Abstract

Coptidis rhizoma and Euodiae fructus have been used as herbal medicine in Asian countries including China for more than two thousand years. The bioactivities of the crude extracts and isolated compounds of both herbs have been widely reported including anti-cancer activity. Dual herbal formulae combined with Coptis and Euodia with different ratio have been clinically used to treat multiple digestive system disorders. The aims of this research are to evaluate the anti-breast cancer potential of Coptis-Euodia formulae, and standard alkaloids berberine and evodiamine, associated with their possible mechanisms as anti-cancer agent candidates. Thereby, a hypothesis of "Interaction and synergy between compounds might enhance, reduce, or even revise the bioactivities of medicinal herbs and their formulae compared with bioactive components isolated from them." is to be proved.

MTT colorimetric assay, scratch wound-healing assay along with adhesion assay have been used to evaluate anti-breast cancer activities of Coptis-Euodia formulae and standard alkaloids. Cell cycle analysis along with topoisomerase I relaxation assay and topoisomerase II decatanation assay were operated to understand the anti-cancer mechanisms of them.

Anti-proliferative activity of *Zuo Jin* pill (Coptis : Euodia = 6 : 1) against both MDA-MB-231 and MCF-7 human breast cancer cell lines has been proved to be the strongest of the three dual herb combination with IC_{50} values of 0.16 and 0.19 mg/mL. Coptis-Euodia inhibited the cellular migration and adhesion ability of MDA-MB-231 cells which suggested the anti-metastasis potential. Results indicated that Coptis dominated the anti-cancer activities of Coptis-Euodia formulae with the contribution of berberine. Cell cycle analysis describes one of the anti-cancer mechanisms of Coptis-Euodia extracts. Berberine (5 μM) arrested over 60% of MDA-MB-231 cells at G0/G1 phase with 24 hrs treatment, while evodiamine (10 μM) arrested over 90% of MDA-MB-231 and 70% of MCF-7 cells at G2/M phase after 24 hrs. However, the cell cycle arresting activity of evodiamine did not reflect on the Euodia included herbal extracts. TCM formulae, with the presence of Coptis, arrested MDA-MB-231 cells at G0/G1 phase to a great extent. The effects of Coptis-Euodia extracts on DAN topoisomerase I and II further explained the possible anti-breast cancer mechanism of these herbal medicine formulae. All Coptis-Euodia extracts exhibited topoisomerase poison like activities in a concentration-dependent manner. Berberine has been confirmed to behave as a DNA intercalator at 100 μM while evodiamine did not show topoisomerase inhibition activity at the same concentration. Chapter six attempted to establish fluorescence based assay targeted sodium and calcium ion channels on cell membrane.

This study is concluded with a discussion of combination therapy potential of herbal medicine and a novel conception of "Artificial Herbal Medicine" which is expected to improve the understanding of traditional used herbal formulae and the standardization of herbal medicine.

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especially my parents and grandfather, who is 95 years old, for their financial support and endless love. I would achieve nothing without them.

Last but not least, I would like to thank myself for being patient with me.

Dedications

I would like to dedicate this research to all the scientists who are doing cancer research. I wish that man can conquer this disease in the near future. This work is also dedicated to the development of herbal medicine.

Declaration

I declare that whilst studying this degree in Biochemistry at London Metropolitan University, I have not been registered for any other award at other university. The work undertaken for this degree has not been submitted elsewhere for any other awards. The results presented in this submission are original work of the author, unless referenced to other sources.

Xinyu Li

31st July 2018

Abbreviations and Symbols

≥	Greater than or equal to
<	Less than
^{o}C	Degree Celsius
a.u.	Arbitrary units
BSA	Bovine serum albumin
C or Coptis	Coptidis Rhizoma or *Coptis chinensis* Franch.
Ca^{2+}	Calcium ions
Cav 3.2	T-type calcium channel subunit 3.2
CPT	Camptothecin
CSs	Cardiotonic steroids
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E or Euodia	Euodiae Fructus or *Euodia rutaecarpa* (Juss.) Benth.
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
ER	Estrogen receptor
FBS	Fetal bovine serum
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
hr or hrs	Hour or hours
IC_{50}	Half maximal inhibitory concentration
kDNA	Kinetoplast DNA
K^{+}	Potassium ion

LB	Lysogeny broth
LC-MS	Liquid chromatography – mass spectrometry
m-AMSA	Amsacrine
mRNA	Messenger ribonucleic acid
mins	Minutes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphemyltetrazolium bromide
Na^+	Sodium ions
Nav 1.5	Voltage-gated sodium channel subunit 1.5
Nck	Nicked DNA
NCS	Newborn calf serum
PBS	Phosphate buffered saline
PI	Propidium iodide
Rel	Relaxed DNA
s	Second
SARS	Severe Acute Respiratory Syndrome
Sc	Supercoiled DNA
S.D.	Standard deviation
SERM	Selective estrogen receptor modulators
TAE	Tris-acetate-EDTA
TCM	Traditional Chinese Medicine
Topo	Topoisomerase
U	Unit
UV	Ultraviolet
VGSCs	Voltage-gated Na^+ channels

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Chapter 1 Introduction

1.1 A brief history of medicinal natural products

Drug discovery is inspired by natural products from the very beginning of the history of medicine. The only source for mankind to looking for medicine was nature, especially plants.[1]

Before the period of Ancient Greek, medical treatment has been through several stages including instinctive, empirical, ghost and soul relevant, magical, astromantic, and religious medicine. Physician (iatros) officially became a profession with assistance of rhizotomist who collect, dried, and mashed herbal rhizoma to prepare medicine. Such position is considered to be the incipient appearance of pharmacist. The first illustrated book of medicinal plants *Herbal* was written by Pseudo-A-puleius (the 5th century) and published in Rome (J. P. de Lignamine, 1481), which became a popular publication about medicinal purpose plants. In the 15th century, with the establishment of pharmacology, the primary research subject was botany. Saladino di Ascoli, a private physician of the Duke of Tarentum, listed medicinal formulae and demonstrated the medicinal part (rhizoma, stem, leaf, flower or fruit etc), weight, dose, and storage of plants in his book *Compendium aromatarioru*, which has been followed by pharmacy to prepare medication. Research on botany not only made a great advancement on medicine, but improved the revival of other related sciences as well. During the period of Renaissance, a pharmacological encyclopedia *Commentary on Discorides* written by P. A. Mattioli has been published in Venice, 1554. Hundreds of plants were elaborated and illustrated in that book, which has been a classical medicinal publication

for more than two centuries. The trend of experiment based medicine started from the 17th century. Many herbal treatments including the bark of *Cinchona* for malaria and the root of *Carapichea ipecacuanha* for dysentery have been introduced into Europe. Those herbal remedies helped people to survive from epidemics.[2]

On the Origin of Species by Means of Natural Selection published in London on 24 November 1895 is a scientific literature about evolutionary biology by Charles Darwin. This book presented a body of evidence gathered from research, correspondence, and experimentation and introduced the scientific theory of natural selection, which has great impact on medical science. Pharmacology became a scientific subject from the second half of 19th century. Researchers started to investigate effects of medicines on animals before applying on human body. Traditional used medications including plants and minerals have been screened to remove ineffective remedies and physicians have been asked to use scientific proved medicine in prescriptions. Tissue culture *in vitro* accomplished by Ross Granville Harrison in 1907 improved the development of both cell biology and pharmacology to a great extent.[2]

It is quite obvious that nature products have been the foundation of medication for human being all over the world for centuries. Moreover, optimized herbal formulae which have contributed to the control and treatment of epidemics including Severe Acute Respiratory Syndrome (SARS) in 2003 and human influenza (H1N1) in 2009 are still serving in clinic.

1.2 Basic theory of Traditional Chinese Medicine [3]

Traditional Chinese Medicine (TCM) is the complex of herbal medicine, acupuncture, massage, exercise etc. which built based on more than 2500 years of Chinese medical practice. TCM has inspired the development of medical treatment in Asia and is still widely used in China. It is believed that around eight thousands of natural products including hundreds of animal or mineral medicines have been employed in the prescriptions and formulae of TCM, among which, more than four thousands of herbs are still clinically used in China. The core theory of TCM is holism, which can be interpreted as unity and integrity. Guided by holism, traditional Chinese medicine attaches great significance to not only the unity and integrity of human body, but also the tight link between humans and nature. As a defining feature of TCM, holism, which synthesizes ancient materialism and dialectics, is reflected on every aspect of TCM, including physiology, pathology, diagnosis and treatment.

1.2.1 Holism of human body

Human body is holistic and composed of tissues and organs. Bearing varying physiological functions, body components are interdependent on each other. Cooperation between core “five *zang*-organs” and supportive “six *fu*-organs” with the interaction of “five sense organs and nine orifices” inside the body through meridians (the longitudinal lines or pathways on the body along which the acupuncture points are distributed) can move essence, blood and body fluid to nurture and regulate all the tissues and organs. In this sense, human body is an organic wholeness. For instance,

the digestion, absorption and excretion of food need the synergy of stomach, spleen and intestines. Yet, the spleen cannot work without the nourishing by the heart, the warming by the kidneys and the clearing by the liver. The function of the stomach and the intestines is input and output respectively. The alteration between fullness and emptiness of these two organs ensures the normal function of the digestive system. Similarly, the body fluids are transported by the spleen, regulated by the lungs and filtered by the kidneys to fulfill the whole metabolic process. The five internal organs, which preserve the vital essence, also provide nutrition to all the tissues and organs of the body. Only if the vital essence in the five organs is sufficient and flourishing can the tissues and organs functionally stable.

This holistic mechanism ensures the overall well-being of the body. However, once a certain tissue or organ becoming diseased, a dysfunction will occur not only to this specific tissue or organ, but also to all the related body parts or even the whole body. For example, the spleen and the stomach are considered as the pivot of health. Disorder of these two organs will break the balance of bodily circulation and further interrupt the absorption. This will subsequently lead to the malnutrition in all the tissues and organs, and ultimately, the weakness of the body. Another example is the running of *qi*. The heart is in charge of blood circulation, while the lungs are in charge of *qi*. *Qi* assists blood circulation, and blood is the carrier of *qi*. The obstruction of *qi* in the lungs clogs the circulation of blood in the heart. In the same way, the blockage of blood in the heart results in the stagnation of *qi* in the lungs. Due to the physiological and pathological interdependence and interaction of tissues and organs, the external changes of the five sense

organs, pulses and other symptoms reflect the inner situation. Therefore, diagnosis can be made based on these changes. Holistic treatment tends to be employed to cure a local lesion and, in the meantime, re-balance the whole body.

1.2.2 Holism of human and nature

Human beings have a close connection with nature. As is said in the ancient Chinese medicine literature *Huang Di Nei Jing*, “human beings are in correspondence with the heaven and the earth”. The changes in nature have impacts on human all year round. As it goes from warm spring to hot summer, active *yang qi* flourishes gradually. In consequences, the blood and *qi* in human body ascend to the surface of the body (muscles and skin), and body fluids are drained by sweat. However, when it turns from cool autumn to cold winter, *yang qi* start to be static. The blood and *qi* descend into the inner body which contracts the tissues and muscles. Body fluids are moved to the lower body by producing more urine. Moreover, the changes throughout a single day can also exert influence on the human body. The morning can be considered as spring while the midday is similar to summer. Likewise, the sunset is thought of as autumn and the night can be taken as winter. The human body can react to these changes correspondingly and therefore functions in a perfect dynamic balance.

1.2.3 Holism of human and other external factors

The geographical and climatic diversity also has certain impacts on the human body. In addition, even social surroundings exert diverse

influences on people psychologically and mentally. Social harmony can benefit health. Contrarily, social badness can be the trigger of diseases. Healthy human body can actively adapt to nature. Normal climate changes cannot produce adverse impact on the human body. However, if the human body is in disorder, or the climate change is intense and beyond the body's self-regulation range, the disease will arise. Thus, so-called common diseases or epidemics normally occur in corresponding seasons. For example, wind diseases are common in spring, heat diseases in summer, dry diseases in autumn, and cold diseases in winter. The sudden changes in surroundings tend to cause low- or non-acclimatization. As the season changes, senior people or patients suffering chronic diseases are less likely to get fully adjusted. They may feel comfortless or experience a disease flare-up or rise-up. For some of the patients, they might undergo a series of syndromes: alleviation in the morning, stability during the day, worsening at dawn and aggregating at night. Therefore, time, climate and geographical surroundings must be taken into consideration when treatment is arranged.

1.2.4 Theory of diagnosis and treatment in TCM

Syndrome, also known as syndrome manifestation, is a holistic reflection of internal and external disorders in human body, which is more complicated than symptom. Syndrome differentiation and identification followed by treatment determination is the basic principle of diagnosis and treatment in TCM. The symptoms and physical signs can be collected and analyzed through four ways of looking, listening, questioning and feeling. The cause, nature, location of disease and the

relationship between healthy and pathogenic factors are identified and summarized as a certain syndrome. This process is called syndrome differentiation. Treatment determination is a process of identifying the treating principles and methods according to the syndrome. Accurate syndrome differentiation may even treat or prevent disease before it arises which lead to the rudiment of preventive medicine.

Syndrome differentiation and treatment determination are interdependent and indispensable to each other. Syndrome differentiation is the premise of determining treatment while treatment determination is the purpose of syndrome differentiation. These two processes serve as a strategy in the clinical application of TCM diagnosis and treatment to understand and handle diseases.

According to the theory of syndrome identification, the dialectical relationship between syndromes and diseases is various. Different syndromes can be the expression of the same disease. Accordingly, different diseases might also lead to the same syndrome. Two clinical treating principles of TCM are created based on the relationship between the disease and the treatment: “treat the same disease in different ways” (due to different syndromes caused by various onset times, regions, body responses or disease stages, different treatments are determined) and “treat different diseases in the same way” (based on the same syndrome emerges in the progress of different diseases, the same treatment can be used accordingly). Obviously, the TCM treatment fixes its attention on distinguishing the syndromes instead of the diseases themselves.

In conclusion, during the practice of TCM syndrome differentiation and treatment determination, the concept of holism is observed to analyze different cases specifically and treat various conditions individually. This strategy is distinguished from the symptomatic treatment which targets the specific regional lesion or disease. The combination of TCM theory and clinical application is considered as the great start of personalized medical treatment from thousands years ago.

1.2.5 Linkage between TCM theory and modern medicine

Personalized treatment and preventive treatment according to individual cases have always been the tenet of TCM. With the development of medicine related research technology, western medicine has been separated with TCM and followed a different pathway for several decades. Many synthetic chemicals have been discovered and passed clinical trial to become officially proved medicines, which have characters including rapid therapeutic effect, consistent quality, and convenient to consume by patient. However, most medical treatments have been designed for the “average patient” with “one-size-fits-all” expectation. Consequently, such medication can be very efficient on most patients but not for others. In order to further develop medical treatment, Precision Medicine, a bold new research effort has been introduced in 2011 by American medical researchers. Precision medicine involves the application of panoramic analysis and system biology to analyze individual case at the molecular level and utilize targeted treatment to solve health problems. The innovation feature of precision medicine is to combine the factors of genome sequence, micro-biome composition, health history, lifestyle, and living environment of specific

patient which allows medication providers to select the most effective treatment for that case. Instead of using “one-size-fits-all” medication, “tailor-made” treatment and prevention strategies for unique individual can be achieved with precision medicine.[4]

Barack Obama, former President of the United States, announced the launching of precision medicine initiative in his 2015 State of the Union address followed with a $215 million investment in the President’s 2016 Budget. The definition of precision medicine happened to coincide with the tenet of TCM which can be considered as a revolution in health improvement and disease treatment. As a forerunner of personalized medicine, TCM might be able to make contribution to precision medicine with experiences on the theory of the holism within human body, and the correlation between human body and nature.

1.3 Modern research of TCM

For thousands of years, TCM was invented and gradually developed by accumulated experience in clinical practice. Modern research of TCM pharmacology can be divided into two basic strategies. Conventionally, in order to investigate and evaluate the active ingredient within a single medicine, that medicine is regarded as medicinal plant or natural product. A chemistry – pharmacology pathway including extraction, isolation, structure analysis and modification of bioactive compounds, followed with pharmacodynamics, pharmacokinetics and toxicology studies of those compounds is performed. This strategy is practiced to find monomers in TCM which can be clinically applied like other western medicine, or be the template of synthetic medicine. Another strategy has

been developed within the recent two decades. Instead of studying bioactive compounds in TCM, traditional used formulae are taken as investigative subjects. With the guidance of TCM theory and the help of scientific analyzing technologies, the scientific mechanism of TCM formulae and the rules of formulae making are expected to be comprehended. Furthermore, pharmacological research can help searching for new application of TCM formulae and provide ideas in developing new medicine. Both strategies are designed to achieve the same goal — take advantage of the wisdom and experience of the ancient TCM to screen clinical used drugs or candidates of drug discovery from natural source.[5]

1.4 Plants and alkaloids

1.4.1 Primary metabolite and secondary metabolite of plant

Plants typically are autotrophic organisms which can fight the menace from other organisms including herbivorous animals, microorganisms, and pathogenic bacteria, fungi, viruses, or parasites. Botanists have found that in order to protect themselves, plants have evolved many physical defenses such as: formation of thick bark in roots to against water loss, presence of the hydrophobic cuticles as a penetration barrier and development of thorns, hooks or spines to fight with herbivores. Additionally, during the life circle of plants, they can produce many compounds to defend themselves against the environment which are known as chemical defenses (also called secondary metabolites).[6-7]

In order to become multifunctional, compounds in plants have been

modulated during evolution. Modern chemistry has proved that even single compound may have several biological functions due to those active functional groups contained in the structure. As an important part of defense, those functional groups allow the compound to interact with more than one cellular or molecular target of enemies organisms of the plants.[8-11]

1.4.2 Alkaloids

Among the secondary metabolites, the earliest isolated pure compounds with biological activity were alkaloids.[12] Alkaloids are a kind of organic compound contains nitrogen. The “alkali-like” name of alkaloid was originally applied to the substances which react with acid to form salts just like inorganic alkalis.[13] Majority of alkaloids have complicated cyclic structure including nitrogen and the nitrogen generally makes the compound basic. Until now, over 21,000 alkaloids have been identified; some of them have been confirmed have anti-bacterial, anti-fungal and anti-viral properties.[12] The most well-known compound is quinine, an anti-malarial agent isolated from *Cinchona succirubra*.[8] Another anti-malarial alkaloid named *Qing Hao Su* (artemisinin) was developed in 1972 in China. Pharmaceutical chemist Tu Youyou, who successfully purified artemisinin from plant *Artemisia annua L*, became a Nobel laureate in medicine in 2015. Phyto-chemistry and medicinal chemistry methodologies enabled a vast array of bioactive secondary metabolites from terrestrial and marine sources to be discovered.[14-15]

1.5 Coptidis Rhizoma (黄连, Huang Lian)

Coptis chinensis Franch., *Coptis deltoidea* C. Y. Cheng *et* Hsiao, *Coptis teeta* Wall., *Coptis omeiensis* (Chen) C. Y. Cheng, and other two species belong to the *Ranunculaceae* family were traditionally used in TCM as Coptidis rhizoma. Coptidis Rhizoma (Huang Lian) is included in crude medicinal material section of Chinese Pharmacopoeia (version 2015) and the most recent version of Japanese Pharmacopoeia (version 2017). Coptis used in this study is *Coptis chinensis* Franch..[16-17]

1.5.1 *Coptis chinensis* Franch.

Dry rhizomes of *Coptis chinensis* Franch., (Figure 1.1) which belongs to genus *Coptis Salisb.*, has been traditionally used to treat *damp-heat* caused syndromes including stuffiness and fullness of abdomen, diarrhea, vomiting, high fever and fidgetiness in early Chinese clinic. Meanwhile, many further pharmacological benefits of Coptis have also been investigated such as anti-inflammatory effects, lowering of blood cholesterol and treatment of diabetes. Coptis is known to be the source of multifunctional isoquinoline alkaloid berberine (Figure 1.2) which is used as standard in the quality control of crude material.[18]

Figure 1.1 Original plants and dry herbal materials of *Coptis chinensis* Franch.

1.5.2 Alkaloids and other compounds isolated from *Coptis chinensis* Franch.

Berberine (Figure 1.2, chemical formula $C_{20}H_{18}NO_4^+$), chemical name as 9,10-Dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-α]quinoliziniu m with a molecular weight of 336.36 g/mol, is a quaternary ammonium isoquinoline alkaloid with natural yellow colour, can be found in several different families and species including *Berberis*. Due to the strong yellow colour of this alkaloid, plants contain berberine were used as fiber dye to stain wool, leather, and wood in Asia.[19] Pharmacology studies proved that berberine and its hydrochloride salt form are cholinesterase, tyrosine decarboxylase and tryptophanase inhibitors, and also antiarrhythmic agents. In spite of Chinese Pharmacopoeia (version 2015), berberine (or berberine hydrochloride) is also included in many other national pharmacopoeias including Japanese Pharmacopoeia (version 2014 to 2017), Korean Pharmacopoeia (version 2010), European Pharmacopoeia (version 9.0) and British Pharmacopoeia (version 2013). Due to a wide

variety of pharmacological effects including antibacterial and antifungal functions, berberine has been used to treat gastrointestinal disorders, cholera and infantile diarrhea in clinic.[20] It is also used as additional medication to treat diabetes and food supplement to help with digestive problems in the US. Other bioactive alkaloids (chemical structure of some of the alkaloids showed in Figure 1.2) including coptisine, jatrorrhizine, palmatine, worenine, epiberberine, magnoflovine, and columbamine, as well as obacunone and obaculactone have been isolated from *Coptis chinensis* Franch.

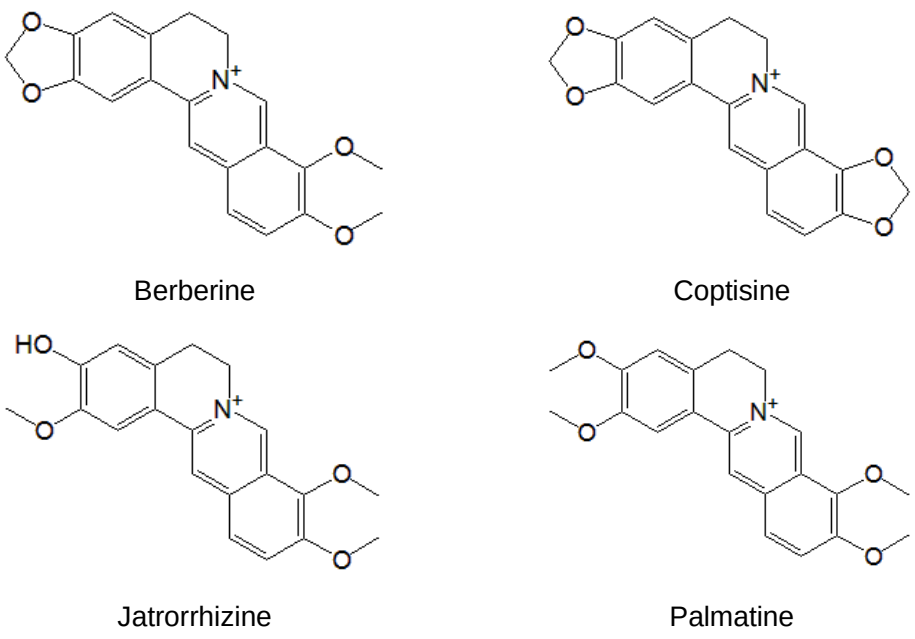


Figure 1.2 Chemical structures of alkaloids isolated from *Coptis chinensis* Franch.

1.5.3 *In vitro* activities of Coptis

Crude extract of Coptis and its pure major alkaloid berberine has been reported to inhibit proliferation and induce apoptosis of MCF-7 breast cancer cells. Moreover, combination treated with Coptis extract can

enhance the cell growth inhibitory effect induced by tamoxifen (an antagonist of the estrogen receptor in breast tissue used in clinic to treat breast cancer) by 39%.[21-24] Such activity was also indicated by the study of Li *et al* group. According to their results, Coptis extract inhibit the growth of cancer cell lines by the suppression of cyclin B1 protein expression which may inhibit the activity of cdc2 kinase.[25] Other targets including upregulation of cytokines interferon-β (IFN-β), tumor necrosis factor-α (IFN-α), as well as interferon-γ (IFN-γ) and inhibition of cyclooxygenase are proved to responsible for the anticancer and cancer preventive activities of Coptis.[26-28]

1.5.4 *In vivo* activities of Coptis

Pharmacology research indicated that the extract of Coptis orally taken by BALB/c mice (1, 2.5, 5, and 10 g/kg) can reduce the blood sugar level of mice in a dose-dependent manner. Berberine (50 mg/kg) has effect on glucose induced diabetes of mice within 2 hrs after orally feeding. The research results of Yu *et al* demonstrated that berberine (200 mg/kg) can affect leukemia *in vivo* by reducing the size and weight of spleen in BALB/c mice injected with WHI-3 leukemia cells. Percentage of MAC-3 and CD11b cells in blood samples have been reduced by berberine with the same dosage.[29] Despite the results of animal test listed above, berberine (1.8 g/day) has been reported clinically to treat patients suffered from hypertension with an effective percentage of 93%.[5]

1.6 Euodiae Fructus (吳茱萸, Wu Zhu Yu)

Euodia rutaecarpa (Juss.) Benth., and other two plants from the same

genus *Euodia rutaecarpa* (Juss.) Benth. *var*. *officinalis* (Dode) Huang, *Euodia. rutaecarpa* (Juss.) Benth. *var*. *bodinieri* (Dode) Huang were traditionally used in TCM as Euodiae Fructus. Euodiae Fructus (Wu Zhu Yu) is included in crude medicinal material section of Chinese Pharmacopoeia (version 2015) and Japanese Pharmacopoeia (version 2015). Euodia selected throughout this study is *Euodia rutaecarpa* (Juss.) Benth..[16,19]

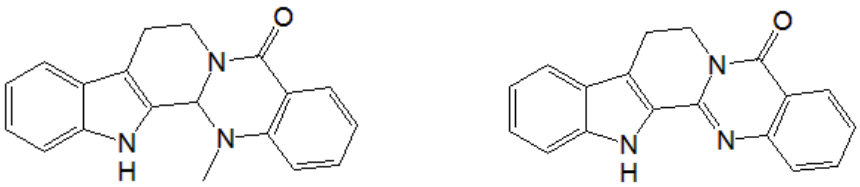
1.6.1 *Euodia rutaecarpa* (Juss.) Benth.

Dry and nearly ripe fruits of *Euodia rutaecarpa* (Juss.) Benth., (Figure 1.3) which belongs to genus *Tetradium,* has been traditionally used for a syndrome characterized by cold hand and feet, migraines and vomiting.[30]

Figure 1.3 Original plants and dry herbal materials of *Euodia rutaecarpa* (Juss.) Benth.

1.6.2 Alkaloids and other compounds isolated from *Euodia rutaecarpa* (Juss.) Benth.

Main constituents purified from *Euodia rutaecarpa* are two alkaloids named evodiamine and rutaecarpine (Figure 1.4). Evodiamine (chemical formula $C_{19}H_{17}N_3O$), with a chemical name of 8,13,13b,14-Tetrahydro-14-methylindolo[2'3'-3,4]pyrido[2,1-b]quinazolin-5-[7H]-one and a molecular weight of 303.36 g/mol, is the major bioactive component from the fruits of *Euodia rutaecarpa* (Juss.) Benth.. This characteristic quinazolinocarbolin alkaloid has been proved to be a diuretic and diaphoretic agent.[20] Other active compounds isolated from Euodia including alkaloids (wuchuyine, hydroxyevodiamine, and evocarpine), essential oils (evoden, ocimene), as well as evodinone, goshuynic acid, limonin and arachidoside *etc*.[16]



Evodiamine Rutaecarpine

Figure 1.4 Chemical structures of alkaloids isolated from *Euodia rutaecarpa* (Juss.) Benth.

1.6.3 *In vitro* activities of Euodia

Euodia extract has strong inhibition activity on gram-negative bacteria *Vibrio cholerae.* 10% methanol extract of Euodia can inhibit *Epidermophyton floccosum* and a variety of fungi efficiently.[5] According

to Chiou *et al*, ethanol extract of Euodia along with evodiamine and rutaeparpin are potent inhibitors of H1N1 (an influenza virus) stimulated chemokines production in A549 lung epithelial cells.[30] Furthermore, evodiamine has been reported to induce apoptosis through the caspase pathway in Hela cells.[31-32] Research of Zhao *et al* proved that evodiamine modulates the activity of the p53 signaling pathway to induce apoptosis and downregulate MMP3 expression by inactivating the JAK2/STAT3 pathway through the downregulation of PGI to inhibit migration of HCT-116 human colorectal cancer cells.[33] Several studies also indicated that Euodia and evodiamine induce apoptosis of cancer cells by regulating protein kinases, caspase, and novel oncoprotein metaderin (MTDH).[34-38] Other in vitro studies also proved that evodiamine inhibit the migratory and invasion activity in several types of cancer cell lines including human breast cancer, prostate cancer, and leukaemia cells.[39-42]

1.6.4 *In vivo* activities of Euodia

Du *et al* indicated that administration of evodiamine (10 mg/kg) significantly reduced MDA-MB-231 breast cancer cells induced tumor growth and pulmonary metastasis on BALB/c mice.[43] Clinical application proved that Euodia decoction is effective on digestive disorder, eczema, and multi-type skin infection. Euodia powder can be used to treat oral ulcers by applying to the affected area directly.[5]

1.7 Coptis-Euodia formulae

1.7.1 Combination in TCM [44]

Combination in TCM is a process that two or more medicines are combined into use according to individual condition and medicinal property. *Shen Nong Ben Cao Jing*, also known as The Classic of Herbal Medicine, is a book of Chinese agriculture and medicinal plants. Although the original text is no longer available, researchers believe that the book was written and edited approx. 200 to 250 AD and has been attributed to the mythical Chinese sovereign *Shen Nong* (approx. 2800 BC). TCM combination is clarified by forerunners into “seven relations of medicinal compatibility”, which first appears in the foreword of *Shen Nong Ben Cao Jing*. “Medicine can be singly used. As such, medicines can also be reinforced, assisted, restrained, inhibited, antagonized, and suppressed. These seven relations should be analyzed comprehensively”. If the condition of the patient is simple or specific, a clearly-targeting single medicine is suggested. Singly used medicine is straight and convenient which can relieve the patient from sickness effectively. However, if the syndrome of the patient is serious and complicated, it is hard for single medicine to exert a comprehensive therapeutic effect. Moreover, some medicines have obvious toxicity which might engender adverse side effects. Such being the case, two or more medicines should be cooperated as a formula to cure the disease and reduce toxicity at the same time. Combination might lead to chemical reactions between medicines followed by potentially enhancement or weaken of therapeutic effect and inhibition or exhibition of toxicity. Thus, the medicinal relations of combination in TCM should be clearly understood before formulating.

Despite of singly used medicine, other six relations of TCM combination can be generalized into four types. First of all, some medicines can coordinate to improve the final therapeutic effect. Such advantage should be taken in clinical practice. Secondly, some medicines can weaken or inhibit the therapeutic effect of each other, which should be carefully examined before clinical practice. Thirdly, some medicines can change the property of each other and subtract the toxicity and side effects. Extra caution should be paid in using toxic and powerful medicines. And finally, working together of some medicine pairs will enhance the toxicity and side effects, which should be avoided as contraindication in combination.

In summary, combination in TCM crystallizes the experience and knowledge accumulated in years of practice. Medicines with specific volume (weight) and standard proportion are made into formulae which represent the classical and developmental outcome of TCM.

1.7.2 Principle of TCM formulae [44]

According to the principles of combination and formulation, medicines in a TCM formula are normally served as monarch, minister, assistant, and guide respectively. By using these principles, TCM formulae are expected to form the best combination of medicinal materials, to maximum the therapeutic effects of each singly used medicine, to minimum the toxicity and side effects of toxic ingredient involved, and to reach disease focus rapidly.[45] The therapeutic effects along with the formulating principles of most TCM formulae have been proved to be reliable by pharmacological research. For example, *Zhuyu* Decoction, a

TCM formula consisting of Euodia, ginger, ginseng and jujube, is believed to have positive effects on warming stomach and stopping vomiting. Pharmacological analysis has proved that the formula alleviates gastrospasm and stops the vomiting by inhibiting the stimulation on gastric mucosa. Formulating mechanism indicated that Euodia dominates the therapeutic effect of the formula as a monarch. Stop vomiting effect is supported by ginger which served as a minister. Ginseng and jujube, which cannot be used to treat vomit or gastrospasm as single medicine, are added to reinforce the total therapeutic effect and reduce toxicity. Another typical formula of mutual reinforcement is the combination of Coptis and scutellaria which is used to treat diarrhea. Result of biology research indicated that the effect of this duo herbal formula in fighting against *Staphylococcus aureus* is several times stronger than that of singly used copits. Application of analytical technology on TCM formulae research allows the scientific mechanism of those precious treasures to be clarified. Moreover, modification and optimization of TCM formulae can be achieved for further clinical application.

1.7.3 Coptis-Euodia formulae and traditional usage

Both Coptis and Euodia were included in *Shen Nong Ben Cao Jing* as noble herb and middle herb respectively. Duo herbal formulae combined with Coptis and Euodia with different ratio are generally used as folk medicine to reduce *liver-heat* and treat stomach ache, vomiting, fever, bleeding and diarrhea. Formula called *Zuo Jin* pill containing Coptis and Euodia (Coptis : Euodia = 6 : 1, w/w) has been recorded in *Dan Xi Xin Fa* (*Ming* Dynasty, 1481 AD).[18, 46-48] Pharmaceutical technics employed

to make “pill” of TCM can be various. In that ancient Chinese medicinal literature, “pill” involved in the formula name of *Zuo Jin* pill was identified as “small pills formed with fine powdered medicinal material mixture and water”. Modern pharmaceutical technics upgraded some TCM pills to “concentrated decoction of medicinal material mixture with appropriate pharmaceutical excipient”. Derivative formulae from this duo herbal formula have been invented including *Zhu Yu* pill (Coptis : Euodia = 1 : 1) and *Fan Zuo Jin* pill (Coptis : Euodia = 1 : 6) to treat specific patients suffered from different types of stomach disorders. Both herbal extracts and/or their isolated single compounds have been reported as novel antineoplastic agents.[22,24] *Zuo Jin* pill has been used as traditional Chinese medicine for a significant period of time and is still officially listed in the traditional Chinese patent medicine section of Chinese Pharmacopoeia (version 2015). New benzylisoquinoline alkaloids and isoindoline alkaloid have been reported to be isolated from this herbal formula and been proved to have inhibitory activities against gastrointestinal cancer cells.[49] However, not many studies have been carried to investigate the bioactivities on these duo herbal formulae.

1.8 Breast cancer and herbal remedy

Breast cancer is the most frequent type of cancer among women which accounts for 32% of the cancer burden in the UK.[50-51] Around one-in-nine women in the UK are diagnosed with breast cancer at some point in their life.[52] Breast cancer incidence increases with age, estrogen exposure, significant radiation exposure, family history and other specific conditions.[53] Breast cancer is rather a conglomerate of diverse molecular syndromes than a single organ-based neoplasm.

Though early stage detection is keen in breast cancer therapy, commonly used imaging detection has low sensitivity and specificity for early breast cancer diagnosis.[54]

The vast majority of breast cancers arise from epithelial cells lining the terminal duct lobular unit. Some tumors show distinct patterns of growth and cellular morphology, and on this basis, certain types of breast cancer can be identified.[53] Breast cancer has a propensity to metastasize to bone, lung pleura, skin, and lymph nodes. However, the metastatic cascade of breast cancer is not a random process, which has been proved to have clear patterns of metastatic preference for specific organ sites or tissues depending on the type of cancer. Increasing knowledge distinguished the genotypic and phenotypic differences between breast cancers and helped to predict the motility of individual breast cancer.[54] Approximately 20% of invasive breast cancer with special types can be classified into tubular, cribriform, medullary-like, mucoid, papillary and classic lobular.[53] Breast cancer can be treated or cured depending on different stages and types. Most breast cancer treatment is surgery before tumor metastasis and with possible adjuvant therapies including radiation therapy, chemotherapy, adjuvant endocrine therapy, and recently, biotherapy.[55]

According to the research of pathology, 50 - 80% of the cases suffer breast cancer are estrogen-dependent. Estrogen receptor has been proved to be a target of breast cancer treatment. Aromatase, the enzyme involved in aromatization process to synthesis estrogen, is another target of breast cancer chemotherapy. Selective estrogen receptor modulators (SERM) and aromatase inhibitors can be used as anti-breast

cancer drugs. Tamoxifen is the most common SERM discovered by AstraZeneca in treating estrogen receptor positive breast cancer patients. However, tamoxifen also cause many serious side effects including cancer and bone loss. Several synthetic aromatase inhibitors such as anastrozole and letrosole are now licensed for clinical use and the side effects occurred at the same time.[52] Investigation of new anti-breast cancer target and high efficient anti-breast cancer agents with low side effects is still arduous.

