE). Various concentration ranged from 0.5 mg/mL to 2.0 mg/mL were applied to HCT-116 cells respectively.

The cell viability percentage were decreased when the concentration of treatment increased. Hence, Figure 4.1 showed a concentration-dependent inhibition of AUFE on HCT-116 viability after 24 hours of incubation period. The highest percentage of cell viability can be observed when treated with the lowest concentration, 0.5mg/mL which was 75.24±12.37% while 68.79±9.14% of cell viability was determined when treated with 0.75mg/mL of AUFE. Remarkably, AUFE treatment at concentrations ranging from 1.25mg/mL to 2.0mg/mL significantly reduced the cell viability from 53.82 ± 4.45% to 15.6 ±6.43% of HCT-116 when compared to untreated cells ($p<0.05$). However, there was no significant difference can be observed at the 1.0mg/mL of treatment when compared to others concentration. Regarding to the cytotoxic effect, 1.125±0.0052mg/mL was obtained as the IC_{50} value of AUFE in HCT-116.

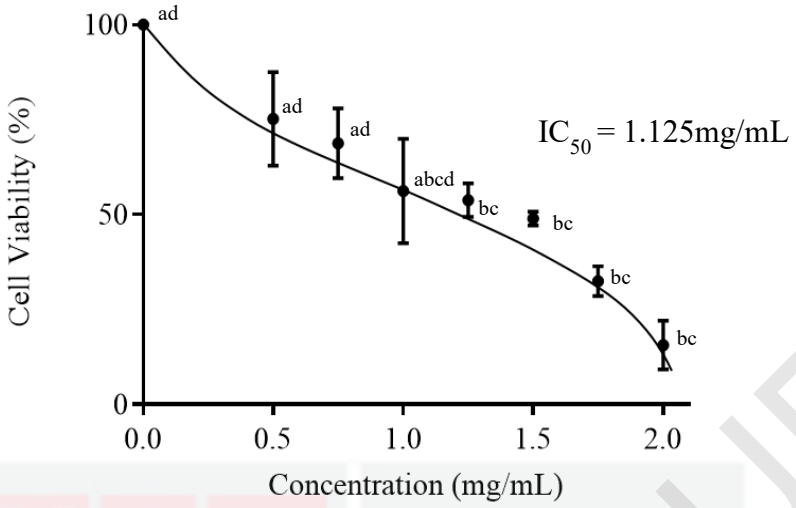

Figure 4.1: Percentages of cell viability of HCT-116 cells treated with AUFE

Data were expressed as mean ± SEM of two independent experiments. Results were analysed by using One-way ANOVA followed by post hoc Tukey HSD test. Different superscript letter between concentrations indicated significant difference (p<0.05).

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4.2 Scratch Assay Analysis in Human Colon Carcinoma Cell HCT-116 after Treatment with AUFE

In wound scratch assay, three different concentration (0.21 mg/mL, 0.42 mg/mL and 0.63 mg/mL) were used to treat the HCT-116 for 24 hours incubation period. The negative control was untreated cells (0mg/mL) while 2 µg/mL of sunitinib was used as the positive control in wound scratch assay to test the validity of the experiment. The difference between the gap area before and after treatment were calculated by using Image J software.

Figure 4.2 illustrated the percentage of cell inhibition when treated with various AUFE concentration and the data were normalised to 0% cell inhibition in negative control. From the bar chart in Figure 4.2, the highest percentage of cell inhibition can be observed when the cells were treated with 0.42mg/mL with 78.89±5.75% of cells were significantly inhibited. Whilst, AUFE at 0.63 mg/mL (68.82 ±9.88%) and 0.21mg/mL (25.97 ±14.01%) showed lesser cell migration when compare to untreated cells (0 mg/mL) respectively. At 0.21mg/mL, AUFE exhibited significant difference when compared to the other 2 concentrations which were 0.42mg/mL and 0.63mg/mL. However, there was no asignificant difference between 0.42mg/mL and 0.63mg/mL of AUFE (Figure 4.3). Therefore, the anti-migratory effect of AUFE was exerted in a concentration-independent manner.

