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CYTOTOXIC ACTIVITIES OF 6-DEOXYCLITORIACETAL AND ITS DERIVATIVES

Miss Nustha Kitprathaung

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	CYTOTOXIC ACTIVITIES OF 6- DEOXYCLITORIACETAL AND ITS DERIVATIVES.
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Field of study	Biotechnology
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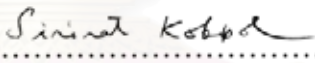

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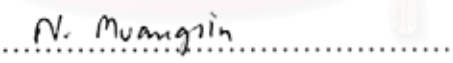

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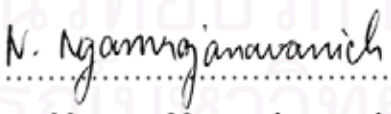

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การเกิดอันตรกิริยาของยารักษาโรคมะเร็งมีปัจจัยสำคัญ 3 ปัจจัย คือ (i) โครงสร้างโมเลกุลของสารประกอบมีรูปร่างโค้งงอคล้ายตัววี (ii) โมเลกุลมีส่วนที่แบนราบ (iii) โมเลกุลมีหมู่ฟังก์ชันที่สามารถเกิดอันตรกิริยาระหว่างโมเลกุลยากับคู่เบสของสาย DNA ได้ เช่น พันธะไฮโดรเจน หรืออันตรกิริยาแบบ π-π. 6-deoxyclitoriacetal เป็นสารที่สกัดได้จากรากของหนอนตายหยาก และมีฤทธิ์ในการยับยั้งเซลล์มะเร็งหลายชนิด วัตถุประสงค์ของงานวิจัยนี้เพื่อต้องการพิสูจน์ปัจจัยที่มีผลต่อการยับยั้งเซลล์มะเร็งแบบ สอดแทรก และเพิ่มประสิทธิภาพในการยับยั้งเซลล์มะเร็งของ 6-deoxyclitoriacetal โดยการเปลี่ยนแปลงสูตรโครงสร้างโมเลกุลของ 6-deoxyclitoriacetal โดยการทำปฏิกิริยาอิพอกซิเดชัน ที่โมเลกุลของ 6-deoxyclitoriacetal และจากนั้นเปิดวงอิพอกไซด์ด้วยมอร์โฟลีน ได้ Compound 1, Compound 2, Compound 3 และ Compound 4 ผลการทดสอบฤทธิ์ในการยับยั้งเซลล์มะเร็งตับ (Hep-G2) พบว่า Compound 4 แสดงฤทธิ์ยับยั้งเซลล์มะเร็งตับที่แรงเมื่อเปรียบเทียบกับ 6-deoxyclitoriacetal โดยมีค่า IC_{50}= 0.038 mg/mL ซึ่งเป็นค่าที่ต่ำที่สุดและต่ำกว่า 6-deoxyclitoriacetal (IC_{50}= 0.108 mg/mL). นอกจากนี้การศึกษาการใช้ยาร่วมกันระหว่าง 6-deoxyclitoriacetal-Predisolone, 6-deoxyclitoriacetal -Podophyllotoxin และ Predisolone-Podophyllotoxin โดยทดสอบกับเซลล์มะเร็งตับ (Hep-G2) พบว่า 6-deoxyclitoriacetal-Podophyllotoxin ให้ผลเสริมฤทธิ์ในการยับยั้งเซลล์มะเร็งตับ (Hep-G2) เมื่อมี 6-deoxyclitoriacetal ในปริมาณ 75-90% โดยน้ำหนัก เมื่อเทียบปริมาณทั้งหมดของสารผสม

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....ณัษฐา กิจประเทือง.....

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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NUSTHA KITPRATHAUNG: CYTOTOXIC ACTIVITIES OF 6-DEOXYCLITORIACETAL AND ITS DERIVATIVES. THESIS ADVISOR: ASSOC. PROF. NONGNUJ MUANGSIN, Ph.D., THESIS COADVISOR: ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph.D., 84 pp.

This thesis proposes that potential DNA-intercalating anti-cancer drugs possess three characteristics: (i) the molecule has a bent shape. (ii) a part of the molecule is planar. (iii) it has at least one functional group that has intermolecular interactions, such as π-π interactions or hydrogen bonding, with DNA.

6-Deoxyclitoriacetal is a substance extracted from the dried root of *Stemona collinsae Craib.* It has been known to have cytotoxic activity against various types of human carcinoma . This research proposes that it has a good chance to be a DNA-intercalating anti-cancer drug. 6-Deoxyclitoriacetal was modified by epoxide ring-opening with morpholine to obtain 4 different structures, Compound 1, Compound 2, Compound 3 and Compound 4. The IC_{50} value of Compound 4 (0.038 mg/mL) was significant lower than that of 6-deoxyclitoriacetal (0.108 mg/mL).

Moreover, the combinations of 6-deoxyclitoriacetal-Prednisolone, 6-deoxyclitoriacetal-Podophyllotoxin and Prednisolone-Podophyllotoxin were tested for cytotoxicity on Hep-G2 (Human hepatoblastoma cell line). The combination of 6-deoxyclitoriacetal-Podophyllotoxin has synergic cytotoxic effects when the amount of 6-deoxyclitoriacetal is between 75-90 %wt, based on the total amount of the two compounds.

Field of study……Biotechnology…….Student's signature……K. Nustha……
Academic year……2006……..Advisor's signature ……N. Muangsin……..
Co-advisor's signature ……N. Ngamrojanavanich……

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	percentage
/	per
bp	base pair
°C	degree celsius
cm	centimeter
CO2	carbon dioxide
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
g	gram
h	hour
HCl	hydrochloric acid
Hep-G2	Human liver hepatoblastoma
Kg	Kilogram
L	litre
M	molar
mg	Milligram
mL	Mililitre
mm	Millimeter
mM	Millimolar
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
wt	weight
µg	Microgram
µl	Microliter
µM	Micromolar
UV	ultraviolet light

CHAPTER I

INTRODUCTION

1.1 Background

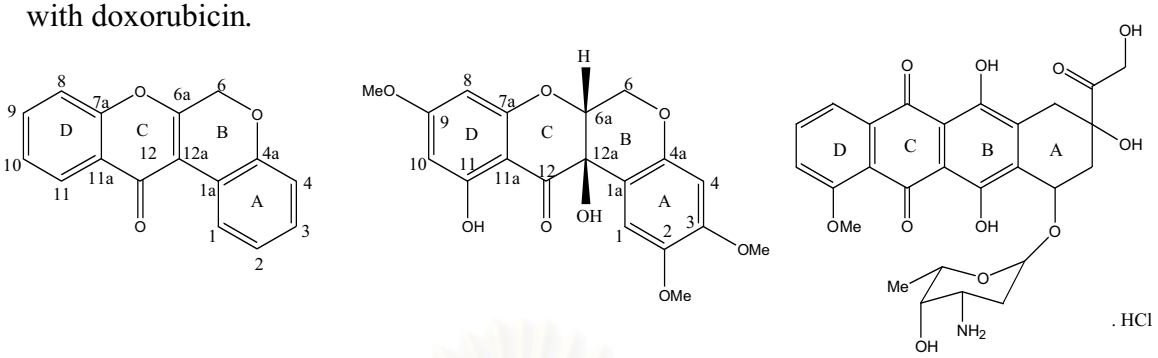
Medicinal plants are rich natural resources. They are cheap, effective and less harmful side-effects than drugs [1]. Thai people know well about herbal medicine for a long time. Nowadays, there are trends towards isolating and identifying the active ingredient in these plants in order to understand the principle behind the therapeutic effects and in search fir more potent drugs. Accordingly, medicinal plants are widely studied by modern techniques in a more scientific way.

Rotenone and rotenoids are the active ingredient of botanical insecticides used for at least 150 years to control crop pests. They are natural toxin produced by several plants and has been used for centuries as a selective fish poison and more recently commercial insecticide.They are known not only as toxicants but also as candidate anticancer agents.

1.2 Rotenoid compounds

Rotenoid, a four fused ring A, B, C and D (Fig. 1.1), possess various bioactivities such as antimicrobial, antiviral action, insecticide and antifeedant properties. One of the interesting activities of rotenoid compounds is anticancer activity. For example, 6-deoxyclitoriacetal from the roots of *Clitoria macrophylla* [2] is known to show strong cytotoxic activity against culture P388 lymphocytic leukemia cell [3]. Rotenoids from *Amorpha fruticosa* are found to be inhibitors of human cancer cell line [4, 5, 6]. In Thailand, the rotenoid compound, 6-deoxyclitoriacetal [Fig. 1.1], was isolated from the dried roots of of *Stemona collinsae* Craib. It has been known to have cytotoxic activity against various types of human carcinoma [7]. 6-deoxyclitoriacetal presents both planar structures, heteroaromatic ring systems corresponding with the important characteristic of intercalation drug molecules. Not only the planar molecule, 6-deoxyclitoacetal present the bent shape as well. This is similar to the structure of known anticancer drug, doxorubicin. Although, 6-

deoxyclitoacetal is not a derivative of doxorubicin, it shares structural similarities with doxorubicin.



(a) (b) (c)

Figure 1.1 Chemical structure of (a) Rotenoid structure,(b) 6-deoxyclitoriacetal (c) Doxorubicin hydrochloride

1.3 Source of Rotenoids

The STEMONA genus, for example, is rich in rotenoids. *Stemona* is a type of perennial climbing plant native to continental Asia and Japan through Southeast Asia to tropical Australia It usually grows in areas of dry vegetation, and usually consists of single thing with alternating, spade-shaped leaves and thick, white tuberous root. *Stemona* is the largest genus with about 25 species occurring as subshrubs or twining herbs mostly with perennial tuberous roots.

1.4 Traditional usages

S. collinsae Craib., *S. tuberosa* Lour., *S. japonica* Miq., and *S. sessilifolia* Miq. have long been used in Thailand, China and Japan for various medicinal and biological properties. Especially extracts from the fleshy tuberous roots are still used to treat cancer, respiratory disorder, including pulmonary tuberculosis and bronchitis and are also recommended to use against different insect pests.

Stemona roots can be used both internally and externally. Externally, it can be applied to the skin as a poultice to rid the body of lice and fungi. Internally, it is used to reduce the incidence of both acute and coughs by relaxing the respiratory system and lowering blood pressure. One components of *Stemona,* tuberostemonine, also has been shown to have some pain-relieving properties. There is also evidence that *Stemona* roots, when taken in a decoction with alcohol, can prohibit growth of the bacteria that causes tuberculosis.

1.5 Intercalative drug

There are two principal modes for non-covalent binding to DNA-intercalation and minor groove binding [8].

The first mode is DNA-intercalation. Intercalating drugs should have planar, heteroaromatic ring systems which insert themselves between two adjacent base pairs in a DNA helix. The drug-DNA complex is stabilized by π-π and van der Waals interactions between the DNA base and the drug molecule. Intercalating drugs also cause structural perturbations in the DNA to accommodate the binding, such as the unwinding of the helix and a lengthening of the DNA, thereby, inhibits the ability of enzymes such as topoisomerase II to interact with DNA (Fig. 1.2). This enzyme cleaves double stranded DNA to reduce the strain that comes from local unwinding. The formation of drug-DNA-enzyme complex leads to breakage of the DNA backbone and inhibits DNA replication and transcription of the target DNA [9]. Intercalators include ethidium, doxorubicin and its derivatives (Fig. 1.3).

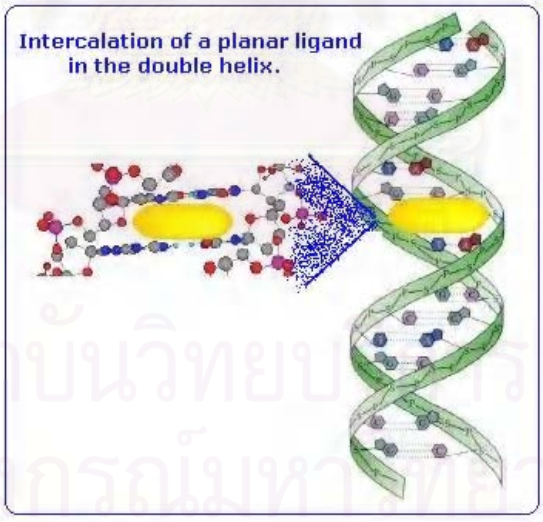
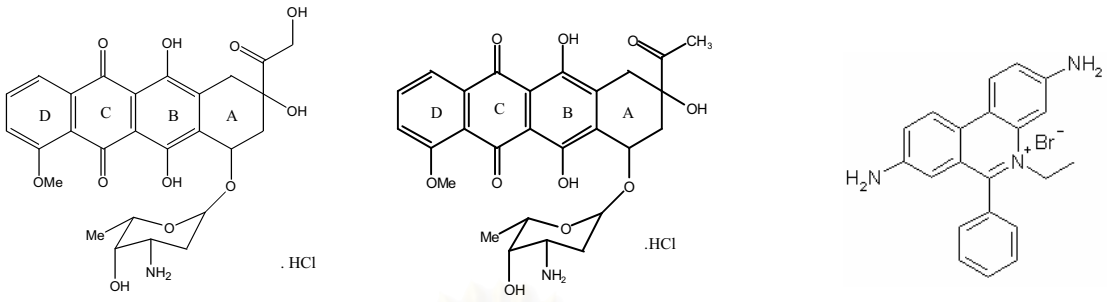


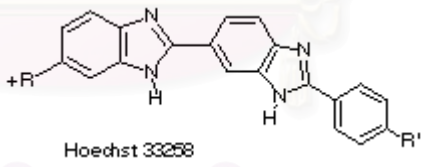
Figure 1.2 Intercalation of a planar ligand in the double helix [10]



(a) (b) (c)

Figure 1.3 Chemical structures of (a) Doxorubicin hydrochloride (b) Daunorubicin hydrochloride (c) Ethidium bromide

The second mode is a minor groove binding. These drugs consist of several aromatic rings (e.g. benzene or pyrrole). The drug-DNA binding is stabilized by hydrophobic interactions, as well as van der Waals interactions and hydrogen bonding. The drug-DNA binding preference is to the A-T base pairs. Minor groove binders do not induce significant structural changes to the DNA. Drugs in this category include Hoechst 33258 (Fig. 1.4).



Hoechst 33258

Figure 1.4 Chemical structure of Hoechst 33258 [10]

Doxorubicin (Fig. 1.3), also known as adriamycin, is an antibiotic used as a treatment for a wide range of solid tumors. This compound possesses an anthracycline chromophore containing four fused rings and a positively charged amino sugar. It interacts with DNA by intercalation and inhibits both DNA replication and transcription [9]. When the drug intercalates with DNA, the cyclohexane 'A' ring resides in the minor groove acting as an anchor, hydrogen bonding to base-pairs above and below. The planar 'D' ring resides in the major

grove. The drug is held in place by the formation of favorable hydrogen bonds to the bases within DNA; for example, the hydroxyl group in the 9 position forms two hydrogen bonds to N2 and N3 of an adjacent guanine [11]. However, this drug does not specific to the base-pair sequence and shows a high level of toxicity as they will also interact with the DNA in many other tissues.

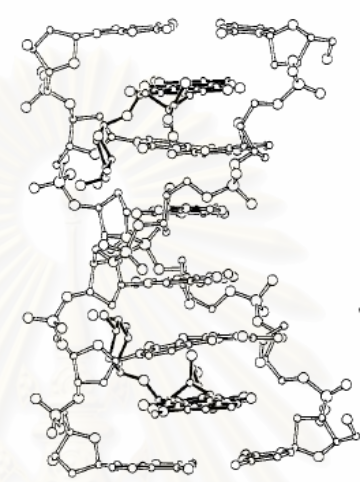


Figure 1.5 Intercalation binding of doxorubicin- d(CGATCG)[12]

1.6 Cytotoxicity

The use of cells models is an indispensable tool of current research into the biological activity of plant extracts. The liver is known to be the main site of xenobiotic biotransformation due to the ability of this organ to express a plethora of enzymes, both quantitatively and qualitatively. Therefore, cell lines of liver origin are widely used in biomedical research involving xenobiotic metabolism including genotoxicity studies. Of these cell lines, the Hep-G2 cell line is the most versatile one. This cell line retains many of the specialized functions normally lost by primary hepatocytes in culture [13]. In the fields of experimental toxicology and pharmacology isolated human hepatocytes have been extensively used for the development and testing of new drugs at both preclinical and clinical stage because of the special *in vivo* role of human hepatocytes in drug metabolism and detoxification [14].

1.7 Combination drug

Cancer is a disease of inappropriate tissue accumulation. Chemotherapeutic agents share one characteristic: they are usually more effective in killing or damaging malignant cells than normal cells. However, the fact that they do harm normal cells indicates their potential for toxicity. Animal tumor investigations and human clinical trials have shown that drug combinations produce higher rates of objective response and longer survival than single agents. Combination drug therapy is the basis for most chemotherapy employed at present [15]. When two drugs are applied in combination as a mixture to a biological system the resulting effect can be equal or different as compared to what is expected from the biological activity of the individual compounds.

Cancer treatment requires inhibitions of variety of faction include tumor cell proliferative, metastatic diddemination of cancer cells to other parts of the body, invasion, tumor-induced neovascularization, and enhancement of host immunological responses and cytotoxicity. Conventional cancer chemotherapeutic agents have often been selected on the basis of their cytotoxicity to tumor cells, However, some anticancer agents have adverse effects on the patient's immune system. Thus it would be greatly advantageous if a cancer therapy or treatment could be developed that would afford non-cytotoxic protection against factors that might lead to progression of tumors.

Mazin A. Moufarj, et al. [16], studies the combination of cisplatin and the nucleoside analog gemcitabine . There was an investigation of hypothesis that synergistic cytotoxicity between gemcitabine and cisplatin in test cell lines may be caused by gemcitabine-mediated pnhibition of cisplatin intraatrand adduct (IA) and interstrand cross-link (ICL) repair. The effect of gemcitabine on the accumulation and repair of cisplatin IA and ICL in each cell lines was then measured directly using gene-specific quantitative polymerase chain reaction and denaturation techniques, respectively. The results show that only 75% IA were removed in the combination treated cells compared with 74% in cispatin control cells. Similarly, repair of cisplatin ICL was inhibited in the gemcitabine-treated cells compared wit the cells treated with cisplatin only (60% versus 72%). These findings demonstrate a direct inhibitory effect of gemcitabine on the repair of the cisplatin IA and ICL and suggest a mechanistic basis for the cytotoxic synergy between the two drugs.

Ossi R. Koechli, et al. [17], studies the combination of paclitaxel (Taxol^{TM}) (TAX) and doxorubicin (Adriamycinl^{TM}) (ADR) in the treatment of breast cancer. This study was done to evaluate the combination effect of TAX and ADR in tree human breast cancer cell lines. Drug synergism/antagonism was shown to be dose-related; synergism was enhanced at higher fractions affected. From the studied they have concluded that TAX-ADR is highly effective and partly synergistic *in vitro*.

A novel class of non-thiol-containing peptidomimetic inhibitors of farnesyltransferase and geranylgeranyltransferase was studied by J. Sun, et al. [18] in 1999. They compared the cfficiency of drug activity between monotherapy and that in combination with the cytotoxic agents cisplatin, Taxol, and gemcitabine. The combination therapy of FTI-2148 and GGTI-2154 with either cisplatin, gemcitabine, or taxol resulted in a greater antitumor efficacy. There were 30- and 33- fold more selective and 30- and 16- fold more potent in whole cells than previous reported thiol-containing FTI-276 and GGTI-297, respectively. Thus, the highly potent and selective novel class of non-thiol-containing peptidometrics inhibits tumor growth was evaluated in combination therapy.

Y. Jounaidi [19]was studied the combination of the bioreductive drugs for P450/P450-reductase-based cancer gene therapy. Tirapazamine (TPZ) exhibits particularly good activity when combined with alkylating agents such as cyclophosphamide (CPA). The results showed that, there was 9L tumor cells were transduced in culture with P450 2B6 and P450 reductase and grown as solid tumors in severe combined immune deficient mice in vivo. Although these tumors showed little response to TPZ treatment alone, tumor growth was significantly delayed, by up to approximately four doubling times, when TPZ was combined with CPA. Some toxicity from the drug combination was apparent, however, as indicated by body weight profiles. These findings suggest the potential benefit of incorporating TPZ, and perhaps other bioreductive drugs, into a P450/P450 reductase-based gene therapy strategy for cancer treatment.

