The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Pharmacological Studies on *Radix et Rhizoma Rhei* (Da Huang) and its active component, Emodin

Li Wing Yan

A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Philosophy

August 2006

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____

Li Wing Yan

ABSTRACT

Cancer and hypertension are two of the most common diseases in the world. It was suggested that hypertension was associated with an increased risk of mortality from cancer. However, a better understanding of the cellular pathway and the relationship between hypertension and cancer may be helpful in elucidating more preventive and therapeutic drugs for the treatment of hypertension and cancer.

Radix et Rhizoma Rhei (Da Huang) has been widely used in traditional Chinese medicines as a laxative, antiphlogistic and hemostatic herb. With high-performance liquid chromatography (HPLC) analysis, it was found that emodin (1,3,8-trihydroxy-6-methylanthraquinone) was one of the most abundant components in the Da Huang species. Emodin has been reported to have diverse biological effects such as anti-inflammatory, antibacterial, anticancer and vasorelaxant effects. However, their anticancer effect and mechanism were involved in the human lung adenocarcinoma A549 and the human breast carcinoma MCF-7 cells, and their vasorelaxant effect on aorta has not been clearly demonstrated. The purpose of this study was to investigate the anticancer effect of the Da Huang water extract, the effect of emodin on growth inhibition, apoptosis induction, and on the expression of apoptotic-related genes in MCF-7 and A549 cells and vasorelaxant effect of emodin on rats' aorta.

For the anticancer study, MCF-7 and A549 cells were treated with Da Huang water extract or emodin at different dosages and time intervals. Growth inhibition was detected by MTS assay and colony formation assay, and cell

apoptosis by microscopic monitoring of cell morphology, DNA fragmentation detection and Comet assays. The expression of apoptotic-related genes was detected by real-time PCR. Results showed that both Da Huang water extract or emodin had growth inhibitory effect on A549 cell and MCF-7 cell. IC_{50} values of Da Huang water extract on A549 cell and MCF-7 cell were 693μg/ml and 583μg/ml, respectively; IC_{50} values of emodin on A549 cell and MCF-7 cell were 16.85μg/ml and 7.22μg/ml, respectively. And their growth inhibitory effect was dose and time-dependent. The cell shrinkage, DNA fragmentation and DNA strand breakage were observed in both the Da Huang water extract or the emodin treated A549 and MCF-7 cells. The expression of apoptotic-related genes was modulated after emodin treatment. The results indicated that emodin induced growth inhibition and apoptosis in MCF-7 and A549 cells through modulation on expression of the apoptotic-related genes.

For the antihypertensive study, the vasorelaxatant effect of emodin was demonstrated by adding emodin to phenylepherine pre-contracted rats' thoracic aorta. The nitric oxide (NO) related mechanism of emodin was investigated by adding L-NAME to exclude the involvement of NO in relaxation, the K^+ channels-controlled mechanism was investigated by adding the ATP-dependent K^+ channel blocker, glibenclamide, and the Ca^{2+}-activated K^+ channel blocker, tetraethyammonium. The results showed that emodin could induce relaxation on aorta partially through the K^+ channels-controlled mechanism but was independent of an endothelium- and NO-related mechanism. In addition, it was shown that emodin worked as an antagonist on Ca^{2+} ion channel as the channel blocker, verapamil to induce relaxation.

The emodin has the potential to be used as drugs for both cancer and hypertension treatment. However, it needs to be further studied in pharmaceutical, toxicological and clinical areas.

PUBLICATIONS ARISING FROM THE THESIS

LI, W.Y., CHAN, S.W., YU, H.F., GUO, D.J., ZHONG, X.Q. Study on the antioxidant and anticancer effects of water extracts of five selected Chinese medicinal herbs. Hong Kong Food and Science Technology Association 8th Anniversary Commemorative Publication. pp.34–39 (2005).

WONG, M.F., LI, W.Y., CHAN, S.W., YU, H.F. The vasorelaxative effect of active component of *Radix et Rhizoma Rhei* (Da Huang). Journal of the Hong Kong College of Cardiology. Vol.13, p.123 (2005).

LI, W.Y., CHAN, S.W., YU, H.F. The study of anticancer effect of Da Huang (*Radix et Rhizoma Rhei*) water extract and its active component, emodin. Proceedings of Hong Kong-Macau Postgraduate Sypmposium on Chinese Medicine, Hong Kong, Abstract, pp.150–151, 12 Aug 2005.

LI, W.Y., CHAN, S.W., YU, P.H.F. Study on the Antioxidant and Anticancer Effects of Water Extracts of Five Selected Chinese Medicinal Herbs. Proceedings of 20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, Kyoto, Japan, p. 595, Jun 18–23 Jun 2006.

LI, W.Y., CHAN, S.W., YU, P.H.F. Effects of emodin on the proliferation inhibition and apoptosis induction in MCF-7 and A549 cells and the

involvement of c-myc and bax gene. Proceedings of The Fukuoka Symposium: New Horizon of Natural Product and Biological Chemistries, Fukuoka, Japan, p. 35, 30–31 Jul 2006.

LI, W.Y., WONG, M.F., CHAN, S.W., YU, H.F. Study on the anticancer effect of *Radix et Rhizoma Rhei* (Da Huang) and its active ingredient, emodin, in MCF-7 and A549 cells. Proceedings of Hong Kong-Macau Postgraduate Sypmposium on Chinese Medicine, Hong Kong, pp.104–105, 17 Aug 2006.

LI, W.Y., GUO, D.J., CHUNG, M.K., LEUNG, T.Y., CHAN, S.W., YU, P.H.F. Effects of Emodin, an active ingredient from *Radix et Rhizoma Rhei* (Da Huang), on the proliferation inhibition and apoptosis induction. Hong Kong Food and Science Technology Association 9th Anniversary Commemorative Publication, pp.45–49 (2006).

LI, W.Y., CHAN, S.W., YU, P.H.F. Correlation between antioxidative power and anticancer activity in herbs from traditional Chinese medicine formulae with anticancer therapeutic effect. Pharmaceutical Biology. Vol. 45, no.7, Article in Press, Accepted (2007).

ACKNOWLEDGEMENTS

In these two years of study, I have been greatly profited from my research project. I have acquired many valuable, enjoyable and unforgettable experiences that enriched my life a lot. Several persons who gave me endless support and encouragement on my study are unforgettable.

I would like to give my deepest gratitude and cordial thanks to my Chief Supervisor Dr. Peter Yu and Dr. S.W. Chan, who continuously gave me both technical and spiritual support throughout my project. Their kind, patient and invaluable advice helped me a lot to overcome many difficulties and solve the problems derived in this challenging, but interesting project.

Thanks are given to the staff of State Key Laboratory of Chinese Medicine and Molecular Pharmocology, Shenzhen, especially to Mr. Guo De-Jian who provide the chemicals, equipment and techniques, and his contributions for the comet assay.

Thanks are also given to Miss Ingrid Wong, member of Dr. Peter Yu's and Dr. SW chan's team, for her valuable contribution for the rat experiments. Also, I would like to thank all the research team members of Dr. Peter Yu and Dr. S.W. Chan, especially Rachel, Emily, Connie and Vincent for giving endless support, providing friendly and kindly help, and offering a good, convenient and warm research environment.

I would like to give many thanks to all the staff and technicians, especially Miss Sarah Yeung, in the Department of Applied Biology and Chemical Technology for their kindly assistance on providing chemicals, equipments and experiment care so that my project could run and complete smoothly. I also would like to thank the Hong Kong Polytechnic University for a stipendship for my study.

Last but not least, thanks are never enough to my parents and friends, Miss KaKa Tang, Miss Panda Au and Mr. Jeremy Yiu who give me endless support, encouragement and solace throughout my difficult time of study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACTB	Actin, beta
ADH	Antidiuretic hormone
AIF	Apoptosis inducing factor
AII	Angiotensin II
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
BAD	BCL2-antagonist of cell death
BAK	BCL2-antagonist/killer
FASL	Fas ligand
BAX	BCL2-associated X protein
BCL-2	B-cell CLL/lymphoma 2
BID	BH3 interacting domain death agonist
BOK	BCL2-related ovarian killer
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium Chloride
cAMP	Cyclic adenosine monophosphate
CCBs	Calcium channel blockers
CCND1	Cyclin D1
CCND2	Cyclin D2
cGMP	Cyclic guanosine 3,5–monophosphate
CHM	Chinese herbal medicine

CK2	Casein kinase 2
C-MYC	v-myc myelocytomatosis viral oncogene homolog
CNP	C-type natriuretic peptide
CO_2	Carbon dioxide
CZE	Capillary zone electrophoresis
DAD	Diode array-multiple wavelength detector
DAG	Diacylglycerol
DISC	Death-inducing signal complex
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDHF	Endothelium-derived hyperpolarizing factor
ECE	Endothelin converting enzyme
EDRFs	Endothelium-derived relaxing factors
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic recticulum
ERK	Extracellular regulated protein kinase
ET-1	Endothelin
FADD	Fas-associated death domain protein
FAK	Focal adhesion kinase
FasL	Fas ligand
FBS	Fetal bovine serum
GA	Glibenclamide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H	Hour

HA	Hyaluronic acid
HPLC	High-performance liquid chromatography
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of capase-activated deoxyribonuclease
IP_3	Inositol triphosphate
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Ca^{2+} -dependent K^+ channels
KCl	Potassium chloride
L-NAME	N^G-nitro-L-arginine methyl ester
LOX	Lipoxygenase
MAP	*Misgurnus anguillicaudatus*
MCL1	Myeloid cell leukemia sequence 1
$MgSO_4$	Magnesium sulphate
MIN	Minute
MLC	Myosin light chains
MLCK	Myosin light chain kinase
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Na^+	Sodium ion
NaCl	Sodium Chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium hydrogen phosphate
NO	Nitric oxide
O_2	Oxygen
p53	Tumor suppressor p53

pCEC	Pressurized capillary electrochromatography
PCR	Polymerase Chain Reaction
PKB	Protein kinase B
PKC	Serine-threonine kinases
PKG	cGMP-dependent protein kinase
PLC	Phospholipase C
PLE	Pressurized liquid extraction
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol
PMS	Phenazine methosulfate
Rb	Retinoblastoma
RPS9	Ribosomal protein S9
SD	Sprague Dawley
sGC	Guanylate cyclase
S.E.M.	Standard errors of means
SR	Sarcoplasmic reticulum
TCM	Traditional Chinese medicines
TEA	Tetraethylammonium chloride
TPA	12-O-tetradecanoylphorbol-13-acetate
TNF	Tumor necrosis factor
VSCC	Voltage-sensitive Ca^{2+} channel

1 INTRODUCTION AND LITERATURE REVIEW

Cancer and hypertension are two leading diseases in the world and they share some common predisposing factors, such as excess alcohol consumption, ageing, obesity and smoking habits (Ray *et al*., 2004).

Angiogenesis is the growth of new blood vessels, and is an important natural process occurring in the body. Any abnormality in angiogenesis can lead to diseases such as cancer and hypertension. Abnormal angiogenesis in cancer provides new blood vessels for the growth and spread of tumors. The walls of blood vessels are formed by vascular endothelial cells, thus abnormal angiogenesis also triggers hypertension.

Chinese herbal medicine (CHM) has long been used in Asia to prevent imbalance of the body. Many herbal extracts such as *Anoectochilus formosanus* Hay., *Gynostemma pentaphyllum* Makino (Lin *et al*., 2000), *Padma lax*, (Hofbauer *et al*., 2006), hawthorn (Walker *et al*., 2006) and *Radix stephaniae* Tetrandrae (Yu *et al*., 2004) have shown to have beneficial action as complementary and alternative medicines for treatment of cancer and hypertension. Interestingly, all these herb extracts were found to be anticancer and antihyptensive due to the presence of its phytochemicals or active components.

Da Huang (Rhubarb, *Radix et Rhizoma Rhei*) is a Chinese herbal medicine that has been widely used as a laxative (Tsai *et al*., 2004), antiphlogistic (Moon *et al*.,

2006; Wu, 1985) and hemostatic (Wang *et al*., 1985) in the treatment of obstipation, gastrointestinal indigestion, diarrhea and jaundice.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is one of the major anthraquinones found in Da Huang with the highest and widest range of pharmacological effects such as anticancer and vasorelaxant effects. Previous study has demonstrated its anticancer effects (Li *et al*., 2005; Lee, 2001) and its relaxant effect on rat's aorta (Huang *et al*., 1991b). However, there is a lack of evidence on its anticancer effect and mechanisms involved on the human lung adenocarcinoma A549 and the human breast carcinoma MCF-7 cell lines, and, the mechanisms involved in its vasorelaxant effect on aorta of rats. If the mechanisms of anticancer and vasorelaxant effects of emodin can be found, a better understanding of common cell biology and the relationship between hypertension and malignancy can be elucidated that may be helpful in developing more preventive and therapeutic avenues to manage hypertension and cancer.

In this project, systematic approaches have been used to explain the function of Da Huang and its active component, emodin, for their beneficial action for prevention or treatment of human lung and breast cancers and also hypertension. HPLC method has also been developed for the identification and quantification of emodin in Da Huang species.

1.1 Apoptosis

Apoptosis refers to the programmed cell death. It is a physiological process that a cell actively degrades itself by destruction of vital cellular components or DNA through various molecular signaling pathways. This process allows the elimination of unwanted or dysfunctional cells from the body during development and other normal biological processes (Lodish *et al.*, 2000).

Apoptosis is different from necrosis which occurs when cells are exposed to extreme variance from physiological conditions causing damage of the plasma membrane. Necrosis begins with the impairment of the ability of cells to maintain homeostasis that results in cell swelling and eventually cell lysis. The break down of plasma membrane causes the release of cytoplasmic content into the extracellular fluid. Thus, necrotic cell death results in an intense inflammatory response. Apoptosis, however, occurs under normal physiological conditions and controlled process, the cytoplasmic content will be kept inside the cells before they are phagocytosed by macrophages. Thus, apoptosis will not cause inflammation (Raff, 1998).

Loss of cell integrity, aggregation of chromatin at nuclear membrane, shrinking of cytoplasm and condensation of nucleus, fragmentation of cells into smaller bodies, non-random mono and oligo-nucleosomal fragmentation of DNA, formation of apoptotic bodies and leaky mitochrondria can be observed when apoptosis occurs.

1.1.1 Apoptotic pathways

There are two major apoptotic pathways: mitochondrion (intrinsic) pathway and the death receptor (extrinsic) pathway.

Mitochondrion (intrinsic) pathway

The intrinsic pathway is triggered by cellular stress such as DNA damage, heat shock exposure of cells to radiation, viral infection, and by oxidative stress caused by free radicals. When there is cellular stress, pro-apoptotic proteins from the Bcl-2 family such as Bax and Bid in the cytoplasm bind to the outer membrane of mitochondria. Another pro-apoptotic protein, BAK, resides within mitochondria. These pro-apoptotic proteins promote the release of intra-membrane content and cytochrome c from the mitochondria (Adrain *et al*., 2002).

Cytochrome c is a key regulator in the intrinsic apoptosis because once it is released from the mitochondria the cell is irreversibly committed to death. Cytochrome c then associates with adenosine triphosphate (ATP), an energy molecule, and Apaf-1, an enzyme, to form a complex in the cytoplasm. This complex activates caspase-9, an initiator protein and form apoptosome by working together with activated caspase-9.

The apoptosome then activates caspase-3, an effector protein, which in turn causes caspase cascade and degradation (Harrison *et al*., 2003). Release of cytochrome c from the intra-membrane of mitochrondria also contains apoptosis inducing factor (AIF) to facilitate the fragmentation of DNA and Smac/Diablo

proteins to inhibit the inhibitor of apoptosis (IAP) (Hague and Paraskeva, 2004). Overall, the Bcl-2 family proteins act as a key regular of apoptosis. The anti-apoptotic members of Bcl-2 family proteins such as Bcl-XL, Bcl-w and Mcl-1 and pro-apoptotic members such as Bax, Bak, Bok, Bad and Bid regulate release of cytochrome c and caspases activation in apoptosis (Gross *et al*., 1999; Burlacu, 2003) (Figure 1).

Death receptor (extrinsic) pathway

The extrinsic pathway is triggered by extracellular ligands or even the removal of growth factors. Extrinsic apoptosis is initiated by the binding of death activators such as Fas ligand (FasL) and tumor necrosis factor (TNF) to their transmembrane death receptors. Binding of FasL to Fas receptor on the target cell causes the aggregation of multiple receptors from the surface of the target cell. This allows interaction between the Fas-associated death domain protein (FADD), a cytoplasmic adaptor protein, on the cytoplasmic side of the receptors and the procaspase-8 forming death-inducing signal complex (DISC). This in turn activates caspase-8, an initiator protein. The activation of caspase-8, similar to caspase-9 in the intrinsic pathway, activates caspase-3, an effector protein, to initiate degradation (Ethell and Buhler, 2003).

The active caspase-8 can also cleave BID protein to tBID that acts as a signal on the membrane of mitochondria. This facilitates the release of cytochrome c in the intrinsic pathway (Adrain *et al*., 2002) (Figure 1).

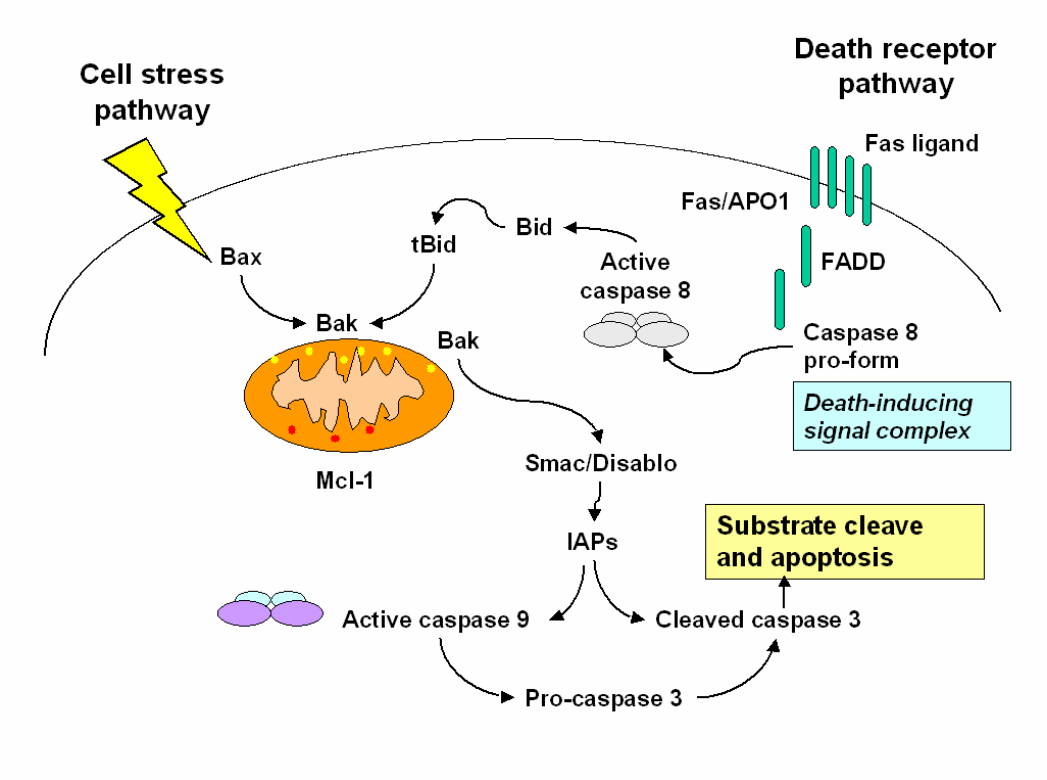


Figure 1. The mechanism of intrinsic (cell stress) and extrinsic (death receptor) apoptotic pathways (The Science Creative Quarterly, Issue 2, Apoptosis by Philip Yau. Available at http://www.scq.ubc.ca/?p=350. Found on August 2006).

p53-mediated apoptosis

p53 is a tumor suppressor which regulates apoptosis, the checkpoints in the cell cycle, DNA repair, senescence and genomic integrity. p53 regulates apoptosis in the intrinsic pathway by regulating Bax which contains p53 binding site in its promoter. Any DNA damage can activate p53 and causes upregulation of Bax to increase the release of cytochrome c and finally initiate apoptosis. Any upregulation of the anti-apoptotic gene such as Bcl-2 and Bcl-X can inhibit p53-mediated apoptosis. Besides, p53 activation also causes an increase in the surface FasL and the binding of FasL to their receptors, resulting in caspase activation and apoptosis (Bennett *et al.*, 1998) (Figure 2).

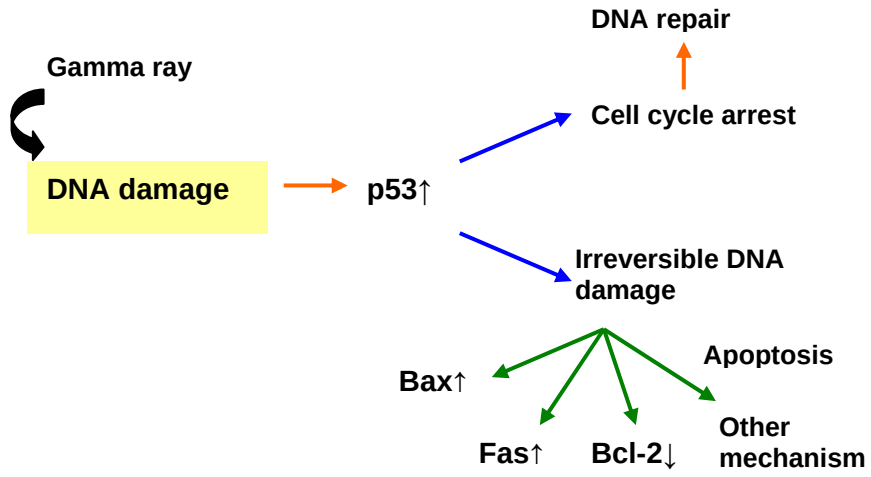


Figure 2. The mechanism of p53-mediated apoptosis (p53-mediated apoptosis research. Available at http://dragon.zoo.utoronto.ca/~B03T0601A/p53.html. Found on August 2006).

1.1.2 Apoptosis and Cancer

Cancer may result from an imbalance of the proliferation of wanted cells and the apoptosis of unwanted cells. Faithful replication and propagation of genetic material by cells to their progeny through cell division, as well as the delicate balance between cell proliferation and apoptotic cell death are two important factors in the maintenance of cell and tissue homeostasis. Any dysfunction in cell proliferation or the apoptotic pathway may result in cancer.

1.2 Cancer

Cancer is a term which refers to a disease that abnormal cells grow in an uncontrolled manner. These abnormal cells are called cancer cells. They are

different from the normal cells that normal cells only divide in most parts of the body to repair injuries or to replace some worn-out or drying cells in adults. Cancer cells grow and divide continuously and have the ability to invade nearby tissues and spread through the bloodstream to other parts of the body.

Usually, the development of cancer cells is caused by DNA damage which cannot be repaired. Cancer can be inherited if people inherited damaged DNA. Cancer can also be developed if people are exposed to carcinogens such as tar and benzene in the environment.

Cancers usually form tumors but some cancers like leukemia does not form tumor. These cancer cells involve blood and blood-forming organs and circulate through other tissues where they grow. It is called metastasis when the cancer cells travel to other parts of body and begin to grow to replace the normal tissue.

There are several different types of cancer including carcinoma, sarcoma, leukemia, lymphoma and multiple myeloma. Carcinoma is a cancer that begins in the skin or in tissues that line or cover the internal organs. Sarcoma is a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is a cancer that starts in the blood-forming tissue such as the bone marrow and causes a large number of abnormal blood cells to be produced and enter the bloodstream. Lymphoma and multiple myeloma are cancers that begin in the cells of the immune system. Although they start from different parts of the body, they all develop because of the out-of-control growth of the abnormal cells.

1.2.1 Breast cancer

Breast cancer is a disease that cancer cells form in the tissues of the breast. There are different types of breast cancer based on the structure of the breast. The breast is made up of lobes, ducts, blood vessels and lymph vessels. The most common type of breast cancer is ductal carcinoma that begins in the cells of ducts. Another type of breast cancer is lobular cancer that begins in the lobes or lobules. An uncommon type of breast cancer is inflammatory breast cancer that the breast looks red and swollen, and feels warm (Association of Cancer Online Resources, Inc. available at http://www.acor.org/index.html. Found on August 2006).

1.2.1.1 Risk Factors

Risk factor is the factor that increases the chance of getting a disease. There are many risk factors of breast cancer as followings (Moore *et al*., 1983; Okobia and Bunker, 2005; Muti, 2005):

1. Heritage such as country of origin, family, race, and any component of lifestyle;
2. Dietary factors such as drinking alcoholic beverages;
3. Ionizing radiation;
4. Hormonal factors such as taking hormones such as estrogen and progesterone;
5. Increasing age;

6. Reproductive characteristics such as breastfeeding, menstruating at an early age, marital state, and parity, older age at first birth or never having given birth;
7. A personal history of breast cancer or benign epithelial diseases of the breast;
8. Psychosomatic factors;
9. Metabolic factors such as glucose metabolism, hyperinsulinemic insulin resistance, and insulin-like growth factor bioavailability;
10. Iatrogenic factors;
11. Immunological factors;
12. And viral aspects of human breast cancer.

Hereditary breast cancer makes up approximately 5% to 10% of all breast cancer. Sometimes, breast cancer is caused by inherited gene mutations. However, it is more common in certain ethic groups.

1.2.1.2 Incidence

According to the report of incidence trend of female breast cancer in Hong Kong from 1993 to 2003 from Hong Kong Cancer Registry, Hospital Authority, the incidence of female breast cancer dramatically increased within ten years. The number of cases of female breast cancer per year was increased from about 1,200 in 1993 to 2,200 in 2003.

Besides, breast cancer is the first leading cancer and the third major causes of cancer death in female that there are about 60 persons suffering breast cancer per 100,000 persons and about 12 persons per 100,000 persons death due to breast

cancer according to the report of leading cancer sites in Hong Kong in 2003 from Hong Kong Cancer Registry, Hospital Authority. Breast cancer also is the second most common cancer occurred in both male and female with about 31 persons suffering breast cancer per 100,000 persons. Since there are about 6 millions people in Hong Kong, it is estimated that there are about 1860 people suffering breast cancer in 2003.

In the United States, breast cancer is the second leading cause of cancer death in female. The incidence of breast cancer is different in different racial and ethic groups. The incidence rate is higher in Whites than African Americans. However, the mortality rate is higher for African Americans than Whites (resources: Surveillance, Epidermiology, and End Results (SEER) Program and the National Center for Health Statistics).

1.2.2 Lung cancer

Lung cancer is a disease that cancer cells form in the tissue of the lung. There are two types of lung cancer: small cell lung cancer and non-small cell lung cancer. There are several different types of non-small cell lung cancer with different kinds of cancer cells. They are squamous cell carcinoma, adenocarcinoma, large cell carcinoma, adenosquamous carcinoma, pleomorphic, sarcomatoid, or sarcomatous carcinoma, carcinoid tumor, salivary gland carcinoma and unclassified carcinoma.

Squamous cell carcinoma is cancer that begins in the squamous cells; adenocarcinoma is cancer that begins in cells with glandular or secretary

properties; large cell carcinoma is cancer that begins in the large cells; adenosquamous carcinoma is cancer that begins in cells that look flattened and with glandular or secretory properties; pleomorphic, sarcomatoid, or sarcomatous carcinoma is a group of cancers that begins in cells that look abnormal; carcinoid tumor is a slow growing neuroendocrine tumor that cancer begins in cells with properties of releasing hormone in response to a signal from the nervous system; unclassified carcinoma is cancer that does not fit into any specific group (Association of Cancer Online Resources, Inc. Available at http://www.acor.org/index.html. Found on August 2006).

1.2.2.1 Risk Factors

There are many risk factors of lung cancer (Lam *et al*., 2004; Chan *et al*., 2003; Zemla *et al*., 1988; Koo *et al*., 1985; Higginson and Jensen, 1977):

1. Tobacco smoking;
2. Indoor exposure to environmental tobacco smoke;
3. Cooking oil vapor;
4. Coal burning;
5. Radon;
6. Outdoor air pollution;
7. Occupational exposure to asbestos and other carcinogens
8. Dietary factors such as consumption of preserved food and fatty food
9. Certain infections such as Mycobacterium tuberculosis, human papilloma virus and Microsporum canis;
10. Exposure to insecticide/pesticide/herbicide;
11. A family history of lung cancer

1.2.2.2 Incidence

According to the report of incidence trend of male lung cancer in Hong Kong from 1993 to 2003 from Hong Kong Cancer Registry, Hospital Authority, the incidence of lung cancer in male is about 1.5 fold higher than that of female. The number of cases of lung cancer in female per year i steadily decreased from 1,400 in 1993 to 1,200 in 2003. However, the number of cases of lung cancer in male in 2003 is nearly the same of that in 1993.

Besides, lung cancer is the most common cancer in male in Hong Kong in 1993. There are 83 males per 100,000 males suffering lung cancer. Lung cancer is also the first major cause of death in both sexes with 71 males death per 100,000 males and about 31 female deaths per 100,000 female according to the report of leading cancer sites in Hong Kong in 2003 from Hong Kong Cancer Registry, Hospital Authority. Since there are about 6 millions people in Hong Kong, it is estimated that there are about 3480 people suffering lung cancer in 2003.

In the United States, lung cancer is the second most common cancer and the first major cause of cancer death in both sexes. From 1982 to 2002, the mortality rate of lung cancer in male has dropped but this is not observed in female, reflecting that smoking has become more common in female. By comparing different race and ethic groups, the incidence and mortality rates of lung cancer are the highest among African American males (resources: Surveillance, Epidermiology, and End Results (SEER) Program and the National Center for Health Statistics).

1.2.3 Treatment of Cancer

Surgery, radiation therapy and chemotherapy are commonly used for the treatment of cancer

Surgery

Surgery is used to remove tumor from the body in most cancer patients. For treatment of breast cancer, there are two main types of surgery. They are breast-conserving surgery and total mastectomy. Breast-conserving surgery is an operation that only removes the cancer from the breast. Total mastectomy is an operation that removes the whole breast containing cancer. Some lymph nodes under arm are removed for biopsy after surgery.

For treatment of lung cancer, there are three types of surgery. They are wedge resection, lobectomy and pneumonectomy. Wedge resection is a surgery that removes a tumor and a small amount of normal tissue around the tumor. Lobectomy is a surgery that removes the whole section of lung. Pneunonectomy is a surgery that removes one whole lung.

Radiation therapy

Radiation therapy is a cancer treatment that uses high-energy x-rays or other types of radiation such as particle radiation to kill the cancer cells. There are two types of radiation therapy, external radiation therapy and internal radiation therapy. In external radiation therapy, radiation is sent toward the cancer but outside the body. In internal radiation therapy, radiation is sent directly into or near the cancer.

Radiation therapy depends on the type of cancer being treated. Radiosurgery may be used if the tumor in patients cannot be removed by surgery. It delivers radiation which directly kill the cancer cells or tumor with little damage to the normal healthy cells or tissues (Association of Cancer Online Resources, Inc. Available at http://www.acor.org/index.html. Found on August 2006).

Chemotherapy

Chemotherapy is a cancer treatment that uses drugs to stop the cancer growth, either by stopping the cancer cells from dividing or by killing the cancer cells. Chemotherapy is better than surgery and radiation (systemic treatments) because it can reach the cancer cells that may have spread to other parts of the body (National Cancer Institute, U.S. National Institutes of Health. Cancer treatment. Available at http://www.cancer.gov. Found on August 2006.).

Other therapies

Hormone therapy is a cancer treatment commonly used to treat breast cancer (Petit, 1972). It involves the removal of hormone or blockage of their action and inhibition of the growth of cancer cells. Tamoxifen is often used to treat early stage of breast cancer and metastatic breast cancer (Nakagawa *et al*., 1992; Rubens, 1995).

Laser therapy is a cancer treatment that uses a laser beam to kill cancer cells. It is commonly used in the treatment of lung metastase (Knappe and Mols, 2004; Inzeo and Haughney, 2004). Chemoprevention is a cancer treatment that uses a

drug and certain type of laser light to kill cancer cells. It is also commonly used in the treatment of lung cancer (Shaipanich *et al*., 2006).

1.2.4 Complementary and alternative medication

Chinese herbal medicine

Chinese herbal medicine (CHM) is a major aspect of traditional Chinese medicines (TCM) that was established by 20BC and has long been used in China and other Asian countries. CHM focuses on nourishing the body, restoring the balance of energy and spirit to maintain health rather than treating a particular disease or medical condition. Some individual herbs or extracts used in disease prevention and treatment are being used as alternatives to Western cancer therapies. CHM is different from Western medicines by treating patients based on the patterns of their symptoms rather than the underlying causes. Thus, CHM usually uses different combinations of herbs to restore the balance of the body, and to prevent and treat hormone disturbances, infections, breathing disorders, and a vast number of other ailments and diseases.

CHM is usually used in combination with conventional cancer treatment such as radiation therapy and chemotherapy. There are many reports that Chinese herbs or their extracts had anticancer activity, could help ease the side effects of conventional cancer therapies, control pain and improve the quality of life.

Antrodia comphorata, a traditional Chinese medicine used in Taiwan, *Brucea javanica* extract, has shown to have anticancer effect on human breast cancer cells (Yang *et al*., 2006; Lau *et al*., 2005).

The use of CHM was beneficial in the majority of patients with breast cancer (Cui *et al*., 2004) and promoted immunological function in the post-treatment of breast cancer patients (Wong *et al*., 2005). Besides, *Scutellaria barbata*, a traditional Chinese herbal medicine native to southern China, the root of *Bupleurum scorzonerifolium*, and *Glycorrhiza glabra, Olenandria diffusa* had anticancer effect on human lung cancer (Yin *et al*., 2004; Cheng *et al*., 2005; Sadava *et al*., 2002).