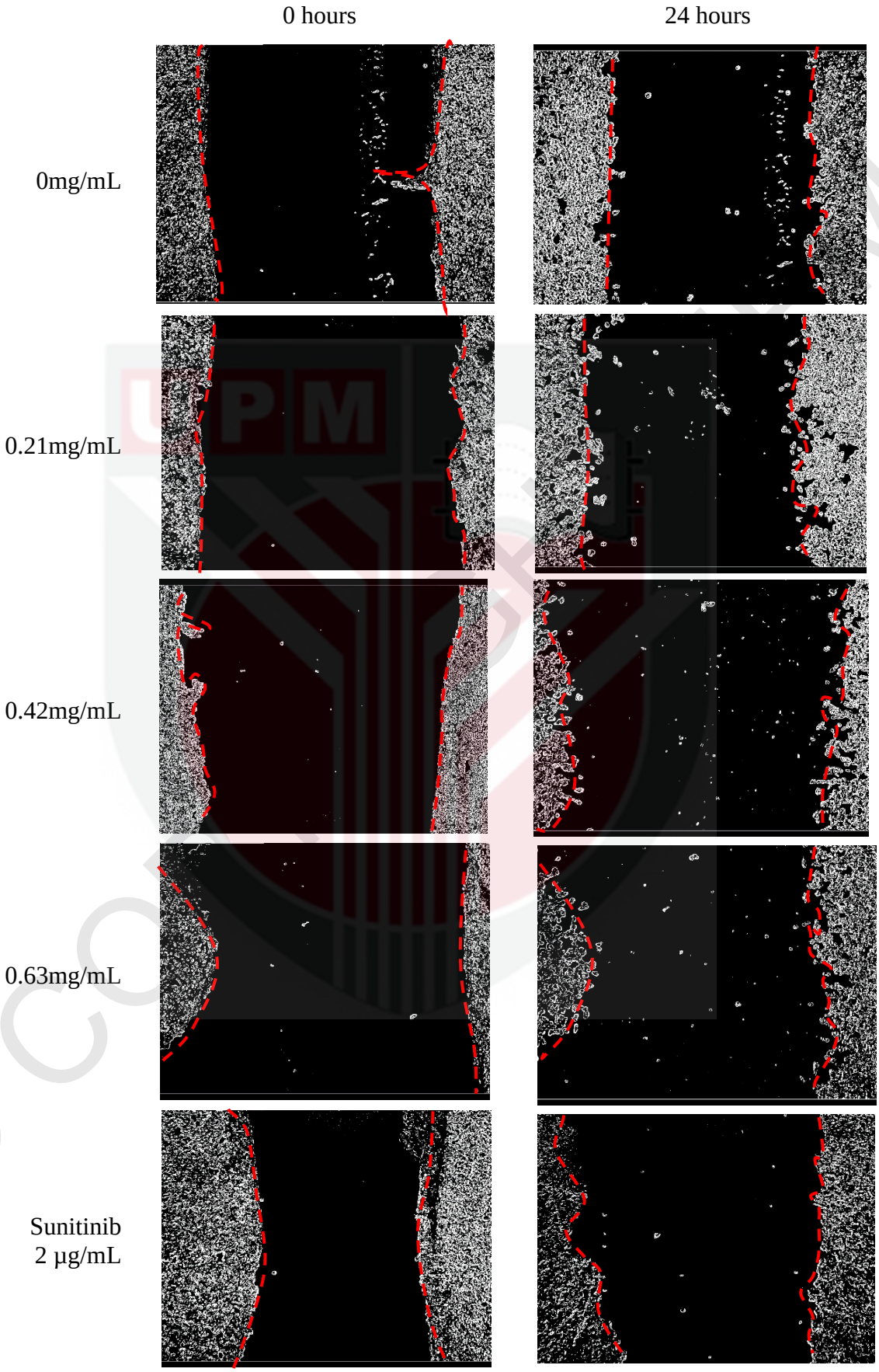

Figure 4.2: The gap captured before and after treatment (4x magnification)