Prednislone [20]

Prednisolone is a non synthetic corticosteroid drug which is usually taken orally but can be delivered by intramuscular injection and can be used for a large number of different conditions. It has a mainly glucocorticoid effect. Prednisone

is a product that is converted by the liver into Prednisolone which is the active drug and also a steroid.

Prednisone is particularly effective as an immunosuppressant and affects virtually all of the immune system. It can therefore be used in autoimmune diseases, inflammatory diseases (such as severe asthma, severe poison ivy dermatitis, ulcerative colitis, Rheumatoid Arthritis and Crohn's disease), various kidney diseases including nephrotic syndrome, and to prevent and treat rejection in organ transplantation. This medicine may also reduce the sex drive.

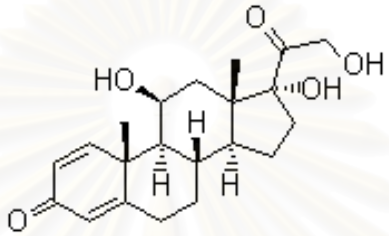


Figure 1.6 Structure of Prednisone

Podophyllotoxin [21]

Extracts of Podophyllum species have been used for diverse cultures since remote times as antidotes against poisons and toxic, cathartic, purgative, antihelminthic, vesicant, and suicide agents [22]. And the use of experimental cancer cells in animals was published too.Also, podophyllotoxin is included in many Pharmacopoeias and used as an antiviral agent in the treatment of Condyloma acuminatum caused by human papillomavirus –HPV- and other venereal and perianal warts. The application of podophyllotoxincured almost all the warts completely in less time than other strategies and with fewerside effects. Podophyllotoxin and analog compounds are also active againstcytomegalovirus and Sindbis virus. Podophyllotoxin is also effective in the treatment of anogenital warts in children and against Molluscum contagiosum, which is generally a self-limiting benign skin disease that affects mostly children, young adults, and HIV patients. Podophyllotoxin has other uses in dermatology: it is a useful agent in psoriasisvulgaris. Antitumor activity is another outstanding property of podophyllotoxin. It is effective in the treatment of Wilms tumors, different types of genital tumors (e.g., carcinoma verrucosus) and in non-Hodgkin’s and other lymphomas. Combination therapies are currently being implemented with other chemotherapeutic agents or with other

techniques useful in the fight against viral infections and cancer. In this sense, condyloma acuminata respond best to thecryotherapy-podophyllotoxin combination; multiple myeloma responds best tohomeotherapy with podophyllotoxin and intermittent local administration ofmethotrexate and systemic polychemotherapy. In combination with interferon, podophyllotoxin is active in genital human infections caused by vulvar pruriticpapillomatosis and together with cis-platin is effective in treating neuroblastomas.The mechanism of action of podophyllotoxin is based on inhibiting thepolymerization of tubulin and arresting the cell cycle in the metaphase.

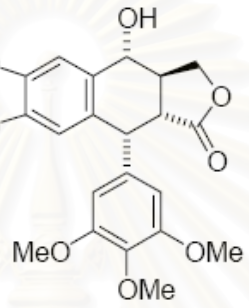


Figure 1.7 Structure of Podophyllotoxin

1.8 The objective of this research

The aim of this work is to study the structure-activity relationship of 6-deoxyclitoacetal and its derivatives on cytotoxic activities for enhancing their anticancer activities by synthesis 6–deoxyclitoriacetal derivatives

Our approach is purposed as follows:

The alternative combination drugs between 6-deoxyclitoacetal and commercial drugs (Predisolone and Podophyllotoxin). In order to investigate the cytotoxicity against HEP-G2. To reduce the drug dosage, to reduce the side effects of chemotherapy, to reduce high cost, to solve the problems about drug resistance and to safe and effective use of the chemotherapy.

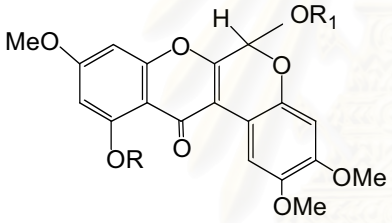
Chapter II

LITERATURE REVIEW

2.1 Isolation

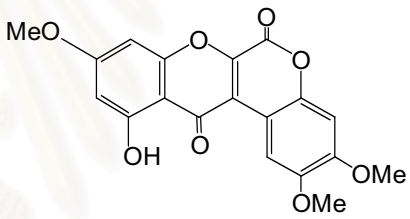
The isolation of rotenoids has been reported from variety pf plants particulary tropical plants are abundant source of them. The following literature are selecyed to review

In 1974, Shienthong [23] and co-workers reported the isolation of four new rotenoid compounds from *Stemona colinsae* Craib. Namely stemonacetal (1), stemonal (2) and stemonone (3) respectively.



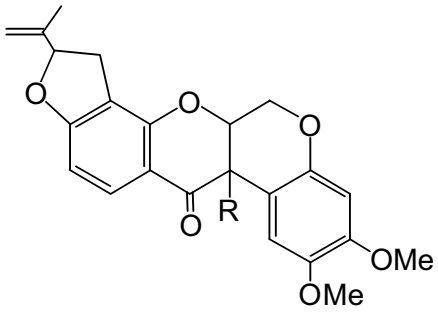
1 R=H, R_1=OCH_2CH_3

2 R=H, R_1=H



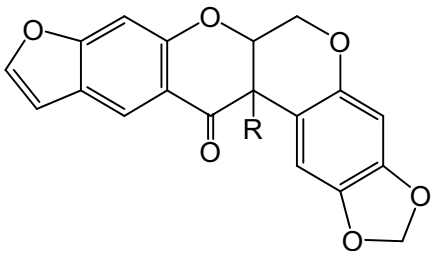
3

Roux and colleagues [24, 25] reporete the isolation of rotenoids compounds from the roots of *Neorautanenia amboenia*. Prelimimaty test showed **(8)** to be toxic to insects.



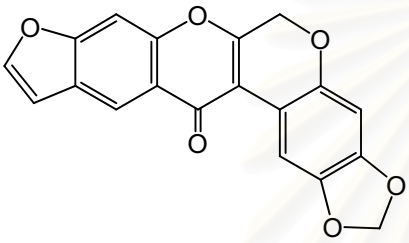
4 R=H

5 R=OH



7 R=H

8 R=OH



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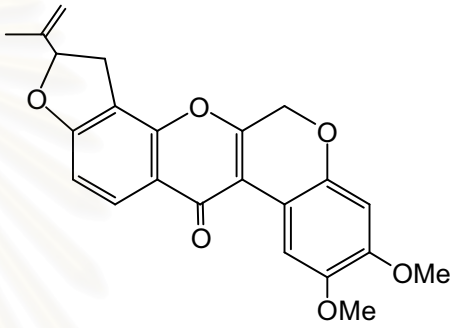

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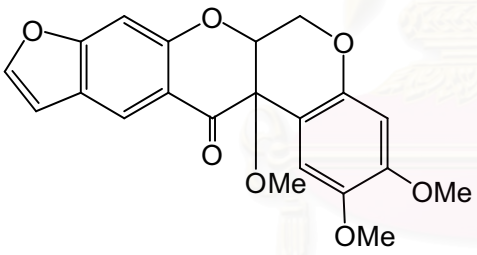

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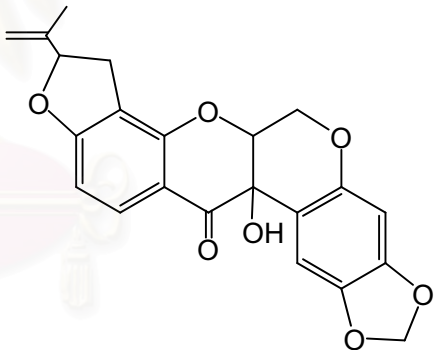

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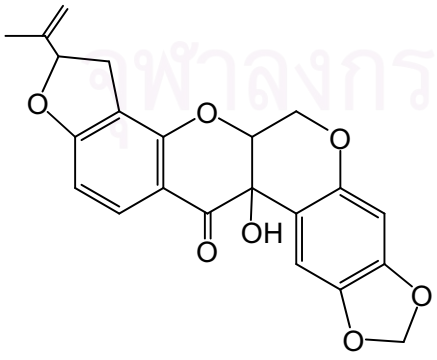

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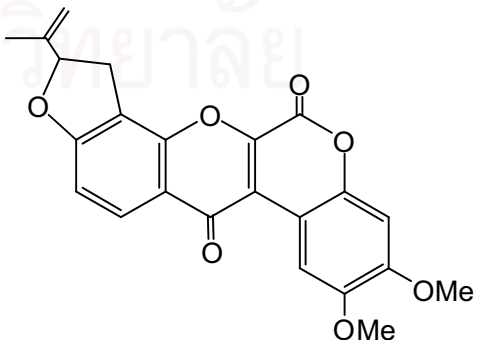
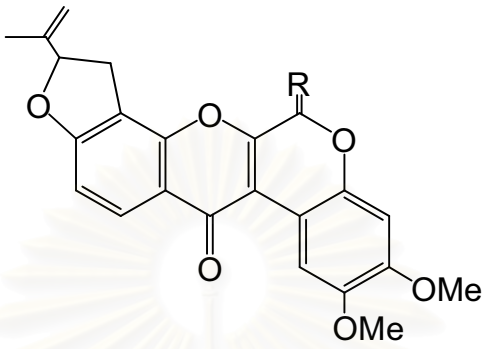


15

A new rotenoid isolated from a hexane extract of *Amorpha canescens* [26] roots and identifie as 6-hydroxydehydrorotenone (**16**). The kniwn two compounds, (**5**) and (**6**) were resulted by chemical conversion to compare their core structure to compound (**16**).

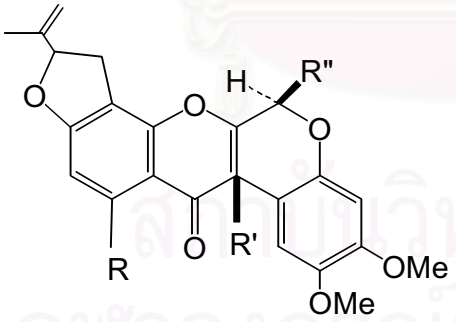


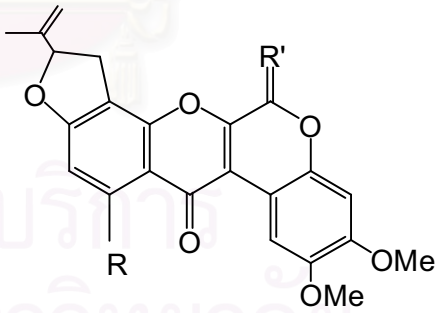
16 R=H, OH

11 R=H_2

15 R=O

Many *Tephrosia* specirs show pesticidal and insecticidal properties due to the presence of rotenoids. In India, the juice of leaves of *T.villosa* [27, 28, 29] is used to treat dropsy and diabetes. The isolation from whole plants of this species found 13 rotenoid compounds and three of them were known.





4 R, R', R"=H

17 R=OH; R', R"=H

18 R, R'=OH; R"=H

19 R, R'=OH; R'=H

20 R,R',R"=OH

11 R=H; R'=H_2

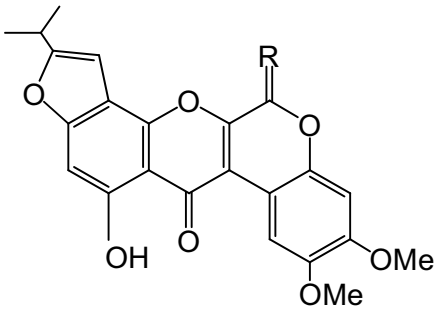
21 R=OH; R'=H_2

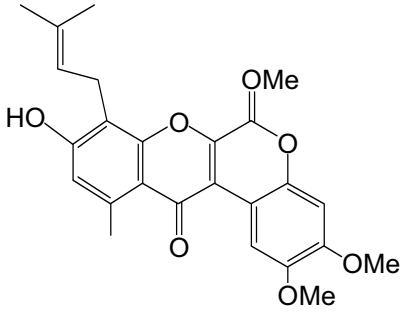
22 R=OH; R'=H, OH

23 R=OH; R'=H, OMe

15 R=H; R'=O

24 R=OH; R'=O



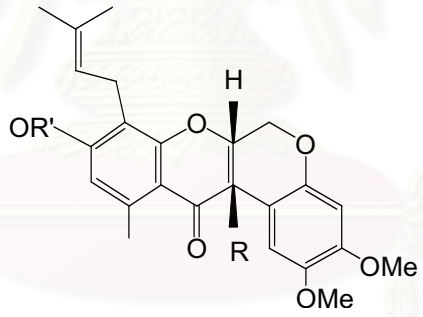


25 R=O

26 R=H, OH

27

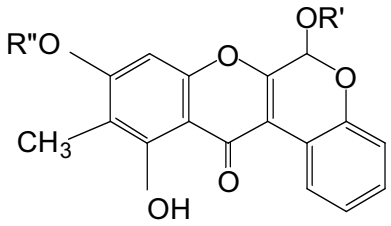
The roots of *Millettia pachycarpa* are occasionally used as a fish poison and are reputed to be insecticidal. Singhal [30] and colleagues reported the isolation if rotenoids from the roots of this species **4, 14, 28a** and **28b**



28a R=R'=H

29b R=OH, R'=H

Boerthaavia diffusa L. [31] is widely used in traditional medicine: in Nepal as a remedy foir back bone pain and as a tonic whole plant) in association with other plant; in Sri Lanka to treat rheumatism and snake bite; in India as amedicine with multiple actions (stomachic, diuretic, antiasthmatic, diaphoretic, anthelminthic, etc.) Investigation on its chemical constituents found two rotenoid analogues

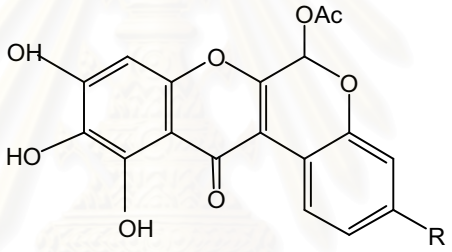


30 R'=CH_3, R"=H

31 R'=R"=H

32 R'=R"=CK_3

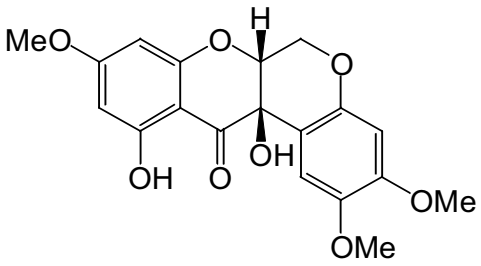
Ahmed et al. [32] reprted two rotenoid derivatives, repenone (**33**) and repenol (**34**), freom *Boerhaauia repens* usually grows in tropical and subtropical regions. This plant is common in Bangladesh and has reputation for versatility.



33 R=H

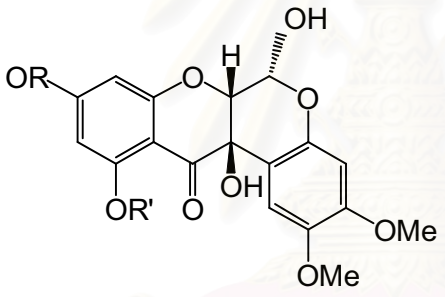
34 R=OH

Clitoria marcrophylla [33] is traditional used in Thailand for skin diseases and for pest control in hortcultureand animal husbandry. It was also reported as anti-inflammatory and antipyretic activities. Phytochemical investigation resulted in the isolation of a new rotenoids, 6-deoyclitoriacetal (**35**), from its roots. In vitro test showed that this compound possessed strong cytotoxic activity against cultured P-388 lymphocytic leukemia cells, but was not active with cultured KB cells.



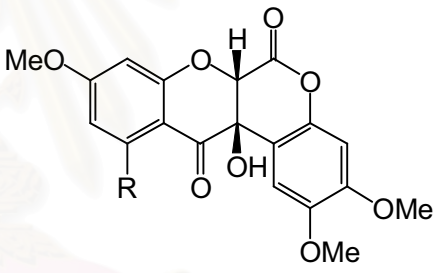
35

The genus *Clitoria* has 60 reported species, some of which possess remarkably anti-inflammatory and antipyretic actives. Moreover, from the roots of *C.macrophylla* has been used for the treatment of skin diseases in Thailand, Preliminary test showed strong cytotoxic activity against cultured P-388 lymphocytic leukemia cells. The isolation of the roots [34, 35] and seeds [36] of Clitoria *Fairchildian* afforded rotenoid derivatives.



36 R=H

38 R=OMe



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2.2 Biological activites

Rotenoids are known as insecticides foe at least 150 years. They have been used even longer as fish poisons by native tribes to obtain food [37] and more recently in fish management to achieve the desired balance of species. The acute toxicity of rotenoids to insects, fish and mammals is attributable to inhibaion of NADH;ubiquinine oxidoreductase (complex I0 activity as the primary target.[38, 39] Rotenoids are known not only as toxicants bus also as candidate anticancer agents base on three observations: exhibition of the formation of the microtubules from tubulin and anti-cancer activities, [40, 41, 42, 43] prevent cell proliferatin induce by a peroxisome proliferators in mouse liver, [44] and ninhibit phorbol ester-induced

ornithine decarboxylase (ODC) avtivity as measure of cancer chemopreventive potency. [45, 46, 47]

2.3 Synthesis

Some of the existing rotenoid syntheses have limitation in term of their ability to accommodate different substitution pattens, and in term of yield. However, a number of synthetic strategies have been used to construct the rotenoid system includind the use of Hoesch condensation, themal condensation of 4 ethoxycarbonylchronan-3-ones with activated phenols, reaction of isoflavones with dimethysulfoxonium methylide, Claisen rearrangement of pro-2-ynyl ethers, aroylation of 4-lithiochromenes, enamines and 4-phenylsulfonylchromans, intramolacular radial cyclization, and combined Wadsworth-Emmons-Mukaiyama aldol methodologies.[48]

In 1998, Gabbutt et al.[48] reported the synthesi of rotenoid core structure using the hypervalent iodine-promoted oxidative ring expansion of the spirocycles as a key step.

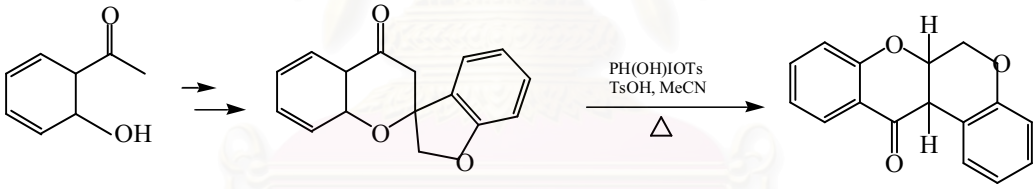


Figure 2.1 Hypervalent iodine in rotenoids synthesis**.**

Lately, Sames et al. [49, 50, 51] have develop a new platinum-catayzed hydroarylation method ahich would be applicable to synthesize rotenoid class of natural products.

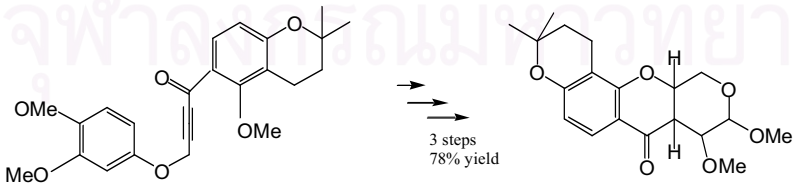


Figure 2.2 $PtCl_2$-catalyzed hydroarylation in rotenoids synthesis**.**

2.4 Cancer overview

• Carcinoma

A carcinoma is a cancer found in body tissue known as epithelial tissue that covers or lines surfaces of organs, glands, or body structures. Many carcinomas affect organs or glands that are involved with secretion, such as breasts that produce milk. Carcinomas account for 80 percent to 90 percent of all cancer cases.