CHM treatment was demonstrated to have contribution to the complete regression of lung carcinoma (Liang *et al*., 2004). Improvement of lung cancer was observed by combined cancer treatment with CHM. Combination of chemotherapy, radiotherapy and adjuvants such as traditional Chinese medicine and immunotherapy showed improved effect on human lung cancer (Cha *et al*., 1997). The therapeutic effect of bronchial arterial infusion chemotherapy on human lung cancer is enhanced by combining it with CHM (Liu *et al*., 2001). The quality of life in patients with advanced non-small cell lung cancer was improved and their survival rate was increased by treatment combining Astragalus and chemotherapy (Zou and Liu, 2003) and treatment combining chemotherapy, radiotherapy and TCM (Han *et al*., 2003). CHM can be a complementary and alternative medicine for cancer treatment.

1.3 Hypertension

Blood pressure is the pressure of blood in blood vessels that is measured in millimeters of mercury (mmHg). There are systolic pressure and diastolic pressure. Systolic pressure is the blood pressure in the arteries when the heart contracts. Diastolic pressure is the blood pressure in the arteries when the heart rests between each heartbeat. The normal systolic and diastolic pressures are below 120 and 80 mmHg, respectively. The normal blood pressure is represented as below 120/80mmHg. Hypertension means high blood pressure or tension that sustained blood pressure is chronically elevated (140/90mmHg or above), measured on both arms is generally regarded as diagnostic. High blood pressure can be just high systolic pressure, distolic pressure or both.

There are two forms of hypertension, primary (essential) hypertension and secondary hypertension. Nearly 95% of hypertension refers to primary hypertension. Secondary hypertension is less common whiand it only accounts for 5% of hypertension. Primary hypertension is caused by the combined effects of several factors while secondary hypertension is caused by a specific abnormality in one of the organs or systems of the body.

1.3.1 Risk Ractors

The development of primary hypertension is associated with the following factors (Williams and Hopkins, 1979; Luma and Spiotta, 2006; Henry, 1988; Schmieder *et al*., 1986; Soubrier *et al*., 1990; Stamler, 1990; Newcomb, 1992):

1. High salt intake such as taking salt exceeding 5.8 grams daily;

2. High levels of saturated fat in the diet;
3. Stress;
4. Tobacco smoking that increases the risk of vascular complications;
5. Overtake of caffeine such as drinking 5 cups of coffee daily that causes a mild increase in blood pressure in elderly people who already had hypertension;
6. Sedentary lifestyle;
7. Low birth weight;
8. Hereditary (genetic) susceptibility such as the genetic factors that affect the renin-angiotensin-aldosterone system that helps to regulate blood pressure by controlling salt balance and the tone (state of elasticity of the arteries);
9. Obesity that losing a kilogram of mass generally reduces blood pressure by 2mmHg;
10. Alcoholism such as drinking alcohol excessively (over two drinks per day);
11. Diabetes mellitus that about 3 in 10 people with Type 1 diabetes and more than half of people with Type 2 diabetes eventually develop high blood pressure;
12. Renal insufficiency;
13. Risk age that making blood vessels stiffer and reduced elasicity, results in smaller cross-sectional area in systole and causes a raised mean arterial blood pressure;
14. African American background;
15. From the Indian sub-continent and

16. Occupational, aircraft and roadway noise exposure factors

In fact, a majority of patients with primary hypertension have in common an increased resistance or lack of elasticity in peripheral arteries or arterioles. Increased the peripheral arteriolar stiffness is present in those individuals whose primary hypertension is associated with obesity, genetic factors, lack of exercise, aging and overuse of salt. Besides, genetic factors attribute for nearly 30% of primary hypertension.

Secondary hypertension is caused by a specific disorder of a particular organ or blood vessel such as kidney, adrenal gland or aortic artery. Disease of kidneys, tumors of adrenal glands, coarctation of the aorta and metabolic syndrome and obesity can cause secondary hypertension.

1.3.2 Incidence

Hypertension is commonly found in the population of Hong Kong. In 2004, the community survey of the Hong Kong Hospital Authority shows that there are 24% of the people aged 40 or above had hypertension. The incidence of hypertension related disease, stoke, had dramatically increased from 21,900 cases in 1997 to 28,600 cases in 2004. A total cost of over HK$0.9 billion and over 300,000 bed days were incurred for serious diseases such as stroke and heart disease caused by hypertension during 2002/2003.

1.3.3 Complications

According to the World Health Organziation, there were 50% of people who did not know they had hypertension. 50% of hypertension patients did not seek any

treatment for their illness and 50% of hypertension patients were under treatment but did not comply with the doctor's instruction. Their hypertension is poorly controlled or untreated which can lead to serious medical problems:

1.3.3.1 Heart Attack

High blood pressure can cause narrowing of coronaray arteries that block the dilverary of oxygen to the heart. The heart muscle cells will died if there is no oxygen and nutrient supply, a heart attack can occur.

1.3.3.2 Atherosclerosis

Hypertension is an important risk factor for atherosclerosis (Spence, 1989). Atherosclersis is the hardening of the arteries. High blood pressure can make the walls of artery thicken and harden, thus, the inside of the blood vessel becomes narrow. Because of the narrowed blood vessels, fats and cholesterol are easier to build up on the wall of the damaged arteries which in turn makes the arteries even narrower. Blood clots can get trapped in the narrowed arteries and results in the blocking of the flow of blood. This keeps blood from flowing through the body effectively and can lead to stroke and heart attack.

1.3.3.3 Enlarged heart

Uncontrolled hypertension can lead to an increased size of heart because the heart grows bigger to compensate for the extra work due to the high blood pressure. Evetually, the heart weakens.

1.3.3.4 Stroke

High blood pressure causes the narrowing of the blood vessels which supplying oxygen to brain. Blood clots form and get trapped in the narrowed blood vessel of the brain and causes bloakage of the supply of oxygen to the brain. This results in stroke. Besides, high blood pressure can cause the burst of arteries in the brain. Such bleeding produces different kinds of stroke.

1.3.3.5 Kidney damage

Uncontrolled hypertension is a risk factor for kidney damage and renal disease (Whelton and Klag, 1989). Sustained high blood pressure can cause the narrowing of blood vessels supplying the kidneys and less waste can be filtered from the blood by the kidneys. Besides, hypertension can damage the kidneys directly and may lead to kidney failure.

1.3.4 Vascular elasticity

1.3.4.1 Vasodilation

Endothelium-dependent relaxation

Endothelium-derived relaxing factors (EDRFs) include prostacyclin (PGI_2), endothelium-derived hyperpolarizing factor (EDHF) and nitric oxide (NO) (Ignarro *et al*., 1987). Among them, NO plays a key role in the endothelium-dependent vascular relaxation.

NO is mainly produced from endothelial nitric oxide synthase (eNOS) in vessels. eNOs is an enzyme constitutively present in the endothelial cells and become active by interaction with Ca^{2+}/calmodulin complex when there are physiological stimuli such as vasoactive agents and arterial shear forces. For example, the presence of vasoactive agent initiates the opening of the Ca^{2+} -dependent K^+ channels (K_{Ca}) and causes the efflux of K^+ from the endothelial cells. This causes the hyperpolarization of endothelial cells and the influx of the transmembrane Ca^{2+} into cells that triggers the generation of NO from L-arginine.

Activation of eNOs results in the production and diffusion of NO from the endothelial cells to the overlying vascular smooth muscle. This activates guanylate cyclase (sGC) to convert guanosine triphosphate into the intracellular second messenger guanosine 3',5'-cyclic monophosphate (cGMP) by stimulation of the cGMP-dependent protein kinase (PKG). This results in vascular relaxation (Rapoport and Murad, 1983; DeFeudis, 1985; Yamashita *et al*., 2000; Hinsbergh, 2001) (Figure 3).

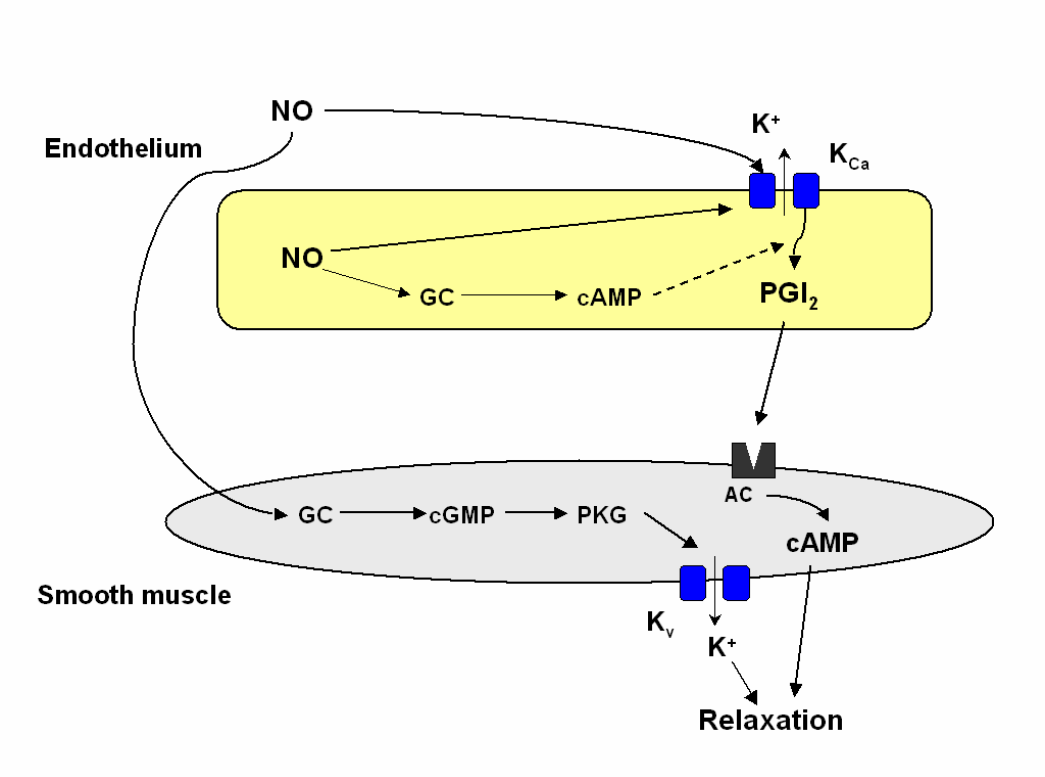


Figure 3. Model showing mechanism of NO-induced vasorelaxation involving interactions between intracellular second messenger cGMP, voltage-dependent potassium (K_v) channels and calcium-dependent potassium (K_{Ca}) channels. AC, GC, and PKG refer to adenylate cyclase, guanylate cyclase, and cGMP-dependent protein kinase, respectively (Hardy *et al*., 1998).

Endothelium-independent relaxation

Endothelium independent relaxation is mediated by vasorelaxants that directly act on the vascular smooth muscle rather than the endothelial cells, causing an increase in the intracellular cAMP/cGMP levels or the opening of the membrane calcium-dependent potassium ion channels (Jain *et al*., 1999).

One example of relaxants causing endothelium independent relaxation is C-type natriuretic peptide (CNP). CNP mediates vasorelaxation through hyperpolarization of the vessel wall independent of the NO and prostaglandin system (Honing *et al*., 2001).

1.3.4.2 Vasoconstriction

Vasoconstriction is triggered by Ca^{2+}/calmodulin-dependent phosphorylation of the regulatory myosin light chain (Valle-Rodriguez *et al*., 2006). Ca^{2+} influx through ion channels and an increase in free intracellular Ca^{2+} from sarcoplasmic reticulum (SR) (Moosmang *et al*., 2003) binds to calmodulin forming Ca^{2+}-calmodulin complex.

This complex activates myosin light chain kinase (MLCK) and results in the phosphorylation of myosin light chains (MLC) in the presence of ATP. Ca^{2+}/calmodulin-dependent phosphorylation of the regulatory myosin light chain causes the formation of a cross-bridge between the myosin heads and the actin filaments. Finally, vasoconstriction occurs.

Vasoconstriction is triggered by different signal transduction pathways including the Gs-protein coupled pathway, the phosphatidylinositol pathway and the nitric oxide-cGMP pathway. All of them involve an increase in intracellular Ca^{2+}.

Gs-protein coupled pathway

The Gs-protein coupled pathway involves Gs protein-coupled receptors (Demoliou-Mason, 1998), which when stimulated, activates the formation of cyclic adenosine monophosphate (cAMP). Increase of cAMP by β_2-adrenoceptor agonist such as epinephrine inhibits MLC phosphorylation that decreases interactions between actin and myosin. This results in vasodilation.

Phosphatidylinositol pathway

Vasoactive agents that acting on α_1-adrenoceptors, angiotensin II (AII) and ET_A receptors can initiate vasoconstriction. Activation of these receptors stimulates phospholipase C (PL-C) to convert phosphatidylinositol (PIP_2) to inositol triphosphate (IP_3). IP_3 in turn stimulates SR to release Ca2+ that causes the formation of diacylglycerol (DAG) and the activation of protein kinase C (PKC). This finally causes vasoconstriction through protein phosphorylation.

Nitric oxide-cGMP pathway

An increase of NO activates guanylyl cyclase to increase the formation of cGMP. An increase in cGMP activates a cGMP-dependent protein kinase that inhibits Ca^{2+} influx, activates K^+ channels and decreases the formation of IP_3. This results in vasodilation.

Endothelin

Endothelin (ET-1) is one of the most powerful vasoconstrictor substances synthesized by the vascular endothelium. Endothelin induces vasoconstriction by triggering the G-protein coupled pathway, phosphatidylinositol pathway and nitric oxide-cGMP pathway. It works by activating the ET_A and ET_B receptors on the vascular smooth muscle cell and endothelial cell membrane.

To initiate vasoconstriction, ET-1 is formed by cleavage of an endothelin precursor (big ET-1) by endothelin converting enzyme (ECE) on the endothelial cell membrane. ET-1 then binds and activates the ET_A receptors on adjacent

vascular smooth muscle cells. The ET_A receptor is coupled to a G-protein which is linked to phospholipase-C and the formation of inositol triphosphate ($IP_{3)}$. The IP_3 then stimulates the sarcoplasmic reticulum (SR) to release calcium. It causes calcium mobilization and the contraction of vascular smooth muscle.

Angiotensin II (AII), antidiuretic hormone (ADH), thrombin, cytokines, reactive oxygen species, and shearing forces acting on the vascular endothelium stimulate the release of ET-1 and hence, the vasoconstriction. Vasodilators such as nitric oxide, prostacyclin and atrial natriuretic peptide inhibit vasoconstriction by inhibiting the release of ET-1.

Besides, ET-1 binds to the ET_B receptors on the vascular endothelium membrane that activates the production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS or NOS). Nitric oxide, a vasodilator, can then modulate the vasoconstriction by the mechanism shown in endothelial dependent vascular relaxation (Klabunde, 2006).

1.3.5 Treatment of hypertension

1.3.5.1 Lifestyle treatments

Lifestyle modifications refer to changes in habits, diet and exercise that can lower the blood pressure and improve the response of patients to blood pressure medications. There are some lifestyle modifications to lower the blood pressure:

1. Lower the intake of salt

The consumption of dietary salt should be less than 6 grams per day for the general population and less than 4 grams for people with hypertension. To reduce the consumption of salt per day, herbs and spices can be used to flavor food instead of salt and limit the amount of salt used in cooking.

2. Modulations of drinking alcohol

The connection between hypertension and alcohol is dose-related. Thus, too much alcohol can be harmful. Men should drink no more than 21 units of alcohol per week and women should drink no more than 14 units of alcohol per week. Drinking 1 to 2 units of alcohol per day may help to protect people from heart disease.

3. Regular physical activity

A regular physical program can help to lower the blood pressure in the long term as well as bringing health benefits. People would be better to do some physical activities such as swimming and cycling on five or more days of the week, each time for at least 30 minutes. It can reduce systolic blood pressure by as much as 5-10mmHg.

4. Weight loss

Weight loss can help reverse problems related to obesity as well as lowering the blood pressure and bringing other health benefits. Blood pressure can be decreased about 2.5/1.5mmHg per each kilogram of weight loss.

5. Limit the intake of caffeine and smoking

   The combination of smoking and the intake of caffeine in hypertension patients may result in an increase in blood pressure. Thus, limiting the amount of caffeine intake and smoking might be beneficial to patients with hypertension.

1.3.5.2 Medications

There are many classes of medications for treating hypertension. They are antihypertensives that act by lowering the blood pressure. Ideally, each drug may reduce the systolic blood pressure by 5 to 10mmHg. Usually, mutiple drugs are necessary to achieve blood pressure control. The commonly used antihypertensives include:

Beta-blockers (Beta-Adrenergic Blocking Drugs)

Beta-adenergic blocking drugs are commonly called beta-blockers. Beta-blockers work by blocking the action of noradrenaline at the beta-receptors in arteries and the heart muscle. Since noradrenaline is a chemical that transmit signals between nerves and muscles, or between one set of nerves and another, beta-blockers can slow down the nerve impulses that travel to the heart by blocking the beta-receptors. It causes the widening of arteries, slowing the heart rate and decreasing the force of heart contraction. This finally results in a fall in blood pressure and reduced work by heart. Examples of beta-blockers include metoprolol, labetalol and atenolol (Prichard, 1966; Seedat, 1975).

Angiotensin converting enzyme inhibitors (ACE Inhibitors)

ACE inhibitors act on the rennin-angiotensin hormonal system that helps to regulate blood pressure. ACE inhibitors work by inhibiting an enzyme in order to inhibiting the conversion of angiotensin I to angiotensin II, which angiotensin II is responsible for narrowing blood vessels and raising the blood pressure. Thus, blood pressure can be lowered. Examples of ACE inhibitors include lisinopril, quinapril and fosinopril (Hilaert and Van den Bossche, 2000; Hillaert *et al*., 2001).

Calcium channel blockers (CCBs)

Calcium channel blockers work by inhibiting the movement of calcium into the muscle cells of heart and arteries. Calcium is vital for these muscles to contract. Calcium channel blockers inhibit the L-type voltage-dependent calcium ion channels that cause a decrease in the intracellular calcium ions in muscles of heart and arteries. The lowered blood pressure reduces the contraction of heart muscle, lowers the amount of electrical impulses generated within the heart to regulate the heartbeat and widens the blood vessels. Examples of calcium channel blockers include amlodipine and verapamil (Katzung *et al*., 2001; Hardman *et al*., 1995).

Diuretics

Diuretics work by preventing Na^+ (salt) entry into the tubular cells of kidneys. It can increase removal of salt from the body. Thus, it can reduce blood pressure by decreasing the blood volume through increasing the volume of urine. Examples

of diuretics include chlortalidone and hydrochlorothiazide (Katzung *et al*., 2001; Hardman *et al*., 1995).

Alpha-blockers

Alpha-blockers work by blocking the alpha-receptors in the smooth muscle of the peripheral arteries throughout the tissues of the body. The alpha-receptors serve to constrict the peripheral arteries. Thus, alpha-blockers can lower the blood pressure by dilating the peripheral arteries. Examples of alpha-blockers include terazosin and prazosin (Reid and Vincent, 1986; Akduman and Crawford, 2001).

Direct vasodilators

Vasodilators are commonly used to lower blood pressure by decreasing the peripheral vascular resistance through relaxing smooth muscle of arterioles. Vasodilators work best in combination with other antihypertensive drugs such as beta-blockers and diuretics that oppose the compensatory responses from the baroreceptors and rennin angiotensin system (Sutton and Bagby, 1992).

1.3.6 Complementary and alternative medications

Chinese herbal medicine

CHM is used to prevent imbalance of the body and the herb, its extract and its active component are commonly used as complementary and alternative medicines for treatment of hypertension.

Lime blossom has blong been used in China for treating hypertension which is associated with arteriosclerosis and anxiety. Hawthorn has been used as a heart tonic for centuries. Viscum album is believed to be a regulator of blood pressure, exerting a healing effect in both hypertension and hypotension. It has often been combined with hawthorn in treating hypertension in Europe.

Chinese studies suggest that *Pueraria lobata* can help normalizing blood pressure. 17 people out of 52 people experienced a marked decline in their blood pressure after drinking tea containing *Pueraria lobata*. An isoflavonoid glycoside from *Puraria lobata* was found to have hypotensive effects and showed excellent clinical results in the treatment of hypertension (Qicheng, 1980). A traditional Chinese medicine, ju-ling-tang has been found to have a beneficial anti-hypertensive effect on renal hypertension (Shih *et al*., 2005). Korean red ginseng could improve the vascular endothelial dysfunction in patients with hypertension possibly through increasing the synthesis of nitric oxide, an endothelial relaxing factor (Sung *et al*., 2000).

Mixture of CHM also showed anti-hypertensive effect and improve the symptoms of hypertension. The Huanglian Fire-Purging Mixture could improve clinical symptoms, the left ventricular diastolic function and myocardial ischemia, correct dyslipoproteinemia and dysglycemia, and reduce blood viscosity. And it was safe and with no obvious adverse reactions (Li, 2005).

1.4 *Radix et Rhizoma Rhei* (Da Huang)

Da Huang (Rhubarb, *Radix et Rhizoma Rhei*) (Figure 4) is the dried root and rhizome derived from Rheum species, and has been widely used as a traditional Chinese medicine since ancient time. Rheum species are distributed in western and northwestern China and some of them are found beyond China and most of them are known to possess drug value (Xiao *et al*., 1984) Da Huang has been recorded in the Shen Nong Ben Chao Jin (神農本草經), a traditional medicinal book.

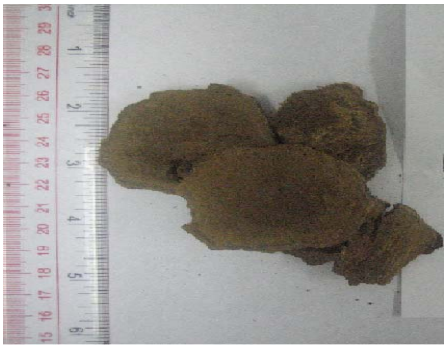
Figure 4. The Figure of *Radix et Rhizoma Rhei* (Xiao *et al*., 1984).

1.4.1 Distribution

Rheum species belong to the family Polygonacea. *Rheum palmatum* Linn (掌葉大黃), *Rheum tanguticum* Maximowicz (甘肅大黃) and *Rheum officinale* Baill.

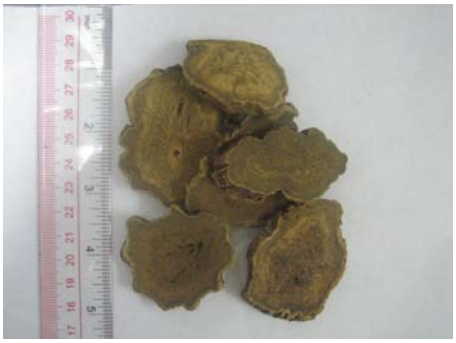
(藥用大黃) are the three main species of Rheum (Figure 5). They are widely distributed in China. *Rheum palmatum* Linn (掌葉大黃) can be found in Sichuan, Gansu, Qinghai, Xikang and other provinces. *Rheum tanguticum* Maximowicz (甘肅大黃) can be found in Sichuan, Gansu, Qinghai, and other provinces. *Rheum officinale* Baill. (藥用大黃) can be found in Hupei, Sichuan, Guizhou, Yunnan and other provinces.

D1: *Rheum tanguticum* Maximowicz from Sichuan (四川野大黃)



D2: *Rheum palmatum* Linn from Gansu (甘肅掌葉大黃)

D3: *Rheum tanguticum* Maximowicz from Gansu (甘肅野大黃)



D4: *Rheum officinale* Baill. from Sichuan (四川藥用大黃)

Figure 5. Photographs of different species of Da Huang from different origins.

1.4.2 Description

Da Huang could be subcylindrical, conical, ovoid or irregular shaped pieces with a length of 3–17 cm and 3–10 cm in diameter. It is externally yellowish-brown

when peeled and mostly with a hole through which a string is passed and with coarse wrinkles. Its texture is compact and fracture is reddish-brown or yellowish-brown. The pith of its rhizome is broad with star spots irregularly scattered or arranged in a ring. The wood of its root is well developed with lined radially, cambium ring without star spots. Da Huang has an aromatic odour and bitter taste. It is slightly stringent, sticky and gritty when chewed.

1.4.3 Chemical components of Da Huang

The chemical components of Da Huang include chrysophanol, physcion, emodin, aloe-emodin, rhein, sennoside, heterodianthrone, chrysophanein, catechin, rhatannin and 2-O-Cinnamoyl-1, 6-di-O-galloyl-β-D-glucoside. Among them, chrysophanol, physcion, emodin, aloe-emodin, and rhein are hydroxyanthraquinones (Ding *et al*., 2003).

1.4.4 Medicinal properties

Da Huang has a bitter taste and a cold property and exerts its effects through entering the heart, large intestine, liver and stomach channels. Its bitter and cold properties help clearing heat from the body. It also has the effects of loosening the bowels, and releasing stagnation in gastro-intestinal tract, fire of excess type and toxic heat. Thus, it is commonly used for constipation due to the accumulation of heat of excess type, and gastro-intestinal fecal impaction due to heat. It also helps removing heat from the blood, clearing toxins, promoting blood circulation to relieve blood stasis (Beijing Xueyuan Press. Available at http://www.tcmtreatment.com/herbs/0-dahuang.htm. Found on July 2005).

1.4.5 Pharmacological effects

Da Huang has been widely used as a laxative (Tsai *et al*., 2004), antiphlogistic (Moon *et al*., 2006; Wu, 1985) and hemostatic (Wang *et al*., 1985) in the treatment of obstipation, gastrointestinal indigestion, diarrhea, and jaundice. Besides, Da Huang has been reported to have vasodilatory (Moon *et al*., 2006) and anticancer effects (Cao *et al*., 2005). However, there are few references on studying the anticancer effect of Da Huang. The pharmacological effects of Da Huang may be due to its active constituent. Emodin is one of the active constituents found in Da Huang and its pharmacological effects have been widely studied.

1.5 Emodin

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a biologically active, naturally occurred anthraquinone derivative. It is an orange crystalline compound and active constituent of many widely used Chinese herbs such as species of *Rheum*. It has the chemical formula $C_{15}H_{10}O_5$ and a molecular weight of 270.24 (Figure 6). Emodin is an inhibitor of NF-κB activation and adhesion molecule expression (Kumar *et al*., 1998), and casein kinase 2 (CK2) (Yim *et al*., 1999).

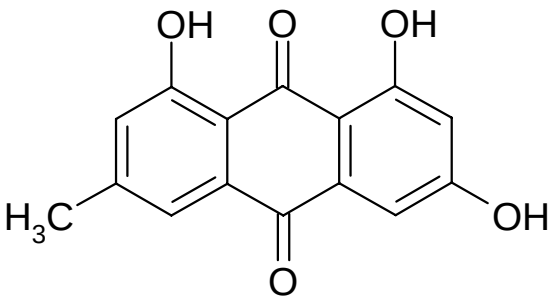


Figure 6. The chemical structure of emodin.

1.5.1 Pharmacological effects

In recent years, emodin has been reported to possess diverse biological effects including anticancer, anti-bacterial, diuretic, immunosuppressive, anti-inflammatory and vasorelaxant activities.

Anticancer activity

Emodin has been reported to inhibit the proliferation of several human cancer cell lines. Emodin showed growth inhibition on human lung adenocarcinoma LACC cells (Li *et al*., 2005) and human lung squamous cell carcinoma CH2 through Bax and Fas death pathway (Lee, 2001).

Emodin also mediated apoptosis on various human cancer cell lines. It induced apoptosis in human hepatoma cell lines including HepG2 and C3A through the activation of p53, p21, Fas/APO-1 and caspase-3 (Huang *et al*., 2004a), human promyeloleukemic HL-60 cells through the activation of caspase 3 casade, but is independent of the reactive oxygen species production (H_2O_2) (Chen *et al*., 2002) and human cervical cancer Bu 25TK cells through the activation of caspases-3, -9 and cleavage of poly (ADP-ribose) polymerase (Srinivas *et al*., 2003).

Emodin inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumor invasion of human cancer HSC5 and MDA-MB-231 cells by suppressing MMP-9 expression through inhibiting the AP-1 and NF-kappaB signaling pathways (Huang *et al*., 2004). Although there are a number of studies on the anticancer effect of emodin on human cancer cell lines, few are on the anticancer effect and its mechanism on human breast MCF-7 and human lung A549 cell lines.

Antibacterial Activity

Emodin has an antibacterial activity on some Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, but no bacteriostatic effect on gram-negative bacteria such as *Klebsiella pneumoniae* and *Escherichia coli* even at a high concentrations (Chukwujekwu *et al*., 2006; Basu *et al*., 2005).

Diuretic activity

Emodin was found to have the ability to enhance the function of the small intestinal peristalsis of mice through raising the secretion of motilin, lowering the content of somatostatin and inhibiting the Na^+-K^+-ATPase activity of the small intestinal mucosae (Zhang *et al*., 2005).

Immunosuppressive activity

Emodin was believed to function as an anti-invasive agent. Emodin could inhibit hyaluronic acid (HA)-induced matrix metalloproteinase and the invasion of human glioma cells through the inhibition of phosphorylation of the focal adhesion kinase (FAK), extracellular regulated protein kinase (ERK) 1/2 and Akt/PKB (Kim *et al*., 2005). Emodin also suppressed the responses of human mononuclear cells to phytohemagglutinin and mixed lymphocyte reaction (Huang *et al*., 1991a).

Anti-inflammatory activity

Emodin showed an inhibitory effect on the proliferation of activated T cells by the impairment of inflammatory cytokine production, IL-2 mRNA level and Ca^{2+} influx in the cells (Kuo *et al*., 2001).

Emodin exhibited its anti-inflammatory effect by regulating inflammatory cytokines, specifically by suppressing NF-kappaB activation (Li *et al*., 2005). It also exhibited an anti-inflammatory effect on carrageenan-induced edema and carrageenin-induced pedal inflammation in rats (Chang *et al*., 1996; Goel *et al*., 1991).

Vasorelaxant activity

Emodin isolated from Polygonum multiflorum exhibited a vasorelaxant effect on rat thoracic aortic rings pre-contracted with phenylephrine (Huang *et al*., 1991a). Emodin also exhibited a dose-dependent relaxation on isolated vascular rings of the human internal mammary artery and saphenous vein, rabbit thoracic aorta, abdominal aorta and mesenteric artery, and rat thoracic aorta. Its vasorelaxant effect might be due to the cGMP accumulation as a result of guanylate cyclase activation by free radicals and/or hydrogen peroxide generated from semiquinone (Huang *et al*., 1991b). However, the mechanism of the vasorelaxant effect of emodin is still not clearly defined.

Others

Emodin showed an anti-proliferative effect on the vascular smooth muscle cells through the p53-mediated apoptotic pathway (Wang *et al*., 2006). It also affected the signal transduction mechanism on the smooth muscle cells. Emodin could activate the contraction of smooth muscle cells by elevating the concentration of Ca^{2+} and thus by modulating the critical Ca^{2+} signal transduction pathways in the pathological setting of multiple organ dysfunction syndrome (MODS). Emodin

might be useful in the treatment of multiple organ dysfunction syndrome (Zheyu *et al*., 2006).

Emodin had the ability to modulate the gating properties of the voltage-dependent potassium channels in a reversible and dose-dependent manner (Li and Ou-Yang, 2005).

2 OBJECTIVES

Cancer and hypertension are two of the leading diseases in the world. Lung and breast cancer are two of major causes of death in men and women, respectively. Therefore, it is of great importance to develop a novel drug that provides beneficial effect on the prevention or even the treatment of both cancer and hypertension. Emodin, an active component of Da Huang, which has the ability to inhibit cancer growth, induce apoptosis and relaxation on aorta, is believed to be anticancer and antihypertensive. It is of value to study its anticancer mechanisms involved in human lung adenocarcinoma A549 cell line and human breast carcinoma MCF-7 cell line, and also the mechanisms involved in its antihypertensive effect.

Thus, there are some questions derived from this study which are as followings:

1. Does the Da Huang water extract and emodin have any anticancer effect on A549 and MCF-7 cells?

2. If the answer for the above question is yes, then what are the mechanisms involved in their anticancer effects?

3. Does emodin present in the Da Huang species?

4. Is there any inter-species and intra-species difference in the content of emodin?

5. Does emodin have any vasorelaxation effect?

6. If the answer for the above question is yes, then what are the mechanisms involved in its vasorelaxation effect?

In order to solve the above problems, the following objectives were set:

1. To confirm the anticancer effect of the Da Huang water extract and emodin.

2. To find out the possible mechanisms for the anticancer effects of the Da Huang water extract and emodin, the following studies will be involved, they are:

   - To find out their effects on cell proliferation.

   - To find out their effects on tumorigenicity.

   - To find out their effects on apoptosis

   - To find out their effects on expression of apoptosis-related genes.

3. To develop an efficient method to identify and quantify emodin.

4. To compare the amount of emodin in inter- and intra- species of Da Huang from different origins.

5. To confirm the vasorelaxant effect of emodin.

6. To find out the possible mechanisms for the emodin-induced vascular relaxation.

    - To find out the relationship between the emodin-induced vascular relaxation and nitric oxide.

    - To find out the relations between the emodin-induced vascular relaxation and potassium ion channel.

    - To find out the relationship between the emodin-induced vascular relaxation and calcium ion channel.

After studying their anticancer and antihypertensive effects and the mechanisms involved, a better understanding of the cell biology in hypertension and malignancy could be elucidated. In addition, emodin from Da Huang could be identified, isolated and purified for development of more preventive and therapeutic avenues to treat hypertension and cancer.

3 METHODOLOGY

3.1 Examination of anticancer effect of Da Huang water extract and emodin

3.1.1 Herbs

The dried Da Huang herb (*Rheum officinale* Baill.) was purchased from Hip Shing Hong Ltd., Hong Kong SAR, China. The voucher samples of herb were stored in Dr. Peter H.F. Yu ‘s laboratory, Department of Applied Biology and Chemical Technology.

3.1.2 Chemicals and Materials

Celltiter 96 aqueous MTS reagent was purchased from Promega Chemicals Co. (Madison, WI, USA). All the other chemicals used were of analytical grade. Cell culture medium, Dulbecco’s modified eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA). Human lung adenocarcinoma A549 cell line (CCL-185) and human breast carcinoma MCF-7 cell line (HTB-22) were purchased from American type Culture Collection (Manassas, USA). Emodin (HPLC grade) was purchased from Sigma-Aldrich (USA).