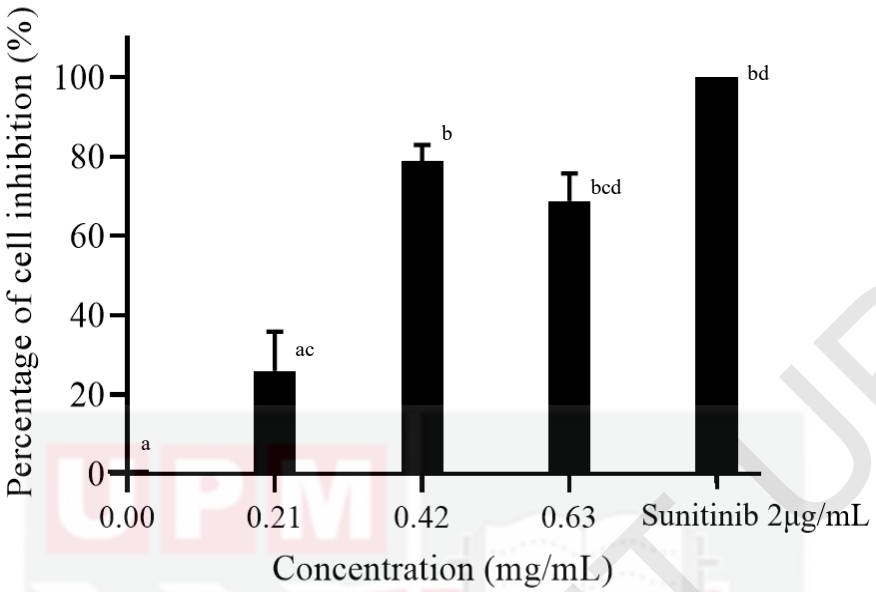

Figure 4.3: Percentages of cell inhibition of HCT-116 cells treated with AUFE after 24 hours incubation period in wound scratch assay

Data were expressed as mean ± SEM of two independent experiments. Results were analysed by using One-way ANOVA followed by post hoc Tukey HSD test. Different superscript letter within groups indicated significant difference (p<0.05).

CHAPTER 5

DISCUSSION

Colorectal cancer (CRC) ranks as the second leading cause of death worldwide (GLOBOCAN, 2018). However, the high toxicities that normally associated with cancer therapy treatments such as chemotherapy always urge the scientists in developing novel drugs for cancer therapy with lesser adverse effects and with greater effectiveness. Therefore, the evaluation and searching of new drugs for cancer therapy are always the prioritized goal for cancer treatment (Demain & Vaishnav, 2011).

There are more than 60% of current anti-cancer drugs were derived from natural sources which include Taxol, vinblastine, and camptothecin (Cragg & Pezzuto, 2015). Hence, in my study, the cytotoxic effect, anti-proliferative effect as well as anti-migratory effects of *Acmella uliginosa* flower extract (AUFE) were evaluated on Human Colorectal Carcinoma Cells (HCT-116).

HCT-116 is one of the colorectal cancer cell line which has been chosen to conduct for this final year project. This is because the doubling time of HCT-116 is very fast, 18hours and this cell line is known as the excellent tools to study tumour growth and metastasis mechanism as well as identify the effect of new drugs and therapies (Rajput et al., 2008).

Firstly, cell viability assay is necessary to carry out for natural products *in-vitro* to test the anti-proliferative effect and efficacy of drug (Fiorentino et al., 2017). Meanwhile, inhibitory concentration (IC) observed at 50% of cell population (IC_{50}) is

important in order to understand the pharmacological and biological characteristics of a drug (He et al., 2016). Therefore, the cell viability activity of AUFE is determined towards colorectal cancer cell line via 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT).