• Sarcoma

A sarcoma is a malignant tumor growing from connective tissues, such as cartilage, fat, muscle, tendons, and bones. The most common sarcoma, a tumor of the bone, usually occurs in young adults. Examples of sarcoma include osteosarcoma (bone) and chondrosarcoma (cartilage).

• Lymphoma

Lymphoma refers to a cancer that originates in the nodes or glands of the lymphatic system, whose job is to produce white blood cells and clean body fluids, or in organs such as the brain and breast. Lymphomas are classified into two categories: Hodgkin's lymphoma and non-Hodgkin's lymphoma.

• Leukemia

Malignant neoplasm of blood-forming tissues; characterized by abnormal proliferation of leukocytes; one of the four major types of cancer. White blood cells are needed to resist infection. Red blood cells are needed to prevent anemia. Platelets keep the body from easily bruising and bleeding. Examples of leukemia include acute myelogenous

leukemia, chronic myelogenous leukemia, acute lymphocytic leukemia, and chronic lymphocytic leukemia. The terms myelogenous and lymphocytic indicate the type of cells that are involved.

• Myeloma

Myeloma is tumor of plasma cells growing in the bone marrow. Multiple myeloma (alsoknown as myeloma or plasma cell myeloma) is a progressive hematologic (blood) disease. It is a cancer of the plasma cell, an important part of the immune system that produces immunoglobulins (antibodies) to help fight infection and disease. Multiple myeloma is characterized by excessive numbers of abnormal plasma cells in the bone marrow and overproduction of immunoglobulin.

Cancer is treated in several ways, depending on each person's medical condition and type of cancer. The traditional treatment of cancer patients involves a combination of surgery, radiotherapy and/or chemotherapy. Although surgery and radiation therapy are both key weapons in the fight against cancer, chemotherapy is also a vital approach and is the only approach possible for treating disseminated cancers. The ultimate goal of cancer chemotherapy is to kill cancer without killing the patient. This requires the development of selective drugs that can kill malignant tumor cells or render them benign without affecting normal cells [22].

Different tumors have different aberrations in signaling and growth stimulation pathways that drive cancer growth. An understanding of these processes is a key to development of new anticancer agents and to identifying optimal treatment strategies and patient populations suitable for specific therapies.

Based on a mechanical approach, we can roughly classify the current anticancer chemotherapeutic approaches or agents as

a) Cytotoxic agents
b) Antibody targeting agents
c) Anti–hormonal agents
d) Signal transduction inhibition agents
e) Ras–inhibition agents
f) Cell cycle modulating agents
g) Apoptosis inducing agents
h) Angiogenesis inhibition agents
i) Anti–invasion agents

Cytotoxic agents are further subdivided as tubulin binders, DNA intercalators, antifolates, alkylating agents, and topoisomerase inhibitors; the most widely used anticancer drugs, such as taxol (paclitaxel or Taxol), doxorubicin, methotrexate, cisplatin and etoposide fall into each of these categories respectively [22].

2.5 Biological activities

2.5.1 Cytotoxicity

Cell death can be defined as the irreversible loss of essential cellular functions and structures. It has long been used as an endpoint to assess both drug efficacy and drug toxicity *in vitro*. In terms of drug toxicity, cytotoxicity assays have been used to

assess the formation of chemically reactive intermediates from drugs that have been implicated in idiosyncratic drug reactions ([52], Fig. 2.3).

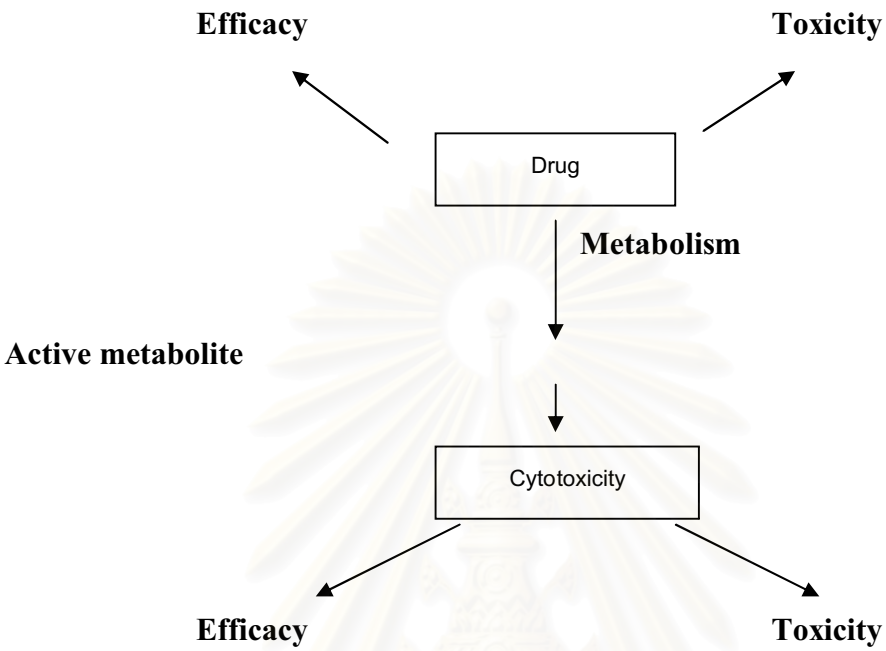


Figure 2.3 The role of metabolism in mediating drug (metabolite) cytotoxicity, which may, in turn, be important for efficacy or toxicity.

a) Methods for the assessment of cell viability

Many methods for the assessment of cell viability have been described (Table 1). These can be divided into direct and indirect methods [53]. The direct assays include clonogenic assays and assays that back extrapolate cell densities in exponentially proliferating cultures; they are sensitive and directly measure surviving fractions of cells, but suffer from the major disadvantages of being labor-intensive, technically difficult, and require several weeks to obtain results. In view of these limitation, various indirect, more rapid assays have been developed. These include measurement of [54]

- Inhibition of incorporation of labeled RNA or DNA precursors, amino acids, or other metabolites
- Exclusion of dyes or enzymes
- Decrease of intracellular ATP content

- Formation of a colored or fluorescent product from a precursor
- Release of a labeled compound or an enzyme from damaged cells
- Total cell biomass

Table 2.1 Some of the methods used to assess cell death [54]

Assay	Type
Trypan blue dye exclusion	Exclusion of dye
Lactate dehydrogenase (LDH) release assay	Release of intracellular enzyme
MTT assay (tetrazolium salt reduction)	Formation of colored product
BCECF proliferative assay	Formation of fluorescent product from precursor
Tritiated thymidine (^{3}H-TdR) release assay	Release of labeled compound
^{51}Chromium (Cr) release assay	Release of radiolabeled compound
Europium (Eu^{3+}) release assay	Release of fluorescent compound
ATP bioluminescence	Release of ATP from damaged cell

Almost any cell can be used as a target in cytotoxicity assays. When toxicity of an agent to a particular organ is being assessed, ideally human cells derived from that organ should be used as targets because they will reflect the content of activating and detoxifying enzymes in that organ. However, this is often not possible to directly extrapolate the data obtained from the animal to humans. So an alternative is to use readily accessible human cells. [55]

b) Mode of cell death

There are two fundamental types of cell death : apoptosis and necrosis[56]. They can be differentiated as indicated in Table 2.2.

Necrosis occurs when the cell is exposed to severe and sudden injury, such as physical and chemical trauma. Because cell injury is extensive, there is often an inflammatory response that is required to clear the debris.

Apoptosis is more subtle, and it is the most important form of programmed cell death. On exposure to the noxious substance, the cell undergoes a complex series of molecular changes (which are not fully understood) that ultimately result in its death in the absence of an inflammatory response. However, in the late stages of apoptosis, changes similar to necrosis may be seen within the cell; hence, simple dye exclusion assays cannot be used to differentiate the two forms of cell death [57]

Table 2.2 Morphological distinction between apoptosis and necrosis

Apoptosis	Necrosis
• Death of isolated cells	• Death of contiguous patches or areas of tissue
• Chromatin condensation, nuclear shrinkage and cell shrinkage	• Nuclear and organelle swelling and whole-cell swelling
• Budding of plasma membrane	• Blebbing of plasma membrane
• Late loss of membrane integrity	• Early loss of membrane integrity
• No inflammatory infiltrate	• Inflammatory infiltrate present
• Phagocytosis of dying cells by neighboring cells	• Phagocytosis of dying cells by professional phagocytes
• DNA laddering on gel electrophoresis	• Nonspecific DNA degradation as a late event

Apoptosis is an energy-dependent process that can run a very fast course (34 min from the onset of budding to complete break-up has been reported) [57]. A biochemical feature of most forms of apoptosis is DNA fragmentation. Initially, DNA fragmentation occurs at 300 or 50 kilobase (Kb) pair intervals; this is followed by cleavage into 180 to 200 bp internucleosomal-sized fragments, which can be visualized on agarose gel as DNA laddering [58]. This is caused by activation of calcium-magnesium-sensitive nucleases, which have not yet been fully characterized. The complex series of molecular events underlying apoptosis are not fully understood; several gene appear to be involved (Table 2.3; [59]).

Table 2.3 Gene products influencing apoptosis

Enhancers of apoptosis	Inhibitors of apoptosis
Bcl-x_s	*Bcl*-2
Bax	*Bcl*-x_L
Bak	*Bcl*-w
Bad	*Mcl*-1
Nbk	p53
Bik 1	Colony-stimulating factors
TNF-□	
Fas/Apo1/CD95	
Interleukin-1□-converting enzyme (ICE)	
c-*myc*	

Necrosis in contrast to apoptosis is an energy-independent process, and is characterized by a progressive reduction in the cellular ATP content [60]. Membrane damage seems to be the key event in the pathogenesis of necrosis; as the injury becomes irreversible, there is a progression from subtle changes in the membrane ionic pumps to nonspecific increases in membrane permeability and, ultimately, to physical membrane disruption.

To date, most of the cytotoxicity assays used in pharmacology and toxicology have used cell death as the endpoint, with differentiating between apoptosis and necrosis.

c) The role of metabolism in drug cytotoxicity

In general, drug metabolism can be considered a detoxification process, in that it converts therapeutically active compounds to inactive metabolites that can then be excreted harmlessly from the body. This process may require one or more than one drug-metabolizing enzyme that may be a phase I and phase II enzyme (Fig. 2.9). Metabolism can lead to the formation of chemically reactive intermediates that, if not adequately detoxified, can bind to various essential cellular macromolecules *in vivo* and, thereby, result in different forms of toxicity. *In vitro* the formation of chemically reactive metabolites will often be manifested as cellular death, irrespective of the

nature of the macromolecule to which the toxic metabolite binds [61]. A drug may undergo sequential phase I and phase II metabolism, or alternatively, it may undergo only phase I or phase II metabolism [62].

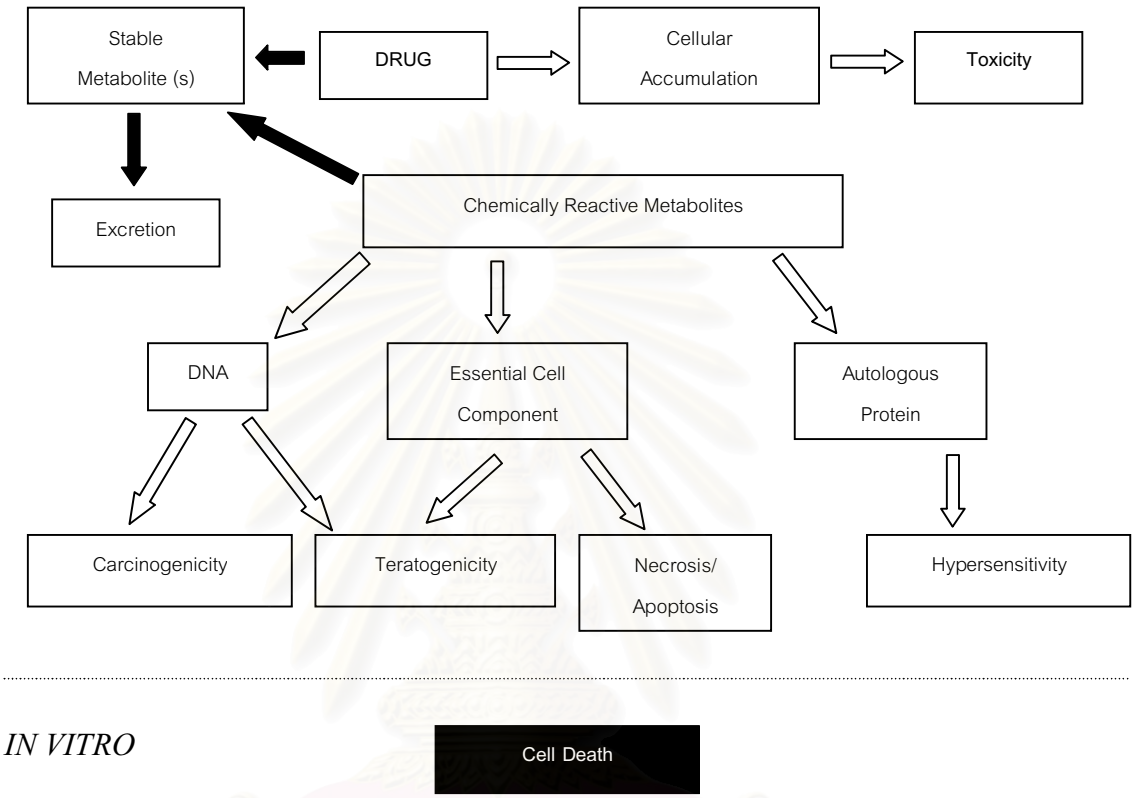


Figure 2.4 The role of metabolism in drug toxicity:

The pharmaceutical industry has also made use of the body's drug-metabolizing enzymes by developing products that are metabolized (by phase I, phase II, or both, enzymes), either within the liver or in the target tissue, to their active components that then mediate the drug's therapeutic action.

d) The use of cytotoxicity as an indicator of drug toxicity

By definition, a chemically reactive metabolite is unstable and it may not be possible to characterize it directly by routine analytical methods, such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS). To assess the formation of these metabolites and their possible role in idiosyncratic drug

toxicity, various methods have been used as indirect markers for their formation [63]. These include the following:

- Covalent binding to proteins
- Immunological detection of drug protein conjugates
- Trapping of the reactive metabolites as thiol adducts and subsequent characterization by nuclear magnetic resonance (NMR) and mass spectroscopy
- Cytotoxicity assays

A two-stage *in vitro* cytotoxicity assay (Fig. 2.5) was devised by Spielberg [55] in an attempt to recreate the *in vivo* generation of chemically reactive drug metabolites, by the incorporation of a drug-metabolite-generating system (microsomes and NADPH) and mononuclear leukocytes (MNL) as a readily available target cell. The drug is incubated with a metabolizing system comprising liver microsomes and NADPH, and incubated with peripheral blood mononuclear leukocytes (MNL) taken from patients with and without idiosyncratic adverse reactions. Cytotoxicity can be determined by various methods, the most common being Trypan blue dye exclusion. Metabolism to stable and protein-reactive metabolites within the system can be assessed by using HPLC and determining covalent binding to the microsomal protein, respectively (Fig. 2.10). The degree of bioactivation of the drug to a chemically reactive metabolite is then assessed by the determination of the viability of the leukocytes. The use of a functional assay is complementary to the chemical and immunochemical methods described in the foregoing. The importance of the assay lies in the fact that several variables can be altered independently or in combination to answer different questions. First, cells from patients with and without adverse drug reactions can be used to determine interindividual variation in cellular detoxification processes [55].

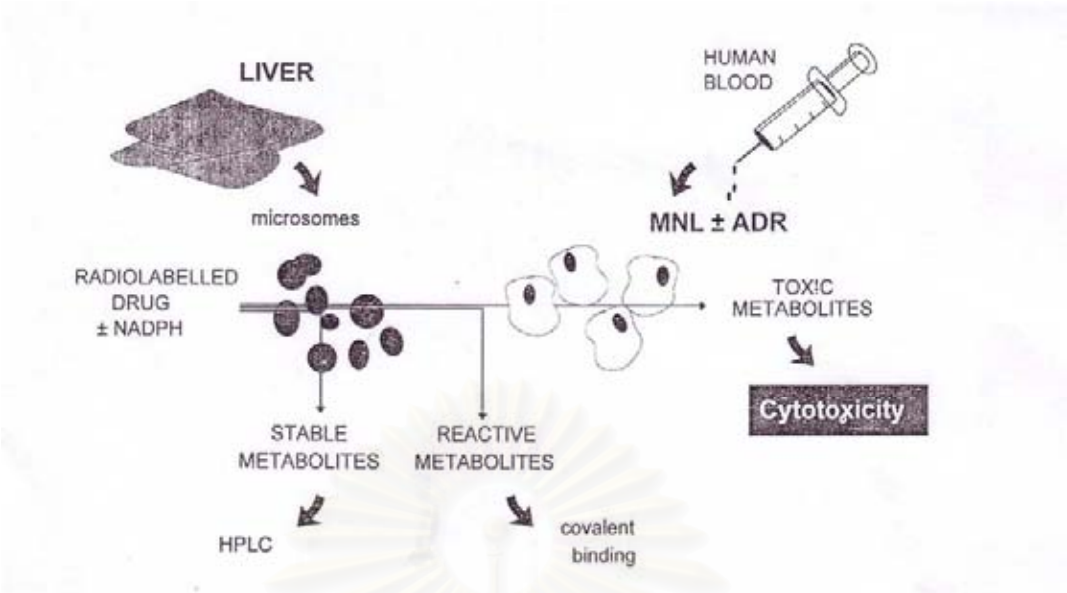


Figure 2.5 A schematic representation of the *in vitro* cytotoxicity assay.

In most of the studies, ether trypan blue dye exclusion or the MTT assay has been used to estimate cell viability. The MTT assay can be used only if a microsomal system is not present in the incubations because microsomes themselves may metabolize MTT. Thus, the MTT assay is suitable only for use when the toxic metabolite itself can be synthesized and then incubated with the cells, as in sulfamethoxazole hydroxylamine [64]. Trypan blue dye exclusion correlates with other indices of cell damage, including lactate dehydrogenase (LDH) release from the cells and loss of ability to respond to concanavalin a blastogenesis [55]

The major problem with the *in vitro* cytotoxicity assay is that it lacks sensitivity in that any changes observed are small [65] and thus, a negative result may not exclude the formation of a toxic metabolite from the coincubated drug. In addition, a chemically reactive metabolite that is not cytotoxic, but may bind irreversibly to protein, may be formed. Therefore, when a radiolabeled compound is available, it may be more useful to combine the determination of both the cytotoxic and protein-reactive metabolites (as well as the stable metabolites) within the same system (Fig. 2.10).

The advantages and disadvantages of using the *in vitro* cytotoxicity assay in elucidating the pathogenesis of idiosyncratic toxicity is illustrated with reference to several compounds that have been associated with different forms of toxicity [54]

2.5.2 Cytotoxic and viability assays

An intensive program to screen plant extracts and other natural materials for anticancer activity began in 1955. Use of *in vitro* assay systems for screening of potential anticancer agents has been common practice almost since the beginnings of cancer chemotherapy. According to the NCI data, many plants extracted posse's cytotoxic activities. Definition of cytotoxicity refers to agents that are toxic to cells *in vitro*. Cytotoxic agents can be further divided into those with cytostatic effect (those that stop cell growth) and cytocidal effect (those that kill cells). The techniques of growing cells as a monolayer have been most frequently applied to the cytotoxicity testing of cancer cell lines. For each compound tested, the IC_{50} (concentration of drug needed to inhibit cell growth by 50%) is generated from the dose-response curves for each cell line. *In vitro* assays are highly useful to evaluate the cytotoxic, mutagenic, and carcinogenic effects of chemical compounds on human cells [66].

a) Parameters which vary among different assays: [67]

ai) Cell culture method

• Seeding density

Seeding density depends on cell size, growth rate, and assay duration. It must be determined individually for each cell type. In a 2-3 day assay, seeding densities are typically in the range of $5\text{-}25\times10^3$ cells per well in 96-well microtitre plates. Time zero values must remain within the linear range of the assay, typically 1.5-2.0 absorbance units.

