การทดสอบฤทธิ์ยับยั้งโทโพไอโซเมอเรส II จากพืชสมุนไพรไทยโดยใช้เซลล์ยีสต์

นางสาวสุภัทรา แสงมาลี

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YEAST CELL-BASED ASSAY OF TOPOISOMERASE II INHIBITORY ACTIVITY OF THAI MEDICINAL PLANTS

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A Thesis Submitted in Partial Fulfillment of the Requirements

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สุภัทรา แสงมาลี: การทดสอบฤทธิ์ยับยั้งโทโพไอโซเมอเรส II จากพืชสมุนไพรไทยโดยใช้เซลล์ยีสต์. (YEAST CELL-BASED ASSAY OF TOPOISOMERASE II INHIBITORY ACTIVITY OF THAI MEDICINAL PLANTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. สุชาดา สุขหร่อง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. อารีรัตน์ ลออปักษา, 79 หน้า.

เอนไซม์โทโพไอโซเมอเรส II (Top2) เป็นเอนไซม์ที่มีความสำคัญในกระบวนการจำลองตัวของเซลล์และมีความสำคัญต่อการดำรงชีวิตของเซลล์ของสิ่งมีชีวิต ในการทดสอบฤทธิ์ยับยั้งเอนไซม์ Top2 ของพืชสมุนไพรไทยโดยการใช้เซลล์ยีสต์ ได้ใช้ยีสต์ *Saccharomyces cerevisiae* ที่กลายพันธุ์และมีความไวต่ออุณหภูมิที่ใช้ในการเพาะเลี้ยง (temperature sensitivity) จากการทดสอบฤทธิ์ยับยั้งเอนไซม์ Top2 ของสารสกัดหยาบจากพืชสมุนไพรไทยจำนวน 30 ตัวอย่าง พบว่าสารสกัดหยาบจากพืชสมุนไพรไทยจำนวน 6 ตัวอย่าง ได้แก่ เหง้าขมิ้นชัน เหง้าขมิ้นอ้อย เถาเถาวัลย์เปรียง ต้นพญามุตติ หัวสบู่เลือด และหัวบอระเพ็ดพุงช้างมีฤทธิ์ยับยั้งเอนไซม์ Top2 สารสกัดจากเถาวัลย์เปรียงถูกเลือกเพื่อทำการศึกษาต่อให้ทราบถึงสารสำคัญที่มีฤทธิ์ดังกล่าว เนื่องจากยังไม่เคยมีรายงานถึงฤทธิ์การยับยั้งเอนไซม์ Top2 ของพืชชนิดนี้มาก่อน

จากการศึกษาองค์ประกอบทางเคมีของเถาวัลย์เปรียงโดยใช้ฤทธิ์ทางชีวภาพในการยับยั้งเอนไซม์ Top2 โดยใช้เซลล์ยีสต์เป็นตัวนำ สามารถแยกสารกลุ่ม isoflavones ได้ 2 ชนิด คือ 5,7,4′ trihydroxy-6,8-diprenylisoflavone และ lupalbigenin ซึ่งได้ทำการพิสูจน์โครงสร้างทางเคมีของสารทั้งสองโดยอาศัยเทคนิคทางสเปคโตสโคปีร่วมกับการเปรียบเทียบข้อมูลจากงานวิจัยที่มีรายงานมาแล้ว การทดสอบฤทธิ์ทางชีวภาพของสารทั้งสองชนิดพบว่า สารทั้งสองชนิดมีฤทธิ์ต้านเซลล์มะเร็งในช่องปากชนิด KB เซลล์มะเร็งเต้านมชนิด MCF-7 และเซลล์มะเร็งปอดชนิด NCI-H187 และยังแสดงฤทธิ์ยับยั้งการคลายเกลียวของสายดีเอ็นเอชนิด supercoiled ค่า IC_{50} ของ 5,7,4′ trihydroxy-6,8-diprenylisoflavone และ lupalbigenin มีค่าเท่ากับ 24.4 และ 18.7 μM ตามลำดับ ข้อมูลที่ได้เป็นการรายงานครั้งแรกถึงกลไกการยับยั้งเอนไซม์ Top2 และการแสดงฤทธิ์ทางชีวภาพของสารสำคัญสองชนิดจากเถาวัลย์เปรียง

ภาควิชา..เภสัชเวทและเภสัชพฤกษศาสตร์... ลายมือชื่อนิสิต.....
สาขาวิชา.....เภสัชเวท..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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SUPHATTRA SANGMALEE: YEAST CELL-BASED ASSAY OF TOPOISOMERASE II INHIBITORY ACTIVITY OF THAI MEDICINAL PLANTS. ADVISOR: ASST. PROF. SUCHADA SUKRONG, Ph.D. CO-ADVISOR: ASSOC. PROF. AREERAT LAORPAKSA, 79 pp.

Topoisomerase II (Top2) is an essential enzyme for survival of growing cells. This study aimed to screen ethnomedicinal plants used in Thai traditional medicine for Top2 poison activity using a yeast cell-based assay. Mutant yeast strains carrying the *top2-1* allele (JN394*t2-1*), which encodes a temperature-sensitive topoisomerase, were developed to address the mechanism of action of agents with Top2 poison activity. Six out of thirty extracts; *Curcuma longa*, *C. zedoaria*, *Derris scandens*, *Grangea maderaspatana*, *Stephania pierrei* and *S. suberosa*, were found to have Top2 poison activity against the yeast cells. Further phytochemical studies are needed for the isolation and identification of the natural Top2 poison agents found in *D. scandens* Benth. (Leguminosae). This is the first time that the Top2 poison activities of *D. scandens* have been studied.

Bioassay-guided fractionation based on Top2 poison activity led to the isolation of two isoflavones from the ethyl acetate extract of the stem of *D. scandens*. The compounds were identified as 5,7,4′-trihydroxy-6,8-diprenylisoflavone and lupalbigenin from spectral evidence. These compounds showed cytotoxicitiy against human epidermoid carcinoma of oral cavity (KB), breast adenocarcinoma (MCF-7) and human small cell lung carcinoma (NCI-H187) cell lines. In DNA Top2 relaxation assay, 5,7,4′-trihydroxy-6,8-diprenylisoflavone and lupalbigenin also showed inhibitory activity with IC_{50} values of 24.4 and 8.7 μM, respectively. This is the first time that Top2 poison acitivity of *D. scandens* has been reported.

Department : Pharmacognosy and Pharmaceutical Botany Student's Signature .....

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%	= percent (part per 100); percentage
μg	= microgram(s)
μl	= microliter(s)
μM	= micromolar
/	= per
α	= alpha
β	= beta
br	= broad (for NMR spectra)
°C	= degree Celsius
$CDCl_3$	= deuterated chloroform
CFU	= colony forming units
cm	= centimeter(s)
^{13}C-NMR	= carbon-13 nuclear magnetic resonance
conc.	= concentration
CPT	= camptothecin
d	= doublet (for NMR spectra)
dd	= doublet of doublet (for NMR spectra)
DEPT	= distortionless enhancement by polarization transfer
δ	= chemical shift
DMSO	= dimethylsulfoxide
DNA	= deoxyribonucleic acid
EIMS	= electron impact mass spectrometry
et. al	= et alii
EtOAc	= ethyl acetate
FCC	= Flash Column Chromatography
g	= gram(s)
^{1}H-NMR	= proton nuclear magnetic resonance
hr	= hour(s)

Hz = hertz

IC_{50} = inhibitory concentration 50%

J = coupling constant

kg = kilogram(s)

L = liter(s)

m = meter(s)

M+ = molecular ion

mg = milligram

MHz = mega hertz

MeOH = methanol

min = minute(s)

ml = milliliter

mM = millimolar

MS = mass spectrum

MW = molecular weight

m/z = mass to charge ratio

O.D. = optical density

ppm = part per million

R_f = retention factor

s = singlet (for NMR spectra)

t = triplet (for NMR spectra)

TLC = thin layer chromatography

Top1 = topoisomerase I

Top2 = topoisomerase II

v/v = volume/volume (concentration)

w/v = weight/volume (concentration)

w/w = weight/weight (concentration)

YPDA = yeast peptone dextrose adenine sulfate

CHAPTER I

INTRODUCTION

The current trend in cancer treatment research is the development of drugs with defined molecular targets. The conceptual idea behind this approach is that knowledge of a drug's mechanism of action provides a target for a specific site of action. These drugs aim to improve clinical results by targeting the cancer cells and minimally affecting healthy cells (Pezzuto, 1997).

DNA topoisomerases have emerged as important targets for anticancer agents. These enzymes catalyse the relaxation of supercoiled DNA via a concerted mechanism for transient DNA cleavage and reannealing (Nitiss *et al*., 1992). DNA topoisomerases can be divided into two classes: type I (Top1) enzymes, which transiently break and rejoin one DNA strand at a time, and type II (Top2) enzymes, which break and rejoin both strands of the double-stranded DNA (Reid *et al*., 1998). The activity of Top2 is different from that of Top1, as it is essential for the viability of growing cells. Top2 enzymes are also essential for DNA-mediated processes and for proliferation, which is characterised by a high level of Top2 expression (Robert and Larsen, 1998; McClendon and Osheroff, 2007). Top2 enzymes have been shown to be clinically important targets for cancer chemotherapy, and their inhibitors are central components of many therapeutic regimens (Reid *et al*., 1998). Many drugs target Top2 by trapping the covalent intermediate, termed the cleavage complex, converting the transient DNA cleavage into a stable DNA break and then covalently attaching the enzyme to the target DNA molecule (Liu 1989; Nitiss *et al*., 1993; Nitiss, 2009). These drugs are often referred to as Top2 poisons because their anticancer activity is due to the trapping of Top2 rather than to the inhibition of Top2 activity. The agents that have been shown to inhibit Top2 by this mechanism include amsacrine, doxorubicin and etoposide (Meczes *et al*., 1997; Patel *et al*., 1997; Hammonds *et al*., 1998).

Recently, many bioactive compounds isolated from plant sources have played an important role in the treatment of cancer (Shoeb, 2006). Thailand has remarkable biodiversity and a rich cultural tradition of medicinal plant use. Many plants in Thailand have a long history of use as alternative treatments for cancer, and Thai folk doctors have extensively documented these uses. However, many of these traditional treatment claims should be investigated because cancer, as a specific disease entity, can be poorly defined by the terms of traditional medicine (Itharat and Ooraikul, 2007). This traditional drug use led us to pursue research into the identification of the natural source of Top2 poison activity in Thai medicinal plants. A bioassay technique was required to screen the large number of plant extracts for Top2 poison activity. We chose a yeast model to screen Top2 activity *in vitro* for the determination of its utility as a molecular target for anticancer agents. This model was selected because it allowed yeast mutations in Top2 to be easily assessed by the consequential drug sensitivity conferred (Reid *et al*., 1998). Furthermore, phytochemical studies are needed for the isolation and identification of the natural Top2 poison agents found in putative hits.

In this study, thirty ethnomedicinal plants used in Thai traditional medicine for cancer treatment were selected and screened using a yeast cell-based assay to identify their Top2 poison activity, followed by bioassay-guided fractionation of the selected plant, *Derris scandens*. Isolation and elucidation of the active compounds were also reported. These results could support the use of Thai medicinal plants as traditional medicine for cancer chemotherapy.

CHAPTER II

LITERATURE REVIEW

2.1 DNA topoisomerases

DNA topoisomerases are nuclear enzymes that catalyze the breakage and rejoining of DNA strands in order to interconvert different topological forms of DNA (Nitiss, 1993). The first DNA topoisomerase was discovered by Dr. James Wang in 1971, from *Escherichia coli* (Wang, 1971). There are two classes of DNA topoisomerases: type I enzymes (Top1) break and rejoin one DNA strand at a time; and type II enzymes (Top2) break and rejoin both strands of a double stranded DNA. Both classes of DNA topoisomerases have been shown to play an important role in a wide variety of DNA metabolic reactions, including replication, transcription, recombination, and DNA repair (Nitiss, 1993).

Every cell type so far examined contains DNA topoisomerases, and where a genetic test has been possible, at least one is essential for cell growth. Examples of the type of organisms in which topoisomerases have been studied include the bacteria *E. coli* and *Staphylococcus aureus*, yeast, the model plants *Arabidopsis*, and *Drosophila*, and man. In addition, several viruses are known to encode a topoisomerase, for example, bacteriophage T4 and animal virus *vaccinia* (Bates and Maxwell, 2005). Examples of DNA topoisomerases and their functions are given in **Table 2.1**.

Table 2.1 Type of DNA topoisomerases (Bates and Maxwell, 2005).

Enzyme	Type	Source	Remarks
Bacterial topoisomerase I (ω protein)	IA	Bacteria (e.g. *E. coli*)	Cannot relax positive supercoils
Eukaryotic topoisomerase I	IB	Eukaryotes (e.g. human)	Can relax both positive and negative supercoils
Vaccinia virus topoisomerase I	IB	*Vaccinia* virus	ATP stimulates topoisomerase activity
Topoisomerase III[a]	IA	Bacteria (e.g. *E. coli*)	Potent decatenating activity
Reverse gyase	IA	Thermophilic Archaea (e.g. *Sulfolobus acidocaldarius*)	Can introduce positive supercoils into DNA (ATP-dependent)
DNA gyrase	IIA	Bacteria (e.g. *E. coli*)	Can introduce negative supercoils into DNA (ATP-dependent)
T4 topoisomerase	IIA	Bacteriophage T4	Can relax, but not supercoil, DNA (ATP-dependent)
Eukaryotic topoisomerase II	IIA	Eukaryotes (e.g. human topoisomerase IIα)	Can relax, but not supercoil, DNA (ATP-dependent)
Topoisomerase IV[a]	IIA	Bacteria (e.g. *E. coli*)	Can relax, but not supercoil, DNA, potent decatenase (ATP-dependent)
Topoisomerase VI	IIB	Archaea (e.g. *Sulfolobus acidocaldarius*)	Can relax, but not supercoil, DNA (ATP-dependent)

[a] Note that topoisomerase III and IV do not represent 'type' of topoisomerase mechanism.

2.2 Structure and mechanism of Topoisomerase II

The primary structure of Top2 can be divided into three domains: the N-terminal domain, the central domain and the C-terminal domain (McClendon and Osheroff, 2007) (**Figure 2.1**). The N-terminal domain (first ~670 amino acids) contains the site of ATP binding and hydrolysis (Berger *et al*., 1998; Champoux, 2001 and Velez-Cruzand and Osheroff, 2004). The central domain (amino acids ~671–1200) contains the active site tyrosine (Y805) required for DNA cleavage and ligation. The C-terminal domain (amino acids ~1201–1521) contains nuclear localization sequences (NLS) and sites of phosphorylation (PO_4) (Shiozaki and Yanagida, 1992).

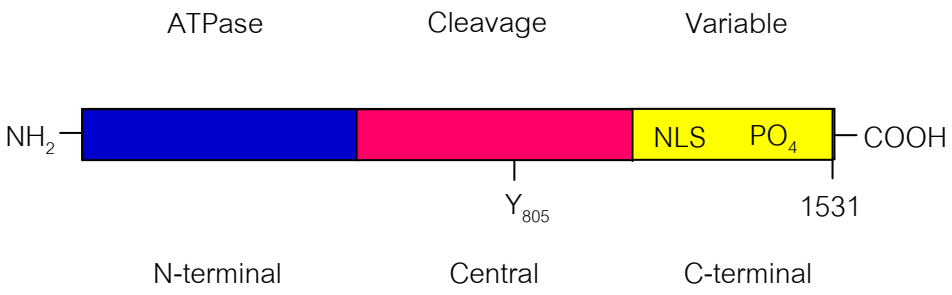


Figure 2.1 Structure of topoisomerase II (modified from McClendon and Osheroff, 2007).

Top2 activity is required to change DNA topology by a double-stranded DNA passage reaction. It is important in the relaxation of DNA supercoils generated by cellular processes, such as transcription and replication, and it is essential for the condensation of chromosomes and their segregation during mitosis (Austin and Marsh, 1998).

Type II topoisomerase acts by generating a transient double-stranded DNA break, followed by a double-stranded DNA passage event in DNA process. The enzyme generates cleaved DNA molecules that contain 4-base single-stranded ends at their 5′-termini (McClendon and Osheroff, 2007). During the cleavage reaction, a tyrosine residue in the active site of each monomer becomes covalently linked to a 5´-phosphate group of one of the DNA strands. This enzyme-bridged intermediate is termed the cleavable complex (Austin and Marsh, 1998) (**Figure 2.2**). Agents that increase levels of Top2-DNA cleavage complexes are known as Top2 poisons because they convert this essential enzyme to a potent cellular toxin. Top2 poisons increase levels of enzyme-DNA cleavage complexes by two non-mutually exclusive mechanisms (Fortune and Osheroff, 2000). Some poisons act by inhibiting the ability of Top2 to ligate the cleaved substrate (Wilstermann and Osheroff, 2003). These agents not only increase the level of cleavage complexes, but also increase the lifetime of these complexes. Other poisons have little effect on the rate of enzyme-mediated ligation and are believed to act primarily by enhancing the forward rate of cleavage complex formation (Baldwin and Osheroff, 2005). The exact mechanism by which this second group of drugs increases levels of DNA cleavage is unknown.