MTT assay is a colorimetric assay which can be used to measure the cell proliferation, evaluate the cytotoxic effects of the particular compounds, as well as the indicator of cell viability (Riss et al., 2016). The proliferation, viability and cytotoxicity can be measured as the cellular metabolic activity will become the indicator for quantitation. The cellular metabolic is the enzyme that will be produced from the viable cells which is succinate dehydrogenase where this enzyme can be produced from mitochondria. The viable cells consists of NAD (P) H-dependent oxidoreductase enzymes are able to reduce the yellow tetrazolium salt to purple formazan crystals (Berridge & Tan, 1993). Theoretically, the intensity of the purple colour will increase proportionally to the concentration of succinate dehydrogenase that has been produced from the cells. This is because in the presence of high concentration of succinate dehydrogenase, the enzyme will reduce more tetrazolium bromide to purple precipitate which also indicate the percentage of cell viability is high. Vice versa, when there is no viable cells or the cells die, the oxidoreductase enzymes are unable to produce which also means that the cells lose the ability to convert the MTT to purple formazan. By the aiding of plate reading spectrophotometer, the absorbance can be read at 570nm to quantify the formation of formazan (Riss et al., 2016). Since MTT assay is cheap and able to access cell viability, so MTT assay is used to evaluate the cytotoxicity and anti-proliferative activity of the extracts towards HCT-116 cells.

According to the result that has been presented in Figure 4.1, the cytotoxic effect of *Acmella uliginosa* flower extract (AUFE) was in the concentration dependent manner where the percentage of cell viability decreased when the concentration of treatment increased. Therefore, the highest cell viability, 75.24%, can be observed when the cells were treated with the lowest dose, 0.5mg/mL; while the lowest percentage of cell viability, 15.6% was observed when the cells were treated with the highest concentration of treatment, 2mg/mL. In addition, the cell viability of HCT-116 cells was significantly inhibited from 53.82% to 15.6% when treated with AUFE at the concentration ranging from 1.25mg/mL to 2.0mg/mL when compared to negative control ($p<0.05$). Regarding to the cytotoxicity of the extract, the IC_{50} that can be determined from the MTT assay was 1.125 ± 0.0052 mg/mL after 24 hours of incubation when there were 50% of cell proliferation was successfully inhibited.

It is believed that AUFE can inhibit the cells viability due to the present of alkylamides. This statement can be supported by the research done by Siti and Intan (2017) which reported that alkylamides not only can help in inhibiting the Cox-1, Cox-2 and 5lipooxygenase (anti-inflammatory activity), but also contribute in inhibiting the development of cancer and metastasis. They also elaborate that alkylamides are the major compounds in *A.uliginosa* flower which having significant effects on human breast cancer MCF-7 cell line (Siti & Intan, 2017; Boontha et al., 2020).

Spilanthol is known as the major bioactive constituent can be isolated from this plant and is categorized as lipophilic alkylamides. Spilanthol is the bioactive compound reported in the anaesthetic and anti-inflammatory activities (Nomura et al., 2013). It has also been reported by Wu et al (2018) to inhibit the expression of cyclooxygenase 2 (COX-2) or also known as prostaglandin endoperoxide synthase-2

(PGE_2). Since aberrant overexpression of COX-2 can contribute to the development of colorectal cancer, previous studies found out that the deregulation of the COX-2 production can provide impact on colorectal cancer (Park & Na, 2019). In other words, when COX-2 expression is not suppressed, it may increase the apoptosis resistance in transformed cells while promoting metastasis process in CRC. Therefore, spilanthol not only can help in suppressing the inflammation, but also can help in lowering the expression of COX-2 which can provide anti-proliferative effect on colorectal cancer development.

In addition, spilanthol was also demonstrated to inhibit nitric oxide production in murine macrophage cell line which also responsible for anti-cancer activity (Rani et al., 2019). The effect of spilanthol also has been tested by the research done by Arriaga-Alba et al. (2013) on *Heliopsis longipes* extract. From the research, they found out that spilanthol exhibits anti-mutagenic properties as it is able to reduce the number of frameshift mutations in the Ames test. It is believed that the expression of CyP450, CYP1A1/2, 2D6 and 3A4 which can lead to development of cancer can be reduced by spilanthol (Arriaga-Alba et al., 2013).