• Drug solubilization

Stock solution of polar compounds are dissolved in water, buffer, or medium then diluted in complete growth medium to the final test concentration. Non-polar compounds are dissolved in a solvent such as dimethylsulfoxide (DMSO), methanol or ethanol (EtOH) and filter sterilized (0.22 μm pores). A 1:1 mixture of DMSO and EtOH is also a good solvent and it evaporates more slowly than EtOH and chemically sterilizes most test materials. DMSO is toxic to cells at concentrations above 0.1-1.0%. Ethanol is usually growth stimulatory in the 1-2% range. Different cells population exhibit different sensitivities to these organics.

• Drug incubation

It is common procedure to incubate cells with drug solutions immediately after enzyme disaggregation of solid tissue, or harvesting of cell monolayer by trypsinization. There is evidence to suggest that susceptibility of cells to drug is altered by enzyme treatment and does not return to control levels until approximately 12 h after enzyme exposure. It may therefore be expedient to include a pre-incubation recovery period for freshly disaggregated cells to allow for this. Maintenance of pH at 7.4 is essential during the incubation period since alterations in pH will alter cell growth, and alkaline pH particularly will reduce cell viability.

aii) Duration of drug exposure and drug concentration

Assay duration is determined by two factors: (i) the length of time cells need to respond to an experimental treatment; and (ii) the length of time that cells can grow before nutrient depletion sets in. Nutrient depletion typically develops within 3-4 days after plating unless cultures are re-fed. Once it begins a progressive deterioration of cellular health and viability develops rapidly, and becomes a major artifact in data interpretation. Nutrient depletion can be calibrated by comparing the day-by-day growth kinetics of cultures that receive no feeding to cultures that are fed daily. The two curves begin to diverge when depletion sets in. This normally sets the upper limit to assay duration if cultures are not fed. With cytotoxic assays, 36-48 h assay period following a 1-day recovery period is usually adequate to detect the effect of a drug while avoiding the need to re-feed in mid experiment.

aiii) End-point used to quantitate drug effect

A variety of methods have been devised for measuring the viability or proliferation of cells *in vitro*. These can be subdivided into four groups:

1) **Reproductive assays** can be used to determine the number of cells in a culture that are capable of forming colonies *in vitro*. In these types of experiments, cells are plated at low densities and the number of colonies is scored after a growth period. These clonogenic assays are the most reliable methods for assessing viable cell number. These methods are very time-consuming and become impractical when many samples have to be analyzed.

2) **Permeability assays** involve staining damaged (leaky) cells with a dye and counting viable cells that exclude the dye. Counts can either be performed manually using a hemocytometer and stained with Trypan blue. This method is quick, inexpensive, and requires only a small fraction of total cells from a cell population. Therefore, this method is generally used to determine the cell concentration (cell number/ml) in batch cell cultures. This is helpful in ensuring that cell cultures have reached the optimal level of growth and cell density before routine sub-culture, freezing, or any experiment. Or counts can be performed mechanically using for example a flow cytometer and propidium iodide. Alternatively, membrane integrity can be assayed by quantifying the release of substances from cells when membrane integrity is lost, *e. g.,* Lactate dehydrogenase (LDH) or 51Cr

3) **Metabolic activity assays**: MTT reduction – a tetrazolium-based colorimetric assay for cell survival and proliferation In 1983, a quantitative colorimetric assay for mammalian cell survival and cell proliferation was proposed by Mosmann. The assay is dependent on the reduction of yellow-colored tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form insoluble formazan which precipitates out of solution in the immediate vicinity of the reaction (Fig. 2.11). The assay measure cell respiration and the amount of formazan product is proportional to the number of living cells present in culture and quantitated with an ELISA plate reader. The assay has been shown to be a simple, rapid alternative to counting cells by dye inclusion/exclusion, monitoring the release of 51Cr from lysed cells, or incorporation of [3H]-thymidine into cellular DNA. The MTT assays have been used with a growing number of cell types including primary cultured cells as well as establish cell lines. This colorimetric microplate assay is cost effective because of the number of tests which can be performed at one time without the problem of radio-isotope and contaminated material disposal.

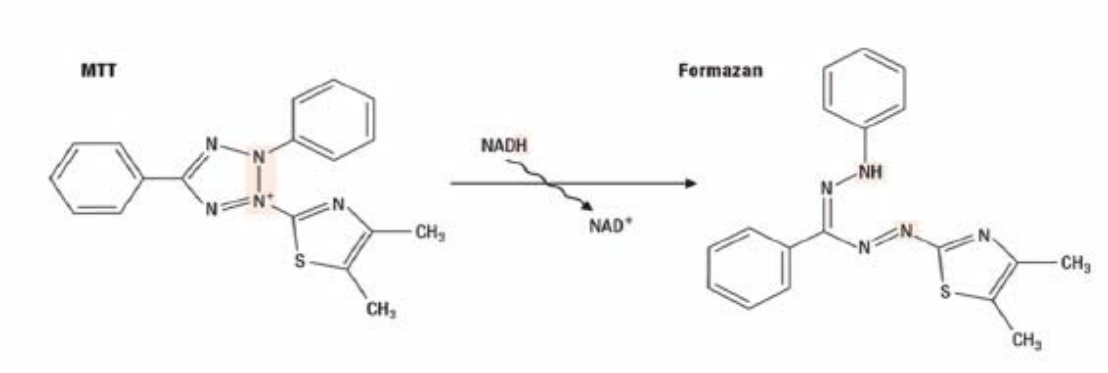


Figure 2.6 Molecular structure of MTT and its corresponding reaction products.

4) **Direct proliferation assays** use DNA synthesis as an indicator of cell growth. These assays are performed using either radioactive or nonradioactive nucleotide analogs. Their incorporation into DNA is then measured.

2.5.3 Human liver hepatoblastoma cells (Hep-G2)

The liver is known to be the main site of xenobiotic biotransformation due to the ability of this organ to express a plethora of enzymes, both quantitatively and qualitatively. Therefore, cell lines of liver origin are widely used in biomedical research involving xenobiotic metabolism including genotoxicity studies. Of these cell lines, the Hep-G2 cell line is the most versatile one. This cell line retains many of the specialized functions normally lost by primary hepatocytes in culture [68]. Hep-G2 has a wide variety of liver-specific metabolic responses to different kind of drug, chemical substances or plant extract (Table 2.4).

Additionally, as reviewed by Knasmuller *et al*. [69], Hep-G2 cells express a wide range of phase I and II enzymes. Phase I enzymes such as cytochrome P450 (CYP) 1A1, 1A2, 2B, 2C, 3A and 2E1, arylhydrocarbon hydrolase, nitroreductase, *N*-demethylase, catalase, peroxidase, NAD(P)H:cytochrome *c* reductase, cytochrome P450 reductase and NAD(P)H: Quinone oxidoreductase. Phase II enzymes such as epoxide hydrolase, sulfotransferase, glutatione *S*-transferase (GST), uridine glucuronosyl tranferase and *N*-acetyl transferase.

Table 2.4 Effects of drugs, chemical substances and plant extracts on human liver hepatoblastoma cells (Hep-G2).

Chemicals	Activity	Concentrations	Incubation time	Results	Reference
Tamoxifen	Anti-breast cancer drugs	0.1 - 20 μM	48 h	• Tamoxifen altered cell cycle of transduced Hep-G2 cells, cells decreased G0/G1 cell numbers, diminished proliferation index. • Induced cell death mostly in cells overexpressing CYP3A4 • Without significant effect on cytotoxicity or proliferation of cells engineered to overexpress CYP2E1 or on empty vector transfected cells.	Holownia and Braszko, 2004
Ebselen [2-phenyl-1,2-cenzoisoseleazol-3- (H)-one]	Anti-inflammatory	1 - 50 μM	24 h	• Reduced the suppression of growth Hep-G2 cells caused by hydrogen peroxide. • Displayed a dose-dependent reduction of lactate dehydrogenase leakage and malondialdehyde formation in hydrogen peroxide-treated cells • Able to reduce the reactive oxygen species formation and DNA damaging effect caused by hydrogen peroxide in a dose-dependent manner.	Yang *et al.*, 1999

Table 2.4 (continued) Effects of drugs, chemical substances and plant extracts on human liver hepatoblastoma cells (Hep-G2).

Chemicals	Activity	Concentrations	Incubation time	Results	References
Silymarin	Component of several comercially produced hepatoprotective remedies.	10 - 100 μM	48 h	• Concentration-dependent cytoprotection against the toxic effects of both allyl alcohol and carbon tetrachloride.	Dvorak *et al*., 2003
Lipiodol (iodinated poppy seed oil)	Chemotherapeutic and radiotherapeutic agents to cancer patients.	2%	72 h	• Lipidol has a selective effect on the uptake of certain cytotoxic agents. • Lipidol -doxorubicin-targeted treatment of Hep-G2 cells may improve the intracellular uptake and hence cytotoxicity of of doxorubicin *in vivo*.	Towu *et al*., 2004
Apigenin	Antioxidant, carbohydrate metabolism promoter, anti-platelet aggregation and anti-inflammatory agent.	25 and 50 μM	48 h	• Induced programme cell death in term of TNF-α, IFN-γ release and induction of caspases activity. • Induced caspase-3, -7, -10 and caspase-9 activity in a dose-dependent manner.	Khan and Sultana, 2006
Acacetin [5,7-dihydroxy-4'-methoxy-flavone]	Antiperoxidative, anti-inflammatory and antiplasmodial effects	1, 5, 10 and 20 μg/ml	72 h	• Inhibited the proliferation of Hep-G2 by inducting apoptosis and blocking cell cycle progression in the G1 phase. • Increased the expression of p53 and p21/WAF1 protein, contributing to cell cycle arrest.	Hsu *et al*.,2004

Table 2.4 (continued) Effects of drugs, chemical substances and plant extracts on human liver hepatoblastoma cells (Hep-G2).

Chemicals	Activity	Concentrations	Incubation time	Results	Refernces
P. peruviana extracts	Anticancer, antimycobacterial, antileukemic, antipyretic, immunomodulatory and treating diseases.	10, 30 and 50 µg/ml	48 h	• Treatment with cabonyl cyanide *m*-chlorophenyl hydrazone shown to induce cell cycle arrest and apoptosis through mitochondrial dysfunction • The extract possesses potent antihepatoma activity and its effect on apoptosis is associated with mitochondrial dysfunction.	Wu *et al*., 2004
Impila	Treat stomach complaints, cough, tapeworm infestations and impotence.	10 mg/ml	24 h	• Induced cytotoxicity in Hep-G2 cells *in vitro* involves depletion of cellular glutathione. • Preventing glutathione depletion by supplementing cells with *N*-acetylcysteine reduces cytotoxicity.	Popat *et al*., 2002
Troglitazone	Antidiabetic agent			• Induced apoptotic cell death characterized by internucleosomal DNA fragmentation and nuclear condensation. • Troglitazone may be one of the factors of liver injury in human.	Yamamoto *et al*., 2001

CHAPTER III

MATERIALS AND METHODS

3.1 General procedures

3.1.1 Analytical Instrument

(a) Nuclear magnetic resonance spectroscopy (NMR)

^{1}H-NMR at 400 MHz was recorded on a Varian Mercury + spectrometer. All chemical shifts were reported in part per million (ppm) using the residual proton signal in deuterated solvents as internal references.

(b) Mass spectrometry (MS)

Mass spectra were recorded on a Agilent 110 Series LC/MD Electrospray mass spectrometer

(c) Rotary vacuum evaporator

The Büchi rotary vacuum evaporator was used for the rapid removal of large amounts of solvents.

(d) HyperChem

Structure Optimization with HyperChem, HyperChem is a sophisticated molecular modeling environment that is known for its quality, flexibility, and ease of use. Uniting 3D visualization and animation with quantum chemical calculations, molecular mechanics, and dynamics, HyperChem puts more molecular modeling tools at your fingertips than any other Windows program. Our newest version, HyperChem Release 7.5, is a full 32-bit application, developed for the Windows 95, 98, NT, ME, 2000 and XP operating systems. HyperChem Release 7.5 incorporates even more powerful computational chemistry tools than ever before, as well as newly incorporated modules,

3.1.2 Materials

All materials and solvents used in this research were standard analytical grade, purchased from Merck, Fluka, and Aldrich. They were used without further purification. dichlorometane and methanol were distilled under reduced pressure.

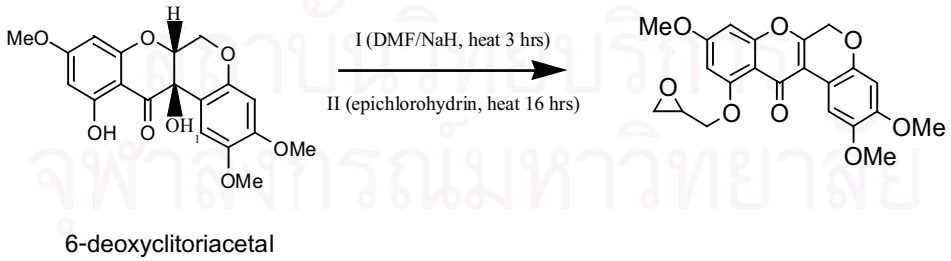
3.2 The plant materials

3.2.1 Extraction of 6-deoxyclitoriacetal from the dried roots of *Stemona collinsae* Craib.

The dried roots of *Stemona collinsae* Craib. were air-dried and pulverized. The powder was extracted with CH_2Cl_2 at room temperature for a week. The CH_2Cl_2 extracts were combined and concentrated to dryness under reduced pressure to obtain a deep brown resinous residue. The crude extract was separated by column chromatography over silica gel column using ethyl acetate : hexane (2:1) as eluent. The fractions showing similar spots were combined and then concentrated to dryness. The CH_2Cl_2 : MeOH (2:1) was used to crystallize 6-deoxyclitoriacetal as colorless needles. The obtained compounds were characterized by spectroscopic methods.

3.3 Synthetic Procedures

3.3.1 Epoxidation of 6-deoxyclitoriacetal



6-deoxyclitoriacetal

Figure 3.1 Epoxidaion of 6-deoxyclitoriacetal

To a solution of 6-deoxyclitoriacetal (0.1715 g, 0.459 mmol) in DMF (20 ml) were added with NaH 0.055 g (2.295 mmol) and stirred at 60 °C for 3 hours. To the above mixture were then added epichlorohydrin 2.754 mmol and heated at 60

°C for 16 hours with stirring. The reaction mixture was poured into a saturated NaCl solution and extracted with dichloromethane. The combined organic extracts were dried over Na_2SO_4, and the solvent was evaporated. The residue was chromatographed by preparative method using a mixture of hexane, dichloromethane and ethyl acetate (1:2:1) as an eluant to give a dark brown solid. Table 3.1 shows the composition of starting materials that varied the base, solvent and time in the epoxidation described above.

Table 3.1 Composition of starting materials in Epoxidaion of 6-deoxyclitoriacetal

Mole ratio of 6-deoxyclitoriacetal : Epichlorohydrin : base	**Solvent**	**Time (hours)**	**Base**
1 : 1 : 1	CH_2Cl_2 :MeOH (1 : 9)	3	NaOH
1 : 1: 1	CH_2Cl_2 :MeOH (1 : 9)	4.5	NaOH
1 : 5 : 5	CH_2Cl_2 :MeOH (1 : 9)	24	NaOH
1 : 5 : 5	CH_2Cl_2 :MeOH (1 : 9)	3	K_2CO_3
1 : 7 : 5	CH_2Cl_2 :MeOH (1 : 9)	8	K_2CO_3
1 : 12 : 5	CH_2Cl_2 :MeOH (1 : 9)	24	K_2CO_3
1 : 1 : 1	DMF	8	NaH
1 : 5 : 5	DMF	24	NaH
1 : 5 : 5	DMF	16	NaH

From Table 3.1 indicates that the best reaction was obtained when using mole ratio of 6-deoxyclitoriacetal : Epichlorohydrin : base = 1 : 5 : 5, using dimethyformamide as a solvent and using sodium hydride as a base at 16 hours. The ne,

dichloromethane and ethyl acetate (1:2:1) as eluant. The obtained compounds were characterized by spectroscopic methods to give Compound 1, Compound 2, Compound 3 and Compound 4

3.3.2 Ring-opening of the epoxide with morpholine.

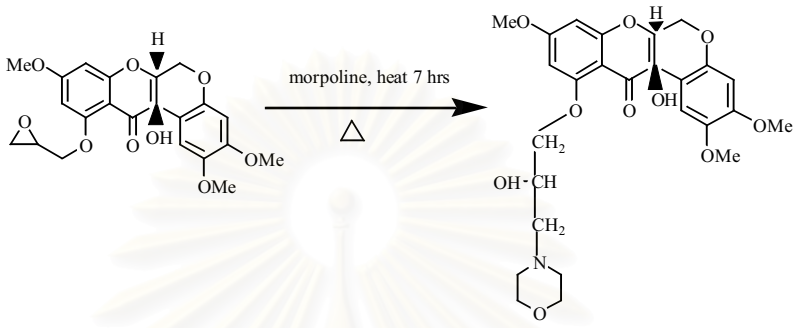


Figure 3.2 Ring-opening of the epoxide

A mixture of Compound 4(from3.3.1), morpoline (0.600 g, 4.615 mmol) in ethanol (6 mL) was heated at reflux for 7 h. Ethanol was removed, and the residue was extracted with ethyl acetate. The extract was washed with brine and dried. The residue was column chromatographed on silica gel using a mixture of hexane, dichloromethane and ethyl acetate (1:1:3) as eluant to give a drak brown solid.

3.4 Bioassays

3.4.1 Cells and cell culture

Hep-G2 characteristics : Morphological of Hep-G2 (Human liver hepatoblastoma) cell lines is epitithelial cells (Fig. 3.4). The Hep G2 cell lines have been isolated from a liver biopsy of a male aged 15 years, with a well differentiated hepatocellular carcinoma. The cells are studied of hepatocyte fuction as an *in vitro* model reflecting possible hepatic metabolism.

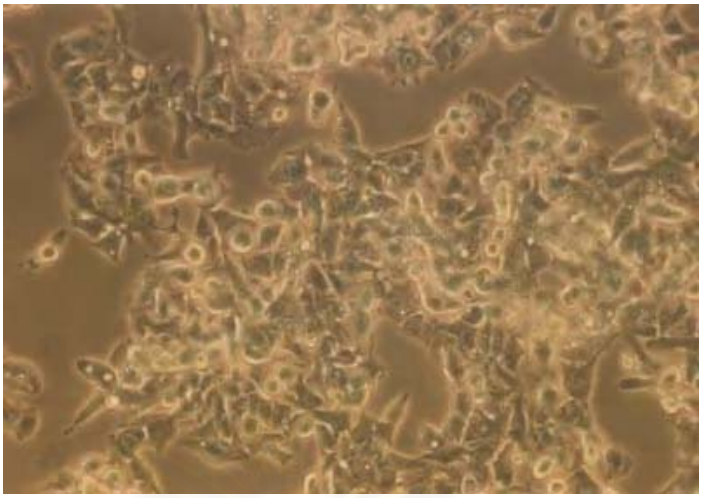


Figure 3.3 Characteristics of human liver hepatoblastoma cells under inverted microscope. (20 x)

Hep-G2 cells were obtained from the American Type Culture Collection; ATCC no. HB8065, storaged at The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. The cell line was cultured in basal medium consisted of RPMI-1640 medium with 2 g/l sodium bicarbonate. Serum supplemented medium was prepared by adding heat-inactivated fetal bovine serum (FBS) to basal medium. The cells were routinely propagated in 5% (v/v) serum-supplemented medium at 37 ^{o}C in a humidified atmosphere of 5% CO_2 incubator. The cells were routinely subcultured once every 3-4 days to maintain the optimum conditions for the exponential growth.

3.4.1.2 Subculturing

Hep-G2 cells were subcultured twice a week. Remove RPMI-1640 medium from the flask by the aid of a 10 ml glass pipette. Exponentially growing cells were detached from the surface of the 25 cm^2 T-flask by trypsinization with 2 ml 0.05% Trypsin solution for 3-4 min or until the cells have rounded up from the surface at room temperature or 37 ^{o}C in incubator. Trypsinize beyond the time required to detach cells to this degree may damage the cells and reduce plating efficiency. The solution was removed. The cells were re-suspended in 5 ml serum-containing RPMI-1640 medium and dispense into the new culture flasks. The medium was added to the final volume of 10 ml and incubated the flask in the incubator.

