TUBULIN-TARGETING ACTIVITY OF PHYTOESTROGENS PRESENT IN *COSMOS CAUDATUS* AQUEOUS EXTRACT AGAINST COLORECTAL CANCER CELL LINE HT-29

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by

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Thank my parents, my grandma, my undergraduate advisors and May 2014.

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LIST OF SYMBOLS

%	Percent
µl	Microlitre
µM	Micromolar
h	Hour
mg	Milligram
min	Minute
ml	Milliliter
nM	Nanomolar
℃	Degree Celsius

LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
AF488	Alexa Fluor 488
AF647	Alexa Fluor 647
BCL2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
CO_2	Carbon dioxide
CDK	Cyclin-dependent kinases
CRC	Colorectal cancer
CFDA	China Food and Drug Administration
C. caudatus	*Cosmos caudatus*
DAPI	4', 6-diamidino-2-phenylindole
ddH_2O	Deionised distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ERK	Extracellular-signal-regulated kinase
FBS	Foetal bovine serum
G1	Gap 1 phase
G2	Gap 2 phase
HADCs	Histone deacetylases
HPLC	High performance liquid chromatography
IC_{50}	Half maximal (50%) inhibitory concentration
JNK	Jun N-terminal kinase
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LC3-II	LC3-phosphatidylethanolamine conjugate
M	Mitotic phase
MTA	Microtubule-associated agents
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mTOR	mammalian target of rapamycin
NF-kB	Nuclear factor kappa B
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
Pen-strep	Penicillin-Streptomycin
PFA	Paraformaldehyde
PI	Propidium iodide
ROS	Reactive oxygen species
rpm	Revolutions per minute
S	Synthesis phase
VEGF	Vascular endothelial growth factor
EGFR	Epidermal growth factor receptor
α	Alpha
β	Beta

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AKTIVITI PENYASARAN TUBULIN BAGI FITOESTROGEN DALAM EKSTRAK AKUES *COSMOS CAUDATUS* KE ATAS SEL KANSER KOLOREKTAL HT-29

ABSTRAK

Cosmos caudatus (*C. caudatus*) merupakan tumbuhan perubatan di Asia Tenggara yang mengandungi fitoestrogen, lakton sesquiterpene dan mikronutrien penting. Fitoestrogen telah dilaporkan mempunyai sifat farmakologi termasuk aktiviti antikanser. Dalam kajian ini kami berhasrat untuk menyiasat aktiviti penyasaran tubulin bagi fitoestrogen utama yang terdapat dalam *C. caudatus* bersama dengan ekstrak akueus *C. caudatus* dalam sel kanser kolorektal HT-29. Ekstrak akueus *C. caudatus*, kaempferol dan kuersetin telah digunakan untuk merawat sel HT-29 selama 24, 48 dan 72 jam dalam ujian 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) untuk menilai kesan rawatan terhadap daya maju sel. Kaempferol dan kuersetin menunjukkan aktiviti sitotoksik dalam sel HT-29 dengan nilai kepekatan penghambatan (IC_{50}) masing-masing pada 143 μM dan 153 μM (43.5 μg/ml dan 46.2 μg/ml), manakala nilai IC_{50} bagi ekstrak akueus *C. caudatus* telah ditentukan pada 150 μg/ml. Sebagai tambahan, kaempferol dan kuersetin telah menunjukkan penghambatan ke atas penghijrahan sel HT-29 dengan mengurangi secara ketara 31.6% dan 32.5% penutupan jurang dalam ujian calar. Sebaliknya, rawatan ekstrak akueus *C. caudatus* pada sel HT-29 tidak mengubah keupayaan penghijrahan sel. Ujian penyebaran sel telah dijalankan untuk menyiasat sama ada perencatan penghijrahan sel yang diperhatikan adalah disebabkan oleh perubahan dalam proses penyebaran sel. Pada nilai IC_{50}, ekstrak akueus *C. caudatus*, kaempferol dan kuersetin mengurangi dengan ketara luas sel dan menambah

lingkaran sel HT-29. Perubahan luas sel dan lingkaran juga telah didapati dalam rawatan dengan kepekatan yang lebih rendah. Penemuan ini mencadangkan bahawa perencatan penghijarahan sel mungkin disebabkan oleh perubahan dalam proses penyebaran sel, selaras dengan organisasi mikrotubul yang berubah semasa penyebaran sel HT-29. Analisis lanjut berkenaan ekspresi tubulin mendedahkan bahawa rawatan ekstrak akueus *C. caudatus*, kaempferol dan kuersetin pada sel HT-29 mengurangi dengan ketara ekspresi α-tubulin dan β-tubulin. Menariknya, perkembangan kitaran sel juga terganggu dengan peningkatan pengumpulan sel dalam fasa G1. Hal ini menyarankan bahawa rawatan ekstrak akueus *C. caudatus*, kaempferol dan kuersetin boleh mengganggu dinamik mikrotubul seterusnya mengakibatkan pembantutan kitaran sel. Secara ringkasnya, ekstrak akueus *C. caudatus* dan fitoestrogen utamanya, kaempferol dan kuersetin, menggunakan sifat-sifat antikanser mereka dengan merencat penghijraham sel, mengubah proses penyebaran sel dan menggalakkan pembantutan kitaran sel dengan menyasarkan tubulin. Penemuan ini membawa kita lebih dekat untuk memahami mekanisme ekstrak akueus *C. caudatus* dan fitoestrogen utamanya ke arah menghasilkan agen penyasaran mikrotubul yang berkesan untuk rawatan kanser.