The cytotoxic effect of AUFE can be observed as it consists of phenolic compounds where Maraya et al. (2015) has explained that some phenolic compounds can kill bacteria and fungi as well as inhibit tumour development. As reported by Boontha et al. (2020), flavonoids possess anticancer activity while alkaloids illustrate anti-proliferative activity on various cancer cells. AU also consists phytochemical constituents that has been tested on human cancer which are alkaloid and phenols. Meanwhile, Gafar et al. (2012) also explained that the compounds such as phenols, saponins and flavonoids are the constituents for antioxidant activities which can help

in protecting the body against cancer by scavenging free radicals. This is due to their effects in preventing the DNA damage caused by the free radicals by scavenging the free radicals. Reactive oxygen species (ROS) can be produced in mitochondrial respiratory chain reaction during aerobic metabolism in eukaryotic cells where energy-source ATP is synthesized. However, normal-by-products synthesis of ROS such as oxygen radical, superoxide, hydroxyl radical, peroxyl and lipid peroxyl are free radicals; in addition, some oxidants like nitric oxide and nitrogen dioxide also can be generated when in low oxygen environment which also can lead to production of free radicals. The free electron from the free radicals are used to react with fundamental biomolecules such as protein, lipids and nucleic acids. As a result, these reaction will lead to pathologic situations which include a variety of chronic diseases such as arthritis, autoimmune disorders as well as various cancers including colorectal cancer (Lalthanpuii &Lalchhandama, 2019).

On the other hands, cell migration is not only a biological fundamental process which the cells are translocated from one location to another location to execute their function but when cell migration goes aberrant, it may contribute to pathologies conditions such as inflammation and cancer metastasis (Trepat et al., 2012; Nikolaou & Machesky, 2020). Due to the correlation of cell migration towards formation of metastasis while metastasis may affect the survival rate among the CRC patients, anti-migratory effect on an alternative therapy is needed. Thus, wound scratch assay has been chosen to determine the anti-migratory effect of AUFE as scratch assay is easy to conduct and inexpensive (Ediriweera et al., 2018).

There were three different concentration used in wound scratch assay which included 0.21mg/mL, 0.42mg/mL and 0.63mg/mL while all these three concentrations

were lower than the IC_{50} value that had been obtained from MTT assay. A lower concentration was used to make sure the cells are still viable when conducting for migration assay to ensure that the cell migration is inhibited due to the effect from treatment but not because of the cells were killed by the concentration treated (Vitor et al., 2019).

For wound scratch assay, the highest percentage of cell inhibition can be observed when the HCT-116 cells were treated with 0.42mg/mL of AUFE where there were up to 78.89 % of cells migrated had been significantly inhibited when compare to negative control (untreated cells) ($p<0.05$). Meanwhile, the cells that treated with 0.21mg/mL and 0.63mg/mL also showing anti-migratory effect, but the effect was lesser which were 25.97% and 68.82% of cell inhibition respectively, when compared to the cells that had been treated with 0.42mg/mL (78.89%) of AUFE. Hence, the anti-migratory effect was observed when treating with AUFE, but the effect was in concentration independent manner.

Since there is anti-migratory effect can be observed when the HCT-116 treated with AUFE, it is postulated that *Acmella uliginosa* is also able to inhibit metastasis. Metastasis is a process where the cancer cells are spread to tissue and organs so a secondary or even the tertiary tumours formed (Martin et al., 2000-2013). Without the occurrence of metastasis, the 5 years survival rate among the patients with localized stage is 90%. However, the survival rate is decreased to 71% when the cancer has spread to surrounding tissues and/or regional lymph nodes; but the 5-year survival rate will be reduced to 14% when metastasis to the distant part of the body occur (CancerNet, 2020). Consequently, Seyfried and Huysentruyt (2013) estimated that metastasis is responsible for about the 90% of cancer death. Moreover, Pretzsch et al.