Since Paclitaxel (Taxol) being isolated and introduced into cancer therapy, there has been a growing interest in using natural products, especially extract from herbs, as a potent source of new therapeutic anticancer drugs. A variety of plant secondary metabolites have been used as agents for chemotherapy and/or chemoprevention of cancer. Such as several known natural aromatase inhibitors based on the flavonoid skeletons.[52] Some herbal alkaloids, for example tetrandrine has been tested to determine the efficient target of its cell proliferation inhibit function.[56]

Epidemiological studies reported that the tumour cell populations from breast cancer patients on cardiotonic steroids (CSs) medication for cardiac problems appeared to have a lower proliferative capacity than tumour cells from patients not on CSs treatment. Such matter of fact interests the researchers to develop CSs as potential anti-cancer agents over the last 10 years.[57] Clinically used drug digoxin, extracted from the most well-known plant *Digitalis* from *Scrophulariaceae* family, was proved to have cytotoxicity effect in human MCF-7 breast cancer cells. G-strophantin (ouabain), a poisonous cardiac glycoside isolated from the

seeds of *Strophanthus gratus* and the bark of *Acokanthera* in the family *Apocynaceae*, was tested in human breast cancer cell line. At concentrations <100 nM, ouabain can produce anti-proliferative effects. The induction of apoptosis was at μM level.[57]

Vincristine (brand name Oncovin®) is a mitotic inhibitor approved by the United States Food and Drug Administration (FDA) in 1963 as cancer chemotherapy to treat acute lymphoblastic leukemia and breast cancer. Vinflunine (brand name Javlor®), a novel fluorinated derivative of vinca alkaloid, has recently been launched in the UK as monotherapy for the treatment of adult patients with advanced or metastatic transitional cell carcinoma of the urothelial tract.[58-60] The anti-breast cancer effect of vinflunine is currently evaluated in clinical trials in combination with other agents.[58]

Breast cancer is moderately sensitive to cytotoxic chemotherapy. Combination treatment of current chemotherapy rapidly reduced the tumor volume but will result in other syndrome including cancer, osteoporosis and coronary heart disease.[54] Since many of the anti-breast cancer drugs were isolated or derived from natural sources, it is most likely that many if not all of them are still hiding in nature. Screening of agents with sufficient anti-breast cancer activities associated with gentle or positive additional effects on human body from TCM formulae appears to be a good choice in anti-breast cancer research.

1.9 Research techniques in cancer research

1.9.1 MTT assay

MTT, also called 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a pale yellow substrate. MTT colorimetric assay is capable of detecting viable cells by the reduction of the tetrazolium salt to formazan. The tetrazolium ring of MTT can be cleaved by the succinate dehydrogenase within cell mitochondria and dark blue precipitant formazan can therefore be accumulated in the cell membrane.(Figure 1.5) Such intercellular reaction can only occurred in living and metabolically active cells, but not dead cells. Formazan can be solubilized in DMSO to generate a purplish solution. Quantified cell viability can therefore be evaluated by spectrophotometric optical density values under 500 to 600 nm. Over more than two decades, MTT assay has been a widely used in *in vitro* testing system for cell proliferation and cytotoxicity quantitation. Moreover, after various modifications, this tetrazolium based method is considered as a versatile and reliable method to examine cell population's response to external factors in cell biology research.[61]

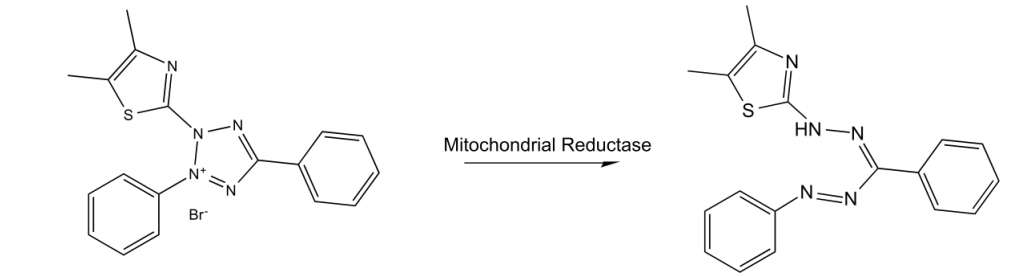


Figure 1.5 Intercellular reduction of MTT

1.9.2 Cell cycle analysis

The process of the replication and division of chromosomes within the nucleus is the basic of cell cycle. Measuring the changes in cell cycle kinetic distribution of cancer cells under different culture condition is one of the ways to evaluate potential cancer therapy. During mitosis, DNA synthesis process leads to different copies of chromosome in cells at different cell cycle stages. Cellular DNA can be labeled with propidium iodide (PI) and the percentage of cells in G0/G1, S and G2/M phases can be discriminated by different fluorescence intensity. Cell cycle distribution can then be determined by flow cytometric analysis.

1.10 Aims and hypothesis

In modern pharmacological studies *in vitro* and *in vivo*, the anti-inflammation activity of Coptis and/or Euodia extracts were considered to link to anti-cancer activity. This study intends to investigate the anti-breast cancer potential of Coptis-Euodia formulae and their possible mechanisms as anti-cancer agent candidates. Partially comprehension and exposure of the TCM formulation principle involved in these duo herbs formulae are expected to be accomplished.

The aim of the anti-proliferative studies of standard alkaloids and Coptis-Euodia formulae 70% methanol extracts on MDA-MB-231 and MCF-7 cell lines were carried out to determine and evaluate the anti-breast cancer ability of these duo herb combinations. Through anti-migration and anti-adhesion studies, the effects of Coptis-Euodia formulae and standard alkaloids on the movement and the behavior of

breast cancer cell would be understood. Cell cycle analysis was employed in this study to investigate the effects of Coptis-Euodia formulae and standard alkaloids on breast cancer cell cycle distribution and improve the understanding of the principles of TCM formulation.

This study is also aimed to examine and evaluate the human topoisomerases I and II inhibition activities of berberine, evodiamine and the crude extracts of Coptis-Euodia formulae compare with known topoisomerases inhibition agents. Topo I relaxation assay and Topo II decatanation assay were used to explain the possible mechanism of Coptis-Euodia extracts and standard alkaloids as potential anti-cancer agents.

The last but not the least, this study was designed to target the voltage Na^{+} and Ca^{2+} ion channels by establishing a florescence based high-throughput assay to screening ion channel blockers from Coptis, Euodia and natural source. Though the work has not been successfully finished, the basic theory would be studied and possible further optimization according to the preliminary results would be discussed.

According to the ‘Holism’ theory of traditional Chinese medicine, hypothesis of this study is: The bioactivities of single used medicinal herb and their formulae might be different from each other which cannot be unilaterally anticipated by the bioactive components. Interaction and synergy between compounds might enhance, reduce, or even revise the bioactivities of medicinal herbs and their formulae. Each of them should be investigated and evaluated as independent candidate in cancer research.

Chapter 2 Methodology

2.1 Materials

2.1.1 Plant materials

Coptis chinensis Franch. was produced in Sichuan province and collected in June 2012. *Euodia rutaecarpa* (Juss.) Benth. was collected in September 2003 from Jiangxi province. Powdered plant materials, along with standard alkaloids berberine hydrochloride (≥ 98%) and evodiamine (≥ 98%) used in this study were generous gifts from Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine (Shanghai, P. R. China) and authenticated by Dr Lihong Wu.

2.1.2 Chemicals, materials and reagents

Methanol used in extraction process and dimethyl sulfoxide (DMSO) employed in the study were obtained from Fisher Scientific (Loughborough, UK). Cell culture medium Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine / penicillin / streptomycin solution, trypsin-EDTA solution (0.25%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphemyltetrazolium bromide (MTT), bovine serum albumin (BSA), along with fibronectin were purchased from Sigma-Aldrich Ltd. (Dorset, UK). Phosphate buffered saline (PBS) tablets, sterilized centrifuge tubes as well as micro-centrifuge tubes, cell culture flasks along with microtiter plates throughout this study were obtained from Fisher Scientific (Loughborough, UK). (*S*)-(+)-Camptothecin (≥ 90%), etoposide (≥ 98%) and amsacrine hydrochloride (≥ 98%) used as positive controls, as well as tris base, ethylenediaminetetraacetic acid (EDTA) solution, tris hydrochloride

solution and sucrose used to prepare buffers in human topoisomerase assays were also purchased from Sigma-Aldrich Ltd. (Dorset, UK). Glacial acetic acid involved in (Tris-acetate-EDTA) TAE buffer, bromophenol blue used to prepare stop buffer, chloroform and isoamyl alcohol used to form extracting mixture along with agarose gel powder were obtained from Fisher Scientific (Loughborough, UK). Human topoisomerase I and II, supercoiled pBR322 DNA, Kinetoplast DNA (kDNA), dilution buffer, relaxation and decatanation assay buffer were obtained from Inspiralis (Norwich, UK).

2.2 Herbal crude extracts and sample preparation

To prepare herbal extracts, fine powdered Coptis (C) and Euodia (E) were carefully weighed and mixed at different ratios (Coptis only, C : E = 6 : 1, 1 : 1, 1 : 6 and Euodia only, w : w) in conical flasks. Each herb mixture (*ca.*14 g) was soaked in 70% methanol (100 mL) for 30 mins and extracted by ultra-sonication for a further 30 mins. Supernatant fluid of each mixture was decanted and filtered under vacuum. The extraction procedure was repeated with fresh 70% methanol (100 mL). The brown coloured suspension was filtered and combined with the previous extract. The solvent was rotary evaporated under reduced pressure to give a dark brown residue, which was then re-suspended in methanol and dried at room temperature under N_2 supply. Standard alkaloids berberine and evodiamine were weighed accurately and dissolved in DMSO to form a 20 M stock solution respectively. Solid herbal crude extract of each mixture and stock solutions of standards were stored at 2 - 8 °C until further use. Herbal extracts were dissolved in 100% DMSO as stock solutions. Stock solutions of herbal extracts and standard alkaloids were

diluted with DMSO and/or cell culture medium on the day of each experiment. The final concentration of DMSO in cell culture did not exceed 1% throughout the study.

2.3 Effects of Coptis-Euodia prescriptions against human breast cancer cell lines

2.3.1 Cell culture

MDA-MB-231 and MCF-7 human breast cancer cell lines were originally purchased from the American Type Culture Collection (Middlesex, UK). Both cell lines were cultured in DMEM supplemented with 10% (v/v) FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (0.1 mg/mL), and maintained at 37 °C in a humidified atmosphere of 5% CO_2 incubator. Passage number of cells used throughout this study was kept lower than 20 in order to maintain the characteristics of working cells close to the reference strain to the greatest extent.

2.3.2 MTT anti-proliferation and cytotoxicity assay

Cells were trypsinized, counted and regrown in 96-well cell culture plates at a concentration of 2×10^5 cells/mL (100 µL per well). After overnight recovery, medium was replaced by freshly prepared medium in the absence or presence of herbal extracts or standard alkaloids.

2.3.2.1 Anti-proliferation assay

In anti-proliferation assay, cells were incubated with herbal extracts at final concentrations from 0.3 µg/mL to 3 mg/mL or standard alkaloids at

concentrations from 0.03 µM to 200 µM. After 1 hr treatment at 37 ºC, medium was removed followed by pre-warmed DMEM (100 µL) and cells were incubated for a further 96 hrs at 37 ºC.

2.3.2.2 Cytotoxicity assay

In cytotoxicity studies, cells were continually treated with herbal extracts (0.1 mg/mL) or standard alkaloids (10 µM) for 24, 48, 72, or 96 hrs at 37 ºC without changing the medium.

2.3.2.3 MTT assay

At the end of respective incubation period, MTT-PBS solution (5 mg/mL, 20 µL) was added to each well and plates were incubated at 37 ºC for 3 hrs. Medium was then carefully aspirated from each well and formazan crystals were dissolved in 100 µL of DMSO. Absorbance was measured with microplate reader (BMG-Labtech, Ortenberg, Germany) at a wavelength of 540 nm. DMSO (1%) treated cells were used as a control.

2.3.2.4 Data analysis and determination of half maximal inhibitory concentration (IC_{50})

Mean absorbance of eight wells of each testing subject was divided by the mean absorbance of 1% DMSO treated sample on the same 96-well microplate to give a percentage viability of that experiment. Percentage viabilities presented in paragraph 3.2 were based on three independent experiments carried out in different days.

The IC_{50} curves were estimated by GraFit software version 5.0.4 (Erithacus Software, Surrey, UK) based on percentage viabilities of nine different concentrations of each testing substances and IC_{50} values were calculated using the 4 full parameter equation.

2.3.3 Scratch wound-healing assay

Cells were trypsinized and plated in 24-well plates with 1.4×10^5 cells/mL (500 µL per well). Plates were incubated at 37 ºC overnight. Cells reached 70-80% confluence as a monolayer before the experiment. The cell monolayer was gently and horizontally scratched with a 200 µL sized pipette tip across the centre of the well. Another straight line was vertically scratched perpendicular to the first wound to create a cross in each well. Wounded cell monolayers were then washed twice with pre-warmed medium to remove the cell debris and replenished with fresh prepared medium (500 µL) in the absence or presence of herbal extracts or standard alkaloids at various concentrations (1 µg/mL to 0.3 mg/mL or 5 µM to 50 µM). DMSO (0.5%) treated cells were used as a control. Cells were incubated at 37 ºC and pictures were taken under Olympus Cell^M inverted microscope (Southend-on-Sea, UK) at various time points (0, 8, 24, or 48 hrs). Width of wound was determined and analyzed by ImageJ software version 1.44p (National Institutes of Health, Maryland, USA). Percentage width of the original wound (0 hr) at particular time points compared with 0.5% DMSO treated cells was used to evaluate the anti-migratory activity of herbal extracts and standard alkaloids.

2.3.4 Adhesion assay

MDA-MB-231 cells were trypsinized and split into 35 mm cell culture dishes and allowed to grow to 70% confluence overnight. After incubation, cells were replenished with fresh prepared medium in the presence of herbal extracts (0.1 mg/mL) or standard alkaloids (10 μM). Cells were then incubated for a further 2, 6 or 12 hrs respectively. Corresponding wells of 96-well plate were coated with fibronectin (16 μg/mL, 20 μL per well) overnight at 37 ºC. On the day of experiment, pre-coated wells were washed twice with 0.1% BSA in PBS and blocked for 30 mins with 0.5% BSA in PBS at 37 ºC. Wells were washed again with 0.1% BSA in PBS after blocking. Cells were trypsinized and re-suspended in serum-free DMEM at 5×10^5 cells/mL. Cell suspensions (100 μL) were added to plates and incubated at 37 ºC for 40 mins. Medium was carefully aspirated from each well and cells were washed three times with PBS solution. The colorimetric MTT assay, as described previously in paragraph 2.3.2.3, was carried out to determine the number of adherent cells. DMSO (0.1%) treated cells were used as a control.

2.4 Cell cycle analysis

MDA-MB-231 or MCF-7 breast cancer cells were trypsinized and split into 35 mm cell culture dishes with 3×10^5 cells per dish and incubated at 37 °C to reach 80% confluence. After recovery, cells were treated with fresh prepared medium in the presence of herbal extracts (0.01 and 0.05 mg/mL), berberine (5 μM) or evodiamine (10 μM) and cultured at 37 °C for further time courses (0, 8, 24, 32, 48 and 72 hrs). At the end of each time point, cells were harvested with trypsin-EDTA along with the culture

medium. Cells were pelleted by centrifuge (150 ×g, 5 min) and washed twice with PBS solution. Cell pellets were then suspended in PBS (300 µL) followed by 700 µL of ice-cold absolute ethanol while vortexing. Cell suspensions were refrigerated at 4 °C for 2 hrs to fix and stored at -20 °C for further experiment.

On the day of analysis, fixed cells were centrifuged (450 ×g, 10 min) and washed with 1 mL of ice-cold PBS twice to remove ethanol. Cell pellets were then suspended in 200 µL of Guava Cell Cycle Reagent (PI) (Millipore Limited , Watford, UK) by pipetting up and down several times and transferred into flat-bottom 96-well plates. Microtiter plates were incubated at room temperature in the dark for 30 mins. Stained cell samples were analyzed by Guava EasyCyte System (Guava Technologies, Hayward, USA). Untreated cells in exponential growth were used to adjust the system and DMSO (1%) treated cells were used as a control.

2.5 Effects of Coptis-Euodia presctiptions against human topoisomerases I and II

2.5.1 Experiment preparation

Both human topoisomerase I and II (10 U/µL) were aliquoted into micro-centrifuge tubes with 20 - 50 U per tube to avoid repeated freeze-thaw cycles. Aliquoted enzymes were stored at -80 °C. Further dilution was made on the day of each experiment with provided dilution buffer. Reaction stop buffer (40% w/v sucrose, 100 mM tris-HCl pH 8.0, 10 mM EDTA, and 0.5 mg/mL bromophenol blue), also called agarose gel loading buffer, was prepared according to the instruction of Inspiralis

(Norwich, UK). The solution was warmed in water bath to dissolve the sucrose and then be covered with foil and stored at room temperature until experiment. Chloroform and isoamyl alcohol were mixed together with a ratio of 24 : 1 (v : v) to form the extracting mixture. To prepare 1 liter of 50× TAE stock solution, 600 mL of HPLC-grade water supplied by Millipore Milli-Q purification system (Watford, UK) was used to dissolve tris base (242 g). Glacial acetic acid (57.1 mL) and EDTA (100 mL, 0.5 M，pH 8.0) were then mixed into the solution. Extra water was added to bring the final volume of the 50× TAE stock solution to 1 liter. The concentrated TAE buffer was diluted to 1× TAE working buffer (40 mM tris, 20 mM glacial acetic acid and 1 mM EDTA) prior to experiments. The 1% (w/v) agarose gel was prepared by melting agarose powder (1.5 g) completely in 1× TAE (150 mL) under microwave. The gel was cooled down before pouring onto the casting tray inserted with 12-well combs.

2.5.2 Titration and human topoisomerase I relaxation assay

Titration of human topoisomerase I (HTopo I) was carried out using 0.02 U to 0.2 U of the enzyme incubated with supercoiled pBR322 DNA (0.3 µg) followed by the standard HTopo I relaxation assay procedures.

Supercoiled pBR322 DNA (0.3 µg) was incubated with 0.1 U of HTopo I in relaxation assay buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.25 mM EDTA and 5% glycerol) at 37 ºC for 30 mins in the presence of herbal extracts (0.1 mg/mL), standard alkaloids berberine or evodiamine (100 µM). Total reaction volume of each sample was 30 µL according to the manufacturer’s instruction. Topo I poison camptothecin (CPT) (100

μM) was used as positive control. Supercoiled pBR322 DNA incubated with or without human topoisomerase I was used as negative control and blank respectively.

2.5.3 Titration and human topoisomerase II decatanation assay

Titration of human topoisomerase II (HTopo II) was carried out using 0.5 U to 4 U of the enzyme incubated with kDNA (0.2 μg) followed by the standard HTopo II decatanation assay procedures.

kDNA (0.2 μg) was incubated with 2 U of HTopo II in decatanation assay buffer (50 mM Tris-HCl pH 7.5, 125 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT and 100 μg/mL albumin) with 1 mM of adenosine triphosphate (ATP) at 37 ºC for 30 mins in the presence of herbal extracts (0.1 mg/mL), standard alkaloids berberine or evodiamine (100 μM). Total reaction volume was 30 μL for each sample refer to the manufacturer's instructions. Topo II poisons amsacrine (m-AMSA) and etoposide (100 μM) were used as positive controls. kDNA incubated with or without human topoisomerase II was used as negative control and blank respectively.

2.5.4 IC_{50} determination of Coptis-Euodia crude extracts against human topoisomerase I and II

The human topoisomerase I relaxation assay and the human topoisomerase II decatanation assay, as described previously, were carried out to determinate the IC_{50} values of Coptis-Euodia crude

extracts. Various concentrations (from 1 to 0.0001 mg/mL) of herbal extracts were used depending on the activity of each sample. CPT (100 μM) or m-AMSA (100 μM) was used as positive control in HTopo I relaxation and HTopo II decatanation assay respectively.

2.5.5 Gel electrophoresis and analysis

Topoisomerase catalytic reaction was stopped by additional 18 μL of stop buffer. DNA samples were then vortically extracted with 30 μL of chloroform / isoamyl alcohol mixture. After centrifuge, 10 μL of samples (upper blue aqueous phase) were loaded into the corresponding wells of 1% agarose gels in TAE working buffer. Gels were then run at 4 V/cm for 15 hrs. 1× TAE buffer containing 0.5 μg/mL ethidium bromide (EtBr) was used to stain gels for 1 hr followed by a destaining process in TAE for a further 30 mins. To separate nicked and relaxed species in human topoisomerase I relaxation assay, DNA samples were run on EtBr contained agarose gel and TAE buffer at a concentration of 0.5 μg/mL. Subsequently, the gel was visualized and photographed by UVP BioSpectrum Imaging System (Ultra-Violet Products Ltd., Cambridge, UK). To evaluate the inhibitory activities of each extract, EtBr fluorescence intensities of supercoiled bands in human topoisomerase I relaxation assay and minicircled bands in human topoisomerase II decatenation assay were quantified by ImageJ software version 1.44p. GraFit software version 5.0.4 was used to estimate the IC_{50} value of each extract.

2.6 Statistical analysis

Each value was presented as mean ± S.D.. Statistical analysis was performed by using SPSS 18.0 software. One-way analysis of variance (ANOVA) was conducted to determine the significance of differences among treatments. Two-way ANOVA analysis was followed to indicate the interaction between factors (time courses and concentrations). $P < 0.05$ was considered as statistically significance.

2.7 Preliminary experiment of fluorescence based assays

2.7.1 Reagents, chemicals and preparations

Newborn calf serum (NCF), anhydrous dimethyl sulfoxide (DMSO) and poly-L-lysine were purchased from Sigma-Aldrich Ltd. (Dorset, UK). Fluorescence indicators Sodium Green tetraacetate, SBFI, $DiBACC_3(4)$, and Calcium Green were obtained from Life Technologies Ltd (Paisley, UK). Fluorescence dyes were dissolved in anhydrous DMSO as stock solutions (2 mM) and stored at -20°C. Stock solutions were then diluted to loading concentration with appropriate buffer before experiment. Chemicals used to prepare buffers in fluorescence based assays, Luria-Bertani (LB) media powder, ampicillin, and Opti-MEM reduced serum medium used in calcium ion fluorescence dye assay along with veratridine, gramicidin and mibefradil were also purchased from Sigma-Aldrich Ltd. (Dorset, UK). QIAGEN plasmid maxi kit and FuGENE HD reagent were obtained from QIAGEN Ltd. (West Sussex, UK) and Roche (Laval, Canada) respectively. Evodiamine and rutaecarpine used

in chapter 6 were isolated and characterized by Dr Xiaobei Pan as generous gifts.

2.7.2 Cell culture and treatment

Human breast cancer cell line MDA-MB-231 and human embryonic kidney cell line HEK 293 were originally purchased from the American Type Culture Collection (Middlesex, UK). Both cell lines were cultured in DMEM supplemented with 10% (v/v) NCS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (0.1 mg/mL), and maintained at 37 °C in a humidified atmosphere of 5% CO_2 incubator.

Before the day of experiment, cells were plated into poly-L-lysine pre-coated 96 well plates with 2×10^4 cells each well and incubated at 37°C, 5% CO_2 overnight.

2.7.3 Sodium ion fluorescence dye assay

2.7.3.1 Sodium green assay and SBFI assay

MDA-MB-231 cells were visualized to ensure an 80% confluence. Growing medium was carefully removed from the well and sodium green or SBFI loading solution (3 - 5 μM, 100 μL) was added to corresponding wells. Plates were covered with foil and incubated at room temperature for 40 mins. Cells were carefully washed with pre-warmed DMEM (100 μL) twice and buffer 1 (100 μL) (Table 2. 1) once. Buffer 1 (60 μL) was loaded to cells followed by veratridine loading solution (10 - 50 μM final). Fluorescence intensity was detected with a microplate reader

(BMG-Labtech, Ortenberg, Germany) with excitation 507 nm and emission 532 nm. Once the base line has been established, 20 µL of buffer 2 (Table 2. 1) was automatically injected. Cells without veratridine treatment were used as control.

Table 2. 1 Composition of buffers used in sodium green assay

	Buffer 1 (mM)	Buffer 2 (mM)
$CaCl_2$	5	5
HEPES pH 7.2	10	10
KCl	2	500
Sorbitol	350	-
NaCl	-	175

2.7.3.2 DiBAC$_3$(4) assay

MDA-MB-231 cells were checked to meet 80% confluence. Growth medium was removed and the cells were washed carefully with Hank's/HEPES buffer pH 7.1 (100 µL) (

Table 2. 2) twice. DiBAC$_4$(3) loading solution (5 µM, 100 µL) was added to corresponding wells and plates were incubated at 37°C for 1 hr in dark.

Veratridine (100 µM final) was loaded to corresponding wells. Fluorescence intensity was detected with microplate reader (well mode with excitation 490 nm and emission 516 nm) and KCl solution (50 mM final) was automatically injected after the base line been established. Cells without veratridine treatment were used as control.

Table 2. 2 Composition of Hank's/HEPES buffer

	Concentration (mM)
NaCl	137
KCl	5.0
Na_2HPO_4	0.25
KH_2PO_4	0.44
$CaCl_2$	1.3
$MgSO_4$	1.0
$NaHCO_3$	4.2
HEPES	20
Glucose	0.2 %

2.7.4 Calcium ion fluorescence dye assay

2.7.4.1 Plasmid purification

Sterile LB broth (5 mL) and ampicillin solution (100 mg/mL, 5 µL) were mixed in a 50 mL tube to make ampicillin final concentration at 100 µg/mL. Pellet size frozen XL1-Blue E.*coli* was suspended in prepared LB brouth. Bacteria were incubated in an orbital shaker for 8 hrs at 37°C with vigorous shaking.

After incubation, the starter culture was diluted 1/500 into LB growing medium with 1‰ ampicillin solution in a 2 L cornical flask. Bacteria were grown in orbital shaker overnight (12 - 16 hrs) at 37°C. The optical density of bacteria sample was checked by spectrophotometer at 600 nm against LB brouth. The value should ideally within 1.0 - 1.5 range.

Bacterial cells were harvested by centrifugation (6000 ×g, 20 min) at 4°C. Supernatant was decanted and bacterial pellets were stored at -20°C.
After harvesting enough bacterial cells, the plasmid DNA isolation was carried out with QIAGEN plasmid purification maxi kit according to the manufacture's instruction. Isolated DNA pellet was air-dried for 10 mins and re-dissolved in a suitable volume of sterile water. The purity and concentration of the DNA were checked by Nano Drop (Thermo Scientific, Wilmington, USA). The $A_{260/280}$ value should be within 1.7 - 1.9 to guarantee the purity of the sample. Isolated plasmid sample was then stored at -20 °C until further experiment.

2.7.4.2 Calcium green assay

Plasmid (Cav 3.2, 2.2 µg) was added to 103 µL of Opti-MEM to form the plasmid solution. FuGENE HD reagent (6.6 µL) was added and mixed carefully. The mixture was incubated at room temperature for 5 - 10 mins. This transfection complex (5 µL) was added to corresponding wells with HEK 293 cells and mixed thoroughly. HEK 293 cells were incubated at 37°C, 5% CO_2 for 48 - 72 hrs. The growing medium was changed 24 hrs after transfection.[62]

After transfection (72 hrs), medium was removed and cells were washed once with buffer 1 (100 µL) (Table 2. 3). Calcium green loading solution (50 µL) was added to each testing well and buffer 1 (50 µL) was added to blank wells. Plates were incubated at 37°C in the dark for 1 hr. Cells were washed with 50 µL of buffer 2 (Table 2. 3) carefully for three times. Gramicidin solution (10 µg/mL, 50 µL), mibefradil solution (100 µM, 50 µL), evodiamine (10 mM, 50 µL) and/or rutaecarpine (10 mM, 50 µL)

were added to corresponding wells and incubated at room temperature in dark for 10 mins. Fluorescence intensity was determined with microplate reader (well mode at extension 485 nm and emission 520 nm). Each sample was read every half second for 60 seconds. KCl solution (60 mM, 20 µL) was automatically injected to each well at the 10^{th} second of reading. Transfected HEK 293 cells labeled with calcium green without further treatment were used as control.

Table 2. 3 Composition of buffers used in calcium green assay

	Buffer 1 (mM)	Buffer 2 (mM)
KCl	2	2
$CaCl_2$	1	1
$MgSO_4$	1	1
Glucose	5	5
HEPES pH7.4 with KOH	10	10
NaCl	138	-
Sorbitol	-	276

Chapter 3 Bioactivities of Coptis-Euodia formulae on breast cancer

3.1 Introduction

3.1.1 Cell apoptosis and metastasis in breast cancer

The number of cells of a multicellular organism is highly regulated by controlling the rate of cell division and cell death. Cell apoptosis refers to the cytologic features of programmed cell death (PCD) which occurs in multicellular organisms. This suicide-like intracellular process of cells is activated automatically if cells are no longer necessary by the organism. The apoptotic process plays a central role in the development and functioning of the immune system which represent a defense mechanism at cellular level against cancer. Interruption of normal apoptosis of cells can lead to cancer and auto-immunity. Anti-cancer agents are expected to stimulate apoptosis of cancer cells by targeting effectors along the death signaling pathway including caspases and protein p53.[63-64]

Metastasis is the natural behavior of invasive cancer cells which may include processes of cell migration, adhesion, and invasion. Molecules which can modulate the capacity of cancer cells to migrate, or to adhere to other cells (or to extracellular matrix) are therefore implicated in cancer therapy.[54,65]

3.1.2 Breast cancer cell lines

The first human cell line HeLa – named after the lady from whom the cell

line was derived, was established over 50 years ago by George Gey. The major benefit of using cultured cell lines is that they offer an infinite supply of a relatively homogeneous cell population that is capable of self-replication in standard cell culture medium in cancer research. The first breast cancer cell line to be established was BT-20 in 1958. MD Anderson series widespread 20 years after and still remains the most commonly used breast cancer cell line in the world.[66]

MDA-MB-231 and MCF-7 breast cancer cell lines are adherent epithelial cells, which obtained from mammary gland and breast of female, who has been diagnosed as adenocarcinoma. However, the difference between those two cell lines is that MDA-MB-231 has proved to be an estrogen receptor (ER) negative breast cancer cell line with high metastatic ability while MCF-7 is an estrogen receptor (ER)-dependent non-metastatic breast cancer cell line. In order to investigate the anti-breast cancer activities of Coptis-Euodia formulae on different types of breast cancer, both MDA-MB-231 and MCF-7 cell lines were used as *in vitro* models through this study.[23,67] Moreover, due to the metastasis behavior of MDA-MB-231 *in vitro*, this cell line was chosen as a perfect model for anti-migration and anti-adhesion investigations.[66]

3.2 Results

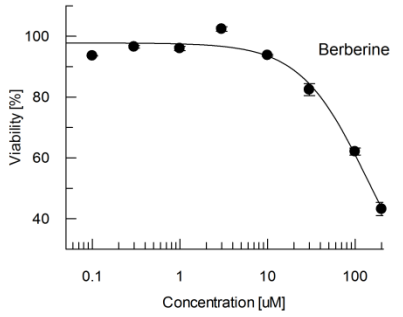
3.2.1 Anti-proliferative and cytotoxicity activities of berberine, evodiamine and Coptis-Euodia crude extracts against human breast cancer cell lines

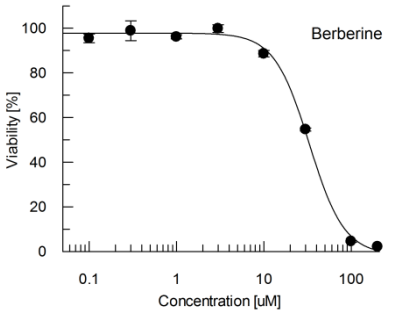
In order to distinguish and evaluate the short term and long term effects of Coptis-Euodia crude extracts and main alkaloid standards isolated

from each herb against human breast cancer cell lines MDA-MB-231 and MCF-7, the MTT-based cell viability assay has been separated into anti-proliferation IC_{50} determination and cytotoxicity evaluation. In anti-proliferation experiments, breast cancer cells were treated with testing compounds at a series of concentrations or 1% DMSO for 1 hour. Medium were then removed and cells were replenished with culture medium for further 96 hrs without disturbance. Short term treatment (1 hour) effects of the testing compounds on the growth of normal breast cancer cells were evaluated by the anti-proliferation IC_{50} values after 96 hrs incubation. Cytotoxicity assay was designed to evaluate the long term effects of anti-cancer candidates at a specific concentration. Percentage cell viabilities from 24 hrs up to 96 hrs in the presence of crude herbal extracts and standard alkaloids compared with 1% DMSO treated cell samples were considered to be the cytotoxicity activities of testing compounds. Percentage viabilities of cells in this section were based on three independent experiments and average absorbance of eight wells of testing substances in each experiment.

3.2.1.1 IC_{50} determination of berberine, evodiamine and Coptis-Euodia crude extracts against breast cancer cell lines

The anti-proliferative IC_{50} curves listed in Figure 3.1 were estimated by GraFit version 5.0.4 (Erithacus Software, Surrey, UK) and IC_{50} values were calculated using the 4 full parameter equation. 1% DMSO treated cells were used as negative control which represents cells with 100% viability and percentage cell viabilities of each testing samples were calculated against it.


Berberine
Viability [%]
100
80
60
40
0.1
1
10
100
Concentration [uM]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Viability [%]
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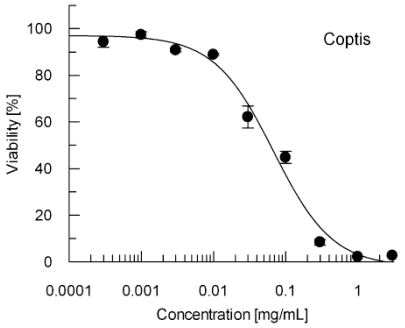
80
60
40
20
0
0.1
1
10
100
Concentration [uM]


Coptis
Viability [%]
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80
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20
0
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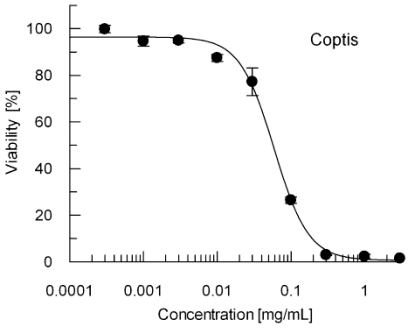
0.001
0.01
0.1
1
Concentration [mg/mL]


Coptis
Viability [%]
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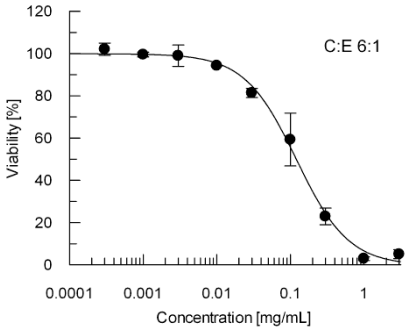
0.001
0.01
0.1
1
Concentration [mg/mL]


C:E 6:1
Viability [%]
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100
80
60
40
20
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0.0001
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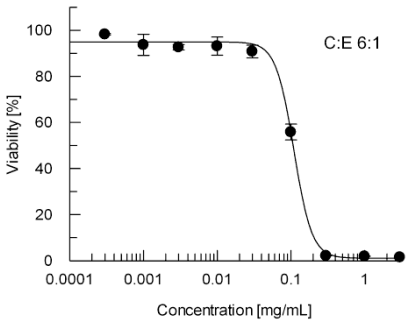
0.01
0.1
1
Concentration [mg/mL]


C:E 6:1
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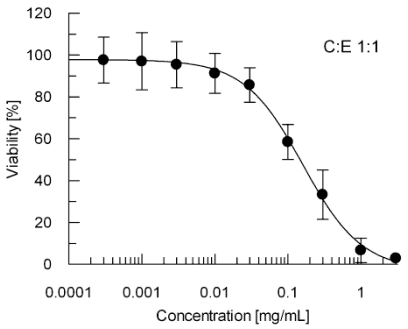
Concentration [mg/mL]


C:E 1:1
Viability [%]
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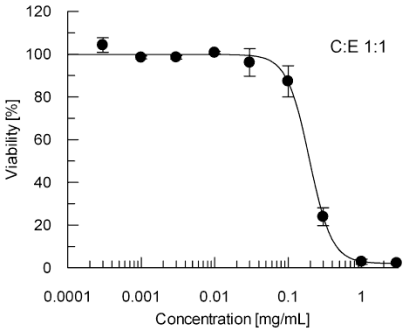
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
Concentration [mg/mL]


C:E 1:1
Viability [%]
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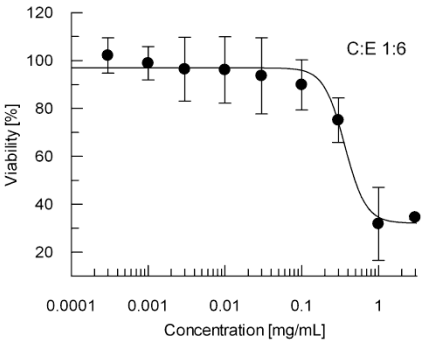
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
Concentration [mg/mL]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0.01
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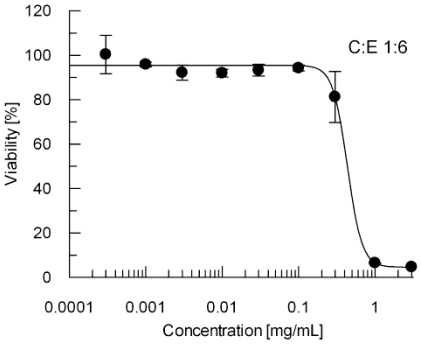
1
Concentration [mg/mL]


C:E 1:6
Viability [%]
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100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
Concentration [mg/mL]

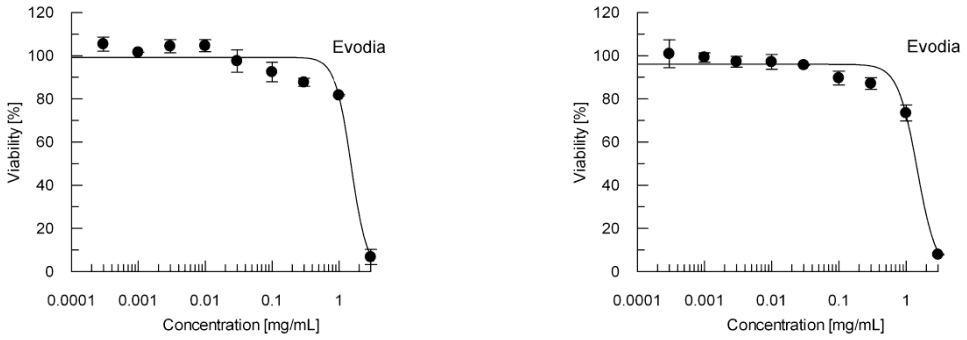


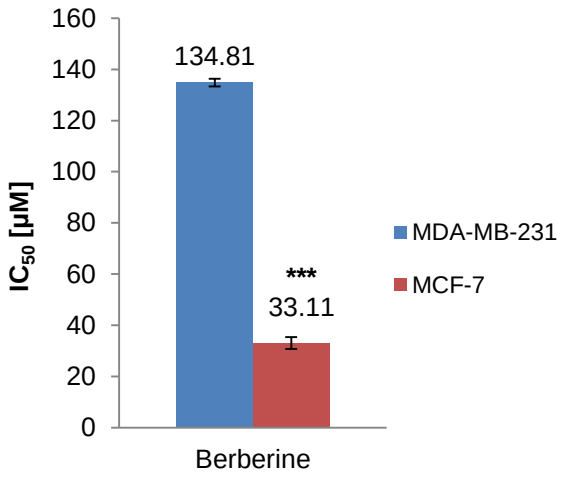
Figure 3.1 Anti-proliferative IC_{50} curves of berberine and Coptis-Euodia crude extracts against MDA-MB-231 (left column) and MCF-7 (right column) human breast cancer cells. Cell viabilities were measured by the MTT assay. Each point represents the mean ± S.D. of multiple wells from three independent experiments (n = 24). The IC_{50} curves were estimated by GraFit version 5.0.4.