TUBULIN-TARGETING ACTIVITY OF PHYTOESTROGENS PRESENT IN *COSMOS CAUDATUS* AQUEOUS EXTRACT AGAINST COLORECTAL CANCER CELL LINE HT-29

ABSTRACT

Cosmos caudatus (*C. caudatus*) is a medicinal plant in Southeast Asia that contains phytoestrogens, sesquiterpene lactones and essential micronutrients. Phytoestrogens have been reported to possess pharmacological properties including anticancer activity. In this study we aimed to investigate tubulin-targeted activity of main phytoestrogens present in *C. caudatus* along with *C. caudatus* aqueous extract in colorectal cancer cell line HT-29. *C. caudatus* aqueous extract, kaempferol and quercetin were used to treat HT-29 cells for 24, 48 and 72 hours in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the effects of the treatments on cell viability. Kaempferol and quercetin showed cytotoxic activity in HT-29 cells with inhibitory concentration (IC_{50}) value of 143 μM and 153 μM (43.5 μg/ml and 46.2 μg/ml), respectively, while the IC_{50} value of *C. caudatus* aqueous extract was determined at 150 μg/ml. In addition, kaempferol and quercetin showed inhibition against HT-29 cell migration by significantly reducing 31.6% and 32.5% gap closure in scratch assay. In contrast, *C. caudatus* aqueous extract treatment in HT-29 cells did not alter cell migratory ability. Cell spreading assay was conducted to investigate whether inhibition in cell migration observed was due to alteration in cell spreading process. At IC_{50} value, *C. caudatus* aqueous extract, kaempferol and quercetin significantly reduced cell area and increased circularity in HT-29 cells. Changes in cell area and circularity had also been found in lower concentration of treatments. This finding suggests that cell migration inhibition could

be due to alteration in cell spreading process, consistent with altered microtubule organization observed in spreading HT-29 cells. Further analysis on expression of tubulin revealed that *C. caudatus* aqueous extract, kaempferol and quercetin treatment in HT-29 cells significantly reduced the expression of α-tubulin and β-tubulin. Intriguingly, cell cycle progression was also interrupted with an increased cell accumulation in G1 phase. This suggests that *C. caudatus* aqueous extract, kaempferol and quercetin treatment may perturb microtubule dynamics hence resulted in cell cycle arrest. In summary, *C. caudatus* aqueous extract and its main phytoestrogens, kaempferol and quercetin, exert their anticancer properties by inhibiting cell migration, altering cell spreading process and promoting cell cycle arrest by targeting tubulin. These findings bring us closer to understanding the mechanisms of *C. caudatus* aqueous extract and its main phytoestrogens towards developing effective microtubule-targeting agents for cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Research background

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States and the fifth worldwide (Benson et al., 2021; Sung et al., 2021). Chemotherapy, immunotherapy, and radiation therapy are typical treatments for colon cancer. In current clinical practise, capecitabine and oxaliplatin combination chemotherapy, 5-fluorouracil, irinotecan, bevacizumab, and cetuximab are examples of first-line therapy (Benson et al., 2021; Zhang et al., 2019). Combination therapy is a new technique for achieving better treatment outcomes that aims to achieve stronger treatment benefits as exemplified in Riahi-Chebbi's study (2019), it can also reduce medication resistance issues, and minimise unwanted effects following cancer treatment. For instance, it has been thoroughly investigated that 5-fluorouracil (5-FU) negatively affects the metabolism of colon cancer cells; when kaempferol is combined with 5-FU, the drug resistance produced by 5-FU can be significantly reduced (Riahi-Chebbi, et al., 2019). Combining two or more therapeutic medications or distinct methods of therapy, such as chemotherapy, immunotherapy, and radiotherapy, constitutes combined therapy.

Paclitaxel is a well-known substance generated from nature that has been used to treat breast cancer and ovarian cancer (Gradishar et al., 2020). Paclitaxel is known to trigger cell death and limit cell migration in numerous cancer types (Peronne et al., 2020). Paclitaxel is thought to exert its anticancer effects via stabilising microtubules and altering their dynamic characteristics by binding to a microtubule component (Peronne et al., 2020; Mukhtar et al., 2014). Paclitaxel and other chemical substances

that bind to tubulin and interfere with microtubule characteristics and functions are known as microtubule-targeting drugs (MTAs).

Due to their easily modifiable architectures and inexpensive manufacture, numerous research have been conducted on small compounds as possible MTAs. Phytoestrogens are tiny polyphenolic compounds that have been researched extensively for their anticancer effects. Other phytoestrogens have indirect effects on microtubule stability, whereas certain phytoestrogens have been observed to bind directly to tubulin and change microtubule dynamics.

Numerous traditional medicinal herbs, including *Cosmos caudatus*, are reported to be rich in flavonoids, particularly quercetin and kaempferol (Andarwulan et al., 2019). Due to their structural resemblance to oestrogen, these chemicals are categorised as phytoestrogens and are hence capable of binding to oestrogen receptors. Other investigations have demonstrated that *C. caudatus* has anticancer capabilities by increasing cell death and inhibiting cell migration in colorectal cancer cells (Moshawih et al., 2017). However, the mechanisms underlying these biological actions of *C. caudatus* remain poorly understood.

1.2 Problem statement

Management of CRC using conventional chemotherapy becomes an economic burden to the country (Simon, 2016). There are major drawbacks of chemotherapy such as drug resistance and toxic effects on non-targeted tissues despite it offers a relief from CRC symptoms. Many studies reported the potential of natural substances that exert selective toxicity against CRC cells and may overcome the drug resistance issue (Riahi-Chebbi et al., 2019; Chakrabarty et al., 2019; Wang et al., 2015). Among the studies carried out, phytoestrogens have been suggested as promising candidates to be developed as MTAs to combat cancer (Xu et al., 2022). Besides, among studies of *C. caudatus,* this plant showed properties as anticancer and antiatherosclerosis by testing its cytotoxic effect as well as antioxidant activity on cancer cells or some animal tissue cells (Moshawih et al., 2017, Sia et al., 2020, Nurhayati et al., 2018, Fauzia et al., 2016). Scanty studies had focused on CRC cells and especially on the activity of cancer cell cytoskeleton, that is also the reason this study decided to throughly figure out the role of microtubule movement in CRC study.

Thus, development of alternative therapy for CRC using local resources that have high content of phytoestrogens is relevant for positive socioeconomic consequences. This study aims to gain a better understanding of the mechanisms that may explain how *C. caudatus* aqueous extract and its main phytoestrogen constituents impact the microtubule organization hence influence cellular processes regulated by microtubules. It is anticipated that promising findings from this study may contribute to a more effective therapy for CRC and at the same time accelerate the country's economic growth.

1.3 Hypothesis

Previous studies have reported the inhibitory activity of *C. caudatus* on CRC cell proliferation and migration (Moshawih et al., 2017). However, there is a meagre understanding to explain and justify the anti-colorectal cancer activities exhibited by *C. caudatus*. Given that *C. caudatus* comprises of bioactive compounds which are known to interrupt microtubule functions, it is to be presumed that *C. caudatus* aqueous extract treatment in CRC HT-29 cells may alter microtubule organization hence impair the proliferation and migration activities of the cancer cells.