Top2 requires two cofactors in order to carry out its catalytic double-stranded DNA passage reaction. First, it needs a divalent cation for all steps beyond enzyme-DNA binding. Second, it uses the energy of adenosine triphosphate (ATP) to drive the overall DNA strand passage reaction (McClendon and Osheroff, 2007).

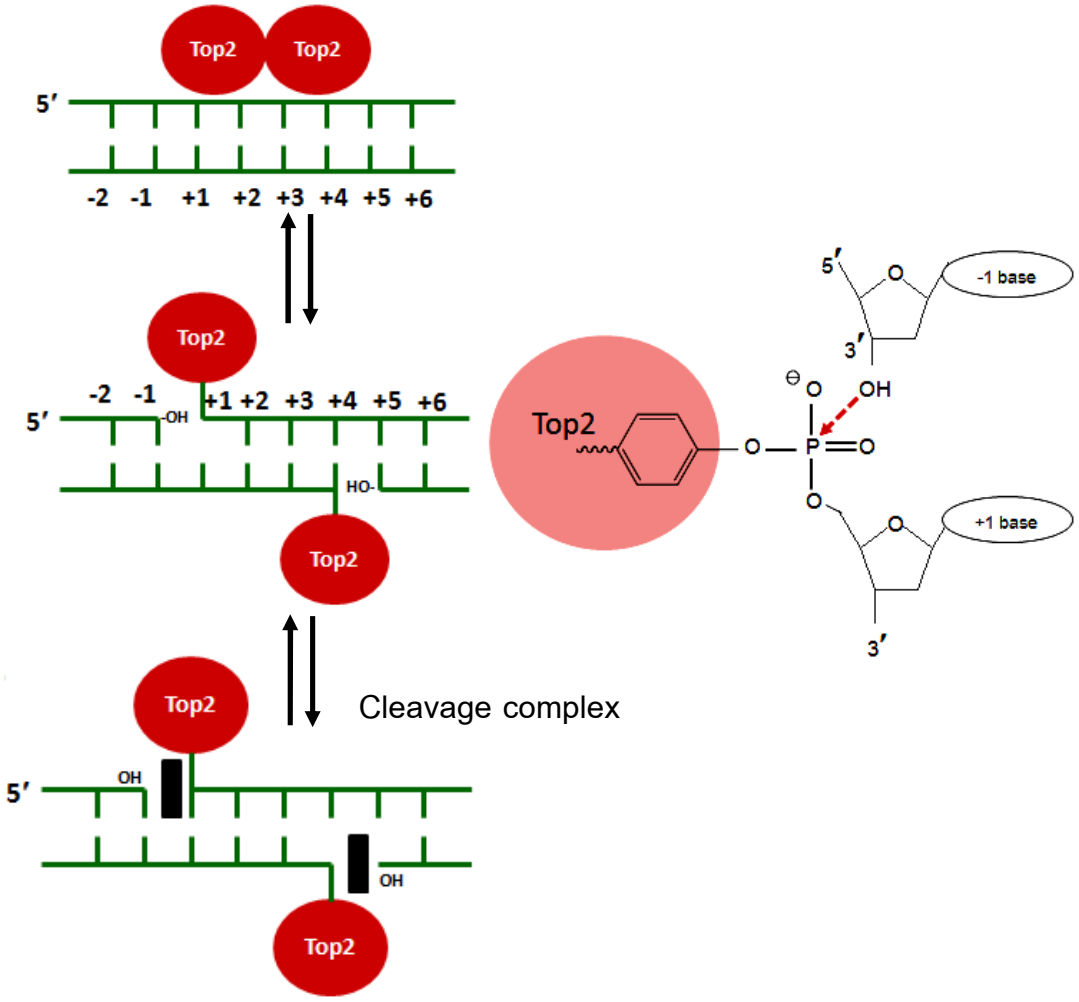


Figure 2.2 DNA topoisomerase II-mediated DNA cleavage and relegation (modified from Pommier *et al.*, 2010).

The activity of Top2 is different from that of Top1, as it is essential for the viability of growing cells. Top2 enzymes are also essential for DNA-mediated processes and for proliferation, which is characterised by a high level of Top2 expression (Robert and Larsen, 1998; McClendon and Osheroff, 2007). Beyond its required physiological functions, this enzyme is a target for some of the most active compounds currently employed for the treatment of human cancer (Elsea *et al*., 1992). For example, doxorubicin, m-AMSA (amsacrine), epipodophyllotoxins, and mitoxantrone, which are termed topoisomerase II poisons (Hammonds *et al*., 1998). These drugs stabilize the cleavable complex stage of the catalytic cycle, increasing the proportion of complexes in which the DNA is cleaved. It is thought that cellular processes such as replication and transcription convert the drug-stabilzed complexes into double-stranded DNA breaks, which are toxic to the cell.

Many eukaryotes, have two isoforms of Top2, Top2α and Top2β. The α -isozyme form has a monomeric molecular mass of 170 kDa and is encoded by a gene on chromosome 17q21-22 (Tsai-Pflugelder *et al*., 1988), whereas the β isoform has a molecular mass of 180 kDa and is encoded by a gene on chromosome 3p24 (Jenkins *et al*., 1992). Although it is known that both isoenzymes can be inhibited by antitumor agents such as etoposide, m-AMSA, and merbarone *in vitro* (Drake *et al*., 1989), the extent to which inhibition of either Top2 α or Top2 β is cytotoxic *in vivo* is unclear. Top2 α is known to be preferentially expressed during mitosis, whereas Top2 β shows little variation in levels during the cell cycle (Woessner *et al*., 1991). One would speculate from these data that Top2 α is the major target of cytotoxic agents.

2.3 Yeast cell-based assay

Yeast has been a great value in demonstrating the specificity of agents that target topoisomerase. The ability to decipher the targets of cell killing using yeast also makes this organism extremely attractive as a screening system to discover new anti-Top2 inhibitors (Nitiss, 1994).

Saccharomyces cerevisia, the budding yeast, is commonly known as "baker's yeast" or "brewer's yeast". It is a single-celled fungus which has thick-walled, oval cells, around 10 μm long by 5 μm wide. Yeast cell can also be propagated as heterothallic and α haploids or a/ α diploids, produced by the fusion of haploid strains of opposite mating type. Under conditions of nutrient deprivation, diploids are induced to undergo meiosis, and the four spore products of a single meiosis can be recovered, grown out and analyzed (Reid *et al*., 1998). It is an ideal model organism for studying the mechanism of action of anti-topoisomerase agents because it is a unicellular eukaryote whose cellular activities are much more like ours than those of a bacterium like *E. coli*. It can be cultured easily, grows rapidly, and entire genome is known and can be easily transformed with genes from other sources (Nitiss, 2001).

S. cerevisiae has a single form of Top2 which has been frequently used as a eukaryotic model in functional studies and in the study of anti-Top2 agents. A mutant temperature sensitive strain of *S. cerevisiae* for Top2 in combination with yeast/human hybrid topoisomerases has been used as a model to study the relative sensitivities of human α and β Top2 enzymes to a variety of Top2 inhibitors both in *in vitro*. Sensitivities to the anti-Top2 drugs were estimated following a short contact inhibition assay based on viable-cell counts (Hammonds *et al*., 1998).

2.3.1 DNA topoisomerase II poisons in yeast

Yeast has a single form of Top2 which has been used a model eukaryote for study activity of Top2-targeted drugs, both to investigate the cellular effect of drugs and to establish Top2 as the cellular target of drugs (Reid *et al*., 1998). The activity of Top2 is different from that of Top1, as it is essential for the viability of growing cells. Deletion of *TOP2* may cause the growing cells death. Thus, the JN394*top2-1* strains, which carry the *top2-1* allele encoding a temperature-sensitive topoisomerase, were constructed, and these strains contain the *ise2* and *rad52* mutations but express the temperature-sensitive *top2-1* mutation instead of the wild-type *TOP2* gene. It has been demonstrated that yeast strains carrying *top2-1* have approximately a wild-type level of activity at 25°C but substantially reduced activity at 30°C. Consequently, the *top2-1* yeast strains proliferate like wild-type (JN 394) cells at the permissive temperature (25°C), and have sufficient Top 2 activity to be viable at the semi-permissive temperature (30°C) (Nitiss *et al*., 1993). Hence, by comparing the cytotoxicity of the anti-Top2 agents on the JN394*top2-1* yeast cells at 25°C and 30°C, it was possible to address the mechanism of action of the anti-Top2 agents.

2.3.2 Yeast strains

Yeast strains used in this study are *S. cerevisiae* strain JN394, genotype *ise2 ura3-52 leu2 trp1 ade2 his7 rad52 : : LEU2* as wild type, strain JN394 Δ *top1,* which has a chromosomal deletion of the topoisomerase I gene (*TOP1*) and strain JN394*top2-1*, a mutant yeast strains carrying the *top2-1* allele, which encodes a temperature-sensitive topoisomerase. Strains carrying this mutation grow normally at 25°C and generally have a wild-type drug sensitivity. These yeast strains are able to grow at 30°C, but the Top2 activity is greatly reduced at this temperature, which causes the strains to be highly drug resistant to anti-Top2 agents.

2.4 Bioassay-guided fractionation

Nature has long been an important source of medicinal agents. An impressive number of modern drugs have been isolated or derived from natural sources, based on their use in traditional medicine (Itharat and Ooraikul, 2007). Many anticancer drugs were isolated from plants for example, camptothecin from *Camptotheca acuminata*, taxol from *Taxus brevifolia*, (Wall and Wani, 1996) and vinblastine and vincristine from *Catharanthus roseus* (Potier, 1980).

The study of bioactive compounds from plants has required the development of bioassay techniques, especially *in vitro* methods which allow a large number of plant extracts to be screened for activity, especially cytotoxicity, against yeast cell-based assay. A bioassay-guided fractionation was performed on the methanolic extracts in order to isolate the bioactive compounds contributing to the Top2-targeted agents. After every fractionation process, the fractions were tested for Top2 inhibitory activity against yeast cell-based assay.

2.5 Botanical aspects and chemical constituents of *Derris scandens* Benth.

D. scandens, known in Thai as Thao-Wan-Priang, is a medicinal plant used in traditional remedies in Thailand. Its dried stem has been used as an expectorant, antitussive, diuretic, and antidysentery agent and for the treatment of muscle aches and pains (Chavalittumrong *et al*., 1999). A hydroalcoholic extract of the stem was reported to have both antimicrobial (Dhawan *et al*., 1977) and immunostimulating activities (Chuthaputti and Chavalittumrong, 1998). In a pharmacological study, the polar fraction when applied resulted in a marked decrease in blood pressure and heart rate (Jansakul *et al*., 1997). So far, there have been no reports on the Top2 poison activity of this plant extract. Coumarins, isoflavones and isoflavone glycosides have been reported as the chemical constituents of the stems (Chuankamnerdkarn *et al*., 2002; Dianpeng *et al*., 1999; Falshaw *et al*., 1969; Johnson *et al*., 1966; Johnson and Pelter, 1966; Mahabusarakam *et al*., 2004; Pelter and Stainton, 1966; Rao *et al*., 1994; Rukachaisirikul *et al*., 2002; Sekine *et al*., 1999). The chemical substances isolated from these plants are summarized in **Table 2.2.**

Table 2.2 Chemical constituents and structures found in *Derris scandens*.

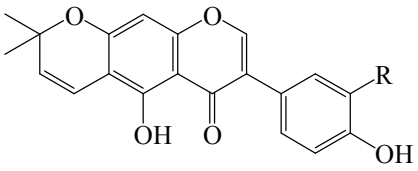
Chemical compound	Category	Plant part	Reference
Chandalone [1]	Isoflavone	Stem	Falshaw *et al*., 1969
Derrisisoflavone A [2]	Isoflavone	Stem	Sekine *et al*., 1999
Derrisisoflavone B [3]	Isoflavone	Stem	Sekine *et al*., 1998
Derrisisoflavone C [4]	Isoflavone	Stem	Sekine *et al*., 1998; Rao *et al*., 2007
Derrisisoflavone D [5]	Isoflavone	Stem	Sekine *et al*., 1998
Derrisisoflavone E [6]	Isoflavone	Stem	Sekine *et al*., 1998
Derrisisoflavone F [7]	Isoflavone	Stem	Sekine *et al*., 1998
Erysenegalensein E [8]	Isoflavone	Stem	Sekine *et al*., 1998
Flemichapparin B [9]	Pterocarpan	Stem	Lin *et al*., 1991
Flemichapparin C [10]	Pterocarpan	Stem	Aditachaudhury and Gupta, 1973
Genistein [11]	Isoflavone	Stem	Mahabusarakam, W., *et al*., 2004
Isochandalone [12]	Isoflavone	Stem	Tahara *et al*., 1989
Isolupalbigenin [13]	Isoflavone	Stem	Tahara *et al*., 1994
Isorobustone [14]	Isoflavone	Stem	Garcia *et al*., 1986

Table 2.2 (Continued).

Chemical substance	Category	Plant part	Reference
Isoscandinone [15]	Isoflavone	Stem	Rao *et al*., 2007
Lupalbigenin [16]	Isoflavone	Stem	Pistelli *et al*., 1996; Sekine *et al*., 1999
Lupinisol A [17]	Isoflavone	Stem	Sekine *et al*., 1998
Lupinisoflavone G [18]	Isoflavone	Stem	Sekine *et al*., 1998
Lupiwighteone [19]	Isoflavone	Stem	Hakamatsuka *et al*., 1991
Maackiain [20]	Pterocarpan	Stem	Obara and Matsubara, 1981
Osajin [21]	Isoflavone	Stem	Mizuno *et al*., 1990
Santal [22]	Isoflavone	Stem	Bohm and Choy, 1987
Scandenal [23]	Isoflavone	Stem	Mahabusarakam *et al*., 2004
Scandenin [24]	Coumarin	Stem	Johnson *et al*., 1966
Scandenin A [25]	Courmarin	Stem	Rao *et al*., 2007
Scandenin B [26]	Isoflavone	Stem	Rao *et al*., 2007
Scanderone [27]	Isoflavone	Stem	Mahabusarakam *et al*., 2004
Scandenone [28]	Isoflavone	Stem	Pelter and Stainton,1966

Table 2.2 (Continued).

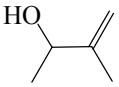
Chemical substance	Category	Plant part	Reference
Scandinone [**29**]	Isoflavone	Stem	Pelter *et al*., 1966; Rao *et al*., 1994; Mahabusarakam *et al*., 2004
Scandione [**30**]	Bibenzyl	Stem	Mahabusarakam *et al*., 2004
Ulexone A [**31**]	Isoflavone	Stem	Maximo *et al*., 2000
5-hydroxy-2″,2″ -dimethylchromeno-[6,7:5″,6″]-2‴,2‴ dimethylchromeno[3′,4′:5‴,6‴]isoflavone [**32**]	Isoflavone	Stem	Maximo *et al*., 2000
5,7,4′ trihydroxy-6,8-diprenylisoflavone [**33**]	Isoflavone	Stem	Shirataki *et al*., 1982; Sekine *et al*., 1999
3′-methylorobol [**34**]	Isoflavone	Stem	Mahabusarakam *et al*., 2004
4′-*O*-methylosajin [**35**]	Isoflavone	Stem	Tahara *et al*., 1989
4′-*O*-methylscandinone [**36**]	Coumarin	Stem	Pelter et al., 1966
4′,4-*O*-dimethylscandenin [**37**]	Isoflavone	Stem	Rao *et al*., 1994



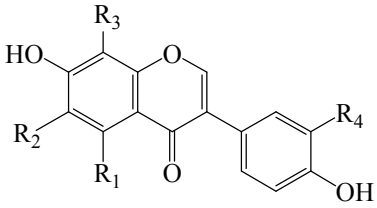
[1] Chandalone R=a

[23] Scandenal R=CHO

a



B



[2] Derrisisoflavone A R1=OMe R2=a R3=a R4=H

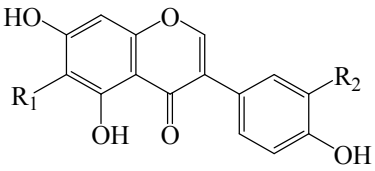
[5] Derrisisoflavone D R1=OMe R2=b R3=a R4=H

[6] Derrisisoflavone E R1=OMe R2=a R3=b R4=H

[8] Erysenegalensein E R1=OH R2=a R3=b R4=H

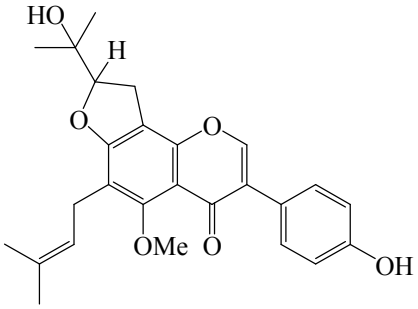
[13] Isolupalbigenin R1=OH R2=H R3=a R4=H