As shown in Figure 3.2, berberine, Coptis, Euodia and three Coptis-Euodia extracts with different mixed ratios gave anti-proliferative activities on both breast cancer cell lines with 1 hour treatment and proliferated for another 96 hrs. Anti-proliferative activity of berberine was four times more effective against the MCF-7 cells with an IC_{50} value of 33.1 μM than MDA-MB-231 cells with an IC_{50} value of 134.81 μM. Breast cancer cell line MDA-MB-231 was more tolerated to berberine than MCF-7 cells ($P < 0.001$). Coptis extract showed the strongest anti-proliferative activity with IC_{50} values of 0.07 and 0.06 mg/mL against MDA-MB-231 and MCF-7 cell line respectively. IC_{50} values of C : E 6 : 1 extract were 0.12 and 0.11 mg/mL, which indicated that 6 : 1 was the most effective ratio of this duo herb extracts against breast cancer cells compared with 1 : 1 (0.16 and 0.19 mg/mL) and 1 : 6 (0.36 and 0.43 mg/mL). Both cell lines were tolerated Euodia extract mostly with IC_{50} values higher than 1.4 mg/mL. However, introducing of Coptis significantly enhanced the anti-proliferative activity against both breast

cancer cell lines with IC_{50} lower than 0.5 mg/mL even with a low ratio (C : E 1 : 6) in the formula ($P < 0.001$). Apart from Coptis, C : E 6 : 1, and C : E 1 : 1 treated samples ($P > 0.05$), the anti-proliferative efficiency of C : E 1 : 6 ($P < 0.01$) and Euodia ($P < 0.05$) extracts on MDA-MB-231 and MCF-7 cells was significant.

The IC_{50} values of evodiamine against both breast cancer cell lines are not presented in this thesis. Under the same experimental circumstances, cell viability of 200 μM evodiamine treated cells was 65% ± 6% on MDA-MB-231 cell line after 96 hrs incubation. Experiment was terminated due to the water solubility limitation of evodiamine and percentage restriction of DMSO in cell culture. According to the results, IC_{50} of evodiamine against MDA-MB-231 was higher than 200 μM. However, the exact value cannot be determined by the MTT-based anti-proliferation assay carried out through this study.

A



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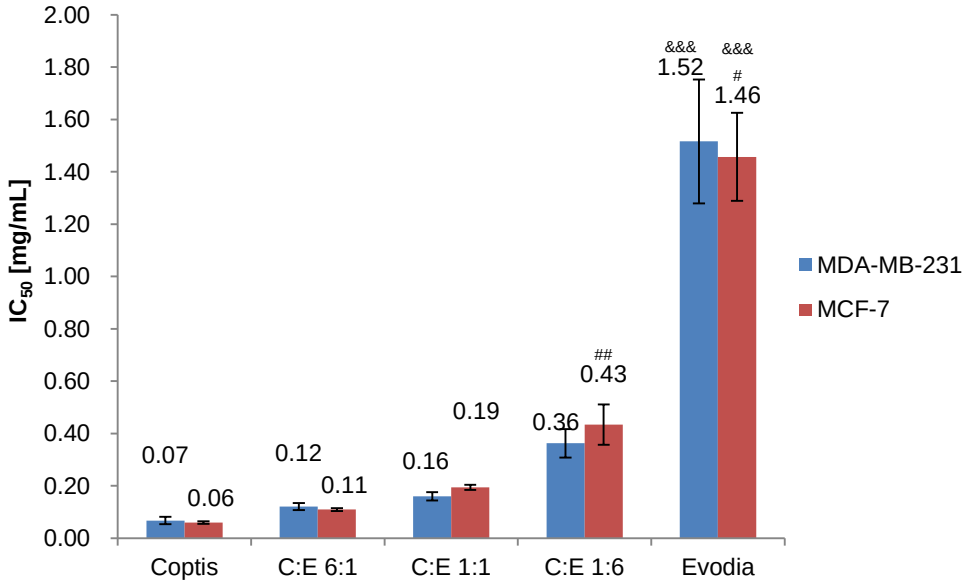


Figure 3.2 Anti-proliferative IC_{50} values of berberine (A) and Coptis-Euodia crude extracts (B) against MDA-MB-231 and MCF-7 cell lines. Cell viabilities were measured by the MTT assay and the IC_{50} values were determined by Grafit 5.4.0. Data are presented as the mean ± S.D. (n = 3). ***P < 0.001 compared with the IC_{50} value of berberine against MDA-MB-231 cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the IC_{50} values of Coptis-Euodia crude extracts against MDA-MB-231 cells. $^{\&\&\&}P < 0.001$ compared with the IC_{50} values of other four Coptis-Euodia crude extracts.

3.2.1.2 Cytotoxicity activities of berberine, evodiamine and Coptis-Euodia crude extracts against breast cancer cell lines

Cytotoxicity activities of berberine (10 μM), evodiamine (10 μM) and Coptis-Euodia extracts (0.1 mg/mL) against MDA-MB-231 and MCF-7 cell lines were evaluated with continuous treatment for up to four days (96 hrs) and followed by MTT assay as described previously. Percentage

viabilities of both cell lines at multi- time points were calculated by using 1% DMSO treated cells for the same period of time as negative control.

Percentage cell viability trends of standard alkaloids treated breast cancer cells from 24 hrs were presented in Figure 3.3. After 24 hrs treatment of evodiamine (10 μM), over 90% cells of both cell lines were still alive. Subsequently, cell viability of MCF-7 dropped to 64.17% after 48 hrs treatment while it of MDA-MB-231 was 73.06%. The time-dependent cytotoxicity activity of evodiamine led the cell viability of MDA-MB-231 cell line to 66.06% and MCF-7 cell line to 47.22% after 96 hrs persistent treatment. Berberine (10 μM) treated MDA-MB-231 and MCF-7 resulted in a great inhibition of cell viability after 24 hrs, with cell viabilitis of 40.62% and 27.57% respectively. The effect of berberine induced cell apoptosis on MCF-7 cell line was very strong after 48 hrs with only 3.86% cells were viable. More than 98% of MCF-7 cells lost viability after 72 hrs exposure to berberine.

According to Figure 3.3, both berberine and evodiamine at 10 μM showed cytotoxicity activity on breast cancer cell lines in a time-dependent manner. Longer exposure to alkaloids led to lower cell viabilities. Moreover, both MDA-MB-231 and MCF-7 cells were tolerated to evodiamine more than to berberine at the same concentration and treatment time. However, the ER^- breast cancer cell line MDA-MB-231 appeared to be more resistant to both alkaloids at all tested time points.

As shown in Figure 3.4, the viability of both breast cancer cell lines were inhibited by 0.1 mg/mL of Coptis-Euodia extracts after 24 hrs treatment. The Coptis-Euodia extracts induced cytotoxicity activities were in a

time-dependent manner. The viabilities of Coptis treated cell lines were lower than 5% compared with the control after 48 hrs. Similar results were also obtained from C : E 6 : 1 and C : E 1 : 1 treated cells lines. In MCF-7 cell line, all five Coptis-Euodia extracts showed strong cytotoxicity activities after 72 hrs with viabilities from 4.45% (Euodia), the highest, to 1.40% (Coptis), the lowest. However, MDA-MB-231 cell line was more tolerant to herbal extracts with higher Euodia ratio in the formulae after long term treatment. Cell viabilities of C : E 1 : 6 and Euodia extracts treated samples were observed to be 26.20% and 49.17% respectively after 72 hrs.

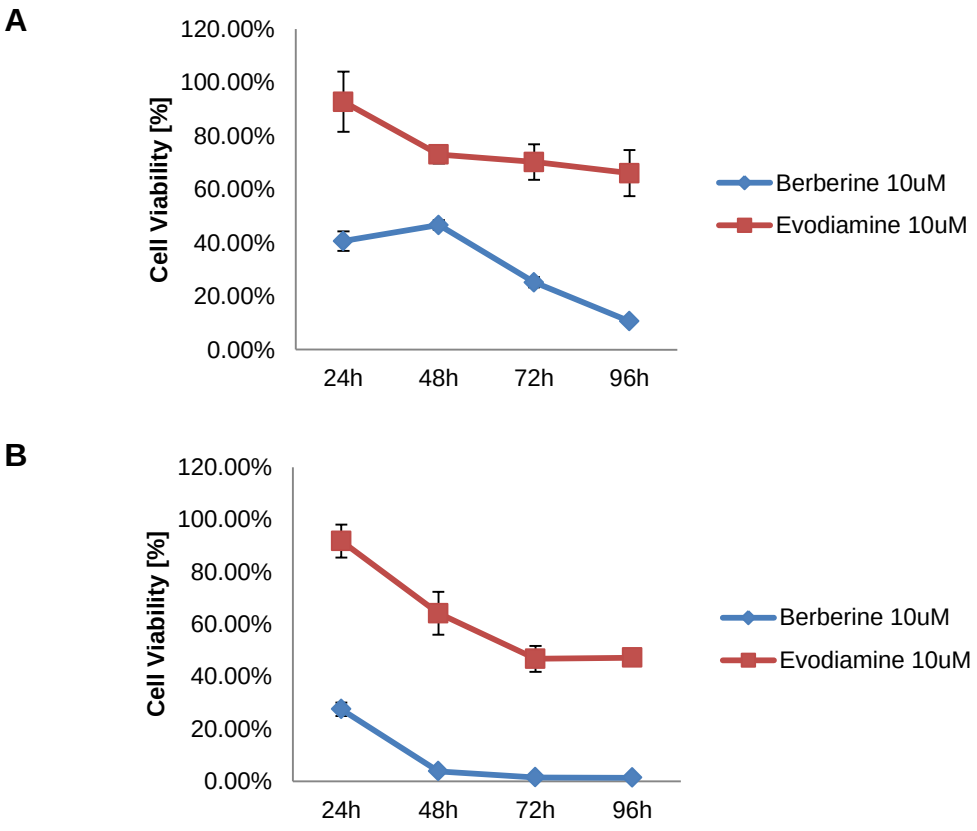


Figure 3.3 Cytotoxicity activities of berberine (10 µM) and evodiamine (10 µM) against MDA-MB-231 (A) and MCF-7 (B) breast cancer cell lines for 24, 48, 72, 96 hrs were determined by MTT assay. Percentage cell viabilities against 1% DMSO

treated cell are presented as mean ± S.D. of multiple wells from three independent experiments.

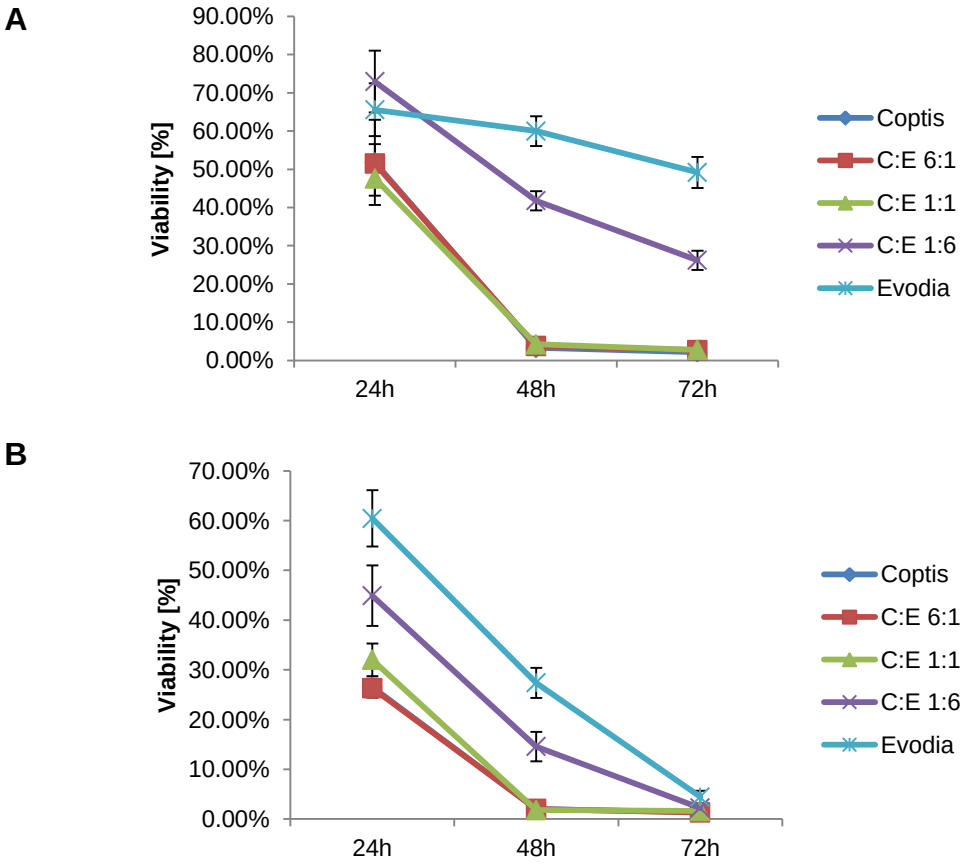


Figure 3.4 Cytotoxicity activities of Coptis-Euodia extracts (0.1 mg/mL) against MDA-MB-231 (A) and MCF-7 (B) breast cancer cell lines for 24, 48, 72 hrs were determined by MTT assay. Percentage cell viabilities against 1% DMSO treated cell are presented as mean ± S.D. of multiple wells from three independent experiments.

3.2.2 Anti-migration activities of berberine, evodiamine and Coptis-Euodia crude extracts against human breast cancer cell line MDA-MB-231

The horizontal motility of MDA-MB-231 human metastatic breast cancer cells was examined by scratch wound-healing migration assay. In order to avoid the cell apoptosis induced anti-migration activities of standard alkaloids and crude herbal extracts, concentrations lower than the IC_{50} value of each sample against MDA-MB-231 were applied and evaluated. After the establishment of the wound, cells were continuously cultured in the absence or presence of berberine (5, 10, and 25 μM), evodiamine (10, 25, and 50 μM) or various concentrations (according to the IC_{50} determination results of section 3.2.1.1) of Coptis (1 and 5 μg/mL), C : E = 6 : 1 (5 μg/mL and 0.01 mg/mL), C : E = 1 : 1 (0.01 and 0.05 mg/mL), C : E = 1 : 6 (0.05 and 0.15 mg/mL) and Euodia (0.05 and 0.15 mg/mL) for at least 24 hrs. Photographs of cell migration behavior at 8, 24, or 48 hrs are represented in Figure 3. 5 (A), Figure 3. 6, and Figure 3. 8.

Photographs presented in Figure 3. 5 (A) demonstrated the motility and the morphologic changes of MDA-MB-231 cells treated with 0.5% DMSO from 0 hr to 48 hrs after wound been established. After being wounded by pipette tip and washed with culture medium twice, cells out of culture environment for a period of time started to shrink. Some round shaped cells can be observed in unwounded areas which indicated that though special cares have been taken, slight stress occurred during the washing process. After 8 hrs recovery in the presence of 0.5% DMSO, most MDA-MB-231 cells in un-wounded areas were in healthy condition with spindle shape and cells at wounded edge started to migrate horizontally to heal the wound. However, a clear boundary of wound can still be observed. The scale bar on the photograph of 24 hrs cultured sample indicated the centre of the original wound. Cells from both sides moved further toward each other to reduce the wound and polygonal shaped

single cells started to crawl and stretch to form linkage between each other. As shown in Figure 3. 5 (B), after 8 and 24 hrs incubation, 0.5% DMSO treated MDA-MB-231 cells (control) migrated and significantly healed the wound ($P < 0.001$) with 53% and 20% width of original wound observed respectively. After 48 hrs incubation, MDA-MB-231 cells treated with 0.5% DMSO healed the wound completely as a confluent monolayer with cells contacted with each other and no uncovered substrate can be observed.

Percentage width of the original wound shown in Figure 3. 7 indicated that both berberine and evodiamine at tested concentrations worked as migration inhibitors against MDA-MB-231 cell line. The anti-migration activities of both alkaloids were in time and dose dependent manner with significant interaction ($P < 0.001$). MDA-MB-231 cells treated with higher concentration of alkaloid for longer time exhibit lower migration ability. Moreover, evodiamine exhibited stronger anti-migration activities compared with berberine against MDA-MB-231 cells at the same corresponding concentrations (10 and 25 μM) ($P < 0.001$).

As shown in Figure 3. 6, cells incubated with higher concentrations (10 and 25 μM) of berberine for 24 hrs can be observed shrunk or exploded with some of the nucleoli disappeared. Obvious clearance areas between cell colonies indicated that cells were acutely stress and started to apoptosis in the presence of external chemical stimulation though slow migration was still in progress. In the meantime, evodiamine treated cells exhibited morphologic change. Considerably amount of cells lost the spindle shape and become round or oval shaped cells. Cells without clear membrane can also be observed in samples incubated with all

three concentrations of evodiamine from 8 hrs.

After 8 hrs treatment with 10 μM of evodiamine, 78% wound width can be detected, while that of berberine was 58%, just 5 percent wider than 0.5% DMSO treated sample. 24 hrs incubated with 10 μM of evodiamine resulted in a 56% wound width, almost 3-fold wider than that of control ($P < 0.001$). Anti-migration activities of both alkaloids at 10 and 25 μM indicated that evodiamine is a much efficient breast cancer metastasis inhibitor than berberine without inducing cytotoxicity on cells ($P < 0.001$).

Migration of the MDA-MB-231 cells was restrained by Coptis crued extract in a time and dose dependent manner ($P < 0.001$). Considerable anti-migration effects can also be observed in the other three Coptis involved Coptis-Euodia extracts at testing concentrations against MDA-MB-231 cells from 8 hrs. Photographs of cells incubated with higher concentration of Coptis (5 μg/mL) and C : E 6 : 1 (5 μg/mL) for 48 hrs indicated unhealthy condition of the cells, with a large amount of oval shaped cells in the visual field. Similar effects can be observed in cell samples treated with C : E 1 : 1 and C : E 1 : 6 extracts after 48 hrs. However, C : E 6 : 1 extract at 0.01 mg/mL exhibited obvious cytotoxicity activity with most of the cells shrunk, detached from the bottom of culture plate and cell debris floated in the medium after 48 hrs incubation (Figure 3. 8). As shown in Figure 3. 9 (A and B), cells treated with extracts with high Coptis ratio in formulae started to show significant anti-migration activity at μg/mL level compared with control ($P < 0.001$). 78% wound width of 0 hr was examined after 8 hrs in 1 μg/mL Coptis extract treated sample. Same strength of inhibition was inspected in cells treated with 0.05 and 0.15 mg/mL of C : E 1 : 6 extract (Figure 3. 9, D).

As shown in Figure 3. 8, MDA-MB-231 cells incubated with both concentrations of Euodia extract migrated faster than cells treated with other extracts from 8 hrs. After 24 hrs incubation, obvious morphologic changes can be observed with some of the cells shrunk and also some loose cell colonies in un-wounded areas. However, healing process of cells at wound edge has not been affected. Data presented in Figure 3. 9 E illustrated that compared with the width of wound at 0 hr, 28% and 30% of wound were observed at 8 hrs with 0.05 and 0.15 mg/mL of Euodia crude extract treated sample respectively. Such results indicated that Euodia crude extract significantly increase the migration ability of MDA-MB-231 cells ($P < 0.001$) which may decrease the anti-metastatic activities of Euodia involved Coptis-Euodia formulae. Though evodiamine worked as a metastatic inhibitor, evodiamine containing extracts of Euodia (0.05 and 0.15 mg/mL) presented the reverse activity. This might due to the low percentage of this quinoline alkaloid in the plant. Quality control of Euodiae fructus in Chinese pharmacopoeia prescribed that the total amount of evodiamine and rutaecarpine (another quinolone alkaloid contained in Euodia) should be no less than 0.15% according to the standard analytical process. Quantitative results of Pan *et al* indicated that 0.024% of evodiamine can be obtained from the dry material of Euodia.[41] Furthermore, other components in the crude extracts of Euodia should responsible for the Euodia induced migration of MDA-MB-231 cells. Thus, Coptis dominated the inhibition of horizontal motility of MDA-MB-231 human metastatic breast cancer cells in these dual herbal formulae according to this wound-healing anti-migration study. Higher concentrations in each Coptis-Euodia crued extracts treated sample (data not shown) which induced apoptosis after 24 hrs incubation may not be considered as anti-migration activity.

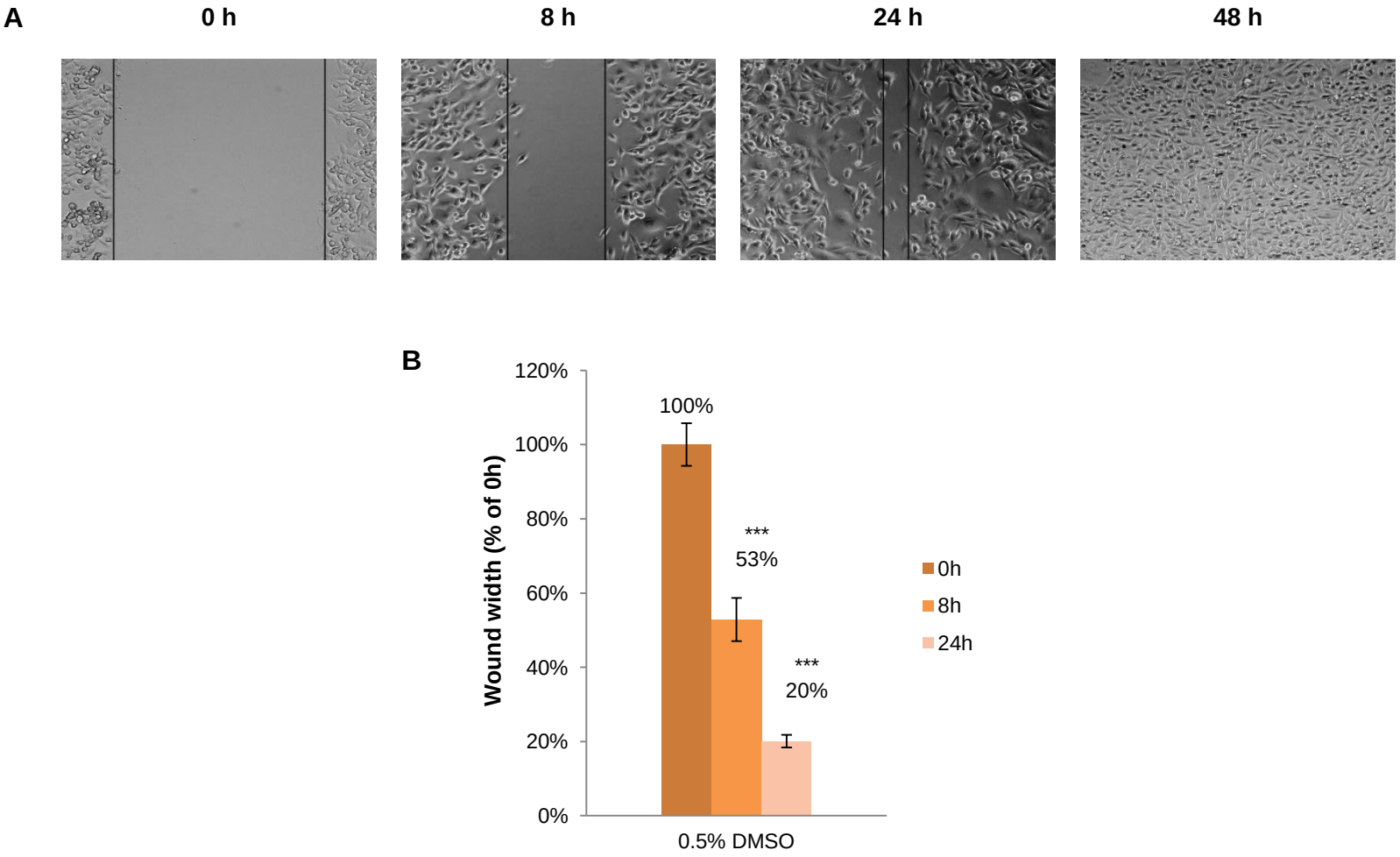
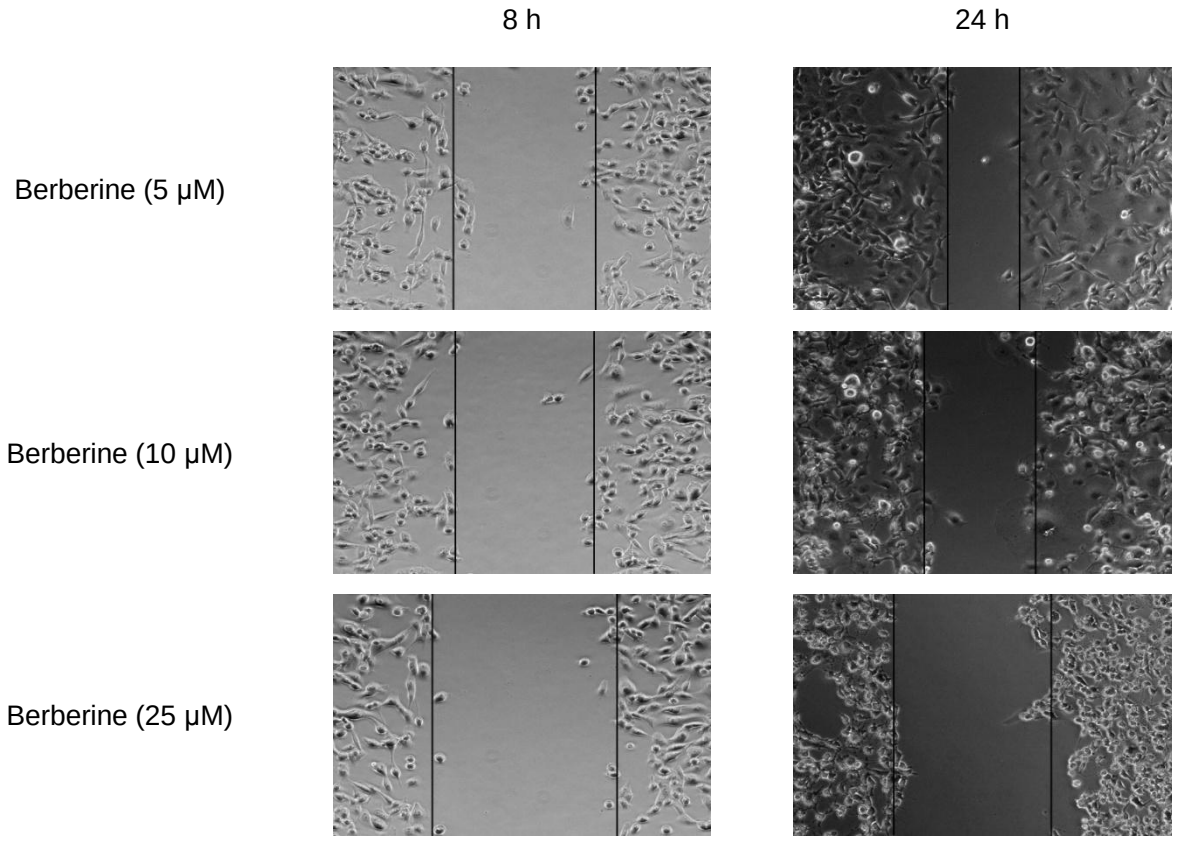

A
0 h
8 h
24 h
48 h
B
Wound width (% of 0h)
120%
100%
80%
60%
40%
20%
0%
100%
***
53%
***
20%
0h
8h
24h
0.5% DMSO

Figure 3. 5 Photographs of wounded MDA-MB-231 cells treated with 0.5% DMSO and incubated at 37 ºC for 0, 8, 24 and 48 hrs (A). Figure 3. 5 (B) is the quantitative effects of 0.5% DMSO on cell migration *in vitro*. Photographs were taken under an Olympus Cell^M inverted microscope. Data are presented as the mean ± S.D. of multiple wells from three independent experiments (n = 12). ***P < 0.001 compared with the width of original wound.


8 h
24 h
Berberine (5 μM)
Berberine (10 μM)
Berberine (25 μM)

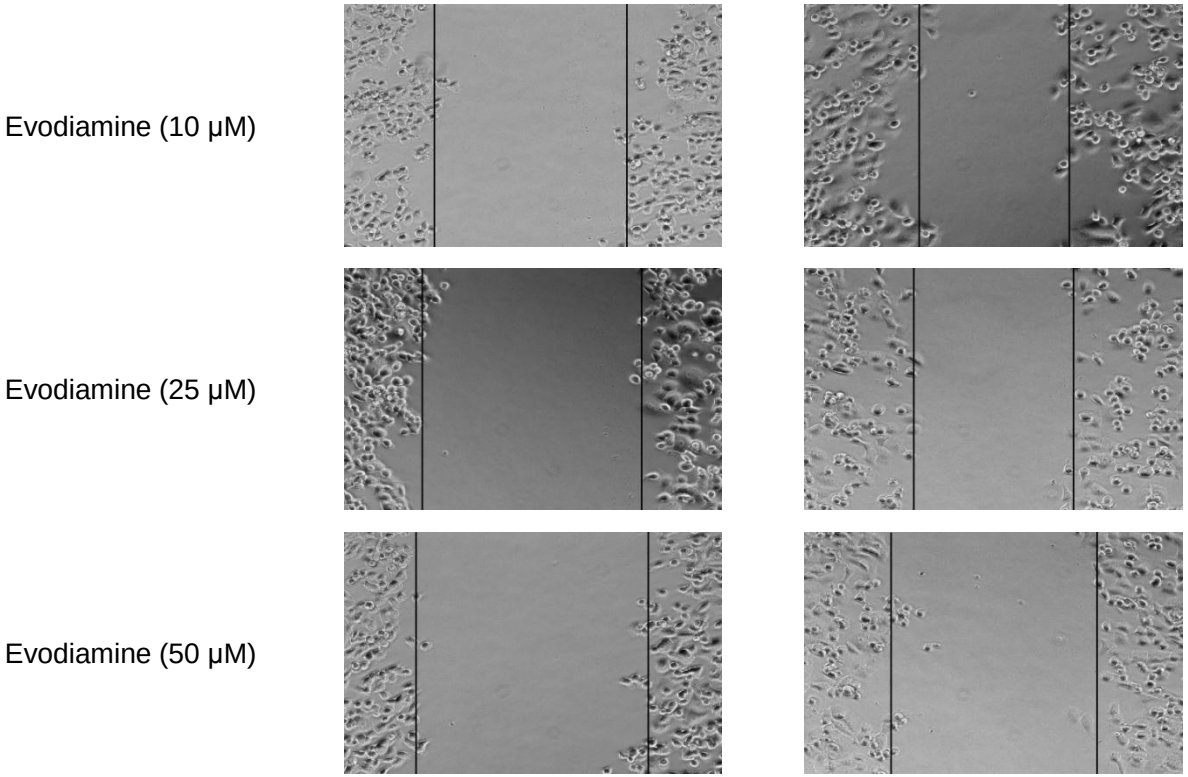


Figure 3. 6 Effects of berberine and evodiamine on MDA-MB-231 cell migration in vitro. Photographs of the wound of cells treated with berberine or evodiamine and incubated at 37 ºC for 8 and 24 hrs were taken under an Olympus Cell^M inverted microscope.

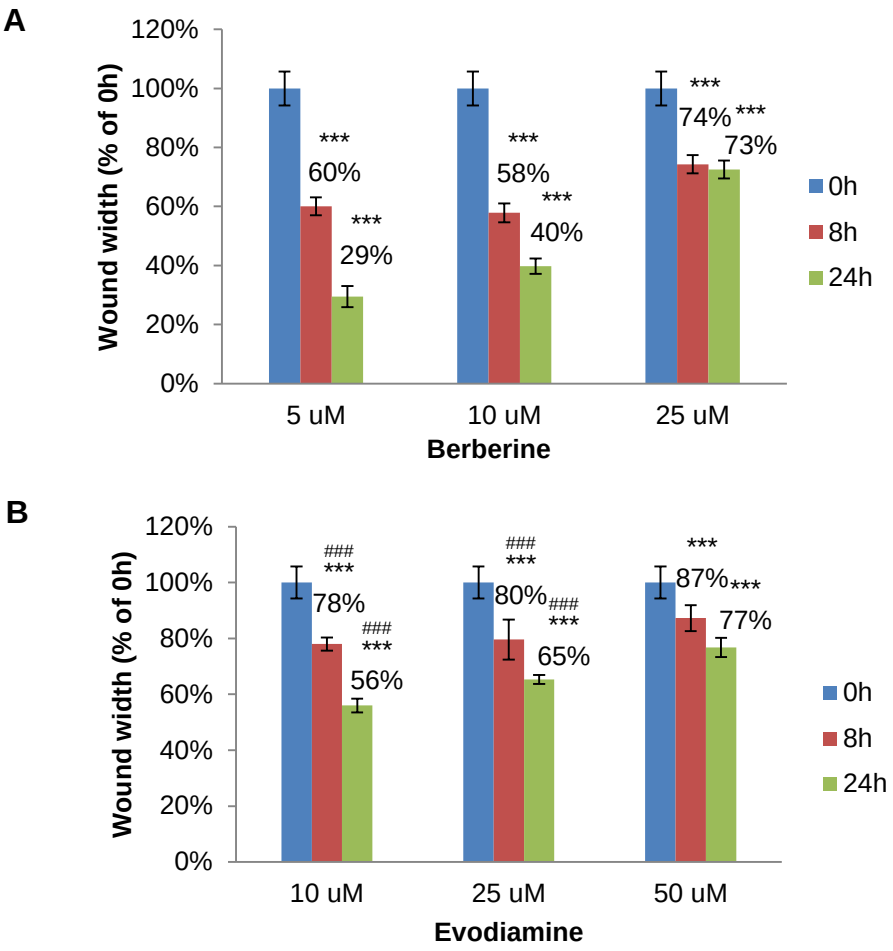
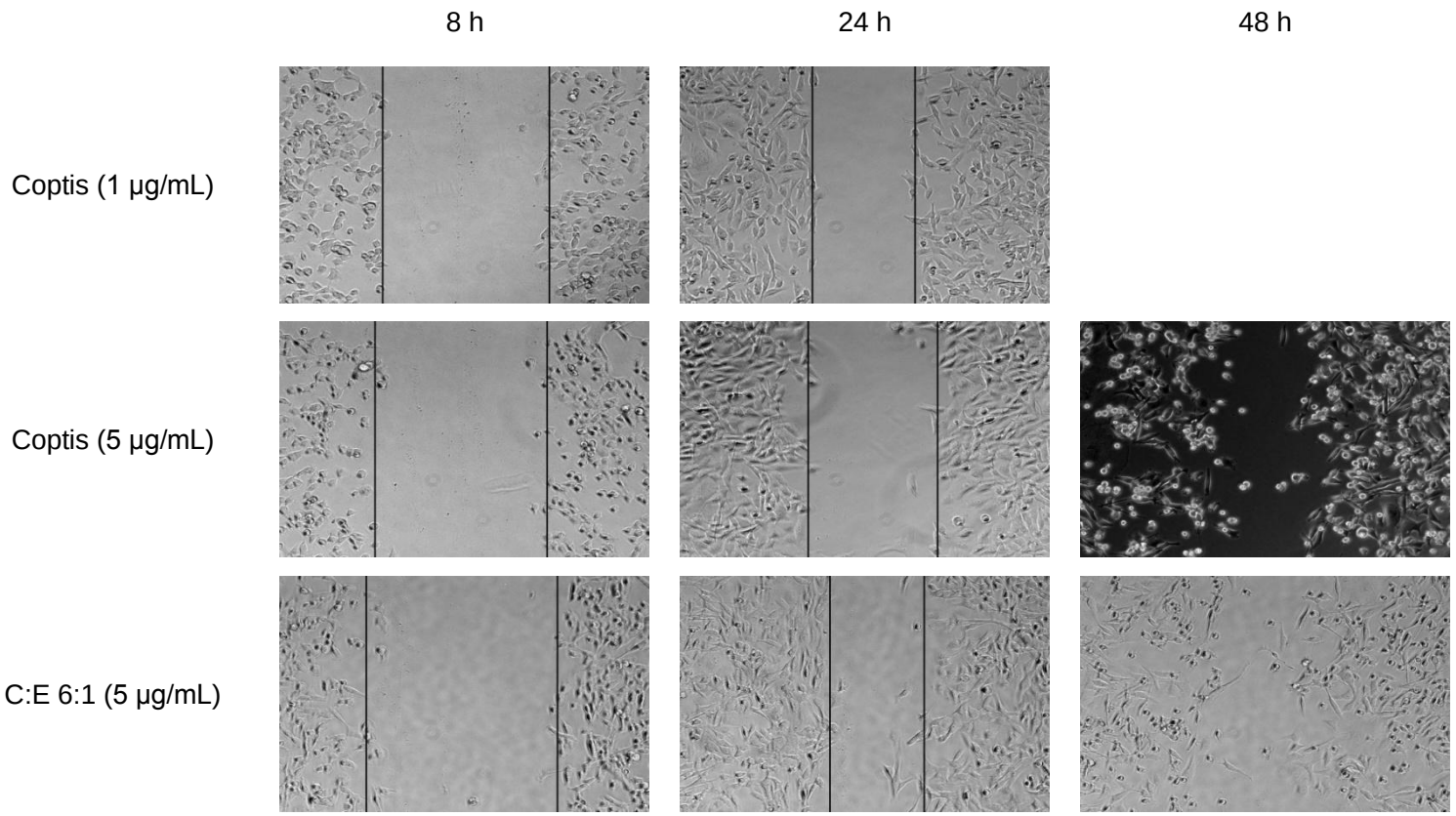
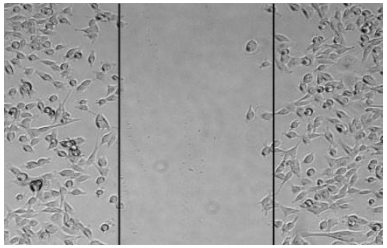
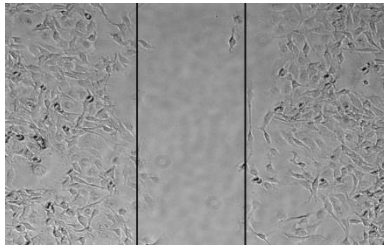
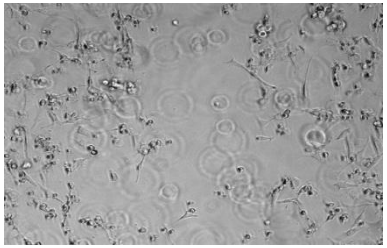


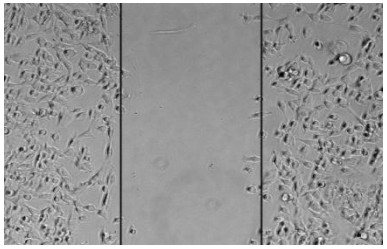
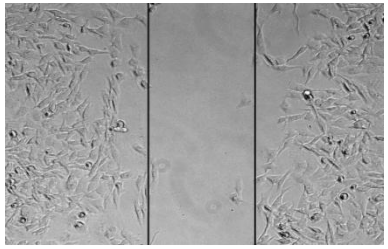
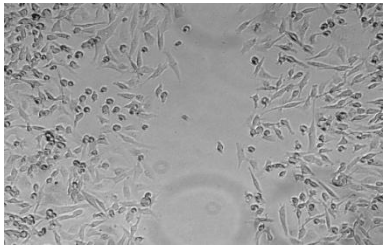
Figure 3. 7 Quantitative effects of berberine (A) and evodiamine (B) on MDA-MB-231 cell migration *in vitro*. Data are presented as the mean ± S.D. of multiple wells from three independent experiments (n = 12). ***P < 0.001 compared with control at the same time point, $^{###}$P < 0.001 compared with berberine treated sample at the same concentration and time point.