1.4 Objectives

1.4.1 To examine the effects of *C. caudatus* aqueous extract, quercetin and kaempferol on HT-29 cell viability

1.4.2 To investigate the effects of *C. caudatus* aqueous extract, quercetin and kaempferol on HT-29 cell morphology and migratory activity

1.4.3 To evaluate the effects of *C. caudatus* aqueous extract, quercetin and kaempferol on α-tubulin and β-tubulin expression in HT-29 cell

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

2.1.1 Definition of colon

Colon is the end of large intestine where is also combined with rectum, cecum and anal canal according to Gray's Anatomy (Standring, 2019). Colon can be divided into ascending colon, transverse colon, descending colon, sigmoid colon and rectum (Mahadevan, 2019). The function of colon is mainly to maintain nutrient absorption and excrete food wastes (Genton et al., 2015).

2.1.2 Colorectal cancer

Colorectal cancer (CRC) or colon cancer is now the second major cause of death in United States and occurs less common in Asia compared with other area. It is estimated that nearly up to 1.9 million occurred cases in year of 2020 worldwide (Benson et al., 2021; Deng & Li, 2019; Zhang et al., 2019; Xi & Xu, 2021). CRC progresses from precancerous polyp with the accumulation of genetic and epigenetic alterations (Simon, 2016). When the polys get neovascularized and linked with lymph, the polyps become pre-cancer form, in which dividing cells in the polyps can pass the bowel wall and invade to nearby organs as well as more metastatic sites thus form the final stage of cancer (Figure 2.1). Cancer progression often takes several years (Simon, 2016).

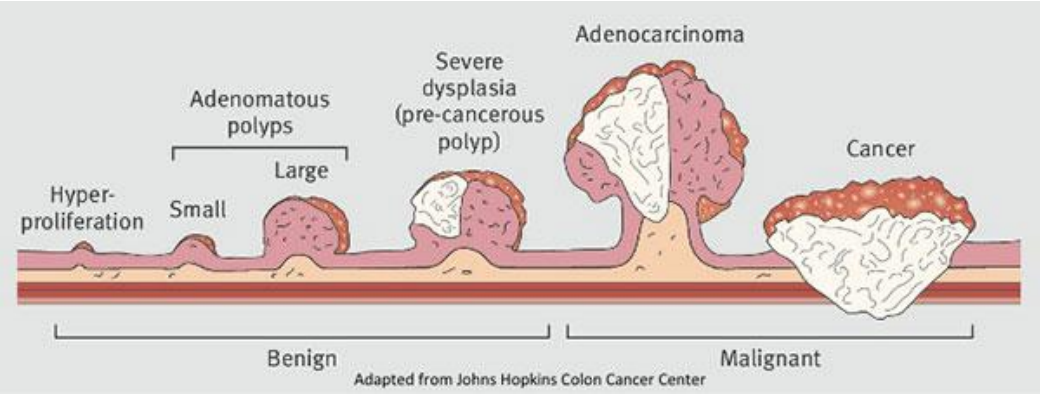

Figure 2.1 Colon cancer development (adapted from Wynder, 2016).

Among all CRC cell lines, the most common types used in experimental works are HCT-116, HCT-8, HT-29, SW-620 and SW-480. They are in different stages of cancer, for example HT-29 is from primary tumor while SW-480 and SW-620 are metastatic colon carcinoma-derived cell lines (Martinez-Bernabe et al., 2023).

2.1.3 Colorectal cancer therapy

Nowadays, capecitabine plus oxaliplatin (shortly named by CAPEOX) is one of the first-line colon cancer chemotherapy in clinical practice and it is commonly utilized in phase III trial. Capecitabine is transferred into 5-Fluorouracil (5-FU) in the body to fight against cancer cells. The mechanism of oxaliplatin is that they can have some cross-linking interactions with guanine residues or the link between guanine and adenine to affect DNA replication and transcription (Martinez-Balibrea et al., 2015). Besides, oxaliplatin shows an increased incidence of neuropathy and gastrointestinal upset (Benson et al., 2021). Because colon is the end of digestive tract, patients have to suffer from side-effect such as diarrhea. Additionally, many phytoestrogens are found to reduce neuron damage via controlling the tight

association between LC3 and Tau in cell autophagy and they have been confirmed such neuroprotection effects in Alzheimer and Parkinson treatment (Hermenean & Ardelean, 2017). Thus, phytoestrogens can be considered as a supplement to control neurological disease in colon cancer treatment though some traditional MTAs might lead to neuropathy.

It has been reported in other studies that 5-FU chemotherapy induces resistance in colon cancer and kaempferols have been observed to diminish the 5-FU drug resistance in LS174 cells (Riahi-Chebbi, et al., 2019). When kaempferols are used together with 5-FU, they exerted a stronger synergistic effect than 5-FU alone to inhibit cell viability. Drug resistance of 5-FU is a result of altered 5-FU metabolism in colon cancer (Riahi-Chebbi, et al., 2019). Given the potential of kaempferol in inhibiting ROS production and influence pathwayssuch as MAPK, PI3K/AKT and NF-κB, introduction of kaempferol with 5-FU may influence 5-FU metabolism to some extent hence overcome drug resistance. In addition, effects of kaempferol on microtubules and microtubule-associated factors also suggest the potential of chemo-suppression.

Additionally, fruquintinib is a newly discovered drug in colon cancer chemotherapy. CRC progression involves vascular endothelial growth factor (VEGF) regulation and fruquintinib has been found to inhibit vascular endothelial growth factor receptor (VEGFR) tyrosine kinase and suppress VEGFR phosphorylation in vivo and in vitro, hence prevent tumor angiogenesis (Zhang et al., 2019). Fruquintinib has already been approved by China Food and Drug Administration (CFDA) in metastatic CRC clinical treatment on September 4, 2018 (Zhang et al., 2019). It is a remedy for mCRC patients who failed in the standard oxaliplatin and

irinotecan therapies (Deng & Li, 2019). Besides, fruquintinib also shows inhibition in lung cancer and gastric cancer as well as manageable toxicity (Zhang et al., 2019).

Apart from chemotherapy, there is immunotherapy such as cetuximab, panitumumab and bevacizumab, which are targeted monoclonal antibodies. Cetuximab and panitumumab are targeting epidermal growth factor receptor (EGFR) signaling pathway while bevacizumab blocks VEGF (Benson et al., 2021). The effects of phytoestrogen on microtubule-associated factors suggest there could be more targets for future immunotherapy. Meanwhile, phytoestrogens also have great potentials in systemic therapy.