[16] Lupalbigenin R1=OH R2=a R3=H R4=a

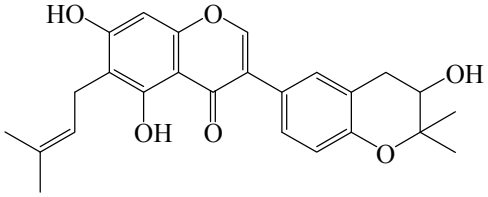


[3] Derrisisoflavone B R1=a R2=b

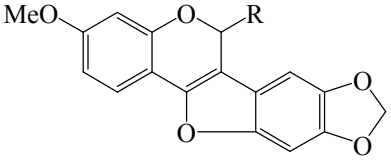
[17] Lupinisol A R1=b R2=a



[4] Derrisisoflavone C

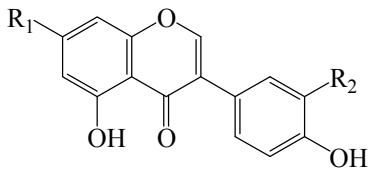


[7] Derrisisoflavone F



[9] Flemichapparin B R=H2

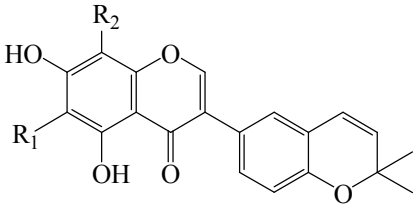
[10] Flemichapparin C R=O



[11] Genistein R1=OH R2=H

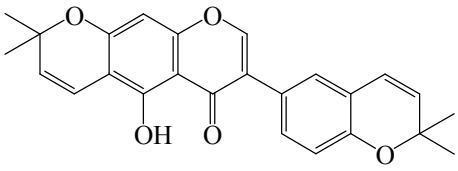
[22] Santal R1=OMe R2=OH

[34] 3′-methylorobol R1=OH R2=Ome

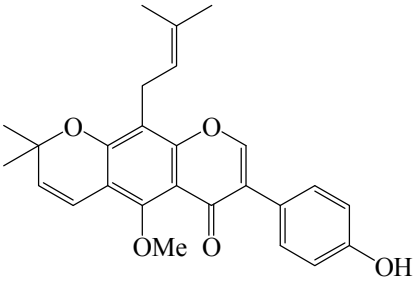


[12] Isochandalone R1=a R2=H

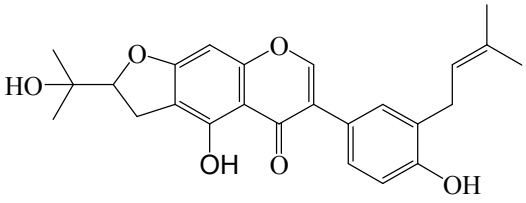
[31] Ulexone A R1=H R2=a



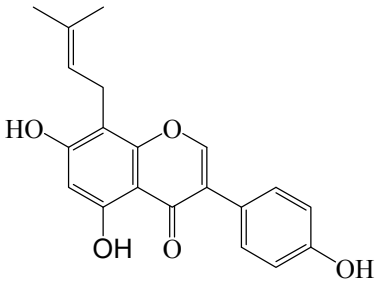
[14] Isorobustone



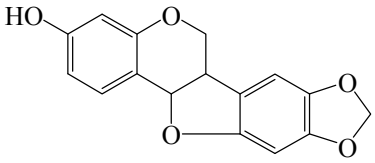
[15] Isoscandinone



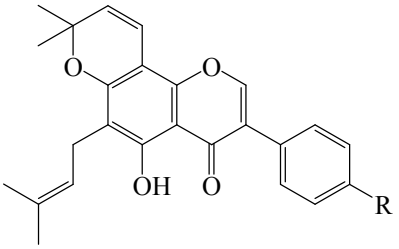
[18] Lupinisoflavone G



[19] Lupiwighteone

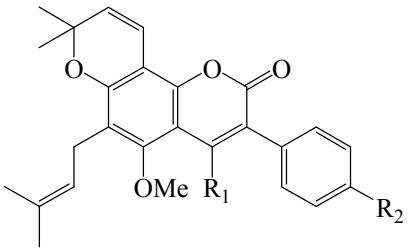


[20] Maackiain



[21] Osajin R=OH

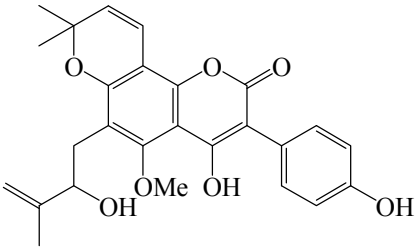
[35] 4′-*O*-methylosajin R=OMe



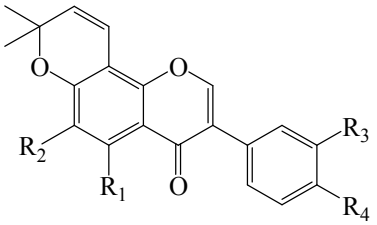
[24] Scandenin R1=OH R2=OH

[25] Scandenin A R1=OH R2=OMe

[37] 4′,4-*O*-dimethylscandenin R1=OMe R2=OMe



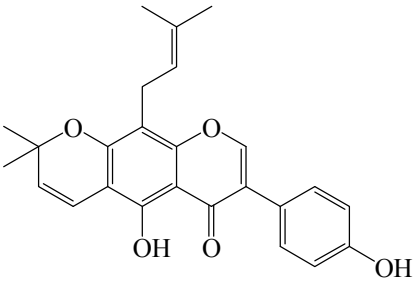
[26] Scandenin B



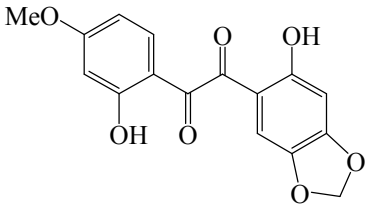
[27] Scanderone R1=OH R2=H R3=a R4=OH

[29] Scandinone R1=OMe R2=a R3=H R4=OH

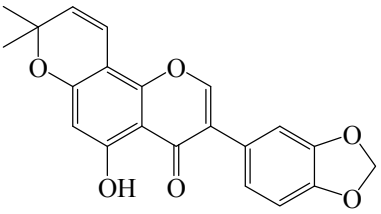
[36] 4′-*O*-methylscandinone R1=OMe R2=a R3=H R4=OMe



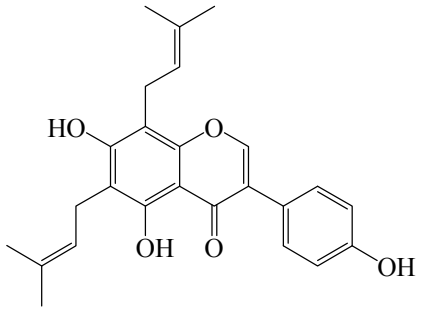
[28] Scandenone



[30] Scandione



[32] 5-hydroxy-2″,2″-dimethylchromeno- [6,7:5″,6″]-2‴,2‴ dimethylchromeno[3′,4′:5‴,6‴]isoflavone



[**33**] 5,7,4′ trihydroxy-6,8 diprenylisoflavone

CHAPTER III

YEAST SCREENING FOR TOPOISOMERASE II POISON ACTIVITY

Top2 is the target of important anticancer drugs, for example, etoposide, anthracyclines (doxorubicin, daunorubicin), and mitoxantrone (Elsea *et al.*, 1992). Many plants in Thailand have a long history of use as alternative treatments for cancer, and Thai folk doctors have extensively documented these uses. However, many of these traditional treatment claims should be investigated because cancer, as a specific disease entity, can be poorly defined by the terms of traditional medicine (Itharat and Ooraikul, 2007). Moreover, the mechanism of these plants for cancer treatment has never been revealed. These traditional drugs led us to pursue research into the identification of the natural source of Top2 poison activity in Thai medicinal plants.

In this chapter, we report Top2 poison activity of selected Thai medicinal plants using a yeast cell-based assay. The results correlated with their anticancer use in Thai traditional medicine and agreed with previous studies.

3.1 Materials and methods

3.1.1 Ethnobotanical search and sample collection

In this study, plant lists were based on the Thai ethnobotanical information collected from different literature sources. Plants were chosen according to their use as an alternative treatment of cancer by Thai folk practitioners, including: analgesic, antimicrobial, antipyretic, cytotoxic properties, a decrease in toxicity and inflammation. Furthermore, the following criteria were applied for plant selection: (1) plants used against any of the symptoms above; (2) plant mentioned in the ethnobotanical literature with full scientific name; (3) native plants of Thailand and (4) plants with cytotoxic activity.

Plant samples (**Table 3.1**) were collected from various locations or purchased from local crude drug markets in Thailand. They were identified by Associate Professor Thatree Phadungcharoen and Associate Professor Dr. Chaiyo Chaichantipyuth at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Voucher specimens were deposited at the Herbarium of Natural Medicine, Chulalongkorn University.

3.1.2 Extraction and preparation methods

The plant materials were cut into small pieces and then dried in a hot air oven at 55°C. The dried plant material was ground into a powder using an electric grinder and stored at -20°C prior to extraction. The extraction was performed by macerating the powdered plant material (100 g) with methanol (500 ml) for 3 days and then filtering the remaining solution. The filtrate was evaporated under reduced pressure by a rotary evaporator until dryness. The crude powder extracts were weighed and stored at -20°C until use.

3.1.3 Preparation of stock solution

Sample of 10 mg of each extract was solubilized in dimethylsulfoxide (DMSO) prior to adding the appropriate culture medium and stocked at a concentration of 10 mg/ml.

Table 3.1 Plant species used for screening of topoisomerase II poison activity.

No.	Plant species	Thai name	Family	Voucher number	Part used	Remedies Used
1.	*Ageratum conyzoides* L.	Sap raeng sap ka	Compositae	SS-SPT001	Leaf	Anticancer (Adebayo *et al*.,2010)
2.	*Artocarpus gomezianus* Wall. Ex Trecul	Hat nun	Moraceae	SS-SPT002	Bark	Cytotoxicity (Bapat *et al*.,2010)
3.	*Bridelia ovata* Decne.	Maka	Euphorbiaceae	SS-SPT003	Leaf	Anticancer (Itharat *et al*., 2007; Saetung *et al*., 2005)
4.	*Curcuma longa* Linn.	Khamin chan	Zingiberaceae	SS-SPT004	Rhizome	Anti-cancer (Mahavorasirikul *et al*.,2010)
5.	*Curcuma zedoaria* (Berg) Roscoe	Khamin ooi	Zingiberaceae	SS-SPT005	Rhizome	Anticancer (Itharat *et al*., 2007; Saetung *et al*., 2005)
6.	*Derris scandens* (Roxb.) Benth.	Thao wan priang	Leguminosae	SS-SPT006	Stem	Anticancer (Saetung *et al*., 2005)
7.	*Dioscorea membranacea* Pierre	Khruea tao hai	Dioscoreaceae	SS-SPT007	Rhizome	Anticancer (Itharat *et al*., 2007; Saetung *et al*., 2005)
8.	*Drynaria quercifolia* (L.) Sm.	Kratae tai mai	Polypodiaceae	SS-SPT008	Stem	Anticancer (Saetung *et al*., 2005)
9.	*Garcinia hanburyi* Hook.f.	Rong thong	Guttiferae	SS-SPT009	Resin	Antibacterial, Anti-cancer (Sukpondma *et al*., 2005; Lu *et al*., 2007)
10.	*Grangea maderaspatana* (L.) Poir.	Phaya mutti	Compositae	SS-SPT010	Whole plant	Cytotoxic activity (Ruangrungsi, 1989)

Table 3.1 (Continued).

No.	Plant species	Thai name	Family	Voucher number	Part used	Remedies Used
11.	*Harrisonia perforata* (Blanco) Merr.	Khontha	Simarubaceae	SS-SPT011	Branch	Cytotoxic activity (Itharat *et al.*, 2010)
12.	*Hydnophytum formicarum* Jack	Hua roi ru	Rubiaceae	SS-SPT012	Stem	Inflammation, cancer (Itharat *et al.*, 1999, 2002)
13.	*Micromelum minutum* (G.Forst.) Wight&Arn.	Hatsa khun	Rutaceae	SS-SPT013	Stem	Antitumor (Itharat *et al.*, 1999)
14.	*Moringa oleifera* Lam.	Marum	Moringaceae	SS-SPT014	Bark	Anticancer (Guevara *et al.*, 1999)
15.	*Phyllanthus amarus* Schumach. & Thonn.	Luk tai bai	Euphorbiaceae	SS-SPT015	Whole plant	Anticancer (Rajeshkumar *et al.*, 2002)
16.	*Phyllanthus urinaria* L.	Ya tai bai	Euphorbiaceae	SS-SPT016	Whole plant	Anticancer (Huang *et al.*, 2006)
17.	*Rauvolfia serpentina* (L.) Benth. ex Kurz	Ra yom	Apocynaceae	SS-SPT017	Root	Anticancer (Dassonneville *et al.*, 1999)
18.	*Salacia chinensis* L.	Khampang jed chan	Celastraceae	SS-SPT018	Stem	Anticancer (Itharat *et al.*, 2007)
19.	*Sapindus rarak* DC.	Ma kham di khwai	Sapindaceae	SS-SPT019	Fruit	Antimicrobial (Wuthi-udomlert *et al.*, 2002)
20.	*Siphonodon celastrineus* Griff.	Ma duk	Celastraceae	SS-SPT020	Leaf	Anticancer (Itharat *et al.*, 2007)

Table 3.1 (Continued).

No.	Plant species	Thai name	Family	Voucher number	Part used	Remedies Used
21.	*Smilax corbularia* Kunth	Khao yen nuea	Smilacaceae	SS-SPT021	Stem	Antitumor (Pornsiriprasert *et al*.,1986)
22.	*Smilax glabra* Roxb.	Khao yen tai	Smilacaceae	SS-SPT022	Stem	Anticancer (Itharat *et al*., 2007)
23.	*Stephania erecta* Criab	Bua bok	Menispermaceae	SS-SPT023	Caudex	Anticancer, Antiplasmodial (Angerhofer, 1999)
24.	*Stephania pierrei* Diels.	Sabu lueat	Menispermaceae	SS-SPT024	Caudex	Anticancer, Antiplasmodial (Angerhofer, 1999)
25.	*Stephania suberosa* L. L. Forman	Bora phet phung chang	Menispermaceae	SS-SPT025	Caudex	Anticancer (Makarasen, 2004)
26.	*Suregada multiflorum* (A.Juss.) Baill.	Khunthong Payabat	Euphorbiaceae	SS-SPT026	Bark	Inflammation, cancer, (Vimunkunakorn, 1979; Itharat *et al*., 1999, 2002; Chayamarit, 1995)
27.	*Terminalia chebula* Retz. var. chebula	Samo thai	Combretaceae	SS-SPT027	Fruit	Anticancer (Subchareon, 1998; Saleem *et al*., 2002)
28.	*Tinospora baenzigeri* Forman	Ching cha chali	Menispermaceae	SS-SPT028	Stem	Antipyretic (Salguero, 2003)
29.	*Willughbeia cochinchinensis* Pierre	Kui	Apocynaceae	SS-SPT029	Stem	Inflammation (Itharat *et al*., 1999)
30.	*Ziziphus attopoensis* Pierre	Kam lang suea khrong	Rhamnaceae	SS-SPT030	Branches	Anti-proliferation (Numchaisermsuk and Cherdshewasart, 2008)

3.1.4 Yeast strain

The *Saccharomyces cerevisiae* strains used in this study were kindly provided by Dr. John Nitiss (St. Jude Children's Research Hospital, USA). The parental yeast strain was *S. cerevisiae* JN394 *ise2 ura3-52 leu2 trp1 ade2 his7 rad52: : LEU2* (Nitiss and Wang, 1988). Two mutant strains derived from JN394 were also employed: JN394 Δ*top1*, which has a chromosomal deletion of the topoisomerase I (*TOP1*) gene (Nitiss and Wang, 1988), and JN394*top2-1*, in which the wild-type *TOP2* gene is replaced with the temperature-sensitive *top2-1* mutant allele (Elsea *et al*., 1992). These two strains are isogenic to JN394 in all other respects (Meng *et al*., 2001).

3.1.5 Media for yeast cell culture

YPDA agar and YPDA broth were used for maintaining and propagating yeasts in molecular microbiology procedures. The compositions of this culture media are described in **Appendix A**.

3.1.6 Storage of yeast cultures

Yeast cultures were stored at -80°C in growth media containing 15% (v/v) glycerol. Yeast cells were recovered from storage by transferring a small frozen sample to YPDA agar plate.

For storage of the mutant yeast strain, they were grown overnight at 25°C and 30°C in YPDA broth media. The 0.5 ml of cultures was mixed with 0.5 ml of sterile 30% (v/v) glycerol in 2 ml vial and transferred to -80°C.