8 h
24 h
48 h
Coptis (1 µg/mL)
Coptis (5 µg/mL)
C:E 6:1 (5 µg/mL)

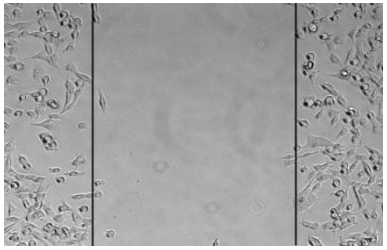
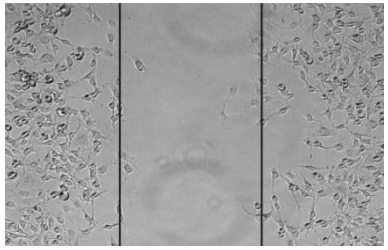
C:E 6:1 (0.01mg/mL)

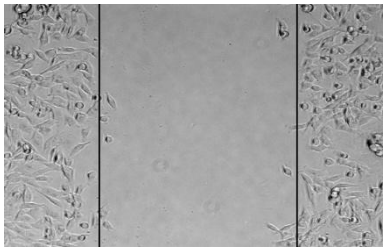
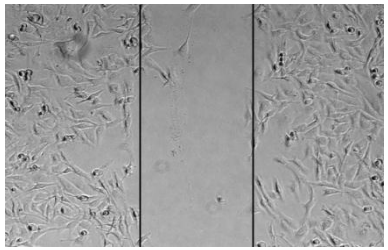
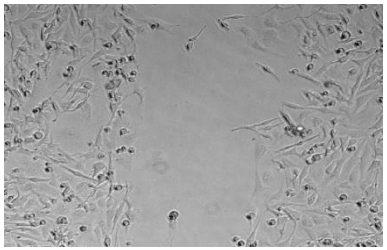
C:E 1:1 (0.01 mg/mL)

C:E 1:1 (0.05 mg/mL)

C:E 1:6 (0.05 mg/mL)

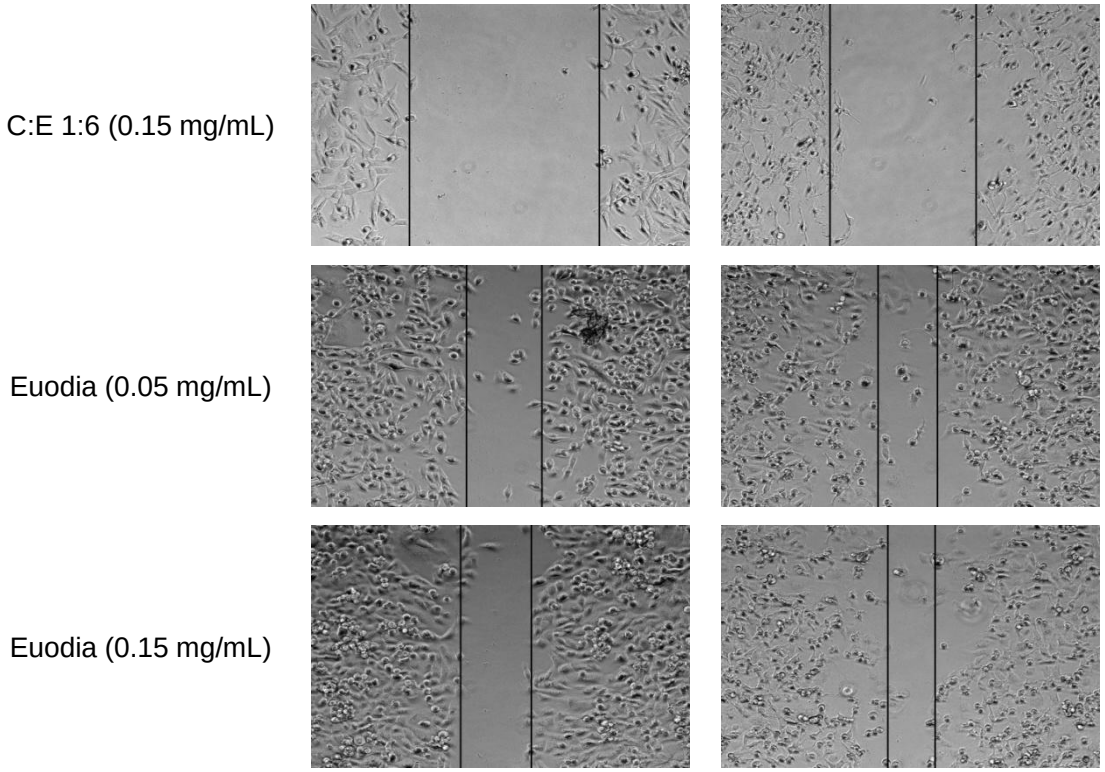
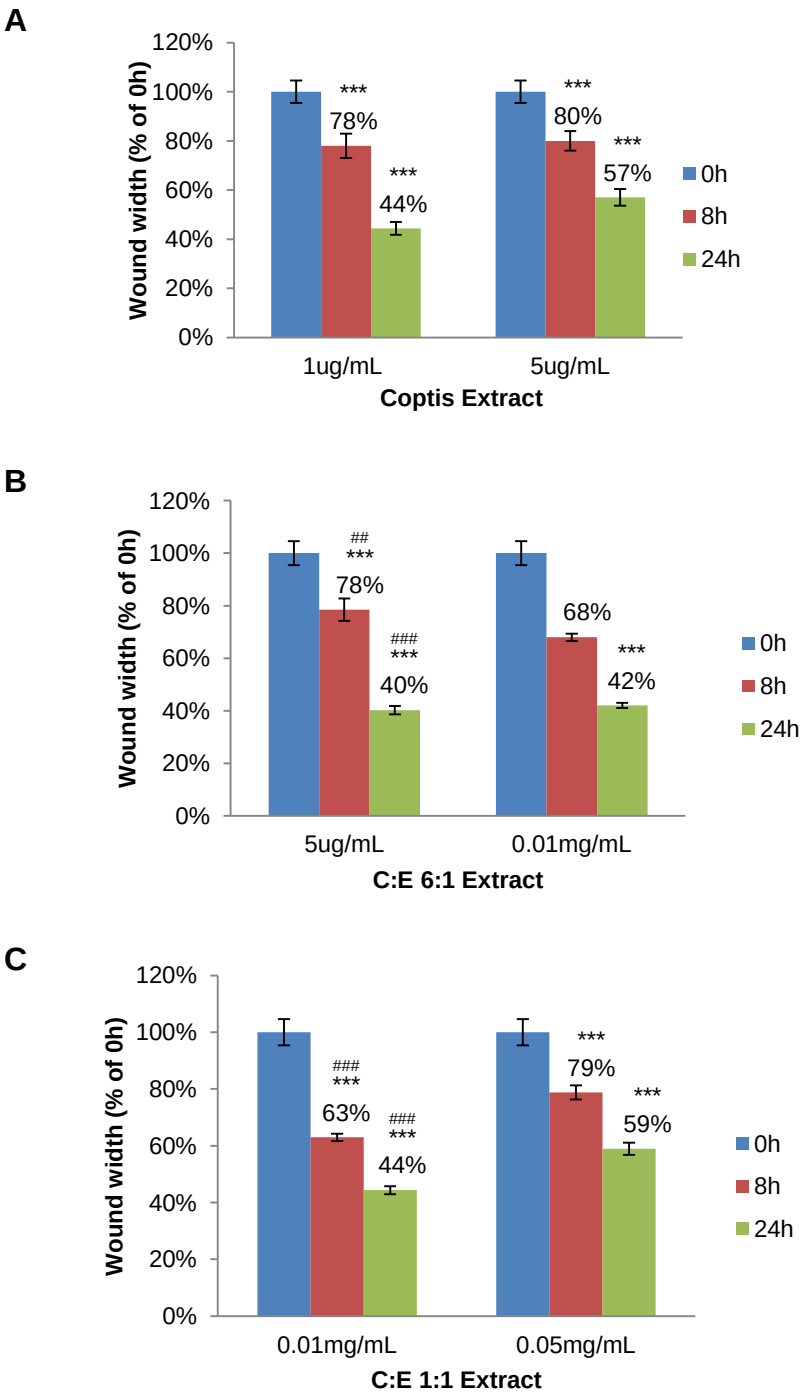


Figure 3. 8 Effects of Coptis-Euodia crude extracts on MDA-MB-231 cell migration *in vitro*. Photographs of the wound of cells treated with Coptis-Euodia crude extracts and incubated at 37 ºC for 8, 24 and 48 hrs were taken under an Olympus Cell^{M} inverted microscope.


A
120%
100%
80%
60%
40%
20%
0%
Wound width (% of 0h)
***
78%
***
44%
***
80%
***
57%
0h
8h
24h
1ug/mL
5ug/mL
Coptis Extract
B
120%
100%
80%
60%
40%
20%
0%
Wound width (% of 0h)
##
***
78%
###
***
40%
68%
***
42%
0h
8h
24h
5ug/mL
0.01mg/mL
C:E 6:1 Extract
C
120%
100%
80%
60%
40%
20%
0%
Wound width (% of 0h)
###
***
63%
###
***
44%
***
79%
***
59%
0h
8h
24h
0.01mg/mL
0.05mg/mL
C:E 1:1 Extract

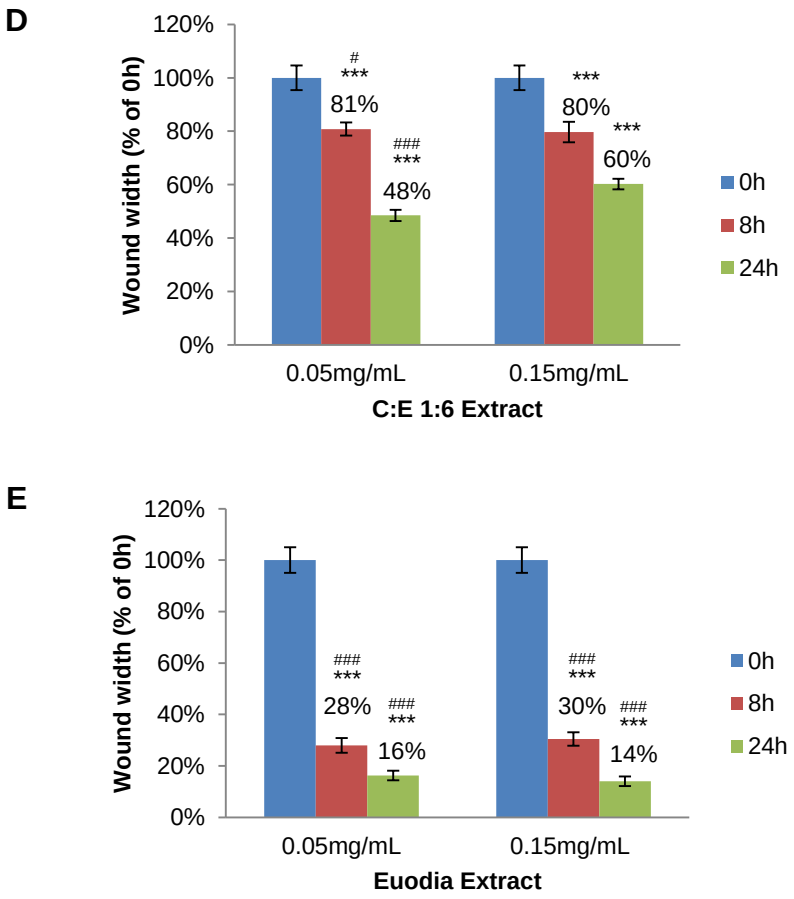


Figure 3. 9 (A to E) Quantitative effects of Coptis-Euodia crude extracts on MDA-MB-231 cell migration *in vitro*. Data are presented as the mean ± S.D. of multiple wells from three independent experiments (n = 12). ***P < 0.001 compared with control at the same time point. $^{\#\#}$P < 0.01, $^{\#}$P = 0.001, $^{\#\#\#}$P < 0.001 compared with Coptis-Euodia crude extracts treated sample at the same concentration and time point.

3.2.3 Anti-adhesion activities of berberine, evodiamine and Coptis-Euodia crude extracts against breast cancer cell line MDA-MB-231

Fibronectin is an extracellular matrix glycoprotein which can enhance

cell adhesion in culture. Employment of fibronectin in cancer cell culture can simulate the *in vivo* cancer cell (tissue) behavior *in vitro*.

Influence of berberien (10 μM), evodiamine (10 μM) and Coptis-Euodia crude extracts (0.1 mg/mL) on the adherent of MDA-MB-231 cells to fibronectin pre-coated surface are shown in Figure 3. 10 and Figure 3. 11 with 0.1% DMSO treated sample as negative control. All tested compounds and herbal extracts decreased the adherent ability of MDA-MB-231 cells. Both berberine and evodiamine at 10 μM exhibited a faint anti-adhesion activity against MDA-MB-231 cell line in a time-dependent manner. However, statistical analysis indicated that there is no significant difference between the anti-adhesion activity of berberine and evodiamine at the same concentration against MDA-MB-231 cells with 2 and 6 hrs treatment. On the other hand, the anti-adhesion activities of Coptis-Euodia crude extracts (0.1 mg/mL) with 2, 6 and 12 hrs exposure were diverse. Time-dependent trends cannot be observed in those herbal extracts treated samples. However, after 12 hrs treatment, adherent ability of MDA-MB-231 breast cancer cells decreased significantly with the increasing proportion of Coptis in Coptis-Euodia formulae ($P < 0.01$). Cells treated with 0.1 mg/mL of Coptis extract for 12 hrs gave the lowest adherent ability of MDA-MB-231 cells. Only 39% adherent cells can be observed by MTT assay compared with control. Viable adherent cells of other Coptis-Euodia extracts treated sample were 45%, 52%, 66%, and 68% respectively after 12 hrs.

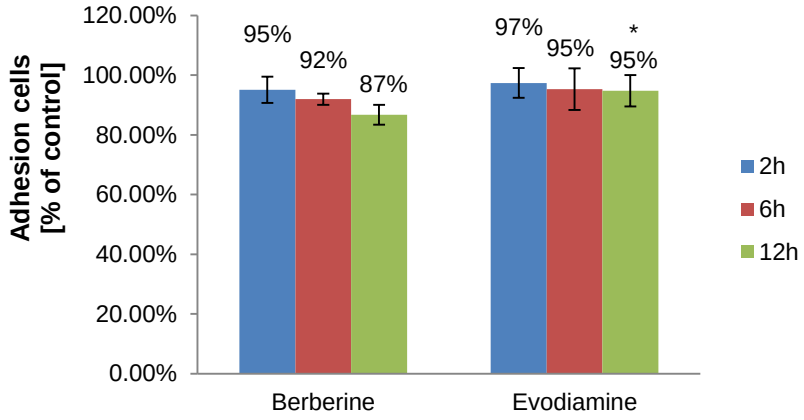


Figure 3. 10 Effects of Berberine (10 μM) and Evodiamine (10 μM) on MDA-MB-231 cells adherent to fibronectin were evaluated by MTT assay. Data are presented as mean ± S.D of multiple wells from two independent experiments (n = 12). *P < 0.05 compared with berberine treated sample at the same time point.

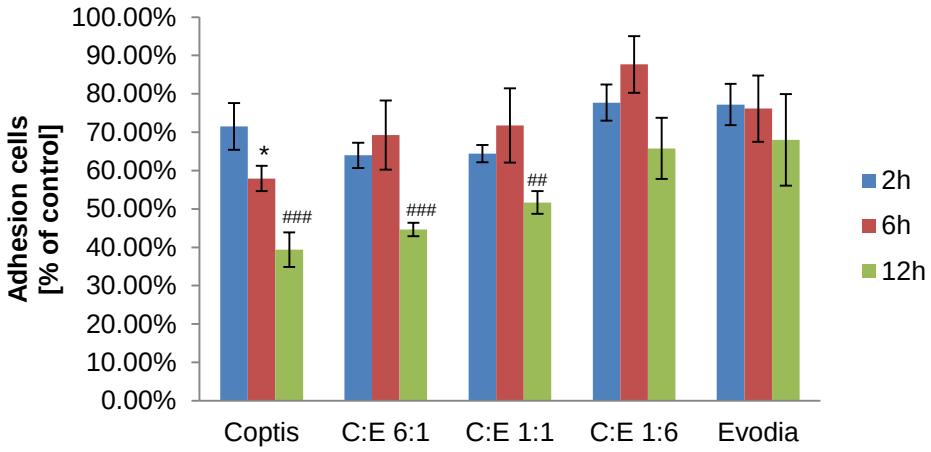


Figure 3. 11 Effects of Coptis-Euodia extracts (0.1 mg/mL) on MDA-MB-231 cells adherent to fibronectin were evaluated by MTT assay. Data are presented as mean ± S.D of multiple wells from two independent experiments (n = 4). *P < 0.05 compared with other four Coptis-Euodia crude extracts treated samples at 6 hrs, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with C : E 1 : 6 and Euodia crude extracts treated samples at 12 hrs.

3.3 Discussion

Commonly, dried medicinal plant believed to be effective for about five years. However, specific effective period of different herbal materials varied due to the storage conditions and the characterization of effective components in the herb. For example, herbal material contains saccharides and/or essential oil may last much shorter than expected due to deterioration of those ingredients. *Euodia rupaecarpa* (Juss.) Benth. used in this study were collected years ago before the extracting procedure started. Though the dry fruits have been sealed and stored in a cool dry place, if degradation of effective components (especially alkaloids) has occurred or not during years of storage remains uncertain. In order to prove the effectiveness of research object, high performance liquid chromatography (HPLC) investigation has been processed. Characteristic peaks of alkaloids in HPLC results (black arrows on chromatogram A of Appendix 2) indicated that the quality of Euodia sample used in this study is acceptable.

As described previously, short term (1 hr) and long term (up to 96 hrs) effects of Coptis-Euodia crude extracts along with standard alkaloids berberine and evodiamine on both human breast cancer cell lines have been investigated in this chapter. IC_{50} determination proved the effect of short term treatment on the growth of cancer cells by testing various concentrations of research candidates (Figure 3.1). Long term cytotoxic activities and trend of specific concentration of each sample on breast cancer cells were then evaluated (Figure 3.3 and Figure 3.4). MTT colorimetric assay applied in both investigations relies on optical density reflected cell viabilities. Thus, cell models used to represent 100% viable

become critical. Passage number of cells, cell culture environment, and other influencing factors during experiments might lead to inaccurate or false accurate results between independent experiments. One steady calibration fits all experiments is difficult. Therefore, instead of creating a calibration curve for the assay in advance, negative control sample treated with 1% DMSO was applied in every single experiment and percentage cell viabilities of testing candidates were calculated by using mean optical density value of multiple wells on the same 96-well plate as 100%. Quantitative analysis based on cell viability in this chapter is then believed to be reliable.

3.4 Conclusion

MTT colorimetric assay based anti-proliferation and cytotoxic evaluation of standard alkaloids and herbal extracts indicated that (a) both berberine and evodiamine has anti-breast cancer activities; (b) anti-proliferative and cytotoxicity activities of Coptis-Euodia formulae against both breast cancer cell lines were in not only a time-dependent manner, but a Coptis ratio-dependent manner as well; (c) stronger anti-proliferative and cytotoxicity effects of both alkaloids and all five Coptis-Euodia formulae have been observed against the ER-dependent MCF-7 than that of the ER negative MDA-MB-231 breast cancer cell line.

Anti-metastasis abilities of berberine, evodiamine and Coptis involved formulae have been confirmed with wound-healing assay and anti-adhesion assay in this study. However, the effects of standard alkaloids against metastasis breast cancer cells cannot represent the anti-cancer ability of neither the TCM formulae, nor the single herb. Due

to the complicacy of bioactive ingredients in single used herb (Coptis and Euodia) and duo herb formulae, bioactivities of herbal extracts cannot be anticipated or evaluated by proven active components isolated from the herb. Such verdict can be concluded from the anti-migration activity study of evodiamine, which has been proved to inhibit the motility of MDA-MB-231 cells while Euodia extracts gave a completely opposite effect on the same cancer cell line. Therefore, though some bioactive ingredients isolated from medicinal herb might contribute to or even dominate the bioactivities of the whole herb, the exact bioactivities of the herb depend on the synergy between every component involved.

Chapter 4 Cell cycle analysis of Coptis-Euodia formulae against breast cancer cell lines

4.1 Introduction

In normal eukaryotic cell cycle, the process involves a complex series of macromolecular coordination. The cell division cycle can be plotted into four stages (Figure 4. 1): the first gap (G1 phase), in which the cell increases in size and prepares to a new round of DNA synthesis; the synthesis (S phase), in which the cell synthesizes DNA; the second gap (G2 phase), in which the cell gets everything ready to divide; and the mitosis (M phase), in which cell division occurs and two viable daughter cells generated. During this crucial replication process, cell stops at one or more checkpoint to avoid mistakes from internal state and external conditions.[68-69]

The first gap phase, which separates previous mitosis and initiation of DNA synthesis, allow the cell to collect and interpret information from both internal and external sources. In most mammalian cell cycle process, significant amount of total cycling time is spent in G1 phase. The second gap phase is defined as period from the end of DNA synthesis to initiation of mitosis. These two gap phases physically and temporally separated the DNA replication and mitosis events. Chromosomes must be duplicated once and only once before mitosis to ensure that each new generation receives an accurate and complete copy of the genome. Any DNA damage occurred throughout cell division must be monitored and/or repaired. Moreover, mitosis must be completed before another round of DNA replication can be re-initiated.

These complex processes ensure a eukaryotic cell to generate a faithful replica of itself without any default.[68-71] This complex process ensured the production of new cells and the death of existing cells. Dysregulation of cell cycle is widely believed to be involved in the development of cancer. Cyclins and cyclin-dependent kinase (CDKs) have been proved to have cell cycle regulatory function. Chemicals targeted these enzymes or proteins are believed to have anti-cancer potential.[54]

Initiative cell cycle arrest presumably provides sufficient time for DNA repairing processes. Within which, DNA lesions can be removed. Chemicals, which can induce cell cycle arrest on either or both checkpoints, are considered to have anti-cancer potential by interrupting the DNA replication process or causing DNA damage. Cell cycle analysis of berberine, evodiamine, and Coptis-Euodia formulae extracts on breast cancer cell lines in this chapter is aimed to understand the effects of those anti-cancer candidates on cell cycle progression which will help to explain the confirmed anti-proliferative and cytotoxicity activities of them.

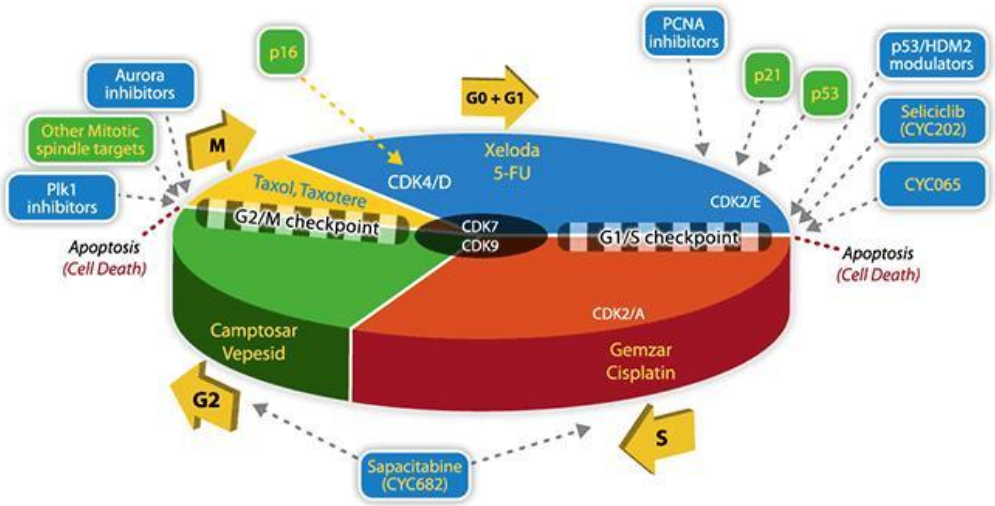


Figure 4. 1 A complete cell cycle process.[71]

4.2 Results

4.2.1 Effects of berberine and evodiamine on cell cycle distribution of MDA-MB-231 breast cancer cell line

An illustration of cell cycle analysis result figures using Guava Cell Cycle Assay has been presented in Figure 4. 2. Gating dot plot (A) demonstrates cell population distribution on three different cell cycle stages. Cells in G0/G1, S, and G2/M phase were distinguished and marked in DNA content histogram (B) with three colors. Statistics for each population within the histogram were analyzed automatically using CytoSoft Software.

Figure 4. 3 shows the cell cycle distribution of 1% DMSO treated MDA-MB-231 human breast cancer cell population after different incubation time points. According to the percentage of proliferative cells in different stages of the cell cycle listed in Table 4. 1. 49.2% of MDA-MB-231 cells were in G0/G1 phase while 16.4% and 34.3% of cells were in S and G2/M phase respectively at 0 hr. Percentage of cells in each cell cycle stage before DMSO treatment indicated that MDA-MB-231 cell line was typically in exponential growth 24 hours after plated. Cells recovered for 24 hours were then chosen to process the cell cycle distribution study and collected as samples of 0 hr.

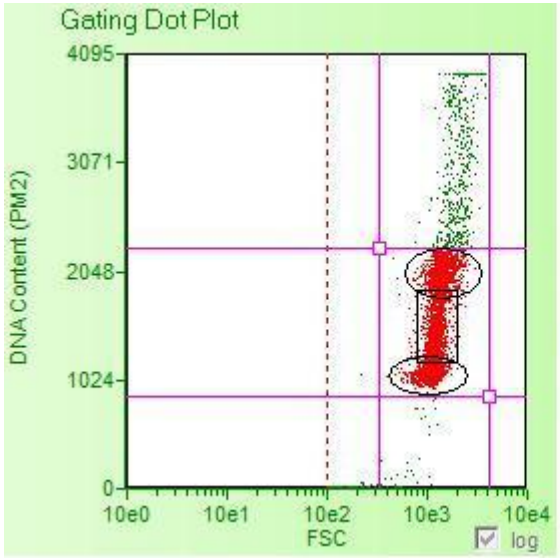
According to the cell cycle distribution of berberine (5 μM) and evodiamine (10 μM) treated MDA-MB-231 cells illustrated in Figure 4. 4 and Figure 4. 5, berberine induced G0/G1 phase arrest can be observed by 24 hrs and persisted for a further 48 hrs, while evodiamine significantly arrested MDA-MB-231 cells at G2/M phase with 8 hrs

treatment. Approximate 60% of cells were observed to be arrested at G0/G1 phase in the presence of berberine, which were about 15% higher than that of samples treated with 1% DMSO for the same period of time (from 24 to 72 hrs). Evodiamine (10 μM) treated MDA-MB-231 cells were arrested at G2/M phase to a great extent, with 57.3% of cells observed on that stage after 8 hrs. G2/M phase arrested activity of evodiamine was increased in a time-dependent manner, more than 90% of cells were arrested with 24 hrs exposure (Figure 4. 6 B). Such effects were persisted until 72 hrs, with 97.6% of cells arrested at G2/M phase, around 55% higher than that of 1% DMSO treated cells. Detailed relative percentage of cell cycle distribution of 1% DMSO, berberine (5 μM), and evodiamine (10 μM) treated MDA-MB-231 cells are presented in Table 4. 1 and Table 4. 2.

Table 4. 1 Relative percentage of cell cycle distribution of MDA-MB-231 cells treated with 1% DMSO

	1% DMSO		
Time (h)	**G0/G1**	**S**	**G2/M**
0	49.2%	16.4%	34.3%
8	55.1%	16.1%	28.7%
24	50.5%	17.6%	31.6%
32	47.0%	13.3%	39.4%
48	45.9%	14.2%	39.9%
72	46.3%	10.2%	43.5%

A



Cell population 3, G2/M phase

Cell population 2, S phase

Cell population 1, G0/G1 phase

B

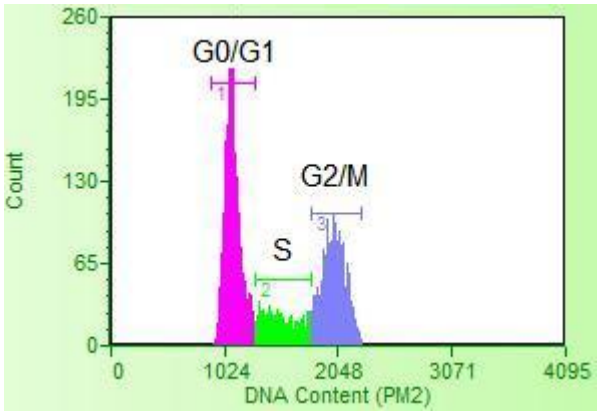


Figure 4. 2 Illustration of cell cycle analysis result figures using Guava Cell Cycle Assay presented in this chapter. Gating dot plot (A) and DNA content histogram (B) represent the result of the same cell sample. Dot plot A indicates the three cell population distribution (red dots) on different cell cycle stages and eliminates cell debris (green dots) from statistics analysis. Peaks with markers 1, 2, and 3 in histogram B represent cells (within the dot plot gate) in G0/G1 phase (pink), S phase (light green), and G2/M phase (blue) respectively.

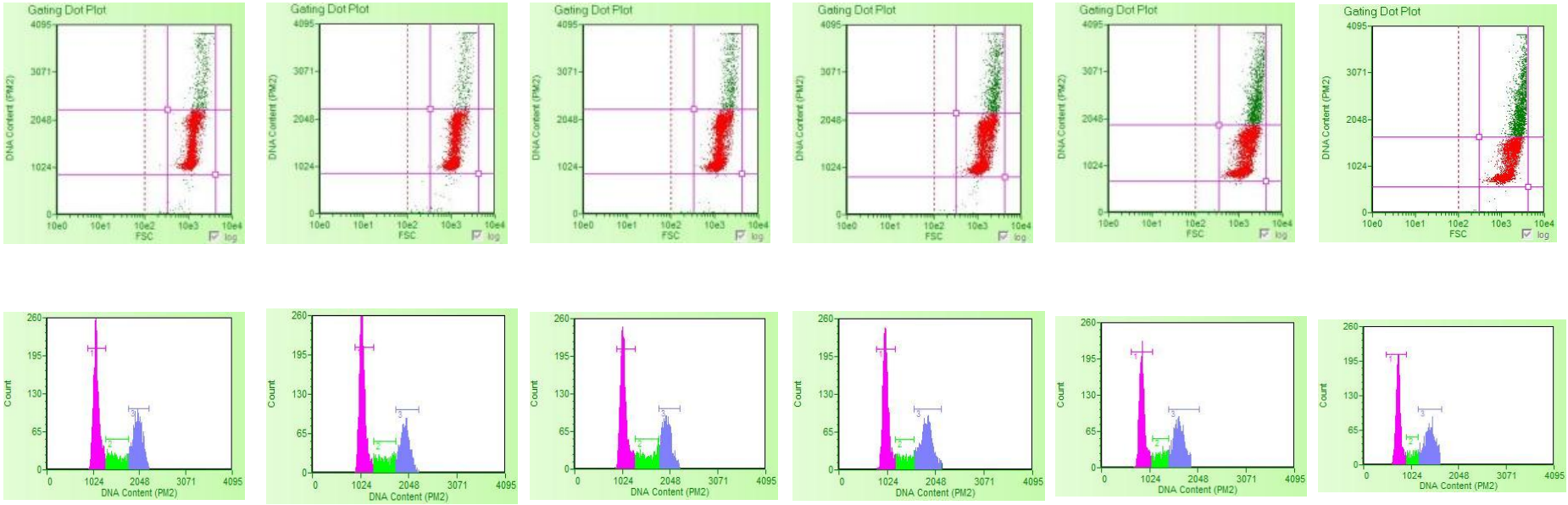
0 h	8 h	24 h	32 h	48 h	72 h
					

Figure 4. 3 Cell population distribution of MDA-MB-231 cells treated with 1% DMSO using Guava Cell Cycle Assay from 0 hr to 72 hrs. The top panel shows the gating dot plots indicated the three cell population distribution (red dots) on different cell cycle stages. Lower panel of DNA content histograms presented cells in G0/G1 phase (pink), S phase (light green), and G2/M phase (blue) respectively.

8 h	24 h	32 h	48 h	72 h

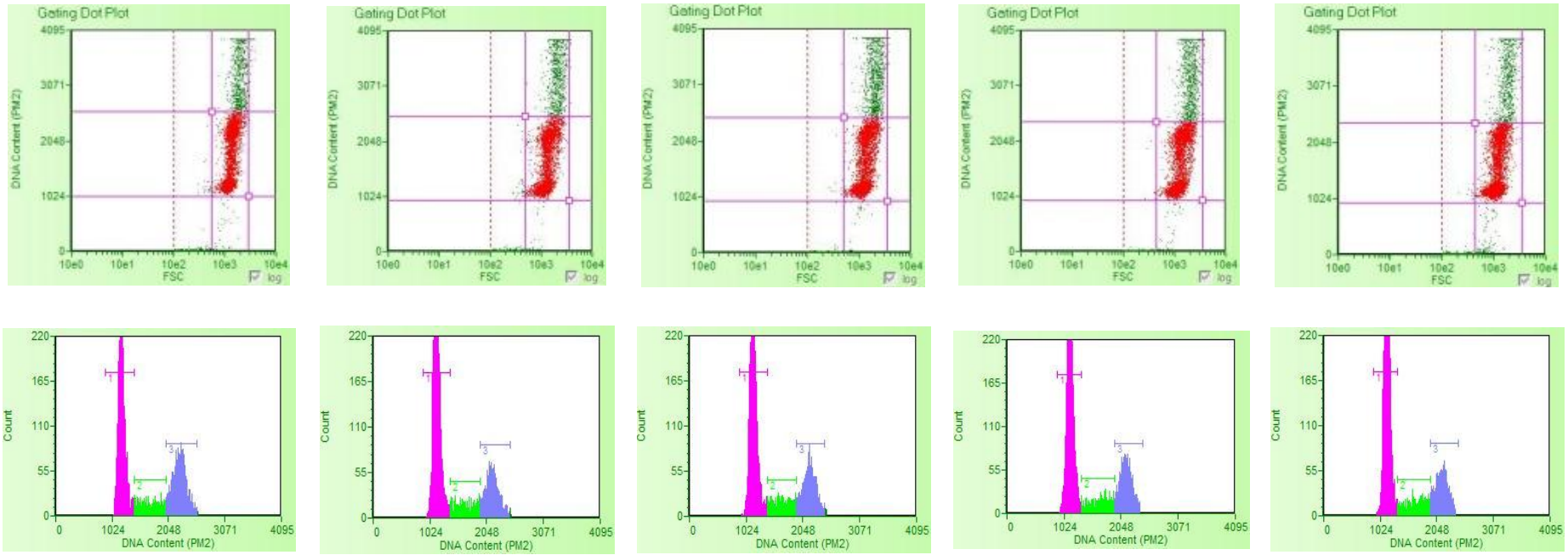


Figure 4. 4 Effects of berberine (5 μM) on cell cycle distribution of MDA-MB-231 breast cancer cell line from 8 hrs to 72 hrs. The top panel shows the gating dot plots indicated the three cell population distribution (red dots) on different cell cycle stages. Lower panel of DNA content histograms presented cells in G0/G1 phase (pink), S phase (light green), and G2/M phase (blue) respectively.