Chemotherapy, immunotherapy and radiotherapy are the common methods in cancer treatment worldwide, however they have been associated with adverse effects. The limitations of chemotherapy include causing damage to healthy tissues, induce inflammation as well as patient suffering from drug resistance (Wang et al., 2015). Microtubule targeted agents (MTAs) bind at specific microtubule domains such as paclitaxel binding domain and colchicine binding domain to exert their anticancer effects. At the same time, MTAs have been reported to cause side effects such as neutropenia, peripheral neuropathy, hair drop, gastrointestinal upset and MDR (Mukhtar et al., 2014). For immunotherapy, the common limitation of the treatment is due to expensive cost that becomes an economic burden to the patient and country (Xu et al., 2022). Moreover, side effects of radiotherapy including nausea, gastrointestinal upset and vomiting may lead to a more serious problems to the patient's health (Lee et al., 2012; Giglio & Gilbert, 2003). Most naturally-derived compounds may cause minimal side effects compared with synthetic compounds because they are more biodegradable and edible (Affat, 2021). Given that they have

great potentials against cancer development, it is worthwhile to explore promising chemotherapeutics from natural products.

2.2 Microtubule cytoskeleton

2.2.1 Component and structure

Microtubules are made up of polymerized dimers, which consist of α and β tubulin, and weight around 55 kDa. They fold into helix structure as shown in Figure 2.2, and further form into long hollow cylinders (Sun et al., 2021). Apart from α and β tubulin, there are also γ, δ and ε tubulin families that mainly present in the centrosome (Čermák et al., 2020). In eukaryotes, microtubules play an important part in the formation of cytoskeleton, they extend from nucleus to the edge of cell as shown in Figure 2.3. They control vital cellular activities ranging from cell movement to intracellular transport, for example, they can segregate chromosomes via spindle stretch during cell division (Čermák et al., 2020). While in protozoans, microtubules are the structural components that form flagella and cilia.

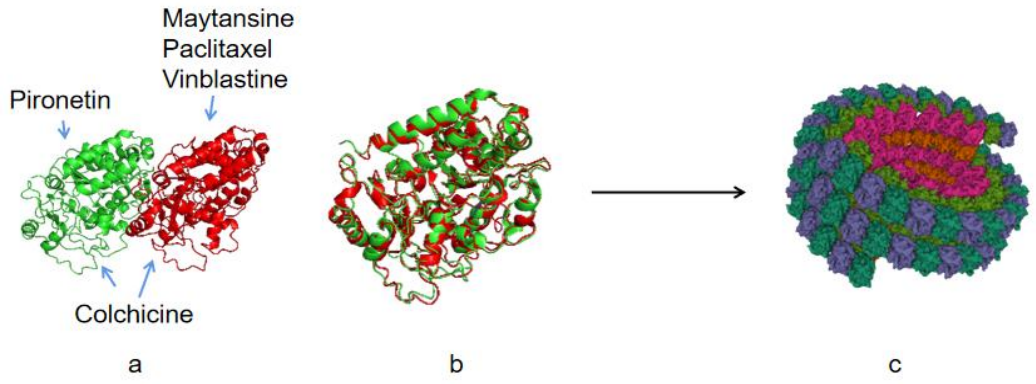

Figure 2.2 (a) Green chain is α-tubulin, red chain is β-tubulin; (b) Tertiary structure of α-tubulin and β-tubulin alignment; (c) Microtubule polymerization structure, 6B0C complex, polymerized with α tubulin, β tubulin and kinesin-like protein (adapted from Benoit et al., 2018)

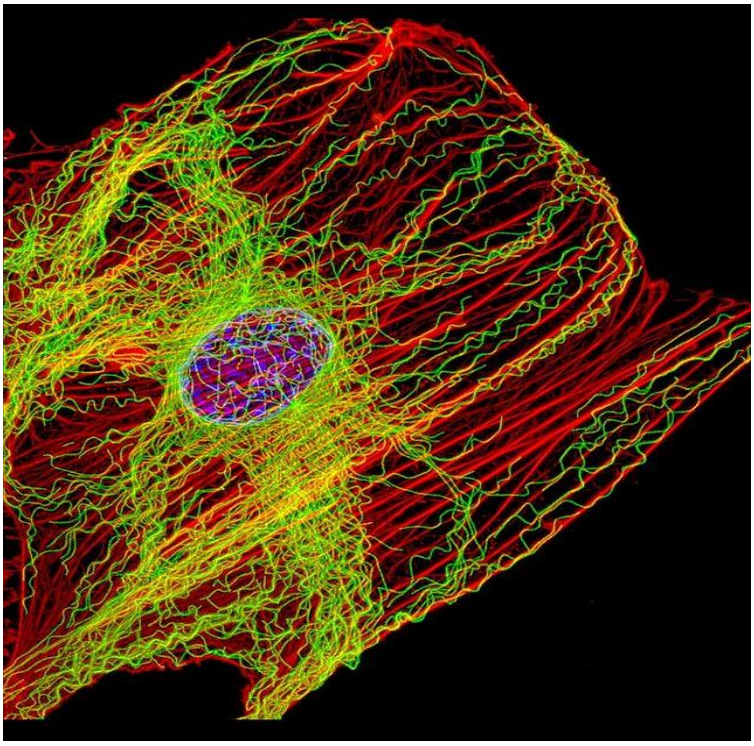

Figure 2.3 Microtubule structure of fibroblast cell. Actin is stained in red and microtubules in green (adapted from Faust & Capco, 2014)

2.2.2 Regulation and dynamics

Microtubules have two main dynamic states, polymerization and depolymerization. Such change of balance between the two states regularly happen in cellular processes, such as cell cycle and cell division. They are dynamic and usually possess some unstable states, for example, treadmilling and bundling during changes (Venkatramani & Panda, 2019). Moreover, Tau proteins, encoded by microtubule-associated protein Tau (MAPT), are essential in stabilizing microtubules. Abnormality occurring in Tau could lead to confusion in composition function of tubulins that further induce microtubule decomposition and influence regular intracellular transport in the neuron (Meng et al., 2021). When hyperphosphorylated

Tau and amyloid-β aggregate insolubly, this reflects the typical feature of Alzheimer's Disease (Rahman et al., 2020).