3.1.3 Preparation of Da Huang water extract and emodin solution

Dried Da Huang herb was ground into fine powder and mixed with distilled water in 1:10 w/v. The rhubarb-water mixture was shaken at 300rpm and 37°C for 2 h. The supernatant was collected after centrifuging the rhubarb-water mixture at 4000rpm for 3 minutes. The residue was re-extracted 2 more times. The supernatant (water extract) was filtered by suction filtration, frozen at -20°C refrigerator overnight, and lyophilized in a freeze dryer (Labconco, Freezone 6) for about 1 week. After lyophilization, the Da Huang water extract was weighed and separated into eppendorfs and centrifuge tubes. Emodin was dissolved in DMEM with 0.1% DMSO. Da Huang water extract and emodin were preserved at –20°C till further use.

3.1.4 Cell culture

Human cancer cell lines, human breast carcinoma MCF-7 and human lung adenocarcinoma A549, were maintained in low glucose DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100µg/ml streptomycin in culture plate at 37°C in humidified incubator with 5% carbon dioxide. The cells were fed every 2–3 days and subcultured once they reached 70–80% confluence.

3.1.5 MTS assay

MTS [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] growth inhibition assay is a non-radioactive cell proliferation assay and a colorimetric method for determining the number of

viable cells. This assay is composed of solutions of a tetrazolium compound (MTS, inner salt) and an electron-coupling reagent, phenazine methosulfate (PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan can be measured at 490nm from 96 well assay plates.

The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of the formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

MTS growth inhibition assay was performed according to the instructions provided by Promega Corporation. Briefly, same number of cells was seeded per well of a 96-well flat-bottomed plate (Falcon) on day 1. On day 2, the cells were treated without or with different concentrations of Da Huang water extract or emodin for a further 72 h or different incubation periods at 37°C with 5% carbon dioxide. At the end of this period, cells were washed with PBS and fresh medium was added. Afterwards, 10ul of MTS solution was added to each well and the plate was further incubated for 2 hours. Optical density was recorded at 490nm using a microplate reader (Bio-Rad, model 550). Each experiment was performed in triplicate. Results are expressed as the percentage growth inhibition with respect to untreated cells (control).

3.1.6 Morphological Monitoring

The effects of Da Huang water extract or emodin on the morphology of A549 and MCF-7 cells were recorded by taking photos under light inverted microscope (Nikon, Japan). On day 1, same number of cancer cells was seeded. On day 2, cancer cells were treated with different concentrations of Da Huang extract /and emodin at different incubation periods. Any cell number or morphological changes on MCF-7 and A549 cells were recorded by Nikon digital camera under an inverted microscope at ×200.

3.1.7 Soft Agar Colony Formation Assay

Soft agar colony formation assays were performed in triplicate in 6-well plates. Agarose gel (DNA grade, 2.5%) was melted in microwave oven and kept at 45 °C in a water bath. Base agar (15ml) for 6 wells was prepared by mixing 7.5ml of 1× DMEM, 3ml of 2× DMEM, 3ml of 2.5% agarose gel and 1.5ml of FBS. Base agar(2ml) was added into each well and allowed to set at room temperature. Top agar (7.5ml) for 6 wells was prepared by mixing 3ml of 2× DMEM, 3ml of 2.5% agarose gel and 1.5ml of FBS and it was kept at 45 °C in a water bath.

Cells were trypsinized and counted, and 120000 cells required per 6-well plate were prepared in 3ml of 1X DMEM.0.5ml of cell suspension (20000 cells) was then aliquot to each tube. It was then mixed gently with 0.5ml of Da Huang water extract/emodin (dissolved in 1× DMEM) or 1× DMEM (control) and 1ml of top agar. The top agar mixture was then added to each well with base agar and allowed to set. Cells were incubated at 37°C in humidified incubator for 7 to 14

days. Colonies with more than 50 cells were counted and recorded by digital camera under light inverted microscope.

3.1.8 DNA Fragmentation Detection Assay

Da Huang water extract/emodin treated or untreated cell pellets were prepared by trypsinization. The cell pellets were rinsed by PBS before used. Aliquot of nuclear lysis buffer (10mM Tris-HCl pH 7.5, 400mM NaCl, 100mM EDTA, 0.6% SDS) (600µl) and RNase (4mg/ml) (10µl) were added to each reaction tube with cell pellet. After gently mixing the tube, they were placed in 37ºC water bath and incubated for 5 min. An aliquot of protein precipitation solution (6M NaCl) (200µl) was then added with vortex to each tube and chilled on ice for 5 min.

After centrifugation in a micro-centrifuge at 13,000 rpm for 10 minutes, supernatant was pipetted to new tube and 600µl isopropanol was added. They were then chilled on ice for 15 min and centrifuged again for 20 min at 13,000 rpm. The supernatant was discarded carefully and the pellet was washed once with 600µl of 70% ethanol to eliminate any salt.

After completely air-drying the DNA pellet, 20µl distilled water was added to dissolve DNA. After measurement of DNA concentration, 10µg/µl of DNA was mixed with loading dye and run in 1.5% agarose gel with ethidium bromide at 100V for 2 h. The fragmentized DNA can be observed under UV illuminator.

3.1.9 Comet Assay (Single Cell Gel Electrophoresis, SCGE)

Drug-induced DNA damage was estimated using the comet assay described by Klaude et al., 1996; Singh et al., 1988 with some modifications. After 2 days, Da Huang water extract or emodin treated cells were separated with EDTA/trypsin and centrifuged at 800 × g for 3 min. Aspirated supernatant was added to 200µl PBS buffer and 800µl of 1% off low melting point agarose, immediately pipetted onto a frosted glass microscope slide pre-coated with a layer of 1.0% normal melting point agarose, prepared in PBS. Coverslips were added and the slides were allowed to solidify at 4 ◦C for 10 min.

Similarly, coated with 0.8% of LMP for 10 min, coverslips were gently removed and cells were lysed in high salt solution (2.5 M NaCl, 10 mM Tris–HCl, 100 mM EDTA, pH 10, with 1% Triton and 10% dimethyl sulfoxide added fresh) for 1 h. After lysis, slides were placed in a horizontal electrophoresis unit containing fresh buffer (1 mM EDTA, 300 mM NaOH pH 13) and incubated for 20 min to allow unwinding of DNA. Electrophoresis was then conducted in fresh electrophoresis buffer (pH 13) for 20 min at 25 V and 300 mA (0.8 V/cm) at 4 °C. Subsequently, the slides were gently washed in neutralization solution (0.4 M Tris–HCl, pH 7.5) for 20min, stained with 20µl ethidium bromide (15µg/ml) and covered with a coverslip. Stained nucleoids were scored visually using a fluorescence microscope (Leica) equipped with a digital camera. Two slides were analysed for each sample and 50 comets on each slide were acquired using

the IM50 software image analysis system. Tail length was calculated and expressed as means ± S.E.M.

3.1.10 Assessment of mRNA expression level of apoptotic-related genes by real-time PCR

To assess the mRNA level of Fas ligand (FASL), myeloid cell leukemia sequence 1 (MCL1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), BCL2-associated X protein (BAX), cyclin D1 (CCND1) and v-myc myelocytomatosis viral oncogene homolog (C-MYC), real-time PCR was performed using ribosomal protein S9 (RPS9) as an internal standard (Table 1). Untreated or emodin treated A549 and MCF-7 cells were trypsinized and total RNA was extracted using Trizol reagent. The RNA was then reverse-transcripted into cDNA by RT-PCR using the SuperScript First-Strand synthesis system. Real-time PCR was performed by SYBR green real-time detection kit using Cepheid SmartCycler.

Table 1. Names and accession number of genes.

Gene Symbol	Gene Full Name	Accession Number
RPS9	Ribosomal protein S9	NM_001013
FASL	Fas ligand	NM_000639
MCL1	Myeloid cell leukemia sequence 1	BC017197
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046
BAX	BCL2-associated X protein	NM_004324
CCND1	Cyclin D1	NM_053056
C-MYC	V-myc myelocytomatosis viral oncogene homolog	NM_002467

Aliquot of real-time PCR mixture (25µl) containing cDNA, iQ SYBR Green Supermix, 10 µM forward primer and reverse primer (Table 2), sterile milli-Q water was prepared. SYBR Green Supermix contained dNTPs (0.4mM of each), *Taq* polymerase (50units/ml), $MgCl_2$ (6 mM), KCl (100 mM), and Tris–HCl at pH 8.4 (40 mM). Real-time PCR was performed under different annealing temperature and cycle number with specific primer concentration (Table 3).

Table 2. Forward and reverse primer sequence and product size of genes.

Gene	Forward Primer and Reverse Primer (5'→ 3')	Product Size(bp)
RPS9	CGTCTCGACCAAGAGCTGA	114
	GGTCCTTCTCATCAAGCGTC	
FASL	CATTTAACAGGCAAGTCCAACTC	326
	TGATCAGCACTGGTAAGATTGAA	
MCL1	CACGAGACGGTCTTCCAAGGCATGCT	496
	CTAGGTTGCTAGGGTGCAACTCTAGGA	
GAPDH	GGTCGGAGTCAACGGTTTGG	1000
	ACCACCCTGTTGCTGTAGCCA	
BAX	TGGAGCTGCAGAGGATGATTG	117
	GAAGTTGCCGTCAGAAACATG	
CCND1	TCTAAGATGAAGGAGACCATC	385
	GCGGTAGTAGGACAGGAAGTTGTTG	
C-MYC	GATTCTCTGCTCTCCTCGACGGAG	274
	GCGCTGCGTAGTTGTGCTGATGTG	

Table 3. The PCR conditions for genes.

Gene	Primer concentration (nM)	Annealing temperature (ºC)	Cycle no.
RPS9	200	60.0	40
FASL	300	65.7	50
MCL1	300	62.0	40
GAPDH	200	60.0	40
BAX	300	60.0	40
CCND1	300	60.0	40
C-MYC	300	67.0	40

Generally, real-time PCR was run with initial denaturation at 95 °C for 3 min, followed by 40-50cycles of denaturation at 95°C for 10 s, primer annealing at 50-67 °C for 30 s and primer extension at 72 °C for 30 s. The melting curves of the final PCR products were analyzed from 60 to 95 °C at 1 °C interval. The mRNA expression level of interest was related to internal standard: ribosomal protein S9 (RPS9) to correct the differences between quantity and quality of different RNA samples. The threshold cycle for each amplification cycle was found and plotted against the standard curve to calculate the concentration of gene product. Values from each group were normalized to gene product of RPS9. A standard curve was generated for each gene for assessing the level of cDNA.

3.2 Quantification of emodin in different species of Da Huang

3.2.1 Chemicals

Emodin (HPLC grade) was purchased from Sigma-Aldrich (USA) and all the solvents including methanol, acetonitrile and acetic acid were of HPLC grade and purchased from International Laboratory (USA).

3.2.2 Preparation of Da Huang extracts

Four samples were purchased from local TCM shop in China. They were *Rheum tanguticum* Maximowicz (四川野大黃), *was Rheum palmatum* Linn (甘肅掌葉

大黃), *Rheum tanguticum* Maximowicz (甘肅野大黃) and *Rheum officinale* Baill. (四川藥用大黃). They were labeled as D1, D2, D3 and D4, respectively. D1 and D4 were originated from Sichuan province and D2 and D3 were from Ganshu. Rhubarbs (100mg) were crushed and extracted with 25ml ethyl acetate. They were placed in ultrasonic bath for 30 min and then allowed to deposit for about 1 day. The residue was collected after the solution was evaporated and dried in a rotary evaporator. Methanol (25ml) was used to re-dissolve the residue and filtered with 0.2μm membrane filter before HPLC analysis.

3.2.3 HPLC analysis of components of Da Huang species

Analysis of components of Da Huang species in an HPLC system (Aligent 1100 series) equipped with a degasser, a quaternary pump, autosampler and a diode array-multiple wavelength detector (DAD). Aliquots of standard or samples (20 μl) were injected into an Alltech Allsphere ODS-25u separation column (250mm × 4.6mm I.D.) with a guard column and eluted at a flow rate of 2.0 ml/min at 25ºC with methanol-acetonitrile-acetic acid (70:15:15, v/v) as mobile phase. The eluant was monitor at 275nm.

Between 1μg/ml to 20μg/ml of HPLC grade emodin dissolved in methanol were used as a standard for the calibration of retention time and identification. The calibration curve was constructed by plotting the peak height against the concentration of emodin and each point in calibration curve was performed at

triplicate. The amount of emodin in samples was measured from the calibration curve.

To increase the accuracy of the HPLC analysis, an internal standard of emodin solution was added to *Rheum tanguticum* Maximowicz (D3) extract in a final concentration of 15μg/ml. Inter- and intra-day HPLC analysis of standard was performed to measure the reliability and accuracy. The reliability was represented by the relative standard deviation (RSD) and calculated by the following equation:

$$\text{RSD (\%)} = (\text{standard deviation/mean}) \times 100\%$$

The accuracy was calculated by the following equation:

$$\text{Accuracy (\%)} = [1-(\text{mean weight measured-weight spiked})/\text{weight spiked}] \times 100\%$$

3.3 Examination of vasorelaxant effect of emodin

3.3.1 Animals

Male Sprague Dawley (SD) rats (250–400g) were supplied by the Animal Centre, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University (HKPU). The animals were kept in animal holding room at room temperature and constant humidity in 12h light-dark cycle with free

access to water and food. The experiment on rats was reviewed and approved by the Committee of Animal Ethics, HKPU.

3.3.2 Chemicals

Acetylcholine, phenylepherine indomethacin, N^G-nitro-L-arginine methyl ester (L-NAME), glibenclamide (GA), tetraethylammonium (TEA) and emodin (HPLC grade) were purchased from Sigma-Aldrich (USA). The pure emodin compound and indomethacin were dissolved in 1% and 100% DMSO, respectively. All other chemicals were dissolved in distilled water.

3.3.3 Rat aorta rings preparation

Male 8-12 week-old SD rats were killed by cervical dislocation and their thoracic aortas were rapidly excised. Their aortas were then placed into Petri dish with Krebs-Ringer bicarbonate solution composed of 4.7mM KCl, 2.5mM $CaCl_2$, 25mM $NaHCO_3$, 1.2mM $MgSO_4$, 1.2mM NaH_2PO_4, 118mM NaCl and 11mM glucose and trimmed free of fat and adherent connective tissues. All aorta rings were cut into 2-3mm-long sections and their endothelia were mechanically removed by gentle rolling with forceps. The endothelium-denuded aorta rings were verified if there was absence of relaxation response on 1μM noradrenaline pre-contracted aorta rings when treated with 10μM acetylcholine.

All isolated aorta rings were fixed onto two parallel hooks maintained in 5ml water-jacked organ baths containing Krebs-Ringer bicarbonate solution, constantly aerating with gas mixture of 95% O_2 and 5% CO_2 and maintaining at

37°C. Resting tension was increased stepwise to reach the final tension of 1.5g, and the aorta rings were allowed to equilibrate for 1h and being replaced with fresh drug-free Krebs Ringer bicarbonate solution every 20 min (Lam *et al.*, 2005). The resting tension was re-adjusted when necessary before commencing the experiment. KCl (40mM) was used to sensitize the aorta rings after equilibration until two consecutive responses were reproducible. The response of aorta rings were detected and recorded by isometric force-displacement transducers connected to a PowerLab data acquisition system.

3.3.4 Measurement of aortic relaxation

After equilibration and sensitization periods, in order to investigate the vasorelaxant effect of emodin on rat aortas, aorta rings were pre-treated with 1μM indomethacin, an inhibitor of cyclo-oxygenase (Cox-1 and Cox-2) enzymes, and then pre-contracted with 1μM phenylepherine. When the contractile response to phenylepherine reached a steady maximal response, emodin was added in progressively increasing cumulative concentrations (1nM–10μM). Any vasorelaxant effect of emodin was assessed as any suppression on phenylepherine-induced contractile response. All data were presented as means ± S.E.M.

3.3.5 Measurement of suppression on contraction

After equilibration and sensitization periods, in order to investigate the suppression effect of emodin on contraction of rat aortas, aorta rings were pre-treated with 10μM emodin and then 1μM indomethacin. When the response of

aorta rings reached a steady state, phenylepherine was added in progressively increasing cumulative concentrations (1nM–100μM). Any suppression effect of emodin on vasoconstriction was assessed as any suppression on phenylepherine-induced contractile response on aortas pre-treated with emodin. All data were presented as means ± S.E.M.

3.3.6 Examination of mechanism of vasorelaxant effect of emodin

3.3.6.1 Study of nitric oxide dependent relaxation

After equilibration and sensitization periods, in order to investigate the effect of NG-nitro-L-arginine methyl ester (L-NAME), a nitric oxide inhibitor, on suppression of emodin-induced vasorelaxation, the aorta rings were pre-treated with 10μM emodin, 20μM L-NAME and then 1μM indomethacin. When the response of aorta rings reached a steady state, phenylepherine was added in progressively increasing cumulative concentrations (1nM–100μM). Any suppression effect of L-NAME on emodin-induced vascular relaxation on aorta rings was assessed as the inhibition of phenylepherine-induced contraction.

3.3.6.2 Study of potassium ion channel related relaxation

After equilibration and sensitization periods, in order to investigate the vasorelaxant effect of emodin on the potassium ion channels blocked aortas, the aorta rings were pre-treated with 3μM glibenclamide (GA), an ATP-sensitive K^+ channels inhibitor, 1mM tetraethylammonium (TEA), a Ca^{2+}-activated K^+

channel blocker, 1μM indomethacin and pre-contracted with 1μM phenylepherine. When the contractile response to phenylepherine reached a steady maximal response, emodin was added in progressively increasing cumulative concentrations (1nM–10μM). Any relaxing effect of emodin on potassium ion channels blocked aorta was assessed as the inhibition of phenylepherine-induced contraction.

3.3.6.3 Study of calcium ion channel related relaxation

In order to investigate the effect of Ca^{2+} channel on the emodin-induced relaxation effect, the control curve must be established in each treatment. For constructing control curve, the aortas were washed with $CaCl_2$ free Krebs-Ringer solution after sensitization following by 40mM KCl. After a steady state was established and cumulative concentration (1μM–3mM) of $CaCl_2$ were added to the organ bath.

For constructing the curve of different treatment, the aortas were washed with $CaCl_2$ free Krebs-Ringer solution after sensitization. Ca^{2+} channels inhibitor, verapamil (1μM), had been added to aortas in different treatments and followed by 40mM KCl. After a steady state was established, cumulative concentration (1μM–3mM) of $CaCl_2$ were added to the organ bath.

3.4 Statistical Analysis

All data were presented as means ± Standard Errors of Means (S.E.M). Statistical analysis was performed by analysis of variance (ANOVA) to detect significant

differences in multiple comparisons with Bonferroni post test: compare all pairs of columns. A value of probability (p)<0.05 was considered to be statistically significant. All statistical analysis tests were performed using Graph Pad Prism 4.02 for windows (GraphPad Software, San Diego California, USA).

4 RESULTS

4.1 Examination of anticancer effect of Da Huang water extract

4.1.1 Inhibitory effect on cell growth

The Da Huang water extract had a growth inhibitory effect on A549 and MCF-7 cells with IC_{50} values, 693μg/ml and 583μg/ml, respectively (Figure 7). To investigate whether the anticancer effect of the Da Huang water extract is time-or dose-dependent, A549 and MCF-7 cells were treated with different concentrations of Da Huang water extract under different incubation periods.

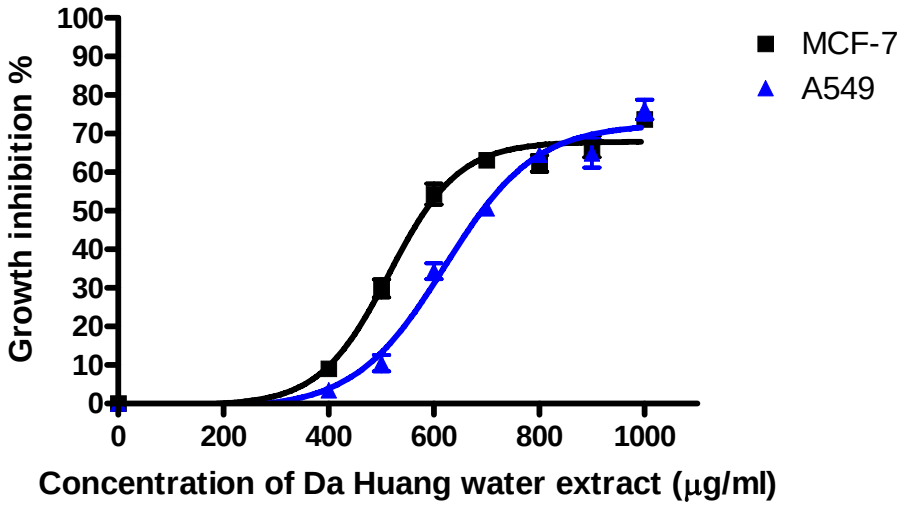


Figure 7. Growth inhibitory effect of Da Huang water extract (400, 500, 600, 700, 800, 900 and 1000μg/ml) on A549 and MCF-7 cells after 72h-treatment. Data are expressed as means ± S.E.M., n=3.

It was found the higher the concentration of or incubation time with Da Huang water extract, the higher was also the growth inhibition on A549 and MCF-7 cells, indicating that the growth inhibitory effect of the Da Huang water extract on A549 and MCF-7 cells was time- and dose- dependent (Figure 8A and 8B).

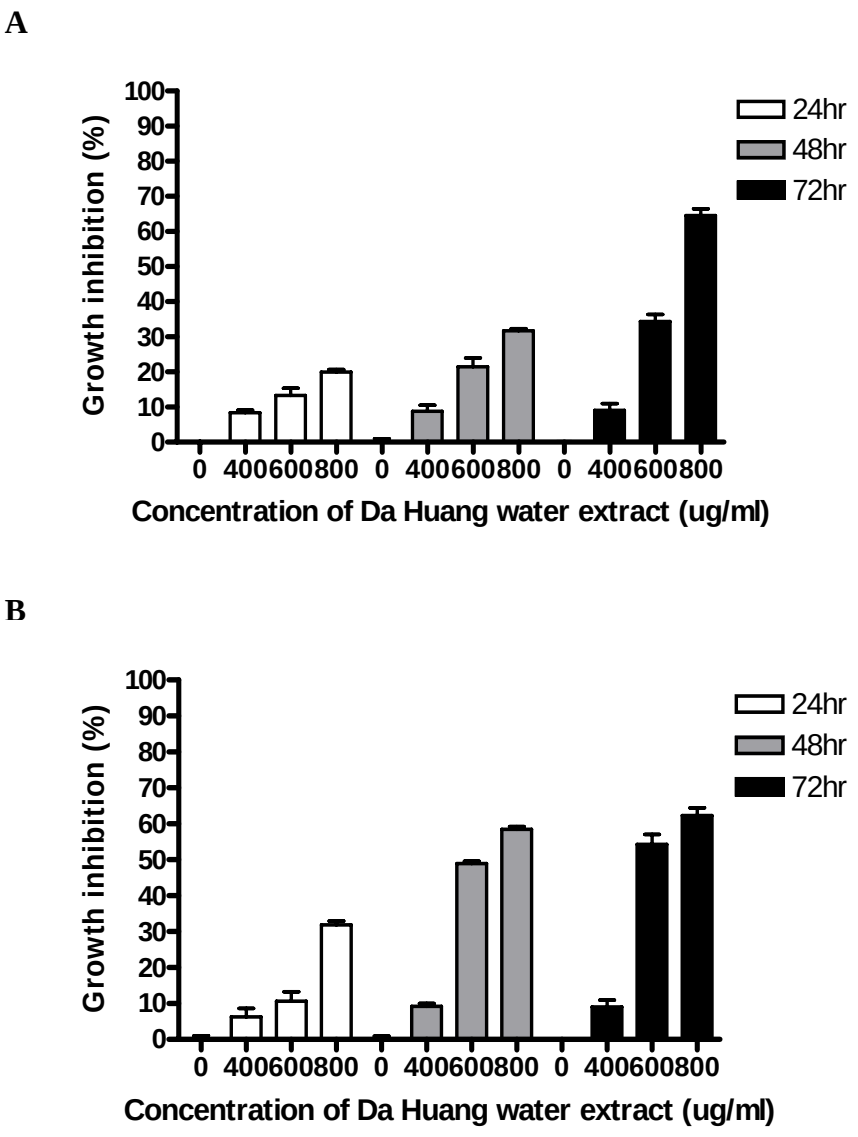
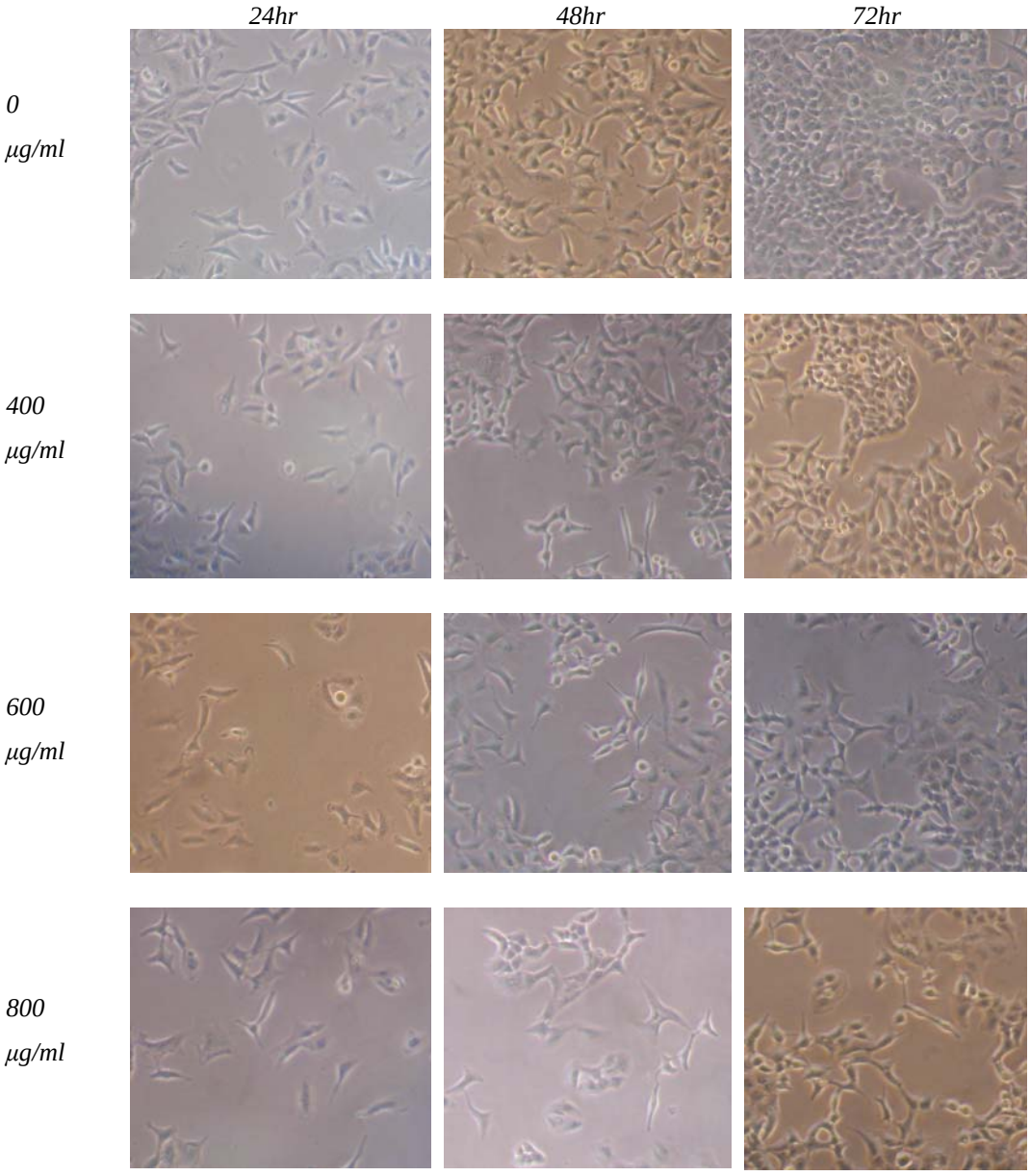


Figure 8. Growth inhibitory effect of Da Huang water extract (400,600 and 800μg/ml) on (A) A549 cells and (B) MCF-7 cells after 24h-, 48h- and 72h- treatments. Data are expressed as means ± S.E.M., n=3.

4.1.2 Change of morphology

The number of A549 and MCF-7 cells was decreased after treatment with the Da Huang water extract when comparing with control. Higher the concentration of the Da Huang water extract used to treat A549 and MCF-7 cells, the fewer the number of cells was observed (Figure 9A and 9B).

A



B

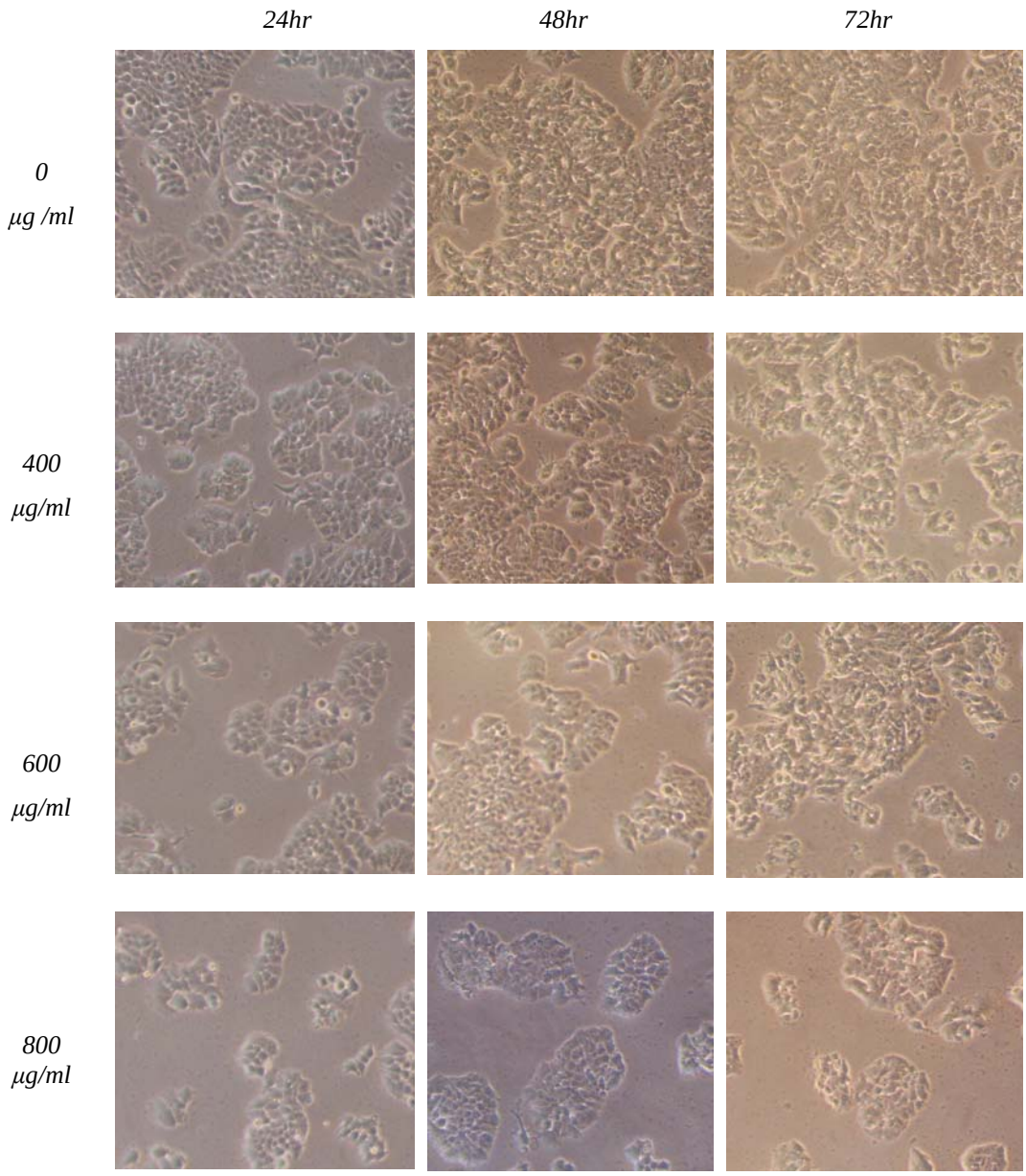


Figure 9. Morphology of Da Huang water extract (0, 400, 600 and 800μg/ml) treated (A) A549cells and (B) MCF-7 cells after 24h-, 48h- and 72h- treatments under light microscope (x200).

4.1.3 Inhibition on colony formation

The Da Huang water extract showed an inhibition on the colony formation of A549 and MCF-7 cells (Figure 10). The inhibitory effect of the Da Huang water extract on colony formation was concentration-dependent.

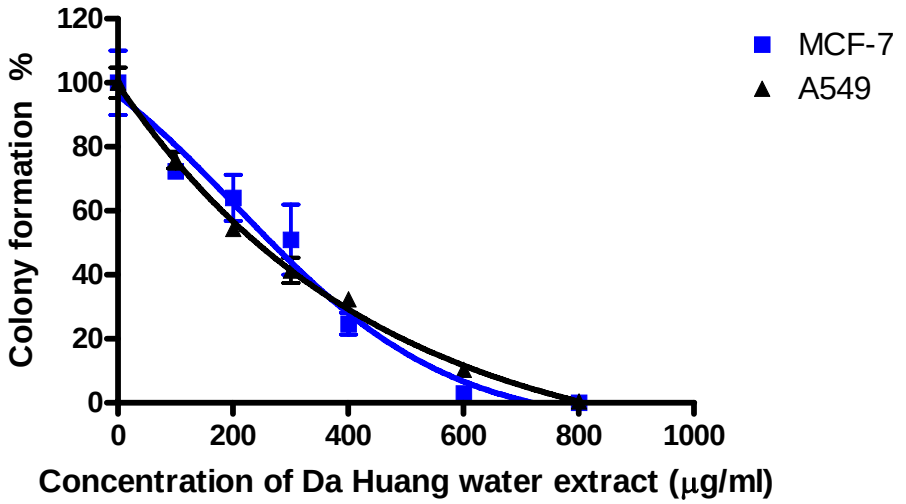
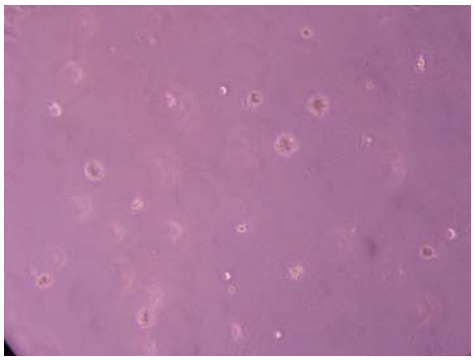


Figure 10. Inhibitory effect of Da Huang water extract (0, 100, 200, 300, 400, 600 and 800μg/ml) on formation of colony of A549 cells and MCF-7 cells after 10 day-treatment. Data are expressed as means ± S.E.M., n=3.