8 h	24 h	32 h	48 h	72 h

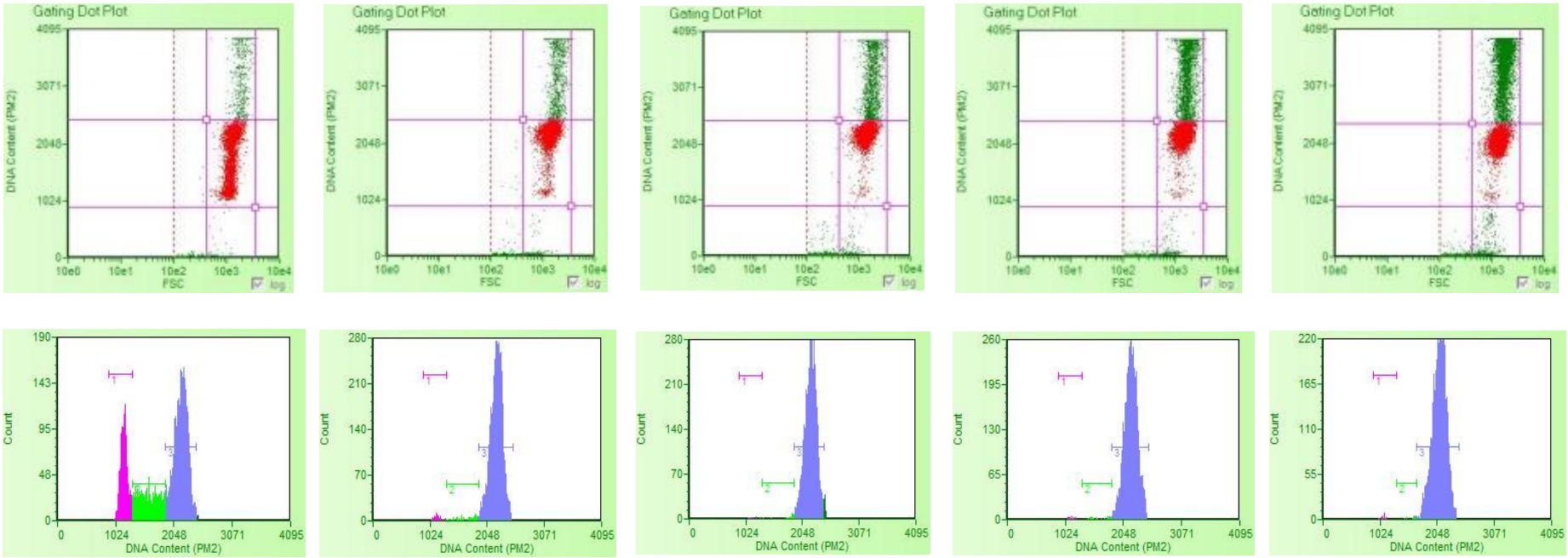


Figure 4. 5 Effects of evodiamine (10 μM) on cell cycle distribution of MDA-MB-231 breast cancer cell line from 8 hrs to 72 hrs. The top panel shows the gating dot plots indicated the three cell population distribution (red dots) on different cell cycle stages. Lower panel of DNA content histograms presented cells in G0/G1 phase (pink), S phase (light green), and G2/M phase (blue) respectively.

Table 4. 2 (A and B) Relative percentage of cell cycle distribution of MDA-MB-231 cells treated with berberine (5 μM) and evodiamine (10 μM)

	A Berberine 5 μM			B Evodiamine 10 μM		
Time (h)	**G0/G1**	**S**	**G2/M**	**G0/G1**	**S**	**G2/M**
8	50.9%	14.3%	34.6%	21.6%	20.6%	57.3%
24	59.9%	14.2%	25.4%	2.0%	3.5%	94.4%
32	57.0%	15.2%	27.2%	0.9%	1.7%	95.8%
48	57.3%	14.9%	27.7%	1.0%	1.7%	97.2%
72	60.0%	15.5%	24.5%	1.4%	1.0%	97.6%

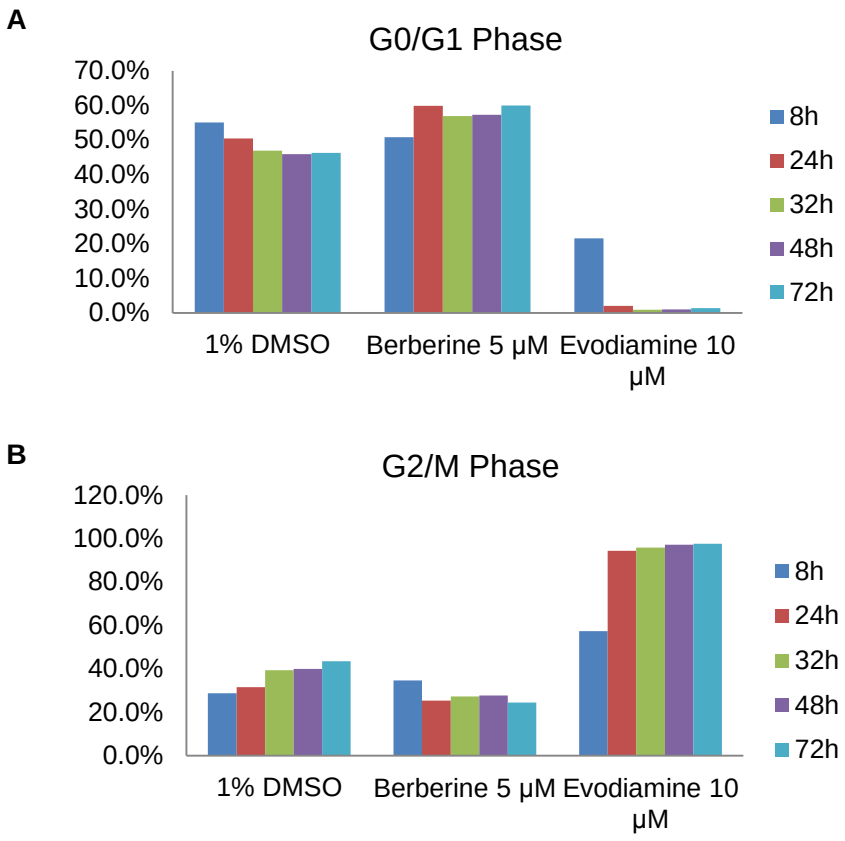


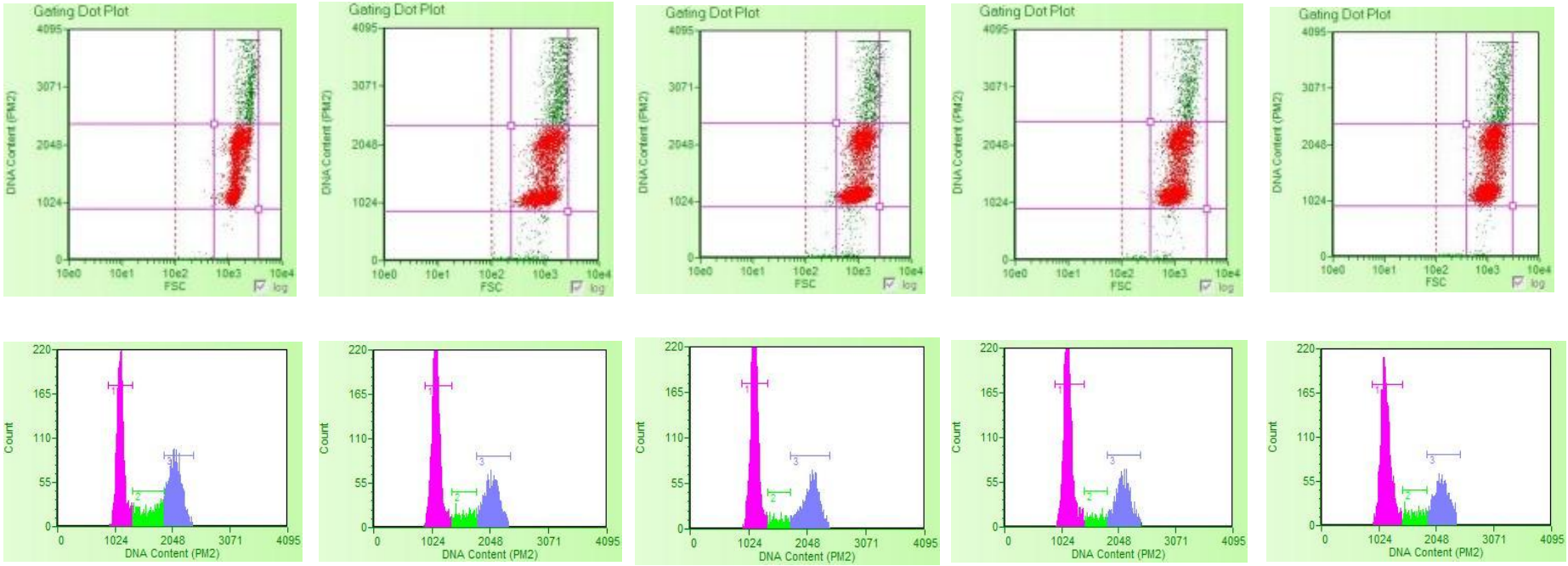
Figure 4. 6 Percentage of MDA-MB-231 cells in G0/G1 phase (A) and G2/M phase (B) after treated with 1% DMSO, berberine (5 μM) or evodiamine (10 μM) for 8, 24, 32, 48 and 72 hrs

4.2.2 Effects of Coptis-Euodia crude extracts on cell cycle distribution of MDA-MB-231 breast cancer cell line

Effects of Coptis-Euodia crude extracts at 0.05 mg/mL on cell cycle distribution of MDA-MB-231 breast cancer cell line evaluated by PI flow cytometric analysis were presented in Figure 4. 7 (A to E) and Figure 4. 8 (A and B), with relative percentage of each cell cycle stage at different time points listed in Table 4. 3 (A to E). Coptis extract started to arrest MDA-MB-231 cells at G0/G1 phase from 24 hrs treatment with 59.1% of viable cells were observed to stay on that stage. Same extent of effects were also presented by C : E 6 : 1 and C : E 1 : 1 treated cells. 24 hrs exposure to 0.05 mg/mL of these two extracts led to 58.0% and 59.0% of viable cells arrested at G0/G1 phase. Euodia extract exhibited G2/M phase arrest activity from 8 hrs. Cells arrested at G2/M phase of Euodia 0.05 mg/mL incubated sample were observed to be 51.9%, which was 23% higher than that of the negative control. Whereas long term exposure to Euodia extract did not extend the activity on cell cycle distribution, the extract resulted in an unhealthy condition of MDA-MB-231 breast cancer cells (Figure 4. 7 E, gating dot plot). Euodia also contributed to the short term (8 hrs) G2/M phase arrest of Coptis-Euodia crude extracts in a Euodia ratio-dependent manner (Figure 4. 8 B). As illustrated in Figure 4. 8 A, the G0/G1 phase arrest activities of Coptis-Euodia extracts were dominated by the presence of Coptis. Among which, C : E 1 : 1 extract appeared to be the best combination of these duo herbal formulae on the cell cycle distribution of MDA-MB-231 breast cancer cell line with 43.5% of cells arrested at G2/M phase by 8 hrs and no less than 59% of cells arrested at G0/G1 phase after 24 hrs.

A **Coptis extract**

8 h	24 h	32 h	48 h	72 h



B **Coptis : Euodia 6 : 1 extract**

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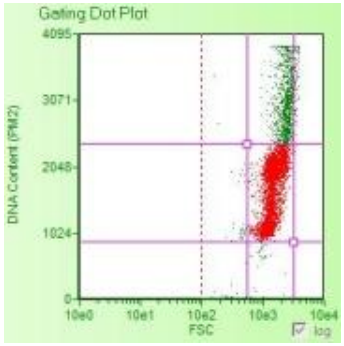	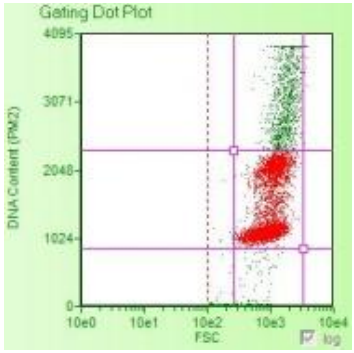	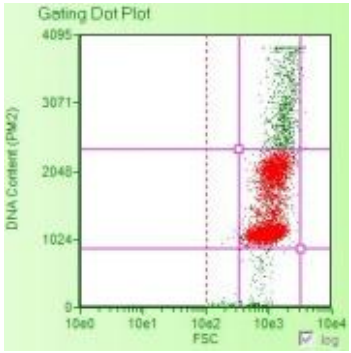	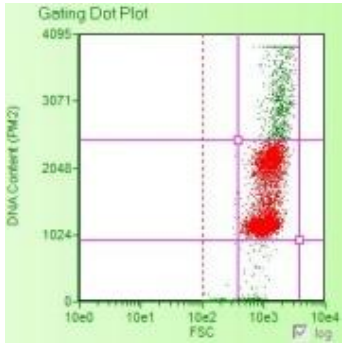	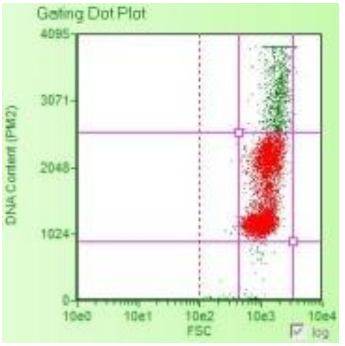
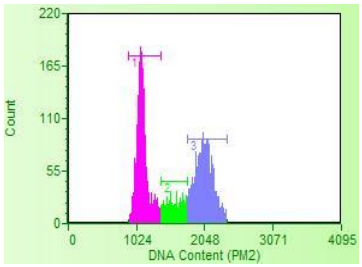	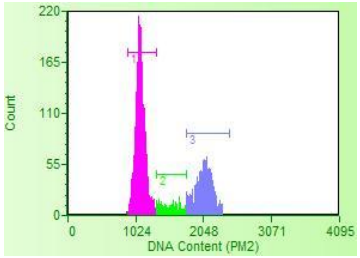	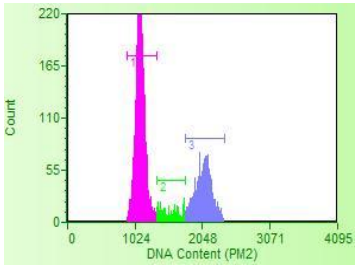	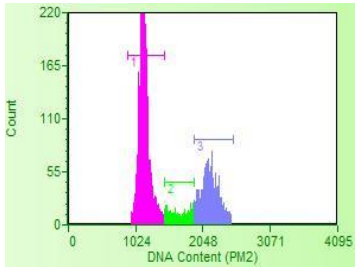	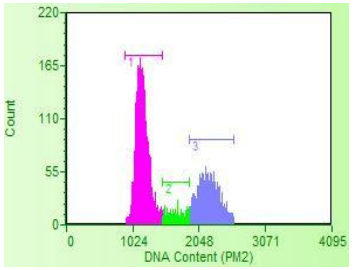

C **Coptis : Euodia 1 : 1 extract**

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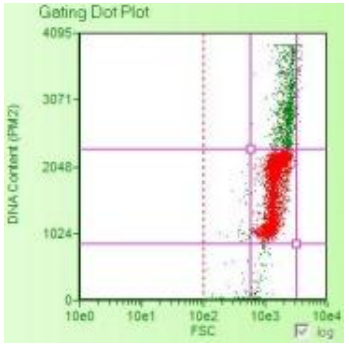	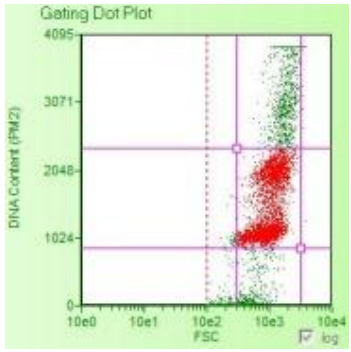	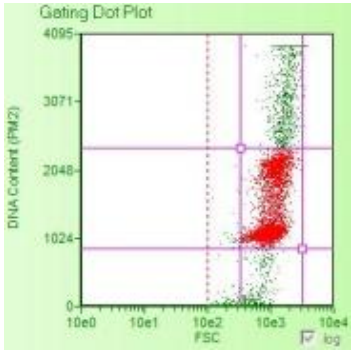	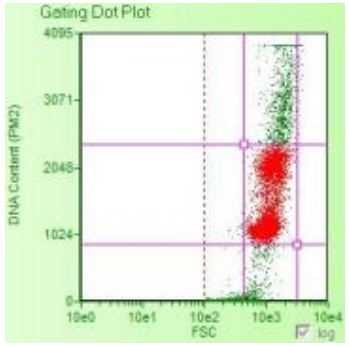	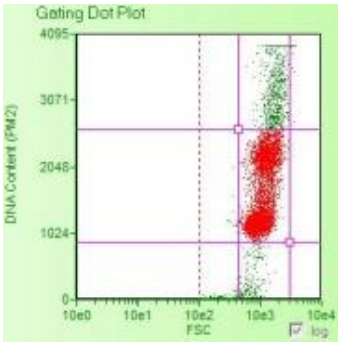
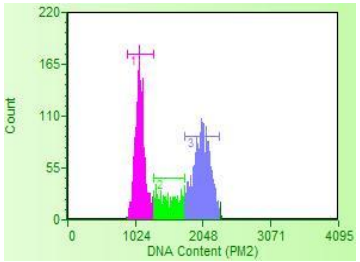	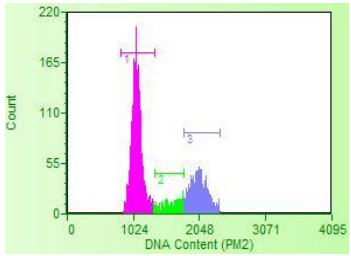	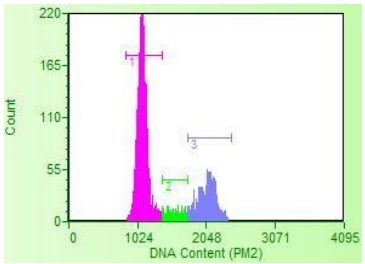	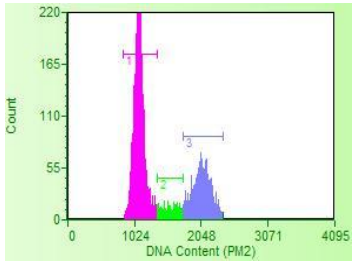	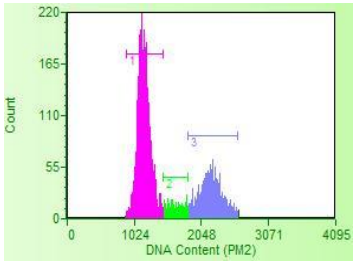

D **Coptis : Euodia 1 : 6 extract**

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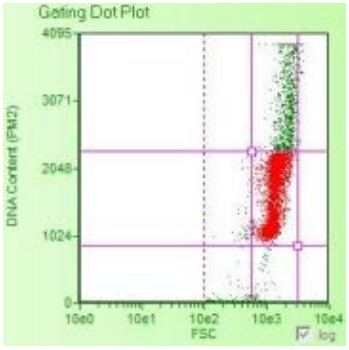
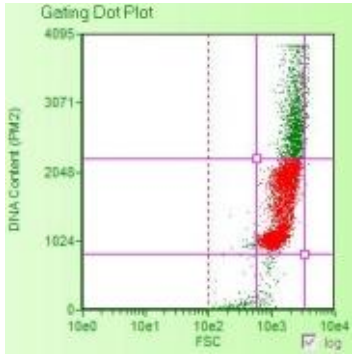
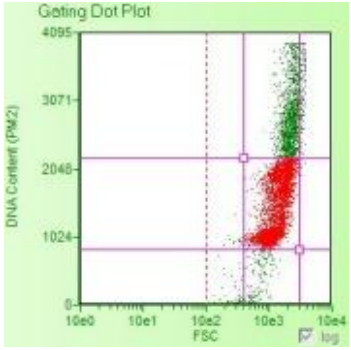
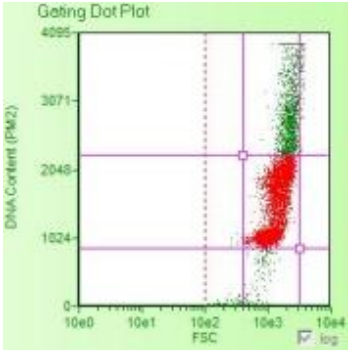
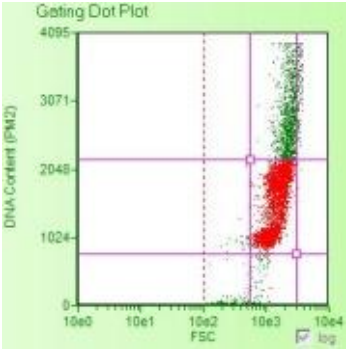
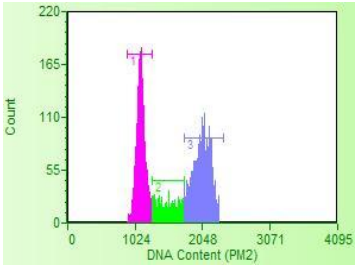
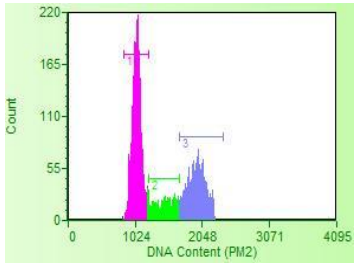
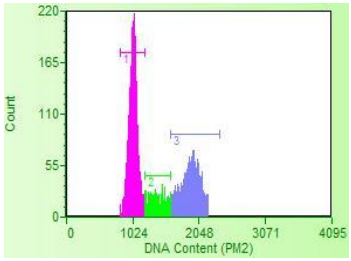
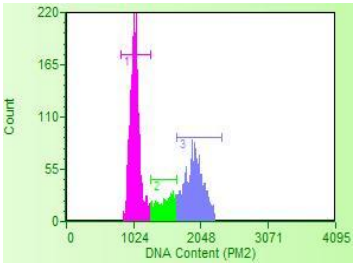
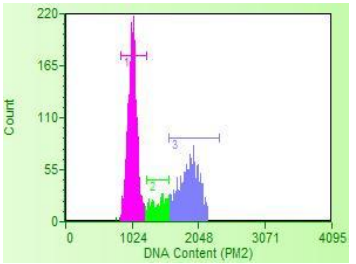
				
				

E **Euodia extract**

8 h	24 h	32 h	48 h	72 h

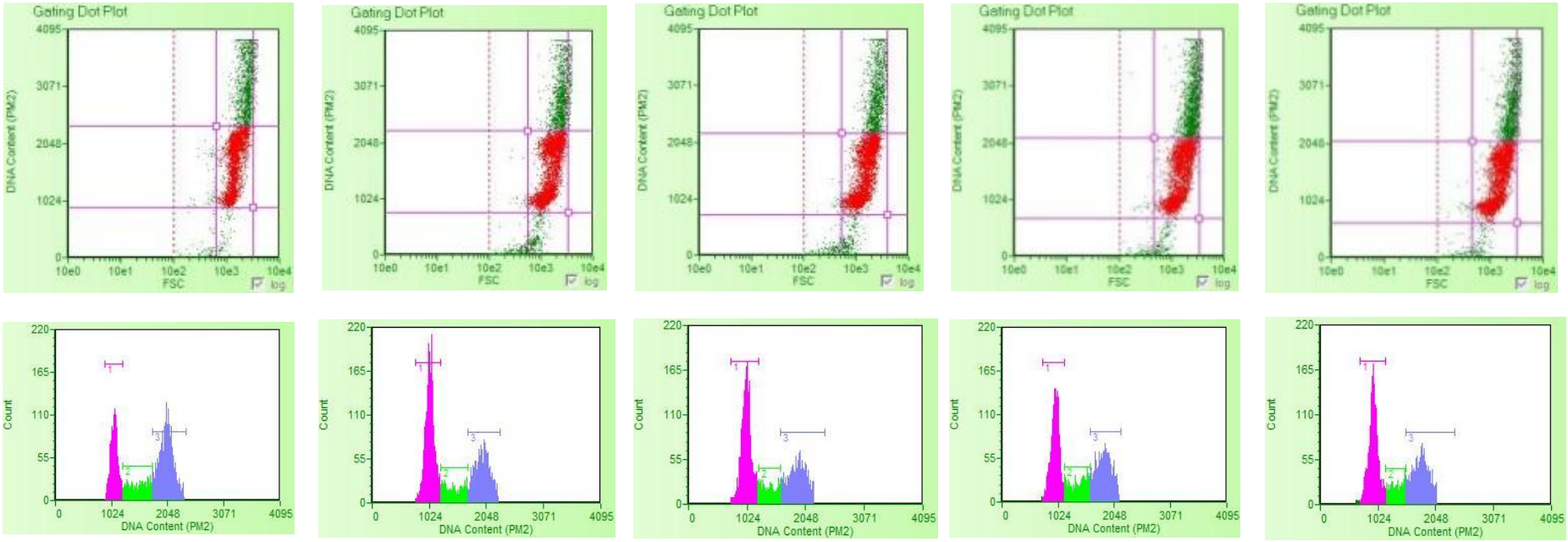


Figure 4. 7 (A to E) Effects of Coptis-Euodia crude extracts (0.05 mg/mL) on cell cycle distribution of MDA-MB-231 breast cancer cell line from 8 hrs to 72 hrs. The top panel shows the gating dot plots indicated the three cell population distribution (red dots) on different cell cycle stages. Lower panel of DNA content histograms presented cells in G0/G1 phase (pink), S phase (light green), and G2/M phase (blue) respectively.

Table 4. 3 (A to E) Relative percentage of cell cycle distribution of MDA-MB-231 cells treated with Coptis-Euodia crude extracts (0.05 mg/mL)

A Coptis extract

Time (h)	G0/G1	S	G2/M
8	45.8%	17.7%	36.5%
24	59.1%	10.7%	30.1%
32	60.5%	7.4%	32.1%
48	60.8%	8.3%	30.6%
72	59.6%	10.6%	29.8%

B Coptis : Euodia 6 : 1

G0/G1	S	G2/M
43.5%	13.8%	42.6%
58.0%	9.6%	32.2%
60.9%	9.0%	30.1%
59.7%	9.7%	30.5%
56.7%	9.2%	34.0%

C Coptis : Euodia 1 : 1

G0/G1	S	G2/M
38.9%	16.8%	43.5%
59.0%	11.3%	29.7%
64.4%	8.1%	27.5%
60.7%	8.6%	30.6%
61.0%	8.5%	30.1%

D Coptis : Euodia 1 : 6

Time (h)	G0/G1	S	G2/M
8	38.0%	15.9%	46.0%
24	51.4%	14.7%	33.8%
32	49.4%	14.7%	35.9%
48	50.8%	13.1%	36.1%
72	51.3%	11.0%	37.3%

E Euodia extract

G0/G1	S	G2/M
29.3%	18.8%	51.9%
50.5%	14.5%	35.0%
50.5%	13.7%	35.8%
41.0%	19.9%	38.7%
46.4%	14.4%	38.4%

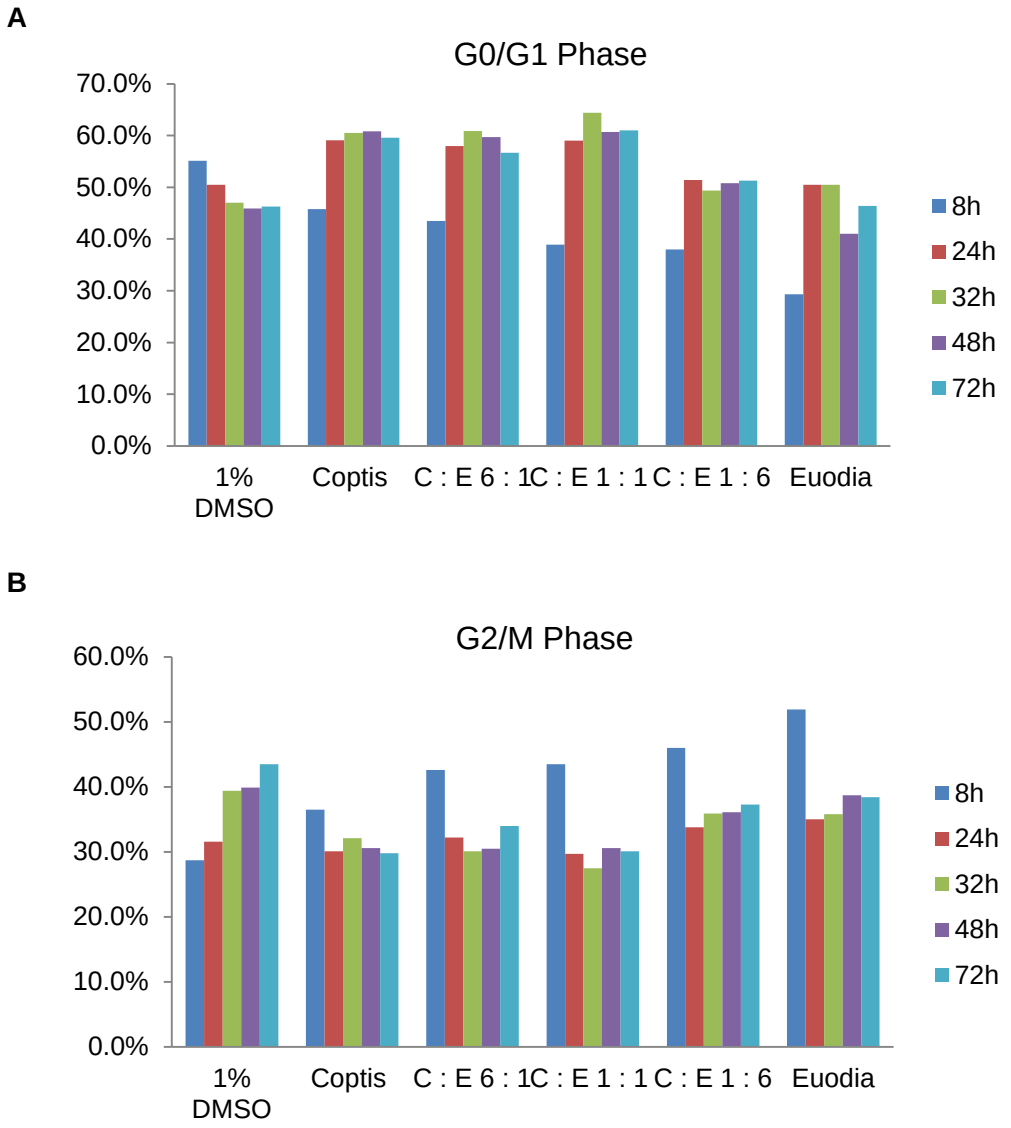


Figure 4. 8 Percentage of MDA-MB-231 cells in G0/G1 phase (A) and G2/M phase (B) after treated with Coptis-Euodia crude extracts (0.05 mg/mL) for 8, 24, 32, 48 and 72 hrs

4.2.3 Effects of evodiamine on cell cycle distribution of MCF-7 breast cancer cell line

Similar to the effect of evodiamine on MDA-MB-231 cell cycle distribution, the alkaloid also arrested MCF-7 cells at G2/M phase by 8 hrs treatment.

As shown in Table 4. 4 and Figure 4. 11, evodiamine at 10 μM arrested 44.6% of MCF-7 cells at G2/M phase which was 12.3% higher than that of the negative control sample. Persisted G2/M phase arrest of evodiamine was in a time-dependent manner up to 48 hrs treatment. No less than 70.6% of cells were arrested at G2/M phase after 24 hrs. Viable cells at G0/G1 phase of evodiamine treated samples were correspondingly decreased at different time points.

Table 4. 4 (A and B) Relative percentage of cell cycle distribution of MCF-7 cells treated with 1% DMSO and evodiamine (10 μM)

	A 1% DMSO			**B Evodiamine (10 μM)**		
Time (h)	**G0/G1**	**S**	**G2/M**	**G0/G1**	**S**	**G2/M**
0	56.9%	18.5%	24.6%	61.1%	16.6%	22.3%
8	42.8%	24.5%	32.3%	33.5%	21.4%	44.6%
24	68.6%	9.9%	20.8%	19.5%	6.8%	73.7%
32	59.4%	11.6%	28.0%	16.2%	5.1%	78.7%
48	61.0%	9.7%	29.3%	13.5%	3.8%	82.7%
72	62.0%	8.2%	29.6%	23.3%	6.1%	70.6%

0 h	8 h	24 h	32 h	48 h	72 h

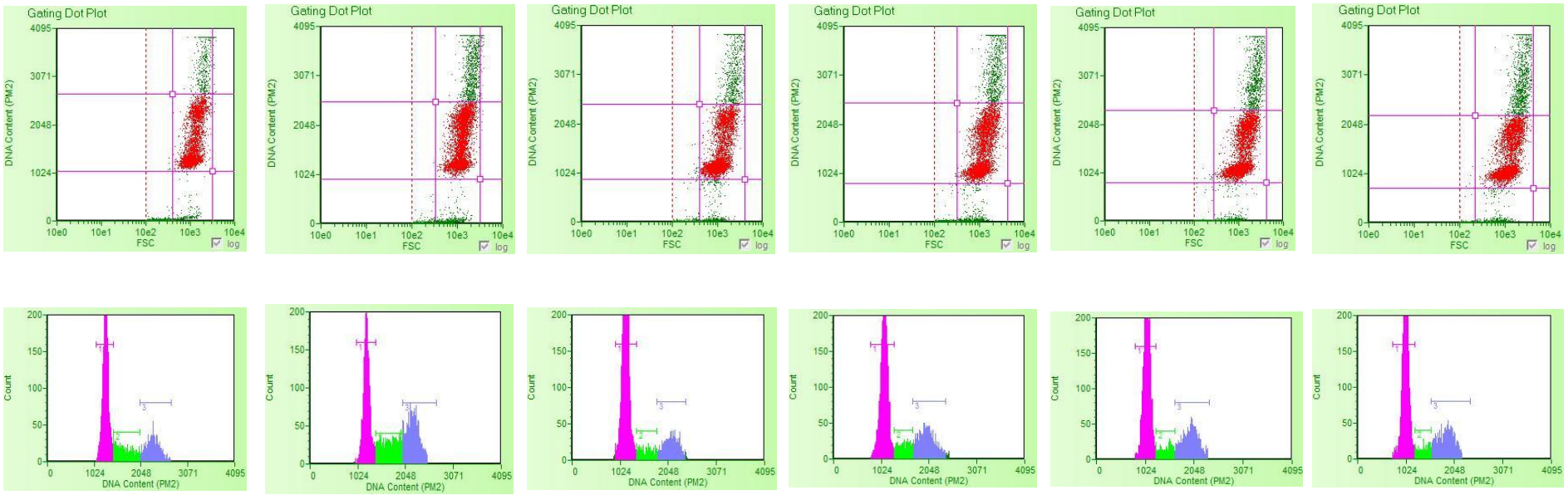


Figure 4. 9 Cell population distribution of MCF-7 cells treated with 1% DMSO using Guava Cell Cycle Assay from 0 hr to 72 hrs. The top panel shows the gating dot plots indicated the three cell population distribution (red dots) on different cell cycle stages. Lower panel of DNA content histograms presented cells in G0/G1 phase (pink), S phase (light green), and G2/M phase (blue) respectively.

0 h	8 h	24 h	32 h	48 h	72 h

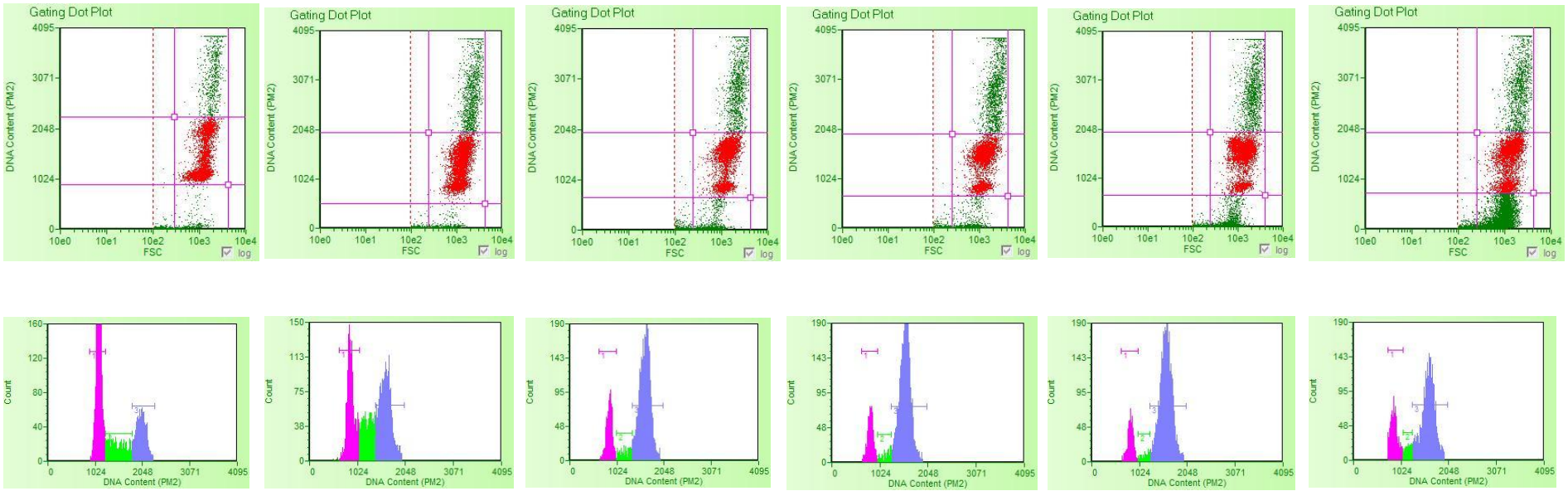


Figure 4. 10 Effects of evodiamine (10 μM) on cell cycle distribution of MCF-7 breast cancer cell line from 0 hr to 72 hrs. The top panel shows the gating dot plots indicated the three cell population distribution (red dots) on different cell cycle stages. Lower panel of DNA content histograms presented cells in G0/G1 phase (pink), S phase (light green), and G2/M phase (blue) respectively.