3.2 Yeast plate count

Yeast is quantitated by pour plate technique (modified from Fankhauser, 2005). The mutant yeasts were grown 24 hr at 25°C and 30°C (25°C for temperature-sensitive *top2* mutants, 30°C otherwise) in a shaking incubator set at 200 rpm in liquid YPDA media. The cultures were adjusted to an OD_{590} of 0.5 and serially diluted 10-fold. A 100 µl of each dilution was mixed with 20 ml of the melted YPDA agar (triplicate) before pouring plates. Culture plates were incubated at 25°C and 30°C for 48 hr. The yeast survival was counted in plates with no more than 300 colony forming units (CFU) and preferably not less than 30 CFUs. The counts of the triplicate plates were averaged. The number of yeast cells in suspension at OD_{590} of 0.5 was calculated by average number of colony forming unit multiply with dilution factor.

3.3 Yeast cell-based assay for topoisomerase II inhibitory activity

For the yeast sensitivity studies, the yeast cells were grown overnight in complete medium (YPDA) containing yeast extract, bacto-peptone, dextrose and adenine sulfate. The cultures were adjusted to an OD_{590} of 0.5, and serially diluted 10-fold, and 5 µl aliquots were spotted onto the YPDA plates. For the primary screening of each plate, the crude extracts were serially diluted to concentrations of 500 µg/ml, 250 µg/ml and 125 µg/ml. For the subsequent screening of putative hits, these crude extracts were serially diluted to concentrations of 200 µg/ml, 100 µg/ml and 50 µg/ml. Etoposide was used as a positive control, and cells incubated with DMSO alone were used as a vehicle control. The final concentration of DMSO never exceeded 2% (v/v) of the culture medium, and this has been shown to be a concentration that has no effect on the growth of the cells (Nitiss and Nitiss, 2001). The plates were incubated for 3 to 4 days at the optimal temperature for cell growth (25°C for temperature-sensitive *TOP2* mutants, 30°C otherwise), and the surviving colonies were observed. The drug sensitivity was determined by comparing the survival of colonies in the crude extract culture with that of those in the drug-treated culture, and the colonies were also photographed. The expected patterns of sensitivity of Top2 poison to mutant yeast strains are shown in **Table 3.2**.

Table 3.2 The expected patterns of sensitivity of Top2 poison to mutant yeast strains.

Test samples	Yeast strains				Interpretation
	JN394 (WT)	JN394 △*top1*	JN394*top2-1*		
	25°C,30°C	25°C,30°C	25°C	30°C	
DMSO (vehicle control)	+ 	+ 	+ 	+ 	Not Top2 poison
Etoposide (positive control)	− 	− 	− 	+ 	Top2 poison
Crude extract no.1	+ 	+ 	+ 	+ 	Not Top2 poison
Crude extract no.2	− 	− 	− 	+ 	Top2 poison
Crude extract no.3	− 	− 	− 	− 	Too high concentration or due to other mechanisms

(+) = growth

(−) = not growth

3.4 Results

3.4.1 Counting of the yeast cell

Yeast cell suspension was counted on YPDA plates. The number of yeast colony forming unit per plate are shown in **Tables A1, A2 and A3** (**Appendix A**). The OD_{590} of 0.5 of yeast suspension were 10^7 CFU/ml.

3.4.2 Topoisomerase II inhibitory activity of Thai medicinal plants

Thirty methanol extracts were screened using mutant yeast system for Top2 poison activity. By comparing the survival colony after exposure to the plant extracts at 25°C and 30°C on the JN394*top2-1* strains, which carry the *top2-1* allele encoding a temperature-sensitive topoisomerase, it was possible to address the mechanism of action of the compounds. To test whether the Top2-DNA cleavable complexes that are induced by the Top2 poisons exhibit a similar property in regards to elevated temperatures, the anti-Top2 agent etoposide was selected for use. The JN394*top2-1* strains were sensitive to etoposide at 25°C, and the level of sensitivity was similar but somewhat greater than that of the wild-type strains. Cell killing was also observed at 30°C, but the cell killing was less than that observed at 25°C. To exclude the possibility that alterations in drug sensitivity were caused by the vehicle, we also tested the sensitivity of the JN394*top2-1* strains to DMSO. At both 25°C and 30°C, the JN394*top2-1* strains were not sensitive to DMSO at any concentration tested (**Figure 3.1 A**).

By this assay, the extracts of 6 out of 30 ethnomedicinal plants, *Curcuma longa*, *C. zedoaria*, *Derris scandens*, *Grangea maderaspatana*, *Stephania pierrei* and *S. suberosa*, were found to have Top2 poison activity against the mutant yeast cells (**Fiure 3.1 B**). The survival colony of mutant yeasts on the agar plates contain individual of other crude extracts are shown (**Figures 3.2-3.3**).

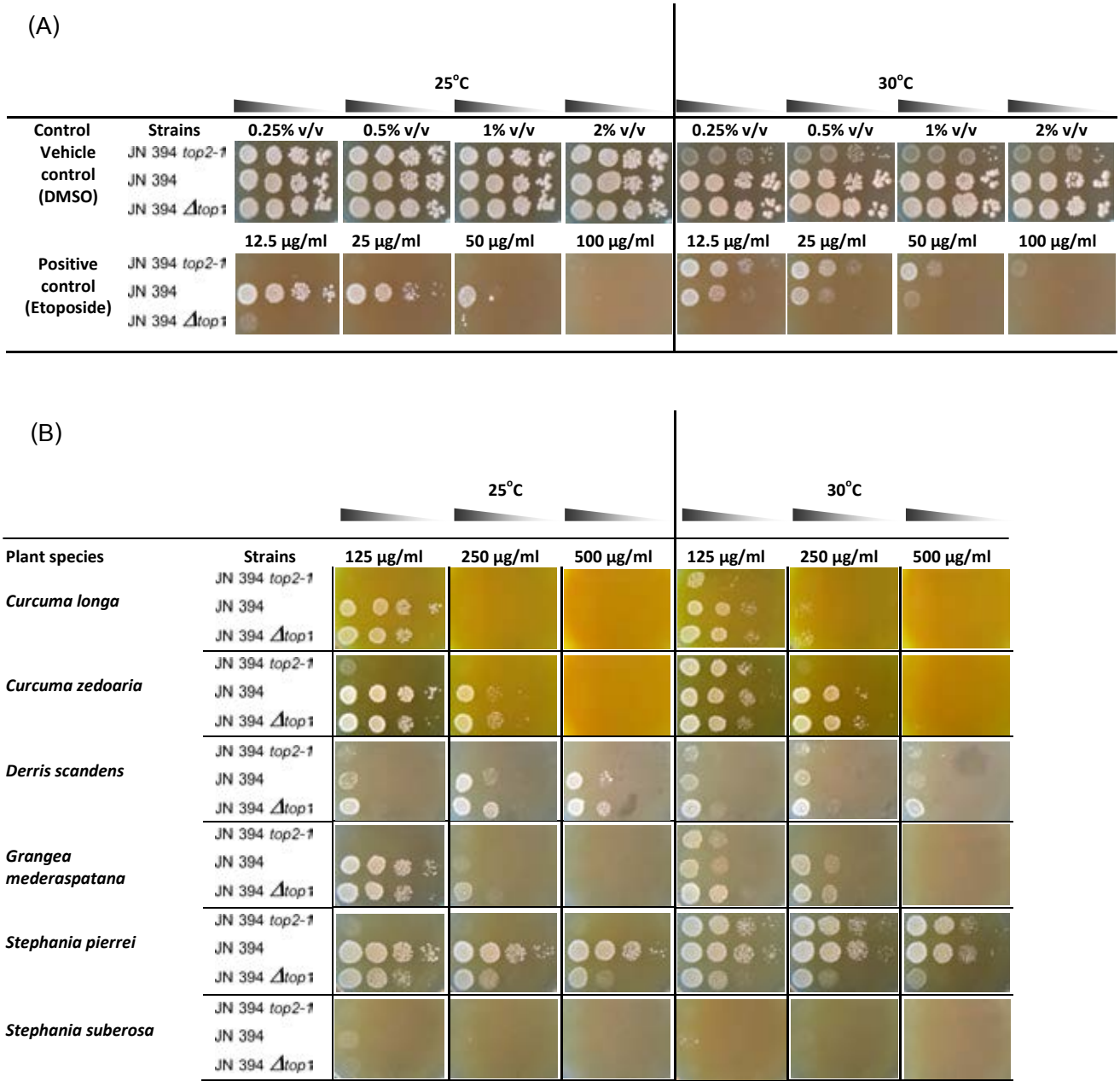


Figure 3.1 The *in vitro* Top2 poison screen of the medicinal plant extracts by a yeast cell-based assay. **(A)** Five microliters of a 10-fold serial dilution (indicated by triangles) of JN394, JN394*top2-1* and JN394 Δ*top1* was spotted onto YPDA plates containing various concentrations of DMSO or etoposide. **(B)** Five microliters of a 10-fold serial dilution of the listed strains was spotted onto YPDA plates containing various concentrations of the crude test extracts. The plates were incubated separately at 25°C (left) or 30°C (right) for 3-4 days, and the cell growth was observed and photographed.

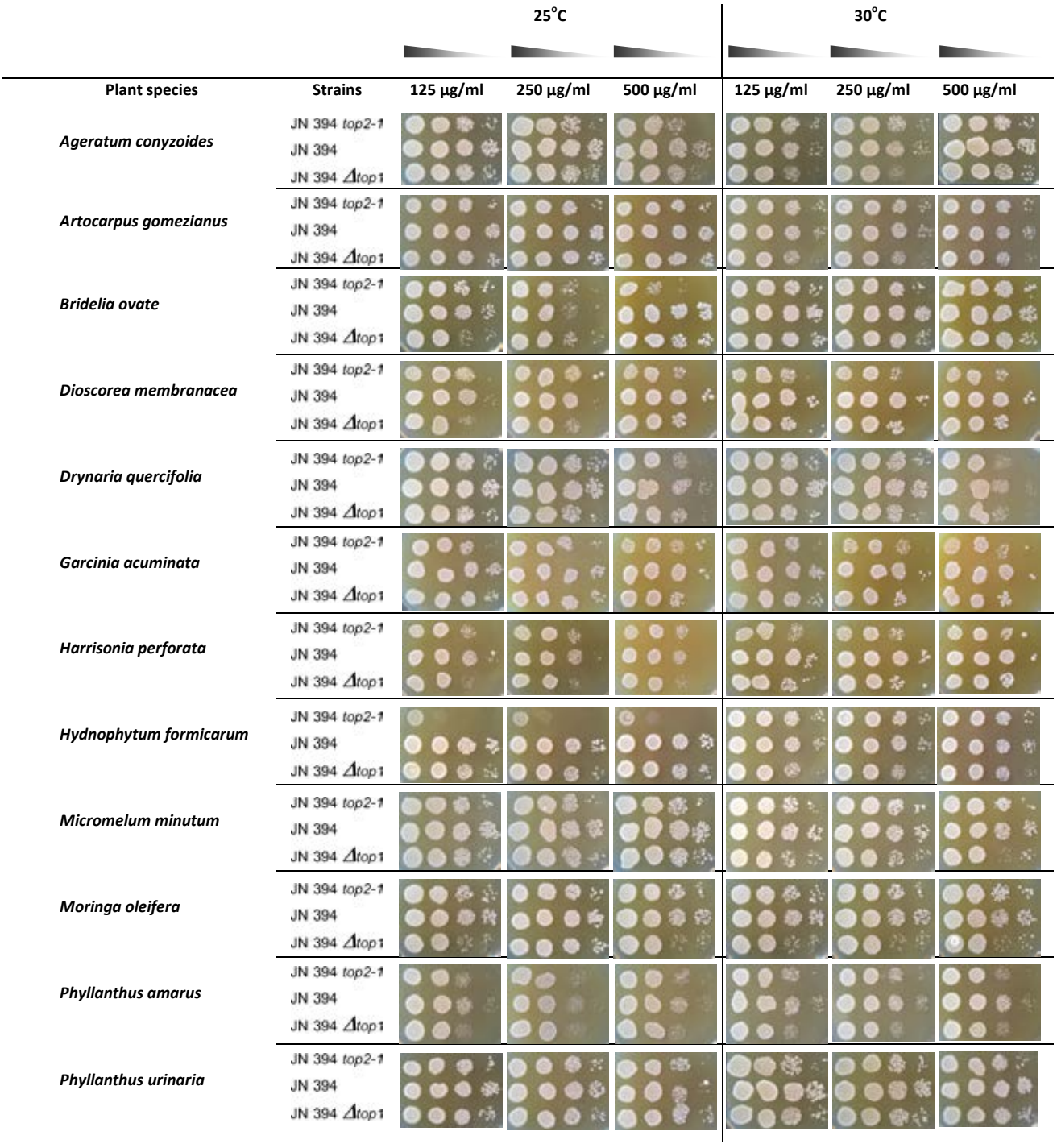


Figure 3.2 The *in vitro* Top2 poison screen of the medicinal plant extracts by a yeast cell-based assay. Five microliters of a 10-fold serial dilution of the listed strains was spotted onto YPDA plates containing various concentrations of the crude test extracts. The plates were incubated separately at 25°C (left) or 30°C (right) for 3-4 days, and the cell growth was observed and photographed.

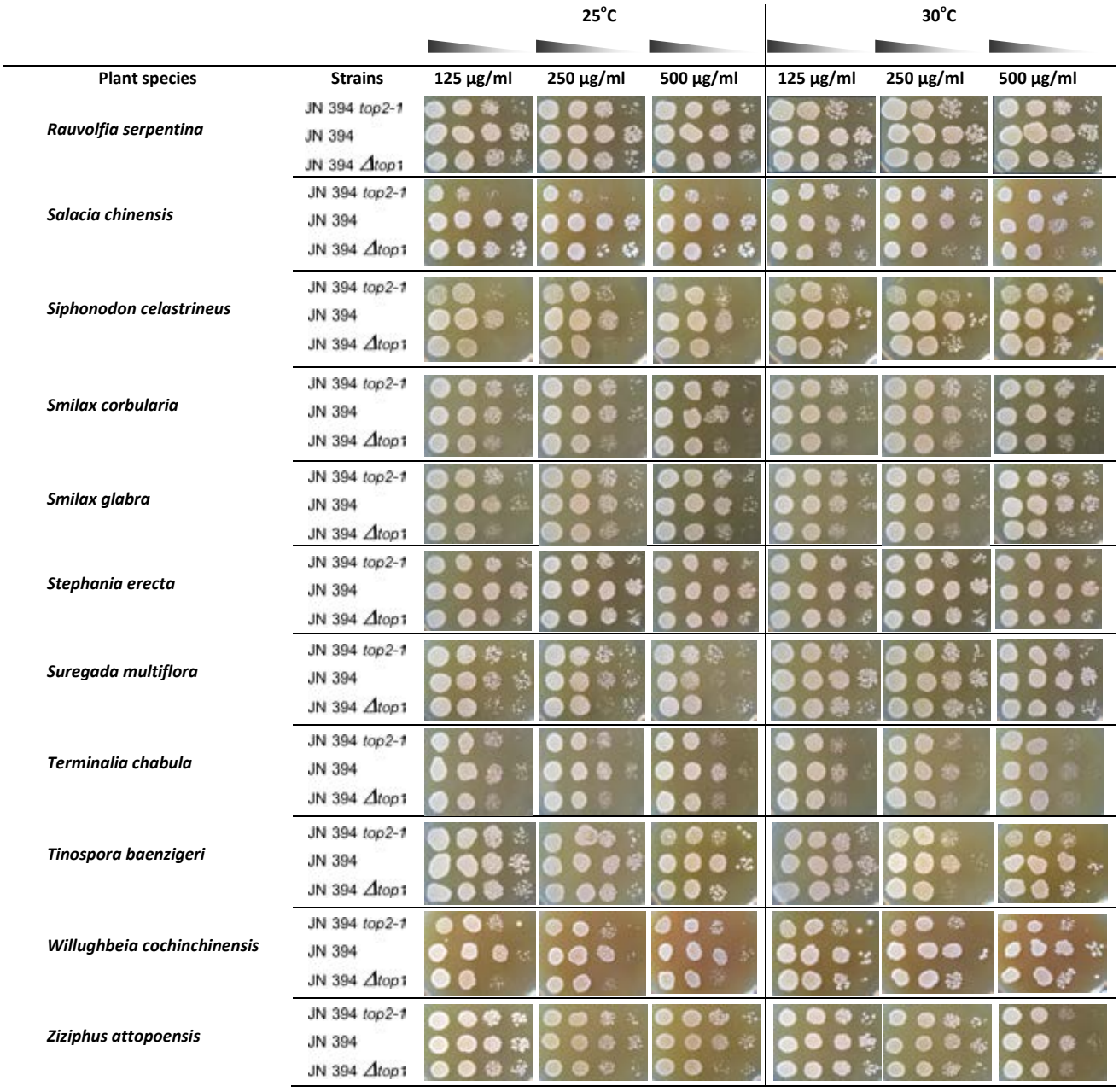


Figure 3.3 The *in vitro* Top2 poison screen of the medicinal plant extracts by a yeast cell-based assay. Five microliters of a 10-fold serial dilution of the listed strains was spotted onto YPDA plates containing various concentrations of the crude test extracts. The plates were incubated separately at 25°C (left) or 30°C (right) for 3-4 days, and the cell growth was observed and photographed.

3.5 Discussion

In this study, thirty plants were chosen based on their ethnomedicinal use by Thai folk doctors (Itharat and Ooraikul, 2007). Our criteria excluded plants without documented traditional use or a previous demonstration of cytotoxic activity.