3.4.1.3 Cell suspension preparation for assay

Hep-G2 cells were propagated 3 days prior to the experiment. After trypsinization, the cells were incubated at room temperature or 37 ^{o}C in incubator for 3-4 min or until the cells have rounded up from the surface. The solution was removed. The RPMI-1640 medium was added and aspirate gently with the aid of a pipette in order to dissociate into single cells.

3.4.1.4 Cell count and dilution

The 0.4% Trypan blue dye solution and hemacytometer were applied to determine the viable cell number. Trypan blue is a stain that will only enter across the membranes of dead/non-viable cells. Make a 1:1 dilution of cell suspension with 0.4% Trypan blue and carefully re-suspended with a Pasture pipette. The haemocytometer and coverslip was thoroughly clean and wipe with 70% alcohol before use. Hemacytometer chamber was covered with coverslip. Sample was drawn into a Pasteur pipette after mixing thoroughly and placed the tip of the pipette at the junction between the counting chamber and the coverslip to aid capillary action. The cell suspension was drawn to fill the chamber with repeat for the other side of the chamber. The chamber was placed on the stage of the microscope with focus on the counting chamber. Non-viable cells became swollen, larger and dark blue. The viable cells, not stained with trypan blue, were counted in 4 of 1-mm square on the corner and on 1-mm square in the middle of hemacytometer (Fig. 3.4).

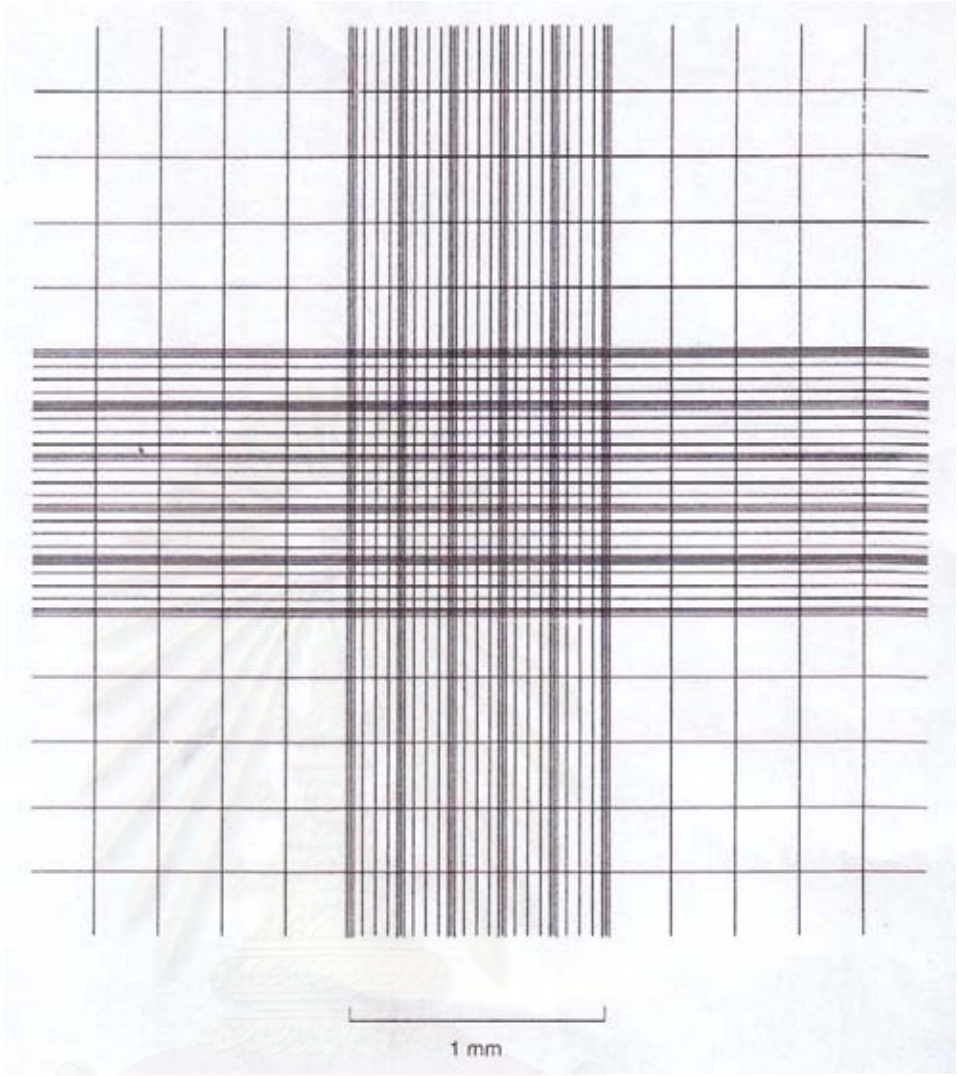


Figure 3.4 Magnified view of the cell counting chamber grid. The central 1-mm square area is divided into 25 smaller aquares, each 1/25 mm square. There are enclosed by triple ruled lines and are further subdivided into 16 squares, each 1/400 mm square.

The cells in each square of the hemocytometer were equivalent to approximately 1 mm, represent a total volume of 0.1 mm^3 and the subsequent cell density per ml was calculated using the following calculation:

Cell density (cell per ml) = (total cell count/5) x 2 $x10^4$

Then, calculate for volume of cell suspension (V_1) (desired cell density = 5 $x10^4$) by using the following calculation:

$C_1V_1 = C_2V_2$

C_1 = Cell density (cell per ml)

C_2 = 5×10^4

V_2 = Final volume

3.5 Cell viability assay

Cell viabillity assay was performed by the MTT (3-(4, 5–dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) colorimetric method (Carmichael et el., 1987 and Twentyman, 1987). After 72 h incubation period, MTT (5 mg/ml) was added at 10 µl/well and incubation was carried out for an additional 3 h at 37 ^{o}C in the dark. The solution was then discarded and 150 µl of DMSO and 25 µl of 0.1 M glycine buffer (pH 10.5) were added into each well to dissolve insoluble formazan crystal. Plates were then kept agitation for 5 minutes at room temperature for complete solubilization. The level of colored formazan derivative was analyzed on a microplate reader at a wavelength of 540 nm. The percentage of cell viability was calculated according to the following formula.

$$\text{The \% of cell viability} = \frac{\text{OD of treated cells} \times 100}{\text{OD of control cells}}$$

The IC_{50} values were obtained by plotting the percentage of cell viability versus the concentrations.

3.6 Cytotoxicity test

(i) Synthesis compound

Prepare 6-deoxclitoriacetal, Doxorubicin, Compound 1, Compound 2, Compound 3 and Compound 4 was dissolved in 100% DMSO and adjusted the concentration to 50 mg/ml. Stock solution was diluted to the test concentrations by 100% DMSO which not exceed 2% of the total volume, an amount that was not toxic to the cells. The assays were prepared by incubationg the cells with the plant extracts (at 0.01, 0.1, 1 and 10 µg/ml, 6 replicate per concentration) and without the extract as a negative control. The 96-well microtitre plates were incubated at 37 ^{o}C in a 5% CO_2 incubator for 3 days (72 h).

(ii) The Combination

6-deoxclitoriacetal, Prednisolone and Podophyllotoxin in combination (the percentage of combined shown in Table below). There was dissolved in 100% DMSO and adjusted the concentration to 50 mg/ml. Stock solution was diluted to the test concentrations by 100% DMSO which not exceed 2% of the total volume, an amount that was not toxic to the cells. The assays were prepared by incubationg the cells with the plant extracts (at 0.01, 0.1, 1 and 10 μg/ml, 6 replicate per concentration) and without the extract as a negative control. The 96-well microtitre plates were incubated at 37 ^{o}C in a 5% CO_2 incubator for 3 days (72 h).

Table 3.2 Combination of 6-deoxyclitoriacetal-Prednisolone (% wt of 6-deoxyclitoriacetal)

6-deoxyclitoriacetal	Prednisolone
100	0
90	10
75	25
50	50
25	75
0	100

Table 3.3 Combination of 6-deoxyclitoriacetal -Podophyllotoxin (% wt of 6-deoxyclitoriacetal)

6-deoxyclitoriacetal	Prednisolone
100	0
90	10
75	25
50	50
25	75
0	100

Table 3.4 Combination of Prednisolone-Podophyllotoxin (% wt Prednisolone)

Prednisolone	Podophyllotoxin
100	0
90	10
75	25
50	50
25	75
0	100

CHAPTER IV

RESULTS AND DISCUSSION

4.1 *Stemona collinsae* Craib.

The dried root of *Stemona collinsae* Craib. (12 kg) was extracted from CH_2Cl_2. The combined CH_2Cl_2 extracts were concentrated to dryness under reduced pressure to obtain a brown crude extract . The crude extract was isolated on silica gel column using ethyl acetate: hexane (2:1) as an eluent. Each fraction was analyzed by TLC visualize using UV light. The fractions showing similar spots were combined and then concentrated to dryness. 6-Deoxyclitoriacetal was obtained from the elution of column chromatography with CH_2Cl_2:Hexane:EtOAc (2:2:1) and re-crystallization with CH_2Cl_2:MeOH (1:2) to obtain a colorless needles (3.12 g). The structure of 6-deoxyclitoriacetal was characterized by the spectroscopic data.

4.2 Modification of 6-deoxyclitoriacetal to improve the cytotoxic potential as an anticancer drug candidate.

4.2.1 Epoxidation of 6-deoxyclitoriacetal

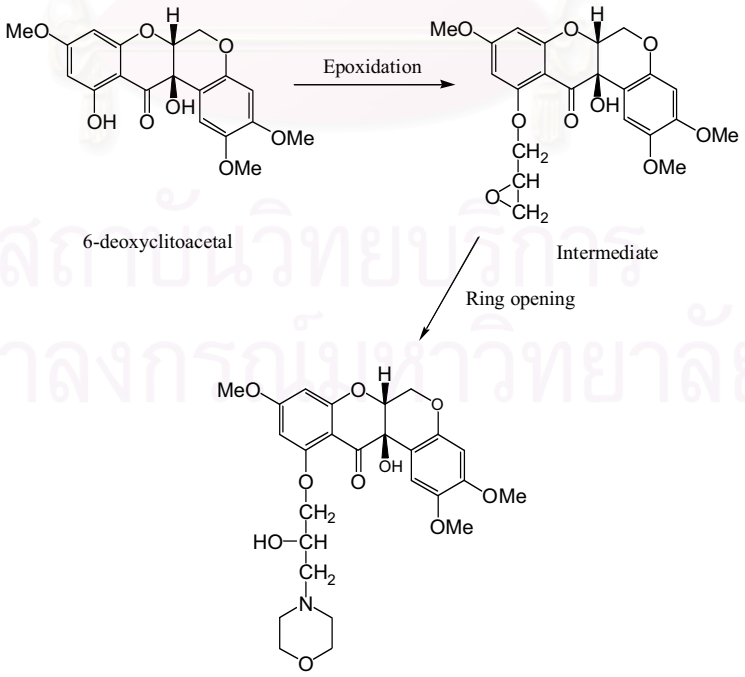


Figure 4.1 Scheme reaction of 6-deoxyclitoriacetal

The epoxidation reaction of 6-deoxyclitoriacetal was carried out by varying the compositions of starting materials; namely base, solvent and the reaction time. The compositions of staring materials are given in Table 4.1. From the results showed that the phenoxyl proton could not be deprotonated when using NaOH and K_2CO_3 as a base. Therefore, the subsequent epoxidation reaction cannot be occurred. The epoxidation reaction of 6-deoxyclitoriacetal, NaH and epichlorohydrin in DMF was carried out 16 hours and gave a mixture of dark brown solid. The mixture were isolated and characterized by NMR and MS analysis.

Table 4.1 Compositions of starting materials for the epoxidation reaction.

Mole ratio of 6-deoxyclitoriacetal : Epichlorohydrinm : base	**Solvent**	**Time (hours)**	**Base**	**The ring-opening of the epoxide**	**Yield of product (%)**
1 : 1 : 1	CH_2Cl_2 :MeOH (1 : 9)	3	NaOH	HCl	0
1 : 1: 1	CH_2Cl_2 :MeOH (1 : 9)	4.5	NaOH	-	0
1 : 5 : 5	CH_2Cl_2 :MeOH (1 : 9)	24	NaOH	H_2SO_4 2 N	0
1 : 5 : 5	CH_2Cl_2 :MeOH (1 : 9)	3	K_2CO_3	-	0
1 : 7 : 5	CH_2Cl_2 :MeOH (1 : 9)	8	K_2CO_3	-	0
1 : 12 : 5	CH_2Cl_2 :MeOH (1 : 9)	24	K_2CO_3	-	0
1 : 1 : 1	DMF	8	NaH	-	✓
1 : 5 : 5	DMF	24	NaH	-	✓
1 : 5 : 5	DMF	16	NaH	morpholine	1.136

The best yield of the epoxidation reaction was obtained when using mole ratio of 6-deoxyclitoriacetal : Epichlorohydrin : base = 1 : 5 : 5, using dimethyformamide as a solvent and using sodium hydride as a base at 16 hours. The mixture was isolated on silica gel column using mixture of hexane, dichloromethane and ethyl acetate (1:2:1) as an eluent. The obtained compounds were characterized by

spectroscopic methods to give Compound 1, Compound 2 and Compound 3 and Compound 4.

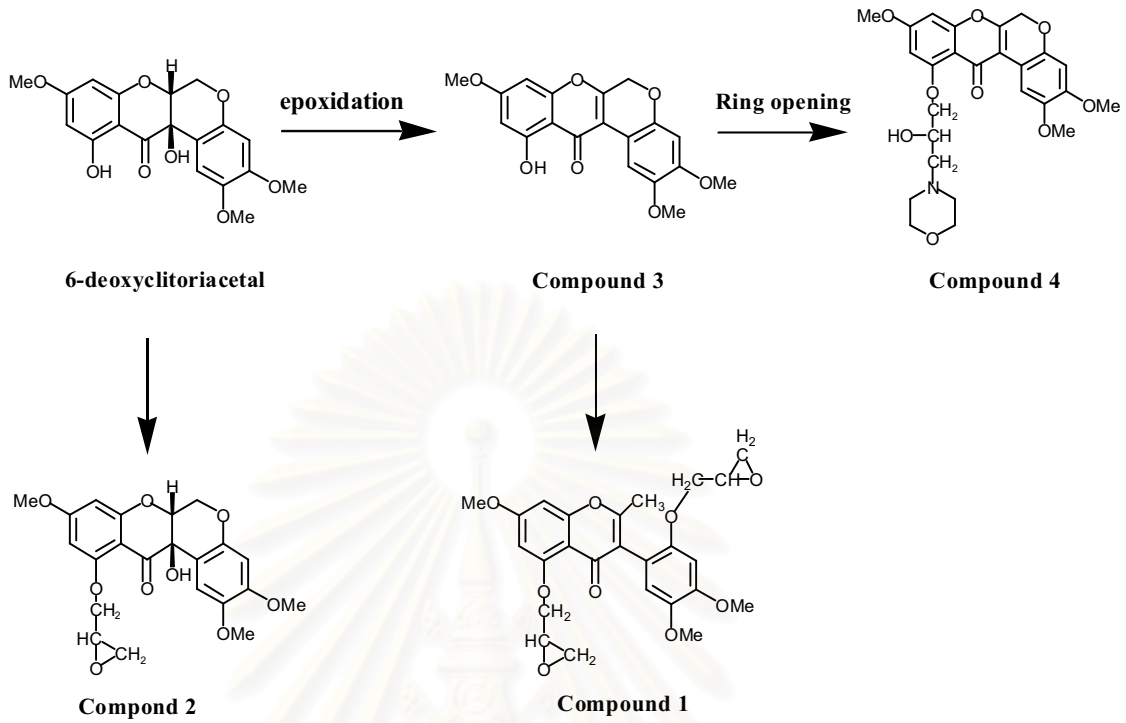


Figure 4.2 Structures of Compound 1, Compound 2, Compound 3and Compound 4

4.2.2 Characterization of 6–deoxyclitoriacetal derivatives modified by the epoxidation reaction

4.2.2.1 Epoxidation of 6-deoxyclitoriacetal

(a) Compound 1 was obtained from the elution of column chromatography with CH_2Cl_2:Hexane:EtOAc (2:2:1) and recrystallization from CH_2Cl_2:MeOH (1:2) to obtain a pale yellow solid (2.2 mg).

The ^{1}H NMR and MS spectrum of Compound 1 indicated that is the chemical structure below:

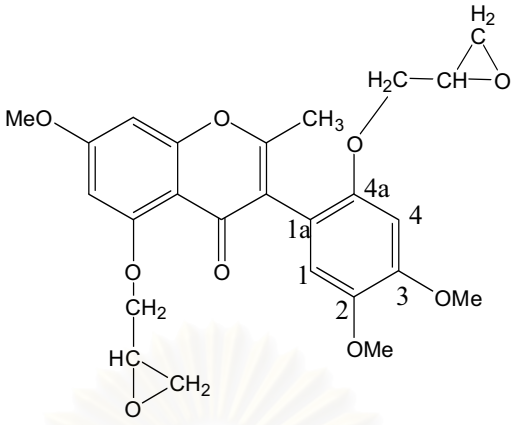


Figure 4.3 Structure of Compound 1

EIMS (*m/z*) (Appendix) $[M+Na]^+$ 494.39 was assigned the molecular formular $C_{24}H_{26}O_9$

^{1}H NMR(Appendix) (CDCl$_3$, 400 MHz.): δ_H 1.183 (H, s, 3-H), 2.990,2.505 (2H, m, H-8'), 2.709, 3.106 (2H, m, H-12'), 3.330 (1H, m, H-7'), 3.696 (3H, s, OMe-3'), 3.707 (3H, S, OMe-7'), 3.768 (3H, s, OMe-4'), 3.850 (1H, m, H-11), 4.444 (2H, m, H-6'), 4.485 (2H, m, H-10), 5.867 (1H, d, J=2.4 Hz, H-8), 5.965 (1H, d, J=2 Hz, H-6), 6.444 (1H, d, J=4 Hz, H-4'), 6.710 (1H, d, J=4 Hz, H-6)

(b) Compound 2 was obtained from the elution of column chromatography with CH_2Cl_2:Hexane:EtOAc (2:2:1) and recrystallization from CH_2Cl_2:MeOH (1:2) to obtain a pale yellow solid (1.9 mg).

The ^{1}H NMR and MS spectrum of Compound 2 indicated that is the chemical structure below:

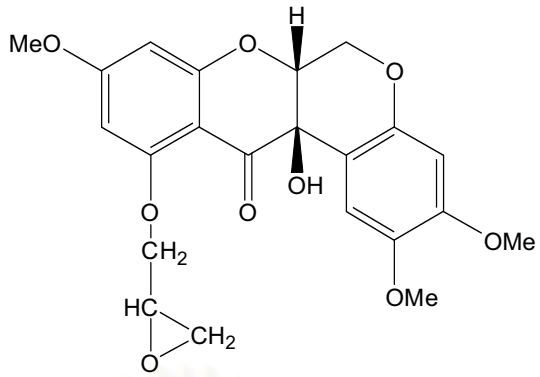


Figure 4.4 Structure of Compound 2

EIMS (*m/z*) (Appendix) $[M+Na]^+$ 452.97 was assigned the molecular formular $C_{22}H_{22}O_9$

^{1}H NMR (Appendix) ($CDCl_3$, 400 MHz.): δ_H 2.818(1H, s, H-14), 2.892(1H, s, H-14), 3.661(3H, s, OMe-2), 3.716(3H, s, OMe-9), 3.6445 (1H, m, H-13), 3.749 (3H, s, OMe-3), 4.422 (2H, m, H-12), 4.489 (1H, d, J= 2Hz, H-6a), 4.522 (2H, dd, J= 2Hz, H-6), 5.982 (1H, d, J= 2.4Hz, H-8), 6.263 (1H, d, J= 2.4Hz, H-10), 6.417 (1H, s, H-4), 6.604 (1H, s, H-1)

(c) Compound 3 was obtained from the elution of column chromatography with CH_2Cl_2:Hexane:EtOAc (2:2:1) and recrystallization from CH_2Cl_2:MeOH (1:2) to obtain a pale yellow solid (2.6 mg).