Tubulin binding sites are being the target of various compounds that can lead to interruption of the microtubule stability (Sun et al., 2021). Most of the tubulin binding sites are located on either α or β tubulin, except the vinca alkaloid binding site which is located at the α, β-heterodimer interface. The paclitaxel and laulimalide/peloruside binding site are discovered with polymerization property (Sun et al., 2021). The site of vinblastine and maytansine are on β-tubulin, but colchicine binds to the surface of α-tubulin and β-tubulin. Additionally, pironetins are found to covalently modify α-tubulin through the unsaturated lactone and potently inhibit microtubule polymerization (Coulup et al., 2019). More α-tubulin binding compounds are waiting to be further disclosed in future.

Paclitaxel is the first natural product discovered to exert microtubular stabilizing effect. Its derivatives, including docetaxel, cabazitaxel and nab-PC, have been commonly used in clinical treatment such as ovarian, prostate and terminal breast cancer therapy. Interaction of paclitaxel with tubulin leads to microtubule polymerization (Peronne et al., 2020). While colchicine, vinblastine, maytansine and their derivatives exhibit depolymerization function (Peronne et al., 2020). Additionally, more novel stabilizing agents, such as epothilone, discodermolides, wangzaozin A and taccalonolides, are increasingly being investigated (Sun et al., 2021; Chen et al., 2021; Yang et al., 2022).

Besides, due to more researches studying on microtubule binding sites, understanding of the mechanism of paclitaxel binding domain, vinblastine binding domain and colchicine binding domain is enhanced. Among the controls on these domains, they usually can lead to some side effects such as neutropenia, peripheral

neuropathy, MDR sensitive, hair drop and constipation in clinical use, even sometimes the treatment might damage normal cells (Mukhtar et al., 2014). In summary, among all the binding sites on microtubules, it is easy to notice that α tubulin is more unrevealed than β tubulin. Therefore, it is necessary to find more unexplored sites for domains on α tubulin in further studies.

2.2.3 Role in cell growth and movement

Cell growth involves cell cycle progression in which increase of cell mass such as DNA synthesis and protein synthesis occurs (Williams & Stoeber, 2011). Cell cycle has four phases which are G1 phase, S phase, G2 phase (also known as interphase) and mitotic (M) phase (Williams & Stoeber, 2011). DNA synthesis happens in S phase and cell growth in G2 phase. Propidium iodide (PI) staining is a common method used in experimental work to detect DNA content in nuclei. Flow cytometry is a technique that can be used to measure DNA content in mitotic phase and analyze specific phases of the cell cycle.

The mitotic phase begins with interphase, prophase, metaphase, anaphase, telophase and cytokinesis as shown in Figure 2.4. Cell division happens in telophase when a cell divides into two daughter cells. The spindles form during prophase is responsible to separate chromatin in later phase. This reflects the important function of microtubules in cell growth. They participate in cell division by forming the mitotic spindles that pull eukaryotic chromosomes apart during mitotic phase (Vale, 2003). Since MTAs may bind to tubulin and alter microtubule stability, it is of great potential to use the MTAs to combat cancer development through cell-cycle aberrations of the cancer cells.

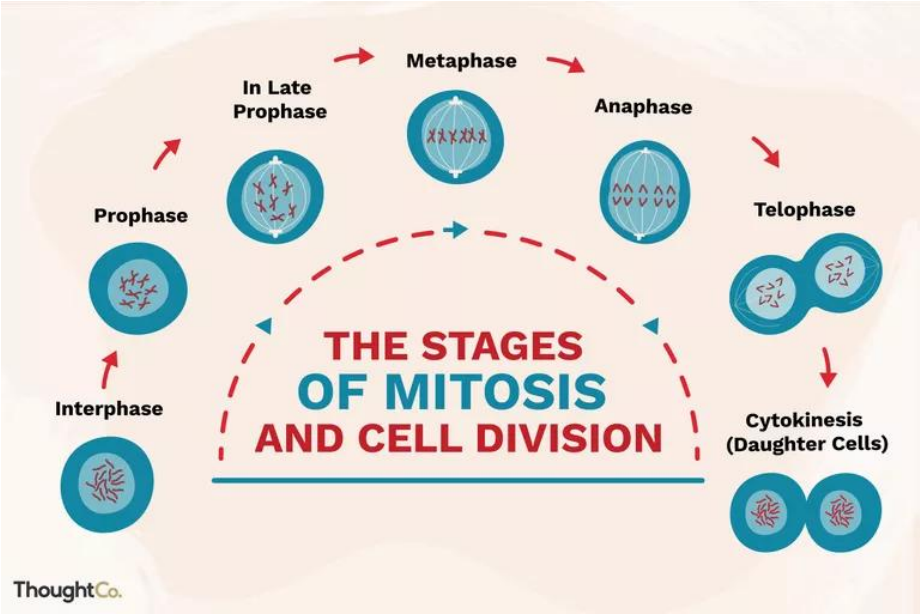

Figure 2.4 Image of mitosis (adapted from Bailey, 2019)

Apoptosis refers to programmed cell death, which is regulated by serious of signaling pathways and apoptosis-related protein such as AKT/mTOR pathway, Bcl-2 family and LC3 (Abotaleb et al., 2018; Jiang et al., 2019). Apoptosis is associated with DNA fragmentation, mRNA degradation, chromatin condensation, cell shrinkage and cell break-down (Abotaleb et al., 2018). Since many reported anticancer agents have been found to induce apoptosis, studying the effects of promising therapeutic compounds on apoptosis has become increasingly popular in the search for cancer cures.

2.2.4 Role in cell migration and cell spreading

Cell migration is crucial for cell growth and maintenance of physiological function. It is triggered by intracellular signalling and extracellular matrix components such as fibrillar structure (Mak et al., 2016), which involves the movement of microtubules. There are four steps for cell to migrate as shown in Figure 2.5 which are; first, formation of lamellipodia and filopodia; second, formation of new adhesions between the cell and matrix; third, the cell shrinks by pulling of actin filaments; fourth, the cell moves forward by release the adhesion of tail with the matrix (Lou et al., 2021). Cytoskeleton morphology changes occur in the first step of cell migration. Cell spreading refers to condition when the suspension of cells firstly attaches onto the substrate and further turn into flatten morphology. This event is the early stage of cell-matrix adhesion and rearrangement of cytoskeletons (Ng et al, 2019). Therefore, microtubules as main component of cytoskeletons, play a vital function in cell migration and cell spreading.