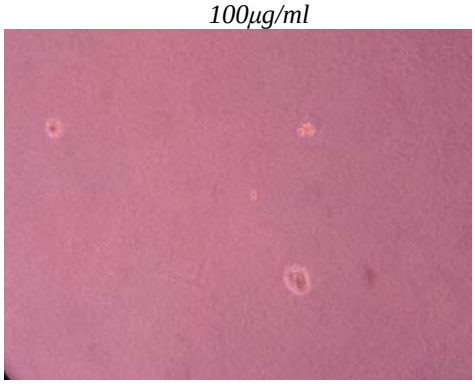
It was found that A549 and MCF-7 cells treated with the higher concentration of Da Huang water extract, the fewer the number of colonies and smallerthe size of the colonies were observed (Figure 11A and 11B).

A

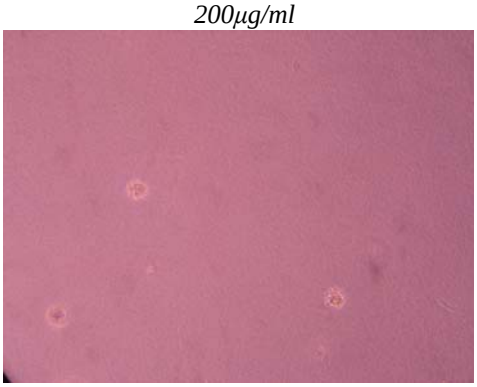
Control



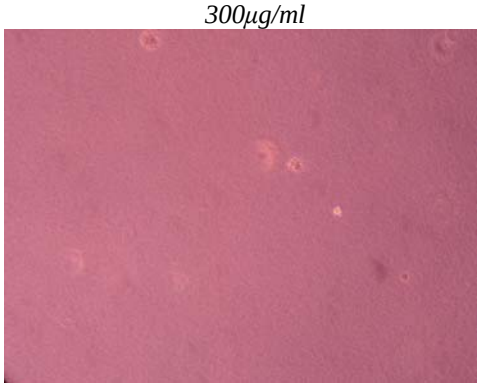
100µg/ml



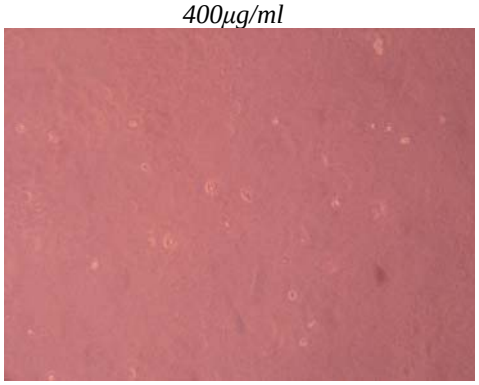
200µg/ml



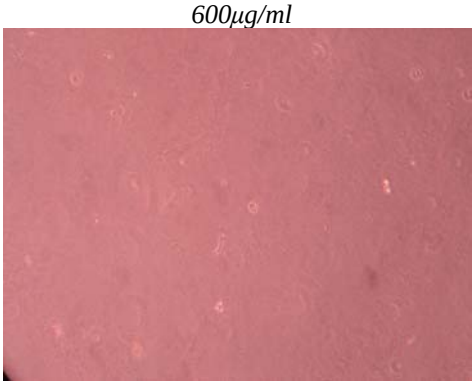
300µg/ml



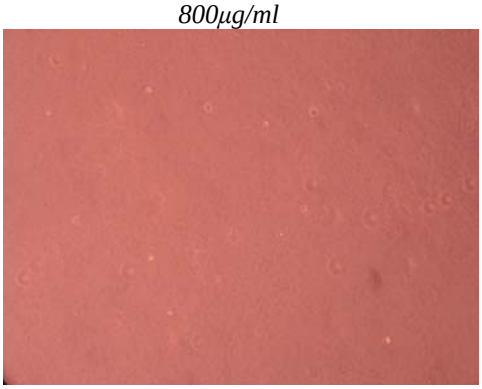
400µg/ml



600µg/ml



800µg/ml



B

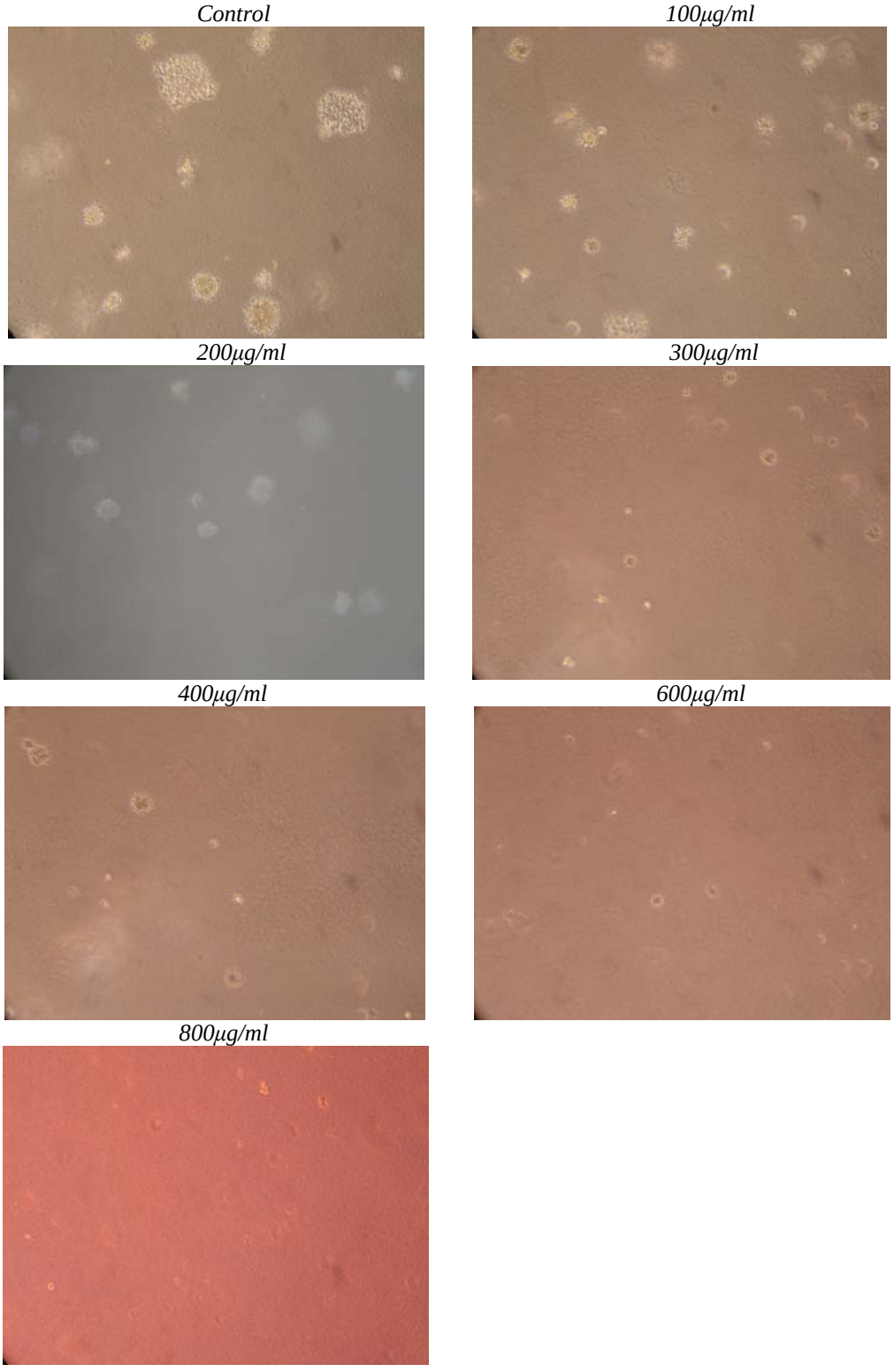


Figure 11. Decrease in colony number and colony size of (A) A549 cells and (B) MCF-7 cells after 10-day treatment with Da Huang water extract (0, 100, 200, 300, 400, 600 and 800μg/ml).

4.1.4 DNA fragmentation

Chromosomal DNA from untreated and Da Huang water extract treated A549 and MCF-7 cells was extracted and resolved in agarose gel under an UV illuminator in order to investigate any fragmentized DNA. It showed that the chromosomal DNA of the Da Huang water extract treated A549 and MCF-7 cells was fragmentized while the chromosomal DNA from untreated cancer cells was not fragmentized with a size greater than 1000bp (Figure 12).

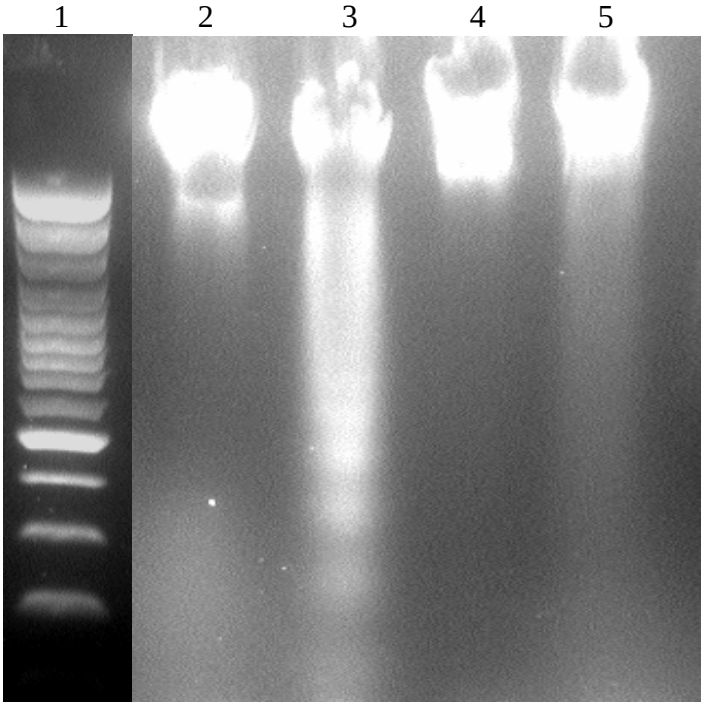


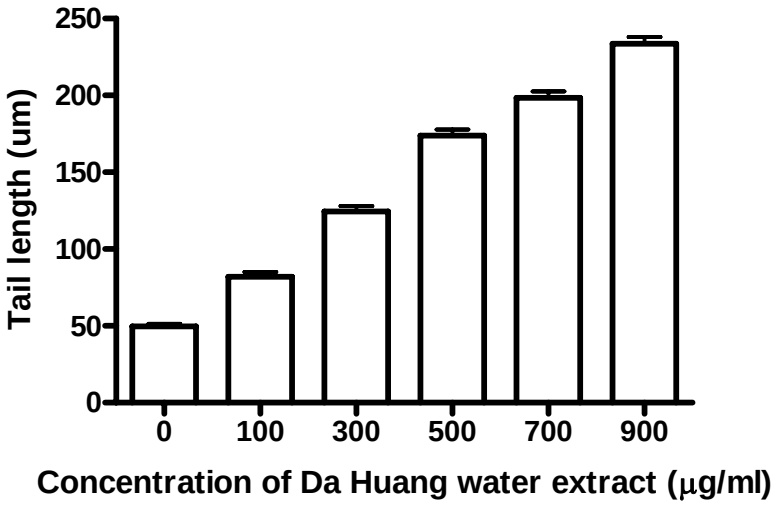
Figure 12. Chromosomal DNA of Da Huang water extract treated or untreated A549 and MCF-7 cells in 1.5% agarose gel under UV illuminator. Lane1: 100bp DNA Marker; lane2: DNA from untreated A549 cells; lane3: DNA from 700μg/ml Da Huang water extract treated A549 cells; lane4: DNA from untreated MCF-7 cells; lane5: DNA from 600μg/ml Da Huang water extract treated MCF-7 cells. All A549 and MCF-7 cells were treated with Da Huang water extract after 72h.

4.1.5 DNA damage

To study the DNA damage in the Da Huang water extract treated A549 and MCF-7 cells under 72h-treatments, comet assay was performed. Comet assay is a sensitive method to monitor DNA single strand breaks at a single-cell level. Any DNA damage is represented as a tail length (tail migration) of the DNA single strand.

When A549 and MCF-7 cells were treated with increasing concentration of the Da Huang water extract for 72h, tail length progressively increased in the Da Huang water extract treated cells (Figure 13A and 13B). In addition, the DNA damage induced by the Da Huang water extract was dose-dependent. The comet assay results showed that the Da Huang water extract induced DNA damage which is one of the features of apoptosis.

A



B

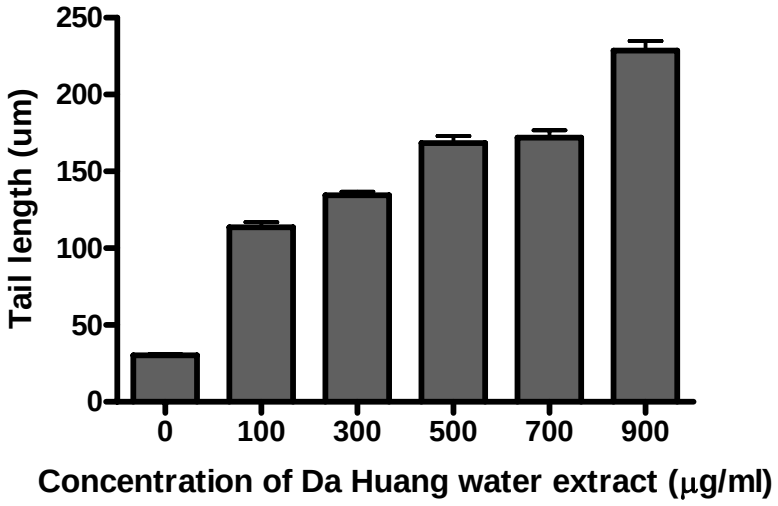


Figure 13. Effect of Da Huang water extract (0,100, 300, 500, 700 and 900μg/ml) on DNA damage on (A) A549 cells and (B) MCF-7 cells after 72h-treatment represented as comet tail length (μm). Data are expressed as means ± S.E.M., n=100.

4.2 Examination of anticancer effect of emodin

4.2.1 Inhibitory effect on cell growth

Emodin had a growth inhibitory effect on A549, MCF-7 and L929 cells but to a different extent. IC_{50} values on A549 and MCF-7 cells were 16.85μg/ml (60μM) and 7.22μg/ml (30μM) of emodin, respectively. Emodin had low cytotoxicity to normal mouse fibroblast L929 cells with IC_{50} value greater than 35μg/ml (Figure 14).

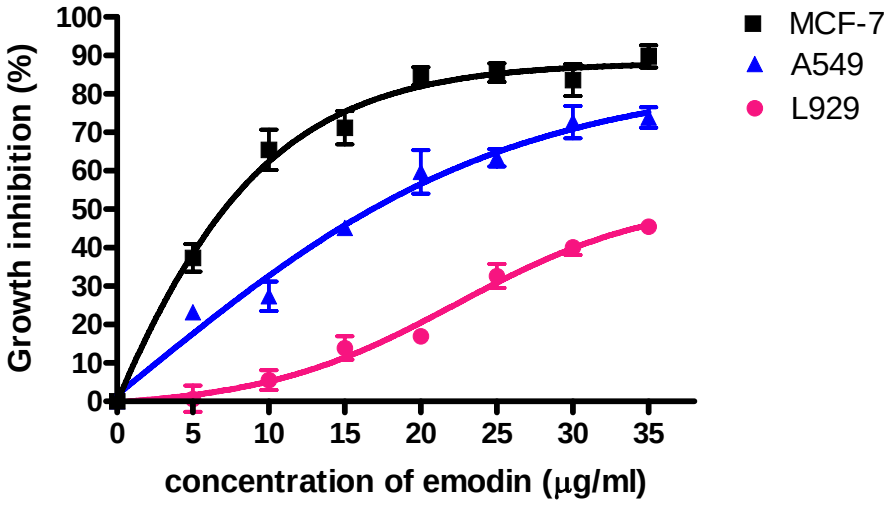
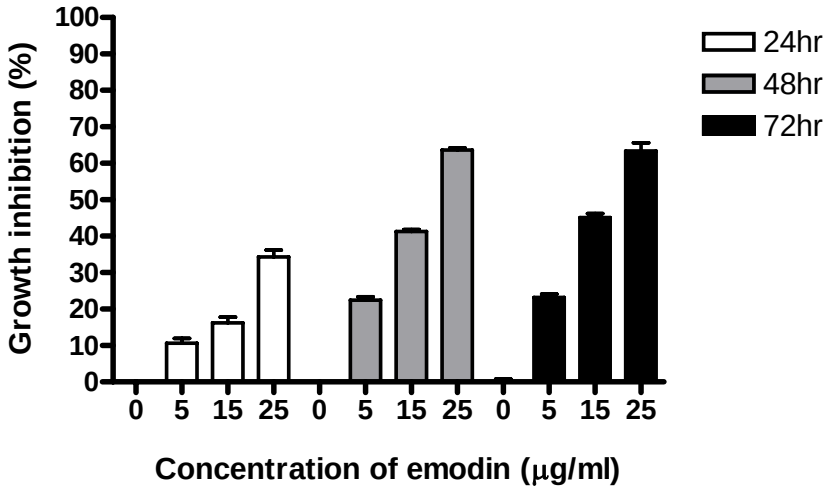


Figure 14. Growth inhibitory effect of emodin (0, 5, 10, 15, 20, 25, 30 and 35μg/ml) on A549, MCF-7and L929 cells after 72h-treatment. Data are expressed as means ± S.E.M., n=3.

The growth inhibitory effect of emodin on A549 and MCF-7 cells was time- and dose- dependent (Figure 15A and 15B).

A



B

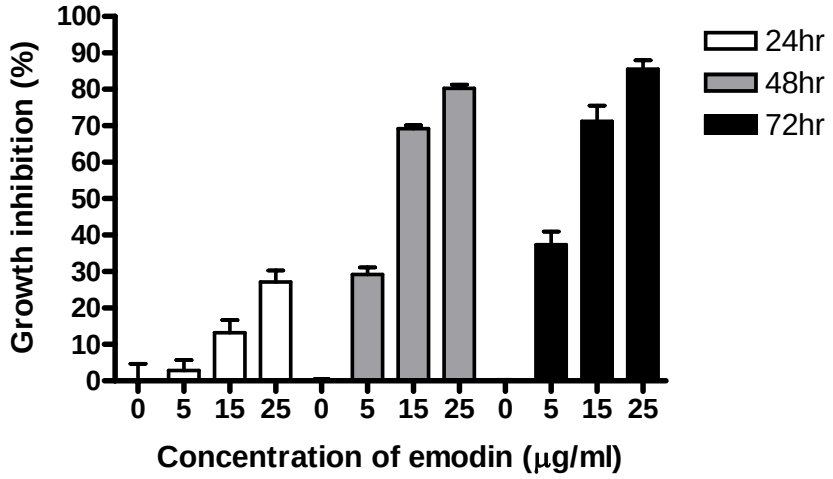


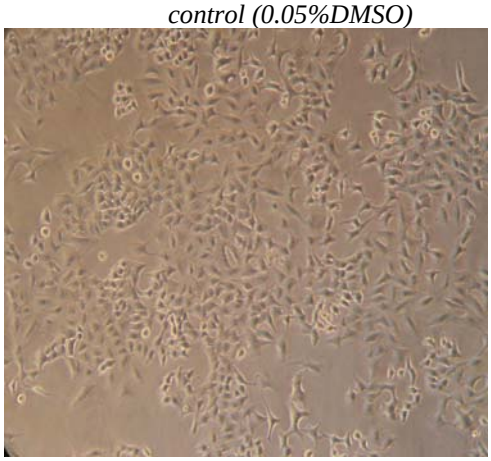
Figure 15. Growth inhibitory effect of emodin (0, 5, 15 and 25μg/ml) on (A) A549 cells and (B) MCF-7 cells after 24h-, 48h- and 72h- treatments. Data are expressed as means ± S.E.M., n=3.

4.2.2 Change of morphology

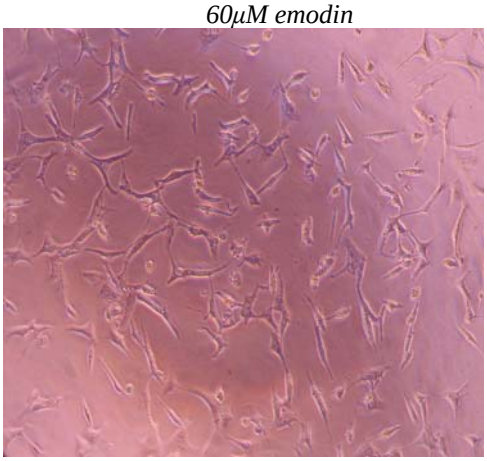
The number of A549 and MCF-7 cells was decreased after treatment with emodin. The higher the concentration of emodin used to treat A549 and MCF-7 cells, the fewer the number of cells was observed. Besides, the morphology of emodin treated A549 and MCF-7 cells was changed. Cell shrinkage was observed indicating that apoptosis might occur (Figure 16A and 16B).

A

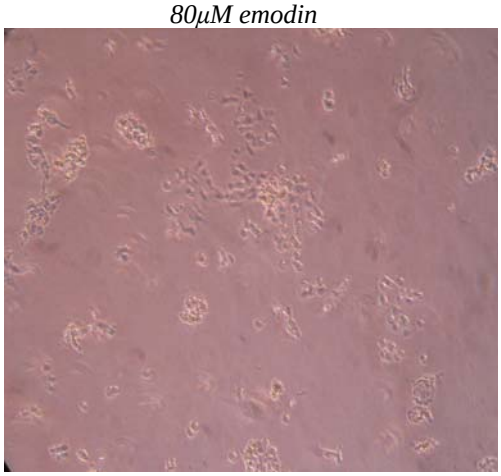
control (0.05%DMSO)



60μM emodin

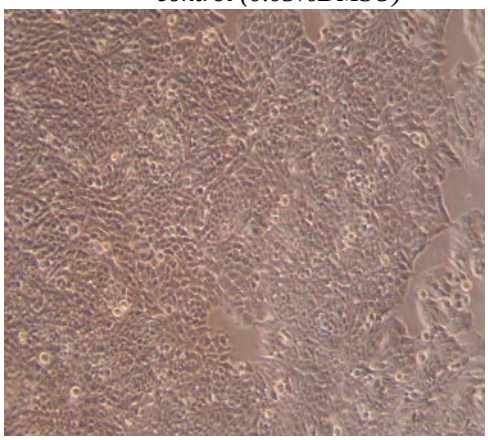


80μM emodin

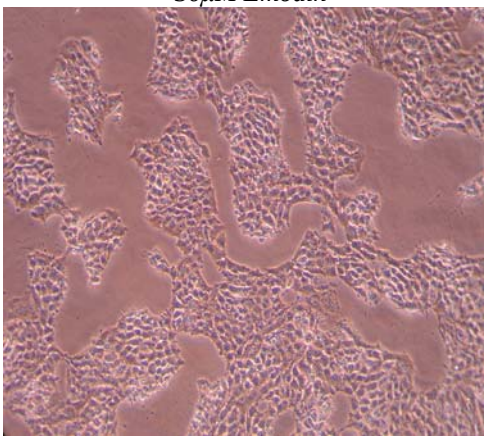


B

control (0.05%DMSO)



30μM Emodin



50μM emodin

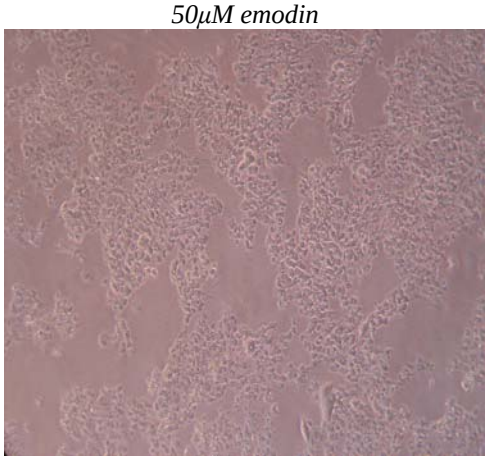


Figure 16. Morphology of (A) emodin (0, 60 and 80μM) treated A549 cells and (B) emodin (0,30 and 50μM) treated MCF-7 cells after 72h-treatment under light microscope (x200).

4.2.3 Inhibition on colony formation

Emodin showed an inhibition on the colony formation of A549 and MCF-7 cells (Figure 17). The inhibitory effect of emodin on colony formation was concentration-dependent.

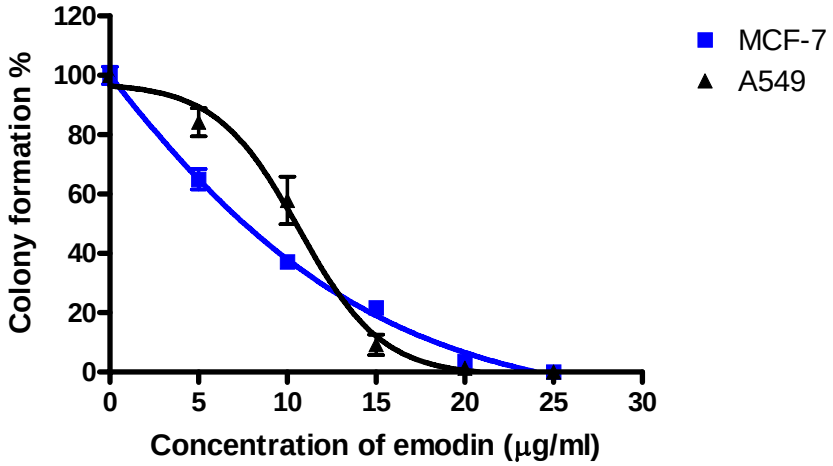
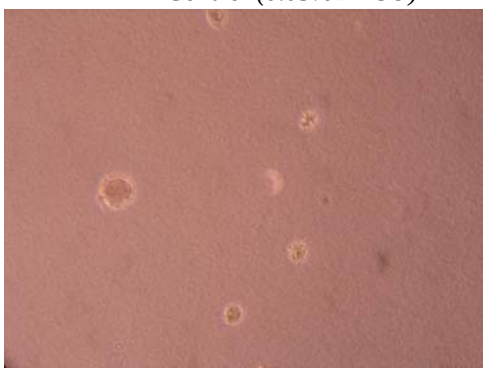


Figure 17. Inhibitory effect of emodin (0, 5, 10, 15, 20 and 25μg/ml) on formation of colony of A549 cells and MCF-7 cells after 10 day-treatment. Data are expressed as means ± S.E.M., n=3.

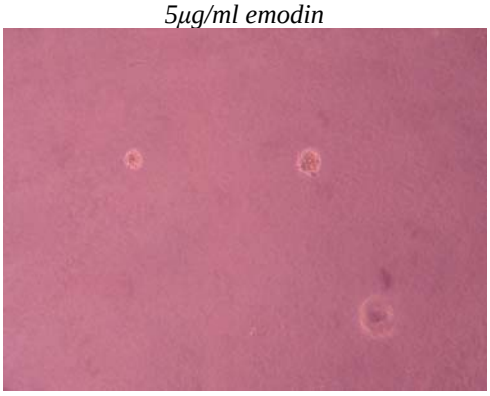
It was found that A549 and MCF-7 cells treated with a higher concentration of emodin, fewer the number of colonies and a smaller size of the colonies were observed (Figure 18A and 18B).

A

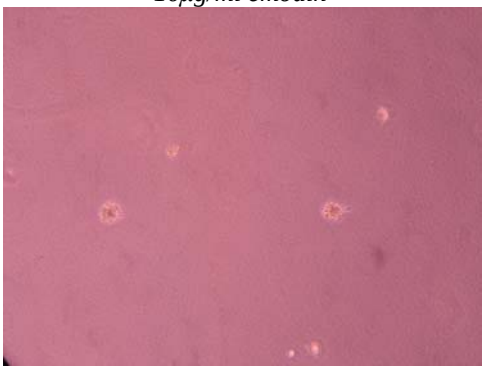
Control (0.05%DMSO)



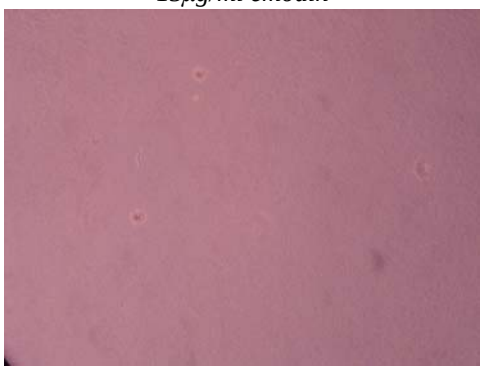
5μg/ml emodin



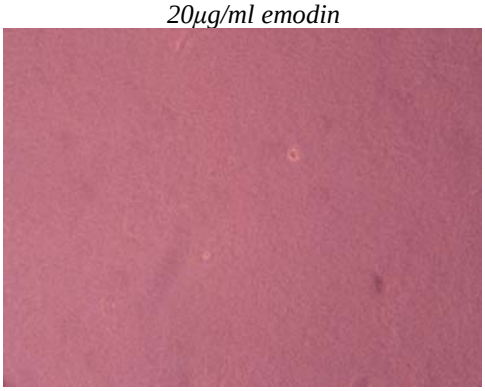
10μg/ml emodin



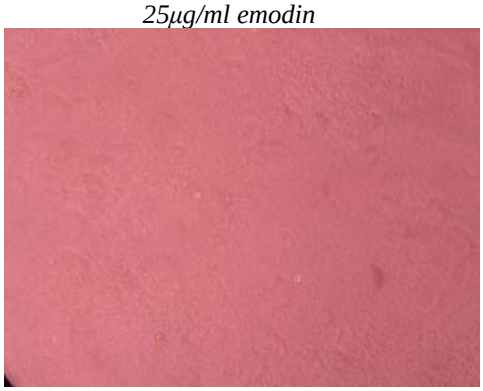
15μg/ml emodin



20μg/ml emodin



25μg/ml emodin



B

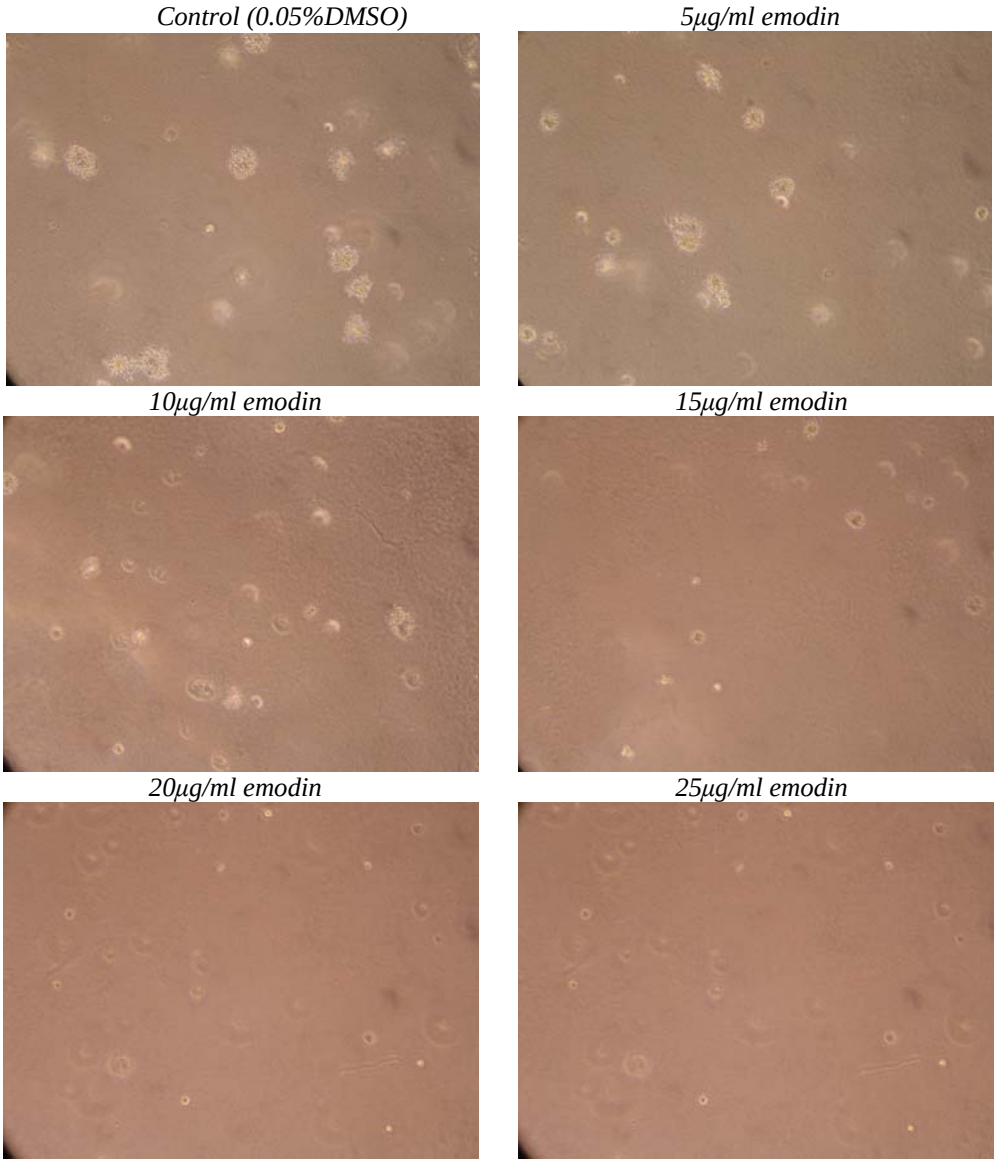


Figure 18. Decrease in colony number and colony size of (A) A549 cells and (B) MCF-7 cells after 10-day treatment with emodin (0, 5, 10, 15, 20 and 25μg/ml).