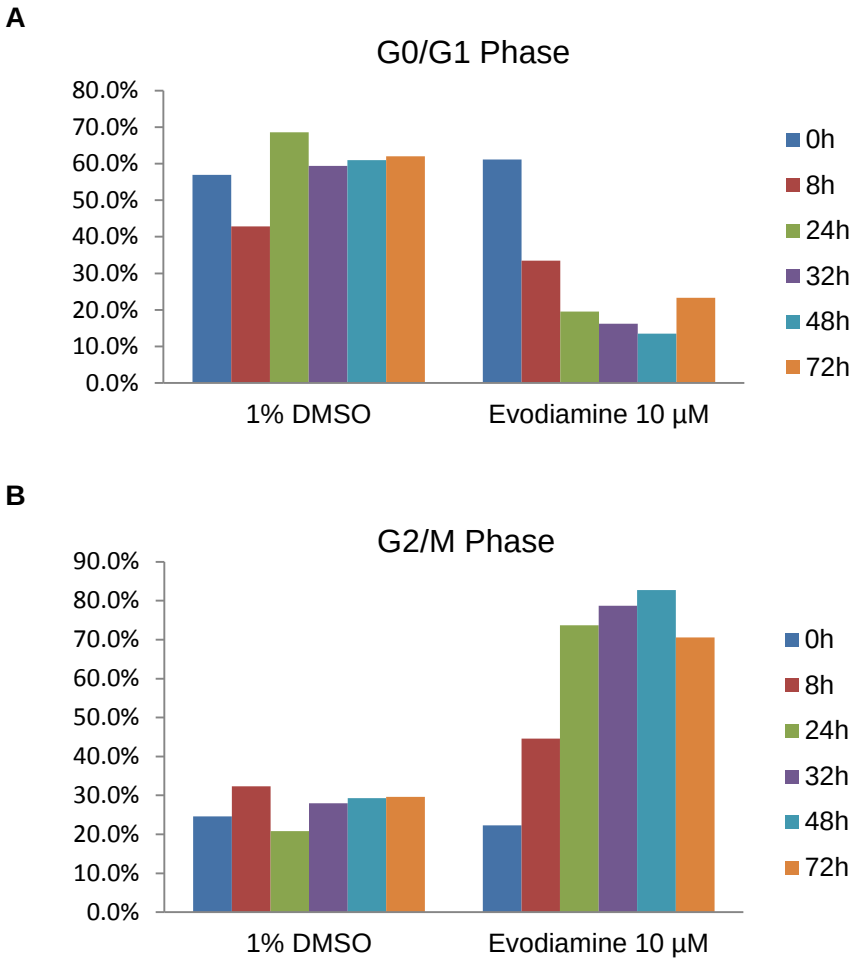


Figure 4. 11 Percentage of MCF-7 cells in G0/G1 phase (A) and G2/M phase (B) after treated with 1% DMSO, or evodiamine (10 μM) for 0, 8, 24, 32, 48 and 72 hrs

4.3 Discussion

Breast cancer cell lines originally derived from patients have been applied as research models in laboratory cancer research to simulate and simplify *in vivo* tumor *in vitro*.[72] As continuous cell lines, both MDA-MB-231 and MCF-7 have unlimited lifespan which can be passaged and proliferate infinitely in theory. However, considerable genetic instability may develop during repeated subculture.[73] Thus, passage number of cells used throughout the thesis has been limited to

20. Relatively fixed passage number of cells used in the same assay not only ensured the phenotype and genotype of cells close to the primary culture to the greatest extent, but close to each other between replications of the same experiment.

According to the growth profile of breast cancer cell lines provided by American Type Culture Collection (ATCC), MDA-MB-231 and MCF-7 cells can be continuously in exponential growth for up to 5 days and 7 days under manufactural culture conditions.[74-77] Therefore, cell sample cultured in growth medium for 72 hrs of each cell line was used to adjust the settings of Guava cell cycle system before each experiment. The adjustment process is applied to eliminate cell debris below the marker and to clarify cell populations by position G0/G1 population at approximately 1024 on the Gating Dot Plot (vertical PM2 axis). Further dot plot gate can be set manually to remove excess debris during cell cycle analysis and to give a more accurate statistical result (Figure 4. 2 A). Each cell cycle analysis investigation of Coptis-Euodia crude extracts and standard alkaloids against breast cell lines has been triplicated. Cell samples of the same research subject from three independent experiments were collected and well mixed before labeling nuclear DNA with propidium iodide (PI). Cells of each testing sample acquired by Guava cell cycle system were stochastic and statistical results for each population within the histogram were provided by CytoSoft Software automatically. The operation reduced the possible error of the instrument and minimized the inaccuracy during repeated system adjustment.

Measurement of changes in cell cycle kinetics in the presence of testing compounds is one of the efficient ways to screen potential therapeutic

drugs. Cell sample along with culture medium should be harvested and collected together for further analysis. The procedure ensured that cells on different cell cycle stages of the same sample are going to be analyzed including cells started to apoptosis, dead cells and cell debris (can be eliminated by setting gate). For example, cell cycle analysis results proved that evodiamine (10 μM) arrested both MDA-MB-231 and MCF-7 breast cancer cells on G2/M phase. Compared with 1% DMSO treated sample, more oval shaped cells can be observed in MDA-MB-231 cells cultured with evodiamine for the same period of time. As in evodiamine treated MCF-7 cells, smaller cell colonies with some loose cells floating in culture medium can be observed from 24 hrs. Cell sample collected after 72 hrs was typical, with most of the cells detached from culture plate and floating in the culture medium. Such differences can also be obtained from the Gating Dot Plots of those samples. G0/G1 population of MDA-MB-231 cells incubated with evodiamine for longer than 24 hrs is hard to be detected with only a great cell population at G2/M phase (Figure 4. 5). However, huge amount of cell debris (green dots below gating field) of MCF-7 cells incubated with the same concentration of evodiamine can be acquired after 24 hrs (Figure 4. 10). The differences between morphology changes observed in evodiamine (10 μM) treated MDA-MB-231 cells and MCF-7 cells indicated that the similar cell cycle disruption might be triggered by different genetic pathways in those two breast cancer cell lines. Therefore, further investigation is necessary to identify the mechanisms of cell cycle arrest induced by berberine, evodiamine, and Coptis-Euodia crude extracts.

4.4 Conclusion

Cell cycle analysis on MDA-MB-231 and MCF-7 breast cancer cells proved that evodiamine significantly arrested both cell lines at G2/M phase at 10 μM by 8 hours treatment. The effect of evodiamine is also responsible for the rapid G2/M phase arrest of Euodia and other Euodia involved Coptis-Euodia extracts on MDA-MB-231 cell cycle. However, evodiamine cannot contribute to cell cycle arrest effect of Euodia extract after 32 hours. Berberine and Coptis extract affected MDA-MB-231 cell cycle distribution by arresting cells at G0/G1 phase after 24 hrs exposure. Furthermore, Coptis dominated the G0/G1 phase arrest activities of Coptis-Euodia dual herb formulae on MDA-MB-231 cell cycle distribution. In summary, evodiamine and Euodia extract can stop cell cycle process after DNA replication process while berberine and other four Coptis-Euodia crude extracts affect the cell cycle before DNA synthesis. Both cell cycle arrest pathways will lead to unsuccessful cell division and induce proliferative difficulties of cancer cells. Results of cell cycle study also indicated that although core bioactive compound isolated from medicinal herb such as berberine and evodiamine play key role in the cell cycle arrest activities of Coptis-Euodia formulae against breast cancer cell lines, synergy between all the components involved in each formulae lead to the final cell cycle arrest activity.

According to the results concluded in this chapter, interest in the mechanism of berberine, evodiamine and Coptis-Euodia formulae affect the cell cycle is arisen. Whether the compound – DNA interaction or other mechanism is involved in the activities of berberine, evodiamine and Coptis-Euodia crude extracts on cell cycle distribution need to be studied. Hence, effects of those investigation subjects on topoisomerase I and II will be studied and discussed in the following chapter.

Chapter 5 Effects of Coptis-Euodia formulae on human topoisomerase I and II

5.1 Introduction

5.1.1 Topoisomerases type I and II

Topoisomerases are enzymes thought to play crucial roles in all living cells by modulate the topological state of DNA. These ubiquitous enzymes are involved in the supercoiling of DNA and participate in many aspects of DNA processing such as replication, transcription, recombination and chromosome condensation. The functions of these enzymes contain regulating DNA over- and under-winding, removing knots and tangles from the genetic material by creating transient breaks in the sugar phosphate backbone of the double helix structure. Previous studies have confirmed that the subclass type I topoisomerase acted by introducing temporary breaks in only one strand of the double helix DNA while subclass type II breaks and relegates both strands of the double-stranded DNA.[78-79] The mechanism involves a covalent linkage between the enzymes and the 3’ or 5’ terminus of the broken DNA strand due to different types of topoisomerases and form cleavage complexes.[78,80]

Such covalent linkage has been shown to be susceptible to interference by certain compounds with distinct mechanisms.[78,80] Many of those compounds stabilize an enzyme-DNA covalent intermediate by forming a ternary DNA-compound-enzyme complex, and hinder the re-closure of DNA breaks which increasing the DNA cleavage levels in living cells as well as in *in vitro* system.[80] If the accumulations of the strand breaks

become overwhelming, they trigger cell death pathway. Such compounds are called “topoisomerases poisons”, one type of topoisomerase inhibitor, because they convert this essential enzyme to a potent cellular toxin and often used as anticancer agents.[78-81]

5.1.2 Topoisomerase inhibitors

Since the identification of mammalian DNA topoisomerases I and II as the targets of anti-cancer drugs in the 1980s, several compounds isolated or derived from natural sources have been proved to have topoisomerases inhibitory activities.[82] Among which, the most famous compound is topo I poison camptothecin (CPT) (Figure 5. 1), the secondary metabolites of *Camptotheca acuminate*. CPT has been derived to other topo I targeting drugs such as irinotecan and topotecan.

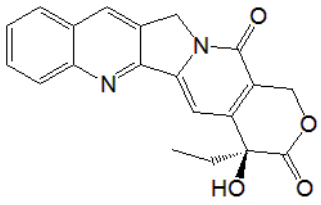


Figure 5. 1 Chemical structure of camptothecin (CPT)

Efforts in synthetic chemistry have also led to numbers of topoisomerase targeting compounds with anti-cancer activities. A type II topoisomerase targeted drug called amsacrine (m-AMSA) (Figure 5. 2) has been used in the treatment of acute lymphoblastic leukemia associated with other anti-cancer agents.[82]

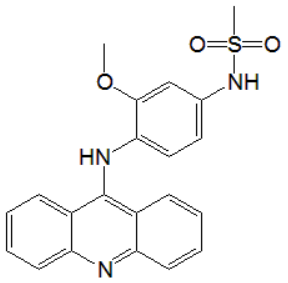


Figure 5. 2 Chemical structure of amsacrine (m-AMSA)

Another topoisomerase poison derived from natural source is etoposide (Figure 5. 3), a semi-synthetic anti-cancer agent used in chemotherapy targeting topo II. It was inspired by podophyllotoxin isolated from the rhizoma of *Podophyllum peltatum*.

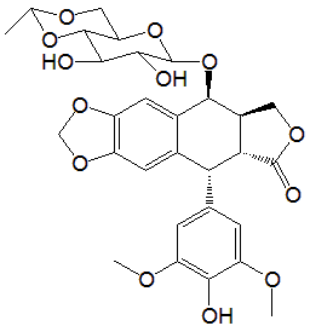


Figure 5. 3 Chemical structure of etoposide

5.1.3 DNA intercalator

Intercalation is the insertion of molecules between the planar bases of double-stranded DNA. DNA intercalator leads to local unwinding of the DNA helix and a decrease in the twist of the DNA. An intercalator usually contains a planar, polycyclic, and aromatic structure in the skeleton. Ethidium bromide (EtBr) (Figure 5. 4), the well-known fluorescence dye,

is a classic example of intercalator which binds tightly to double-stranded DNA.[83]

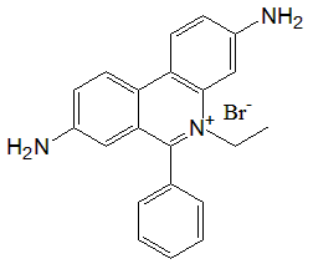


Figure 5. 4 Chemical structure of ethidium bromide (EtBr)

This chapter is designed to investigate whether topoisomerases are one of the targets involved in the cell cycle arrest ability of Coptis-Euodia crude extracts along with standard alkaloids berberine and evodiamine. Potential activities of Coptis-Euodia crude extracts on DNA and human topoisomerase type I and II are going to be evaluated and discussed in this chapter.

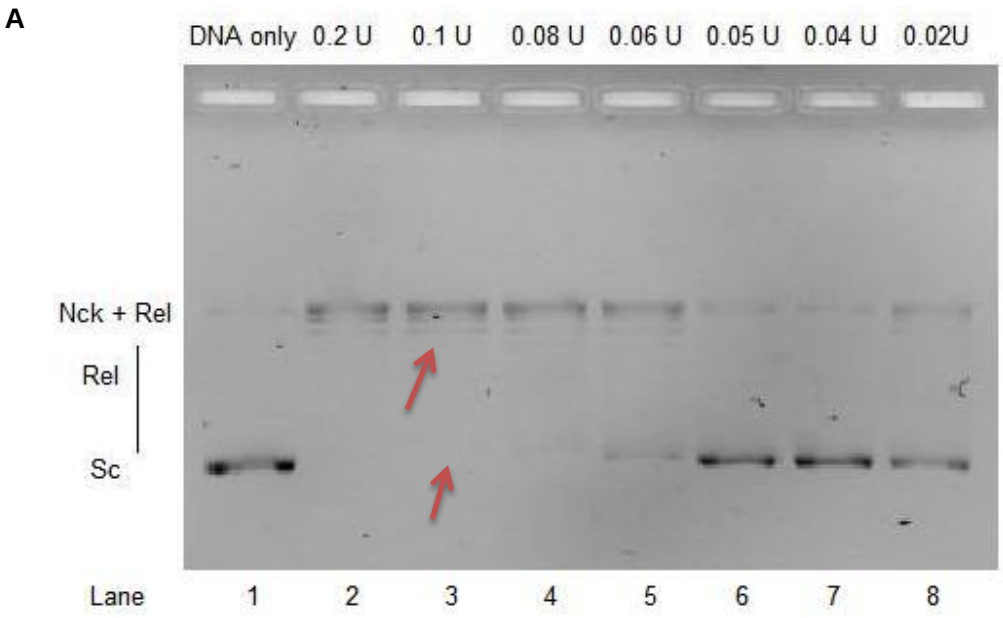
5.2 Results

5.2.1 Titration of human topoisomerase I and II

Before any topoisomerase inhibitory activity investigation of herbal extracts, the activity of the purchased batch of topoisomerase has to be evaluated in advance. Therefore, titration assays were carried out by testing different units of topo I (0.02 to 4 U) and topo II (0.5 to 4 U) with topoisomerase relaxation assay or topoisomerase II decatenation assay respectively (methods refer to sections 2.5.2 and 2.5.3 of Chapter 2). As presented in Figure 5. 5 A, compared with the clear supercoiled DNA band of the DNA only sample (Figure 5. 5 A, lane 1), no supercoiled

band but sharp nicked and relaxed DNA bands can be observed in samples incubated with 0.08, 0.1, or 0.2 U of topo I (Figure 5. 5, lane 2 to 4, and the red arrows). Samples incubated with enzyme less than 0.08 U started to show supercoiled DNA band on the bottom of the gel which indicated the incompletely interaction between topo I and plasmid DNA (Figure 5. 5 A, lane 5 to 8). The activity of topo II is showed in Figure 5. 5 B. Different from the kDNA only and samples incubated with 0.5, 0.8, or 1 U of topo II with kDNA (Figure 5. 5 B, lane 1 and 4 to 6), sharp DNA bands of decatenated mini circles can be observed in samples incubated with 2.5 and 4 U of topo II with no kDNA left in sampling wells (Figure 5. 5 B, lane 2 and 3, and the red arrow).

According to the results of titration assay, 0.1 U of topo I can relax 0.3 μg of supercoiled pBR322 DNA, while 2 U of topo II can decatenate 0.2 μg of kDNA under the experimental conditions. Therefore, both enzymes have been proved to be active and 0.1 U of topo I and 2 U of topo II were appropriate for further topoisomerase inhibitor investigation studies.



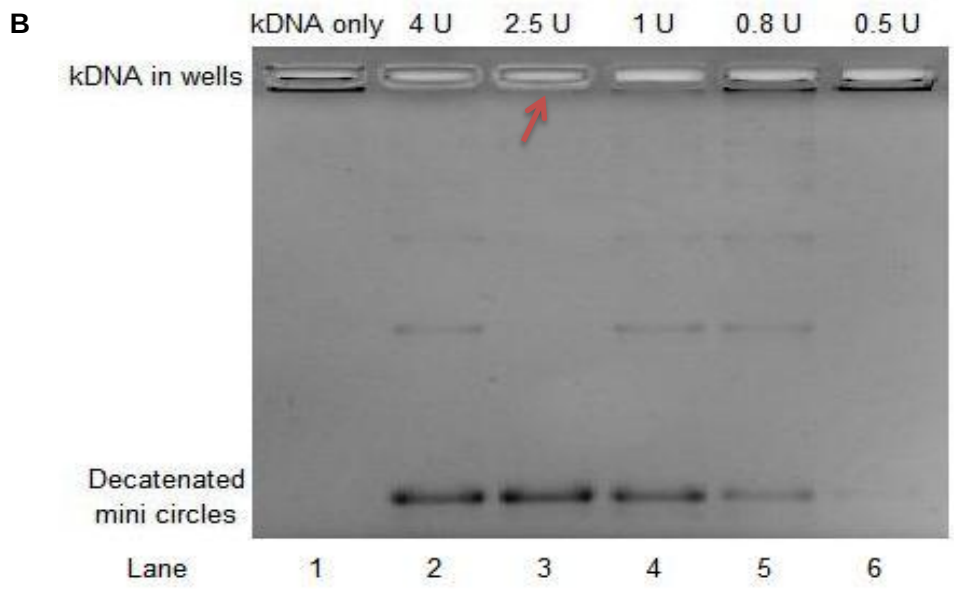


Figure 5. 5 Titration results of HTopo I from 0.02 to 0.2 U reacted with 0.3 μg of supercoiled pBR322 DNA (A) and HTopo II from 0.5 to 4 U reacted with 0.2 μg of kinetoplast DNA (B)

Nicked (Nck); Relaxed (Rel); Supercoiled (Sc)

5.2.2 Effects of Coptis-Euodia crude extracts on human topoisomerase I and II

Human topoisomerase I inhibition activity of Coptis-Euodia crude extracts (0.1 mg/mL) was evaluated by measuring the relaxation of supercoiled plasmid pBR322 DNA. As shown in Figure 5. 6 A lane 3, known Topo I inhibitor CPT blocked the activity of topo I and gave a supercoiled DNA band on the bottom of the gel at the concentration of 100 μM. Compared with the effect of positive control, DNA supercoiled band can also be detected in samples co-incubated with topo I and 0.1 mg/mL of C : E 1 : 1, C : E 1 : 6, or Euodia extracts (lane 8, 9 and 10). In contrast to Euodia extract, evodiamine at 100 μM did not exhibit activity on Topo I with only a band of nicked (+ relaxed) DNA on the gel (lane 5).

Though higher concentration of evodiamine (300 μM) started to exhibit topo I inhibitory activity (gel not presented), the compound cannot be properly dissolved in sample preparation process due to the poor water solubility and reaction volume limitation of topo I relaxation assay (section 2.5.2 of chapter 2). Berberine (100 μM), Coptis, and C : E 6 : 1 (0.1 mg/mL) samples behaved as DNA intercalator which inserted themselves into double helix of supercoiled DNA and caused a series of relaxed bands with lower mobility between nicked and supercoiled band on the middle part of the gel (lane 4, 6 and 7).

In order to indicate whether the inhibiting activity of herbal extracts was due to the effects on the Topo I – DNA cleavage complexes or not, nicked and relaxed DNA band has to be separated on gel. Ethidium bromide (EtBr) was then introduced into agarose gel. On EtBr containing gel (Figure 5. 6 B), the relaxation band of supercoiled DNA was partially (land 8) or totally (lane 9 and 10) inhibited compared with Topo I + DNA sample (lane 2). Such results indicated that those three herbal extracts at 0.1 mg/mL exhibited human topoisomerase I inhibitory activities which depended on the proportion of Euodia involved in the formula.

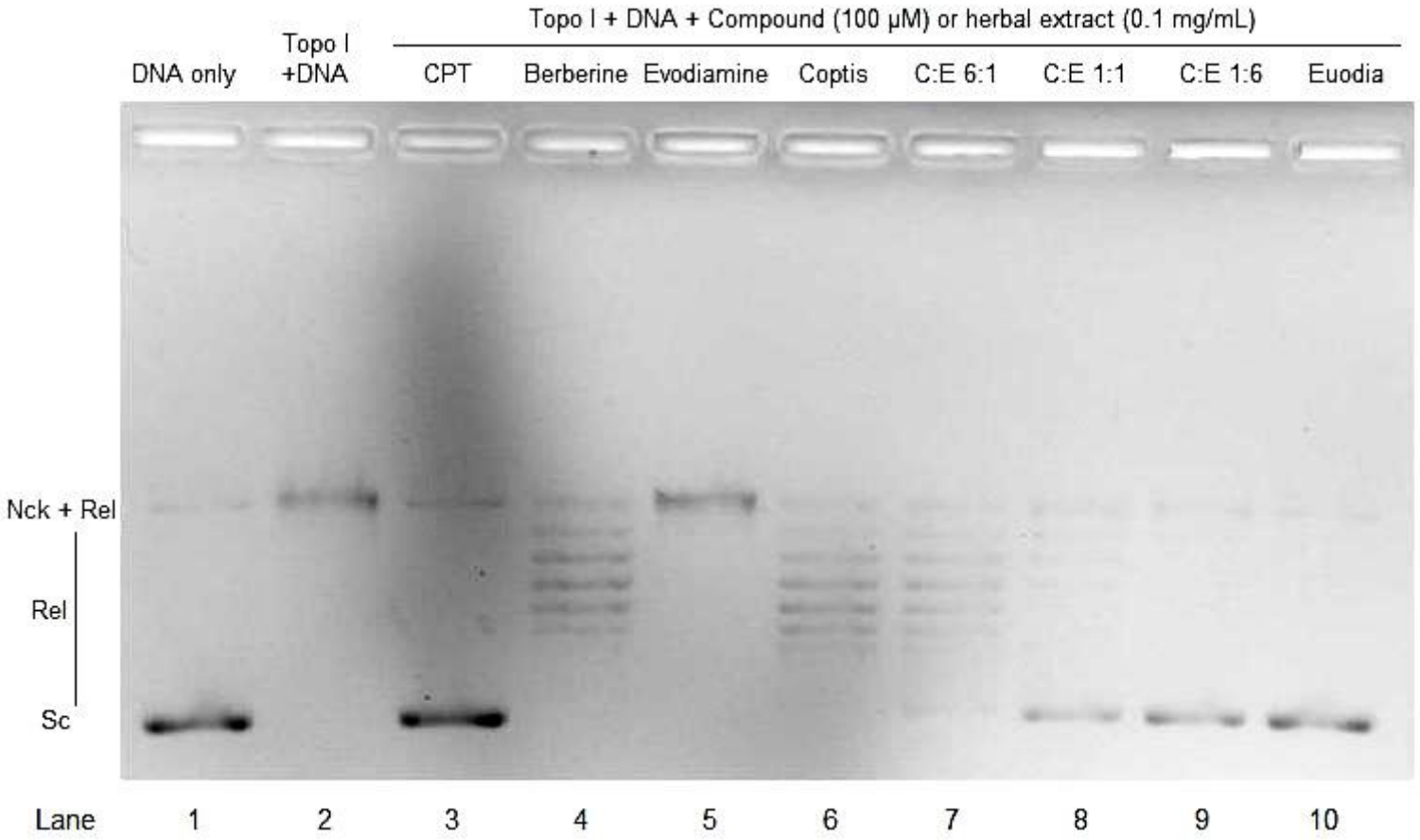
In human topoisomerase II decatenation assay, inhibition activity of Coptis-Euodia crude extracts (0.1 mg/mL) was examined by the anti-decatenation of kinetoplast DNA by topo II enzyme. Catenated kDNA has very low mobility which remained in the original sampling well and formed a single sharp DNA band on top of the gel (Figure 5. 7, lane 1). Sample of kDNA incubated with topo II indicated that the enzyme decatenated kDNA and decatenated mini circles with high mobility on agarose gel formed a clear band on the bottom of the gel (lane 2). Such

decatenation activity can be reduced in the presence of topo II inhibitor. Compared with the known topo II poison m-AMSA (lane 4), extracts of C : E 1 : 1, C : E 1 : 6 and Euodia showed fully topo II inhibitory activity while 100% of catenated kDNA remained in sampling wells and no decatenated DNA mini circle band can be observed on the bottom of those tracks (lane 9, 10 and 11). Moreover, berberine (100 μM), Coptis, and C : E 6 : 1 extracts inhibited topo II efficiently (lane 5, 7 and 8) with 80% catenated kDNA remained in wells.

Both results of relaxation and decatenation assay indicated that herbal extracts C : E 1 : 1, C : E 1 : 6 and Euodia are dual inhibitor against human topoisomerase I and II. The inhibitory activities of those three extracts against topo I were weaker than CPT (100 μM) with fainter supercoiled DNA bands on the bottom of the gel, and stronger than etoposide and m-AMSA (100 μM) with sharper catenated kDNA bands on the top of the gel at the concentration of 0.1 mg/mL.

Evodiamine, the main alkaloid in Euodia, shows no inhibiting activity on both topo I and II at a concentration of 100 μM. Such results indicated that evodiamine does not responsible for the topo I and II inhibitory activities of Euodia extract. Other compound(s) in Euodia may lead(s) to the inhibitory ability of the extract. DNA intercalator berberine contributed to the intercalator-like activity of Coptis and C : E 6 : 1 extracts at 0.1 mg/mL. Inhibitory activities of Coptis-Euodia formulae crude extract at the same concentration (0.1 mg/mL) on human topoisomerase I and II were in a Euodia ratio-dependent manner. Higher proportion of Euodia in the formulae leads to stronger topoisomerases inhibitory activities.

A


Topo I + DNA + Compound (100 μM) or herbal extract (0.1 mg/mL)
DNA only
Topo I +DNA
CPT
Berberine
Evodiamine
Coptis
C:E 6:1
C:E 1:1
C:E 1:6
Euodia
Nck + Rel
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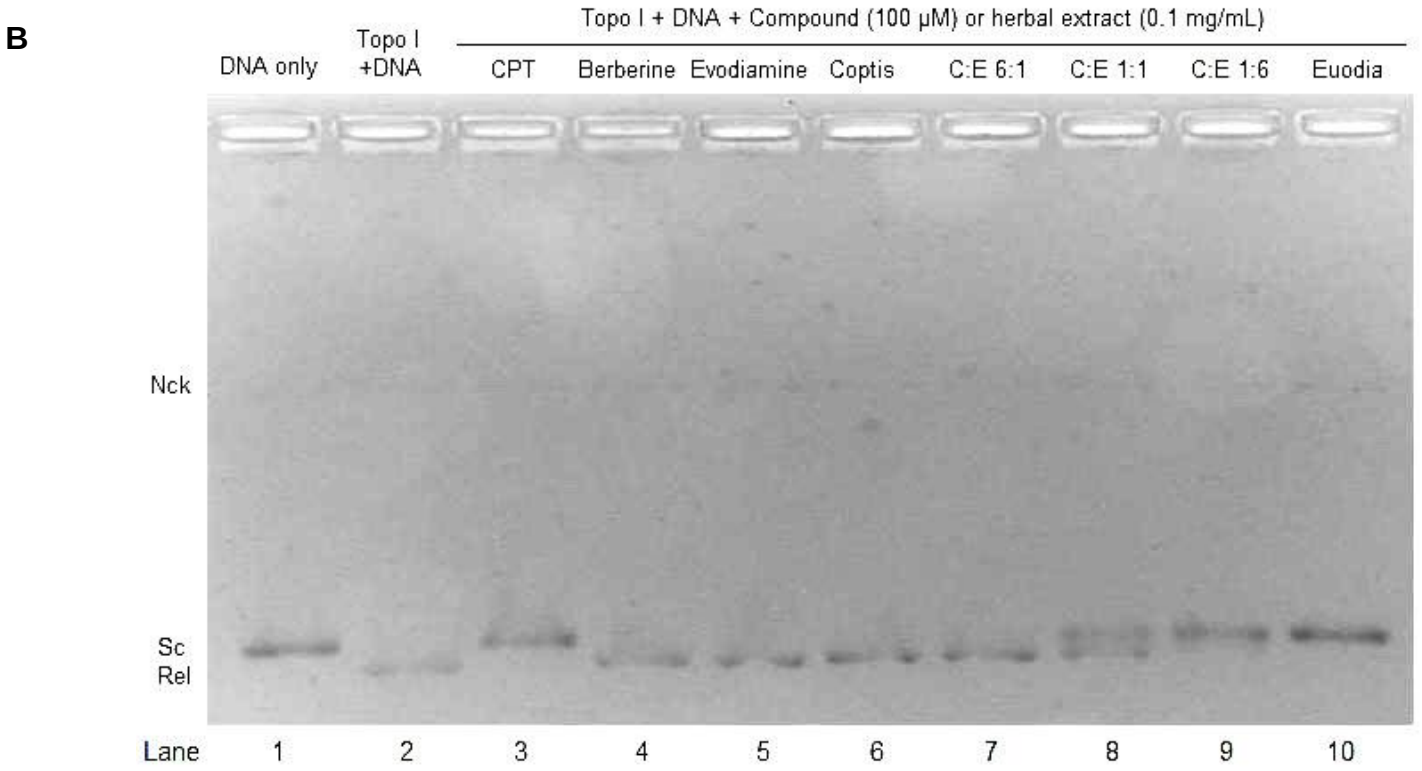


Figure 5. 6 Effects of Coptis-Euodia crude extracts (0.1 mg/mL) and standard alkaloids (100 μM) on the relaxation of plasmid pBR322 DNA (0.3 μg) incubated with human topoisomerase I (0.1 U). CPT (100 μM) was used as positive control. DNA samples were separated by electrophoresis on the 1% agarose gel without (A) or with (B) the presents of ethidium bromide. Nicked (Nck); Relaxed (Rel); Supercoiled (Sc)

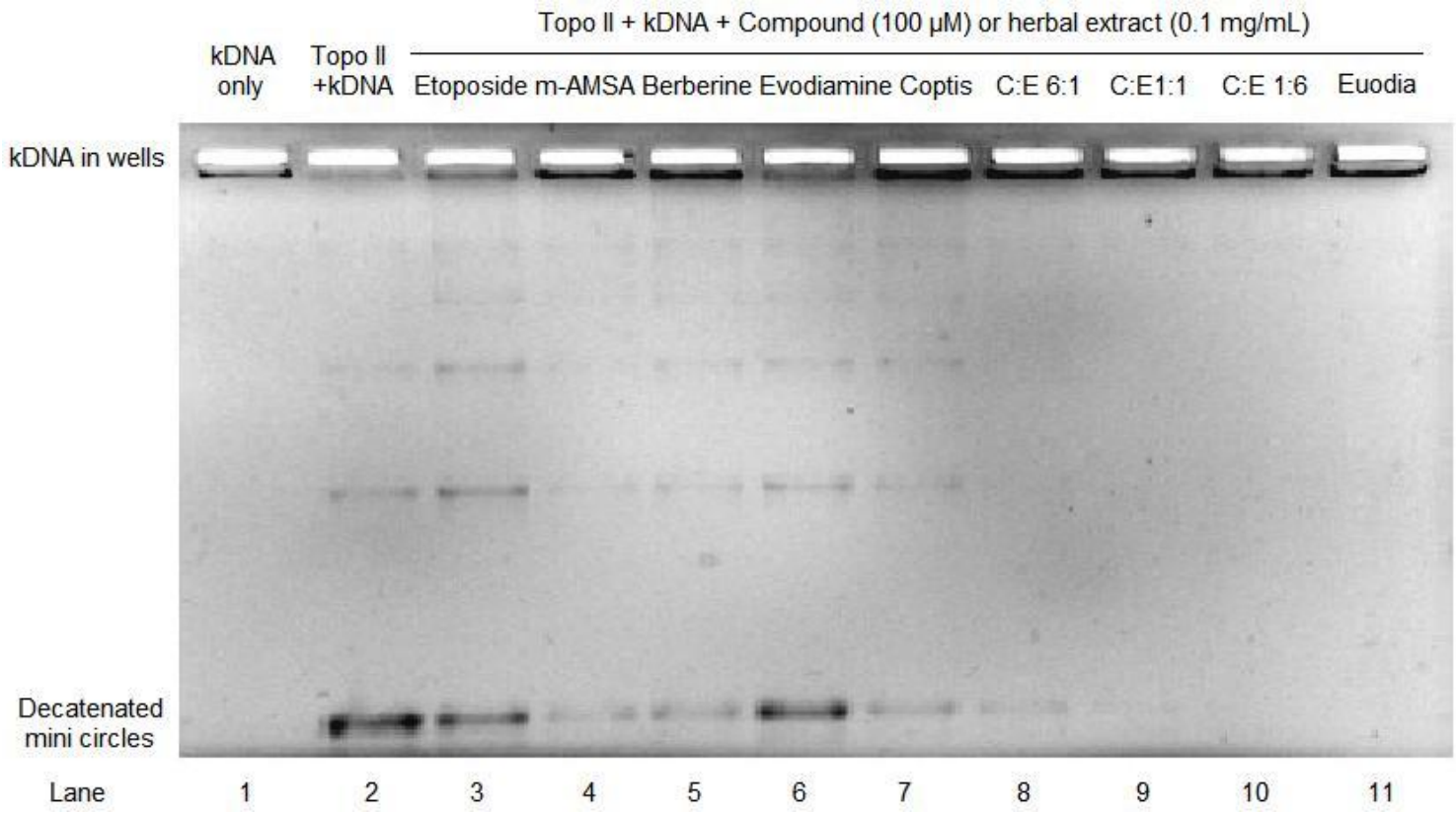


Figure 5. 7 Effects of Coptis-Euodia crude extracts (0.1 mg/mL) and standard alkaloids (100 μM) on the decatenation of kDNA (0.2 μg) incubated with human topoisomerase II (2 U). Etoposide and m-AMSA (100 μM) were used as positive control.

5.2.3 Inhibitory activities of Coptis-Euodia crude extracts on human topoisomerase I and II

Inhibitory activities of various concentrations of Coptis-Euodia formulae crude extracts on human topoisomerase I and II were evaluated by topo I relaxation assay and topo II decatenation assay respectively. As shown in Figure 5. 7, lane 3 and 4, activity of m-AMSA was stronger than that of etoposide as a topo II inhibitor at the same concentration (100 μM) with a sharper kDNA band remaining in wells on the top of the gel and a fainter decatenated mini circle band on the bottom of the gel. Hence, CPT (100 μM) and m-AMSA (100 μM) were selected as positive controls in the following inhibitory activity evaluation study.

In topo I relaxation assay, the intensity of supercoiled band was measured by ImageJ. Percentage intensity of each testing candidates against DNA only sample (lane 1 of agarose gel A in Figure 5. 8 to Figure 5. 12) on that gel represented the inhibitory activity of that concentration. And in topo II decatenation assay, the intensity of decatenated mini circle band was measured by ImageJ. Percentage intensity of each testing candidates against kDNA incubated with topo II sample (lane 2 of agarose gel B in Figure 5. 8 to Figure 5. 12) on that gel represented the inhibitory activity of that concentration. Each dot on the IC_{50} curves represented the mean value of three reads. Agarose gel results, associated with the IC_{50} curves of each Coptis-Euodia crude extract are presented in Figure 5. 8 to Figure 5. 12. Corresponding IC_{50} value of Coptis-Euodia formulae against topo I and II calculated by GraFit are listed in Table 5. 1. Data not shown if IC_{50} value cannot be estimated by GraFit software.

According to agarose gel A of Figure 5. 8, Coptis extract start to show topo I inhibitory activity from 0.6 mg/mL with an obvious supercoiled pBR322 DNA band on the bottom of the track (lane 8). Samples incubated with higher concentrations of Coptis (up to 1 mg/mL) demonstrated stronger inhibitory activity on topo I. Such result indicated that though berberine, the DNA intercalator, is the main alkaloid in Coptis, there are other compound(s) in Coptis crude extract worked as topoisomerase I inhibitor(s) and dominated the activities with concentrations higher than 0.6 mg/mL. The IC_{50} value of Coptis extract on topo I is 823.64 ± 41.56 μg/mL (*i.e.* 0.82 mg/mL). Similar results can be observed in C : E 6 : 1 samples. Increase the ratio of Euodia in the formulae leads to stronger inhibitory activity on both topo I and II enzymes. IC_{50} of Euodia extract on topo II was estimated to be 0.56 ± 0.26 μg/mL, which was almost 200 times more efficient than that of Coptis extract.