The ^{1}H NMR and MS spectrum of Compound 3 indicated that is the chemical structure below:

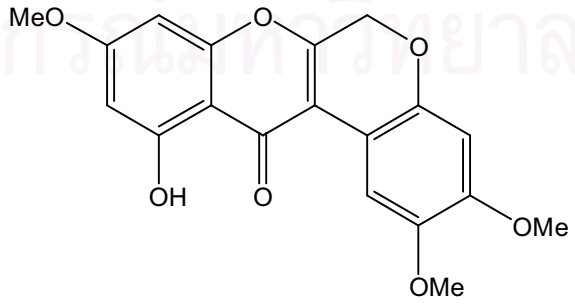


Figure 4.5 Structure of Compound 3

EIMS (*m/z*) (Appendix) $[M+H]^+$ 356.94 was assigned the molecular formular $C_{19}H_{16}O_7$

^{1}H NMR (Appendix) ($CDCl_3$, 400 MHz.): δ_H 3.757 (3H, s, H-9), 3.771 (3H, s, H-2), 3.821 (3H, s, H-3), 4.513 (1H, d, J=2.4 Hz, H-6), 5.977 (1H, d, J=2.4 Hz, H-8), 6.054 (1H, d, J=2.4 Hz, H-10), 6.493 (1H, S, H-1), 6.684 (1H, S, H-4), 11.506 (1H, S, OH-11)

(d) **Compound 4** was obtained from the elution of column chromatography with CH_2Cl_2:Hexane:EtOAc (2:2:1) and recrystallization from CH_2Cl_2:MeOH (1:2) to obtain a pale yellow solid (2.5 mg).

The ^{1}H NMR and MS spectrum of Compound 4 indicated that is the chemical structure below:

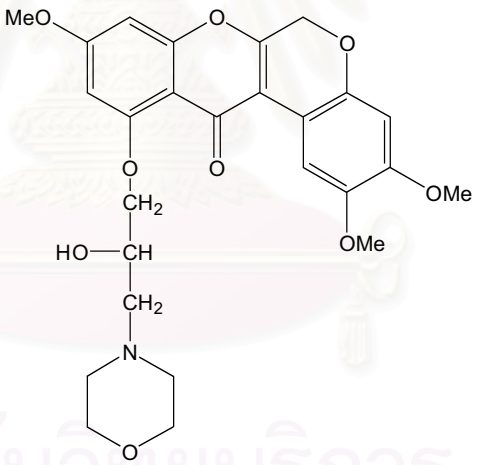


Figure 4.6 Structure of Compound 4

EIMS (*m/z*) (Appendix) $[M+Na]^+$ 522.48 was assigned the molecular formular $C_{26}H_{29}O_9N$

^{1}H NMR (Appendix) ($CDCl_3$, 400 MHz.): δ_H 2.942(4H, d, H-15,16), 3.699(4H, d, H17,18), 3.753(3H, s, OMe-2), 3.772(3H, s, OMe-9), 3.817(1H, s, OMe-3), 4.288(2H, d, J=2.4Hz, H-8), 4.533(2H, d, J=2.4Hz, H-6), 5.970Z1H, d, J=2.4Hz, H-10), 6.053(1H, d, J=2.4Hz, H-10), 6.493(1H, s, H-4), 6.682(1H, s, H-1

4.3 Cytotoxic assay on HEP-G2 cell of 6–deoxyclitoriacetal derivatives

In vitro cytotoxic activity of Compound 1, Compound 2, Compound 3 and Compound 4 against HEP-G2 (Hepatoma) was shown in Table 4.2

Table 4.2 The growth response and cytotoxicity (IC_{50}, mg/mL) against Hep-G2 of 6-deoxyclitoriacetal, Doxorubicin, Prednisolone, Podophyllotoxcin, Compound 1, Compound 2, Compound 3 and Compound 4**.**

Compound	IC_{50} (mg/mL)
6-deoxyclitoriacetal	**0.1081**
Doxorubicin HCl	**5.1×10^{-4}**
Compound 1	**3.0278**
Compound 2	**0.1518**
Compound 3	**0.0800**
Compound 4	**0.0380**

DNA-intercalation drugs should have a planar moiety that can intercalate into base pairs of a DNA helix. These results in the deformation of a DNA, and hence inhibited both DNA replication and transcription. However, the preliminary studies of our group by P.Chimsook [78] have revealed that being planar is not enough to make a molecule to be an anti-cancer drug. The DNA-intercalating anti-cancer drug should have three characteristics: (i) molecule has a bent shape, (ii) a part of the molecule is planar and (iii) it has functional groups that have intermolecular interactions, such as π-π interactions, hydrogen bonding, with DNA. In that studies have shown that the ring A (Fig 4.8) is a key molecular part to intercalate between the DNA base pairs and the –OH group at C11 to participate the hydrogen bonding to

DNA and then stabilizing the DNA-drug complex. Therefore, in this work, the discussions will be focused on these three characteristics.

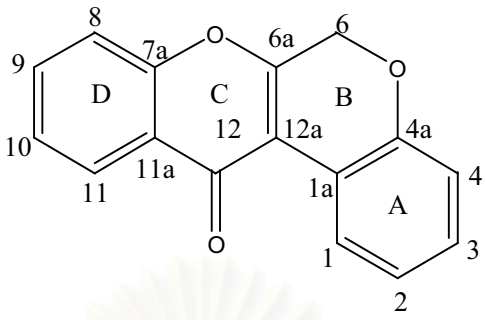
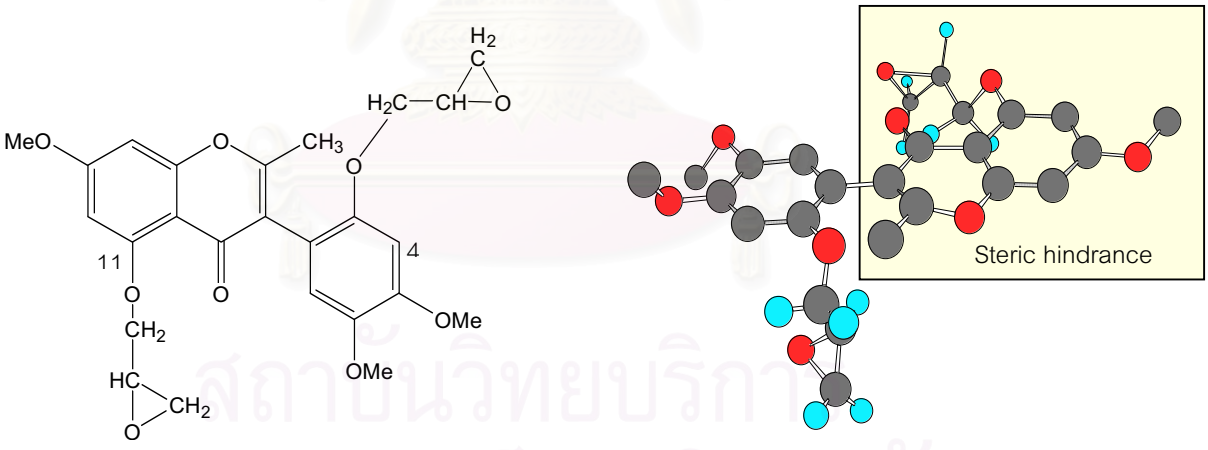


Figure 4.7 Structure of a rotenoid core

Compound 1

Compound 1 is a diepoxide compound which is two epoxypropyl groups at C4a and C11 in the molecule. The epoxypropyl group at C4a makes the structure is not planar shape and has a steric hindrance (Fig. 4.8). As a result it can not intercalate between the base pairs of the DNA double stranded helix. This is constistent with the IC_{50} of Compound 1 (3.027 mg/mL) which is significant higher than that of 6-deoxyclitoriacetal (IC_{50} = 0.1081 mg/ml).

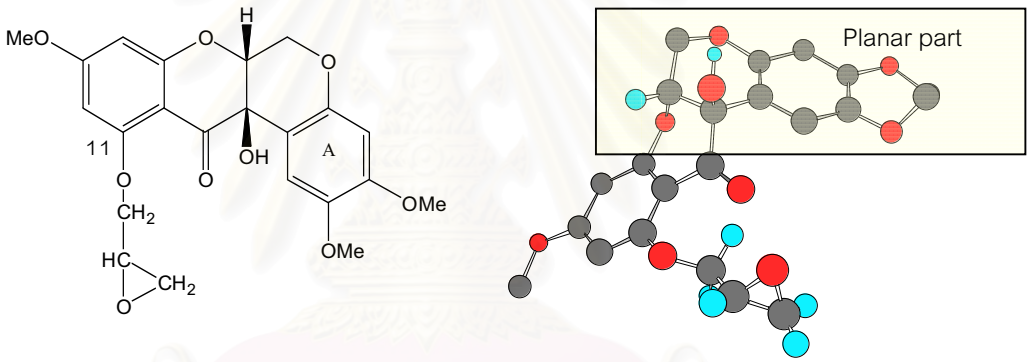


(a) **(b)**

Figure 4.8 (a) Chemical structure of Compound 1 and (b) 3D-structure of Compound 1 generated from Hyperchem

Compound 2

Compound 2 is a major product of the reaction of epicholohydrin with 6-deoxyclitoriacetal. There is only one epoxypropyl group at C11 in the molecule. The overall structure is similar to 6-deoxyclitoriacetal. Except the functional group at C11 is a hydroxyl group in 6-deoxyclitoriacetal, and is an epoxypropyl group at C11 in the compound 2. From the previously studied by P.Chimsook, the ring A is a part that intercalate into the DNA base pairs, therefore in this case it is assumed that the ring A should able to intercalate into the DNA as well. However, the epoxypropyl part has a possibility to participate hydrogen bonding with DNA less than the hydroxyl group (Fig. 4.10). Thereby, the binding between Compound 2 and DNA is not stable. It can be seen that the IC_{50} value (IC_{50} = 0.1518 mg/mL) is not less than the 6-deoxyclitoriacetal (IC_{50} = 0.1081 mg/mL).



(a) **(b)**

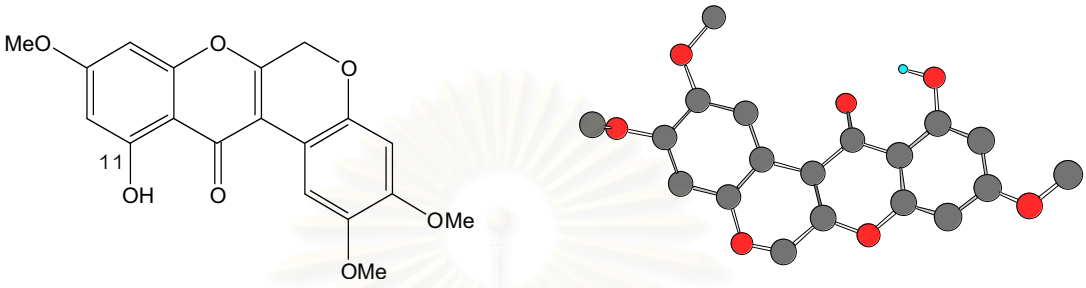
Figure 4.9 (a) Chemical structure of Compound 2 and (b) 3D-structure of Compound 2 generated from Hyperchem

Compound 3

For the structure of Compound 3 (Fig. 4.10), the water molecule is lost from the starting material, making the molecule is planar. This molecule should be able to insert the whole molecule into the DNA helix and hence the IC_{50} should be significant less than that of 6-deoxyclitoriacetal and the others. However, the IC_{50} of Compound 4 is 0.0800 mg/mL which is not significant less than the expected value, probably the DNA-compound complex is not stable.

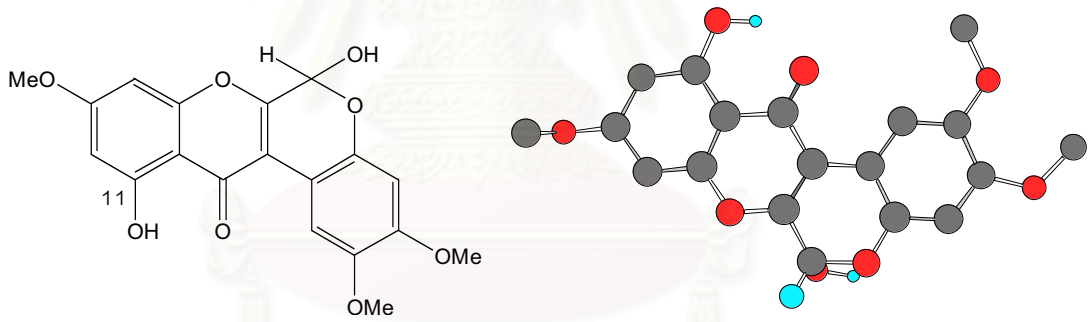
Sremonal (Fig.4.11) is anothercompound that has a planar shape. However, it is inactive. Also, its OH group at C11 has a strongly intramolecular

hydrogen bond to the C=O group. Therefore, it has no functional group to hydrogen bond to the DNA. This may be a good evidence that be planar is not enough for a molecule to intercalate to the DNA. The molecule should also present a bent shape, because the bent shape may make the part of the molecule that can hydrogen bonding to the DNA fit into a major groove or a minor groove of DNA



(a) (b)

Figure 4.10 (a) Chemical structure of Compound 4
and (b) 3D-structure of Compound 4 generated from Hyperchem



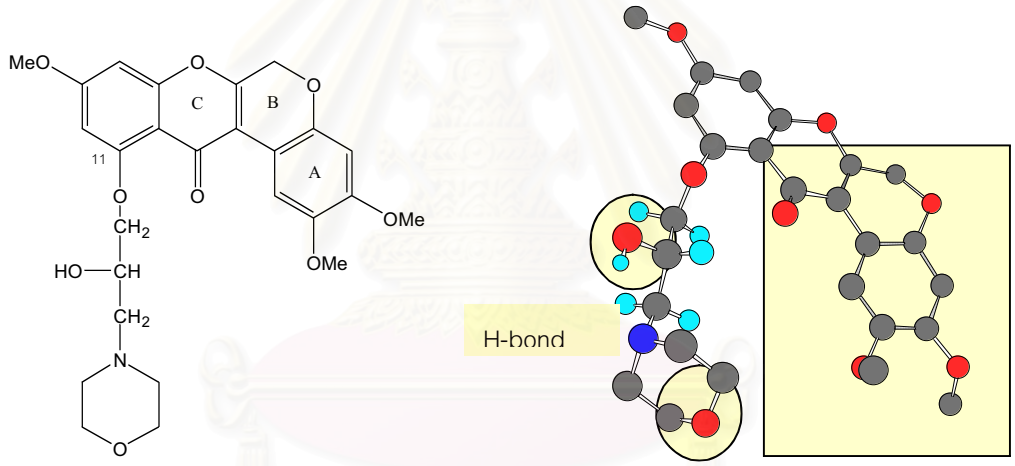
(a) (b)

Figure 4.11 (a) Chemical structure of stemonal
and (b) 3D-structure of stemonal generated from Hyperchem

Compound 4

Compound 4 (Fig. 4.12) is the product of the epoxide ring-opening with morpholine. The IC_{50} of 0.3796 mg/mL is significant lower than that of 6-deoxyclitoriacetal (0.1081 mg/mL). This is probably due to the planar ring A to C system is able to intercalate between the base pairs of DNA. Moreover, the DNA-Compound 4 complex is stabilized by the hydrogen bonding to the DNA by the

hydroxyl group and the morpholine group of Compound 4. This is an evidence that the functional group at C11 is a key factor for inhibit the cytotoxic activity. It is proposed that the hydroxyl group at C11 of 6-deoxyclitoriacetal is rigid to participate hydrogen bonding with DNA, so that the binding of DNA-6-deoxyclitoriacetal is not promising stable. When the hydroxyl group was modified to a longer chain of morpholinopropoxy, it is expected that the chain is more flexible than the unmodified one. The hydroxyl group and the oxygen atom at morpholine may promote the hydrogen bonding and hence enhance the stability of the DNA-Compound 4 complex. The structure of Compound 4 is similar to the structure of known anticancer drug, doxorubicin which can intercalate between base pairs of the DNA helix which cause single strand and double strand breaks in DNA, thereby inhibition of the replication and transformation process and thence death. Therefore Compound 4 exhibited cytotoxic activity against HEP-G2 with lowest concentration.



(a) (b)

Figure 4.12 (a) Chemical structure of Compound 4
and (b) 3D-structure of Compound 4 generated from Hyperchem

As a result of the structure-activity relationship on cytoxicity of 6-deoxyclitoriacetal and its derivatives mentioned above, these findings indicated that the structures of 6-deoxyclitoriacetal play an important role in inhibiting the cytotoxicity. The IC_{50} was significantly effected not only by the ability of molecule to intercalate into DNA, but also the strong binding between the molecule and the DNA. For example, Intermadiate 1 have two epoxypropyl groups at C4a and C11 in the molecule that makes the structure is not planar shape and have a steric hindrance. As a result it can not intercalate between the base pairs of the DNA double stranded helix.

Consequently, the IC_{50} of 3.0278 mg/mL of Compound 1 is significant higher than that of 0.1081 mg/mL of 6-deoxyclitoriacetal.

In summary, it can be concluded that the DNA-intercalating anti-cancer drug should have three characteristics: (i) molecule has a bent shape, (ii) a part of the molecule is planar and (iii) it has functional groups that have intermolecular interactions. Thereby in this studies, Compound 4 which is the product of the epoxide ring opening with morpholine can exhibited cytotoxic activity against HEP-G2 with lowest concentration, IC_{50} of 0.3796 mg/mL is significant lower than that of 6-deoxyclitoriacetal (0.1081 mg/mL). The inhibition performance may be attributed by the structure of Compound 4 is suitable intercalate and strong binding between the DNA helix, therefore inhibition of the replication and transformation process is more pronounced.

4.4 The Combination drugs inhibited cell proliferation *in vitro*

As 6-deoxyclitoriacetal is a potential anticancer drug, therefore in this work is concerned about the opportunity to use the new generation combination drugs to enhance the cytotoxic activities of the chemotherapy drugs, reduce the drug dosage, reduce the side effects of chemotherapy, reduce high cost, solve the problems about drug resistance and safe and effective use of the chemotherapy. The effects of various combinations of 6-deoxyclitoriacetal-Prednisolone, 6-deoxyclitoriacetal-Podophyllotoxin and Prednisolone-Podophyllotoxin on Hep-G2 were studies.

Prednisolone is a corticosteroid drug with predominantly glucocorticoid and low mineralocorticoid activity, making it useful for the treatment of a wide range of inflammatory and auto-immune conditions such as asthma, rheumatoid arthritis, Ulcerative Colitis and Crohn's disease, multiple sclerosis, cluster headaches and Systemic Lupus Erythematosus. It can also be used as an immunosuppressive drug for organ transplants. Predisolone acetate opthamolic suspension is a sterile corticosteroid used to help to reduce swelling, redness, itching, and allergic reactions affecting the eye.

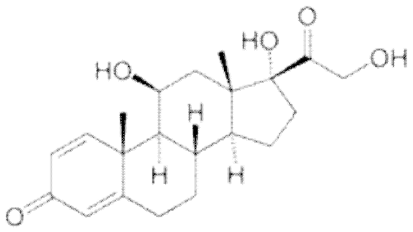


Figure 4.13 Chemical structure of Prednisolone

Podophyllotoxin, otherwise known as podofilox is a non-alkaloid toxin in the lignan family present at concentrations of 0.3 to 1.0% by mass in the rhizome of American Mayapple Podophyllum peltatum. Podophyllotoxin is the pharmacological base for the important anti-cancer drug Etoposide.