4.2.4 DNA fragmentation

It was shown that chromosomal DNA extracted from emodin treated A549 and MCF-7 cells was fragmentized. Laddering DNA was observed in lane 3 and lane 5 (Figure 19).

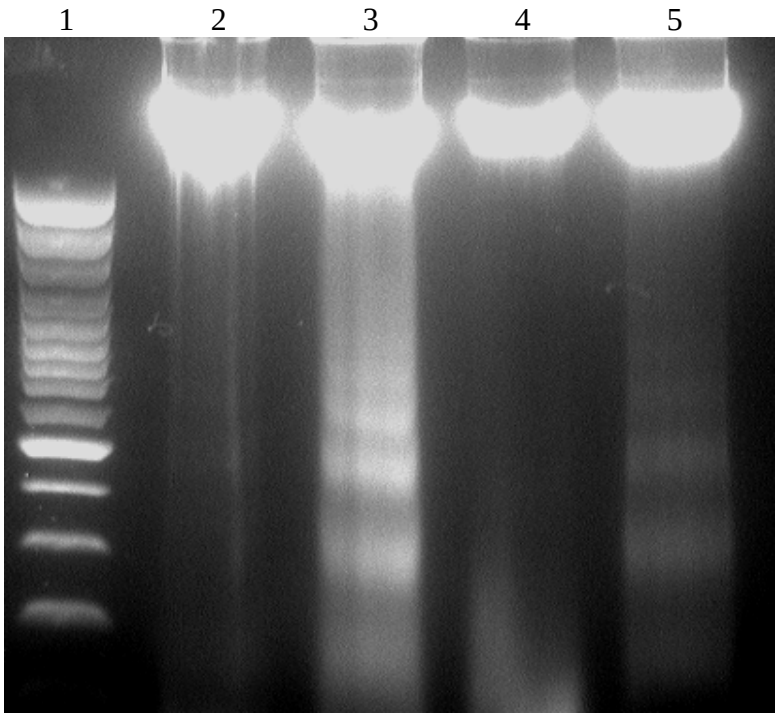


Figure 19. Chromosomal DNA of emodin treated or untreated A549 and MCF-7 cells in 1.5% agarose gel under UV illuminator. Lane1: 100bp DNA Marker; lane2: DNA from 0.05% DMSO treated A549 cells; lane3: DNA from 0.05% DMSO and 60μM emodin treated A549 cells; lane4: DNA from 0.05% DMSO treated MCF-7 cells; lane5: DNA from 0.05% DMSO and 30μM emodin treated MCF-7 cells. All A549 and MCF-7 cells were treated with emodin after 72h.

4.2.5 DNA damage

To study the DNA damage in the emodin treated A549 and MCF-7 cells under 72h-treatment, comet assay was performed. When A549 and MCF-7 cells were treated with 5, 15, 25, 35 and 45μg/ml of emodin under 72h, as indicated by increased tail length, the DNA damage was significantly observed in emodin treated cells compared with the control (Figure 20A and 20B). In addition, the DNA damage induced by emodin was dose-dependent. The comet assay results

in A549 and MCF-7 cells showed that emodin induced DNA damage which is one of the features of apoptosis.

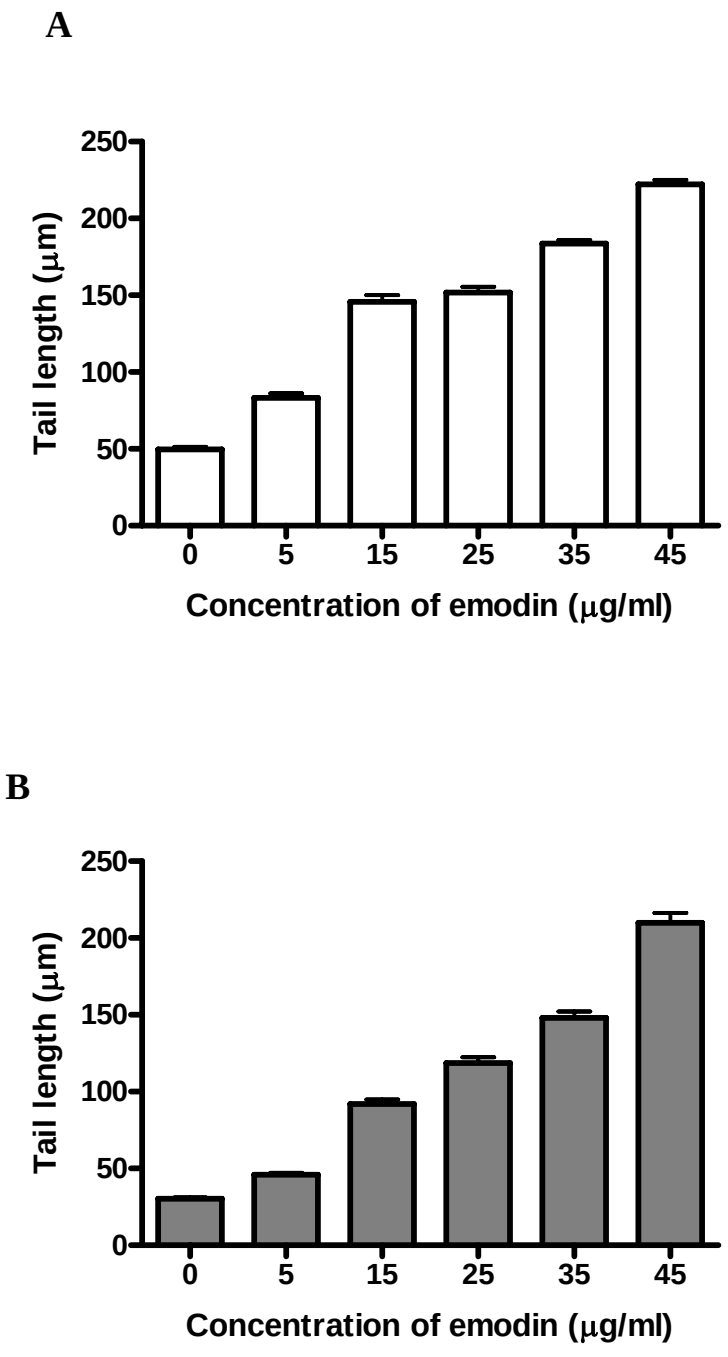


Figure 20. Effect of emodin (0,5, 15, 25, 35 and 45μg/ml) on DNA damage on (A) A549 cells and (B) MCF-7 cells after 72h-treatment represented as comet tail length (μm). Data are expressed as means ± S.E.M., n=100.

4.2.6 mRNA level of apoptosis-related genes

In order to obtain a molecular insight into the induction of apoptosis, the mRNA levels of some apoptosis-related genes including FASL, MCL1, GAPDH, BAX, CCND1 and C-MYC in A549 and MCF7 cells after emodin treatment were analyzed. RNA was extracted from emodin treated A549 and MCF-7 cells and reverse-transcripted into cDNA. The cDNA was then amplified by specific primers by real-time PCR to produce specific gene product. By comparing the mRNA levels of the genes between the control and the emodin treated samples, the effect of emodin on gene expression of the apoptosis-related genes could be examined. All the mRNA levels of genes were normalized to that of the internal standard RPS9.

From Table 4A, the fold change of the genes, FASL and GAPDH, were greater than 1.200, the genes MCL1, CCND1 and C-MYC were smaller than 1.200 and the gene BAX was between 0.800 and 1.200. It indicated that FASL and GAPDH were upregulated, MCL1, CCND1 and C-MYC were downregulated and the gene expression of BAX was not changed in A549 cells after treated with 60μM emodin. The same gene expression profile was observed in MCF-7 cells treated with 30μM emodin (Table 4B). Emodin affected the gene expression of the apoptosis related genes.

Table 4. Effects of (A) 60μM emodin on Gene expression of A549 cells and (B) 30μM emodin on Gene expression of MCF-7 cells after 72h-treatment.

A

Gene	Control		Emodin treated		$Gene_N$ relative to control[c] (Fold change[d])
	Relative copies of mRNA[a]	$Gene_N$ normalized to RPS9[b]	Relative copies of mRNA[a]	$Gene_N$ normalized to RPS9[b]	
FASL	0.707±0.0008	2.022±0.873	4.963±1.091	12.683±3.093	7.400±1.897***
MCL1	0.155±0.050	0.351±0.066	0.062±0.018	0.155±0.044	0.505±0.194
GAPDH	0.235±0.022	0.617±0.200	0.237±0.040	0.602±0.065	1.216±0.381
BAX	0.864±0.263	1.822±0.015	0.682±0.125	1.693±0.242	0.928±0.128
CCND1	0.409±0.050	1.045±0.298	0.257±0.033	0.659±0.119	0.713±0.214
C-MYC	0.163±0.015	0.432±0.144	0.114±0.011	0.285±0.020	0.762±0.156

B

Gene	Control		Emodin treated		$Gene_N$ relative to control[c] (Fold change[d])
	Relative copies of mRNA[a]	$Gene_N$ normalized to RPS9[b]	Relative copies of mRNA[a]	$Gene_N$ normalized to RPS9[b]	
FASL	0.381±0.047	2.034±0.684	3.682±0.131	60.708±20.326	40.866±21.397***
MCL1	0.477±0.145	2.475±0.834	0.029±0.009	0.390±0.021	0.217±0.900
GAPDH	0.054±0.020	0.356±0.222	0.012±0.003	0.289±0.047	1.528±0.617
BAX	0.299±0.037	1.745±0.722	0.133±0.004	1.243±0.176	0.964±0.304
CCND1	0.393±0.052	2.016±0.531	0.036±0.007	0.563±0.151	0.325±0.148
C-MYC	0.234±0.043	1.138±0.190	0.013±0.001	0.202±0.053	0.201±0.079

a . The relative copies of mRNA are determined by entering the threshold value (Ct) into the equation of standard curve of specific gene.

b. The normalized amount of mRNA of specific gene is determined by diving the amount of mRNA of specific gene by the amount of mRNA of RPS9.

c. The fold change of gene expression is determined by diving normalized amount of mRNA of specific gene after emodin treatment by the normalized amount of mRNA that gene of control.

d. The expression of gene is upregulated if the fold change is >1.200; no change if the fold change is between 0.800-1.200; downregulated if the fold change is <0.800.

Where the relative copies of mRNA of RPS9 of control are 0.472±0.142, that of 60μM emodin treated A549 cells are 0.400±0.024 and that of 30μM emodin treated MCF-7 cells are

0.074±0.021; data are expressed as means ± S.E.M., n=3. ***Differ significantly from control at $p < 0.001$.

4.3 Quantification of emodin in different species of Da Huang

4.3.1 HPLC chromatograms of different species of Da Huang

Four species of Da Huang were employed. They were labeled as D1, *Rheum tanguticum* Maximowicz (from Sichuan); D2, *Rheum palmatum* Linn (from Ganshu); D3, *Rheum tanguticum* Maximowicz (from Ganshu) and D4, *Rheum officinale* Baill. (from Sichuan). Results showed that emodin was eluted from column at about 11.5 minutes. From the HPLC chromatograms of D1 to D4, all of them possessed emodin but in different amount (Figure 21).

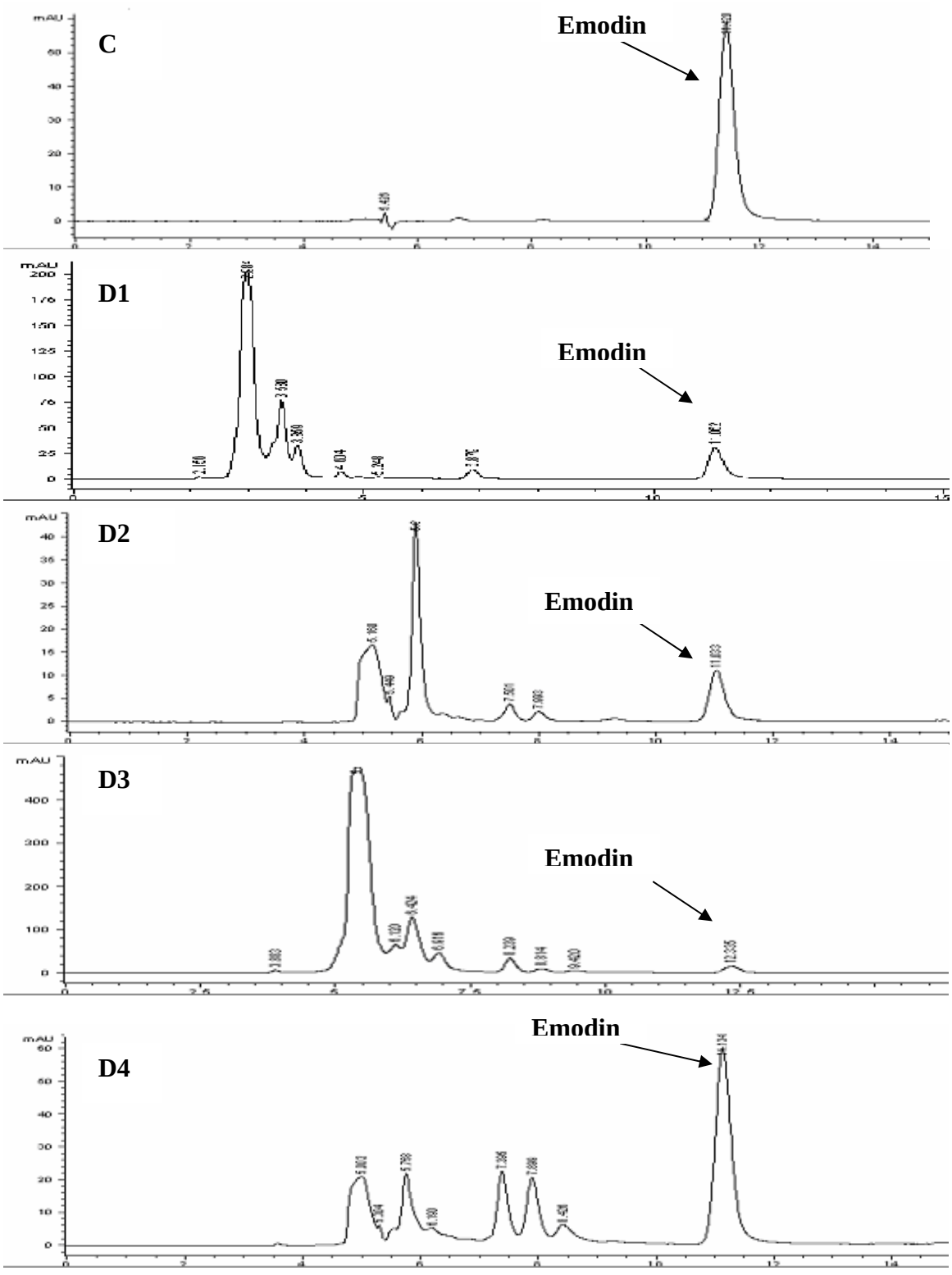


Figure 21. The HPLC chromatograms of four different samples labeled as D1 to D4. D1 was *Rheum tanguticum* Maximowicz; D2 was *Rheum palmatum* Linn; D3 was *Rheum tanguticum* Maximowicz and D4 was *Rheum officinale* Baill.

4.3.2 Emodin content in different species of Da Huang

All species of Da Huang we examined contained emodin but in different amount. After calculating the amount of emodin in each species of Da Huang, it was found that there was variation in the amount of emodin among these four species. D4 was found to have the highest amount of emodin among D1, D2 and D4 (Table 5).

Table 5. Content of emodin in Da Huang (Rheum) species.

***Rheum* species**	**Label**	**Emodin concentration (μg/ml)**
Rheum tanguticum Maximowicz	D1	2.74 ± 0.09
Rheum palmatum Linn.	D2	1.85 ± 0.03
Rheum officinale Baill.	D4	10.33 ± 0.53

Data were expressed as mean ± S.E.M., n=3-4.

Besides, variation was also found in the same species from different origins. By comparing the content of emodin in D1 and D3, it was found that the content of emodin was higher in D1 than D3 even through they were the same species but with different origins (Table 6).

Table 6. Content of emodin in *Rheum tanguticum* Maximowicz from different origins.

Origins	**Label**	**Emodin concentration (μg/ml))**
Sichuan	D1	2.74 ± 0.09 **
Gansu	D3	1.68 ± 0.02

Data were expressed as mean ± S.E.M., n=3–4, ** Differ significantly from D3 at $p < 0.01$.

By t-test analysis, the content of emodin in D1 and D3 was significantly different, indicating that the content of emodin in the Da Huang species might be affected by environmental factors such as climate and humidity.

4.3.3 Accuracy and reliability of the HPLC system and extraction method

To test the accuracy and reliability of the HPLC system, a calibration curve of standard emodin was constructed with concentration ranging from 1μg/ml to 20μg/ml (Figure 22) and inter-day and intra-day HPLC analysis was performed at any concentration of emodin on the standard curve. For inter-day and intra-day HPLC analysis, a point of 8μg/ml was chosen for assessment of accuracy and reliability.

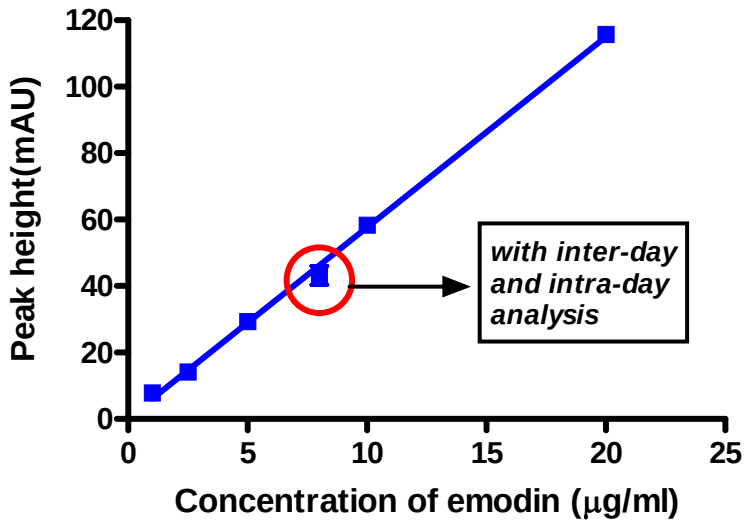


Figure 22. Calibration curve of emodin ranged from 1μg/ml to 20μg/ml. Data were expressed as means ± S.E.M., n=3.

By statistical analysis, the equation, r-squared value, test range, yield of recovery and limit of detection of the calibration curve of emodin could be calculated

(Table 7). From the table, the value of r-squared showed that the calibrate curve was linear and the detection limit was as low as 0.5μg/ml of emodin. Besides, the extraction method of the Da Huang species showed good recovery yield, which was as high as 76%.

Table 7. Information of calibration curve of emodin.

Equation	y = 5.7339x + 0.3547
r^2	0.9952
Test range (μg/ml)	1–20
Limit of detection (μg/ml)	0.5
Yield of recovery (%)	76.19 ± 1.387

By measuring the inter-day and intra-day variability of the HPLC analysis, the accuracy for inter-day variability was about 88% and for intra-day variability, it was about 98%. The values of the relative standard deviation (RSD) were small, indicating that the HPLC system was highly reliable (Table 8).

Table 8. Calculations of reliability and accuracy of intra- and inter-day for the emodin quantification.

Emodin (8μg/ml)	**Intra-day variability**	**Inter-day variability**
Detected (n=3)	8.18±0.39	6.99 ± 0.73
RSD[a] (%)	4.72	10.40
Accuracy[b](%)	97.780	87.435

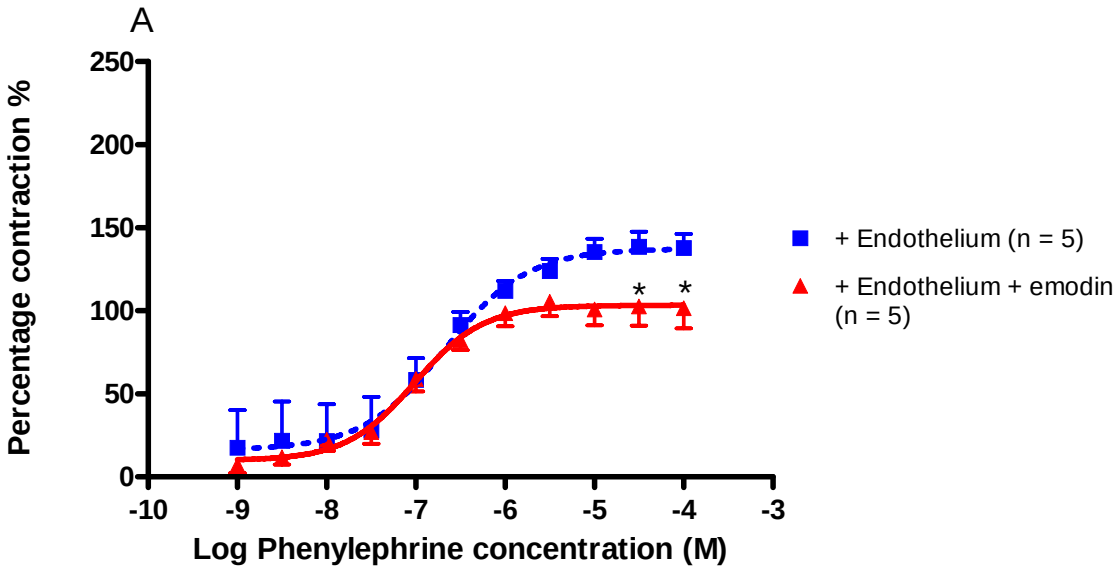
[a]RSD (%) (relative standard deviation) = (Standard deviation/mean)x100.

[b]Accuracy (%) = [1– (mean concentration measured – concentration injected)]/ concentration injected] x 100.

4.4 Examination of vasorelaxant effect of emodin

4.4.1 Effect of emodin on tension development of aorta rings with or without endothelium

To investigate whether emodin could induce vascular relaxation, the endothelium intact and denuded aorta were pre-contracted with phenylephrine. Results showed that emodin (10μM) could induce vascular relaxation on both endothelium intact (Figure 23A) (p<0.05) and denuded aorta (Figure 23B) (p<0.01) and it could significantly suppress the contractile response of aorta induced by phenylephrine.



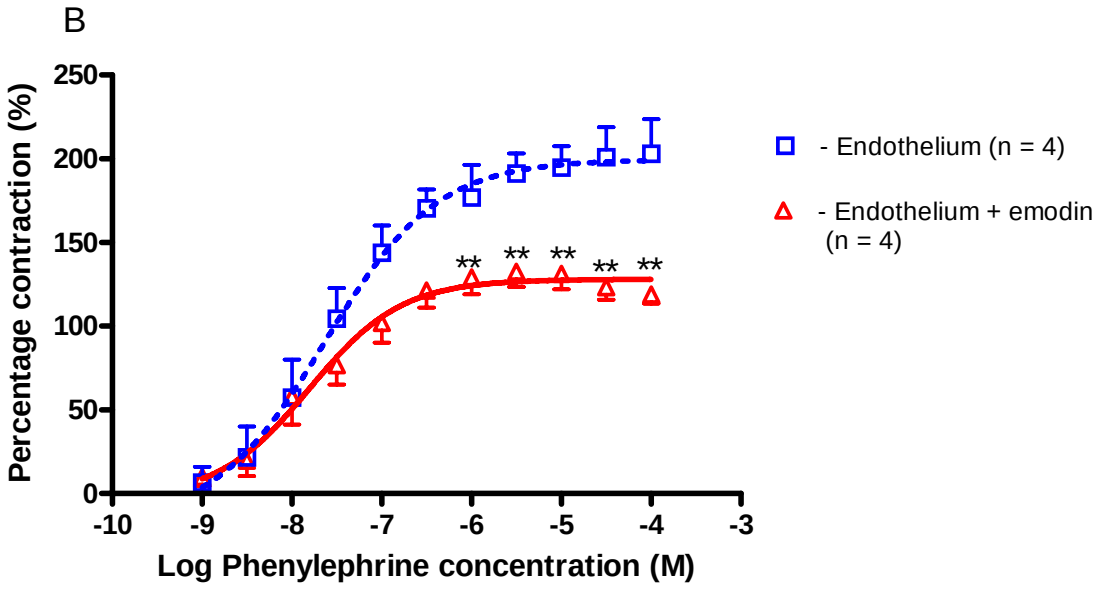


Figure 23. Suppressing effect of emodin (10μM) on phenylephrine-induced vascular contraction on (A) endothelium intact and (B) endothelium denuded aortas. Data are expressed as means ± S.E.M., n=4–5. *Differ significantly at $p<0.05$;**Differ significantly at $p<0.01$.

By measuring the relaxant effect of emodin, endothelium intact and denuded aortas were pre-contracted with phenylepherine and treated with cumulative concentration of emodin. Result showed that emodin exerted a greater relaxant effect on phenylephrine pre-contracted endothelium intact aorta than that of endothelium-denuded aorta (Figure 24A). By measuring the suppressing effect of emodin on phenylepherine-induced contraction, endothelium intact and denuded aortas were pre-treated with emodin followed by cumulative concentrations of phenylepherine. Result showed that emodin exerted a greater suppressing effect on phenylephrine-induced contraction on endothelium intact aorta than that of endothelium denuded aorta (Figure 24B).

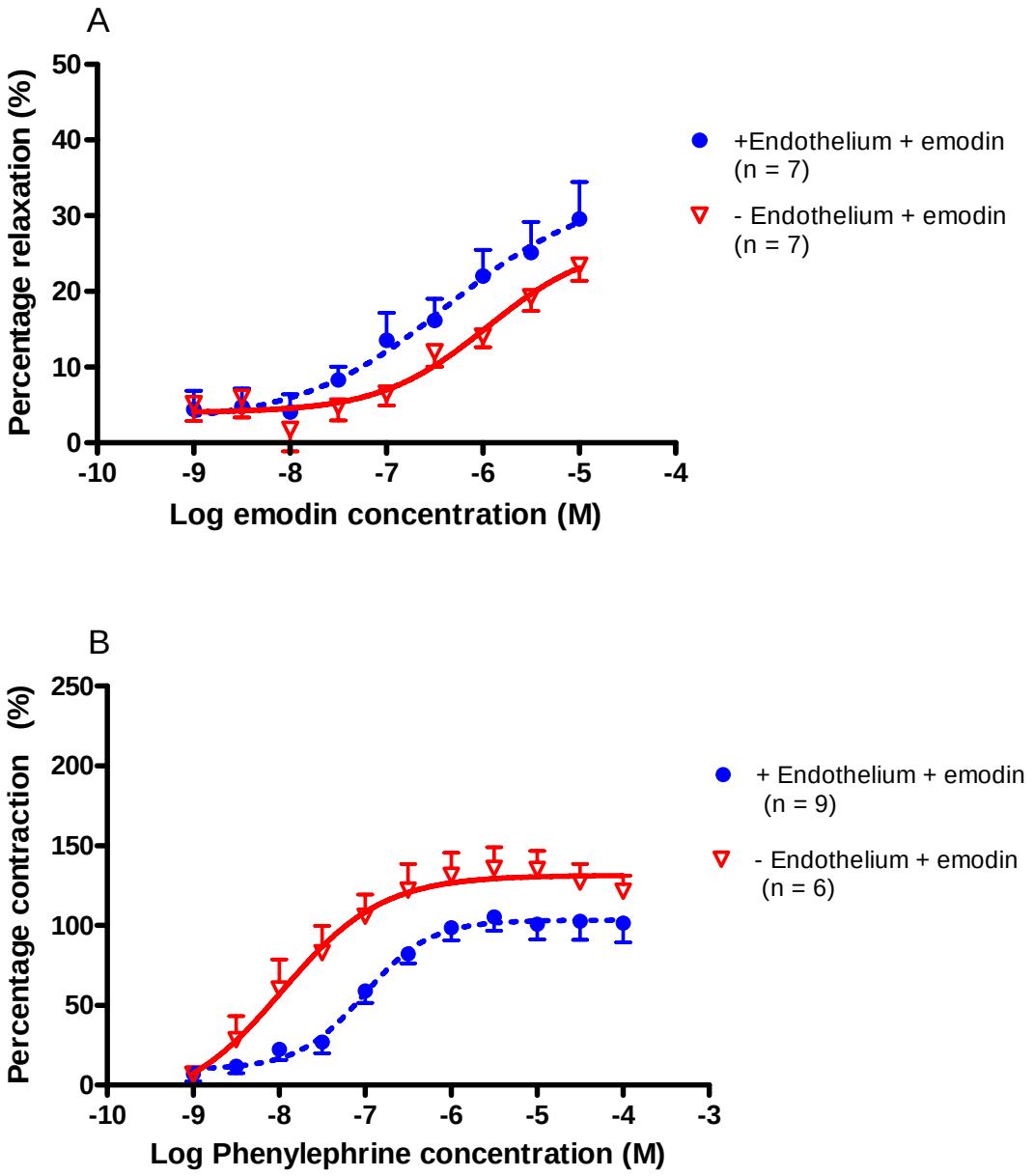


Figure 24. The vasorelxant effect of emodin on phenylephrine-induced vascular contraction on endothelium intact and denuded aorta. (A) The relaxant effect of emodin on phenylephrine pre-contracted endothelium intact and denuded aortas. (B) The suppressing effect of emodin on contractile response of emodin pre-treated endothelium intact and denuded aortas. Data are expressed as means ± S.E.M., n= 6–9.

4.4.2 Examination of mechanism of emodin-induced vascular relaxation

4.4.2.1 Effect of endothelial mediator on emodin-induced vascular relaxation

From a previous study, Emodin-induced vascular relaxation found to be concentration dependent. Emodin showed greater relaxant effect on phenylephrine pre-contracted endothelium intact aorta than that of endothelium-denuded aorta, suggesting that emodin-induced vascular relaxation might be endothelium dependent. However, the presence of endothelium might release nitric oxide, a vasorelaxing factor, which would result in vascular relaxation. Thus, to investigate whether the emodin-induced vascular relaxation was endothelium dependent, a nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) was added to inhibit the release of nitric oxide from endothelial cells.

Results showed no significant difference in emodin-induced vasorelaxant effect between L-NAME pretreated endothelium intact aorta and untreated endothelium-denuded aorta (Figure 25). It indicated that emodin-induced vascular relaxation was concentration-dependent but endothelium- and nitric oxide- independent.

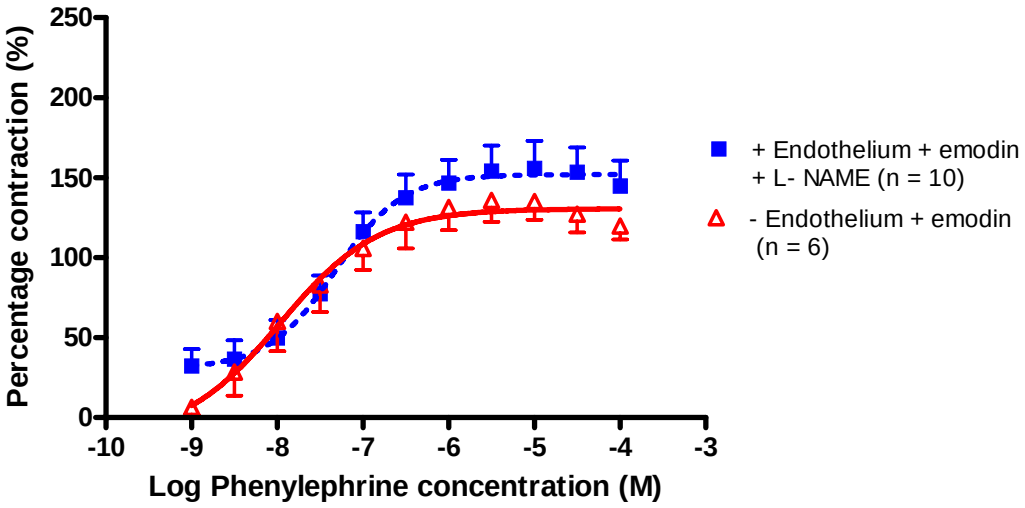


Figure 25. Suppressing effect of N^G-nitro-L-arginine methyl ester (L-NAME) (20μM) on phenylephrine-induced vascular contraction on endothelium intact aortas. Data are expressed as means ± S.E.M., n= 6–10.

4.4.2.2 Effect of potassium channel blocker on emodin-induced vascular relaxation

Results showed that the ATP-sensitive potassium ion channels (K_{ATP}) inhibitor, glibenclamide (GA) (3μM), and the calcium-activated potassium ion channel (K_{Ca}) blocker, tetraethylammonium (TEA) (1mM) could not completely reduce the emodin-induced vascular relaxation on phenylephrine pre-contracted endothelium intact aorta (Figure 26). It indicated that both types of potassium ion channel blocker were involved in the vascular relaxation induced by emodin. Since emodin-induced vascular relaxation is potassium ion channel dependent, it was believed that emodin might affect the flow of calcium ions or the openings of calcium ion channels.