(2019) claim that metastasis is the main causes of colorectal cancer death among majority of the patients while liver and peritoneum are the common metastasis distant sites. Hence, Valastyan and Weinberg (2011) also clarify that anti-metastatic is an essential effect in order to use as the therapeutic agents for carcinoma patients that have a number of disseminated tumour cells in their blood, bone marrow and distant organ sites. In the other words, anti-metastatic is known as one of the key consideration when designing drugs for cancer.

It is also believed that phenolics and flavonoids content also are known as the bioactive constituents which have contributed to anti-migratory potential of *Acmella uliginosa* or also known as *Spilanthes acmella* where these compounds had shown that metastasis was inhibited on MCF-7 cancer cells (Boontha et al., 2020). Generally, phenolics are reported to have strong radical scavengers, metal chelators as well as endogenous defence mechanism modifiers. In terms of phenolic compound structure, the key structural that contributes to the anti-cancer effects are the aromatic ring, unsaturated substituted chains as well as the number and position of free hydroxyl groups. Therefore, phenolics are believed to that have potential as anticancer compound due to their antioxidant effects (Abotaleb et al., 2020). As an example, the phenolics constituents that presence in *Tragopogon porrifolius* portrays significant antioxidant effects on several experiments which include FRAP assay, CUPRAC Assay, DPPH Assay and other towards breast and colon cancer cell lines (Al-Rimawi et al., 2016).

Other than antioxidant effect, phenolic compound particularly phenolic acid, also reported to possess anti-carcinogenic activity towards colon cancer by inhibiting the transcription factors that can be linked to inflammation; inhibit cell proliferation

by D-type cyclins, and cyclin-dependent kinases (CDK); induce apoptosis and inhibit cell migration as well as exhibit anti-metastatic effect (Rosa et al., 2016).

Furthermore, alkaloid also known as one of the secondary metabolites that can be detected in Subang nenek. Uraku and Ogbanhi (2015) claim that alkaloids can help in eliminating and reducing human cancer cell lines. The anticancer effect from alkaloid can be observed as it targets the DNA replication or protein synthesis towards the mechanism action on tumour cells where will lead to apoptosis of the neoplastic cells (Isah, 2016). Other than inducing apoptosis, alkaloids also can prevent metastasis to occur by inhibiting some cell signalling pathways and proteins that favour tumour cell growth such as MMP-2, MMP-9 and AKT (Dey et al., 2018).

CHAPTER 6

CONCLUSION AND FUTURE RECOMMENDATION

6.1 Conclusion

In evaluating the anti-proliferating and cytotoxic activity, the viability of cells had been inhibited by the treatment of the extract while when the cells were treated with the concentration ranging from 1.25mg/mL to 2.0mg/mL had significantly inhibited the cell proliferation and the effect was in concentration dependent manner. The IC_{50} value obtained at 1.125mg/mL of AUFE. For wound scratch assay, all three different concentrations which were 0.21mg/mL, 0.42mg/mL and 0.63mg/mL of treatments showed anti-migratory effect but 0.42mg/mL of treatment showed the highest percentage of cells inhibition. It is postulated that the cytotoxicity, anti-proliferative as well as anti-migratory effect of AUFE are due to the phytochemical profile of the plants such as the presence of spilanthol, phenolics, flavonoids, alkaloids and others.

In conclusion, *Acmella uliginosa* flower extract (AUFE) exhibits cytotoxic effects on human colorectal carcinoma HCT-116 cells. Preliminary study on the effects of AUFE in wound scratch assay also illustrates that this extract can inhibit the migration of HCT-116 cells.

6.2 Future Recommendation

For future studies, it is recommended that other metastasis assays such as transwell assay, apoptosis assay, invasion assay and others can be conducted in order to evaluate the anti-migratory effect of this extract. This is because metastasis is a multi-complex mechanism which consists of several processes such as invasion, angiogenesis, migration and others. Meanwhile, there is a wide range of *in-vitro* assays or techniques that have been developed to access and provide the initial platform for cancer drug discovery approaches. Therefore, with the quantification and proves from other assays, this extract can be further confirmed that it exhibiting anti-migratory effects which is effective for inhibiting metastasis.