To screen the Top2 poison activity of the plant extracts, a yeast cell-based assay was used. A yeast model system for the *in vitro* analysis of the interaction between Top2 and various anticancer inhibitors was previously developed by Nitiss and colleagues (Nitiss *et al*., 1993). This system has been used extensively to analyse the effects of specific anticancer agents on yeast Top2 (Elsea *et al*., 1992; Nitiss *et al*., 1993; Froelich-Ammon *et al*., 1995; Meng *et al*., 2001). However, this was the first time that the system was utilised as an *in vitro* method to screen a large number of plant extracts for Top2 poison activity.

In this system, increased levels of Top2 lead to increased DNA damage and therefore a greater sensitivity to cell death, while a decrease in the Top2 level or activity leads to drug resistance as a result of the reduced levels of DNA damage (Nitiss and Nitiss, 2001). JN394*top2-1* strains, which carry the *top2-1* allele encoding a temperature-sensitive topoisomerase, were constructed, and these strains contain the *ISE2* and *rad52* mutations described earlier but express the temperature-sensitive *top2-1* mutation instead of the wild-type *TOP2* gene. It has been demonstrated that yeast strains carrying *top2-1* have approximately a wild-type level of activity at 25°C but substantially reduced activity at 30°C. Consequently, the *top2-1* yeast strains proliferate like wild-type (JN 394) cells at the permissive temperature (25°C), are non-viable at 37°C and have sufficient Top 2 activity to be viable at the semi-permissive temperature (30°C) (Nitiss *et al*., 1993). Therefore, by comparing the cytotoxicity of the plant extracts on the JN394*top2-1* yeast cells at 25°C and 30°C, it was possible to address the mechanism of action of the extracts. If the extracts from the medicinal plants were to kill the cells by acting as a poison, then the sensitivity of the yeast to such extracts should be decreased in cells with lower enzyme activity at 30°C. This system enabled us to dissect

the mechanism of action behind the Top2 poison activity and also demonstrated that the conversion of Top2 into a cellular poison plays a critical role in cell killing (Nitiss, 1994).

The JN394 Δ*top1* strains were also tested to prove that the death of the yeast cells was not related to the activity of Top1. When Top1 was deleted, only expression of Top2 could be demonstrated. Yeast strains that lack Top1 will be as sensitive to the Top2 poison agents as the wild-type strains (Nitiss and Wang, 1988). To test whether the Top2-DNA cleavable complexes that are induced by the Top2 poisons exhibit a similar property in regards to elevated temperatures, the anti-Top2 agent etoposide was selected for use. The JN394*top2-1* strains were sensitive to etoposide at 25°C, and the level of sensitivity was similar but somewhat greater than that of the wild-type strains. At 30°C, the JN394*top2-1* cells were able to grow in the presence of 50 µg/ml etoposide. At a higher drug concentration (100 µg/ml etoposide), cell killing was also observed at 30°C, but the cell killing was less than that observed at 25°C. To exclude the possibility that alterations in drug sensitivity were caused by the vehicle, we also tested the sensitivity of the JN394*top2-1* strains to DMSO. At both 25°C and 30°C, the JN394*top2-1* strains were not sensitive to DMSO at any concentration tested (**Figure 3.1 A**). These results demonstrated that the drug resistance conferred by *top2-1* was recessive to that of wild-type Top2, that the temperature-sensitive Top2 was necessary for drug resistance at 30°C and that the vehicle did not affect the drug sensitivity of the yeast strains.

The addition of aliquots of serially diluted culture medium onto agar plates provided a rapid and semi-quantitative measure of drug-induced cytotoxicity. Using this approach, one- to four-log decreases in cell viability have been shown to be a consequence of the Top2-targeting agents (Woo *et al*., 2001). Therefore, this is a valuable model to use for the establishment of eukaryotic Top2 DNA as the cellular target of specific anticancer agents. There are several advantages regarding the use of yeast in the study anti-topoisomerase drugs; yeast genes are well-defined, and the ability to carry out targeted gene disruption and gene replacement in yeast makes it possible to assess changes in drug sensitivity (Nitiss, 1994). Previous studies have

reported the use of yeast for studying drugs that act on DNA topoisomerase (Eng *et al*., 1988; Nitiss and Wang, 1988; Sirikantaramas *et al*., 2008). These studies demonstrated that camptothecin, a Top1 inhibitor, was specific for Top1 by showing that yeast *top1* mutants were completely resistant to the cytotoxic action of the drug. These experiments also demonstrated that the conversion of the enzyme into a cellular poison may have been responsible for the cellular killing activity of camptothecin, as this drug can kill yeast cells even though Top1 is not essential for yeast viability (Sirikantaramas *et al*., 2008). These results support the use of a yeast system to screen drugs that specifically target topoisomerase.

The recorded anticancer medicinal uses of the studied plant species are shown in **Table 3.1**. Thirty methanol extracts were screened and the results revealed that six out of the thirty extracts showed Top2 poison activity (**Figure 3.1 B**). At 30°C, the JN394*top2-1* cells were not able to grow in the presence of 500 µg/ml or 250 µg/ml of crude extract. When the extract concentrations were decreased to 200 µg/ml, 100 µg/ml and 50 µg/ml, cell death was observed at 30°C but was observed at a lesser extent than it was at 25°C. As the plant extracts were not randomly selected, a high hit rate of 20% for the plant species with specific Top2 poison was observed. This specific activity was seen for the methanol extracts of the stem of *D. scandens*, the whole plant of *G. maderaspatana*, the caudex of *S. pierrei*, the caudex of *S. suberosa*, the rhizome of *C. longa* and the rhizome of *C. zedoaria*. The effect of other extracts without Top2 poison activity are shown in **Figures 3.2-3.3**. As far as we know, this is the first time that the Top2 poison activities of *D. scandens and G. maderaspatana* have been reported.

The Top2 poison screen results of the plant extracts from *S. suberosa*, *S. pierrei*, *C. longa* and *C. zedoaria* correlated with their anticancer use in Thai traditional medicine and agreed with previous studies. The results were also in agreement with the previously published work that validated this yeast screening system. The results of this study revealed that the methanol extract from *S. suberosa* had potent Top2 poison activity in mutant yeast models. In a previous study, the aporphine alkaloid compounds that were isolated from this plant exhibited anti-proliferative activity against a human

cancer cell line by targeting DNA topoisomerase II (Woo *et al*., 1999). In this study, the crude extracts from the rhizomes of *C. longa* and *C. zedoaria* exhibited Top2 poison activity, and an extensive body of literature is available on the bioactivity of *C. longa* and *C. zedoaria*. Several previous studies have shown that curcumin, the active component of the *Curcuma* species, displayed Top2 poison activity (Cordero *et al*., 2003; Chattopadhyay *et al*., 2004). A previous study also found that the fractionation of the ethyl acetate extract from the rhizomes of *C. longa* rhizomes exhibited topoisomerase I and II inhibition activity (Roth *et al*., 1998). In addition, the ethanol extract of *C. zedoaria* was isolated and demonstrated to be cytotoxic against human ovarian cancer OVCAR-3 cells (Syu *et al*., 1998). The caudex extract of *S. pierrei* also demonstrated slight Top2 poison activity in our screen. In a previous study, *S. pierrei* extract was shown to be cytotoxic for a variety of mammalian cancer cell lines (Stévigny *et al*., 2005). Interestingly, the yeast cells in our study were not able to grow at either 30°C or 25°C in any concentration of the methanol extract of *S. rarak* (**Figure 3.1 B**). Based on this result, the cell death caused by the *S. rarak* extract may not be due to Top2 poison activity. Due to the demonstrated Top2 poison activity of *D. scandens* and *G. maderaspatana,* as well as their corresponding uses in Thai folk medicine, further phytochemical studies are needed for the isolation and identification of the natural Top2 poison agents found in these two plants.

3.6 Conclusions

Our study demonstrated that the mutant yeast system was an effective and useful model for identifying the cell killing activity of Top2 poisons. This yeast model is a powerful tool because it is simple, highly specific, easy to implement and cost-effective. Thirty ethnomedicinal plants were selected based on their documented use as alternative treatments for cancer provided by Thai folk practitioners. The extracts of six out of thirty ethnomedicinal plants, *C. longa*, *C. zedoaria*, *D. scandens*, *G. maderaspatana*, *S. pierrei* and *S. suberosa*, were found to have Top2 poison activity against the yeast cells. The use of drug screens can validate the traditional use of medicinal plant species and can bolster the cultural and medicinal significance of these taxa.

CHAPTER IV

BIOASSAY-GUIDED ISOLATION OF TOPOISOMERASE II INHIBITING COMPOUNDS FROM *DERRIS SCANDENS* (Roxb.) Benth

In the present study, six of thirty methanol plant extracts showed potent Top2 inhibitory activity: rhizome of *Curcuma longa*, rhizome of *Curcuma zedoaria,* stem of *Derris scandens,* whole plant of *Grangea maderaspatana*, *caudex of Stephania pierrei* and caudex of *Stephania suberosa*. Interestingly, prior to this study the extract of *D. scandens* has never been reported for their Top2 poison activity. Therefore, *D. scandens* was then selected for further study through bioassay-guided fractionation, using several chromatographic techniques. In this chapter, we report the isolation of Top2-targeted compounds from *D. scandens*. The results from this study may give scientific evidence to support the traditional use of this plant as an anticancer remedy.

4.1 Materials and Methods

4.1.1 Plant meterials

D. scandens samples were purchased from a local crude drug market, Bangkok, in November 2010. They were identified by Associate Professor Thatree Phadungcharoen at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Authentication was performed by comparison with a herbarium specimen (BKF No. 126882) at the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment. Voucher specimens (SS-SPT006) have been deposited at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

4.2 General techniques

4.2.1 Analytical thin layer chromatography (TLC)

Technique : One dimension, ascending

Absorbent : Silica gel 60 F254 (E.Merck) precoated plate

Layer thickness : 0.2 mm

Distance : 6.5 cm

Temperature : Laboratory temperature (30-35^{o}C)

Detection : 1. Ultraviolet light at wavelengths of 254 and 365 nm

2. Anisaldehyde and heating at 105°C for 10 min.

4.2.2 Column chromatography

4.2.2.1 Vacuum liquid column chromatography (VLC)

Absorbent : Silica gel 60 (No.7734) particle size 0.063-0.200 nm (70-230 mesh ASTM) (E. Merck)

Packing method : Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvents, mixed with a small quantity of adsorbent, triturated, dried and then placed gently on top of the column.

Detection : 1. Fractions were examined by TLC observing under UV light at the wavelength of 254 and 365 nm.

2. Fractions were examined by TLC observing after spray with anisaldehyde and heating at 105°C for 10 min.

4.2.2.2 Flash column chromatography

Absorbent : Silica gel 60 (No.9385) particle size 0.040-0.063 nm (70-230 mesh ASTM) (E. Merck)

Packing method : Wet packing

Sample loading : The sample was dissolved in a small amount eluent and then applied gently on top of the column.

Detection : Fractions were examined in the same way as described in section **4.2.2.1.**

4.2.3 Spectroscopic techniques

4.2.3.1 Mass spectra (MS)

Mass spectra were recorded on a Micromass LCT mass spectrometer (ESI-TOF-MS). (National Center for Genetic Engineering and Biotechnology).

4.2.3.2 Proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectra

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained on a Bruker Avance DPX-300 FT-NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

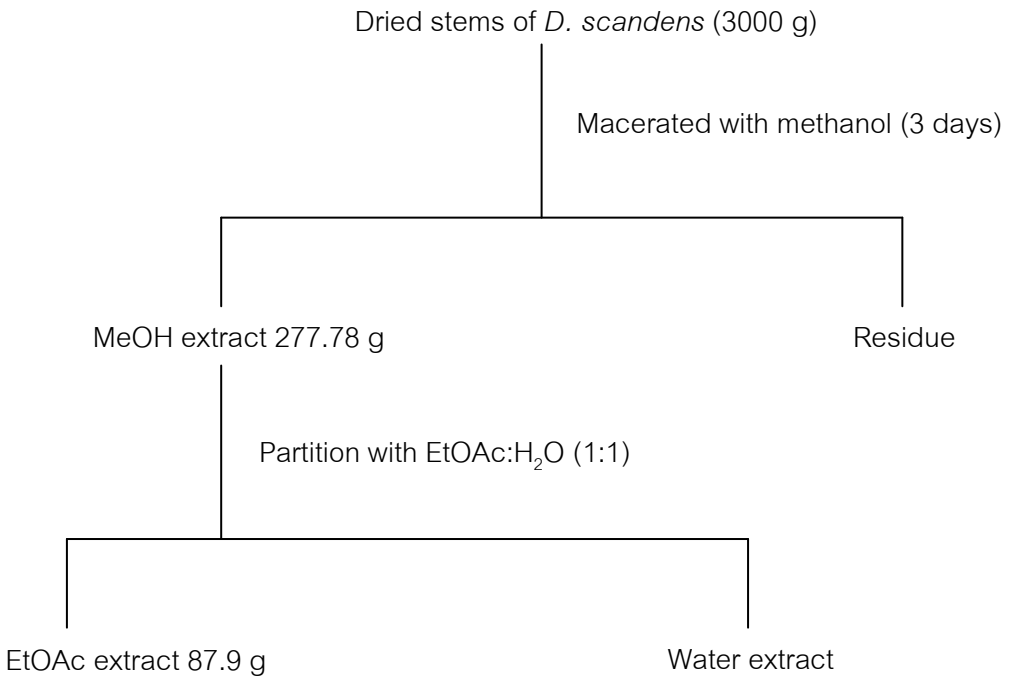
Solvents for NMR spectra were deuterated chloroform (chloroform-*d*) and deuterated acetone (acetone-d_6). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

4.2.3.3 Solvents

All organic solvents employed throughout this work were of commercial grade and were redistilled prior to use.

4.3 Extraction and isolation

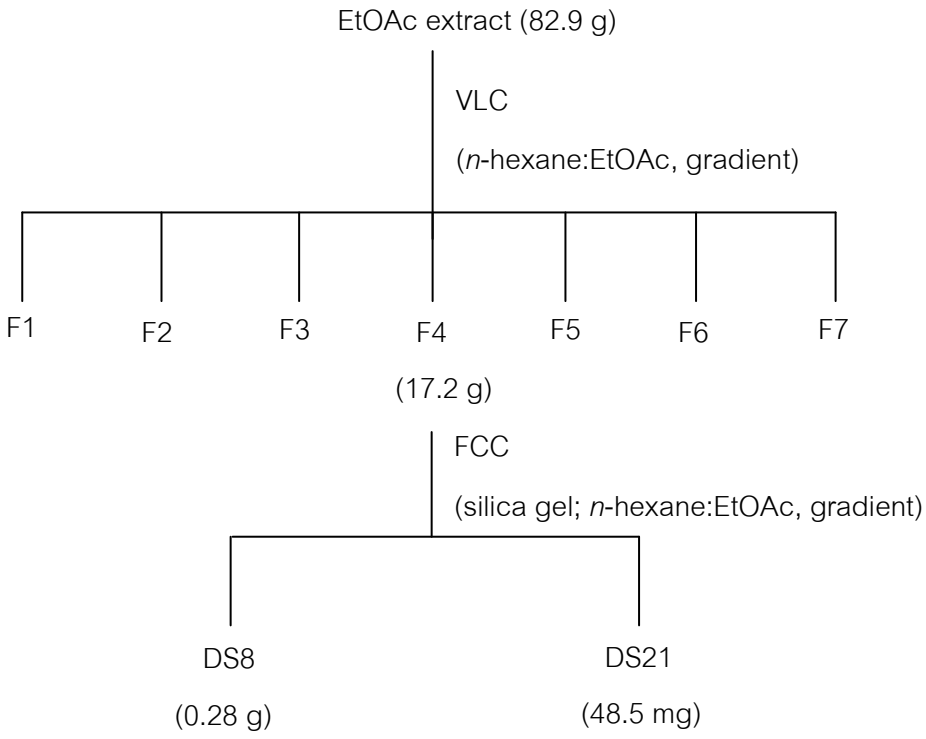
Plants material was cut into small pieces and dried in a hot air oven at 55°C. Dried plant materials were ground into powder with an electric grinder and stored at -20°C prior to extraction. Extraction was carried out by macerating the powdered plant material (3000 g) with methanol (12 L) for 3 days and filtered. The filtrate was evaporated under reduced pressure by rotary evaporator until dryness. The methanol extract (277.78 g) was partition with ethyl acetate (EtOAc) and water (4x300 ml), to give an EtOAc extract (87.9 g) and water extract, respectively. (**Scheme 4.1**)