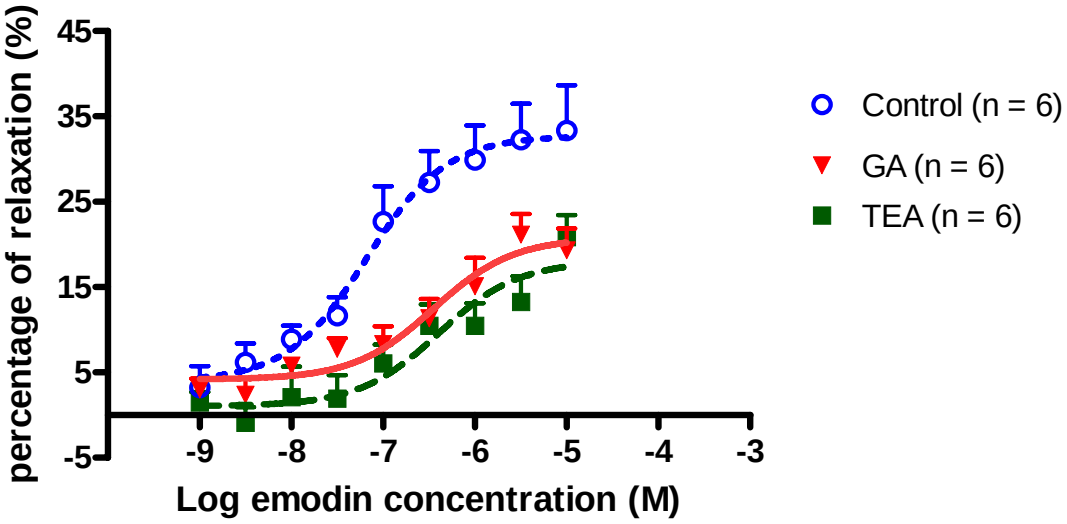


Figure 26. Suppressing effect of the potassium ion channel blockers, glibenclamide (GA) (3μM) and tetraethylammonium (TEA) (1mM) on the percentage of emodin (1nM–10μM) induced relaxation on endothelium intact aorta. Data are expressed as means ± S.E.M., n=6.

4.4.2.3 Effect of emodin on extracellular calcium ion-induced vasoconstriction

Results showed that both verapamil and emodin suppressed the calcium chloride induced contraction on endothelium intact aorta in similar extent (Figure 27A and 27B). They produced a significant antagonism with calcium ions and caused a shift of the contractile curve to the right and downward.

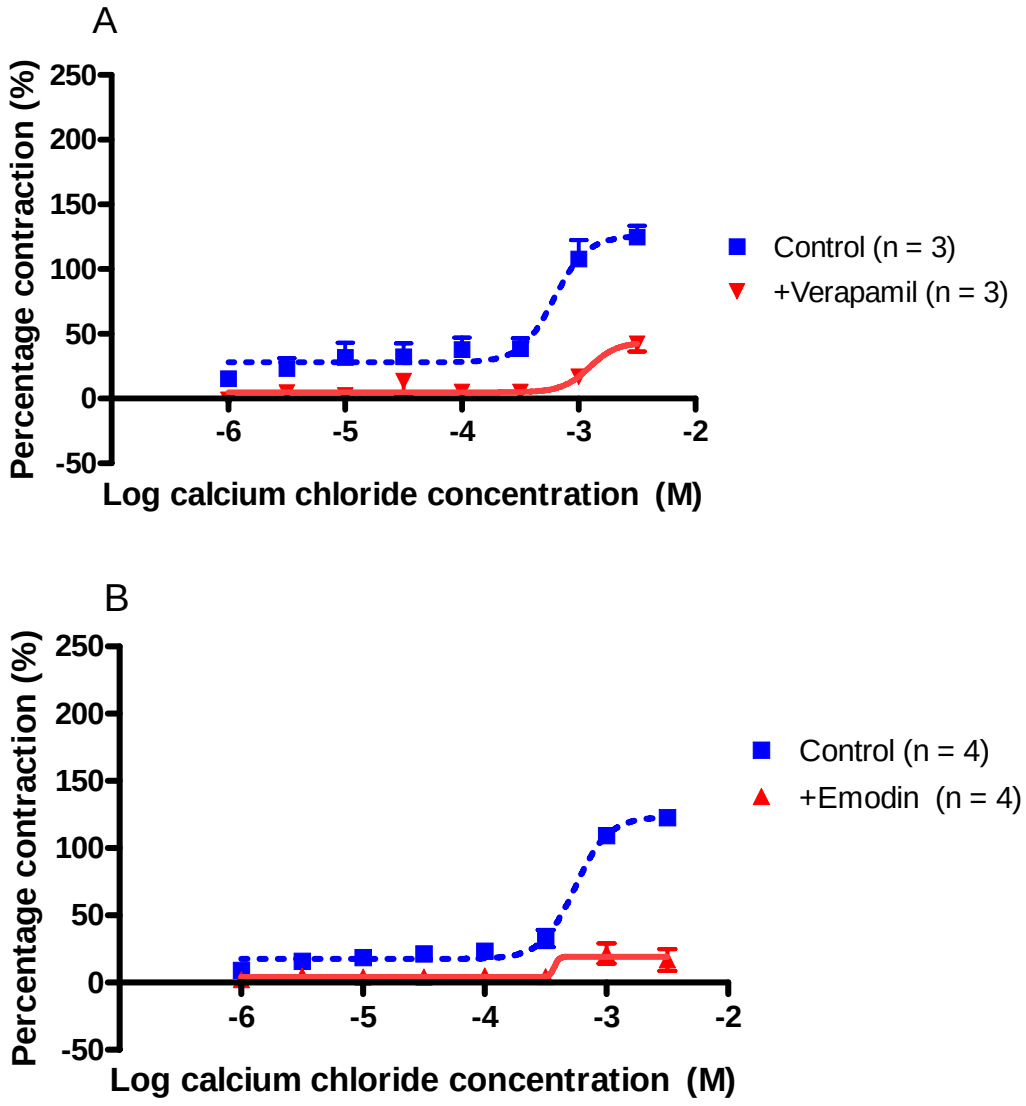


Figure 27. Suppressing effect of (A) verapamil (0.1μM) and (B) emodin (33μM) on calcium chloride ($CaCl_2$) induced contraction on endothelium intact aorta. Data are expressed as means ± S.E.M., n=3–4.

Both of verapamil and emodin exerted a higher suppressing effect than the control on calcium chloride-induced contraction. However, there was no significant difference in suppressing effect between the two compounds (Figure 28). It indicated that emodin could induce vasorelaxation by working as calcium ion channel blocker like verapamil.

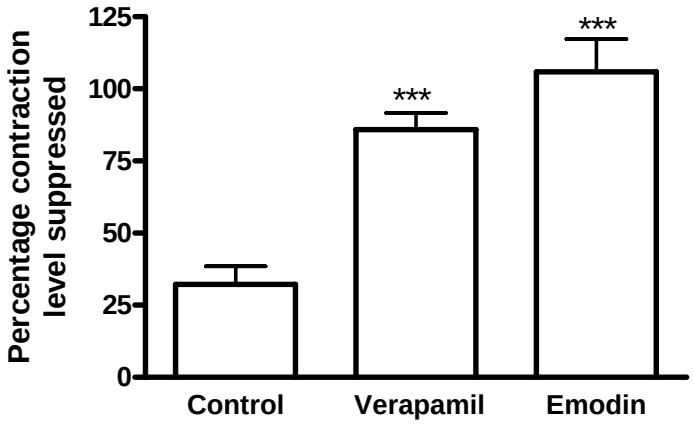


Figure 28. Comparison of suppressing effect of verapamil (0.1μM) and emodin (33μM) on calcium chloride-induced contraction with control. Data are expressed as means ± S.E.M., n=3–4. ***Differ significantly from control at $p < 0.001$.

5 DISCUSSION

The present study provides the evidence to show Da Huang (Rhubarb, *Radix et Rhizoma Rhei*) water extract had anticancer effect on human lung adenocarcinoma A549 cells and human breast carcinoma MCF-7 cells, confirmed by the inhibition of cancer cell growth, colony formation, cell shrinkage, DNA fragmentation and DNA damage. The study also suggests emodin might be an active component with anticancer effect. Da Huang water extract exhibits growth inhibitory effect on A549 and MCF-7 cells, and emodin has been shown to be an active component in the Da Huang species and exhibited on overall similar anticancer effect. The study shows that emodin inhibits the growth of A549 cells and MCF-7 cells by regulating the gene expression level of the apoptosis-related genes. Besides, emodin was found to have vasorelaxant effect on rat's aorta. The present study suggests the possible mechanisms involved in its anti-hypertensive effect.

5.1 Examination of anticancer effect of Da Huang water extract

Chinese herbal medicines have long been used in Asia. They are usually cooked on a low boil for an hour or more and consumed as a liquid decoction. Sometimes, Chinese herbal medicines are immersed in alcohol as a health supplement. In this study, Da Huang herb was extracted by distilled water based on the traditional method of preparing Chinese herbal medicines. Patients usually take Chinese herbal medicines in a liquid form and it is believed that the active components of Da Huang herb are water-soluble and may synergistically act with one another. Da Huang was not extracted by hot water because heat might cause decomposition or degradation of the active components.

Da Huang is a herb widely grown in many provinces of China and has been used as laxative (Tsai *et al*., 2004), antiphlogistic (Moon *et al*., 2006; Wu, 1985) and hemostatic (Wang and Jiao, 1985) in the treatment of obstipation, gastrointestinal indigestion, diarrhea, and jaundice. Da Huang has an ability to inhibit cancer cell growth. One study reported that bushen huayu jiedu recipe had antitumor activity on hepatocarcinoma and Da Huang was one of the components of the recipe (Cao *et al*., 2005). There were few reports on the anticancer effect of Da Huang water extract on human lung adenocarcinoma A549 cell line and the human breast carcinoma MCF-7 cell line.

In this study, the in vitro anticancer activities of the Da Huang water extract on A549 and MCF-7 cells were investigated. To elucidate in more details how the

Da Huang water extract exert its anticancer effect, the growth, colony formation, morphology and chromosomal DNA fragmentation and single DNA strands breaks of the Da Huang water extract treated cancer cells were monitored.

Da Huang water extract was demonstrated to have significant inhibitory effects on cancer cell growth and colony formation, and can induce cancer cell apoptosis. The present study confirmed that the Da Huang water extract had strong dose- and time- dependent anticancer activity against A549 and MCF-7 cells (Figure 8), with IC_{50} at 693μg/ml and 583μg/ml, respectively (Figure 7). The Da Huang water extract also inhibited the colony growth potential and decreased the colony size of A549 and MCF-7 cells in a dose-dependent manner (Figure 10 and 11).

Inhibiting tumor cell growth and inducing tumor cell death are two major parameters to inhibit tumor growth, and in turn the growth of cancer. In this study, the Da Huang water extract showed significant inhibition on the growth of human lung adenocarcinoma A549 cell line and human breast carcinoma MCF-7 cell line (Figure 7). These cancer cell lines were derived from different tissues of the body. The growth inhibitory effect of the Da Huang water extract indicates that it has a general function in anti-tumor cell growth.

In fact, tumor pathogenesis and progression involve several signaling pathways and molecular mechanisms. These include elevated external growth factors, STAT proteins, lipoxygenase (LOX), chemokines, mitogen-activated protein (MAP) kinases (Aggarwal and Shishodia, 2006), Ras protein, serine-threonine kinases (PKC), protein kinase B (PKB) and proteasome (Roberto *et al*., 2006). Abnormal growth signaling pathways and regulation of these molecules lead to

the invasion and destruction of the neighboring tissues and metastases. These finally result in cancer with cell proliferation, differentiation and survival of tumor cells. Cancer may result from several abnormal growth signaling pathways but one pathway may play a more important role than the others in specific tumor.

In this study, the Da Huang water extract exhibited growth inhibitory effect on both A549 and MCF-7 cells, and their growth were anchorage-dependent. This implies that the Da Huang water extract may interfere with the interaction of tumor cells.

Besides, A549 and MCF-7 cells showed different sensitivities to treatment of the Da Huang water extract. MCF-7 cells were more sensitive to the growth inhibitory effect of Da Huang water extract than A549 cells. A smaller amount of the Da Huang water extract was required to cause a 50% growth inhibition on MCF-7 cells than that of A549 cells (Figure 7). These results imply that the Da Huang water extract may act on more than one pathway in carcinogensis. Since different signaling pathways are involved in the development of A549 and MCF-7 cells.

The first phase of cancer occurs when normal cells mutate to tumor cells. They divide and grow continuously in an uncontrolled manner and finally form a tumor. To investigate how the Da Huang water extract affects the tumorigenicity of A549 and MCF-7 cells in vitro, soft agar colony formation assay was used. Soft agar colony formation assay is a more sensitive parameter of toxicity then cell viability to determine the growth and drug sensitivity of cancer cells,

because the number and the size of colonies are assessed when the cells are in the state of proliferation and are thus more sensitive to any toxic agents. Soft agar colony formation assay was carried out by using a semisolid agarose bilayer in 6-well plates and involved counting of colonies relative to the control under microscope (Wylie and Bowen, 2005). In this study, A549 and MCF-7 cells were grown in the soft agar and no Da Huang water extract was added to the control. The Da Huang water extract inhibited colony formation and reduced size of colonies of both A549 and MCF-7 cells in a dose-dependent manner (Figure 10 and 11). This implies that Da Huang water extract could inhibit the tumorigenicity of A549 and MCF-7 cells. The growth inhibitory effect of Da Huang water extract on colony formation explains its anti-cancer effect in clinical application.

Apoptosis is the controlled elimination of excess and unwanted cells which including damaged and infected cells under normal physiological condition. It is an important process in the structural development of the embryo and tissues homeostasis in the adult animal. Any dysregulation of apoptosis can result in abnormality, disease and death (Wilson *et al*., 1998). Cancer is a result of uncontrolled cell proliferation as well as the dysregulation of apoptosis. Most cancer cells are resistant to various mechanisms that lead to cell death. To kill cancer cells, the agent should be able to induce the apoptosis of tumor cells. Da Huang water extract is believed to induce apoptosis on A549 and MCF-7 cells because several features of apoptosis, such as DNA fragmentation (Figure 12) and DNA strand-breakage (Figure 13), were observed.

At this stage, our data suggest that Da Huang water extract had anti-tumor effect and might induce apoptosis on A549 and MCF-7 cells. It is not possible to say which compounds contained in the Da Huang water extract are responsible for the observed effects. However, our data suggest that the biological effects of Da Huang water extract under these experimental conditions could be related to the overall effects of its active component, emodin evidenced in this extract. The anti-tumor activity of emodin has been widely reported in literature.

5.2 Examination of anticancer effect of emodin

To have a successful cancer treatment, chemotherapeutic agents should have the ability to induce cell death in the tumor cells because poor prognostic outcome of human lung and breast cancers is greatly due to their resistance to the current cancer therapies. Thus, development of novel inducers of apoptosis is of importance to provide a new therapeutic approach for any anti-cancer design.

CHM have been utilized for thousands of years and several previous studies demonstrated the potential use of their phytochemicals as chemotherapeutic agents in cancer treatment. Usually, phytochemicals present in medicinal herbs such as berberine (Letasiova *et al*., 2006), guieranone A (Fiot *et al*., 2006) and saikosaponin D (Hsu *et al*., 2004) exert anti-tumorigenic activity by affecting the subcellular signaling pathways and, induction of cell-cycle arrest and apoptosis in cancer cells.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a major anthraquinone usually found in the species of *Rheum* such as Da Huang. Emodin has diverse pharmacological effects and its anti-cancer effects were widely reported. Emodin has been reported to inhibit proliferation of several human cancer cell lines (Li *et al*., 2005; Lee, 2001; Huang *et al*., 2004a; Chen *et al*., 2002; Srinivas *et al*., 2003; Huang *et al*., 2004b) through different molecular mechanisms.

In this study, in vitro the anticancer effects of emodin on other human cancer cell lines including human lung adenocarcinoma A549 cell line and human breast carcinoma MCF-7 cell line were investigated through monitoring the growth, colony formation, morphology, chromosomal DNA, the mRNA level of apoptosis-related genes of the emodin treated cancer cells. The study demonstrated that emodin had significant effects on inhibiting cancer cell growth, colony formation and inducing cancer cell apoptosis through the modulation of the expression of apoptosis related genes.

The present study confirmed that emodin had strong dose- and time- dependent anticancer activity against A549 and MCF-7 cells (Figure 15), with IC_{50} at 16.85μg/ml (60μM) and 7.22μg/ml (30μM), respectively (Figure 14). Emodin also inhibited the colony growth potential and decreased the colony size of A549 and MCF-7 cells in a dose-dependent manner (Figure 17 and 18).

Inhibiting tumor cell growth is one of the major means for successful cancer treatment. In this study, we demonstrated that emodin had significant inhibition on the growth of A549 and MCF-7 cells (Figure 14 and 15). This implies that

emodin has a general function in anti-tumor cell growth because A549 and MCF-7 cell lines were derived from different tissues of the body. Furthermore, emodin may interfere with the interaction of A549 and MCF-7 cells since their growth were anchorage-dependent.

A549 and MCF-7 cells also showed different sensitivities to emodin and MCF-7 cells were more sensitive to emodin than that of A549 cells (Figure 14). This implies that emodin may act on more than one pathway involved in carcinogensis because different signaling pathways are involved in the development of A549 and MCF-7 cells.

Inhibiting the formation of tumor is important in cancer treatment. To elucidate how emodin affects the tumorigenicity of A549 and MCF-7 cells in vitro, soft agar colony formation assay was performed. The number of colonies observed in the soft agar culture with emodin added relative to the control were counted and monitored under microscope. It was found that emodin inhibited colony formation and reduced the size of colonies of both A549 and MCF-7 cells in a dose-dependent manner (Figure 17 and 18). This implies that emodin could inhibit the tumorigenicity of A549 and MCF-7 cells and also explains its clinical importance for cancer treatment.

Apoptosis and necrosis are two important types of cell death. Apoptosis refers to the programmed cell death occurs in physiological condition. It is characterized by cell shrinkage, condensation of the cytoplasm and nuclear chromatin, fragmentation of the cells into a cluster of membrane-bound structure, formation of apoptotic bodies and break up of DNA at the internucleosomal spaces into oligome fragments. Necrosis is another type of cell death. It refers to the

progressive and complete degradation of cell structure that occurs after death. Various stimuli such as hypoxia, viral infection and corrosive chemicals induce necrosis through damaging the cellular membrane irreversibly. Necrosis is characterized by a marked swelling of mitochondria, and the appearance of dense strictures in their matrix followed by the progressive dissolution of the entire cell (Figure 29).

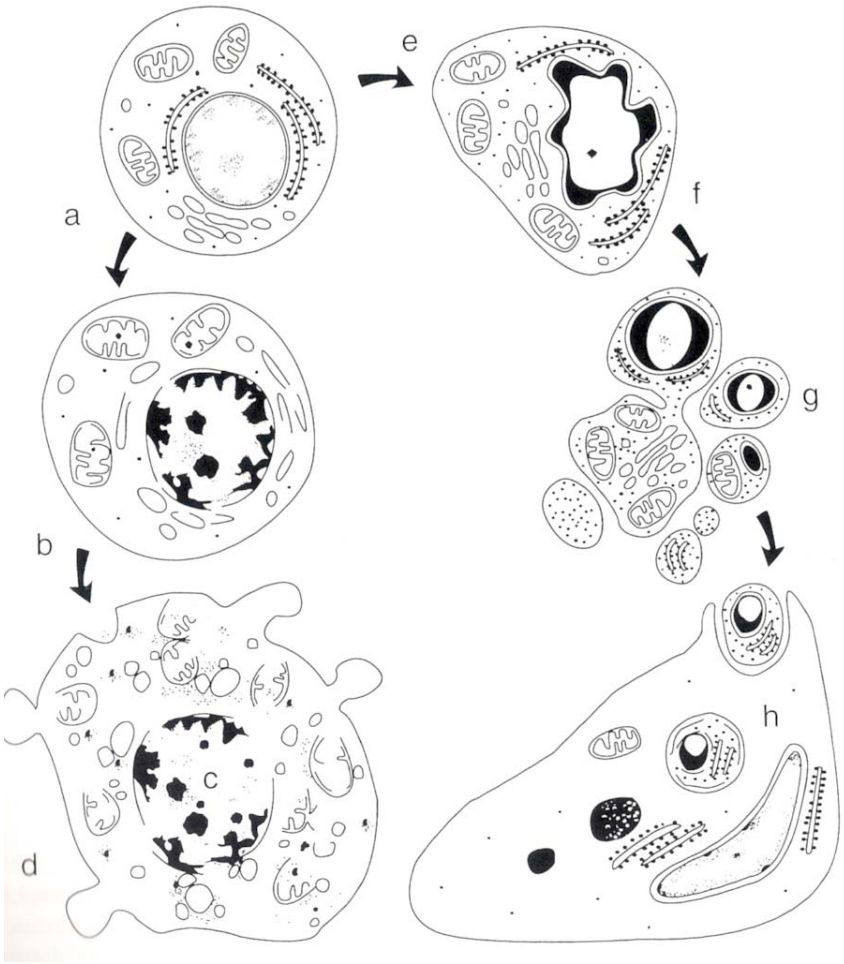


Figure 29. Mechanism of necrosis or apoptosis. In necrosis, the first step is (a) an increase of intracellular volume, mitochondrial swelling (b) followed by vacuolization, dilatation of endoplasmic reticulum, blebbing, increased permeability, and condensed nuclei, (c) coagulation and karyolusis, (d) elimination of the cell by inflammation and phagocytosis, (f) followed by

budding and karyorrhexis and (g) break up and formation of apoptotic bodies which (h) may be destroyed by phagocytosis by macrophages (Cameron and Feuer, 2000).

Apoptosis and necrosis plays a fundamental role in the regulation of normal tissue turnover in most pathological processes through eliminating all debris formed from the aged and dying cells (Cameron and Feuer, 2000). In a healthy state, cell degeneration and cell death are balanced by cell renewal or proliferation. Any imbalance between these two processes leads to many diseases such as cancer, which is a result of uncontrolled cell proliferation and abnormal regulation of cell death. Thus, anti-cancer agents should activate apoptosis in tumor cells.

In this study, the morphology of the emodin treated A549 and MCF-7 cells was monitored under microscope, their chromosomal DNA were monitored in 1.5% agarose gel with ethidium bromide under an UV illuminator and any DNA strand breakage at a singe cell level was monitored by comet assay. The emodin treated A549 and MCF-7 cells showed cell shrinkage (Figure 16), DNA fragmentation (Figure 19) and internucleosomal DNA damage at single cell level (Figure 20) were observed. These morphological changes were hallmarks of apoptosis. It also indicates that emodin could be able to activate the activity of the DNA endonuclease on the double-strand cleavage of chromosomal DNA into fragments.

Similar observations have been reported in the emodin-treated human lung adenocarcinoma LACC cells (Li *et al*., 2005), human lung squamous cell carcinoma CH2 (Lee, 2001.), human hepatoma cell lines HepG2 and C3A (Huang *et al*., 2004a), human promyeloleukemic HL-60 cells (Chen *et al*., 2002),

human cervical cancer Bu 25TK cells (Srinivas *et al.*, 2003), and human cancer HSC5 and MDA-MB-231 cells (Huang *et al.*, 2004b). In addition, the present data showed that non-tumor cells such as mouse fibroblast L929 cells were more resistant to emodin-induced cytotoxic effect with IC_{50} at greater than 35μg/ml (Figure 21). Similar observations have been reported in emodin treated non-tumor cells including human fibroblast-like lung WI-38 cells, rat heart endothelial cells, rat hepatic stellate cells, and rat hepatocytes (Su *et al*., 2005).

The present results do not agree with the results reported by Matsuda *et al.* (2001) and Demirezer *et al.* (2001). They claimed that emodin enhanced the proliferation or showed no cytotoxic effect to MCF-7 cells. However, they did not mention the methods to investigate the effect of emodin on MCF-7 cells. The differences in the findings may be due to the different sources, purities and concentractions of emodin applied to MCF-7 cells. Nevertheless, this is the first report showed the antiproliferative effect of emodin on MCF-7 cell.

By observing the overall biological effects of emodin, it is coincident with the findings that Da Huang water extract inhibited the proliferation of A549 and MCF-7 cells. Thus, it is believed that the anticancer effect of Da Huang water extract is related to the overall biological effect of emodin evidenced in it.

In fact, apoptosis can be triggered by a variety of physiological and stress stimuli that initiate one or several distinct signaling pathways by gene regulation. These stimuli may alter the gene expression that activates various signaling pathways in apoptosis. The most common apoptotic pathways are the mitochrondrion (intrinsic), the death receptor (extrinsic) and the p53-mediated pathways. The

activation of various apoptotic pathways activates a common effector mechansim, caspase casade, started at caspase-3. This causes structural alterations that are typical of apoptosis (Figure 30). Activation of a specific pathway in apoptosis is greatly dependent on the types of cells and stress.

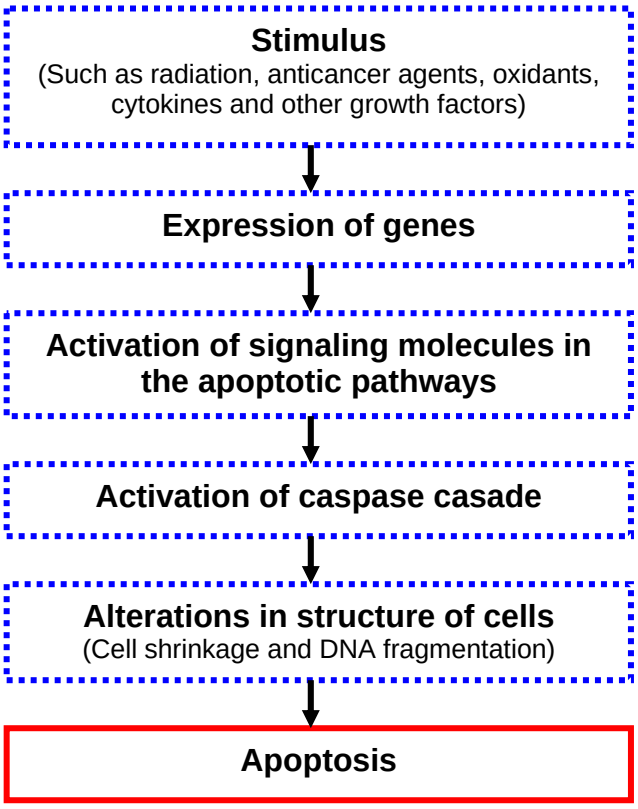


Figure 30. Schematic illustration of various steps of apoptosis.

To elucidate how emodin induce apoptosis in A549 and MCF-7 cells, quantitative real-time PCR using SYBR Green dye was performed. The mRNA level (expression) of genes including FASL, MCL1, GAPDH, BAX, CCND1 and C-MYC involved in apoptosis were investigated. All of the gene expression was normalized to the reference gene, RPS9.

In the present study, the mRNA level of FasL was significantly elevated in A549 cells (Table 4A) and MCF-7 cells (Table 4B) after 72hr-treatment of emodin when compared with the control. FasL is a gene which encods Fas ligand (CD95 ligand). Fas ligand is a membrane glycoprotein which belongs to the tumor necrosis factor family (TNF family) involved in the extrinsic pathways of apoptosis. Upregulation of gene expression of FasL enhanced apoptosis through the extrinsic pathway. Bindings of the Fas ligands to Fas receptors cause trimerization of Fas. The trimerized cytoplasmic region of Fas receptor recruits FADD.

The death effector domain of FADD serves as adaptor molecules to recruit procaspase-8 and form death-inducing signal complex (DISC). The activated caspase-8 then activates caspase-3 that finally leads to the cutting of some target proteins such as the inhibitor of capase-activated deoxyribonuclease (ICAD) presented in the inactive cytosolic complex and/or nuclear complex. Cleavage of capase-activated deoxyribonuclease (ICAD) and other proteins by caspase casade cause internucleosomal DNA fragmentation and other structural alternation (Cameron and Feuer, 2000). It implies that emodin could inhibit the growth of A549 and MCF-7 cells through the Fas-mediated apoptotic pathway.

Similar observations have been reported in emodin treated human hepatoma cells (Shieh *et al*., 2004), platycodin D treated keratinocytes (Ahn *et al*., 2006) and safrole oxide treated human lung cancer A549 cells (Du *et al*., 2006).

MCL1 and BAX belong to member of the Bcl-2 family of proteins. MCL1 is a gene which refers to the myeloid cell leukemia sequence 1. It is an apoptotic B cell leukemia-2 (Bcl-2) homologue that plays a key role in the regulation of the

cell death process with particular members such as Bcl-2. It serves as an anti-apoptotic activator that inhibits apoptosis through blocking cytochrome *c* released from the mitochondria in intrinsic pathway. Thus, down regulation of MCL1 expression leads to apoptosis. It can be observed in hydroxymethylglutaryl-coenzyme A reductase inhibitors treated human cardiac myocytes (Demyanets *et al*., 2006), cinnamaldehyde treated human PLC/PRF/5 cells (Wu *et al*., 2005) and leptomycin B treated U937 leukemia cells (Jang *et al*., 2004).

BAX is a gene which encodes BCL2-associated X protein that forms a heterodimer with BCL2. BAX functions as an apoptotic activator and regulates apoptosis in the intrinsic pathway. Activation of BAX enhances the release of cytochrome c from mitochondria and the loss in membrane potential. It is due to the interaction of BAX with the mitochondrial voltage-dependent anion channel and its effect on increasing the opening of the mitochondrial voltage-dependent anion channel. The expression of BAX is regulated by the tumor suppressor p53 and BAX involves in p53-mediated apoptosis. Upregulation of BAX expression results in apoptosis. It can be observed in beauvericin treated human lung cancer (Lin *et al*., 2005), a polysaccharide, from the loach *Misgurnus anguillicaudatus* (MAP), treated human hepatocellular carcinoma cells (Zhang and Huang, 2006), curcumin treated human ovarian cancer cells (Shi *et al*., 2006), puerarin treated colon cancer HT-29 cells (Yu and Li, 2006) and squamocin treated T24 bladder cancer cells (Yuan *et al*., 2006).

In the present study, emodin induced the down-regulation of MCL1 expression but did not change the gene expression of BAX in A549 and MCF-7 cells after

72h-treatments (Table 4). Emodin induced the down-regulation of MCL1 expression promotes cytochrome c release froms mitochrondria in the intrinsic pathway. This results in the formation of a complex in the cytoplasm that activates caspase-9 and forms apoptosome. The apoptosome then activates caspase-3 and in turn caspase cascade and degradation (Harrison and Link, 2003; Gross *et al*., 1999; Burlacu, 2003).

These results do not agree with the results of Su *et al*. (2005) and Lee (2001) in which an up-regulation of the BAX expression was observed after emodin treatment on human lung cancer cells. It might be due to treatment with different concentrations of emodin under different incubation periods. However, the data agree with the results of Chen *et al*. (2002) which showed the expression of BAX gene remained unchanged in emodin-treated human promyeloleukemic HL-60 cells.

Interestingly, tumor suppressor p53 is a direct transcriptional activator of the human BAX gene and BAX plays an important role in the p53-mediated apoptosis (Zhan *et al*., 1994; Miyashita and Reed, 1995). To elucidate whether the expression of BAX is regulated by p53, Miyashita *et al*. (1994) examined the expression of BAX in p53-deficient cells. They obtained evidence that low expression of bax was observed in p53 deficient cells. To further confirm the important role of BAX in p53-dependent apoptosis, McCurrach *et al*. (1997) investigated the effects of BAX deficiency in primary fibroblasts expressing the E1A oncogene, a setting where apoptosis is dependent on endogenous p53. They demonstrated that BAX could function as an effector of p53 in chemotherapy-induced apoptosis and contributed to a p53 pathway in suppression of oncogenic

transformation. It implies that emodin did not affect gene expression of tumor suppressor p53. Emodin might induce apoptosis in A549 and MCF-7 cells through modulation of MCL1 gene in the intrinsic apoptotic pathway but independent of the p53-mediated apoptotic pathway.

GAPDH is a gene which encoding glyceraldehyde-3-phosphate dehydrogenase, a key enzyme, and catalyzes an important energy-yielding step in glycolysis, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide. It is an abundant RNA species so it is often used as a potential internal RNA standard or housekeeping gene. However, it has been reported that a wide variation in expression of GAPDH was observed in different tissues, insulin, dexamethasone and mitogens treated cells, virally transformed or oncogene-transfected fibroblasts and human pancreatic or colon adenocarcinoma (Mogal and Abdulkadir, 2006).

In addition, recent studies suggested that GAPDH exhibits diverse nonglycolytic functions depending on its subcellular localization. One of the most intriguing was the induction of apoptosis. Overexpression of GAPDH was reported to induce apoptosis through the initiation of one or more apoptotic cascades (Ishitani *et al*., 2003). It is the first report that emodin upregulated GAPDH expression A549 (Table 4A) and MCF-7 (Table 4B) cells after 72hr-treatment. It implies that emodin-induced apoptosis is GAPDH dependent.

Any cell cycle arrest may lead to the induction of apoptosis. CCND1 refers to the cyclin D1 gene. The protein encoded by CCND1 belongs to the highly conserved

cyclin family, whose members are characterized by a dramatic periodicity in abundance of protein throughout the cell cycle (Figure 31).