Table 5. 1 IC_{50} of Coptis-Euodia crude extracts against HTopo I and HTopo II. Data are presented as the mean ± S.D. (n=3)

		HTopo I	HTopo II
Concentration (μg/mL)	Coptis	823.64 ± 41.56	102.11 ± 4.51
	C : E 6 : 1	963.45 ± 34.09	-
	C : E 1 : 1	49.95 ± 4.95	55.49 ± 1.92
	C : E 1 : 6	1.98 ± 0.15	6.80 ± 0.11
	Euodia	-	0.56 ± 0.26

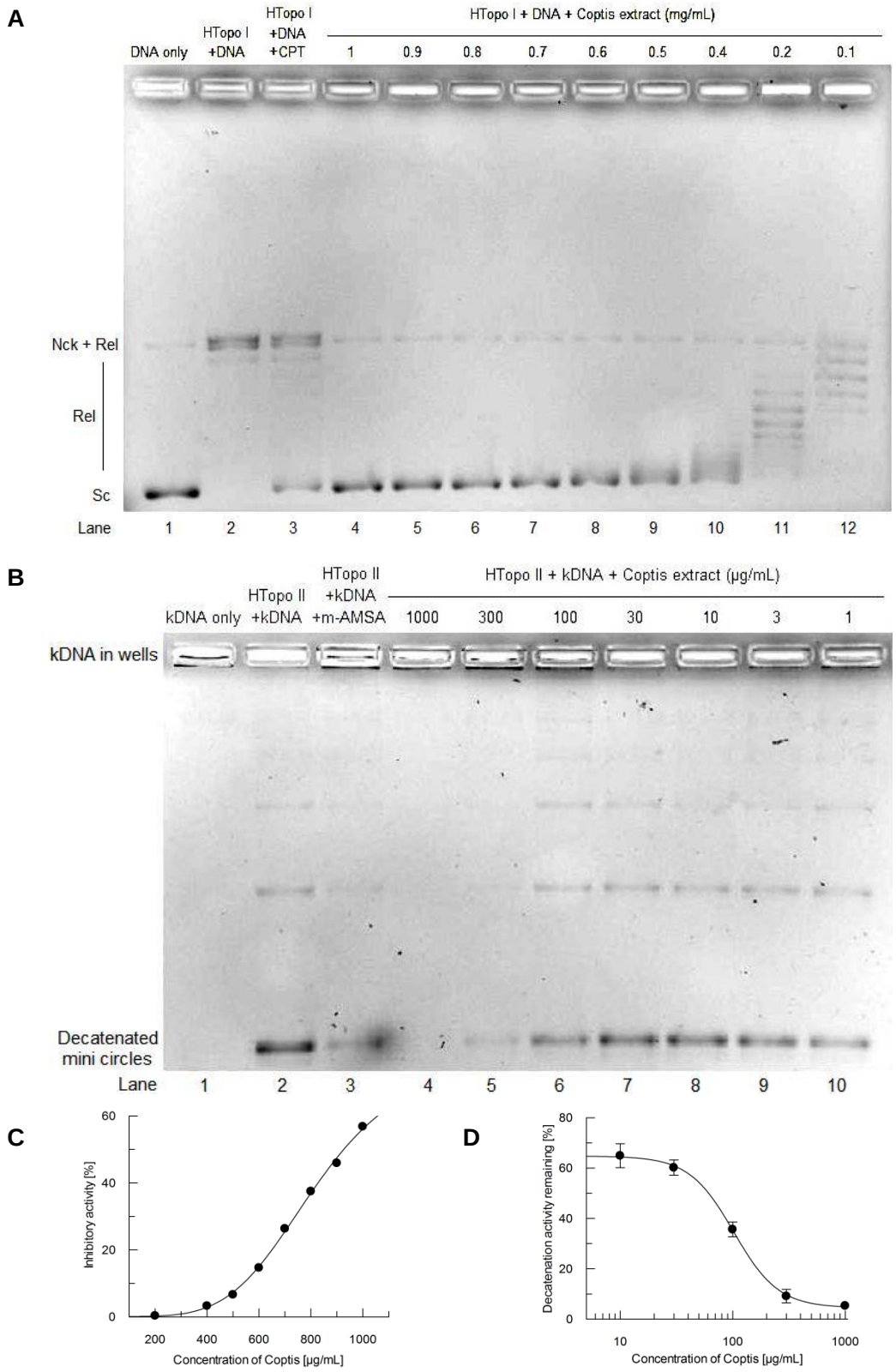


Figure 5. 8 Inhibitory activities of Coptis crude extract against HTopo I (A and C) using the relaxation assay and HTopo II (B and D) using the decatenation assay.

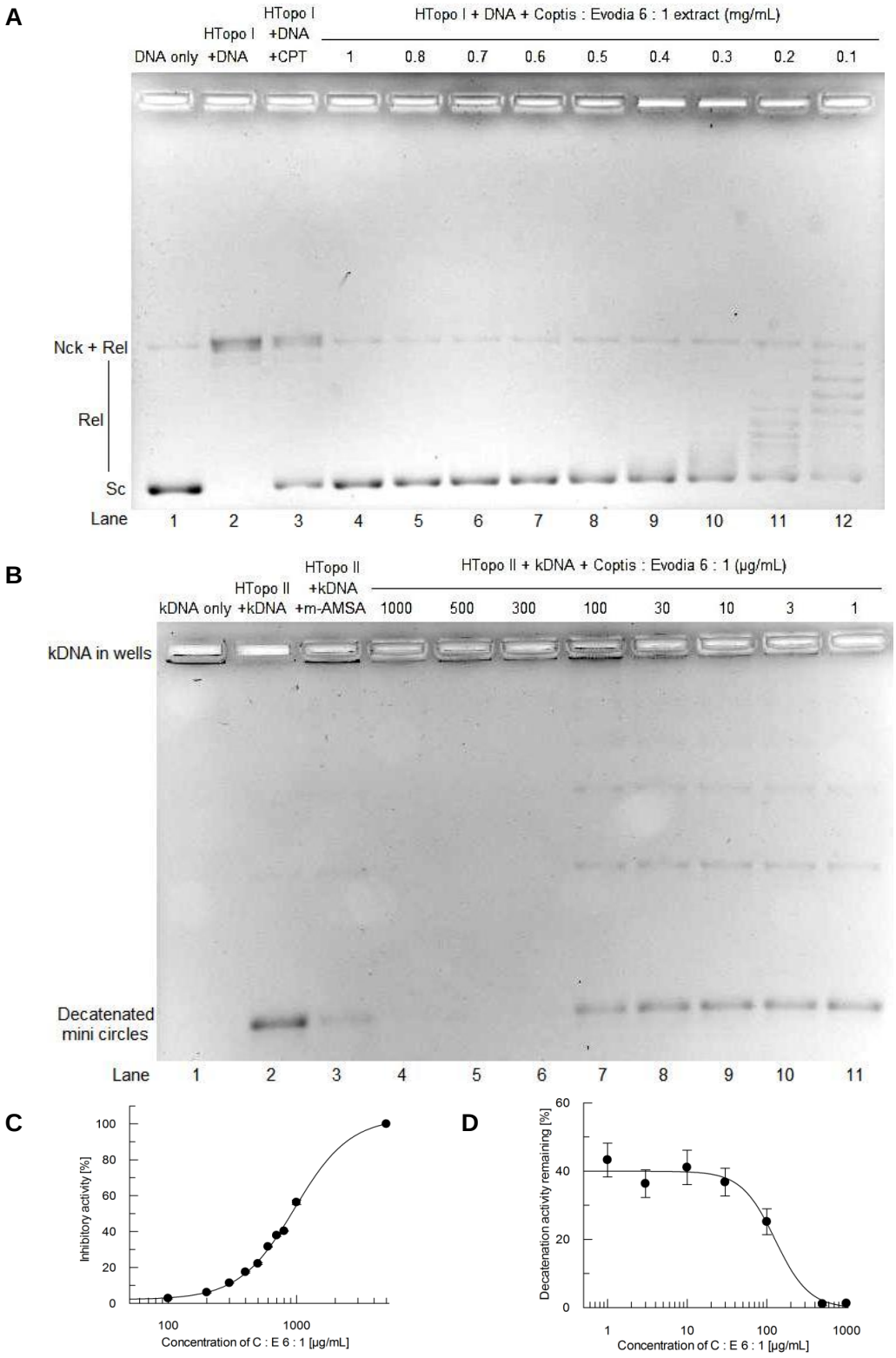


Figure 5. 9 Inhibitory activities of Coptis : Euodia 6 : 1 crude extract against HTopo I (A and C) using the relaxation assay and HTopo II (B and D) using the decatenation assay.

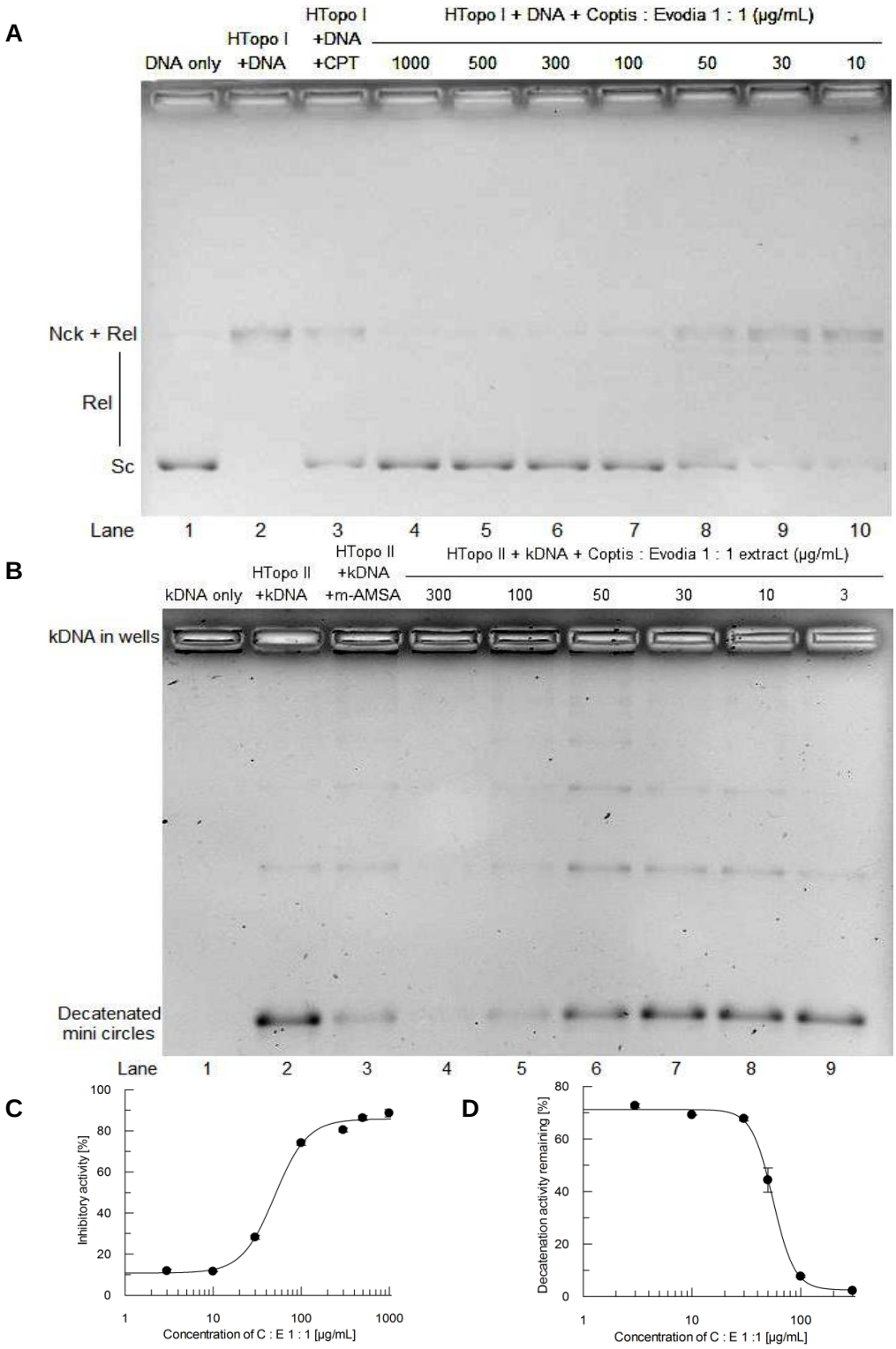


Figure 5. 10 Inhibitory activities of Coptis : Euodia 1 : 1 crude extract against HTopo I (A and C) using the relaxation assay and HTopo II (B and D) using the decatenation assay.

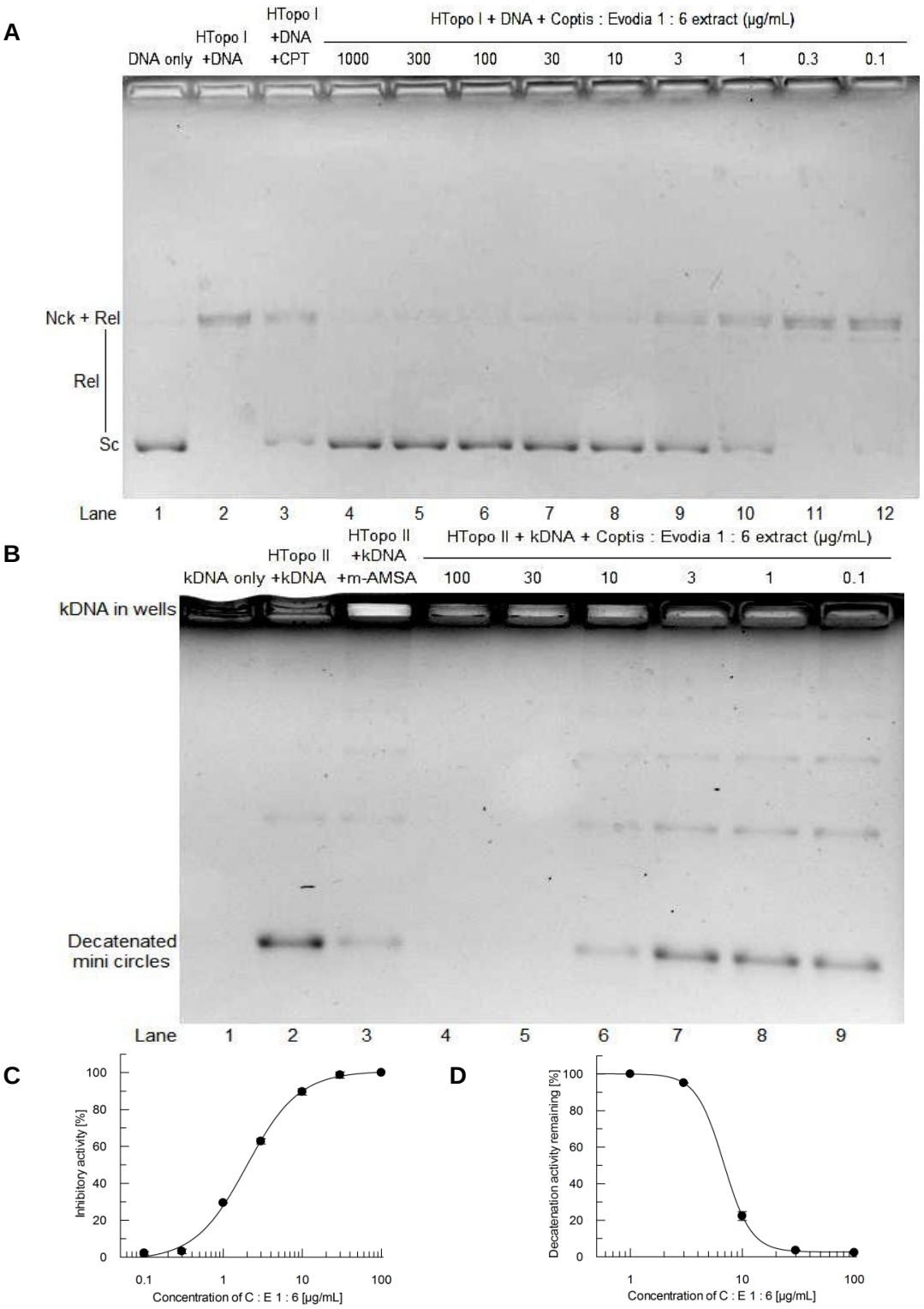


Figure 5. 11 Inhibitory activities of Coptis : Euodia 1 : 6 crude extract against HTopo I (A and C) using the relaxation assay and HTopo II (B and D) using the decatenation assay.

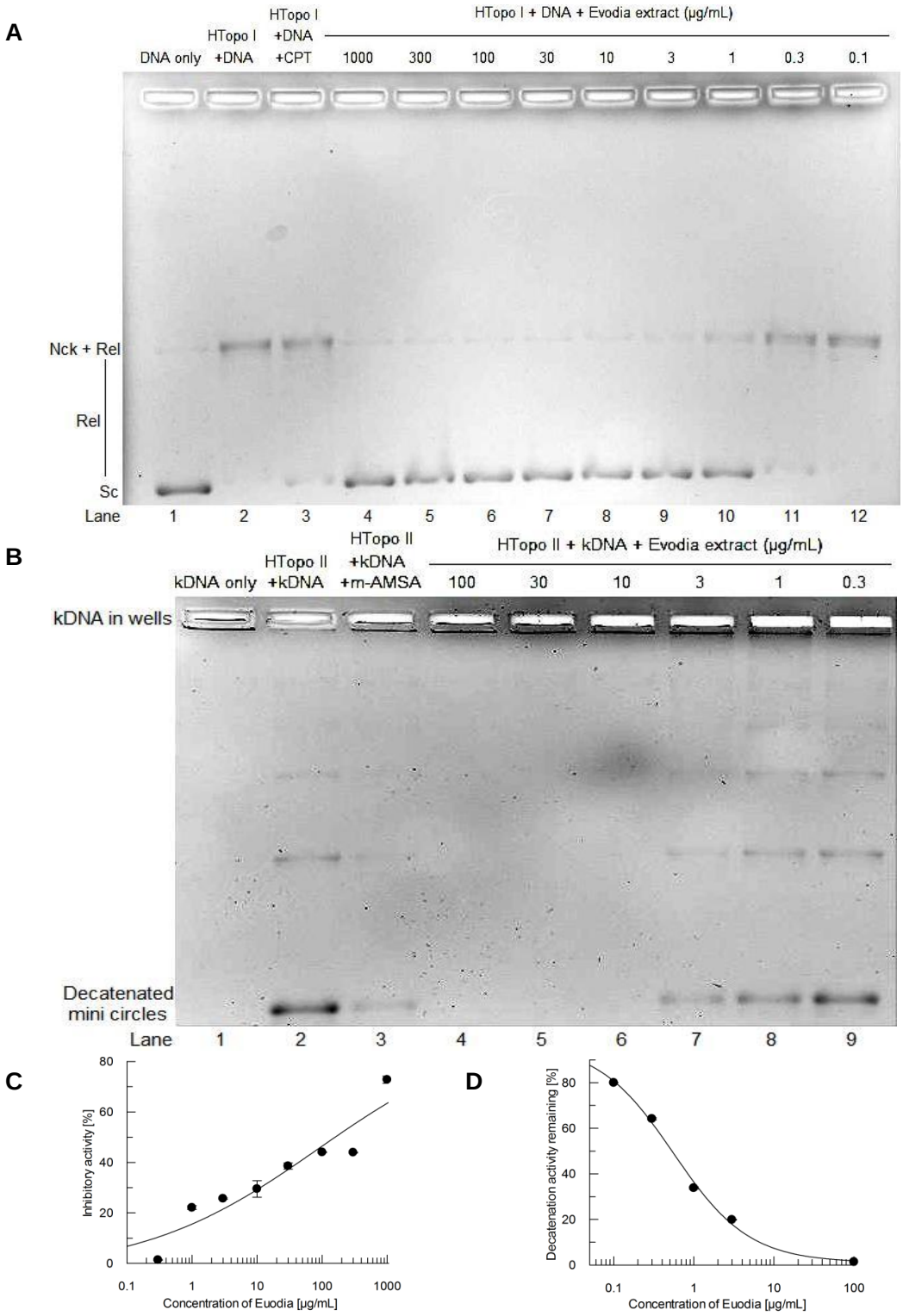


Figure 5. 12 Inhibitory activities of Euodia crude extract against HTopo I (A and C) using the relaxation assay and HTopo II (B and D) using the decatenation assay.

5.3 Discussion

The *E.coli* plasmid pBR322 DNA is a typical negatively supercoiled plasmid which migrates through the gel as a sharp single band with high mobility due to the compact and writhed structure.[83] An intercalating molecule can affect the twist and helical repeat by insert itself between two base pairs of double-stranded DNA and lead to a local unwind of the helix. The classic DNA intercalator is ethidium bromide, which changes the agarose gel electrophoresis profile of pBR322 DNA significantly by producing a ladder of relaxed DNA bands with low mobility at critical concentration. However, the effect of DNA intercalator varies depending on the concentration. As the concentration of intercalator increases, the twist of initially negatively supercoiled DNA reduces until the DNA become fully relaxed. Higher concentration of intercalator will lead to further wind of relaxed DNA in the opposite direction and finally generate positively supercoiled DNA. Hence, agarose gel profiles also changes correspondingly according to the increasing concentration of DNA intercalator react with supercoiled pBR322 DNA. A typical intercalating molecule should give characteristic agarose gel electrophoresis profiles of: firstly, a series of relaxed DNA bands (negatively writhed) with lower mobility; and then, relaxed DNA band with very low mobility; afterwards, a ladder of relaxed DNA bands (positively writhed) with higher mobility (compare with relaxed DNA band); and finally a sharp single band of positively supercoiled DNA with high mobility close to the position of negatively writhed DNA band.[41,83-84]

Inhibitory activity results of Coptis-Euodia crude extracts with high Coptis proportion against topo I showed similar gel electrophoresis profile to

DNA intercalating molecule. For example, a ladder of relaxed DNA bands can be observed on gel A (lane 12) of Figure 5. 8 when incubated with 0.1 mg/mL of Coptis crude extract. The higher the concentration of Coptis crude extract in sample, the lower the mobility of relaxed DNA bands appeared on gel (lane 11 and 10). Only a single supercoiled DNA band can be detected in samples with the concentration of Coptis crude extract higher than 0.5 mg/mL (from lane 9 leftward to lane 4). Analogous characteristic relaxed and supercoiled DNA bands can also be found on gel A of Figure 5. 9 (lane 9 to 12) and Figure 5. 10 (lane 7 to 10). Since berberine, the main alkaloid in Coptis, has been proved to be an DNA intercalating molecule, whether the supercoiled bands of samples incubated with higher concentrations of Coptis contained herbal extracts were negatively supercoiled DNA bands which caused by the inhibitory activities against topo I or positively supercoiled DNA bands which caused by the intercalating molecules involved in the extracts cannot be clearly identified due to the complexity of compounds in herbal extract. The relaxation ability of topoisomerase I made the interaction between negatively supercoiled plasmid pBR322, DNA intercalator, and the enzyme even more complicated. Further investigation is necessary to clarify the specific targets and mechanisms involved in human topoisomerase I inhibitory activity of compounds in Coptis and Euodia extracts.

The IC_{50} for inhibition of the relaxation or decatenation ability of topoisomerases can be visually assessed as the concentration of Coptis-Euodia crude extract which leads to a 50% reduction in the amount of supercoiled plasmid DNA detected in topo I assay and mini circles kDNA produced in topo II assay respectively. The result of

agarose gel electrophoresis is sensitive to many factors during the processes of experiment preparation including temperature, different activity of enzyme between batches and the conditions of electrophoresis. Moreover, the quantitative analysis is fully depending on the quality of gel documentation. Thus, the IC_{50} results presented in section 5.2.3 were obtained under the specific experimental conditions described previously in this study. Comparison of IC_{50} values of Coptis-Euodia crude extracts against human topoisomerase I and II between studies may have difficulties.

5.4 Conclusion

Berberine has been confirmed to be an effective DNA intercalator in this study with sufficient inhibitory activity on topoisomerase II. Furthermore, all five Coptis-Euodia crude extracts behaved as inhibitors on both human topoisomerase types I and II at various concentrations with IC_{50} values at μg/mL level. Topo I and II inhibitory activities of herbal formulae extracts at the same concentration were dominated by the proportion of Euodia contained in the prescription. However, evodiamine at tested concentration (100 μM) presented no effect on both topo I and II.

Hence, standard alkaloids berberine and evodiamine do not responsible for the inhibitory activities of Coptis-Euodia crude extracts on topo I. Interaction and/or synergy between components in Coptis-Euodia fulfilled the distinct topo I and topo II inhibition activities of herbal formulae. However, the specific chemical compound in the extracts of Coptis-Euodia formulae worked as topoisomerases inhibitor is yet to be elucidated.

Chapter 6 Preliminary experiments of fluorescence based assays to screen potential sodium and calcium channel blockers

6.1 Introduction

Ion channels are integral membrane proteins that open in response to a depolarizing stimulus, thereby allowing ion travel between the extracellular space and the cell interior. Ion channels are major signalling complexes expressed in many tissues, where they have diverse involvement in cellular activity.[85-86] Most channels are specific for one ion such as potassium, calcium and sodium.

6.1.1 Voltage-gated sodium channels

Sodium channels conducting sodium ions (Na^+) through the cell plasma membrane. Channel opening is transient, allowing the flow of sodium ions down their concentration gradient, thus generating an inward current, and most channels rapidly inactivate, within milliseconds of opening, and undergo conformational changes to recover form inactivation. Sodium ion channels are responsible for the rising phase of action potentials in excitable cells, for example, neurons and myocytes.[86]

There are two types of sodium ion channels: voltage-gated Na^+ channels (VGSCs) and ligand-gated Na^+ channels. Voltage-gated Na^+ channels are heteromultimers of α and β protein subunits. The α subunit is sufficient to form the core of a functional channel when expressed by a

cell, whereas the modulatory β subunit has an auxiliary status. The α subunits of sodium channels (VGSCα) are large polypeptides that are organized into four homologous domains (DI - DIV), each consisting of six trans-membrane segments that are connected by intra- and extracellular linkers.[86] VGSCs are expressed in a wide variety of excitable and non-excitable tissues, and display a range of functional forms.[87] Depolarization produced a rapid transient increase in sodium conductance and a slower maintained increase in potassium conductance. The particular sites for ionic flow were opened as a result of membrane potential changes are voltage-gated sodium channels.

Sodium pump isoform expression is specifically altered in a tissue-specific manner in diseases such as hyper- and hypothyroidism, hypokalemia, hypertension, heart failure and also in cancer cells and tissues.[57] Ion channels are increasingly being suggested to be involved in different stages of cancer process including cell proliferation, migration and survival.[85]

6.1.2 Nav 1.5 and breast cancer

According to the recent investigation, voltage-gated ion channels are widely expressed in a range of cancer cells.[85] Moreover, increasing evidence suggests that expression of voltage-gated ion channels become epigenetically abnormal in metastatic cancer cells of epithelial origin.[88] As such, these channels are becoming the targets of significant drug development efforts to block or reduce voltage-gated ion channel activity in order to prevent or combat malignant disease. Components with specific voltage-gated Na^+ channels (VGSCs)

inhibition property may have anti-metastatic cancer potential.[87]

Voltage-gated Na^+ channels have been detected in different cell lines including human breast cancer. Importantly, up-regulation of functional voltage-gated Na^+ channels has been found in metastatic human breast cancer *in vitro* and *in vivo*.[85,87] Nav 1.5 expression strongly correlated with lymph node metastasis in breast cancer *in vivo* shows that Nav 1.5 may play a key role in metastasis. Sufficient evidences indicate that voltage-gated Na^+ channels can be a target in breast cancer treatment.

Previous work of Banu Isbilen *et al* proved that docosahexaenoic acid (DHA) helps to reduce the migration of MDA-MB-231 human breast cancer cells to the same extent as tetrodotoxin (a specific VGSCs inhibitor with highly toxicity) by suppressed VGSCs activity *in vitro*. The results showed that a VGSC inhibitor such as DHA can down-regulate mRNA and plasma membrane protein levels of neonatal Nav 1.5 voltage-gated Na^+ channel. Nav 1.5 is known to be predominant in migration MDA-MB-231 cell line.[89]

6.1.3 Sodium ion fluorescence dye assay

6.1.3.1 Sodium Green indicator and SBFI

Sodium Green tetraacetate (Figure 6. 1) is a cell-permeant probe which can determine intracellular Na^+ concentration. There are two fluorescein analogs link to those two nitrogens of the crown ether. This visible light-excitable compound has sufficient selectivity for the Na^+ ion in the presence of other monovalent cations. The probe can freely diffuse across the cell membrane and is cleaved by intracellular esterases to

convert the acetate into the sodium-responsive acidic form. The peak excitation and emission wavelengths for Sodium Green are 507 and 532 nm respectively.

SBFI is also a sodium-sensitive cell-permeant probe. The working mechanism is similar to sodium green, but this indicator has a bigger difference between the excitation and emission wavelengths which are 340 and 500 nm respectively.

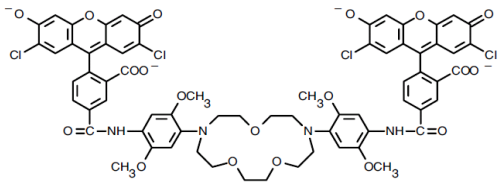


Figure 6. 1 Sodium Green indicator

6.1.3.2 DiBAC$_4$(3) dyes

DiBAC$_4$(3) is one of the most used bis-barbituric acid oxonols in publications which is primarily sensitive to plasma membrane potential. The dye enters depolarized cells and bind to intracellular proteins or membranes and exhibits enhanced fluorescence. Increased depolarization results in more influx of the anionic dye and gives an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. The peak excitation and emission wavelengths for DiBAC$_4$(3) are 490 and 516 nm respectively.

6.1.3.3 Veratridine

Veratridine is a steroid-derived alkaloid exists in plants of *Liliaceae* family. The neurotoxin has molecular function by activating sodium ion channels and induces an influx of sodium ion into cells. It can also keep the activated ion channels responsive to further stimulations. Such activity can be detected with the presence of proper cell-permeant fluorescence dye.[90]

6.1.4 Voltage-gated calcium channels (Cav 3.2) and cancer

Calcium channels are mainly responsible for voltage-gated depolarizations and maintain the depolarization during the plateau of the action potential in different kind of muscle cells. They also can be found in brain, heart, nerve terminals and many other tissues. Calcium channels play major role in controlling the internal calcium ion concentration which acts as an intracellular second messenger for a wide variety of cellular functions. Since the discovery of voltage-gated calcium channels in 1950s, the classification of them has developed broadly in three stages. They have now being distinguished by cloning and genetic analysis.[91-92] Voltage-gated calcium channels can be classified into different types: T-type, L-type, N-type, P/Q-type and R-type Ca^{2+} channels. T-type Ca^{2+} channels which belong to LVA (rapid and voltage-dependent inactivation) class have a tiny conductance and make a transient current. Three broad gene subfamilies—Cav1, Cav2 and Cav3 have been distinguished by comparison of sequence similarities and differences.[92]

Unlike normal cells, tumor cells acquire significant growth autonomy which leads to an abnormal activity of proliferation. Such self-dependent growth signals including abnormal expansion of neuroendocrine differentiated cells. This growth signal appears in many common carcinomas including colorectal, lung, breast and prostate which requires Ca^{2+} signaling to progress. Previous works of Mariot, P. *et al* and Gackiere, F. *et al* indicated that Cav 3.2 is involved in calcium-dependent neuroendocrine differentiation of human prostate cancer cells LNCaP.[93]

6.1.5 Calcium ion fluorescence dye assay

6.1.5.1 Transfection

Transfection is a valuable technology to study gene function and protein expression by introducing nucleic acids into eukaryotic cells. The process can be achieved by several nonviral technique including chemical, lipid or physical methods.[94] FuGENE HD transfection reagent is a novel, nonliposomal formulation designed to transfect DNA into a wide variety of cell lines with high efficiency and low toxicity. In this research, human embryonic kidney cell line HEK 293 was used to express Cav 3.2.[62]

6.1.5.2 Calcium Green indicator

Long-wavelength calcium indicator calcium green in this research is in the cell-permeant acetoxymethyl (AM) ester form. The indicator exhibits an increase in fluorescence intensity by binding to calcium ion. Calcium green^{TM}-1 gives more fluorescent at low calcium concentrations than

fluo-3 which can contribute to determine the baseline calcium ion levels and can help to increase the visibility of resting cells. The excitation and emission wavelengths for calcium green™-1 (AM) are 506 and 531 nm respectively.

6.1.5.3 Calcium ion fluorescence dye assay

Gramicidin is a linear pore-forming pentadecapeptide antibiotic which can insert into the plasma membrane easily and form ions conducted pores. These pores can driving cells' membrane voltage to potassium equilibrium, thereby convert the T-type Ca^{2+} channels from inactivated state to activated state. Ca^{2+} influx can be induced by changing the extracellular K^{+} ion concentration, and the intracellular fluorescence change can be measured by pre-loaded cell permeant Ca^{2+} fluorescence dye.[62]

Quinoline alkaloids isolated from *Euodia rutaecarpa* was reported to modulate intracellular Ca^{2+} levels.[95] In order to test the Ca^{2+} mediation ability of alkaloids from natural source, mibefradil, a clinical used calcium channel blocker, is introduced as a positive control.

6.2 Results

6.2.1 Sodium ion fluorescence dye assay

6.2.1.1 Sodium green assay and SBFI assay

According to previous research, in order to help expressing Nav 1.5 on MDA-MB-231 cells, sodium ion channel activator veratridine was introduced in this study. Figure 6. 2 and Table 6. 1 demonstrated the

fluorescence intensity changes of sodium green labeled breast cancer cells treated with different concentrations of veratridine (10, 20, 40 or 50 μM) for 2 hrs or overnight. Compared with negative control, higher fluorescence intensity can be detected in veratridine (40 μM) treated MDA-MB-231 cells either for 2 hrs or before experiment (Figure 6. 2). Long term or higher concentration treatment with veratridine did not show considerable fluorescence intensity increase. Results indicated that veratridine has a rapid effect on sodium ion channel as an activator.

Fluorescence intensity of MDA-MB-231 cells loaded with veratridine (40 μM) followed by extra potassium ion injection were shown in Figure 6. 3. Negative control sample gave an obvious fluorescence increase from 10 seconds. Though veratridine treated cells showed much higher fluorescence intensity from the very beginning of the reading process, extra potassium ion did not result in any change after injection.

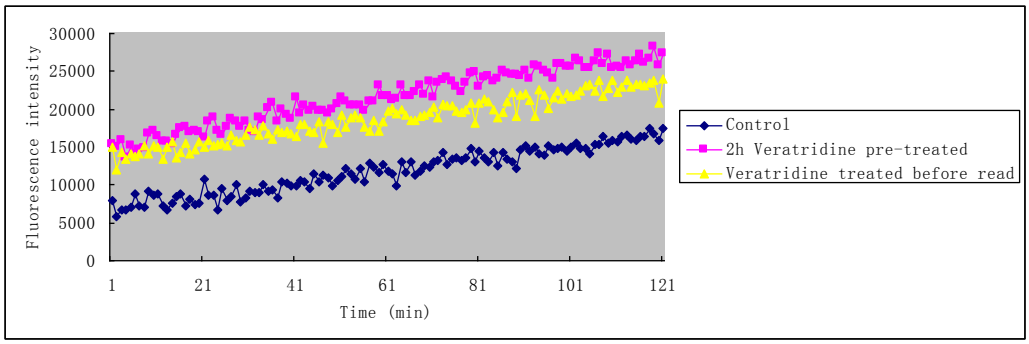


Figure 6. 2 Fluorescence intensity (a.u.) kinetic (plate mode) of veratridine (40 μM) treated MDA-MB-231 cells with different treatment time. Sodium green labeled cells were used as negative control.

Table 6. 1 Fluorescence intensity (a.u.) of MDA-MB-231 cells treated with veratridine at various concentrations overnight.

	Veratridine (μM)		
	10	20	50
Fluorescence intensity (a.u.)	112706.6	119138.4	123787.4

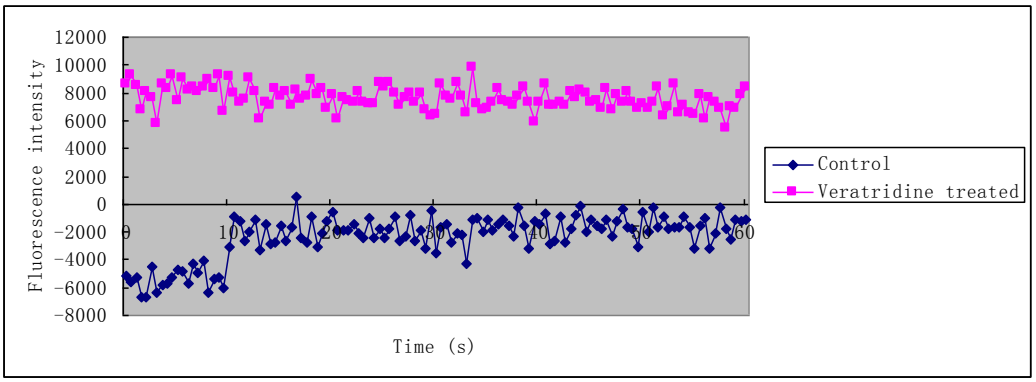


Figure 6. 3 Fluorescence intensity (a.u.) kinetic (well mode) of veratridine (40 μM) treated MDA-MB-231 cells compared with sodium green labeled cell sample (control). Buffer 2 (referred to Table 2. 1) (20 μL) was injected automatically at the 10^{th} second by microplate reader.

In order to compare the differences between sodium ion fluorescence dyes SBFI and sodium green, a test with the same treatment on MDA-MB-231 has been carried out with the microplate reader endpoint mode. Compare the fluorescence intensity of SBFI (5 μM) labeled MDA-MB-231 cells with sodium green (3 μM) (Table 6. 2), both sodium ion fluorescence dyes worked perfectly on MDA-MB-231 cells at testing concentration.

Table 6. 2 Fluorescence intensity (a.u.) of sodium green (3 μM) and SBFI (5 μM) labeled MDA-MB-231 cells. Data were presented as mean ± S.D. (n=5).