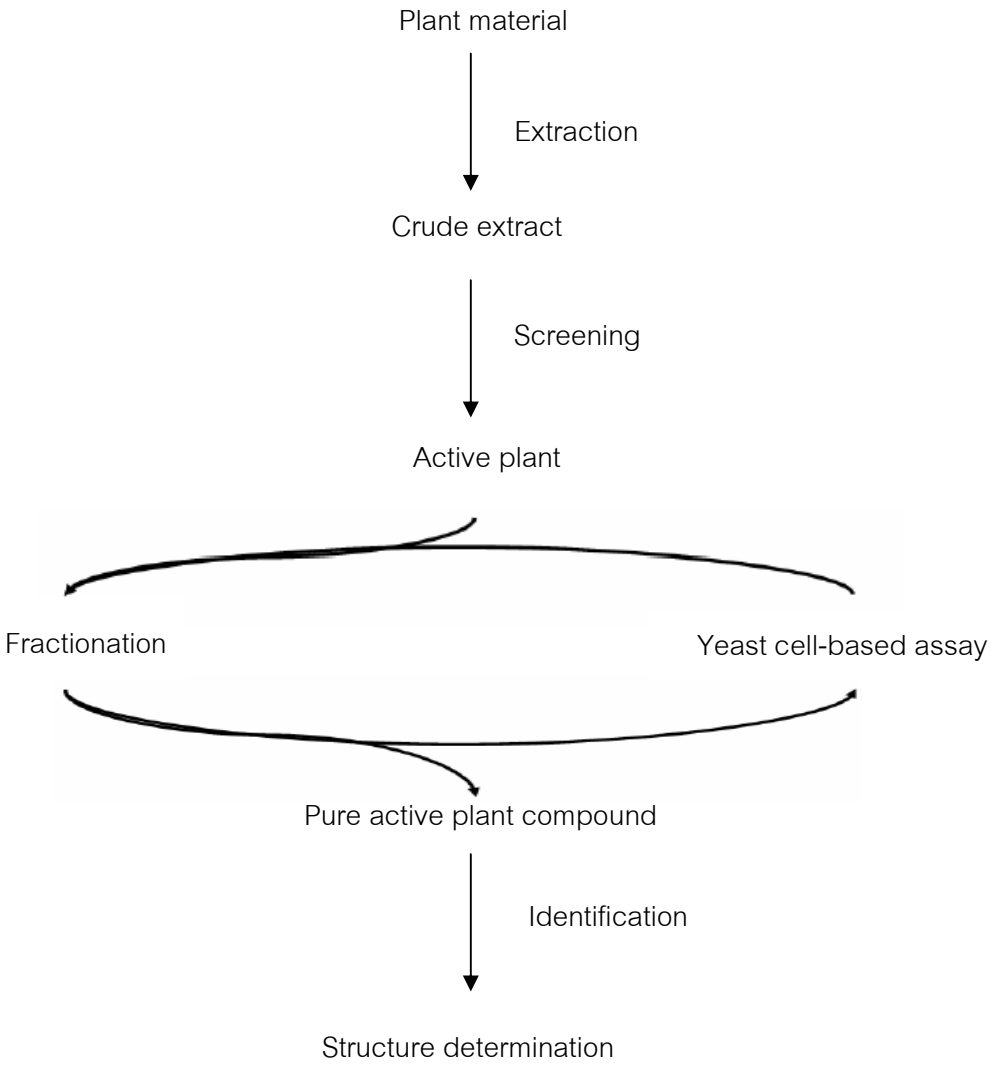
Scheme 4.1 Extraction scheme of dried stems of *D. scandens*.

4.4 Bioassay-guided fractionation process

The EtOAc extract of *D. scandens* was fractionated by vacuum liquid column chromatography. Then, all fractions were tested against Top2 poison activity by yeast-cell based assay. The most active fractions were then subjected to repeat chromatography. This procedure finally led to the isolation of two pure compounds. The procedure from the EtOAc extract to the isolation of pure compounds is shown in **Scheme 4.2**. The bioassay-guided fractionation protocol is shown in **Scheme 4.3**.



Scheme 4.2 Separation of the EtOAc extract of *D. scandens*.



Scheme 4.3 Bioassay- guided fractionation protocol.

4.5 Separation and isolation

4.5.1 Isoaltion of EtOAc extract

The EtOAC extract (82.9 g) was separated by vacuum liquid column chromatography using a sintered glass filter column of silica gel (No.7734). The EtOAC extract was dissolved in a small amount of EtOAc, triturated with silica gel (No. 7734) and dried under vacuum. Elution was performed in a polarity gradient manner with mixtures of *n*-hexane and EtOAc (10:0 to 0:10). The eluants were collected 250 ml per fraction and examined by TLC (silica gel, *n*-hexane- EtOAc 6:4). Fractions with similar chromatographic patterns were combined to yield 7 fractions: F1 107.1 mg), F2 (28.4 mg), F3 (451.9 mg), F4 (17.2 g), F5 (702.9 mg), F6 (255.6 mg), and F7 (51.8 mg)

4.5.2 Isoaltion of compound DS8 ans DS21

Fraction F4 (4 g) was purified on a silica gel column. (silica gel No. 9385, gradient mixture of *n*-hexane and EtOAc 10:0 to 0:10). The isolation gave compounds DS8 (280 mg, R_f 0.85, silica gel *n*-hexane and EtOAc 6:4) and DS21 (48.5 mg, R_f 0.5, silica gel *n*-hexane and EtOAc 6:4). Compounds DS8 and DS21 were identified as 5,7,4′-trihydroxy-6,8-diprenylisoflavone and lupalbigenin, respectively.

4.6 Topoisomerase II poison activity assay

For the yeast sensitivity studies, the yeast cells were grown overnight in YPDA broth. The cultures were adjusted to an OD_{590} of 0.5, and serially diluted 10-fold, and 5 µl aliquots were spotted onto the YPDA plates. For the screening of the isolated compounds, these compounds were serially diluted to concentrations of 200 µg/ml, 100 µg/ml and 50 µg/ml. Etoposide was used as a positive control, and cells incubated with DMSO alone were used as a vehicle control. The final concentration of DMSO never exceeded 2% (v/v) of the culture medium, and this has been shown to be a concentration that has no effect on the growth of the cells (Nitiss and Nitiss, 2001). The plates were incubated for 3 to 4 days at the optimal temperature for cell growth (25°C for temperature-sensitive *TOP2* mutants, 30°C otherwise), and the surviving colonies were observed. The drug sensitivity was determined by comparing the survival of colonies in the crude extract culture with that of those in the drug-treated culture, and the colonies were also photographed.

4.7 Cytotoxicity assay

Cytotoxicity against cancer cell growth inhibitory activities were tested by National Center for Genetic Engineering and Biotechnology (BIOTECH), National Science and Technology Development Agency (NSTDA). The bioassay laboratory protocol for all testing is described in **Appendix B**.

4.8 Results

4.8.1 Bioassay-guided fractionation of *D. scandens* extract

Bioassay-guided fractionation was performed on the EtOAc extract in order to isolate the bioactive compounds from *D. scandens*. In the fractionation process, the fractions were tested against Top2 poison activity by yeast cell-based assay. The growing cultures of mutant yeasts at 25°C and 30°C on YPDA agar containing 100 μg/ml, fixing concentration, of 7 fractions of crude EtOAc showed three active fractions (**Figure 4.1**). The three fractions that exhibited Top2 poison activities were F3, F4 and F5. Fraction F4 showed the highest Top2 poison activity. The most active fractions were then used for further fractionation step. This procedure finally led to the isolation of two pure compounds, DS8 (5,7,4′-trihydroxy-6,8-diprenylisoflavone) and DS21 (lupalbigenin). Both DS8 and DS21 demonstrated Top2 poison activity (**Figure 4.2**). The procedure from crude extract to the isolation of pure compounds is shown in **Schemes 4.1 - 4.3.**

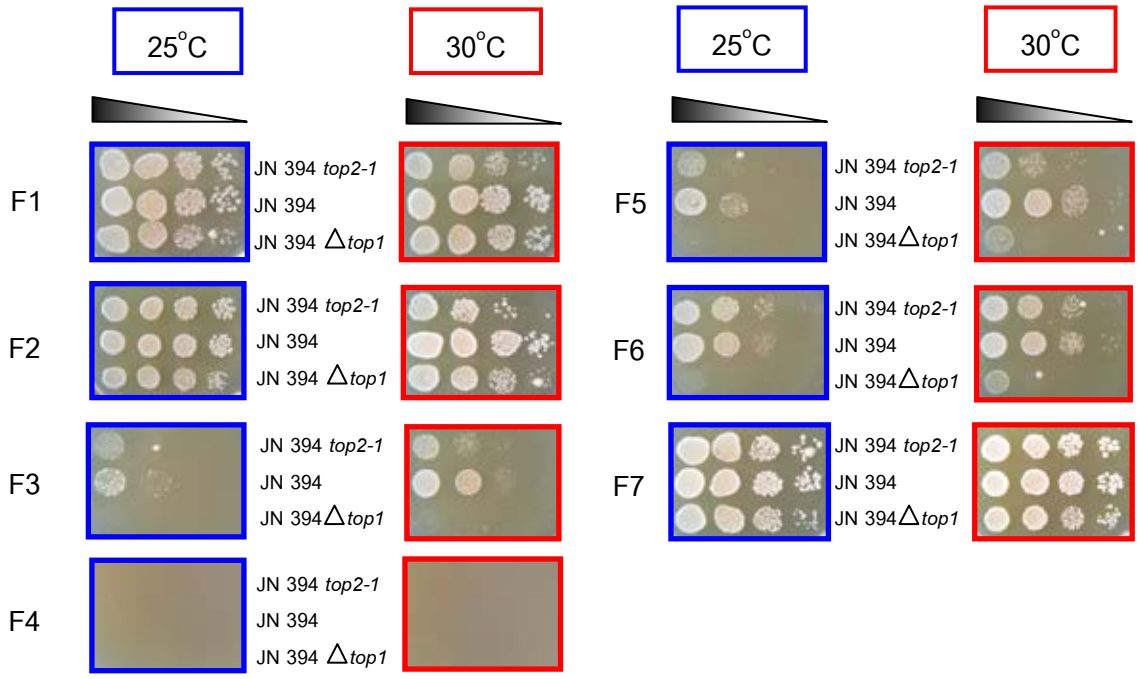


Figure 4.1 The *in vitro* Top2 poison screen of the medicinal plant extracts by a yeast cell-based assay. At 100 μg/ml of fractions (F) from *D.scandens* stem extract with exponentially growing of mutant yeast straians. Five microliters of ten-fold serial dilutions was spotted on plates which containing 100 μg/ml of F 1-7. The plates were incubated separately at 25°C (left) or 30°C (right) for 72 hr, and the cell growth was observed and photographed.

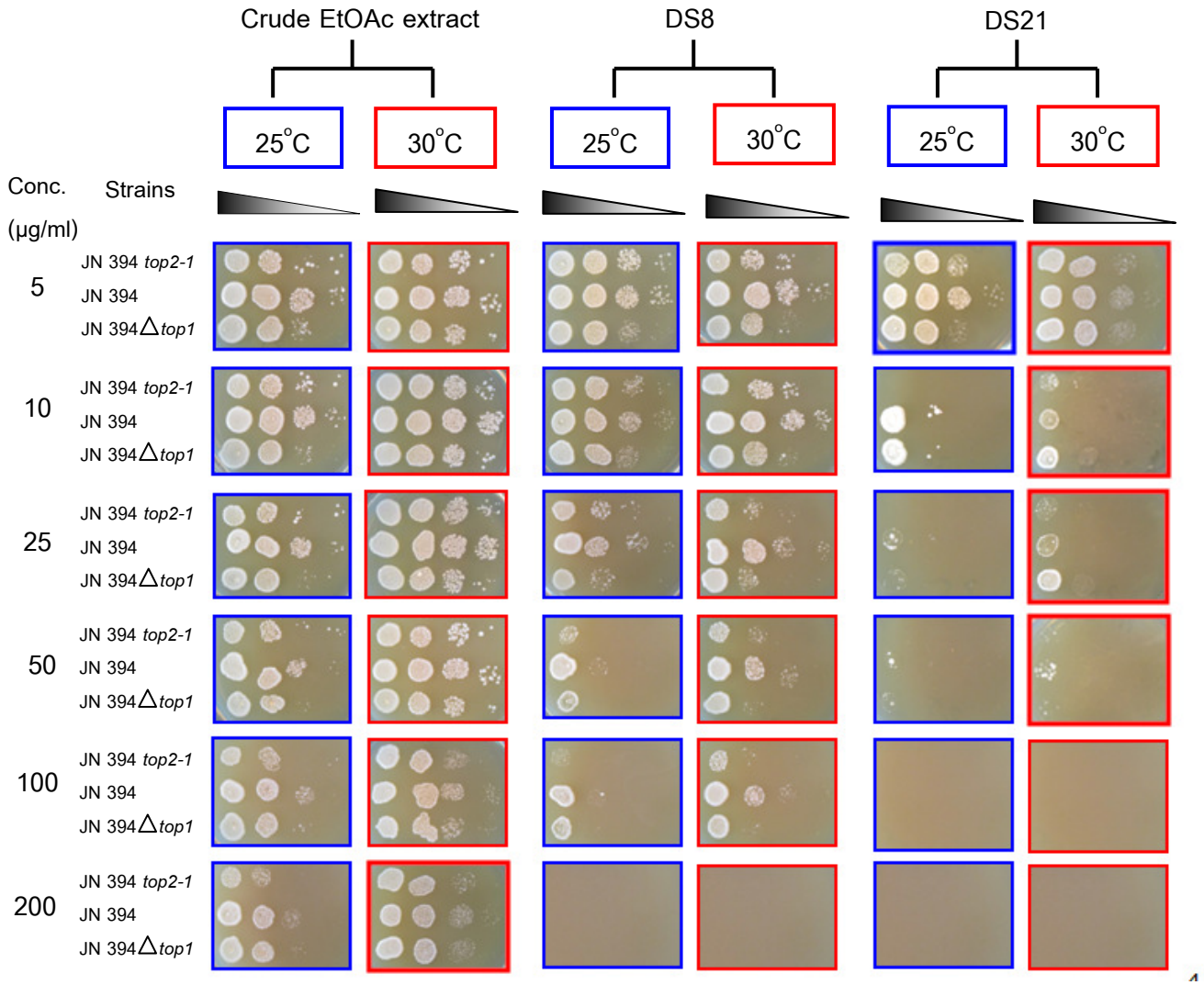


Figure 4.2 The *in vitro* Top2 poison screen of the medicinal plant extracts by a yeast cell-based assay. Crude EtOAc extract, compound DS8 and compound DS21 isolated from *stem of D. scandens* at various concentrations with exponentially growing of mutant yeast straians. Five microliters of ten-fold serial dilutions was spotted on plates which containing various concentrations of crude EtOAc extract, compound DS8 and compound DS21. The plates were incubated separately at 25°C (left) or 30°C (right) for 72 hr, and the cell growth was observed and photographed.

4.8.2 Physical and spectral data of the isolate compounds

4.8.2.1 Compound DS8

Compound DS8 was obtained as a yellow solid, soluble in EtOAc (280 mg, 0.09 % base on dried weight of stem).

ESI-MS: *m/z* (% relative intensity); **Figure C1**

^{1}H NMR: δ ppm, 300 MHz, in $CDCl_3$; see **Table 4.1**, **Figure C2**

^{13}C NMR: δ ppm, 75 MHz, in $CDCl_3$; see **Table 4.1**, **Figures C3** and **C4**

4.8.2.2 Compound DS21

Compound DS21 was obtained as a yellow solid, soluble in EtOAc (48.5 mg, 0.02 % base on dried weight of stem).

ESI-MS: *m/z* (% relative intensity); **Figure C5**

^{1}H NMR: δ ppm, 300 MHz, in acetone-d_6; see **Table 4.2**, **Figure C6**

^{13}C NMR: δ ppm, 75 MHz, in acetone-d_6; see **Table 4.2**, **Figures C7** and **C8**

4.8.3 Structure determination of isolate compounds

4.8.3.1 Structure determination of compound DS8 (5,7,4′-trihydroxy-6, 8-diprenylisoflavone)

Compound DS8 was obtained as a yellow solid. The ESI mass spectrum (**Figure C1**) showed a pseudomolecular ion $[M+H]^+$ at *m/z* 407.19, corresponding to $C_{25}H_{26}O_5$. The ^{1}H NMR spectrum (Figure C2 and Table 4.1) of compound DS8 showed signals at δ 7.87 (1H, s, H-2), 13.05 (1H, s, 5-OH), 6.37 (1H, s, 7-OH), 7.28 (2H, d, *J* = 8.4 Hz, H-2′, 6′), 6.76 (2H, d, *J* = 8.4 Hz, H-3′, 5′), 3.45 (2H, br s, H-1″), 5.23 (2H, t-like, *J* = 5.5 Hz, H-2″, H-2‴), 1.81 (6H, s, H-4″, H-4‴), 1.75 (3H, s, H-5″), 3.45 (2H, br s, H-1‴), and 1.72 (3H, s, H-5‴)

The ^{13}C NMR, DEPT 90 and DEPT 135 spectra (**Figures C3** and **C4** and **Table 4.1**) exhibited 25 carbon signals, corresponding to 4 methyls, 2 methylenes, 7 methines and 12 quaternary carbons. Through comparison of its ^{1}H and ^{13}C NMR data and MS data with previously published data, compound DS8 was identified as 5,7,4′-trihydroxy-6,8-diprenylisoflavone (Sekine *et al*., 1999).