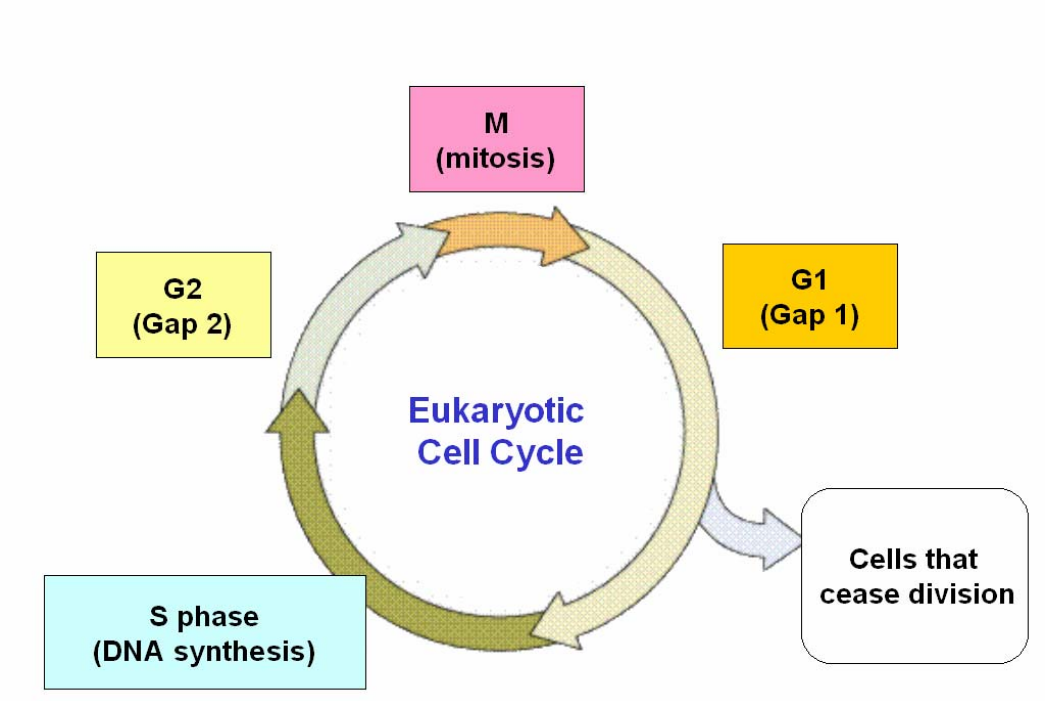


Figure 31. The cell cycle. Gap 0 (G0): There are times when a cell will leave the cell cycle and quit dividing that may be a temporary resting period or more permanent period; Gap 1(G1): Cells increase in size, produce RNA and protein. It is an important checkpoint to ensure everything is ready for DNA synthesis; S phase: DNA replication occurs. Complete DNA instruction in cells is duplicated to produce two similar daughter cells; Gap 2 (G2): During the gap between DNA synthesis and mitosis, cells will continuously grow to produce new proteins. The end of G2is another checkpoint to determine whether the cells can enter M phase and divide; Mitosis (M): Protein production and cell growth stop. Mitosis occurs (Life: The Science of Biology. Available at http://www.sinauer.com. Found on August 2006).

Cyclins serve as regulators of CDK kinases that different cyclins exhibit distinct expression and degradation patterns. Cyclin D1 functions as a regulatory subunit of CDK4 or CDK6. Cyclin D1 cooperates with CDK4 or CDK6 and forms cyclin D-Cdk4/Cdk6 complexes that the activity of cyclin is required for cell cycle

G1/S phase transition. Cyclin D1 has been shown to interact with retinoblastoma gene product Rb protein that serves as a checkpoint that restricts entry into the S phase by binding to the transcription factor E2F and blocks the transcription of the S phase genes.

Any abnormalities in Rb pathway lead to uncontrolled cell proliferation and tumorigenesis in various types of cell lines, animal models, and human tumors (Liu *et al*., 2005). Inhibition of the cell cycle has been appreciated as a target for the management of cancer in recent years (McDonald and El-Deiry, 2000; Owa *et al*., 2001).

C-MYC refers to the v-myc myelocytomatosis viral oncogene homolog. The mutations, overexpression, rearrangement and translocation of this oncogene have been associated with a variety of tumor (Zaharieva *et al*., 2005; Toncheva and Zaharieva, 2005; Tornillo *et al*., 2005; Konopka *at al*., 2004). The protein encoded by C-MYC also functions as a transcriptional factor of specific target genes which are involved in many cellular processes such as proliferation, differentiation and apoptosis (Pelengaris and Khan, 2003). C-MYC protein also serves as checkpoint of cell cycle like cyclin D1. It enhances phosphorylation of retinoblastoma gene product Rb involved in the cell cycle, regulates expression of CCND2 (encodes cyclin D2) and CDK4 and induces cyclin E–CDK2 activity early in the G1 phase of the cell cycle that is an essential event in the C-MYC induced G1-S progression. Thus, suppression of C-MYC activity can lead to the arrest of G_0/G_1 in the cell cycle. C-MYC also cooperates with several genes such as BCL2, PIM1 and V-RAF in tumorigenesis which have been shown to suppress apoptosis. It suggests that the suppression of C-MYC activity can

induce cell cycle arrest, apoptosis and suppress tumorigenesis (Pelengaris *et al*., 2002). Cyclin D1 and CMYC provide a therapeutic and chemoprevention target for the treatment of cancers through the induction of cell cycle arrest and apoptosis (Chang *et al*., 2004; Petty *et al*., 2003; Way *et al*., 2005; Lee *et al*., 2005).

This is the first study demonstrating that emodin could lower the expression of CCND1 and CMYC in A549 (Table 4A) and MCF-7 (Table 4B) cells. Down-regulation of CCND1 and CMYC imposes a blockage of the G1 phase to the S phase, causing the G0/G1 phase arrest of the cell cycle. This G0/G1 phase arrest in the cell cycle is an irreversible process where the cells are unable to repair these damages and ultimately undergo apoptosis. This explains how emodin inhibited growth and induced apoptosis on A549 and MCF-7 cells.

Overall, emodin could induce growth inhibition on A549 and MCF-7 cells through the upregulation of FASL and GAPDH expression and the downregulation of MCL1, CCND1 and C-MYC expression. The growth inhibitory effect of emodin might involve both the intrinsic and the extrinsic apoptotic pathways and the cell cycle arrest. However, further experiments are needed to elucidate the mechanism involved.

5.3 Quantification of emodin in different species of Da Huang

Plant extracts such as ginkgo biloba, echinacea, ginseng, grape seed, green tea, lemon, lavender, rosemary, thuja, sarsaparilla, soy, prickly pear, sagebrush, jojoba, aloe vera, allantoin, feverwort, bloodroot, apache plume, and papaya, (Hsu, 2005) and herbal extracts such as *Scutellaria barbatae, Paeoniae radix* and *Angelica sinensis* (Powell *et al*., 2003; Lee *et al*., 2002; Ye *et al*., 2003) have been widely used as topical applications for wound-healing, anti-aging, treatments of cancer and other diseases.

All these plants possess phytochemicals that might be act synergistically and are highly reactive with other compounds, such as reactive oxygen species and biologic macromolecules, to neutralize free radicals or initiate diverse biological effects. Many experimental studies have provided growing evidence for the beneficial actions of phytochemicals on human health and treatment of diseases. One of the most common phytochemicals found in plants is flavonoid, a plant phenolic (Marchand, 2002).

To identify, quantify and purify the active components or phytochemicals in herbs, an advanced analytical method should be developed. High performance liquid chromatography (HPLC), gas chromatography with mass spectrometry detection and high performance capillary electrophoresis are some of the methods used for the analysis of components in herbal extracts (Yang *et al*., 2005; Gong *et al*., 2004; Yan *et al*., 2006; Sun *et al*., 2001). Among them, HPLC is the most common method to analyze the chemical constituents in herbal extracts.

Emodin commonly found in the Rheum species was found to have diverse biological effects. Thus, HPLC was employed to analyze emodin in the Da Huang species in this experiment. Since there is a great variation in the amount of chemical constituents between species and sources, the contents of emodin in Da Huang extracts from different species and origins were also analyzed.

The five hydroxyanthraquinones, aloe-emodin, rhein, emodin, chrysophanol, and physcion, in Da Huang extract had been analyzed by HPLC by Ding *et al*. (2003). They used a Zorbax SB-C18 column and methanol-0.5% acetic acid (85:15, v/v) as the mobile phase, and the detection wavelength was set at 254 nm for separation and detection of hydroxyanthraquinones, respectively. In addition, 90 minutes and 12 minutes were used for the extraction of Da Huang and the elution of emodin, respectively. In the present study, the HPLC method was modified. An Alltech Allshpere ODS-25u separation column (250mm × 4.6mm I.D.) and a methanol-acetonitrile-acetic acid (70:15:15, v/v) as the mobile phase were used for separation, and the detection wavelength was set at 275nm.The use of acetic acid in the mobile phase could improve the peak tailing and detection limit of the HPLC system (Ding *et al*., 2004). In this experiment, acetonitrile was used together with methanol and acetic acid in the mobile phase because it was believed to increase the polarity and shorten the elution time of emodin. The extraction time for Da Huang extract was 60 minutes which was faster than that reported by Ding *et al*. (2003). However, the elution time of emodin was found to be similar to that reported by Ding *et al*. (2003). About 11.5 minutes were used for the elution of emodin under the HPLC system used in this experiment (Figure 21).

From studying chromatograms of Da Huang extract including D1, *Rheum tanguticum* Maximowicz (from Sichuan); D2, *Rheum palmatum* Linn (from Ganshu); D3, *Rheum tanguticum* Maximowicz (from Ganshu) and D4, *Rheum officinale* Baill. (from Sichuan), it was proved that emodin was present in all these extracts (Figure 21). By studying content of emodin in different species of Da Huang, it was found that the extract of *Rheum officinale* Baill. possessed the highest amount of emodin (10.33 ± 0.53μg/ml) (Table 5). The content of emodin was three fold more than that of the inter-species.

By comparing the content of emodin in the same species of Da Huang but from different origins, it was found that the extract of *Rheum tanguticum* Maximowicz from the Sichuan province in southern China had a higher amount of emodin (2.74 ± 0.09μg/ml) than that from Gansu province in northern China (Table 6). The content of emodin was about one fold more than that in intra-species from different origins. Higher content of emodin found in extract of *Rheum tanguticum* Maximowicz from Sichuan province in the South of China. It might be due to more favorable environmental factors such as rainfall, temperature, climate, weather and light-dark period for the growth of Da Huang in Sichuan province than that in Gansu province.

Variation in the content of emodin among inter-species and intra-species of Da Huang from different origins was reported in this study. It implies that the bioactivities of the Da Huang extracts might be greatly dependent on their species and origins because of their contents of biologically active emodin.

As the HPLC results showed ethyl acetate extract of *Rheum officinale* Baill had the highest amount of emodin, it suggests that the water extract of *Rheum*

officinale Baill used in the anticancer study also contains high amount of emodin. It is because emodin is soluble in both aqueous and organic solvents. Therefore, the overall anticancer effect of Da Huang water extract might be due to its content of emodin which showed a related anticancer activity on A549 and MCF-7 cells. From HPLC analysis of Da Huang species, other UV-sensitive components were also detected. They may be other anthraquiones in Da Huang species with similar structure to emodin. Thus, the anticancer effects of Da Huang water extract might also due to any synergistic actions of emodin with other anthrquiones.

In fact, in recent years, there are some other advanced analytical methods to detect emodin in Da Huang. A pressurized liquid extraction (PLE) and capillary zone electrophoresis (CZE) separation were developed for the detection of emodin in Da Huang as reported by Gong *et al.* (2005). The extraction could be performed in 5 minutes. However, a high pressure of 1500 psi and a high temperature of 140 degrees C, were required for the extraction. The high pressure and temperature used in the extraction might affect the components of the extracts that were heat sensitive. Moreover, a separation voltage at 25 kV and a temperature at 20 degrees C were used for separation.

Besides, a pressurized capillary electrochromatography (pCEC) with monolithic column was used for the determination of the structurally related anthraquinones in Da Huang as reported by Lu *et al.* (2006)**.** The anthraquinones in Da Huang was baseline-separated within 5 minutes with a high percentage of recoveries of Da Huang samples (81.3–86.4%) and low variation in the analysis (RSD less than or equal to 5.2%). However, a voltage of −20 kV and high pressure were

used for the extraction. The extraction of Da Huang by high pressure and high temperature reported in Gong *et al*. (2005) and Lu *et al*. (2006) were more expensive and dangerous than the HPLC method used in this study. Moreover, these analytical methods were less convenient to use because more preparation work and determining factors were needed and controlled in the separation process.

The calibration curve of this HPLC method was linear ($r^2 \geq 0.9952$) in the concentration range of 1μg/ml to 20μg/ml of emodin (Table 7). The method also showed a satisfactory sensitivity with a detection limit as small as 0.5μg/ml of emodin, recovery (76%) (Table 8), precision (RSD ≤ 10.40%) and selectivity (Table 8). Overall, the HPLC method for the quantification of emodin in the Da Huang samples used in this study was an accurate, simple, fast, convenient and reproducible for the detection of emodin in the Da Huang samples. It could be used for controlling the quality of other Chinese medicinal herbs and medical preparations.

5.4 Examination of vasorelaxant effect of emodin

Apart from its anticancer activity, biologically active emodin also showed vasorelaxant effect in this study. This provided evidence that emodin could serve as a therapeutic agent targeted for the treatment of human lung and breast cancers and hypertension, which both of them are the two major leading diseases in the world. Hypertension refers to a high blood pressure which is

140/90mmHg or above. Hypertension is commonly found in the population and any uncontrolled or untreated hypertension leads to many serious diseases such as atherosclerosis, heart disease and stroke. To lower the blood pressure, the drug should be responsible to cause vasodilation. Thus, development of novel inducers of vasodilation is important to provide a new therapeutic approach for any anti-hypertensive design.

To study the vasorelaxant effect of emodin, the percentage of vascular relaxation on phenylephrine-pretreated aorta and the percentage of suppression on phenylephrine-induced aortic contraction were measured. Phenyephrine is a α-adrenergic receptor agonist that is commonly used as a vasopresser to increase blood pressure by the induction of vasoconstriction. Chies *et al*. (2004) and Silverman *et al*. (1994) also had reported that use of phenylephrine to mediate vasoconstriction.

In the present study, experiment results suggested that emodin (10μM) could induce vascular relaxation on both endothelium intact (Figure 23A) ($p<0.05$) and denuded aorta (Figure 23B) ($p<0.01$). This was consistent to the results reported in Huang *et al*. (1999a and 1999b), which claimed that emodin could suppress the contractile response of rat aorta.

Vasodilation is of importance to control blood pressure and it could be endothelium dependent and endothelium independent. Endothelium-derived relaxing factors (EDRFs) such as prostacyclin (PGI_2), endothelium-derived hyperpolarizing factor (EDHF) and nitric oxide (NO) (Ignarro *et al*., 1987) play important roles in the endothelium dependent vascular relaxation. NO was

produced by endothelial nitric oxide synthase (eNOS) from L-arginine in vessels in the presence of physiological stimuli for vascular relaxation.

Increased production of NO could induce the activity of guanylate cyclase (sGC) to convert guanosine triphosphate into the intracellular second messenger guanosine 3',5'-cyclic monophosphate (cGMP) in smooth muscle cells. An increase in the intracellular concentration of cGMP caused vascular relaxation (Rapoport and Murad, 1983; DeFeudis, 1985; Yamashita *et al*., 2000; Hinsbergh, 2001) (Figure 32). It is important to investigate some methods to block the release of NO from endothelial cells so that it can eliminate the effect of NO on vascular relaxation.

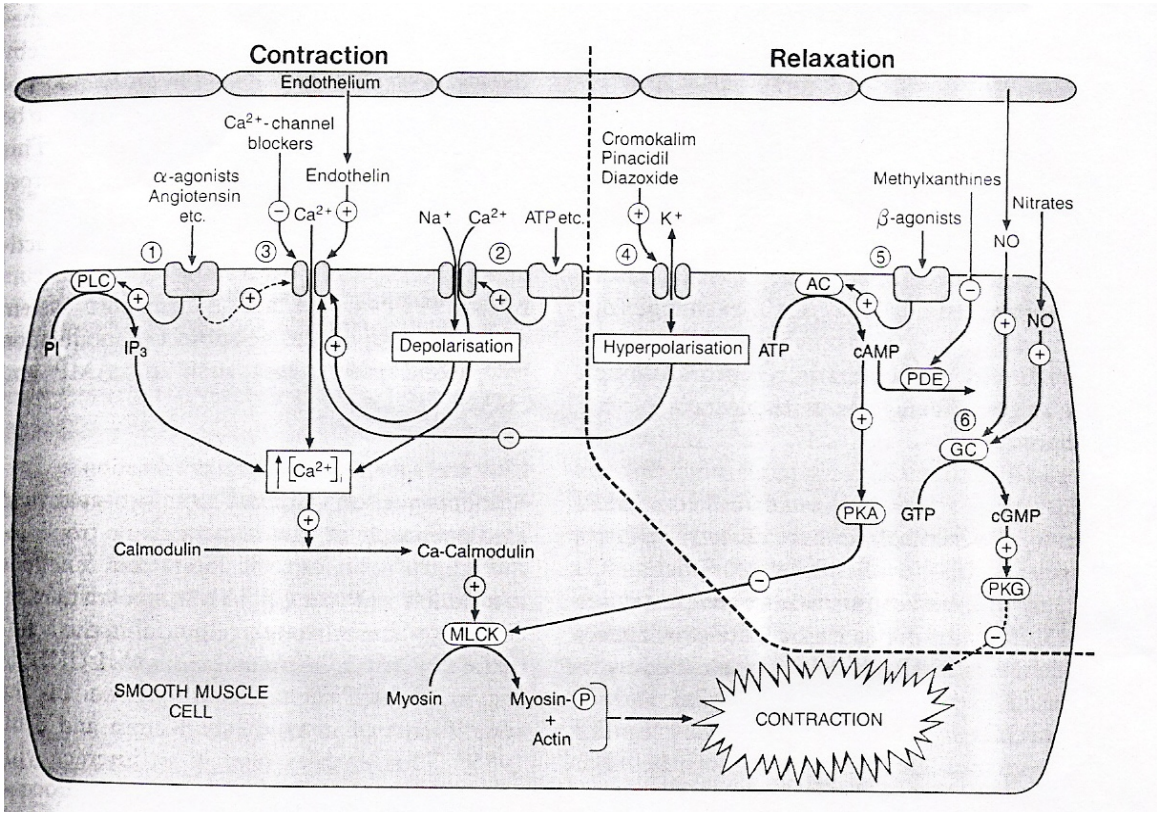


Figure 32. Control of vascular smooth muscle (Levick, 2003).

To study whether the emodin-induced vascular relaxation was endothelium dependent, indomethacin (1μM) was used to eliminate the effect of prostacyclin produced from endothelium followed by L-NAME (20μM) to exclude the effect of NO. Indomethacin is a non-selective cyclooxygenase inhibitor which is commonly used as a non-steroidal anti-inflammatory drug through inhibiting the production of by blocking the production of vasoconstrictor prostaglandins (Connolly *et al*., 1998).

L-NAME is a non-selective inhibitor of nitric oxide synthase which is commonly used to induce hypertension by eliminating the effect of NO on vascular relaxation (Santos *et al*., 2003).

In the present study, no significant difference was found in the emodin-induced vasorelexant effect between the L-NAME pretreated endothelium intact aorta and the untreated endothelium-denuded aorta (Figure 25). It implies that emodin-induced vascular relaxation was concentration-dependent but endothelium- and NO- independent. Similar observations were found in Terminalia superba methanol extract induced relaxation on rat thoracic aorta (Dimo *et al*., 2006), hydrogen peroxide induced relaxation on human vascular smooth muscle cells (Palen *et al*., 2006), carbachol and acetylcholine induced relaxation on rat isolated renal arteries (Jiang *et al*., 2000), and histamine induced relaxation on human dorsal penile artery (Martinez *et al*., 2000).

As the emodin-induced vascular relaxation is NO independent, other mechanisms such as potassium and calcium ion channels had been investigated in this study. To evaluate the contribution of potassium ion channels in emodin-induced vascular relaxation, glibenclamide (GA) (3μM) and tetraethylammonium

chloride (TEA) (1mM) were recruited to block the agonistic effect acting on the potassium ion channels. GA and TEA had been often used to study the role of potassium channels in vascular relaxation (Usta *et al*., 2006; Ozdem *et al*., 2006; Ohashi *et al*., 2005; Prieto *et al*., 2006).

Glibenclamide is an ATP-sensitive K^+ channel (K_{ATP}) blocker and tetraethylammonium chloride is a Ca^{2+}-activated K^+ channel blocker (K_{Ca}). Blockage of K_{ATP} and K_{Ca} could eliminate their effect on vascular relaxation. Opening of these potassium channels causes membrane hyperpolarisation and prevents the voltage-sensitive Ca^{2+} channel (VSCC) from opening. This in turn prevents the influx of Ca^{2+} through the opening of voltage-sensitive Ca^{2+} channel (VSCC), which triggers vasoconstriction (Figure 32).

In the present study, GA and TEA could not completely reduce the emodin-induced vascular relaxation on phenylephrine pre-contracted endothelium intact aorta (Figure 26). This implies that potassium ion channels, K_{ATP} and K_{Ca}, only contributed partly in the emodin-induced vascular relaxation. As there was a high interaction among K_{ATP}, K_{Ca} and VSCC in the control of vascular relaxation, we investigated the contribution of calcium ion channels in the emodin-induced vascular relaxation.

To investigate whether emodin serves as an antagonist on calcium ion channel to exhibit its vasorelexant effect on aorta, a calcium chloride concentration dependent curve was constructed under verapamil and emodin treatments. Verapamil (Figure 33) is a common medical drug that acts as an calcium ion channel blocker.

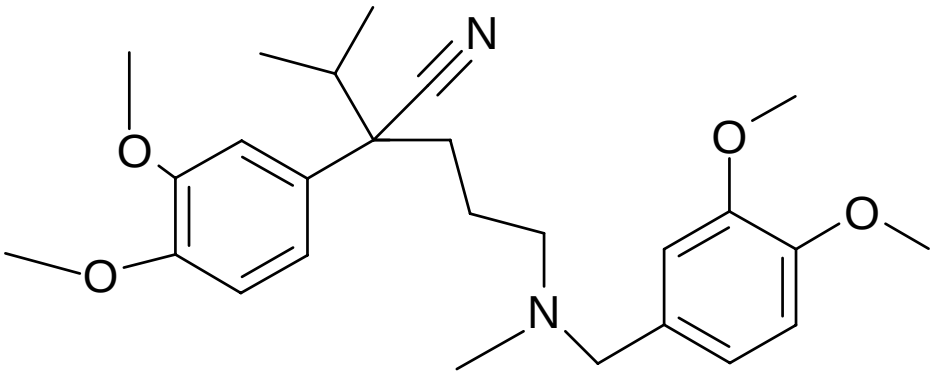


Figure 33. The chemical structure of verapamil.

Calcium ion channels such as VSCC play an important role in the control of vascular contraction and relaxation. Inhibition on calcium ion channels leads to an decrease influx of Ca^{2+} through VSCC in the vascular smooth muscle cells that favours vascular relaxation (Figure 32). In the present study, the calcium chloride induced contraction on endothelium intact aorta was suppressed by both verapamil (Figure 27A) and emodin in similar extent (Figure 27B).

Both of the calcium chloride contractile curves shifted to the right and down. This indicated that they produced a significant antagonism with calcium ion channels. It implies that emodin could serve as an antagonist on VSCC to induce its vasorelaxant effect and it behaved as a VSCC blocker like verapamil. It is believed that similarity in the chemical structures of emodin (Figure 6) and verapamil (Figure 33) contributes to their similar antagonistic effect on calcium ion channels.

By comparing their chemical structures, it was found that both emodin and verapamil consisted a bulky structure with benzene rings. Overall, this study

provided evidence that emodin induced vascular relaxation on aorta of rats through acting as a VSCC antagonist.

Similar observations were found in raloxifene (Tsang *et al*., 2004.), polyamines and acetylpolyamines (Myung *et al*., 2000) and 17-beta-estradiol (Salom *et al*., 2001) induced vascular relaxations.

Ca^{2+} channel blockers (CCBs) such as amlodipine and verapamil (Katzung *et al*., 2001; Hardman *et al*., 1995) have long been used for treatment of hypertension by lowering the blood pressure. They are also prescribed to hypertensive patients with heart disease because most CCBs can decrease the force of contraction of myocardium and slow down the conduction of electrical activity within the heart. Thus, CCBs can be used for treatment of heart disease such as irregular heart rate by lowering the heart rate. Emodin acting as calcium channel blocker could be beneficial for the treatment of hypertension as well as heart disease, which is one of the complications of hypertension.

Interestingly, calcium ion channel antagonists had been reported to have inhibitory effect on cancer cell growth by Bertolesi *et al*. (2002). As emodin was found to be a calcium ion channel antagonist and exhibit both anticancer and antihypertensive effects, it is believed that emodin-induced anticancer and antihypertensive effects are calcium ion channel dependent. However, more experiments should be conducted to elucidate the relationship between its anticancer effect and the calcium ion channels.

6 CONCLUSION

In this study, the anti-cancer effects of Da Huang water extract and emodin in human lung adenocarcinoma A549 cell line and human breast carcinoma MCF-7 cell line were demonstrated. Both Da Huang water extract and emodin showed significant inhibition on cancer cell growth and tumorigenicity. In addtion, the Da Huang water extract and emodin were possible to induce apoptosis in these cancer cell lines as confirmed by cell shrinkage, DNA fragmentation and DNA strand breakage at single cell level. Modulation of expression of apoptosis-related genes including FASL, GAPDH, MCL1, CCND1 and CMYC was also found in the emodin-treated A549 and MCF-7 cells. It was believed that the emodin exhibited its anticancer effect on A549 and MCF-7 cells through induction of apoptosis by regulating the expression of apoptosis-related genes.

Emodin showed anti-cancer effect similar to the Da Huang water extract. As the presence of emodin in Da Huang was confirmed by HPLC analysis, it was believed that the anticancer effect of the Da Huang water extract was closely related to the overall biological effect of its active component, emodin.

Apart from its anti-cancer effect, emodin was demonstrated to have vasorelaxant effect on rats' thoracic aorta in this study by measuring the percentages of relaxation and suppression on phenylephrine induced contractile response. The emodin-induced vascular relaxation was endothelium- and nitric oxide-independent. Potassium ion channels partially contributed to the emodin-induced vascular relaxation. Emodin was found to serve as a calcium ion channel antagonist to induce vascular relaxation similar to verapamil.

The present study provided evidence for the potential use of emodin in the prevention or even treatment of human lung and breast cancers, and hypertension. Also, this study provided a sensitive and reliable HPLC analysis method for the quantification of emodin in the Da Hunag species and even active components in other Chinese medicines, which may be helpful for the quality control of Chinese medicines in pharmaceutical usage and even clinic application.

7 RECOMMENDATIONS FOR FUTURE WORK

This study indicated that the Da Huang water extract and emodin had anticancer effects on A549 and MCF-7 cells which is believed to be dependent on their ability to induce apoptosis. However, many factors and mechanisms involved in apoptosis are still not yet studied. Other features of apoptosis such as activation of caspase, release of cytochrome c, change of mitochondrial membrane potential, formation of apoptotic bodies and condensation of chromatin, induction of cell cycle arrest should be further investigated in order to further confirm their ability on the induction of apoptosis.

Besides, emodin was proven to have vasorelexant activity on phenylephrine pre-contracted rat's aorta through acting as a calcium ion channel antagonist. However, this is only a rat model, which cannot represent the real situation in human. Study on the effect of emodin on human blood vessels or vascular smooth muscle cells would be suggested.

Emodin was found to inhibit the growth of A549 and MCF-7 cells and exhibit vasorelaxant effect. Interestingly, Bertolesi *et al*. (2002) has reported that calcium ion channel antagonists could inhibit cancer cell growth. As it was dounf that emodin was a calcium ion channel antagonist, apart from its ability of inducing apoptosis, it is believe that its anticancer effect on A549 and MCF-7 cells is greatly dependent on its ability of blocking the calcium ion channels. Since there are no reports on the relationship between the antihypertensive and anticancer activities of emodin, it is worthy to study the role of calcium ion

channels in cancer cells, and the relationship between the emodin-induced anticancer effect and the blockage of calcium ion channels. These suggested the future work might help to explain why emodin possessed both anticancer and antihypertensive effects.

8 REFERENCES

Adrain, C., Creagh, E.M., Martin, S.J., Ed. Marek, L., Henning, W., 2002. Caspase Cascades in Apoptosis. Caspases-their role in cell death and cell survival. Moleculare Biology Intelligence. New York, 41–51.

Ahn, K.S., Hahn, B.S., Kwack, K.B., Lee, E.B., Kim, Y.S., 2006. Platycodin D-induced apoptosis through nuclear factor-κB activation in immortalized keratinocytes. European Journal of Pharmacology 537 (1-3), 1–11.

Aggarwal, B.B., Shishodia, S., 2006. Molecular targets of dietary agents for prevention and therapy of cancer. Biochemical Pharmacology 71(10), 1397–1421.

Akduman, B., Crawford, E.D., 2001. Terazosin, doxazosin, and prazosin: current clinical experience. Urology 58 (1), 49–54.

Basu, S., Ghosh, A., Hazra, B., 2005. Evaluation of the antibacterial activity of *Ventilago madraspatana* Gaertn., *Rubia cordifolia* Linn. and *Lantana camara* Linn.: isolation of emodin and physcion as active antibacterial agents. Phytotheray Research 19 (10), 888–894.

Bennett, M., Macdonald, K., Chan, S.W., Luzio, J.P., Simari, R., Weissberg, P., 1998. Cell Surface Trafficking of Fas: A Rapid Mechanism of p53-Mediated Apoptosis. Science 282, 290–293.

Bertolesi, G.E., Shi, C., Elbaum, L., Jollimore, C., Rozenberg, G., Barnes, S., Kelly, M.E., 2002. The Ca^{2+} channel antagonists mibefradil and pimozide inhibit cell growth via different cytotoxic mechanisms. Molecular Pharmacology 62(2), 210–219.

Bianco, R., Melisi, D., Ciardiello, F., Tortora, G., 2006. Key cancer cell signal transduction pathways as therapeutic targets. European Journal of Cancer 42 (3), 290–294.

Burlacu, A., 2003. Regulation of apoptosis by Bcl-2 family proteins. Journal of Cellular and Molecular Medicine 7 (3), 249–257.

Cao, Y., Xia, Q.H., Meng, H., Zhong, A.P., 2005. Antitumor and synergistic effect of Chinese medicine "bushen huayu jiedu recipe" and chemotherapy on transplanted animal hepatocarcinoma. World Journal of Gastroenteroloy 11 (33), 5218–5220.

Cameron, R.G., Feuer, G., 2000. Apoptosis and Its Modulation by Drugs. Springer-Verlag Berlin Heidelberg, New York.

Cha, R.J., Zeng, D.W., Chang, Q.S., 1997. Non-surgical treatment of small cell lung cancer with chemo-radio-immunotherapy and traditional Chinese medicine. Zhonghua Nei Ke Za Zhi 33 (7), 462–466.

Chan, Y.M., Koo, L.C., Ho, J.C.M., Tsang, K.W.T., Chau, W.S., Chiu, S.W., Ip, M.S.M., Lam, W.K., 2003. Risk factors associated with lung cancer in Hong Kong. Lung Cancer 40 (2), 131–140.

Chang, C.H., Lin, C.C., Yang, J.J., Namba, T., Hattori, M., 1996. Anti-inflammatory effects of emodin from ventilago leiocarpa. American Journal of Chinese Medicine 24 (2), 139–142.

Chang, G.C., Hsu, S.L., Tsai, J.R., Liang, F.P., Lin, S.Y., Sheu, G.T., Chen, C.Y., 2004. Molecular mechanisms of ZD1839-induced G1-cell cycle arrest and apoptosis in human lung adenocarcinoma A549 cells. Biochemical Pharmacology 68 (7), 1453–1464.

Chen, Y.C., Shen, S.C., Lee, W.R., Hsu, F.L., Lin, H.Y., Ko, C.H., Tseng, S.W., 2002. Emodin induces apoptosis in human promyeloleukemic HL-60 cells accompanied by activation of caspase 3 cascade but independent of reactive oxygen species production. Biochemical Pharmacology 64 (12), 1713–1724.

Cheng, Y.L., Lee, S.C., Lin, S.Z., Chang, W.L., Chen, Y.L., Tsai, N.M., Liu, Y.C., Tzao, C., Yu, D.S., Harn, H.J., 2005. Anti-proliferative activity of Bupleurum scrozonerifolium in A549 human lung cancer cells in vitro and in vivo. Cancer Letter 222 (2), 183–193.

Chies, A.B., de Oliveira, A.M., Pereira, F.C., de Andrade, C.R., Correa, F.M., 2004. Phenylephrine-induced vasoconstriction of the rat superior

mesenteric artery is decreased after repeated swimming. Journal of Smooth Muscle Research 40 (6), 249–258.

Chukwujekwu, J.C., Coombes, P.H., Mulholland, D.A., Staden, J.V., 2006. Emodin, an antibacterial anthraquinone from the roots of Cassia occidentalis. South African Journal of Botany 72 (2), 295–297.

Connolly, C., McCormick, P.A., Docherty, J.R., 1998. Effects of the selective cyclooxygenase-2 inhibitor nimesulide on vascular contractions in endothelium-denuded rat aorta. European Journal of Pharmacology 352 (1), 53–58.

Cui, Y., Shu, X.O., Gao, Y., Wen, W., Ruan, Z.X., Jin, F., Zheng, W., 2004. Use of complementary and alternative medicine by Chinese women with breast cancer. Breast Cancer Research and Treatment 85 (3), 263–270.

DeFeudis, F.V., 1985. Endothelium-derived relaxing factor (EDRF). Agents Actions 17 (2), 181–183.

Demirezer, L.O., Kuruuzum-Uz, A., Bergere, I., Schiewe, H.J., Zeeck, A., 2001. The structures of antioxidant and cytotoxic agents from natural source: anthraquinones and tannins from roots of *Rumex patientia*. Phytochemistry 58, 1213–1217.

Demoliou-Mason, C.D., 1998. G-protein-coupled receptors in vascular smooth muscle cells. Biological Signals and Receptors 7 (2), 90–97.

Demyanets, S., Kaun, C., Pfaffenberger, S., Hohensinner, P.J., Rega, G., Pammer, J., Maurer, G., Huber, K., Wojta, J., 2006. Hydroxymethylglutaryl-coenzyme A reductase inhibitors induce apoptosis in human cardiac myocytes in vitro. Biochemical Pharmacology 71 (9), 1324–1330.

Dimo, T., Laurent, F., Rakotonirina, S.V., Tan, P.V., Kamtchouing, P., Dongo, E., Cros, G., 2006. Methanol extract of Terminalia superba induces endothelium-independent relaxation of rat thoracic aorta. Pharmazie 61 (5), 470–473.

Ding, M.Y., Ma, S.W., Liu, D.L., 2003. Simultaneous determination of hydroxyanthraquinones in Rhubarb and experimental animal bodies by High-Performance Liquid Chromatography. Analytical Sciences 19, 1163–1165.