	Fluorescence intensity (a.u.)
Sodium green (3 μM)	8860 ± 3131
SBFI (5 μM)	11607 ± 3923

6.2.1.2 DiBAC3(4) assay

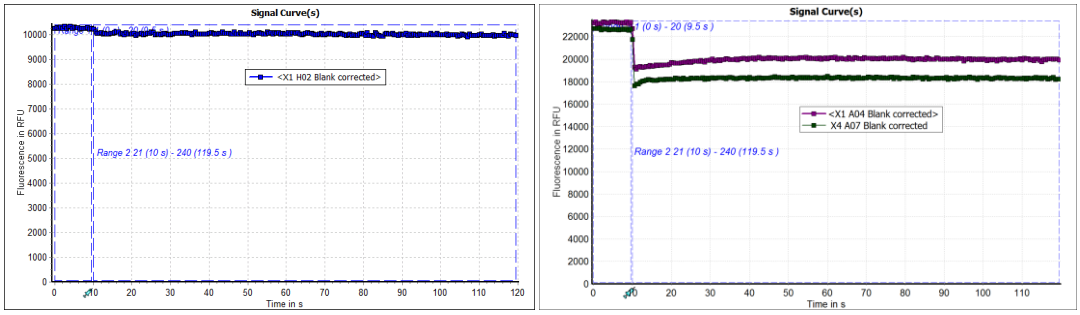


Figure 6. 4 Fluorescence intensity (a.u.) kinetic (well mode) of DiBAC3(4) working solution (5 μM, 100 μL) (left) and veratridine (40 μM) treated MDA-MB-231 cells labeled with 5 μM of DiBAC3(4) (right, green line). KCl solution (50 mM final) was injected automatically at 10th second by microplate reader. Cell sample without veratridine treatment were used as negative control (right, purple line)

Figure 6. 4 (left) shows the kinetic fluorescence intensity of $DiBAC_3(4)$ working solution (5 μM, 100 μL) in 96-well. KCl solution was injected at 10th second to change the concentration of extracellular K^+ ion and induce the Ca^{2+} influx as described in the assay. A decrease in fluorescence can be found just after the injection. However, in Figure 6. 4 (right), MDA-MB-231 cells with (dark green line) and without (purple line) veratridine treatment indicated an obvious decrease with extra potassium ion. According to the working hypothesis of $DiBAC_3(4)$ and

Figure 6. 4 (right), cell memberane of MDA-MB-231 cells were in hyperpolarization state after stimulated with extra potassium ion in which case may lead to the decrease in fluorescence.[96] Fluorescence intensity of veratridine, a sodium channels activator, treated cells dropped to an even lower level than untreated cells. The results still cannot be explained so far.

6.2.2 Calcium ion fluorescence dye assay

6.2.2.1 Plasmid purification

Plasmid Cav3.2 was isolated and purified from XL1-Blue E.*coli* contains that plasmid. The Nano Drop spectrum results (Table 6. 3) showed that both batch of the plasmid were within the purity range and with good concentration which was reliable in transfection process.

Table 6. 3 Purity and concentration of plasmid Cav 3.2

	$A_{260/280}$	Concentration ($\mu g \cdot \mu L^{-1}$)
Batch 1	1.89	419.7
Batch 2	1.87	482.9

6.2.2.2 Calcium green assay

To determine the potassium induced activation of calcium channel in HEK 293 cells, a blank sample labeled with calcium green without further treatment were read in buffer 2 (referred to Table 2.3). The results of blank (Figure 6. 5, dark blue line) gives a stable intracellular fluorescence from 0 to 10 seconds around 38000 a.u.. After the injection of KCl (60 mM, 20 µL) at the 10^{th} second, the fluorescence changing

formed an obvious peak from 11 to 30 second with a maximum 51081 a.u. at 20.5 second. The trend line proved the HEK 293 cells can express cloned T-type calcium channel 3.2 and the Cav 3.2 channel can be activated successfully by additional extracellular potassium ion. Thereby, the preloaded cell permeant fluorescence probe showed the increase of intracellular Ca^{2+} ion concentration with fluorescence intensity. The original fluorescence level at 0 second was about 4000 a.u. higher than the blank sample when the cells treated with gramicidin (10 μg/mL) before read. The pink line in Figure 6. 5 proved that gramicidin can increase the permeability of plasma membrane which led to a higher fluorescence signal through the reading.

Clinically used calcium channel blocker mibefradil was used in the study as a positive control. The yellow and light blue lines of Figure 6. 5 showed a clear effect of mibefradil (100 μM) treatment. The fluorescence change did not form a clear peak after KCl injection in both samples. Cells loaded with gramicidin (10 μg/mL) associated with mibefradil (100 μM) exhibited a higher fluorescence level at 66000 a.u. while mibefradil (100 μM) treated sample gave 53000 a.u.. Such results further proved the pore-forming activity of gramicidine at μg/mL level and calcium channel blockage ability of mibefradil.

In order to demonstrate the effect of Euodia extract on calcium channel, evodiamine and rutaecarpine which isolated from Euodia were performed. Cell sample treated with evodiamine (10 mM) showed a flat fluorescence signal (Figure 6. 6 A) through the reading. The additional extracellular K^+ ion did not induce the activation of Ca^{2+} channel. Cells loaded with mibefradil and evodiamine gave a similar fluorescence

signal around 51000 a.u. with a same trend. The results indicated that evodiamine might be a calcium channel blocker. Contrarily, rutaecarpine treated cells gave higher initial fluorescence intensity at 50000 a.u. and a low peak after 10 seconds (Figure 6. 6 B). Compared with the blank, the increase of fluorescence intensity after KCl injection was flatter with the maximum of 56509 a.u.. The effect of KCl was fully blocked in mibefradil and rutaecarpine treated sample. The results indicated that rutaecarpine might not responsible for T-type calcium channel 3.2 blockage.

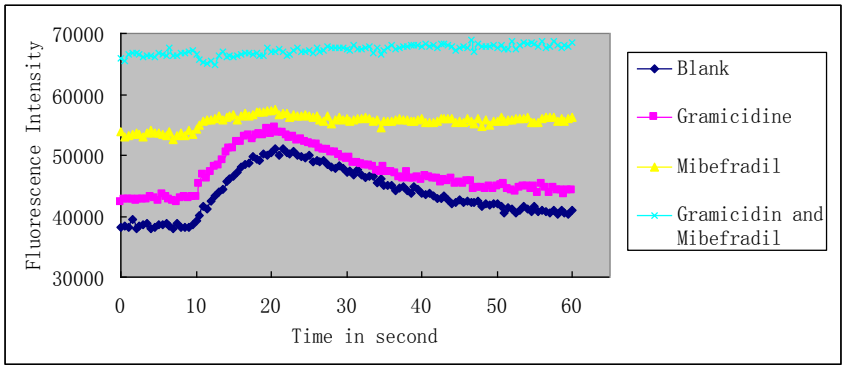
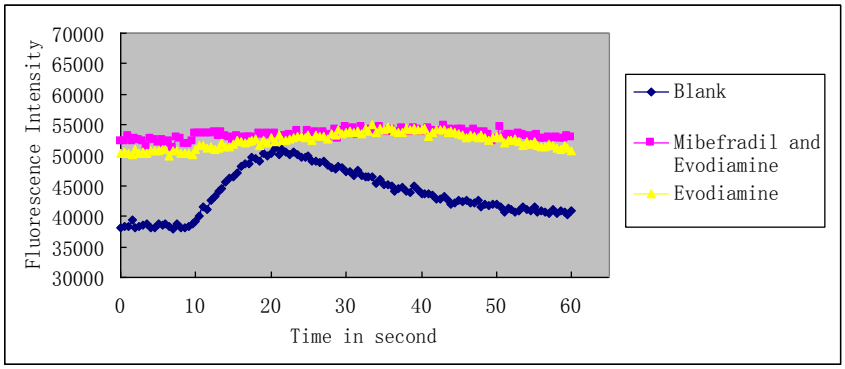


Figure 6. 5 Effects of gramicidine (10 μg/mL), mibefradil (100 μM), and gramicidine (10 μg/mL) associated with mibefradil (100 μM) on intracellular Ca^{2+} level in HEK 293 cells. KCl solution (60 mM, 20 μL) was injected automatically at the 10^{th} second by microplate reader. Cell sample labeled with calcium green was used as negative control (blank).

A



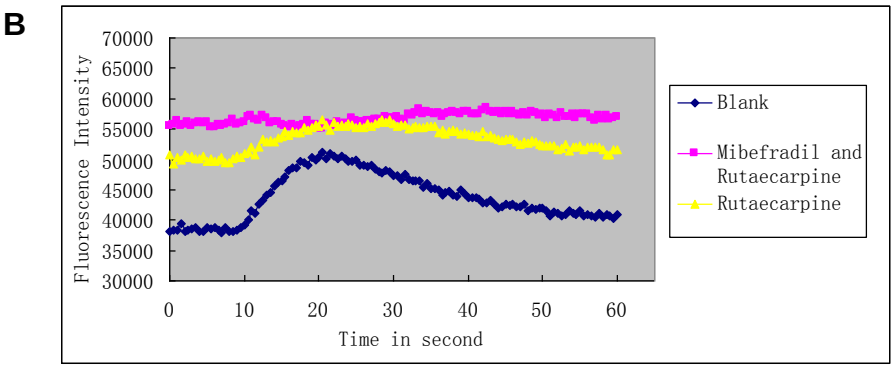


Figure 6. 6 Effects of evodiamine (10 mM) (A) and rutaecarpine (10 mM) (B) on intracellular Ca^{2+} level in HEK 293 cells in the absence or presence of mibefradil (100 μM). KCl solution (60 mM, 20 μL) was injected automatically at the 10^{th} second by microplate reader. Cell sample labeled with calcium green was used as negative control (blank).

6.3 Discussion and further work

Preliminary results of sodium green assay demonstrated in Figure 6. 3 and other preliminary experiments did not lead to a reliable fluorescence based assay. One possible reason is the voltage-gated sodium ion channels Nav 1.5 of MDA-MB-231 cells were already fully responded to sodium channel activator veratridine before the fluorescence intensity detecting started. Further potassium ion stimulation will not result in higher fluorescence intensity signal. Therefore, kinetic changes of fluorescence intensity with the voltage-gated sodium ion channel Nav 1.5 on the cell membrane of MDA-MB-231 cells responds to ion channel activator or inhibitor cannot be detected by the assay described in this study. Further optimization is necessary to establish a reliable fluorescence based assay to screen sodium ion channel blockers.

Calcium green assay operated in this study has been proved to have the calcium channel blocker screening potential according to the preliminary results demonstrated in this chapter. However, all the results have not been repeated or statistically analyzed to prove the reliability of the assay. Therefore, further work need to be done to achieve the establishment of a reliable fluorescence based calcium ion channel blocker screening assay.

Chapter 7 General discussions

7.1 Conclusion of this study

This research investigated the anti-proliferative activities with short term treatment at various concentrations and determined the IC_{50} values of standard alkaloids berberine and evodiamine, along with the 70% methanol extracts of TCM formulae Coptis-Euodia against MDA-MB-231 and MCF-7 human breast cancer cell lines by MTT assay. Long term treatment cytotoxic activities of those research subjects against both breast cancer cell lines have also been evaluated. All seven candidates exhibited anti-breast cancer activities by inhibiting the growth of cancer cells with short term treatment and inducing apoptosis of cancer cells after long term culture. Anti-metastasis activity of those anti-cancer candidates from natural source against high metastatic breast cancer cell line MDA-MB-231 has been investigated in this study by scratch wound-healing assay and anti-adhesion assay. Standard alkaloids along with Coptis involved crude extracts affected the 2-D migration and adhesion behavior of MDA-MB-231 cells at μM and μg/mL level in a time-dependent manner. Though evodiamine successfully inhibited the motility of breast cancer cells, same activity cannot be observed from Euodia extract on tested concentrations. Research results suggested that apart from the cytotoxicity activities of them, metastasis of breast cancer cells can also be inhibited by standard alkaloids and Coptis contained formulae extracts at concentrations below anti-proliferative IC_{50} values.

Despite the MTT colorimetric assay employed in this research, many other techniques can also be used to detect and evaluate viable cells.

Dead cells in a cell sample can be stained by trypan blue due to the permeability of dead cell membrane. Cell viability can then be determined by counting the unstained cells under microscope with hemocytometer or cell counter. However, trypan blue assay is not suitable for large numbers of sample and manual error may occur during repeat when counting cells with microscope.[97] Another way to detect and quantify viable cells is flow cytometry, which appears to be more accurate but expensive. As in cell motility inhibition study, cell migration can also be evaluated by chemotaxis assays such as 3-dimensional Boyden chamber assay. Migration rate can be determined by the number of cells migrate through the pores of membrane from the upper compartment into the lower chamber. Boyden chamber assay is more suitable for measuring the chemo-attractant on cell migration and real-time evaluation of migration rates cannot achieve with this assay.[98]

In order to clarify the anti-cancer mechanism of Coptis-Euodia extracts on breast cancer cells, especially on the cell cycle distribution, a fluorescence based flow cytometric cell cycle analysis has been employed in this study. Berberine, the primary alkaloid of Coptis, considerably arrested MDA-MB-231 cells at G0/G1 phase. The G0/G1 phase arrested activity of berberine contributed to the effects of Coptis and Coptis contained extracts on cell cycle distribution of MDA-MB-231 cell line to a great extent. Evodiamine has been proved to arrest both MDA-MB-231 and MCF-7 breast cancer cells at G2/M phase significantly. However, such activity did not reflect on the effects of Euodia and Euodia involved extracts on cell cycle distribution of MDA-MB-231 cell line after 24 hrs.

Research models of cancer applied throughout this study are breast cancer cell lines, which have been widely used in laboratory. Diverse models such as primary tumors, paraffin-embedded samples, xenografts, tumor primary cell cultures, and genetically engineered mice have also been used for different types of cancer research.[99-104] The benefits of using cell lines are obvious including infinite supply of research object especially cell lines derived from a human cancer. This ‘easy to manipulate’ *in vitro* cancer model allows researchers to evaluate presently used anticancer drugs, screen potential anticancer therapies, and understand genetic and cellular pathways involved. Another unneglectable reason of using cell model is the difficulty of animal drug testing procedure ethically. However, disadvantages of using cancer cell lines including genomic instability and different growth environment from *in vivo* tumor are also apparent.[105] Among which, low clinical relevance become the main shortcoming of human cancer-derived cell lines. Some research pointed the radical changes in gene expression profiles brought on by extended periods of cell culture.[106-108] Though the limitation of passage number of most continuous cell lines can undergo has not been determined, genetic uncertainty cannot be ignored. Therefore, it is important to observe the morphological change during subculture and track the maintenance of cancer cell line.[109] Despite of the genetic uncertainty, one cancer cell line may only represent a specific subtype of the original tumor. Thus, a laboratory proved effective anti-cancer candidate by using certain cancer cell line as research model may not ensure the same activity on actual clinical trial. Nevertheless, cell lines are still the most sufficient research model and the front line of cancer research.

DNA topoisomerases have been proved to be involved in the anti-breast cancer mechanism of Coptis-Euodia formulae crude extracts by employing human topoisomerase I relaxation assay and human topoisomerase II decatenation assay. Berberine has been confirmed to behave as a DNA intercalator at 100 μM while evodiamine did not show topoisomerase inhibition activity until 300 μM. All Coptis-Euodia extracts exhibited topoisomerase poison like activities on both topo I and II in a concentration-dependent manner. The IC_{50} value of each extract has also been estimated in this study. Results indicated that other topoisomerases inhibitor and/or synergy between components in Coptis-Euodia formulae might lead to the topoisomerases inhibitory activities since berberine and evodiamine are not fully responsible for it.

In the chemical structure based point of view: most topoisomerase targeting molecules, either isolated from natural source or synthesized, contain basic chemical structure of either quinoline or isoquinoline (Figure 7. 1).[82] Phytochemistry research proved that most alkaloids isolated from Coptidis rhizma are protoberberine (Figure 7. 1, middle), which can be considered as duo isoquinoline. Moreover, many quinoline alkaloids have been purified from Euodiae fructus. In spite of berberine and evodiamine, it is highly possible that other alkaloids in both herbal medicines were contributed to the topoisomerase inhibition activities of Coptis-Euodia formulae crude extracts.

In a TCM formulae combination point of view: results of this study suggested that, (a) introducing of Coptis significantly enhanced the anti-cancer activities of the duo herbal formulae; (b) Euodia in the formulae reduced the toxicity of Coptis; (c) advantages (bioactivities) of

singly used Coptis and Euodia have been inherited by the formulae of these two herbal medicine; (d) cooperation between these two herbal medicine showed considerable activities on breast cancer cells and human topoisomerases. For instance, *Zhu Yu* Pill (Coptis : Euodia = 1 : 1) demonstrated anti-proliferative activity against both MDA-MB-231 and MCF-7 breast cancer cell lines with IC_{50} values of 0.16 and 0.19 mg/mL respectively, which was almost 10 times more efficient than that of singly used Euodia. The extract also sufficiently arrested MDA-MB-231 cells at G0/G1 phase compared with the effect of singly used Coptis. Furthermore, C : E 1 : 1 extract worked as dual inhibitor against human topoisomerase I and II with IC_{50} values of 49.95 and 55.49 μg/mL respectively.

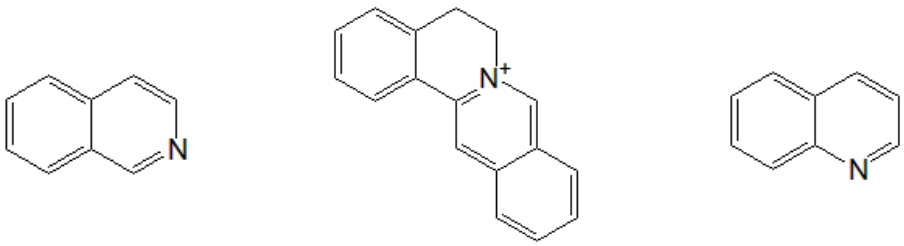


Figure 7. 1 Chemical skeleton of isoquinoline (left), protoberberine (middle), and quinoline (right)

In conclusion, this study clarified that DNA intercalator berberine insert itself between the base pair of DNA strand of cancer cells, which converted the structure of DNA. This molecule – DNA binding action interrupted DNA synthesis process of cancer cells and stopped cells at G0/G1 checkpoint of cell cycle. The whole process resulted in the apoptosis of cancer cells. Such mechanism also contributed to the anti-cancer activities of Coptis and Coptis containing herbal formulae.

Euodia worked as topoisomerase poison on both topo I and topo II by stabilizing the covalent topo-DNA cleavage complexes and preventing the re-ligation of DNA. The topoisomerase poisoning activities of other Euodia involving herbal formulae were dominated by components of Euodia but not evodiamine. Evodiamine affected breast cancer distribution by arresting cancer cells at G2/M phase. However, the topoisomerase poisoning activities were not considerable even at concentrations over 300 μM. Further molecular biology studies are necessary to investigate other therapeutic target involved in the anti-breast cancer activities of Coptis-Euodia formulae.

Conclusion of the whole study proved the hypothesis stated in Chapter 1. The specific bioactivities of medicinal herb and herbal formulae cannot be concluded by single or multiple bioactive ingredients in it, even with similar results obtained from the same assay. The investigation of isolated chemical compound is to understand part of the mechanism and target involved in the bioactivities of the whole herb or formula. Full mechanism profile was the outcome of interaction and synergy between hundreds of compounds in single used medicinal herb and their formulae which should be investigated and evaluated independently.

7.2 Potential of TCM and formulae as combination therapy candidates in cancer treatment

Combination therapy, also known as polytherapy, is a broad therapeutic way with more than one medication or non-medical therapies involved. Cellular pathways operate more like complicated webs than straight superhighways. There are multiple redundancies, or alternate routes,

that may be activated in response to the inhibition of the same pathway. Therefore, the one-size-fits-all medication mode easily results in drug resistance and clinical relapse due to the resistant strains of disease and genetic differences in patients. Combination therapies are necessary to effectively treat infectious diseases such as malaria, tuberculosis, cancer, and HIV.[110] This therapeutic way is expected to avoid the evolution of drug resistance, reduce side effects, and increase response rates by targeting different pathways of the same condition.[111] Recent precision medicine research have focused on targeting multiple biomarkers found in individual tumors by using combinations of FDA-approved anticancer drugs. The basic theory of using combination therapy happened to share the same point of view with the principle of traditional Chinese medicine combination (known as TCM formulae), which has been practiced for hundreds of years. (Relative content can be found in section 1.7 of Chapter 1 of this thesis.) Different from the synthesis chemical medication, one single used bioactive medicinal plant contains hundreds of components with multiple bio-targets involved. The particularity of medicinal plant expended the possibility of TCM and TCM formulae as combination therapy in cancer research. There are three potential stages of medicinal plant in combination therapy application: Single used medicinal plant, which has been proved to be multi-targeted, can be treated as natural combination therapy; Herbal prescriptions (also known as TCM formulae), allow the synergy between components, can be the perfect source of combination therapy research; and Medicinal plant or herbal prescriptions associate with existing anticancer medicine as combination therapy. Potentially, multi-targeted medicinal herb involved in TCM formulae might work together as a team in series or in parallel, with or without additional chemotherapy, in cancer treatment. Moreover,

combination therapy of TCM formulae can also reduce the risk of drug interactions with the support of clinical experience. However, every bio-target involved in anticancer activity and the efficiency of each bioactive ingredient in TCM formulae need to be clarified before the combination therapy investigation can actually started.

7.3 Modern research of TCM and formulae

7.3.1 Current research of TCM and formulae

In spite of the fact that plants have been used for therapeutic purposes for millennia, only a relatively few plants or plant derivatives are currently officially recognized worldwide as effective drugs. Yet many of these continue to be used as complimentary and folk medicines, or in pharmaceutical preparations.

Traditionally, after a prescription of TCM being made by therapist, the crude herbal material mixture was socking and cooking with water, and a TCM decoction with medicinal effect is prepared. With the development of the pharmaceutical science of TCM, more and more ready-made TCM pills, tablets, capsules and concentrated drink according to traditionally used formulae have been produced. Ready-made TCM simplified the preparation process of medicine and improved the consistency of TCM. However, the therapeutic effects of ready-made TCM are believed to be weakened (less effective) compared with TCM decoction due to the different formulation of medicine. Furthermore, pharmacy shelf available TCM is no longer personalized or even traditional.

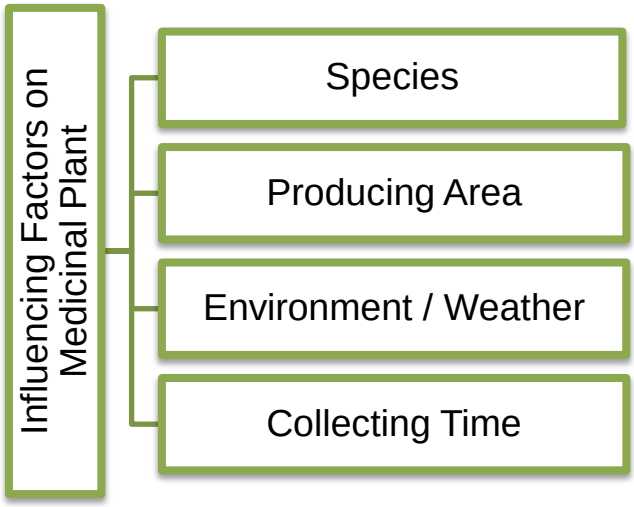


Figure 7. 2 Influencing factors on medicinal plant

Herbal medicine is difficult to standardization due to the complicated chemical compositions and multiple influencing factors during the processes of the growth and harvest of medicinal plant. Figure 7. 2 listed the possible internal and external influencing factors on medicinal plants including species, producing area, environment, weather, and collecting time. Those factors will affect the percentage of secondary metabolites (bioactive components) in the plant, and even the total therapeutic effects of it. Appendix 1 to Appendix 3 demonstrated an example of influencing factors on Euodiae fructus (one of the main research subject of this thesis). Different species of Euodiae fructus sonication extracted by ethyl acetate resulted in different color of extractions. HPLC (high performance liquid chromatography) chromatograms in Appendix 2 were obtained by the same gradient mobile phase. Detected and well separated peaks with the same retention time but different peak area can be observed in chromatograms A and B (different species) of Appendix 2. However, even the same species with the same injection volume can give difference in peak areas (chromatograms B and C). Analytical results helped to explain why both Euodia rutaecarpa (Juss.)

Benth. and Euodia rutaecarpa (Juss.) Benth. var. officinalis (Dode) Huang can be used as Euodiae fructus, which also indicated the core difficulty of TCM standardization.

Quality standard of synthesis medicine is consistent with a clear and specific describe. For example, quality control (content) of Etoposide capsule in the British pharmacopoeia is described as “95% to 105% of the stated amount” according to the standard analytical procedures. In the crude medicinal material section of the Chinese Pharmacopoeia, quality standard of an herbal medicine is often described according to the percentage of main bioactive component in the plant which is no more than 10% (w/w) in most cases. For example, quality control of Coptidis rhizoma (another main research subject of this thesis) is standardized by berberine, which should be no less than 5.5% to 7.0% in the crude material according to the standard analytical procedures demonstrated in Chinese pharmacopoeia, percentage varies with the species. Percentage of other alkaloids including coptisine (no less than 1.6%) and palmatine (no less than 1.5%) can be used as additional standards in the quality control of Coptidis rhizoma.[17] The difference of quality standard between synthetic medicine and natural herbal medicine is the main impediment in the further development and application of TCM. The inconsistency of natural products resulted in an implicit impression of the herbal medicines employed in TCM – untrustworthy, which is obviously a misunderstanding. In order to change the prejudice of TCM, many standardization quality control methods have been established. The most recent standardization of TCM is called fingerprint chromatography by using either HPLC or HPTLC (high performance thin layer chromatography) technique (Appendix 3) to

prove the relatively consistency of TCM. Though the establishment of HPLC or HPTLC related standardization has been widely accepted in China and many Asia countries, it still not detailed and consistency enough to satisfy the whole world.

Common research routine of single TCM is listed in Figure 7. 3. After a series of extraction and isolation procedures, bioactivities screening of those separated single compounds begins. Subsequently, the chemical structures of bioactive compounds are to be identified. A series of further research will be employed on those compounds as candidates or chemical template of drug discovery. Other compounds with non- or less bioactivity will be neglected. However, can bioactive single compound isolated from a medicinal plant represent the complete therapeutic effects and pharmaceutical potential of that TCM? The answer is “Not always”. This point of view has been discussed and proved in previous chapters 3 to 5. Moreover, therapeutic effective compounds can also be produced in human body after consuming the TCM formula. More and more researchers realized such facts and started to extend research subjects from singly used medicinal plant to combined TCM formulae. However, the unstable quality of each plant involved in the formula will result in a more complicated circumstance when mix them together as one research subject. Consequently, majority studies of TCM formulae followed an intuitionistic research routine directly from *in vitro* to *in vivo* (animal test) (Figure 7. 4). Quite often, premature publicity on unconfirmed research data has tainted the reputation of many herbal medicine and formulae. Unfortunately, TCM formulae involved in clinical cancer therapy is still rare outside China.

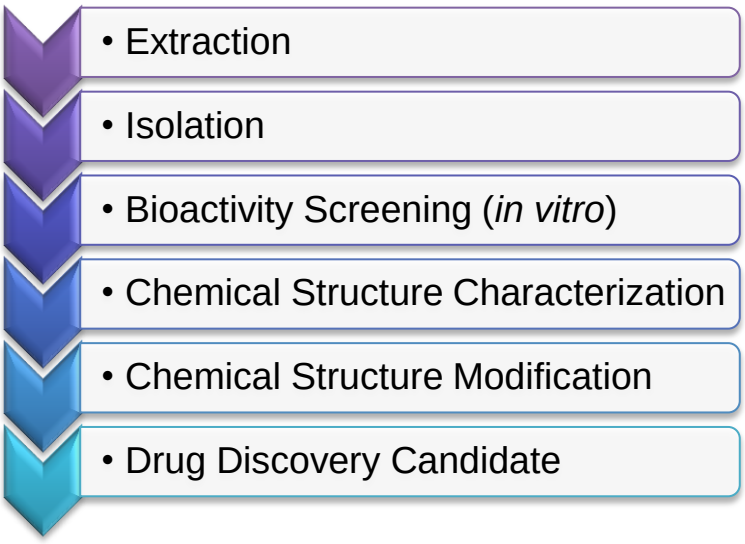


Figure 7. 3 Research routine of single TCM

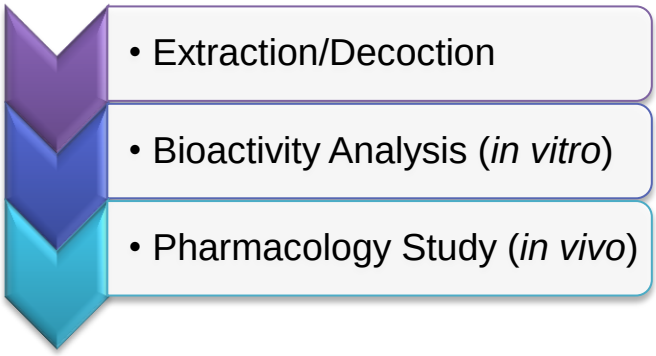


Figure 7. 4 Research routine of TCM formulae

7.3.2 Potential future research of TCM and formulae

A novel concept called “Artificial Herbal Medicine” is now been brought out in this thesis. The concept of artificial herbal medicine combines the holism of natural herbal medicine and the consistency of synthesis medicine together. After the “chemical structure characterization” step of the current single medicinal plant research routine (Figure 7. 3), each component of that medicinal plant is to be synthesized and mixed back together according the proportion of its original plant model (a plant

model with the best therapeutic effects) to form an artificial herbal medicine without the appearance of the plant. Hence, the quality control of both singly used herbal medicine and combined formulae can then be standardized with a complete, detailed, and consistent component list. It may sound like a mission impossible, but science also needs a hint of imagination.

In order to achieve the hypothesis of artificial herbal medicine, cooperation between many scientific subjects including botany, phytochemistry, analytical chemistry, cell biology, molecular biology, pharmacology, organic chemistry and research techniques involved in these subjects are necessary. Though the cost must be beyond imagination, the benefits are quite remarkable.

Some diseases, especially cancer, require a long term (or even lifetime) treatment. Acute side effects of treatment always weaken the patient and result in other serious disorder in the body. According to the principle of TCM formulae combination, most formulae are designed not only to treat the disease, but also to improve the health condition of patient. Additional affects including immune system building up, therapeutic side effects recovery, mentally relaxation, and quality of life improvement. In colloquial language, personalized TCM formulae are expected to be a kind of therapy, which can work as fighter and defender at the same time. Such medication is perfect for patients who are not suitable for surgery or on the stage of post-surgery recovery.

In conclusion, achievement of the standardization of herbal medicine will upgrade TCM formulae from complementary and alternative medicine to

primary medicine. Furthermore, employment of TCM formulae will transform cancer therapy from a tough and resistant pathway (surgery, radiation therapy, or chemotherapy) to a gentle and natural pathway. With the understanding and development of traditional Chinese medicine and TCM formulae, natural products will continuously contribute to or even lead the cancer therapy in the coming future.

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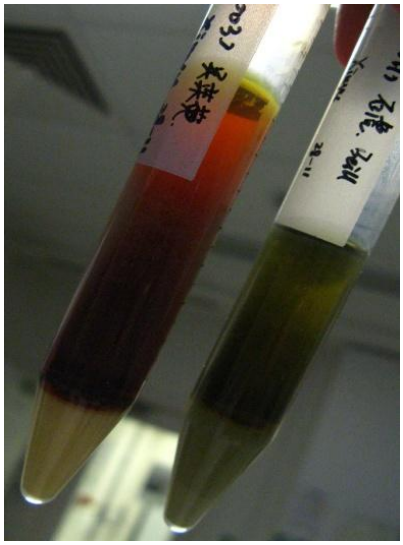
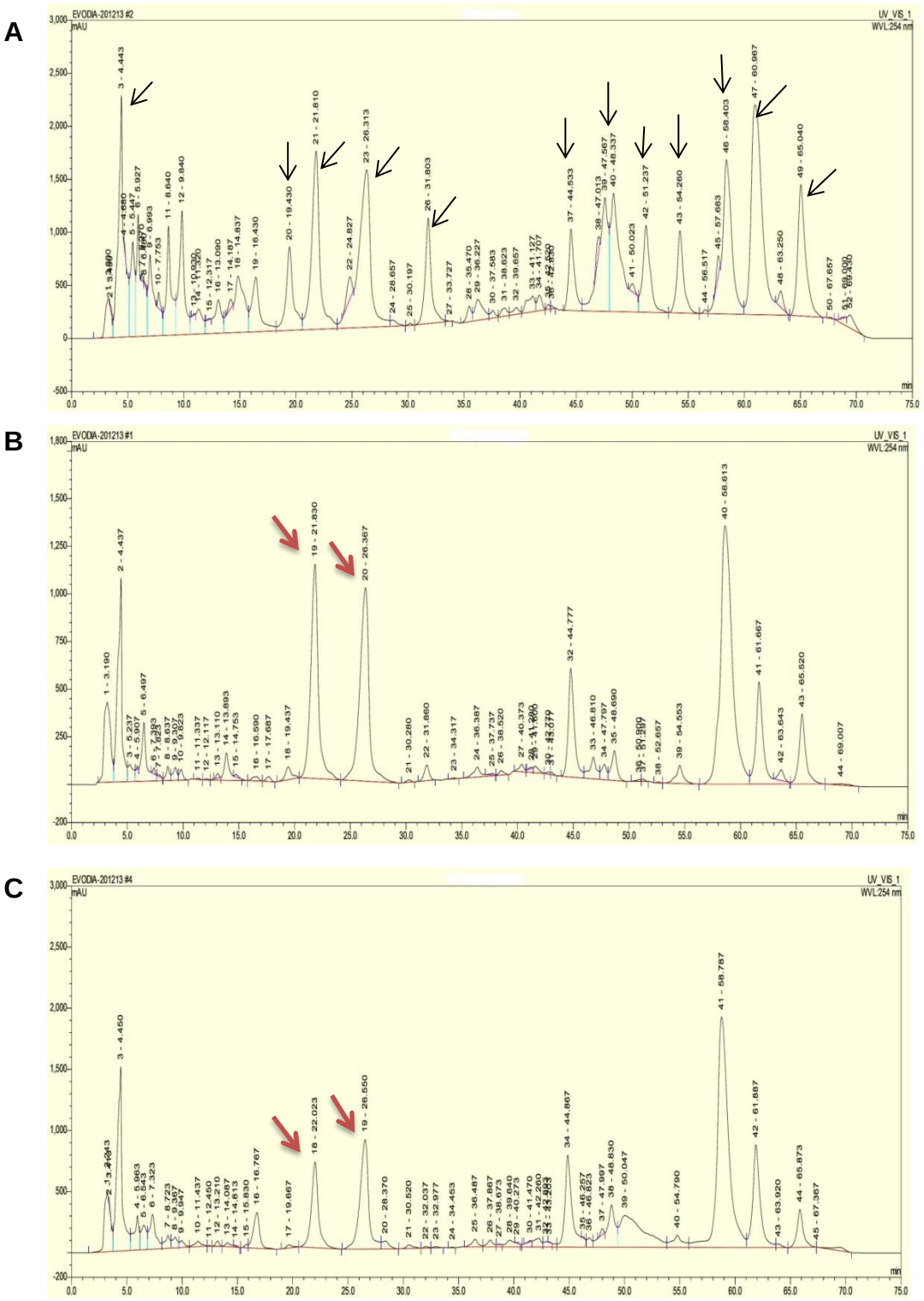
Appendix

Appendix 1



Ethyl acetate extracts of *Euodia rutaecarpa* (Juss.) Benth. (left brownish solution) and *Euodia rutaecarpa* (Juss.) Benth. *var*. *officinalis* (Dode) Huang (right greenish solution)

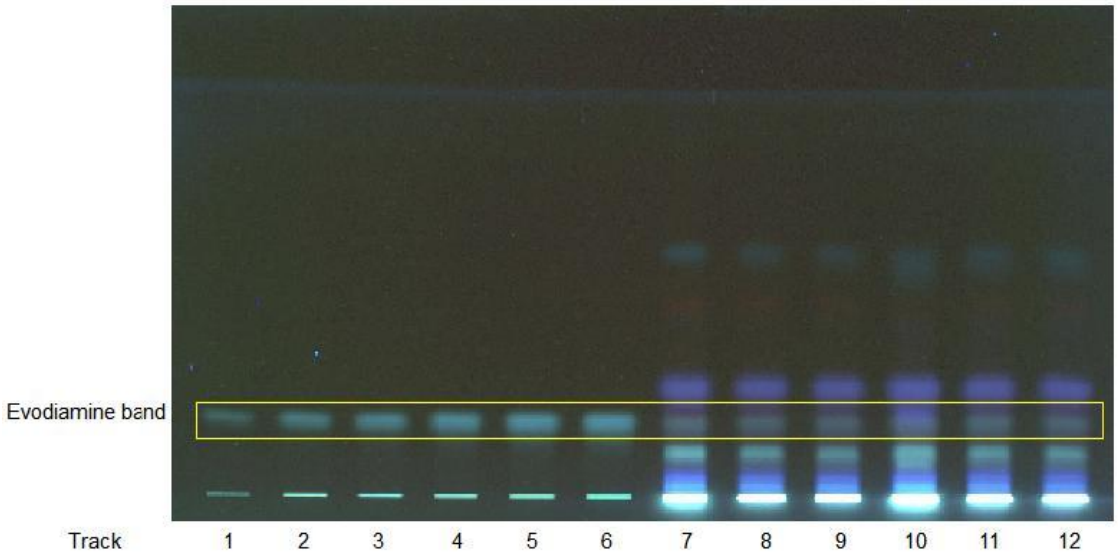
Appendix 2



HPLC chromatograms of *Euodia rutaecarpa* (Juss.) Benth. (A), *Euodia rutaecarpa*

(Juss.) Benth. *var*. *officinalis* (Dode) Huang batch one and two (B and C) at 254 nm with the same gradient mobile phase (Methanol : Acetonitrile : H_2O = 0 min 2% : 38% : 60%, 0 – 22 mins 5% : 38% : 57%, 22 – 35 mins 30% : 38% : 32%, 35 – 75 mins 45% : 38% : 17%) and stationary phase (C_{18} analytical column)

Appendix 3



Developed HPTLC fingerprint chromatogram of evodiamine at different concentrations (0.25, 0.5, 0.75, 1, 1.5, 2 mg/mL, tracks 1 to 6) and *Euodia rutaecarpa* (Juss.) Benth. *var*. *officinalis* (Dode) Huang extract at two concentrations (30 mg/mL, tracks 7 to 9 and 40 mg/mL, tracks 10 to 12) at 366 nm