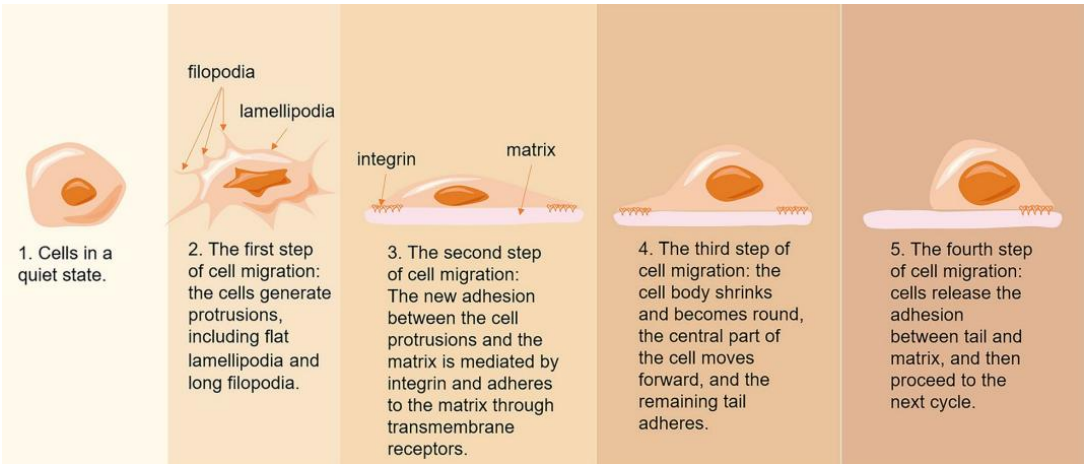

Figure 2.5 Image of cell migration (adapted from Lou et al., 2021)

2.3 *Cosmos caudatus* (Ulam raja)

Cosmos caudatus (*C. caudatus*) from Asteraceae family is a native plant widely cultivated in Indonesia and Malaysia as shown in Figure 2.6. The fresh leaves of this plant are usually consumed raw. According to folk medicine, *C. caudatus* has great potentials as anticancer, anti-parasites, antioxidant, anti-inflammation, anti-allergy, anti-mutagen and anti-tumorgenicity, it also potentially improves blood circulation, promotes bone formation and ameliorates infectious diseases (Safitri et al., 2020; Sharifuldin, 2014; Moshawih et al., 2017). Scientific research findings suggest the benefits of *C. caudatus* as therapy for hypertension and diabetes (Chan et al., 2016). The plant contains phytochemical compounds such as phytoestrogens, sesquiterpene lactones and essential micronutrients (Ramadhan et al., 2018; Moshawih et al., 2017). Anticancer agents such as curcumin and lutein are also the main compounds present in *C. caudatus*, by which they target factors in carcinogenesis pathway including initiator caspases (Safitri et al., 2020). Other components, such as rutin, quercitrin and quercetin, are present in approximately 0.13-0.94%, 1.51-13.78% and 0.18-0.92% in the extract of *C. caudatus* when evaluated by high performance thin layer

chromatography (HPTLC) analysis (Sharifuldin, 2014). Quercetin and its derivatives exhibit strong antioxidant properties to reduce cellular oxidative stress, and there are more than 20 other antioxidants identified in *C. caudatus* by similar study (Sharifuldin, 2014). In vitro study of *C. caudatus*, it is one of common methods to test its anticancer effects by antioxidant activity assay. Extract/fractions of *C. caudatus* show favourable antioxidant activity in nomral muscle cells and cancer cells such as cervical cancer and leukaemia cells though the antioxidant potency may be weaker than pure component (Moshawih et al, 2017; Nurhayati et al, 2018; Yahya & Latif, 2022).

Figure 2.6 Photo of *C. caudatus* (adapted from Chan et al., 2016)

2.4 Phytoestrogen

2.4.1 Structure and classification

Phytoestrogens are naturally occurring compounds that have similar molecular structures to human estrogen (Figure 2.8). This characteristic allows phytoestrogens bind to estrogen receptors and exert estrogenic activity (Sayed & Elfiky, 2018). Phytoestrogens can be found in diverse food sources such as soy products, coffee, tea,

wine, fruits and many plant species (Torrens-Mas & Roca, 2020; Kashyap et al., 2017). They are also present in popular medicinal plants like *Pueraria mirifica* and red clover (Lee et al., 2002; Geller et al., 2009), which are utilized for the treatment of particular diseases. Phytoestrogens can be classified as flavonoids and non-flavonoids (Figure 2.7). Flavonoids comprise of several main subtypes, namely prenylflavonoids, coumestans, isoflavones, flavones, chalcones and anthocyanidins. While for non-flavonoids, the well-known groups are stilbenes, lignans and tannins, which are commonly found in nuts, legumes and whole grains (Das & Devi, 2019; Nichenametla et al., 2006).