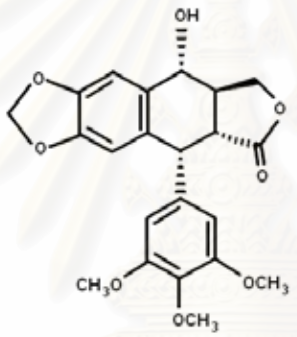


Figure 4.14 Chemical structure of Podophyllotoxin

4.4.1. Control groups

The samples was dissolved in 100% DMSO and adjusted the concentration to 50 mg/mL. Stock solution was diluted to the test concentrations by 100% DMSO which not exceed 2% of the total volume, an amount that was not toxic to the cells. The assays were prepared by incubation the cells with the samples (at 0.01, 0.1, 1, and 10 μg/mL; 6 replicate per concentration).

4.4.2 Combination of 6-deoxyclitoriacetal-Prednisolone

The IC_{50} value of the combination of 6-deoxyclitoriacetal-Prednisolone with various amount of Prednisolone from 0-100 %wt are given in Table 4.3

Table 4.3 The percentage of combined and cytotoxicity (IC_{50}, mg/mL) against Hep-G2 of 6-deoxyclitoriacetal-Prednisolone

Compound		IC_{50} (mg/mL)
6-deoxyclitoriacetal (%wt)	Prednisolone (%wt)	
100	0	0.1081
90	10	0.0153
75	25	0.0948
50	50	0.0883
25	75	0.0362
10	90	0.0206
0	100	Proliferative

The best IC_{50} of combination 6-deoxyclitoriacetal with Prednisolone is 90% of 6-deoxyclitoriacetal and 10% of Prednisolone (IC_{50}=0.0153 mg/mL) which is less than used of 6-deoxyclitoriacetal (IC_{50}=0.1081 mg/mL.) and Prednisolone alone (proliferative). However, These combination drugs do not appear to differ significantly in efficacy or adverse effect.

4.4.3 Combination of 6-deoxyclitoriacetal-Podophyllotoxin

The IC_{50} value of the combination of 6-deoxyclitoriacetal- Podophyllotoxin with various amount of Podophyllotoxin from 0-100 %wt are given in Table 4.4

Table 4.4 The growth response and cytotoxicity against Hep-G2 of 6-deoxyclitoriacetal-Podophyllotoxin

Compound		IC_{50} (mg/mL)
6-deoxyclitoriacetal (%wt)	Podophyllotoxin (%wt)	
100	0	0.1081
90	10	1.5×10^{-4}
75	25	1.0×10^{-4}
50	50	inactive
25	75	inactive
10	90	inactive
0	100	inactive

The results showed that the combination of 6-deoxyclitoriacetal with Podophyllotpxin showed obviously synergic effects when the amount of 6-deoxyclitoriacetal is between 75-90 %wt based on the total amount of two compounds.

4.4.4 Combination of Prednisolone and Podophyllotoxin

The IC_{50} value of the combination of Prednisolone-Podophyllotoxin with various amount of Podophyllotoxin from 0-100 %wt are given in Table 4.7

Table 4.5 The growth response and cytotoxicity against Hep-G2 of Prednisolone-Podophyllotoxin

Compound		IC_{50} (mg/mL)
Prednisolone (%wt)	Podophyllotoxin (%wt)	
100	0	proliferative
90	10	3.29×10^{-3}
75	25	7.66×10^{-3}
50	50	4.73×10^{-3}
25	75	inactive
10	90	inactive
0	100	inactive

The combination of Prednisolone- Podophyllotoxin is effective against Hep-G2 at 50-90 %wt based on the total amount of two compounds.

The results showed that the combination of Prednisolone-Podophyllotocin showed obviously synergic effects when the amount of Prednisolone is between 50-90 %wt based on the total amount of two compounds.

CHAPTER V

CONCLUSION

6-Deoxyclitoriacetal is a substance extracted from the dried root of *Stemona collinsae Craib.* It has been known to have cytotoxic activity against various types of human carcinoma . This study proposes that the substance has a good chance to be a DNA-intercalating anti-cancer drug. Structure of 6-deoxyclitoriacetal was modified by epoxide and then ring-opening with morpholine to obtain Compound 1, Compound 2, Compound 3, and Compound 4. Their IC_{50} values are 3.0278, 0.1518, 0.0800 and 0.0380 mg/mL, respectively. The IC_{50} value of Compound 4 (0.0380 mg/mL) is significant lower than that of 6-deoxyclitoriacetal (0.1081 mg/mL). Therefore, Compound A exhibits cytotoxic activity against HEP-G2 with the lowest concentration.

The result of the structure-activity relationship on cytoxicity of 6-deoxyclitoriacetal and its derivatives, indicated that the structures of 6-deoxyclitoriacetal play an important role in inhibiting the cytotoxicity. The IC_{50} was significantly effected not only by the ability of molecule to intercalate into DNA, but also the strong binding between the molecule and the DNA. For example, Intermadiate 1 have two epoxypropyl groups at C4a and C11 in the molecule that makes the structure is not planar shape and have a steric hindrance. As a result it can not intercalate between the base pairs of the DNA double stranded helix. Consequently, the IC_{50} of 3.0278 mg/mL of Compound 1 is significant higher than that of 0.1081 mg/mL of 6-deoxyclitoriacetal.

In summary, it can be concluded that the DNA-intercalating anti-cancer drug should have three characteristics: (i) molecule has a bent shape, (ii) a part of the molecule is planar and (iii) it has functional groups that have intermolecular interactions with DNA. Thereby in this studies, Compound 4 which is the product of the epoxide ring opening with morpholine can exhibited cytotoxic activity against Hep-G2 with lowest concentration. Its IC_{50} of 0.0380 mg/mL is significant lower than that of 6-deoxy (0.1081 mg/mL). The inhibition performance may be attributed to the structure of Compound 4 that can intercalate and has strong binding between the

DNA helix. Therefore, its inhibition of the replication and transformation process is more pronounced.

Moreover, the combinations of 6-deoxyclitoriacetal-Prednisolone, 6-deoxyclitoriacetal-Podophyllotoxin and Prednisolone-Podophyllotoxin were tested with Hep-G2. The combination of 6-deoxyclitoriacetal-Podophyllotoxin has synergic effects when the amount of 6-deoxyclitoriacetal is between 75-90 %wt, based on the total amount of the two compounds.

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Perspectives

The study in this thesis shown many interesting characteristics of 6-deoxyclitoriacetal . Therefore, there are many possibilities for further research on this substance and its complexes. For example,

1. The modification 6-deoxyclitoriacetal, may be modified to enhance its effectiveness as an anti-cancer drug candidate.

2. The combination drugs are increase dose qualitative in patient. When there are demands to manufacture the plant materials into commercialized products, more and more data is still needed to ensure for the efficacy and safety of such products.

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APPENDICES

Appendix A

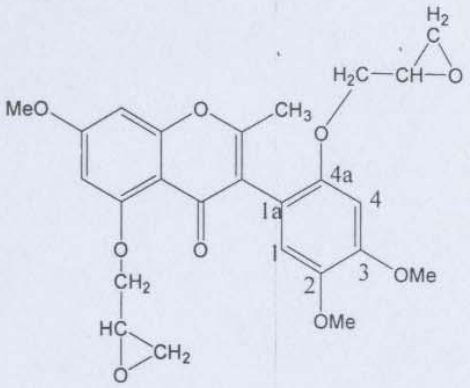


Figure 1 ^{1}H-NMR (400 MHz) of Compound 1

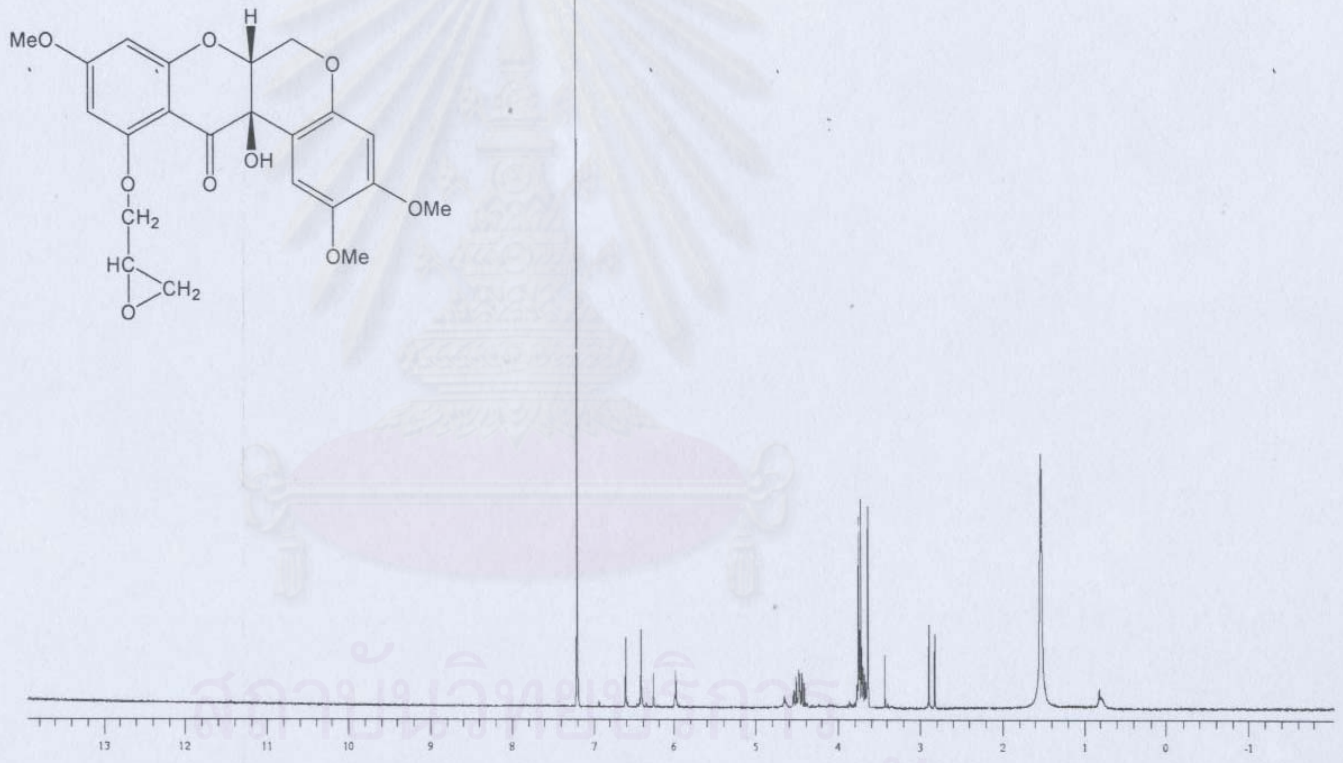


Figure 2 ^{1}H-NMR (400 MHz) of Compound 2

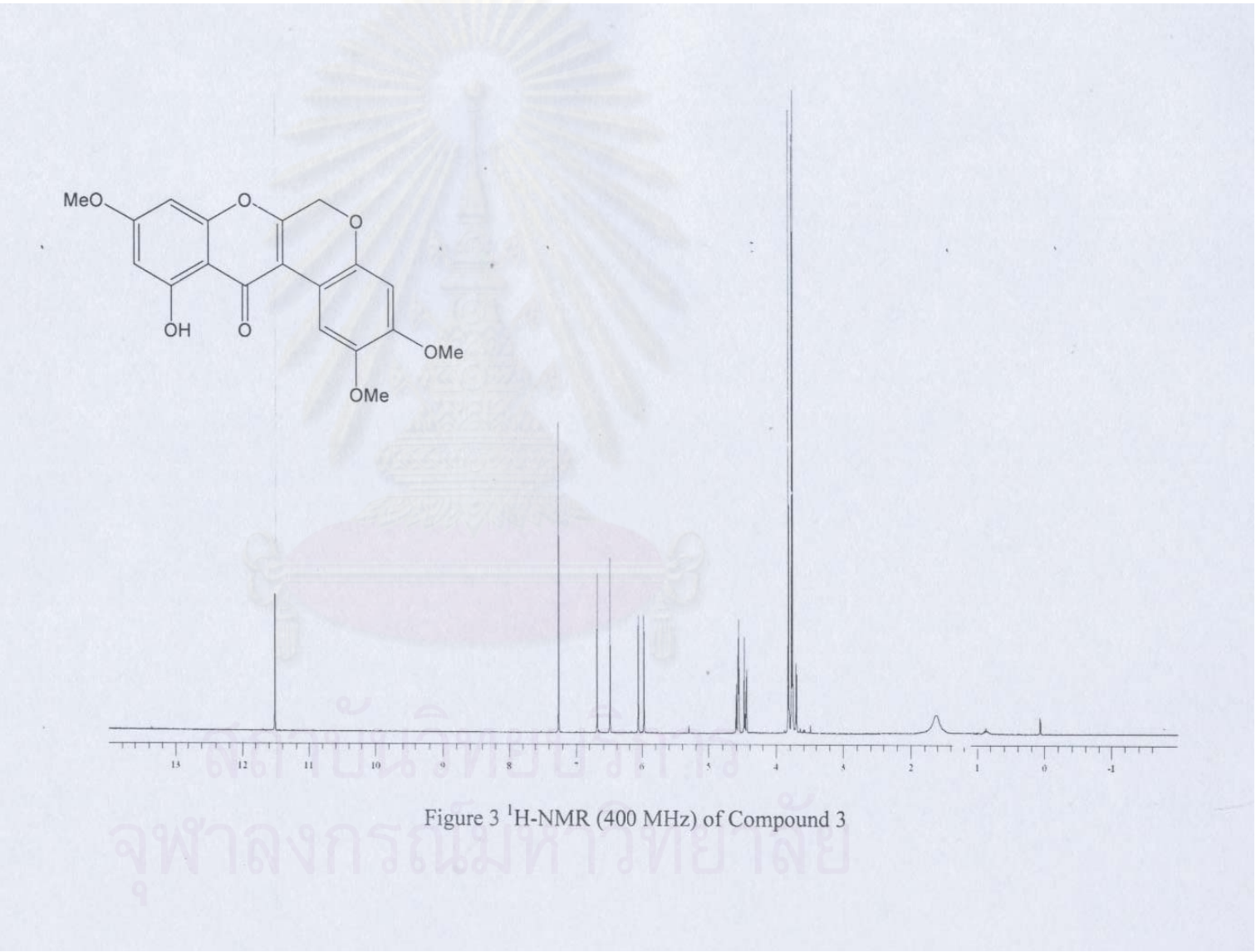


Figure 3 ^{1}H-NMR (400 MHz) of Compound 3

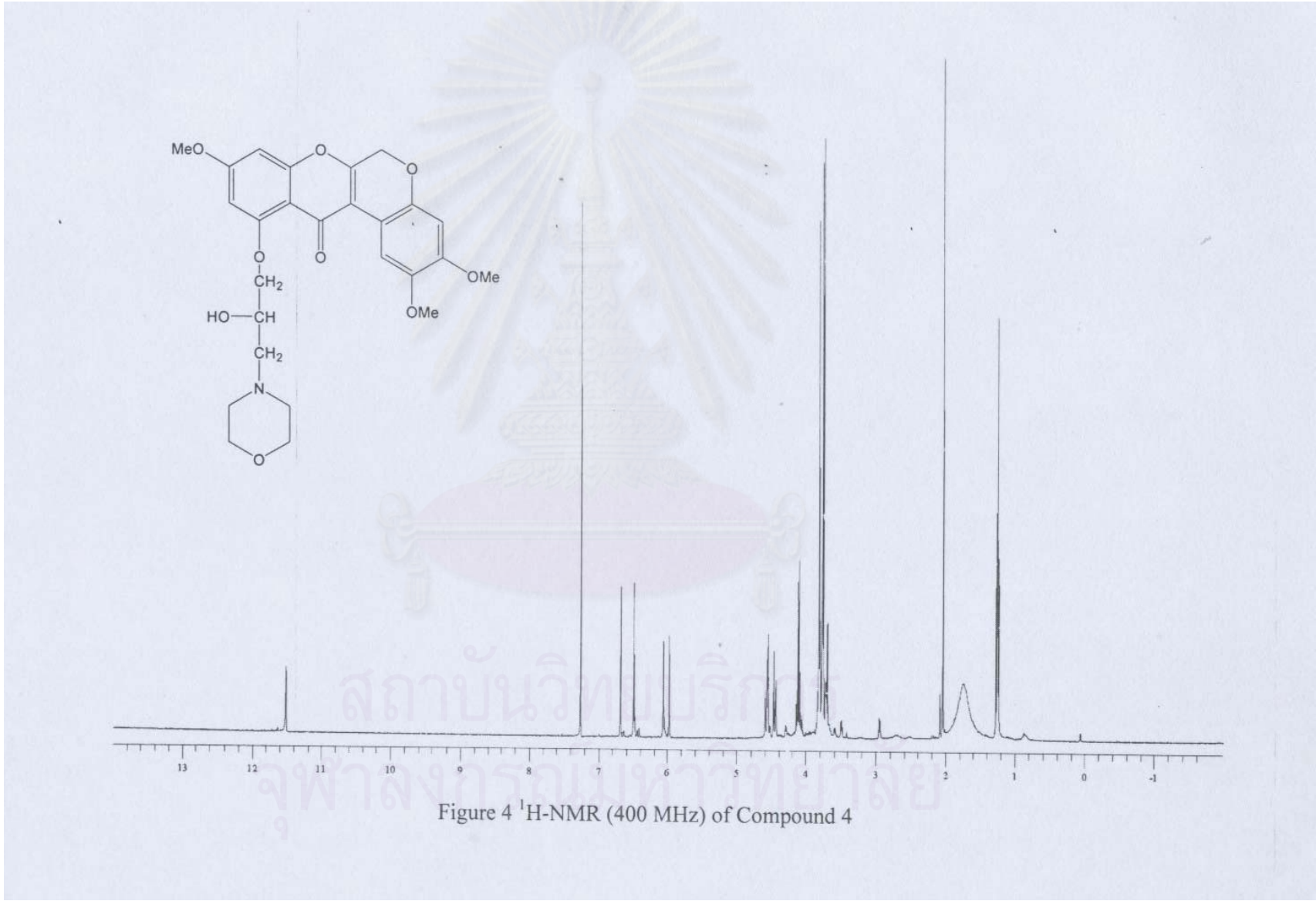
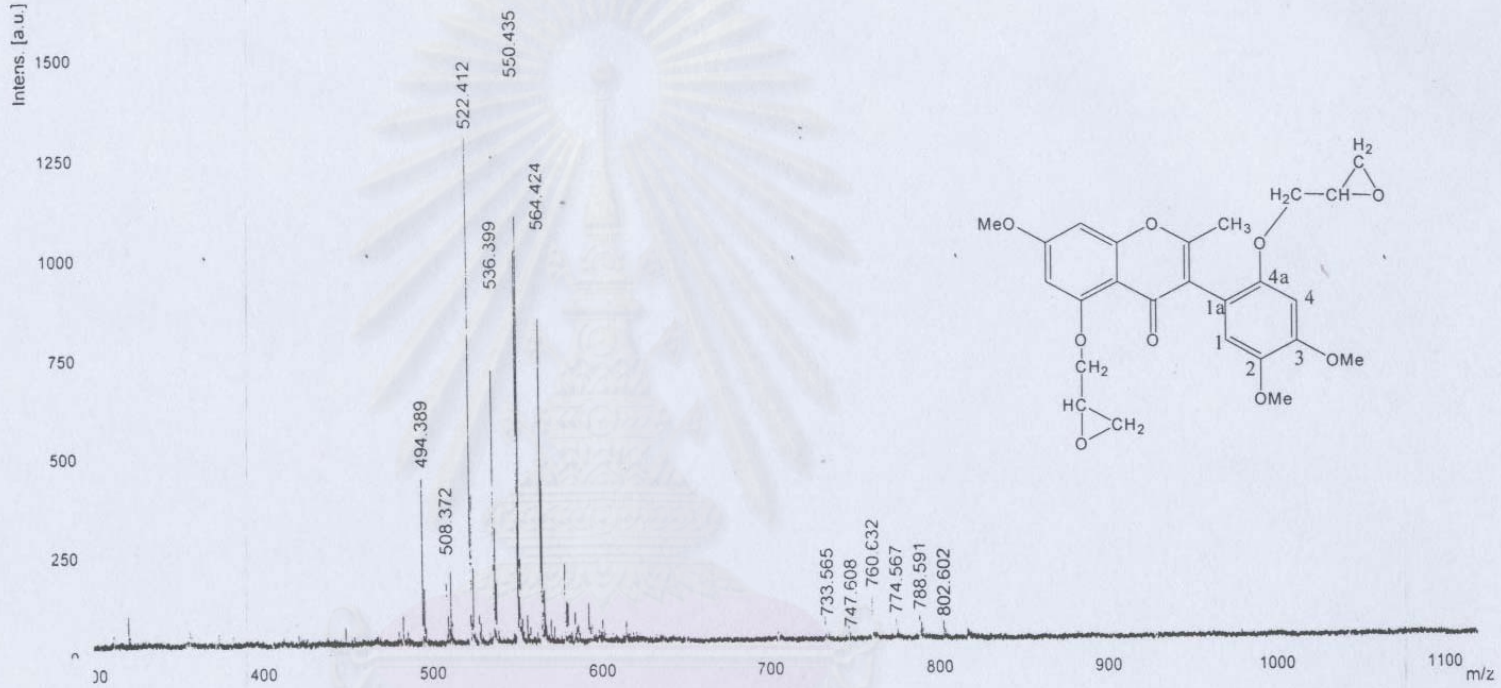