Since there are many bioactive compounds that shown to be contributed to the anti-proliferative of this extract, it is also suggested that a high throughput screening (HTS) drug discovery can be carried out to investigate the most potent compound or even identifying the pharmacophore of the compound (Macarron et al., 2011). On the contrary, a computer-aided drug design (CADD), a combination of advanced computational approaches, biological science and chemical synthesis also can be used to facilitate the drug discovery process (Baig et al., 2018).

In the other perspective, this extract also can be used to test on other cancer cell line except for human breast cancer cell line and human colorectal carcinoma cell line. As both breast cancer cell, MCF-7 and colorectal carcinoma cell, HCT-116 are solid-based tumour, so it is recommended that the anti-proliferative effect or other properties from this extract can be evaluated on liquid cancers such as myeloma and lymphoma.

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APPENDICES

Supplementary Table 1

Table S1: Percentage of cell viability of HCT116 cells treated with different AUFE concentrations after 24hours incubation

Concentration (mg/mL)	Cell Viability ± SEM (%)
0.00	100.00 ± 0.00^{ad}
0.50	75.24 ± 12.37^{ad}
0.75	68.79 ± 9.14^{ad}
1.00	56.21 ± 13.78^{abcd}
1.25	53.82 ± 4.45^{bc}
1.50	48.94 ± 1.9^{bc}
1.75	32.48 ± 3.91^{bc}
2.00	15.6 ± 6.43^{bc}

Data were expressed as mean ± SEM of two independent experiments. Results were analysed by using One-way ANOVA followed by post hoc Tukey HSD test. Different superscript letter within groups indicated significant difference ($p<0.05$).

Supplementary Table 2

Table S3: Percentage of cell viability of HCT-116 in each independent experiment

Concentration (mg/mL)	Percentage of cell viability (%)		Cell Viability ± SEM (%)
	Trial 1	Trial 2	
0.00	100	100	100.00 ± 0.00^{ad}
0.50	87.6	62.87	75.24 ± 12.37^{ad}
0.75	77.93	59.65	68.79 ± 9.14^{ad}
1.00	69.98	42.43	56.21 ± 13.78^{abcd}
1.25	49.37	58.26	53.82 ± 4.45^{bc}
1.50	50.83	47.04	48.94 ± 1.9^{bc}
1.75	36.38	28.57	32.48 ± 3.91^{bc}
2.00	22.03	9.17	15.6 ± 6.43^{bc}

Data were expressed as mean ± SEM of two independent experiments. Results were analysed by using One-way ANOVA followed by post hoc Tukey HSD test. Different superscript letter within groups indicated significant difference ($p<0.05$).

Supplementary Table 3

Table S3: Percentages of cell inhibition of HCT116 cells treated with AUFE after 24 hours incubation period

Concentration (mg/mL)	Cell Inhibition ± SEM (%)
0.00	0.00 ± 0.00^{a}
0.21	25.97 ± 14.01^{ac}
0.42	78.89 ± 5.75^{b}
0.63	68.82 ± 9.88^{bcd}
Sunitinib 2µg/mL	100.00 ± 0.00^{bd}

Data were expressed as mean ± SEM of two independent experiments. Results were analysed by using One-way ANOVA followed by post hoc Tukey HSD test. Different superscript letter within groups indicated significant difference (p<0.05).

Supplementary Table 4

Table S4: Percentage of wound closure of HCT116 cells treated with different concentrations of AUFE after 24 hours in scratch assay analysis.

Concentration (mg/mL)	Percentage of wound closure (%)		Mean of wound closure (%)
	Trial 1	Trial 2	
0.00	15.63	18.24	16.94
0.21	13.67	10.95	12.36
0.42	2.4	4.9	3.65
0.63	3.33	7.49	5.41
2µg/mL of sunitinib	-11.67	-29.22	-20.45