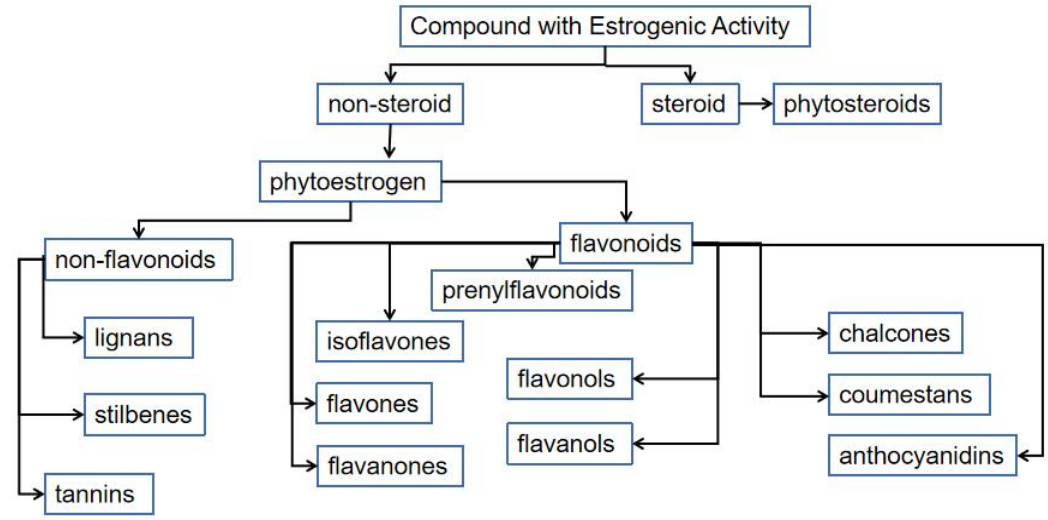

Figure 2.7 Types of phytoestrogens

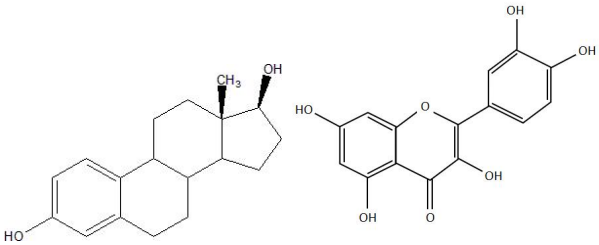

Figure 2.8 Chemical structure of 17β-estradiol (left) and quercetin (right)

Many studies have reported on phytoestrogens ability in combating cancer through activities ranging from reducing tumor cell growth to inhibiting cancer cell migration and triggering cell death. For example, silibinin has been shown to prevent migration of metastatic breast cancer cells (Lashgarian et al., 2020) while some lignans suppress mitotic spindle formation and tubulin polymerization in lung adenocarcinoma, ovarian cancer, and neuroblastoma-derived cells (Esfandiari et al., 2017).

2.4.2 Role of phytoestrogen in cancer therapy

Many studies have been carried out to evaluate cancer preventive effects of phytoestrogens and the promising findings thereby offer alternative treatment against cancer. This part summarizes the role of phytoestrogens in various cancer cells focusing on their effects on microtubule properties and functions. Phytoestrogens may either promote microtubule polymerization or depolymerization. They are able to interact directly with tubulin at different sites hence interrupt the microtubule stability and properties in different ways. For instance, quercetin and combretastatin A4 are found to bind at the colchicine site of tubulins and perturb microtubule polymerization in prostate, breast and lung cancer cells (Wright et al., 2012; McNulty et al., 2015), which have a similar anticancer mechanism as paclitaxel affecting microtubule dynamics. Besides, for podophyllotoxin derivatives, they can bind on α-tubulin and lead to disruption of microtubule polymerization in breast cancer cells (Khaled et al., 2017). Phytoestrogens may also interact with microtubule-associated proteins and influence signaling pathways such as MAPK/ERK pathway, histone deacetylase (HDAC), 1A/1B-light chain 3 (LC3) and cyclin-dependent kinases (CDK) family in many cancer cells (Talib et al., 2020;

Jiang et al., 2019; Bai et al., 2018; Boojar et al., 2020; Sundaram et al., 2018). Through regulating microtubule-associated factors, phytoestrogens affect microtubule movement and formation, cell migration, cell proliferation, cell apoptosis and cell cycle (Imran et al., 2019; Abotaleb et al., 2018; Talib et al., 2020).

2.4.3 Phytoestrogens in *C. caudatus*

Kaempferol and quercetin are the main flavonoids found in *C. caudatus,* in which they comprise more than 60% of the total flavonoids as evaluated by HPLC analysis (Andarwulan et al., 2010). *C. caudatus* ethanolic extracts contain quercetin in a higher content than kaempferol (Reihani et al., 2016). Quercetin is a bioactive flavonoid that has been reported to influence various signaling pathways and cytoskeleton components (Rauf et al., 2018). Besides, quercetin and kaempferol has the ability to work against oxidative stress, which is associated with cancer suppression reported in breast cancer, colon cancer, lung cancer, prostate cancer and cervical cancer (Kashyap et al., 2017).

Quercetin has been found to bind at the colchicine site of tubulin and interact with two cysteine residues. The binding stimulates microtubule depolymerization and causes cell cycle arrest at G/M phase in prostate and breast carcinoma cells (Almatroodi et al., 2021; Wright et al., 2012). Moreover, quercetins are found to inhibit cell proliferation, promote G1 and G2 phase arrest by decreasing cyclin D1 expression and alter microtubule arrangement in colorectal cancer (CRC) cells (Hashemzaei et al., 2017; Zhao et al., 2017). Other studies have reported quercetin induces caspase-dependent apoptosis through MAPKs pathway via phosphorylation of MAPK signaling pathway and p53 (Hashemzaei et al., 2017; Kee et al., 2016; Yang et al., 2019; Darband et al., 2018). Conversely, quercetins diminish the efficacy

of microtubule-targeting drugs such as taxol and nocodazole in arresting HCT116 cells at G2/M (Chahal et al., 2018, Samuel et al., 2010), which might be owing to competitive effects between quercetins and these compounds.

Kaempferol has been found to significantly disrupt the formation of tube-like structures and networks as well as exhibit anti-angiogenesis effects in human umbilical vein endothelial cells (Chin et al., 2018). By using fluorescence imaging, it has been shown that kaempferols destroy the regular networks of microtubules and alter the morphology of cell cytoskeletons in cervical cancer cells (Tu et al., 2016). Recent studies demonstrate that kaempferol induces cell cycle arrest and reduces the survival of colorectal cancer cells via the inhibition of thymidylate synthase, lowering of p-Akt activation and activation of cell autophagy (Riahi-Chebbi et al., 2019; Ashrafizadeh et al., 2019; Kim & Park, 2020). Mechanisms that involve in kaempferol-induced apoptosis and cell cycle arrest include prevention of reactive oxygen species (ROS) accumulation, activation of MAPK cascades and modulation expression of MAPK, PI3K/AKT, JAK/STAT3, H2A histone family member X (H2AX), CDK family, ERK-1/2, Bcl-2, p53 and NF-κB (Imran et al., 2019; Choi et al., 2018). In addition, kaempferol raises the amount of LC3-II in human neuroblastoma cells (Filomeni et al., 2012), where the neuroprotective effects of kaempferols are attributed to the tight association between LC3 and Tau in cell autophagy, hence becomes an alternative to overcome side effects of MTAs in clinical use.