Figure 4 ^{1}H-NMR (400 MHz) of Compound 4



m/z	SN	Quality Fac.	Res.	Intens.	Area
494.389	41.8	18715	3207	465.00	93
508.372	21.9	10492	2805	244.00	59
522.412	117.9	61794	2862	1313.00	297
536.399	82.0	40940	3501	913.00	179
550.435	129.3	60213	2677	1440.00	354
564.424	95.1	74031	3736	1059.00	224
733.565	7.1	3904	3241	79.00	29
747.608	4.6	1453	2046	51.00	20
760.632	13.5	6306	2720	150.00	5

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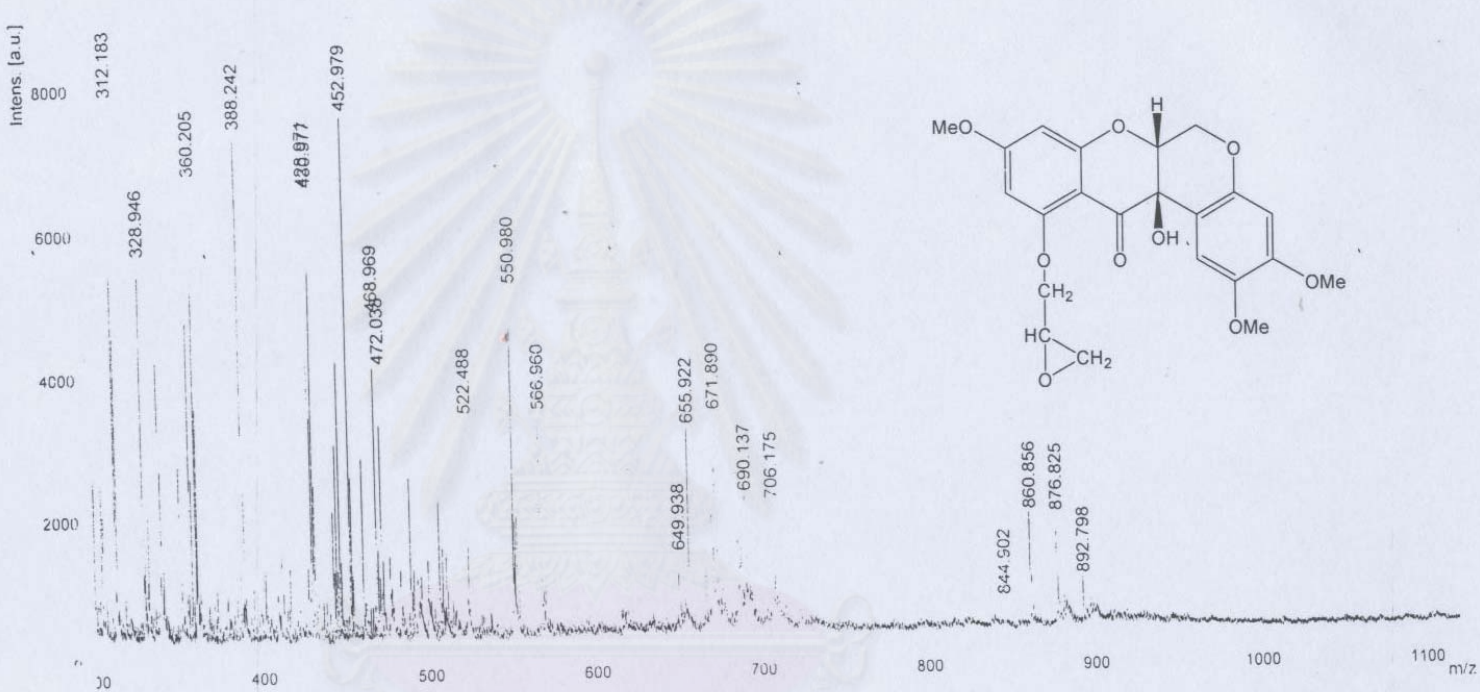
Figure 5 MS spectrum of compound 1



m/z	SN	Quality Fac.	Res.	Intens.	Area
312.183	54.5	428119	1731	7887.00	1716
328.946	39.2	199038	1765	5673.00	1197
360.205	46.7	316150	2108	6762.00	1422
388.242	51.1	432907	2155	7403.00	1695
428.971	45.5	513279	2421	6581.00	1684
430.977	45.4	512284	2430	6577.00	1652
452.979	52.7	290265	2368	7631.00	1682
468.969	33.5	436919	2865	4844.00	1233
472.038	28.4	195028	2975	4109.00	884

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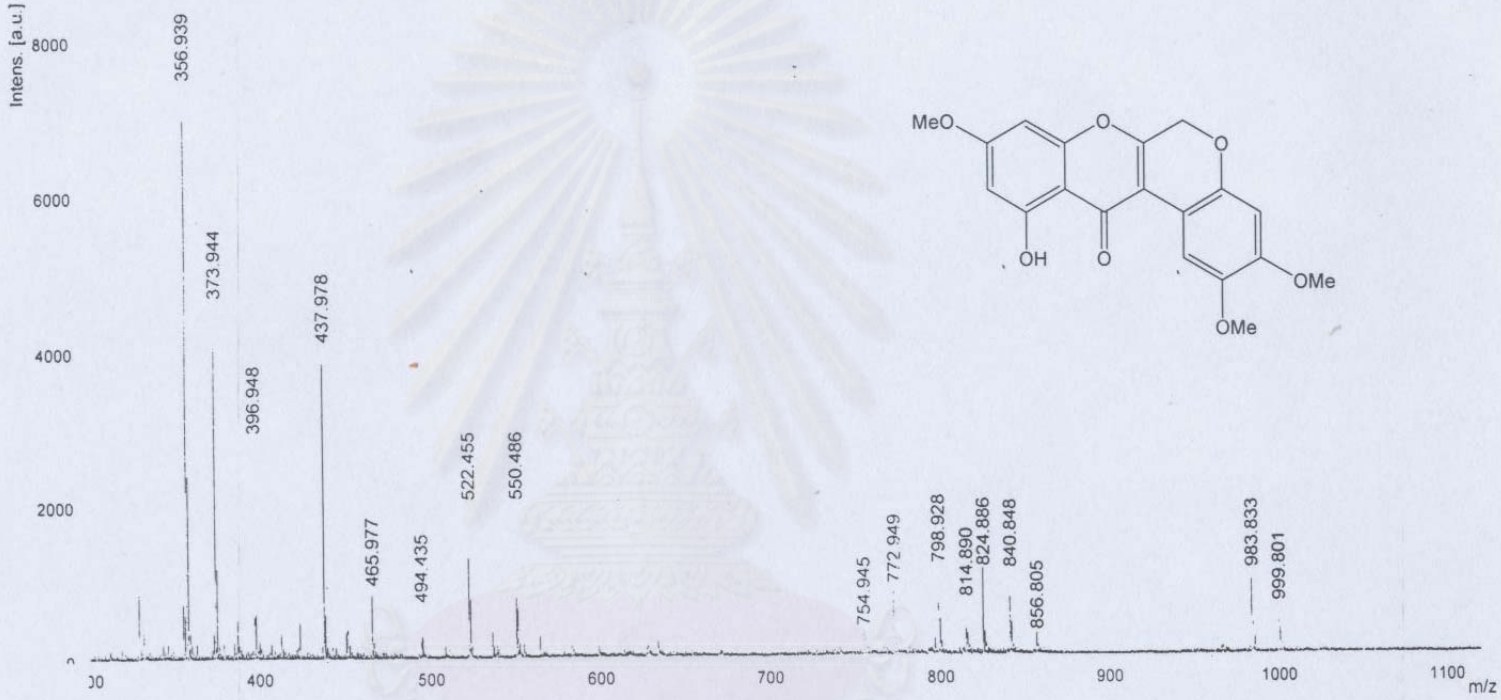
Figure 6 MS spectrum of compound 2



m/z	SN	Quality Fac.	Res.	Intens.	Area
356.939	164.8	562982	3060	7487.00	1132
373.944	103.1	402654	3459	4686.00	680
396.948	64.5	215272	3444	2932.00	460
437.978	90.6	307585	3452	4118.00	710
465.977	21.0	40976	3496	953.00	166
494.435	16.3	26532	3773	740.00	129
522.455	45.3	86155	3805	2059.00	382
550.486	45.5	77375	3738	2066.00	392
754.945	9.1	21943	3716	414.00	127

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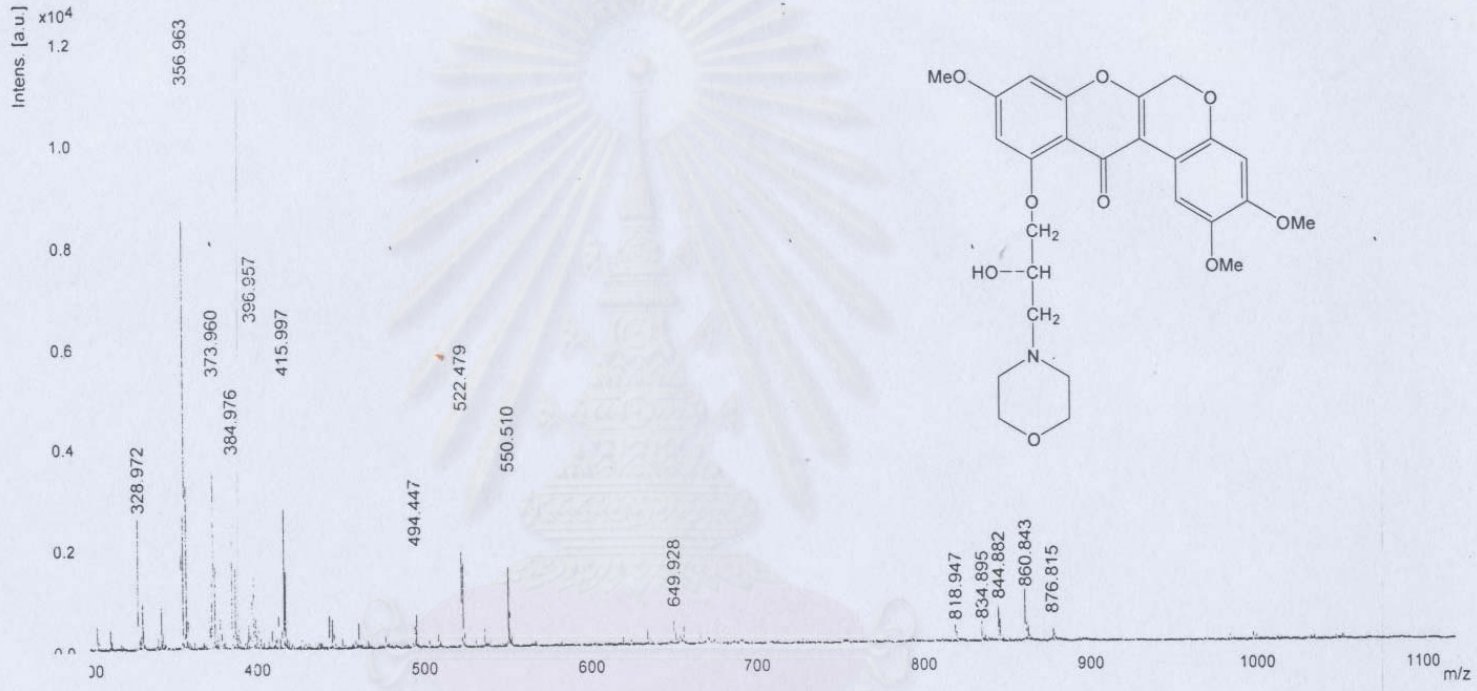
Figure 7 MS spectrum of compound 3



m/z	SN	Quality Fac.	Res.	Intens.	Area
328.972	46.0	255948	3005	2699.00	424
356.963	190.0	416611	3162	11146.00	1470
373.960	92.2	218542	3395	5409.00	729
384.976	65.4	253938	2778	3840.00	640
396.957	110.3	246595	3430	6473.00	892
415.997	92.4	232975	3806	5420.00	728
494.447	34.3	82537	3505	2010.00	353
522.479	79.7	166935	3713	4674.00	783
550.510	58.6	106620	3779	3440.00	59

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Figure 8 MS spectrum of compound 4

APPENDIX B

Table B1 The growth response against Hep-G2 of 6-deoxyclitoriacetal, Doxorubicin, Compound 1, Compound 2, Compound 3 and Compound 4

Compound	Concentration (µg/ml)			
	0.01	**0.1**	**1**	**10**
6-deoxyclitoriacetal	96.04 ± 8.00	90.61 ± 4.05	68.01 ± 3.02	64.41 ± 4.83
Doxorubicin HCl	96.67 ± 10.71	67.49 ± 3.92	38.40 ± 1.98	19.10 ± 1.52
Compound 1	93.70 ± 7.64	89.15 ± 2.37	83.10 ± 6.91	67.73 ± 3.22
Compound 2	119.90 ± 1.85	110.93 ± 4.03	96.79 ± 1.31	64.81 ± 8.48
Compound 3	92.90 ± 9.96	90.04 ± 2.01	77.48 ± 2.77	56.53 ± 1.94
Compound 4	115.65 ± 5.07	92.52 ± 6.32	77.02 ± 1.94	61.88 ± 2.78

Table B2 The growth response and cytotoxicity against Hep-G2 of 6-deoxyclitoriacetal-Prednisolone

Compound		Concentration (µg/ml)			
6-deoxy clitoriacetal (%wt)	**Prednisolone (%wt)**	**0.01**	**0.1**	**1**	**10**
100	**0**	96.04 ± 8.00	90.61 ± 4.05	68.01 ± 3.02	64.41 ± 4.83
90	**10**	103.29 ± 5.61	81.73 ± 5.14	62.40 ± 2.50	57.71 ± 1.57
75	**25**	92.44 ± 5.21	82.75 ± 9.88	65.13 ± 2.05	63.41 ± 2.41
50	**50**	94.40 ± 5.65	84.32 ± 6.12	65.90 ± 4.45	63.65 ± 3.40
25	**75**	89.08 ± 7.05	83.89 ± 4.71	65.42 ± 3.17	56.74 ± 2.49
10	**90**	111.62 ± 8.10	91.73 ± 7.90	72.74 ± 5.32	56.89 ± 3.47
0	**100**	114.48 ± 3.79	108.92 ± 4.01	106.28 ± 11.69	102.52 ± 2.06

Table B3 The growth response and cytotoxicity against Hep-G2 of 6-deoxyclitoriacetal-Podophyllotoxin

Compound		Concentration (µg/ml)			
6-deoxy clitoriacetal (%wt)	**Podophyllotoxin (%wt)**	**0.01**	**0.1**	**1**	**10**
100	**0**	96.04 ± 8.00	90.61 ± 4.05	68.01 ± 3.02	64.41 ± 4.83
90	**10**	90.91 ± 2.02	49.73 ± 2.18	51.35 ± 1.82	52.95 ± 2.13
75	**25**	84.76 ± 8.20	50.37 ± 2.36	53.88 ± 1.83	59.47 ± 2.35
50	**50**	65.93 ± 0.75	62.42 ± 0.43	62.12 ± 1.60	57.44 ± 1.23
25	**75**	58.24 ± 1.49	56.27 ± 2.30	57.63 ± 1.05	59.50 ± 2.68
10	**90**	47.20 ± 3.36	51.48 ± 2.10	57.69 ± 1.08	57.75 ± 2.58
0	**100**	58.43 ± 1.30	44.91 ± 1.13	48.12 ± 0.68	49.39 ± 3.25

Table B4 The growth response and cytotoxicity against Hep-G2 of Prednisolone-Podophyllotoxin

Compound		Concentration (µg/ml)			
Prednisolone	**Podophyllotoxin (%wt)**	**0.01**	**0.1**	**1**	**10**
100	**0**	114.48 ± 3.79	108.92 ± 4.01	106.28 ± 11.69	102.52 ± 2.06
90	**10**	97.05 ± 6.97	69.31 ± 8.82	52.03 ± 5.32	48.22 ± 2.78
75	**25**	59.38 ± 6.13	54.80 ± 6.36	51.51 ± 3.06	50.44 ± 2.01
50	**50**	132.75 ± 3.75	80.38 ± 3.18	69.93 ± 4.34	45.24 ± 2.70
25	**75**	102.89 ± 4.27	65.81 ± 1.75	66.61 ± 4.03	65.41 ± 3.54
10	**90**	73.86 ± 7.80	50.43 ± 1.97	75.32 ± 4.37	63.22 ± 3.22
0	**100**	58.43 ± 1.30	44.91 ± 1.13	48.12 ± 0.68	49.39 ± 3.25

APPENDIX C

CYTOTOXICITY STUDY

EMEM Medium

EMEM powder medium (Biowitaker)	19.15 g
HEPES	3g
NaHCO3	2g
Penicillin G (stock solution)	10,000 units
Streptomycin (stock solution)	10,000 units
Sterile water	2 L

Weight and mix all ingredients in sterile water. Adjust pH to 7.0.Filtrate with 0.22 μm membrane (Whatman). Dispense the filtrate into bottles. All bottled mediums are stored in 37 °C incubator for 24 hr. for sterility test.

0.25 Trypsin (in HEPES-Buffer Saline)

HEPES-buffer saline

NaCl	8 g
KCl	0.4 g
Na_2HPO_4	0.1 g
Dextrose	1.0 g
HEPES	2.38 g
Distilled water	1 L

All ingredients were mixed in 1lt volumetric flask and stirred with magnetic stirrer until all ingredients were completely dissolved. Then 2.5g of Trypsin powder (Gibco) was added. The solution was stirred until Trypsin was completely dissolved. Then adjust pH to 7.0 (by add 7.5% $NaHCO_3$ and/or 1% HCl). The solution was filtrated (through 0.22 μm membrane) and dispensed into bottles.

The bottled trypsin was stored in 37°C incubator for 24 hr. for sterility test.

0.4% Trypan Blue Dye

Trypan Blue	1.6 g
NaCl	3.24 g
KH2PO4	0.24 g
Distilled water	400 ml

All ingredients were mixed altogether, heat and stirred with magnetic stirrer until completely dissolved. Adjust pH to 7.2-7.3 (by add 7.5% $NaHCO_3$ and/or 1% HCl). Then dispensed into light protecting bottles.

Phosphate buffer solution

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distill water	1 L

All ingredients were mixed and dispensed into bottles. All bottles were autoclaved for 15 minute.

Sorensen' s glycine buffer

0.1 M Glycine	100 ml
0.1 M NaCl	100 ml

All ingredients were mixed. Adjusted to pH 10.5 with 1 M NaOH

MTT solution

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma)	0.5 mg
DMEM	1 ml

Add MTT 0.5 mg into DMEM 1 ml. All ingredients were mixed and sterilized by filter. Then dispensed into light protecting bottles and freshly prepared for every experiment.

BIOGRAPHY

Miss Nustha Kitprathaung was born on August 8, 1978 in Saraburi, Thailand. She received Bachelor Degree of Science (Biotechnology), Faculty of Industrail Technology, Silpakonn University in 2002. She has enrolled in the Graduate school, Chulalongkorn University for Master Degree of Science in Biotechnology during 2003-2007.

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