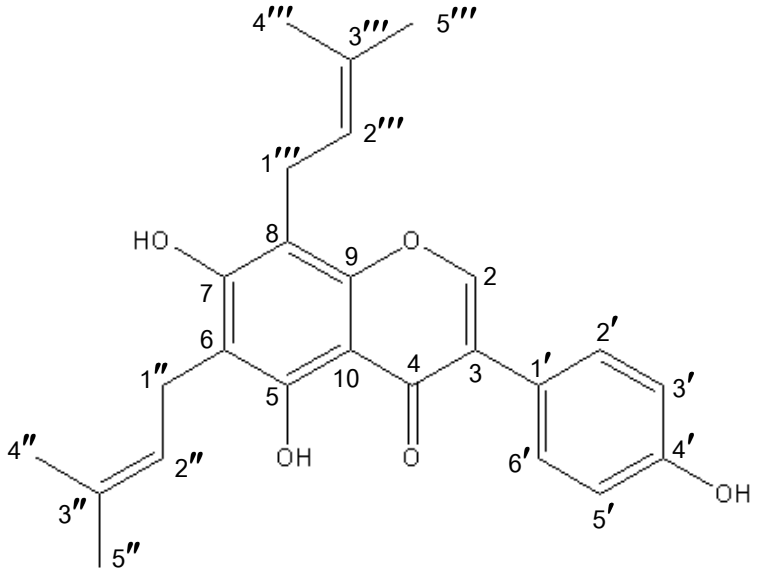


Figure 4.5 Chemical structure of DS8, 5,7,4′-trihydroxy-6, 8-diprenylisoflavone.

Table 4.1 NMR spectral data of compound DS8 ($CDCl_3$) and 5,7,4′-trihydroxy-6,8 diprenylisoflavone ($CDCl_3$).

Position	DS8		5, 7, 4′-trihydroxy-6,8-diprenylisoflavone	
	^{1}H (mult., *J* in Hz)	^{13}C	^{1}H (mult., *J* in Hz)	^{13}C
C-2	7.87 (s)	152.8	7.82 (s)	152.6
C-3	-	121.3	-	121.2
C-4	-	181.5	-	181.3
C-5	-	159.7	-	157.4
C-6	-	110.3	-	110.1
C-7	-	157.4	-	156.9
C-8	-	105.5	-	105.3
C-9	-	153.4	-	153.3
C-10	-	105.5	-	105.3
C-1′	-	121.3	-	121.1
C-2′	7.28 (d), *J* = 8.4	130.3	7.29 (d), *J* = 8.6	130.3
C-3′	6.76 (d), *J* = 8.4	115.8	6.78 (d), *J* = 8.6	115.6
C-4′	-	156.1	-	155.8
C-5′	6.76 (d), *J* = 8.4	115.8	6.78 (d), *J* = 8.6	115.6
C-6′	7.28 (d), *J* = 8.4	130.3	7.29 (d), *J* = 8.6	130.3
C-1″	3.45 (br s)	21.7	3.39 (d), *J* = 5.4	21.6
C-2″	5.23 (t-like), *J* = 5.5	121.3	5.19 (t-like), *J* = 5.4	121.2
C-3″	-	135.4	-	135.6
C-4″	1.81 (s)	17.9	1.77 (s)	17.9
C-5″	1.75 (s)	25.8	1.69 (s)	25.8
C-1‴	3.45 (br s)	21.7	3.41 (d), *J* = 5.4	21.6
C-2‴	5.23 (t-like), *J* =5.5	121.5	5.15 (t-like), *J* = 5.4	121.4
C-3‴	-	134.1	-	134.1
C-4‴	1.81 (s)	17.8	1.76 (s)	17.9
C-5‴	1.72 (s)	25.7	1.67 (s)	25.7
5-OH	13.05 (s)	-	13.04 (s)	-
7-OH	6.37 (s)	-	6.28 (s)	-

4.8.3.2 Structure determination of compound DS21 (lupalbigenin)

Compound DS21 was obtained as a yellow solid. The ESI mass spectrum (**Figure C5**) showed a pseudomolecular ion $[M+H]^+$ at *m/z* 407.18, corresponding to $C_{25}H_{26}O_5$. The ^{1}H NMR spectrum (**Figure C6** and **Table 4.1**) of compound DS21 showed signals at δ 8.4 (1H, s, H-2), 13.66 (1H, s, OH-5), 7.64 (1H, s, H-2′), 7.57 (1H, d, *J*= 8.2, H-6′), 7.20 (1H, d, *J* = 8.2 Hz, H-5′), 6.79 (1H, s, H-8), 5.69 (1H, t-like m, H-2‴), 5.59 (1H, t-like m, H-2″), 3.67 (4H, d, *J* = 6.9 Hz, H-1‴, H-1″), 2.04 (3H, s, H-5″), 2.09 (3H, s, H-5‴), 1.96 (3H, s, H-4‴), and 2.02 (3H, s, H-4″)

The ^{13}C NMR, DEPT 90 and DEPT 135 spectra (**Figure C7** and **C8** and **Table 4.1**) exhibited 25 carbon signals, corresponding to 4 methyls, 2 methylenes, 7 methines and 12 quaternary carbons. Through comparison of its ^{1}H and ^{13}C NMR data and MS data with previously published data, compound DS21 was identified as lupalbigenin (Sekine, T., *et al*., 1999).

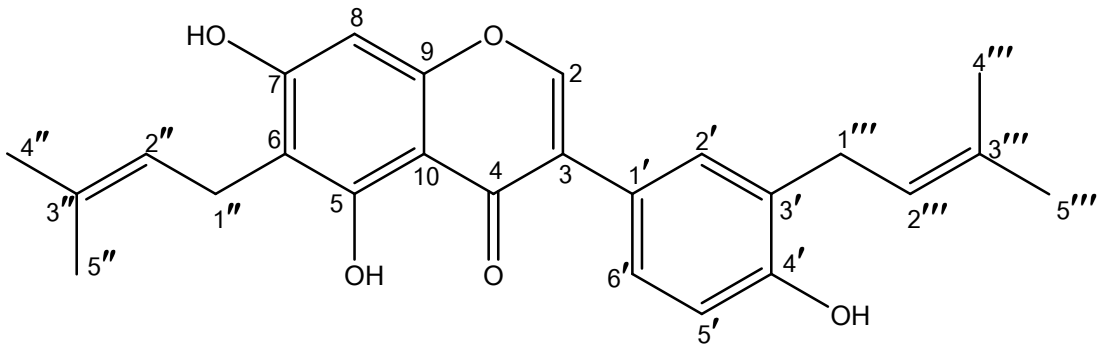


Figure 4.5 Chemical structure of DS21, lupalbigenin.

Table 4.2 NMR spectral data of compound DS21 (Acetone-d_6) and lupalbigenin (Acetone-d_6).

Position	DS21		Lupalbigenin	
	^{1}H (mult., *J* in Hz)	^{13}C	^{1}H (mult., *J* in Hz)	^{13}C
C-2	8.4 (s)	153.9	8.1 (s)	153.9
C-3	-	123.3	-	123.2
C-4	-	181.7	-	181.7
C-5	-	160.6	-	160.5
C-6	-	112.3	-	112.3
C-7	-	162.5	-	162.5
C-8	6.79 (s)	93.7	6.48 (s)	93.7
C-9	-	155.8	-	155.8
C-10	-	106.0	-	106.0
C-1′	-	123.6	-	123.3
C-2′	7.64 (s)	131.2	7.33 (d), *J* = 2.1	131.2
C-3′	-	128.5	-	124.1
C-4′	-	156.7	-	156.7
C-5′	7.20 (d), *J* = 8.2	115.5	6.88 (d), *J* = 8.2	115.5
C-6′	7.57 (d), *J* = 8.2	128.6	7.27 (dd), *J* = 8.2, 2.3	128.5
C-1″	3.67 (d), *J* = 6.9	29.1	3.36 (d), *J* = 7.2	29.1
C-2″	5.59 (t-like m)	123.1	5.28 (t-like m), *J* = 7.2	123.1
C-3″	-	132.4	-	131.6
C-4″	2.02 (s)	25.8	1.65 (br s)	25.8
C-5″	2.04 (s)	17.9	1.74 (br s)	17.8
C-1‴	3.67 (d), *J* = 6.9	22.0	3.36 (d), *J* = 7.2	22.0
C-2‴	5.69 (t-like m)	124.1	5.37 (t-like m), *J* = 7.2	123.6
C-3‴	-	131.6	-	131.5
C-4‴	1.96 (s)	25.9	1.71 (br s)	25.8
C-5‴	2.09 (s)	17.8	1.73 (br s)	17.6
5-OH	13.66 (s)	-	13.17 (s)	-

4.8.4 Cytotoxicity activity

Table 4.3 Cytotoxicity of isolated compounds from *D. scandens* against vero cell line and cancer cell lines.

	IC_{50}			
Cell lines / Samples	KB	MCF-7	NCI-H187	Vero cells
Crude extract	Active	active	Active	Cytotoxicity
Water	Inactive	inactive	Inactive	non- cytotoxicity
EtOAc extract	Active	active	Active	Cytotoxicity
DS-8	8.61 μM	9.85 μM	5.74 μM	15.53 μM
DS-21	9.22 μM	4.26 μM	7.71 μM	10.72 μM
Ellipticine	0.717 μM	n.d.	0.723 μM	1.36 μM
Doxorubicin	0.564 μM	8.43 μM	0.025 μM	n.d.

n.d. = not determined

4.9 Discussions

Chemical constituents of *D. scandens* have been reported previously (Chuankamnerdkarn *et al*., 2002; Dianpeng *et al*., 1999; Falshaw *et al*., 1969; Johnson *et al*., 1966; Johnson and Pelter, 1966; Mahabusarakam *et al*., 2004; Pelter and Stainton, 1966; Rao *et al*., 1994; Rukachaisirikul *et al*., 2002; Sekine *et al*., 1999). Some reports demonstrated cytotoxicity of crude extract from this plant against cancer cell lines (Laupattarakasem *et al*., 2007). However, no previous studies have looked into the detail of the mechanism. In this study, the methanol extract of *D. scandens* exhibited Top2 poison activity against yeast cell-based assay. Bioassay-guided fractionation and isolation led to the identification of two bioactive compounds, DS8 and DS21. Comparison of their ^{1}H and ^{13}C NMR and MS data with previously publications (Sekine, T., *et al*., 1999), compound DS8 was identified as 5,7,4′-trihydroxy-6,8-diprenylisoflavone and compound DS21 was identified as lupalbigenin.

The cytotoxicity activities of DS8 and DS21 showed their potential as anti-cancer principles from *D. scandens*. The two compounds exhibited cytotoxicity activities against KB oral cavity cancer cell lines (IC_{50} = 8.61 and 9.22 μM respectively), MCF-7 breast cancer cell lines (IC_{50} = 9.85 and 4.26 μM respectively), and NCI-H187 small lung cancer cell lines (IC_{50} = 5.74 and 7.71 μM respectively). Compound DS8 showed higher cytotoxicity against KB and NCI-H187 cell lines than DS21. However, lower cytotoxicity activity against MCF-7 cell lines was found in DS8. Both DS8 and DS21 showed cytotoxicity activities against vero cells with IC_{50} = 15.53 and 10.72 μM, respectively. When compared with ellipticine (IC_{50} = 1.36 μM), Top2-targeted drug, DS8 and DS 21 exhibited lower cytotoxicity. This study is the first report of the cytotoxicity mechanism (Top2 poison) of these two compounds from *D. scandens.*

CHAPTER V

TOPOISOMERASE II–MEDIATED SUPERCOILED DNA RELAXATION ASSAY OF *DERRIS SCANDENS* (Roxb.) Benth

Top2 is an enzyme involved in the relaxation of DNA during a number of critical cellular processes (Wada *et al*., 2001). A catalytic intermediate stage involving a covalent linkage between Top2 and the 5′ end of the broken DNA strand has been shown to be susceptible to interference by certain compounds which essentially prevent the relegation of the broken strand and the release of bound enzyme. Such compounds are referred to as Top2 poison, of which action refers to interference with supercoil relaxation activity of the enzyme. Identification of Top2 poison requires tractable biochemical assays employing purified enzyme (Webb and Ebeler, 2003). In this chapter, the conversion of supercoiled plasmid DNA to relaxed DNA by human Top2 was examined in the presence of compounds from *D. scandens*.

5.1 Materials and method

Reagents were purchased from TopoGEN (Columbus, OH, USA). DNA Top2 activity was measured by assessing relaxation of supercoiled pHOT1 DNA. The reaction mixture contained 30 mM Tris-HCl (pH 8), 50 mM NaCl, 10 mM $MgCl_2$, 3 mM ATP, 15 mM dithiothreitol, pHOT1 supercoiled (250 ng), the indicated drug concentrations, and 2 units of human Top2 in a final volume of 20 μl. Reactions were carried out at 37°C for 1 h and then terminated by the addition of 2 mL of 10% sodium dodecyl sulfate (SDS) and 2 mL of 50 μg/mL proteinase K. The reaction mixture was subjected to electrophoresis through a 1% agarose gel in TAE buffer (90 mM Tris-acetate and 2 mM EDTA). After electrophoresis, the gels were stained with ethidium bromide 30 min and were then destained in distilled water prior photographed under ultraviolet light. Images were taken with Bio-Rad Gel documentation and analyzed with Quantity One 1-D Analysis Software. The supercoiled DNA, linear DNA and relaxed DNA were identified from their

mobilities on the gel. Etoposide was used as positive control. Concentration for 50% inhibition (IC_{50}) were determined by interpolation from plots of Top2 activity versus inhibitor concentration.

5.2 Results and discussion

The effect of compounds isolated from *D. scandens* on the strand passage activity of Top2 was determined by the enzyme-mediated supercoiled DNA relaxation. Etoposide was included for comparison since it is a well established inhibitor of Top2. As shown in **Figure 5.1**, the EtOAc extract, compound DS8, and compound DS21 displayed significant inhibition of this reaction in a concentration-dependent manner at 2.5-100 μM (**Figure 5.1A**, **B**, and **C**, respectively, *lanes 7-12*). Etoposide caused similar inhibition at lower concentrations (**Figure 5.1**, *lanes 1-3*). Inhibitory activities of the compounds were presented as micromolar concentrations that cause 50% inhibition per unit of enzyme (IC_{50}), under the assay conditions. From the plots obtained with six different concentrations of the drugs, IC_{50} values were obtained and the results are the average of three estimations.

In this study, the crude EtoAc extract, DS8 and DS21 showed complete inhibition of the relaxation reaction by Top2 at concentrations of 100, 50 and 100 μM, respectively. The inhibitory effects of crude EtOAc, DS8 and DS21 were dose-dependent. Crude EtOAc extract DS8 and DS21 have IC_{50} values of 40.4, 24.4, 18.7 μM for the Top2 relaxation activity, respectively. These facts indicate that compounds DS8 and DS21 are selective inhibitors against Top2 inhibitory activity. The result correlated with the previous test using yeast cell-based assay, D21 showed the stronger inhibitory activity than DS8.

Top2 poisons such as etoposide (IC_{50} 9.3 μM) are known to stimulate the stabilization of covalent complexes between Top2 and DNA, the so-called “cleavable complex”, thus generating DNA double-strand breaks and consequently causing cytotoxicity. Compounds DS8 and DS21 may stabilize the cleavable complex similar to etopoisde. This result suggests that compound DS8 and DS21 may inhibit the enzyme activity in the same manner as etoposide.