CHAPTER 3

METHODOLOGY

3.1 Materials and equipments

3.1.1 General chemicals, media and reagents

Chemicals and reagents were purchased from respective suppliers; paclitaxel was purchased from Macklin (SH, CHN, catalogue number: p875571), kaempferol (purity > 98%) was purchased from TOCRIS (Bristol, UK, catalogue number: 3603/50), quercetin (purity > 95%) was purchased from Sigma-aldrich (Burlington, MA, USA, catalogue number: Q4951).

Cell culture items such as Fetal Bovine Serum (FBS, cytiva, AU, catalogue number: SV30160.03), 0.25% (w/v) trypsin-EDTA solution (cytiva, UT, US, catalogue number: SH30042.02) and penicillin (gibco, NY, US, catalogue number: 15140-122, 10000 U/ml)/streptomycin (10000 μg/ml) were all cell culture grade. Dulbecco's Modified Eagle's Medium (DMEM) and Dulbecco's phosphate buffered saline (D-PBS) were purchased from Sigma-Aldrich (Welwyn Garden, Herts, UK, catalogue number: D5796, D8537), cell culture grade dimethylsulfoxide (DMSO) was purchased from BioBasic (NY, US, catalogue number: D0231) and DMEM medium without phenol red was bought from Simply (Taipei, CHN, catalogue number: CC107-0500).

Colorectal cancer cell line HT-29 was generously provided by Dr Nozlena Abdul Samad (IPPT, USM).

3.1.2 Equipments

All sterile operations were performed in biosafety cabinet class 2, SASTEC ST-BSC411A (Selangor, Malaysia). Growing HT-29 cells were incubated in incubator NUAIRE (Plymouth, MN, USA) in the condition of 37 ℃, 5% CO_2. Microscope OLYMPUS IX51 under 5x (Tokyo, Japan) was used for imaging. For item storage at 4 ℃, -20 ℃ and -80 ℃, fridge LG Electronics (Seoul, Korean), Haier (Qingdao, CHN) and Sanyo Electric (Osaka, Japan) were used. Data of MTT assay was acquired using microplate reader BioTek Elx808 (Santa Clara, CA, USA). Flow cytometer used for tubulin expression and cell cycle experiments was from BD FACSCalibur (St. Louis, MO, USA). Concentrator (IKA MS 3, Selangor, Malaysia), vertex (Dragonlab MX-S, Beijing, CHN) and centrifuge Megafuge 16 (Thermo Scientific, Waltham, Massachusetts, USA) were used in necessary steps. Sterilization of consumables was done using autoclave HIRAYAMA HV-50P (Saitama, Japan).

3.2 Methods

3.2.1 Preparation of stock and working solutions

The stock of *C. caudatus* aqueous extract, kaempferol and quercetin were dissolved in DMSO/medium mixture (DMSO/medium, 1:1), and kept at -80 °C until use. Kaempferol and quercetin were diluted in cell culture medium to prepare a stock solution at 1 mg/ml. Stock solution of *C. caudatus* aqueous extract was prepared at concentration of 4 mg/ml. Working concentration for paclitaxel (positive control) was prepared at 10 nM and DMSO (<1% in cell culture) was used as vehicle control. The working concentration for crystal violet was 0.25% (m/v) and mitomycin C was 4 µg/ml. The working concentrations of PI and RNAseA in cell cycle kit were 50 µg/ml and 100 U/ml.

3.2.2 *Cosmos caudatus* aqueous extract

Cosmos caudatus aqueous extract (voucher number, USMP 11377 by Sharifuldin, 2014) was prepared by a research assistant, Fathihah Athasya Zainol Abidin. Dried *C. caudatus* leaf powder was purchased from HERBagus Sdn. Bhd., Kepala Batas, Penang. Extraction was conducted according to the method described by Seyedreihani (2017) with slight modifications. 3.5 g of powdered *C. caudatus* leaf was added into 2.5 ml of deionized water and mixed well. The mixture was swirled on the horizontal platform of an orbital shaker for 2 h. *C. caudatus* aqueous extract was obtained by filtering the aqueous mixture using Whatman No. 1 filter paper. The extraction process was repeated two times. All extract was freeze dried and stored at 4 °C until further study.

3.2.3 Culture and maintenance of HT-29 cells

HT-29 cells were grown in DMEM media containing 10% FBS/1% Pen-strep at 37 °C, 5% CO_2. HT-29 cells grown in T75 flask were trypsinized when achieved 80% -90% confluence. Old media was removed and cells were washed with PBS before the addition of trypsin. Trypsin action was neutralized by adding fresh media and the cells were spun at 1000 rpm for 5 min. Supernatant was discarded and pellet was dissolved in fresh growth media. An appropriate number of cells were placed into T75 flask and incubated at 37 °C, 5% CO_2.

3.2.4 MTT assay

3.2.4(a) Materials for MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder (TOCRIS, Bristol, UK, catalogue number: 5224/500);

3.2.4(b) Method of MTT assay

1*10^4 cell/well of cells were seeded in 96-well plate and incubated at 37 ℃, 5% CO_2 overnight. Then the treatment was added into each well. For kaempferol and quercetin, cells were treated with a series of concentrations, 15 μM, 30 μM, 60 μM, 120 μM and 240 μM (4.5 μg/ml, 9.1 μg/ml, 18.2 μg/ml, 36.4 μg/ml and 72.8 μg/ml). The concentration series for extract should be 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml and 400 μg/ml. HT-29 cells were treated for 24 h, 48 h and 72 h in three plates separately. After treatment, the treatment was removed, and fresh media with 10% MTT was added into every well. The plate was incubated for 4 h. Then the solution in every well was removed after centrifuge. DMSO was used to dissolve the purple pellet, after that detected by spectrometer at 490 nm. The rest two plates were treated by MTT and detected in the second and third days.

3.2.5 Scratch wound assay

3.2.5(a) Materials for scratch wound assay

Mitomycin C (Roche, Mannheim, Germany, catalogue number: 10107409001);

Crystal violet (Sigma-aldrich, st. Louis, MO, USA, catalogue number: C6158);

3.2.5(b) Method of scratch wound assay

Cells were seeded in 24-well plate. The plate was incubated until surface of each well fully occupied with cells and formed a single layer. Then cells were incubated in 4 μg/ml of mitomycin C at 37 ℃, 5% CO_2 for 2 h after old media was removed. Next, the scratch was made by 1 ml pipet tip in every well. Mitomycin C was removed and 0 h wells were stained by 200 μl crystal violet, then other treatments were added into the remaining wells and the plate was incubated at 37 ℃,