Du, A.Y., Zhao, B.X., Miao, J.Y., Yin, D.L., Zhang, S.L., 2006. Safrole oxide induces apoptosis by up-regulating Fas and FasL instead of integrin β4 in A549 human lung cancer cells. Bioorganic and Medicinal Chemistry 14 (7), 2438 – 2445.

Ethell, D.W., Buhler, L.A., 2003. Fas Ligand-Mediated Apoptosis in Degenerative Disorders of the Brain. Journal of Clinical Immunology 23 (6), 439 – 446 (2003).

Fiot, J., Sanon, S., Azas, N., Mahiou, V., Jansen, O., Angenot, L., Balansard, G., Ollivier, E., 2006. Phytochemical and pharmacological study of roots and

leaves of Guiera senegalensis J.F. Gmel (Combretaceae). Journal of Ethnopharmacology 106 (2), 173–178.

Goel, R.K., Das Gupta, G., Ram, S.N., Pandey, V.B., 1991. Antiulcerogenic and anti-inflammatory effects of emodin, isolated from Rhamnus triquerta wall. Indian Journal of Experimental Biology 29 (3), 230–232.

Gong, F., Fung, Y.S., Liang, Y.Z., 2004. Determination of volatile components in ginger using gas chromatography-mass spectrometry with resolution improved by data processing techniques. Journal of Agricultural Food Chemistry 52 (21), 6378–6383.

Gong, Y.X., Li, S.P., Wang, Y.T., Li, P., Yang, F.Q., 2005. Simultaneous determination of anthraquinones in Rhubarb by pressurized liquid extraction and capillary zone electrophoresis. Electrophoresis 26, 1778–1782.

Gross, A., McDonnell, J.M., Korsmeyer, S.J., 1999. BCL-2 family members and the mitochondria in apoptosis. Genes and Development 13 (15), 1899–1911.

Hague, A., Paraskeva, C., 2004. Apoptosis and disease: a matter of cell fate. Nature Cell Death and Differentiation, 1–7.

Han, M.Q., Su, J.M., Huang, H.Y., Long, W.H., Qin, L.P., Qin, P., Liu, W., Sheng, Z.Y., 2003. Prognostic analysis of advanced non-small cell lung cancer treated by sequential chemo-radiation therapy combined with traditional Chinese medicine: a report of 54 cases. Zhong Xi Yi Jie He Xue Bao 1 (3), 195–198.

Hardman, J.G., Gilman, A.G., Limbird, L.E., 1995. The Pharmacological Basis of Therapeutics (9th edition). McGraw-Hill, New York.

Hardy, P., Abran, D., Hou, X., Lahaie, I., Peri, K.G., Asselin, P., Varma, D.R., Chemtob, S., 1998. A Major Role for Prostacyclin in Nitric Oxide–Induced Ocular Vasorelaxation in the Piglet. Circulation Research 83, 721–729.

Harrison, J.D., Link, M., 2003. To live or die - a cell's choice. Essays in Biochemistry 37, 109–119.

Henry, J.P., 1988. Stress, salt and hypertension. Social Science and Medicine 26 (3), 293–302.

Hillaert, S., Van den Bossche, W., 2000. Optimization of capillary electrophoretic separation of several inhibitors of the angiotensin-converting enzyme. Journal of Chromatogr A 895 (1-2), 33–42.

Hillaert, S., De Grauwe, K., Van den Bossche, W., 2001. Simultaneous determination of hydrochlorothiazide and several inhibitors of angiotensin-

converting enzyme by capillary electrophoresis. Journal of Chromatography A 924 (1-2), 439–449.

Higginson, J., Jensen, O.M., 1997. Epidemiological review of lung cancer in man. IARC Scientific Publications 16, 169–189.

Hinsbergh, V.W.M.V., 2001. NO or H_2O_2 for Endothelium-Dependent Vasorelaxation Tetrahydrobiopterin Makes the Difference. Arteriosclerosis, Thrombosis, and Vascular Biology 21, 719.

Hofbauer, S., Kainz, V., Golser, L., Klappacher, M., Kiesslich, T., Heidegger, W., Krammer, B., Hermann, A., Weiger, T.M., 2006. Antiproliferative properties of Padma Lax and its components ginger and elecampane. Forschende Komplementarmedizin 13 (1), 18–22.

Honing, M.L., Smits, P., Morrison, P.J., Burnett, J.C.Jr., Rabelink, T.J., 2001. C-type natriuretic peptide-induced vasodilation is dependent on hyperpolarization in human forearm resistance vessels. Hypertension 37 (4), 1179–1183.

Hsu, S., 2005. Green tea and the skin. Journal of the American Academy of Dermatology 52 (6), 1049–1059.

Hsu, Y.L., Kuo, P.L., Lin, C.C., 2004. The proliferative inhibition and apoptotic mechanism of Saikosaponin D in human non-small cell lung cancer A549 cells. Life Sciences 75 (10), 1231–1242.

Huang, H.C., Chu, S.H., Chao, P.D., 1991a. Vasorelaxants from Chinese herbs, emodin and scoparone, possess immunosuppressive properties. European Journal of Pharmacology 198, (2-3), 211–213.

Huang, H.C., Lee, C.R., Chao, P.D., Chen, C.C., Chu, S.H., 1991b. Vasorelaxant effect of emodin, an anthraquinone from a Chinese herb. European Journal of Pharmacology 205, 289–294.

Huang, Q., Shen, H.M., Ong, C.N., 2004a. Emodin-induced apoptosis through p53-dependent pathway in human hepatoma cells. Life Sciences 74 (18), 2279–2290.

Huang, Q., Shen, H.M., Ong, C.N., 2004b. Inhibitory effect of emodin on tumor invasion through suppression of activator protein-1 and nuclear factor-kappaB. Biochemical Pharmacology 68 (2), 361–371.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G., 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proceedings of the National Academy of Sciences of the United States of America 84 (24), 9265–9269.

Inzeo, D., Haughney, A., 2004. Laser therapy in the management of lung cancer. German. Clinical Journal of Oncology Nursing 8 (1), 94–95.

Ishitani, R., Tajima, H., Takata, H., Tsuchiya, K., Kuwae, T., Yamada, M., Takahashi, H., Tatton, N.A., Katsube, N., 2003. Proapoptotic protein glyceraldehyde-3-phosphate dehydrogenase: a possible site of action of antiapoptotic drugs. Progress in Neuro-Psychopharmacology and Biological Psychiatry 27 (2), 291–301.

Jain, V., Vedernikov, Y.P., Saade, G.R., Chwalisz, K., Garfield, R.E., 1999. Endothelium-Dependent and -Independent Mechanisms of Vasorelaxation by Corticotropin-Releasing Factor in Pregnant Rat Uterine Artery. Pharmacology and the Experimental Therapeutics 288 (2), 407–413.

Jang, B.C., Paik, J.H., Jeong, H.Y., Oh, H.J., Park, J.W., Kwon, T.K., Song, D.K., Park, J.G., Kim, S.P., Bae, J.H., 2004. Leptomycin B-induced apoptosis is mediated through caspase activation and down-regulation of Mcl-1 and XIAP expression, but not through the generation of ROS in U937 leukemia cells. Biochemical Pharmacology 68(2), 263–274.

Jiang, F., Li, C.G., Rand, M.J., 2000. Mechanisms of nitric oxide-independent relaxations induced by carbachol and acetylcholine in rat isolated renal arteries. British Journal of Pharmacology 130 (6), 1191–1200.

Katzung, B.G., Appleton & Lange, Norwalk, C.N., 2001. Basic and Clinical Pharmacology (8th edition).

Kim, M.S., Park, M.J., Kim, S.J., Lee, C.H., Yoo, H., Shin, S.H., Song, E.S., Lee, S.H., 2005. Emodin suppresses hyaluronic acid-induced MMP-9 secretion and invasion of glioma cells. International Journal of Oncology 27 (3), 839–846.

Klabunde, R.E., 2006. Vascular Smooth Muscle Contraction and Relaxation. Cardiovascular Physiology Concepts. Available at http://www.cvphysiology.com. Found on August 2006.

Klaude, M., Eriksson, S., Nygren, J., Ahnstorm, G., 1996. The comet assay: mechanisms and technical considerations. Mutation Research 363 (2), 89–96.

Knappe, V., Mols, A., 2004. Laser therapy of the lung: biophysical background. Radiologe 44 (7), 677–683.

Konopka, B., Janiec-Jankowska, A., Paszko, Z., Goluda, M., 2004. The coexistence of ERBB2, INT2, and CMYC oncogene amplifications and PTEN gene mutations in endometrial carcinoma. Journal of Cancer Research and Clinical Oncology 130 (2), 114–121.

Koo, L.C., Ho, J.H., Lee, N., 1985. An analysis of some risk factors for lung cancer in Hong Kong. International Journal of Cancer 35 (2), 149–155 (1985).

Kumar, A., Dhawan, S., Aggarwal, B.B., 1998. Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) inhibits TNF-induced NF-kappaB activation, IkappaB degradation, and expression of cell surface adhesion proteins in human vascular endothelial cells. Oncogene 17, 913–918.

Kuo, Y.C., Meng, H.C., Tsai, W.J., 2001. Regulation of cell proliferation, inflammatory cytokine production and calcium mobilization in primary human T lymphocytes by emodin from Polygonum hypoleucum Ohwi. Inflammation Research 50 (2), 73–82.

Lam, F.F., Yeung, J.H., Cheung, J.H., 2005. Mechanisms of the dilator action of Danshen (*Salvia miltiorrhiza*) on rat isolated femoral artery. Journal of Cardiovascular Pharmacology 16, 361–368.

Lam, W.K., White, N.W., Chan-Yeung, M.M., 2004. Lung cancer epidemiology and risk factors in Asia and Africa. The international journal of tuberculosis and lung disease: the official journal of the International Union against Tuberculosis and Lung Disease 8 (9), 1045–1057.

Lau, F.Y., Chui, C.H., Gambari, R., Kok, S.H., Kan, K.L., Cheng, G.Y., Wong, R.S., Teo, I.T., Cheng, C.H., Wan, T.S., Chan, A.S., Tang, J.C., 2005.

Antiproliferative and apoptosis-inducing activity of Brucea javanica extract on human carcinoma cells. International Journal of Molecular Medicine 16 (6), 1157–1162.

Lee, E.J., Min, H.Y., Chung, H.J., Park, E.J., Shin, D.H., Jeong, L.S., Lee, S.K., 2005. A novel adenosine analog, thio-Cl-IB-MECA, induces G_0/G_1 cell cycle arrest and apoptosis in human promyelocytic leukemia HL-60 cells. Biochemical Pharmacology 70 (6), 918–924.

Lee, S.M.Y., Li, M.L.Y., Tse, Y.C., Leung, S.C.L., Lee, M.M.S., Tsui, S.K.W., Fung, K.P., Lee, C.Y., Waye, M.M.Y., 2002. Paeoniae Radix, a Chinese herbal extract, inhibit hepatoma cells growth by inducing apoptosis in a p53 independent pathway. Life Sciences 71 (19), 2267–2277.

Lee, H.Z., 2001. Effects and mechanisms of emodin on cell death in human lung squamous cell carcinoma. British Journal of Pharmacology 134 (1), 11–20.

Letasiova, S., Jantova, S., Cipak, L., Muckova, M., 2006. Berberine—antiproliferative activity in vitro and induction of apoptosis/necrosis of the U937 and B16 cells. Cancer Letters 239 (2), 254–262.

Levick, J.R., 2003. An introduction to cardiovascular physiology (fourth edition). London, Arnold, New York.

Li, H.L., Chen, H.L., Li, H., Zhang, K.L., Chen, X.Y., Wang, X.W., Kong, Q.Y., Liu, J., 2005a. Regulatory effects of emodin on NF-kappaB activation and inflammatory cytokine expression in RAW 264.7 macrophages. International Journal of Molecular Medicine 16 (1), 41–47.

Li, J.N., Lu, F.Z., Xiao, J.L., Bao, Y., 2005b. Inhibitory effect of emodin on proliferation of human lung adenocarcinoma cells in vitro. Zhongguo Zhong Xi Yi Jie He Za Zhi 25 (7), 640–642.

Li, S.Y., Ou-Yang, S., 2005. Emodin blocks voltage dependent potassium channels in rat proximal colon smooth muscle cells. Yao Xue Xue Bao 40 (9), 804–809.

Li, Y., 2005. A clinical study on haunglian fire-purging mixture in treatment of 46 cases of primary hypertension. Journal of Traditional Chinese Medicine 25 (1), 29–33.

Liang, H.L., Xue, C.C., Li, C.G., 2004. Regression of squamous cell carcinoma of the lung by Chinese herbal medicine: a case with an 8-year follow-up. Lung Cancer 43 (3), 355–360.

Lin, C.C., Huang, P.C., Lin, J.M., 2000. Antioxidant and hepatoprotective effects of Anoectochilus formosanus and Gynostemma pentaphyllum. American Journal of Chinese Medicine 28 (1), 87–96.

Lin, H.I., Lee, Y.J., Chen, B.F., Tsai, M.C., Lu, J.L., Chou, C.J., Jow, G.M., 2005. Involvement of Bcl-2 family, cytochrome c and caspase 3 in induction of apoptosis by beauvericin in human non-small cell lung cancer cells. Cancer Letters 230 (2), 248–259.

Liu, C.L., Wang, Y.D., Jin, X.J., 2001. Clinical observation on treatment of non-small cell lung cancer with Chinese herbal medicine combined with bronchial arterial infusion chemotherapy. Zhongguo Zhong Xi Yi Jie He Za Zhi 21 (8), 579–581.

Liu, C.L., Zhu, P.L., Liu, M.C., 1999. Computer-aided development of a high-performance liquid chromatographic method for the determination of hydroxyanthraquinone derivatives in Chinese herb medicine rhubarb. Journal of Chromatography A 857 (1–2), 167–174.

Liu, T.J., Zhu, E., Wang, L.H., Okada, T., Yamaguchi, A., Okada, N., 2005. Abnormal expression of Rb pathway–related proteins in salivary gland acinic cell carcinoma. Human Pathology 36 (9), 962–970.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J.E., 2000. Molecular and Cell Biology (4th Edition), Freeman and Company, New York.

Lu, H., Wang, J., Wang, X., Lin, X., Wu, X., Xie, Z., 2006. Rapid separation and determination of structurally related anthraquinones in Rhubarb by

pressurized capillary electrochromatography. Journal of Pharmaceutical and Biomedical Analysis, In Press, Corrected Proof. Available at the National Center for Biotechnology Information.

Luma, G.B., Spiotta, R.T., 2006. Hypertension in children and adolescents. American Family Physician 73 (9), 1558–1568.

Martinez, A.C., Prieto, D., Raposo, R., Delgado, J.A., Resel, L., 2000. Garcia-Sacristan A, Benedito S. Endothelium-independent relaxation induced by histamine in human dorsal penile artery. Clinical and Experimental Pharmacology and Physiology 27 (7), 500–507.

Matsuda, H., Shimoda, H., Morikawa, T., Yoshikawa, M., 2001. Phytoestrogens from the roots of *Polygonum cuspidatum* (Polygonaceae): structure-requirement of hydroxyanthraquinones for estrogenic activity. Bioorganic and Medicinal Chemistry Letters 11, 1839–1842.

McCurrach, M.E., Connor, T.M., Knudson, C.M., Korsmeyer, S.J., Lowe, S.W., 1997. Bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proceedings of the National Academy of Sciences of the United States of America 94 (6), 2345–2349.

McDonald, E.R.III, El-Deiry, W.S., 2000. Cell cycle control as a basis for cancer drug development. International Journal of Oncology 16, 871–886.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., Reed, J.C., 1994. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9 (6), 1799–1805.

Miyashita, T., Reed, J.C., 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80 (2), 293–299.

Mogal, A., Abdulkadir, S.A., 2006. Effects of Histone Deacetylase Inhibitor (HDACi); Trichostatin-A (TSA) on the expression of housekeeping genes. Molecular and Cellular Probes 20 (2), 81–86.

Moon, M.K., Kang, D.G., Lee, J.K., Kim, J.S., Lee, H.S., 2006. Vasodilatory and anti-inflammatory effects of the aqueous extract of rhubarb via a NO-cGMP pathway. Life Science 78 (14), 1550–1557.

Moore, D.H., Moore, D.H.II, Moore, C.T., 1983. Breast carcinoma etiological factors. Advanced Cancer Research 40, 189–253.

Moosmang, S., Schulla, V., Welling, A., Feil, R., Feil, S., Wegener, J.W., Hofmann, F., Klugbauer, N., 2003. Dominant role of smooth muscle L-type calcium channel $Ca_v1.2$ for blood pressure regulation. EMBO Journal 22 (22), 6027–6034.

Muti, P., 2005. The role of endogenous hormones in the etiology and prevention of breast cancer: the epidemiological evidence. Recent Results Cancer Research 166, 245–256.

Myung, C.S., Blankenship, J.W., Meerdink, D.J., 2000. A mechanism of vasodilatory action of polyamines and acetylpolyamines: possible involvement of their Ca^{2+} antagonistic properties. Journal of Pharmacy and Pharmacology 52 (6), 695–707.

Marchand, L.L., 2002. Cancer preventive effects of flavonoids-a review. Biomedecine and Pharmacotherapy 56 (6), 296–301.

Nakagawa, K., Shibata, C., Momono, S., Sasaki, Y., Furusawa, A., Sato, T., 1992. A case of advanced breast cancer effectively treated with tamoxifen. Gan To Kagaku Ryoho 19 (9), 1391–1394.

Newcomb, R.D., 1992. Epidemiology of essential hypertension and diabetes mellitus. Optometry Clinics 2 (2), 1–13.

Ohashi, M., Faraci, F., Heistad, D., 2005. Peroxynitrite hyperpolarizes smooth muscle and relaxes internal carotid artery in rabbit via ATP-sensitive K^+ channels. American Journal of Physiology: Heart and Circulatory Physiology 289 (5), 2244–2250.

Okobia, M.N., Bunker, C.H., 2005. Epidemiological risk factors for breast cancer–a review. Nigerian Journal of Clinical Practice 8 (9), 35–42.

Owa, T., Yoshino, H., Yoshimatsu, K., Nagasu, T., 2001. Cell cycle regulation in the G1 phase: a promising target for the development of new chemotherapeutic anticancer agents. Current Medicinal Chemistry 8, 1487–1503.

Ozdem, S.S., Yalcin, O., Meiselman, H.J., Baskurt, O.K., Usta, C, 2006. The role of potassium channels in relaxant effect of levosimendan in rat small mesenteric arteries. Cardiovascular drugs and therapy 20 (2), 123–127.

Palen, D.I., Ouhtit, A., Belmadani, S., Lucchesi, P.A., Matrougui, K., 2006. Hydrogen peroxide acts as relaxing factor in human vascular smooth muscle cells independent of map-kinase and nitric oxide. Frontiers in Bioscience 11, 2526–2534.

Pelengaris, S., Khan, M., 2003. The many faces of c-MYC. Archives of Biochemistry and Biophysics 416 (2), 129–136.

Pelengaris, S., Khan, M., Evan, G.I., 2002. This paper demonstrates the multiple innate oncogenic properties of c-Myc activation in vivo, when c-Myc-induced apoptosis is suppressed. Cell 109 (3), 321–334.

Petty, W.J., Dragnev, K.H., Dmitrovsky, E., 2003. Cyclin D1 as a target for chemoprevention. Lung Cancer 41 (1), 155–161.

Petit, J.C., 1972. Hormone therapy of breast cancer. Journal de médecine de Strasbourg 2 (10), 655–659.

Powell, C.B., Fung, P., Jackson, J., Dall'Era, J., Lewkowicz, D., Cohen, I., Smith-McCune, K., 2003. Aqueous extract of herba *Scutellaria barbatae*, a chinese herb used for ovarian cancer, induces apoptosis of ovarian cancer cell lines. Gynecologic Oncology 91 (2), 332–340.

Prieto, D., Rivera, L., Benedito, S., Recio, P., Villalba, N., Hernandez, M., Garcia-Sacristan, A., 2006. Ca^{2+}-activated K^{+} (K_{Ca}) channels are involved in the relaxations elicited by sildenafil in penile resistance arteries. European Journal of Pharmacology 531 (1-3), 232–237.

Prichard, B.N., 1966. The treatment of hypertension by beta-adrenergic blocking drugs. Angiologica 3 (5), 318–329.

Qicheng, F., 1980. Some current study and research approaches relating to the use of plants in the traditional Chinese medicine. Journal of Ethnopharmacology 2 (1), 57–63.

Raff, M., 1988. Cell suicide for beginners. Nature 396, 119–122.

Rapoport, R.M., Murad, F., 1983. Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role of cyclic GMP. Journal of Cyclic Nucleotide and Protein Phosphorylation Sesearch 9 (4-5), 281–296.

Ray, A., Ray, S., Koner, B.C., 2004. Hypertension, cancer and angiogenesis: Relevant epidemiological and pharmacological aspects. Indian Journal of Pharmacology 36 (6), 341–347.

Reid, J.L., Vincent, J., 1986. Clinical pharmacology and therapeutic role of prazosin and related alpha-adrenoceptor antagonists. Cardiology 73 (3), 164–174.

Rubens, R.D., 1995. Metastatic breast cancer. Current Opinion in Oncology 7 (6), 523–536.

Sadava, D., Ahn, J., Zhan, M., Pang, M.L., Ding, J., Kane, S.E., 2002. Effects of four Chinese herbal extracts on drug-sensitive and multidrug-resistant small-cell lung carcinoma cells. Cancer Chemotherapy and Pharmacology 49 (4), 261 – 266.

Salom, J.B., Burguete, M.C., Perez-Asensio, F.J., Torregrosa, G., Alborch, E., 2001. Relaxant effects of 17-beta-estradiol in cerebral arteries through

Ca(2+) entry inhibition. Journal of cerebral blood flow and metabolism 21 (4), 422–429.

Santos, L.D., Xavier, F.E., Vassallo, D.V., Rossoni, L.V., 2003. Cyclooxygenase pathway is involved in the vascular reactivity and inhibition of the Na^+, K^+-ATPase activity in the tail artery from L-NAME-treated rats. Life Sciences 74 (5), 613–627.

Schmieder, R.E., Messerli, F.H., Ruddel, H., 1986. Risks for arterial hypertension. Cardiology Clinics 4 (1), 57–66.

Seedat, Y.K., 1975. Treatment of hypertension with the aid of beta-adrenergic blocking drugs. South African Medical Journal 49 (21), 846–848.

Shaipanich, T., McWilliams, A., Lam, S., 2006. Early detection and chemoprevention of lung cancer. Respirology 11 (4), 366–372.

Shi, M.X., Cai, Q.F., Yao, L.M., Mao, Y.B., Ming, Y.L., Ouyang, G.L., 2006. Antiproliferation and apoptosis induced by curcumin in human ovarian cancer cells. Cell Biology International 30 (3), 221–222.

Shih, H.C., Lee, T.H., Chen, S.C., Li, C.Y., Shibuya, T., 2005. Anti-hypertension effects of traditional Chinese medicine ju-ling-tang on renal hypertensive rats. American Journal of Chinese Medicine 33 (6), 913–921.

Shieh, D.E., Chen, Y.Y., Yen, M.H., Chiang, L.C., Lin, C.C., 2004. Emodin-induced apoptosis through p53-dependent pathway in human hepatoma cells. Life Sciences 74 (18), 2279–2290.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175 (1), 184–191.

Silverman, D.G., Jotkowitz, A.B., Freemer, M., Gutter, V., O'Connor, T.Z., Braverman, I.M., 1994. Peripheral assessment of phenylephrine-induced vasoconstriction by laser Doppler flowmetry and its potential relevance to homeostatic mechanisms. Circulation 90, 23–26.

Soubrier, F., Jeunemaitre, X., Corvol, P., 1990. Hereditary factors of essential arterial hypertension. La Revue du praticien 40 (23), 2156–2160.

Spence, J.D., 1989. Pathogenesis of atherosclerosis and its complications: effects of antihypertensive drugs. Journal of Human Hypertension 3 (2), 63–68.

Srinivas, G., Anto, R.J., Srinivas, P., Vidhyalakshmi, S., Senan, V.P., Karunagaran, D., 2003. Emodin induces apoptosis of human cervical cancer cells through poly (ADP-ribose) polymerase cleavage and activation of caspase-9. European Journal of Pharmacology 473 (2-3), 117–125.

Stamler, J., 1991. Blood pressure and high blood pressure. Aspects of risk. Hypertension 18 (3), 95–107.

Su, Y.T., Chang, H.L., Shyue, S.K., Hsu, S.L., 2005. Emodin induces apoptosis in human lung adenocarcinoma cells through a reactive oxygen species-dependent mitochondrial signaling pathway. Biochemical Pharmacology 70 (2), 229–241.

Sun, L., Meng, L., Chen, J., Ma, J., Hu, R., Jia, D.Z., 2001. Determination of rutin and quercetin in mulberry leaves by high performance capillary electrophoresis. Se Pu 19 (5), 395–397.

Sung, J., Han, K.H., Zo, J.H., Park, H.J., Kim, C.H., Oh, B.H., 2000. Effects of red ginseng upon vascular endothelial function in patients with essential hypertension. American Journal of Chinese Medicine 28 (2), 205–216.

Sutton, J.M., Bagby, S.P., 1992. Nontraditional combination pharmacotherapy of hypertension. Cleveland Clinic Journal of Medicine 59 (5), 459–468.

Toncheva, D., Zaharieva, B., 2005. Coexistence of copy number changes of different genes (INK4A, erbB-1, erbB-2, CMYC, CCND1 and ZNF217) in urothelial tumors. Tumour Biology 26 (2), 88–93.

Tornillo, L., Duchini, G., Carafa, V., Lugli, A., Dirnhofer, S., Di Vizio, D., Boscaino, A., Russo, R., Tapia, C., Schneider-Stock, R., Sauter, G.,

Insabato, L., Terracciano, L.M., 2005. Patterns of gene amplification in gastrointestinal stromal tumors (GIST). Laboratory Investment 85 (7), 921–931.

Tsai, J.C., Tsai, S., Chang, W.C., 2004. Effect of ethanol extracts of three Chinese medicinal plants with laxative properties on ion transport of the rat intestinal epithelia. Biological and Pharmaceutical Bulletin 27 (2), 162–165.

Tsang, S.Y., Yao, X., Essin, K., Wong, C.M., Chan, F.L., Gollasch, M., Huang, Y., 2004. Raloxifene relaxes rat cerebral arteries in vitro and inhibits L-type voltage-sensitive Ca2+ channels. Stroke 35 (7), 1709–1714.

Usta, C., Eksert, B., Golbasi, I., Bigat, Z., Ozdem, S.S., 2006. The role of potassium channels in the vasodilatory effect of levosimendan in human internal thoracic arteries. European Journal of Cardio-thoracic Surgery 30 (2), 329–332.

Valle-Rodriguez, A.D., Calderon, E., Ruiz, M., Lopez-Barneo, A.O.J., Urena, J., 2006. Metabotropic Ca^{2+} channel-induced Ca^{2+} release and ATP-dependent facilitation of arterial myocyte contraction. Biological Sciences/Physiology 103 (11), 4316–4321.

Walker, A.F., Marakis, G., Simpson, E., Hope, J.L., Robinson, P.A., Hassanein, M., Simpson, H.C., 2006. Hypotensive effects of hawthorn for patients with

diabetes taking prescription drugs: a randomised controlled trial. The British Journal of General Practice 56 (527), 437–443.

Wang, H.L., Jiao, D.H., 1985. A study of rhubarb's constituents and the mechanism of their hemostatic effect. Zhong Xi Yi Jie He Za Zhi 5 (9), 555–557.

Wang, X.F., Ge, J.B., Sun, A.J., Xu, D.L., Wang, K.Q., 2006. Involvement of p53-dependent pathway in the antiproliferative activity of emodin in human smooth muscle cell. Zhonghua Xin Xue Guan Bing Za Zhi 34 (1), 44–49.

Way, T.D., Kao, M.C., Lin, J.K., 2005. Degradation of HER2/*neu* by apigenin induces apoptosis through cytochrome *c* release and caspase-3 activation in HER2/*neu*-overexpressing breast cancer cells. FEBS Letters 579 (1), 145–152.

Whelton, P.K., Klag, M.J., 1989. Hypertension as a risk factor for renal disease. Review of clinical and epidemiological evidence. Hypertension 13 (5), I19–I27.

Williams, R.R., Hopkins, P.N., 1979. Salt, hypertension, and genetic-environmental interactions. Progress in Clinical and Biological Research 32, 183–194.

Wilson, J.W., Booth, C., Potten, C.S., 1998. Apoptosis gene. Kluwer Academic Publisher, Boston, Dordrecht/ London.

Wong, C.K., Bao, Y.X., Wong, E.L., Leung, P.C., Fung, K.P., Lam, C.W., 2005. Immunomodulatory activities of Yunzhi and Danshen in post-treatment breast cancer patients. American Journal of Chinese Medicine 33 (3), 381–395.

Wu, L.Y., 1985. Effect of rhubarb processing on its anti-inflammatory action. Zhong Yao Tong Bao 10 (6), 18–21.

Wu, S.J., Ng, L.T., Lin, C.C., 2005. Cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins and MAPK pathway. Life Sciences 77 (8), 938–995.

Wylie, P.G., Bowen, W.P., 2005. Determination of Cell Colony Formation in a High-Content Screening Assay. Journal of the Association for Laboratory Automation 10 (4), 203–206.

Xiao, P.G., He, L.I., Wang, L.W., 1984. Enthopharmacology study of Chinese Rhubarb. Journal of Enthopharmacology 10, 275–293.

Yan, S., Xin, W., Luo, G., Wang, Y., Cheng, Y., 2006. Chemical fingerprinting of Gardenia jasminoides fruit using direct sample introduction and gas

chromatography with mass spectrometry detection. Journal of AOAC International 89 (1), 40–45.

Yang, H.L., Chen, C.S., Chang, W.H., Lu, F.J., Lai, Y.C., Chen, C.C., Hseu, T.H., Kuo, C.T., Hseu, Y.C., 2006. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by Antrodia camphorata. Cancer Letters 231 (2), 215–227.

Yang, X., Zhao, Y., Wang, Q., Wang, H., Mei, Q., 2005. Analysis of the monosaccharide components in Angelica polysaccharides by high performance liquid chromatography. Analytical sciences: the international journal of the Japan Society for Analytical Chemistry 21 (10), 1177–1180.

Yamashita, T., Kawashima, S., Ohashi, Y., Ozaki, M., Rikitake, Y., Inoue, N., Hirata, K.I., Akita, H., Yokoyama, M., 2000. Mechanisms of Reduced Nitric Oxide/cGMP–Mediated Vasorelaxation in Transgenic Mice Overexpressing Endothelial Nitric Oxide Synthase. Hypertension 36, 97.

Ye, Y.N., So, H.L., Liu, E.S., Shin, V.Y., Cho, C.H., 2003. Effect of polysaccharides from Angelica sinensis on gastric ulcer healing. Life Sciences 72 (8), 925–932.

Yim, H., Lee, Y.H., Lee, C.H., Lee, S.K., 1999. Emodin, an anthraquinone derivative isolated from the rhizomes of Rheum palmatum, selectively

inhibits the activity of casein kinase II as a competitive inhibitor. Planta Medica 65, 9–13 .

Yin, X., Zhou, J., Jie, C., Xing, D., Zhang, Y., 2004. Anticancer activity and mechanism of Scutellaria barbata extract on human lung cancer cell line A549. Life Sciences 75 (18), 2233–2244.

Yu, X.C., Wu, S., Chen, C.F., Pang, K.T., Wong, T.M., 2004. Antihypertensive and anti-arrhythmic effects of an extract of Radix Stephaniae Tetrandrae in the rat. Journal of Pharmacy and Pharmacology 56 (1), 115–122.

Yu, Z.L., Li, W.J., 2006. Induction of apoptosis by puerarin in colon cancer HT-29 cells. Cancer Letters 238 (1), 53–60.

Yuan, S.S.F., Chang, H.L., Chen, H.W., Kuo, F.C., Liaw, C.C., Su, J.H., Wu, Y.C., 2006. Selective cytotoxicity of squamocin on T24 bladder cancer cells at the S-phase via a Bax-, Bad-, and caspase-3-related pathways. Life Sciences 78 (8), 869–874.

Zaharieva, B., Simon, R., Ruiz, C., Oeggerli, M., Mihatsch, M.J., Gasser, T., Sauter, G., Toncheva, D., 2005. High-throughput tissue microarray analysis of CMYC amplificationin urinary bladder cancer. International Journal of Cancer 117 (6), 952–956.

Zemla, B., Zielonka, I., Kolosza, Z., 1998. Tobacco smoking and exposure to dust and gas pollution in the place of work and lung cancer risk. Neoplasma 35 (2), 135–143.

Zhan, Q., Fan, S., Bae, I., Guillouf, C., Liebermann, D.A., O'Connor, P.M., Fornace, A.J. Jr., 1994. Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. Oncogene 9 (12), 3743–3751.

Zhang, C.X., Huang, K.X., 2006. Mechanism of apoptosis induced by a polysaccharide, from the loach *Misgurnus anguillicaudatus* (MAP) in human hepatocellular carcinoma cells. Toxicology and Applied Pharmacology 210 (3), 236–245.

Zhang, H.Q., Zhou, C.H., Wu, Y.Q., 2005. Effect of emodin on small intestinal peristalsis of mice and relevant mechanism. World Journal of Gastroenterology 11 (20), 3147–3150.

Zheyu, C., Qinghui, Q.I., Lixin, L., Tao, M.A., Xu, J., Zhang, L., Lunan, Y., 2006. Effects of emodin on Ca^{2+} signal transduction of smooth muscle cells in multiple organ dysfunction syndrome. The Journal of Surgical Research 131 (1), 80–85.

Zou, Y.H., Liu, X.M., 2003. Effect of astragalus injection combined with chemotherapy on quality of life in patients with advanced non-small cell lung cancer. Zhongguo Zhong Xi Yi Jie He Za Zhi 23 (10), 733–735.