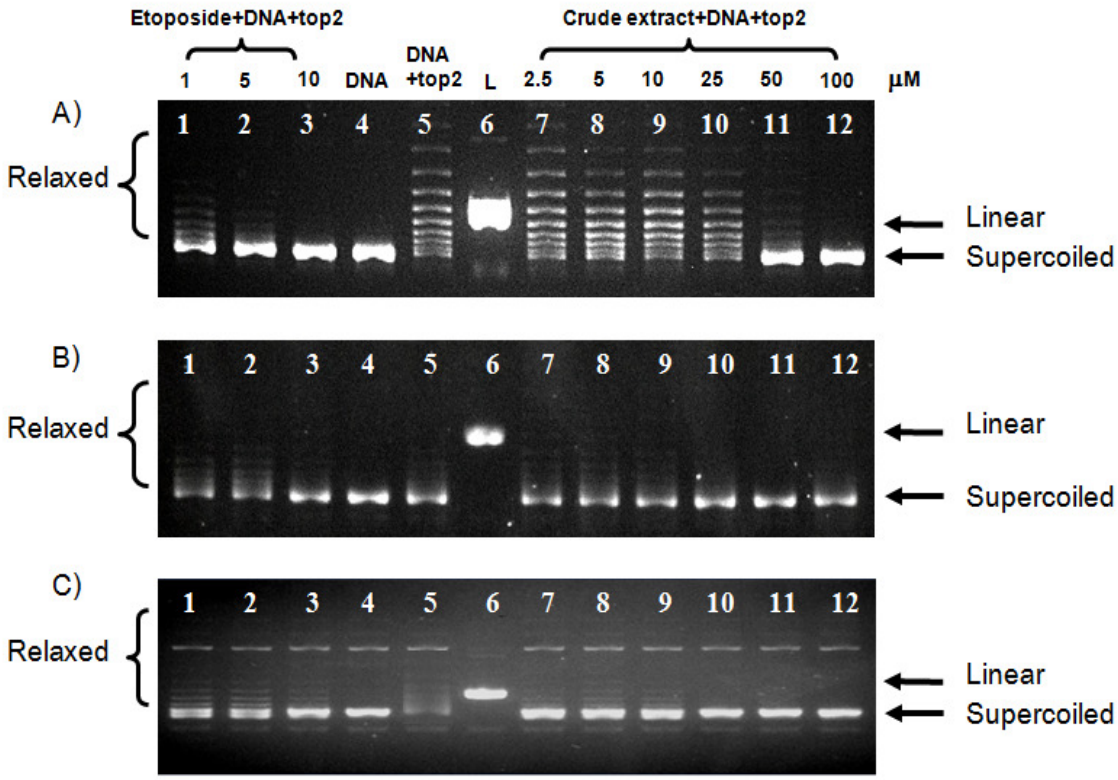


Figure 5.1 Effect of crude EtOAc extract, DS8 (5,7,4′-trihydroxy-6,8-diprenylisoflavone) and DS 21 (lupalbigenin) from *D. scandens* on the DNA relaxation activity by human DNA top2. Purified enzymes 2 units were incubated with 250 ng of supercoiled DNA with and without test compound (drug or bioactive compounds) and incubated for 30 min at 37 °C. *Lane 1-3* human top2 with etoposide at 1, 5 and 10 µM, respectively; *lane 4* supercoiled DNA alone; *lane 5* human top2 with no drug; *lane 6* linear DNA maker; *lane 7-12* human top2 with crude extract or bioactive compounds at 2.5, 5, 10, 25, 50 and 100 µM, respectively.

CHAPTER VI

CONCLUSIONS

Our study demonstrated that the mutant yeast system was an effective and useful model for identifying the cell killing activity of Top2 poisons. We have successfully developed an *in vitro* assay that uses a mutant yeast screening system to evaluate the cellular target for the extracts and compounds with Top2 poison activity. This yeast model is a powerful tool because it is simple, highly specific, easy to implement and cost-effective. Thirty ethnomedicinal plants were selected based on their documented use as alternative treatments for cancer provided by Thai folk practitioners. The results determined that six, *C. longa*, *C. zedoaria*, *D. scandens*, *G. maderaspatana*, *S. pierrei* and *S. suberosa*, of the test extracts functioned as Top2 poisons.

Interestingly, this is the first time that the Top2 poison activity of *D. scandens* has been reported. Therefore, *D. scandens* was selected for further phytochemical studies. Bioassay-guided fractionation based on Top2 II poison activity led to the isolation of two isoflavones from the ethyl acetate extract of the stem of *D. scandens*. The compounds were identified as 5,7,4′-trihydroxy-6,8-diprenylisoflavone and lupalbigenin by spectral evidence and compared with previously published data. All these compounds showed cytotoxicitiy against human epidermoid carcinoma of oral cavity (KB), breast adenocarcinoma (MCF-7) and human small cell lung carcinoma (NCI-H187) cell lines. In DNA relaxation assay, 5,7,4′-trihydroxy-6,8-diprenylisoflavone and lupalbigenin were found be selective inhibitors of human Top2 activity with IC_{50} value 24.4 and 18.7 μM activity, respectively.

Plants remain a prime source of new drugs for the treatment of cancer and will likely be the basis of novel anticancer agents. The use of drug screens can validate the traditional use of medicinal plant species and can bolster the cultural and medicinal significance of these taxa.

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APPENDIX

Appendix A

1. YPDA media (Difco™)

Broth formula

Formula per Liter	
Yeast Extract	10.0 g
Peptone	20.0 g
Dextrose	20.0 g
Adenine sulfate	0.5 g

Agar formula

Formula per Liter	
Yeast Extract	10.0 g
Peptone	20.0 g
Dextrose	20.0 g
Adenine sulfate	0.5 g
Agar	17.5 g

Media preparation

1. Dissolve the media components in distilled water.
2. Heat the agar medium with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave the agar and broth media at 121°C for 15 minutes.

Table A1 The number of yeast colony forming unit of JN394.

Serial dilutions of yeast cultures	Yeast colony forming unit per plate				
	Plate 1	Plate 2	Plate 3	Average	CFU/ml
1 ($O.D._{590}$ = 0.5)	>300	>300	>300	>300	-
10^{-1}	>300	>300	>300	>300	-
10^{-2}	>300	>300	>300	>300	-
10^{-3}	>300	>300	>300	>300	-
10^{-4}	>300	>300	>300	>300	-
10^{-5}	89	96	116	100.33	$1x10^{7}$
10^{-6}	11	13	17	13.67	-
10^{-7}	0	0	0	0	-

Table A2 The number of yeast colony forming unit of JN394 Δ*top1*.

Serial dilutions of yeast cultures	Yeast colony forming unit per plate				
	Plate 1	Plate 2	Plate 3	Average	CFU/ml
1 ($O.D._{590}$ = 0.5)	>300	>300	>300	>300	-
10^{-1}	>300	>300	>300	>300	-
10^{-2}	>300	>300	>300	>300	-
10^{-3}	>300	>300	>300	>300	-
10^{-4}	>300	>300	>300	>300	-
10^{-5}	98	100	104	100.67	$1x10^{7}$
10^{-6}	7	11	16	11.33	-
10^{-7}	0	0	0	0	-

Table A3 The number of yeast colony forming unit of JN394*top2-1*.

Serial dilutions of yeast cultures	Yeast colony forming unit per plate				
	Plate 1	Plate 2	Plate 3	Average	CFU/ml
1 ($O.D._{590}$ = 0.5)	>300	>300	>300	>300	-
10^{-1}	>300	>300	>300	>300	-
10^{-2}	>300	>300	>300	>300	-
10^{-3}	>300	>300	>300	>300	-
10^{-4}	>300	>300	>300	>300	-
10^{-5}	139	153	151	147.67	$1.48x10^{7}$
10^{-6}	15	11	13	13	-
10^{-7}	0	0	0	0	-

Appendix B

Bioassay laboratory protocol

1. Cytotoxic against primate cell line (Vero)

Method:	Green fluorescence protein (GFP) detection
Positive control:	Ellipticine
Negative control:	0.5% DMSO
Maximum final test concentration:	50 µg/ml

The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N1 plasmid (Clontech). The cell line is maintained in minimal essential medium supplemented with 10% heat- inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, at 37°C in a humidified incubator with 5% CO_2.

The assay is carry out by adding 45 µl of cell suspension at $3.3x10^4$ cells/ml to each well plates containing 5 µl of test compounds previously diluted in 0.5% DMSO, and then incubating for 4 days at 37°C incubator with 5% CO_2. Fluorescence signals are measured by using SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signal at day 4 is subtracted with background fluorescence at day 0. The percentage of cytotoxic is calculated by the following equation, where FU_T and FU_C represent the fluorescence units of cells treated with test compound and untreated cells, respectively.

$$\% \text{ cytotoxicity} = [1-(FU_T/FU_C)] \times 100$$

IC_{50} values are derived from dose-response curves, using 6 concentrations of 2-flod serially diluted samples, by the SOFTMax Pro software (Molecular device). Ellipticine and 0.5% DMSO are used as a positive and a negative control, respectively.

2. Cancer cell growth inhibition

Method:	Resazurin microplate assay (REMA)
Positive control:	Ellipticine and doxorubicin
Negative control:	0.5% DMSO
Maximum final test concentration :	50 µg/ml

Three cancerous human-cell lines are available for this assay:

1) KB cell line (epidermoid carcinoma of oral cavity, ATCC CCL-17)

2) MCF-7 cell line (breast adenocarcinoma, ATCC HTB-22)

3) NCI-H187 (small cell lung carcinoma, ATCC CRL-5804)

This assay is performed using the method described by Brien *et al*. (2000). In brief, cells at a logarithmic growth phase are harvested and diluted to $7x10^4$ cells/ml for KB and $9x10^4$ cells/ml for MCF-7 and NCI-H187, in fresh medium. Successively, 5 µl of test sample diluted in 5% DMSO, and 45 µl of cell suspension are added to 384-well plates, incubated at 37°C in 5% CO_2 incubator. After the incubation period (3 days for KB and MCF-7, and 5 days for NCI-H187), 12.5 µl of 62.5 µg/ml resazurin solution is added to each well, and the plates are then incubated at 37°C for 4 hour. Fluorescence signal is measured by using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 and 590 nm. Percentage inhibition of cell growth is calculated by the following equation, where FU_T and FU_C represent the mean fluorescence units from treated and untreated conditions, respectively.

$$\% \text{ inhibition} = [1-(FU_T/FU_C)] \times 100$$

Dose-response curves are plotted from 6 concentrations of 2-flod serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be derived using the SOFTMax Pro software (Molecular device, USA).

Appendix C

BIORESOURCES RESEARCH UNIT

Low resolution report

Acquisition Date 3/29/2011 4.10.27 PM

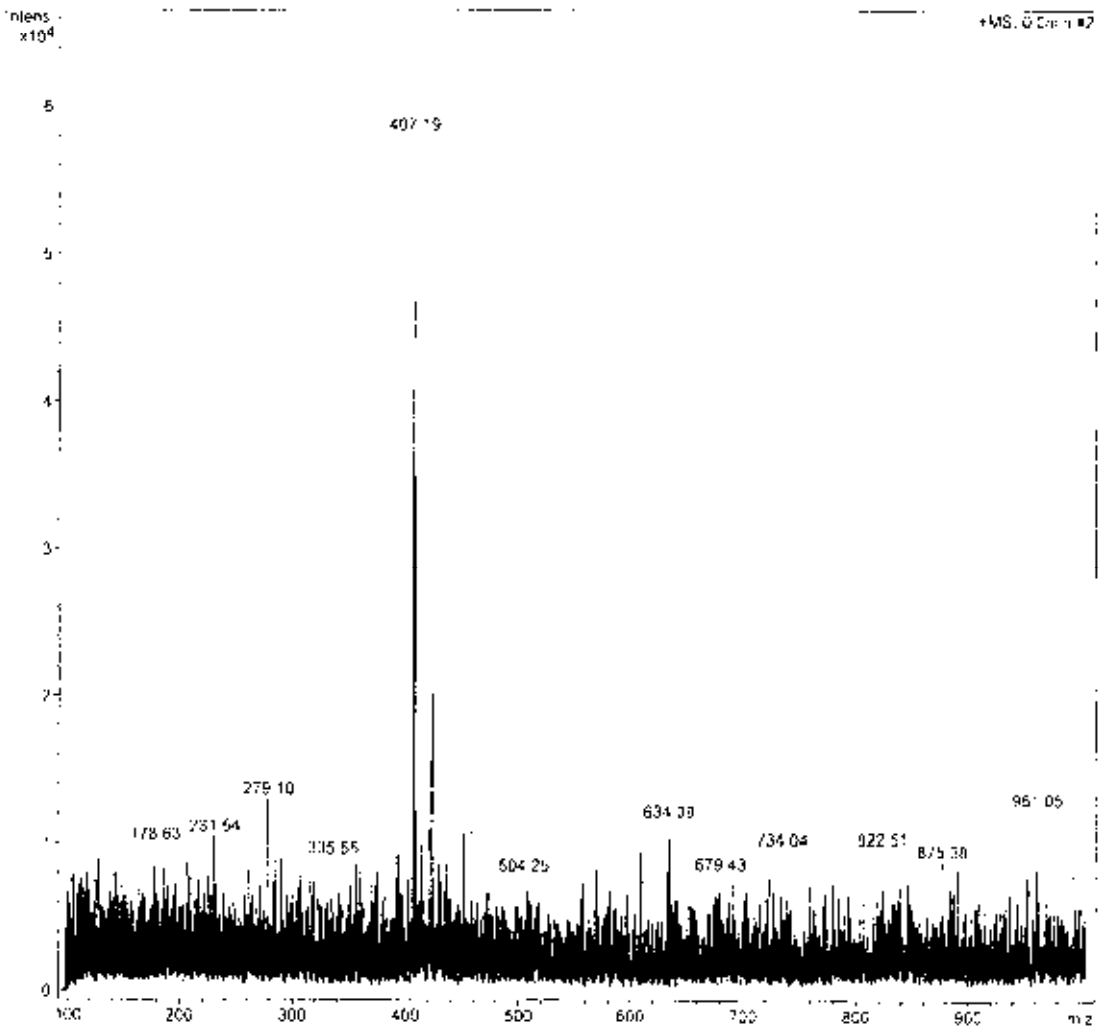
Analysis Name D:\Data\customer\OR F 8.d
Method NaFormate_pos_infusion.m
Sample Name OR F 8

Operator Suttichai
Instrument micrOTOF

Ext 3560
Bruker

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.0 Bar
Focus	Not active			Set Dry Heater	150 °C
Scan Begin	100 m/z	Set Capillary	4000 V	Set Dry Gas	6.0 l/min
Scan End	1000 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Source



Bruker Daltonics DataAnalysis 3.4 printed: 3/29/2011 4.16.06 PM Page 1 of 1

Figure C1 Mass spectrum of compound DS8.

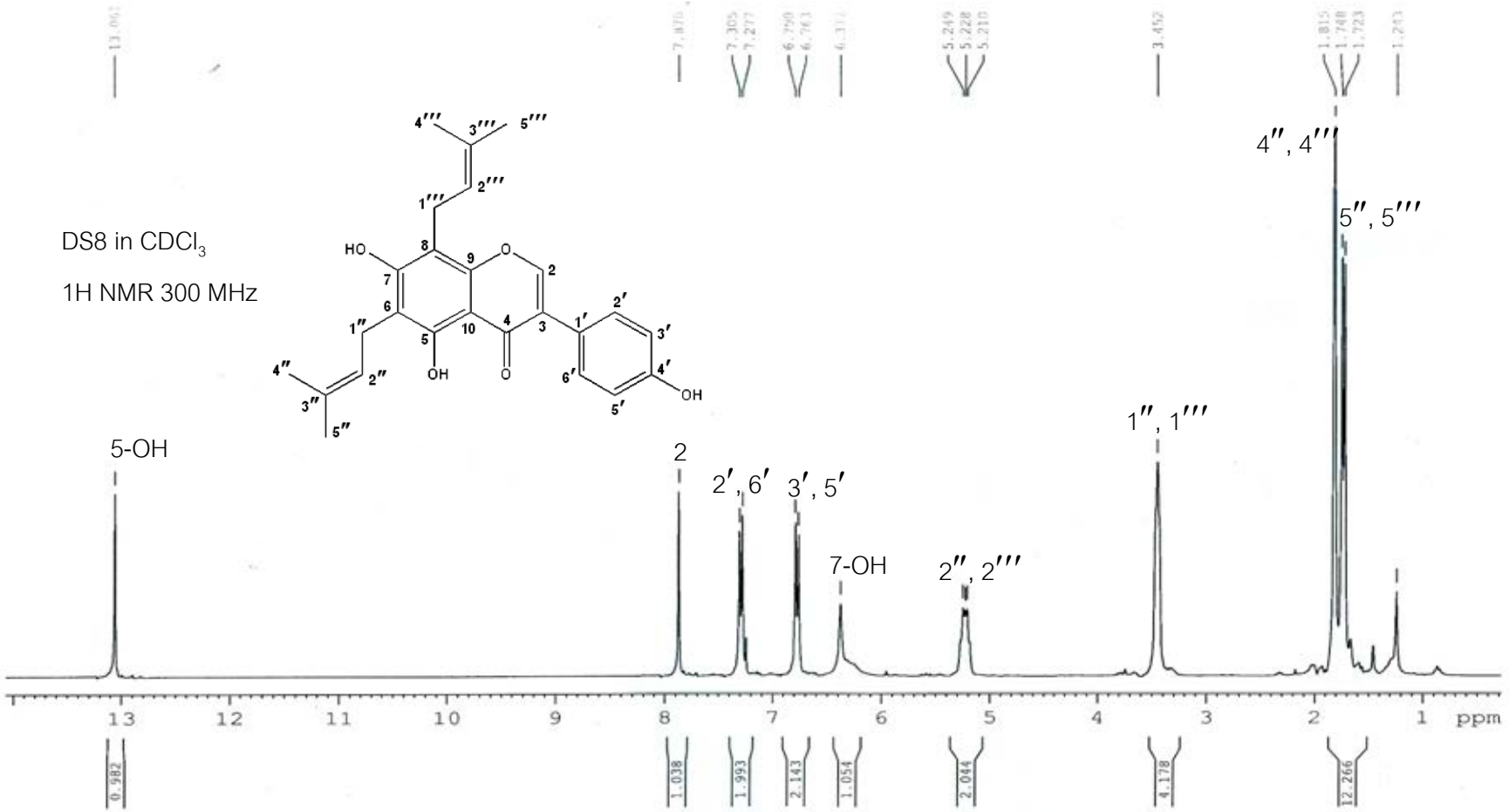


Figure C2 ^{1}H NMR (300 MHz) spectrum of compound DS8 ($CDCl_3$).

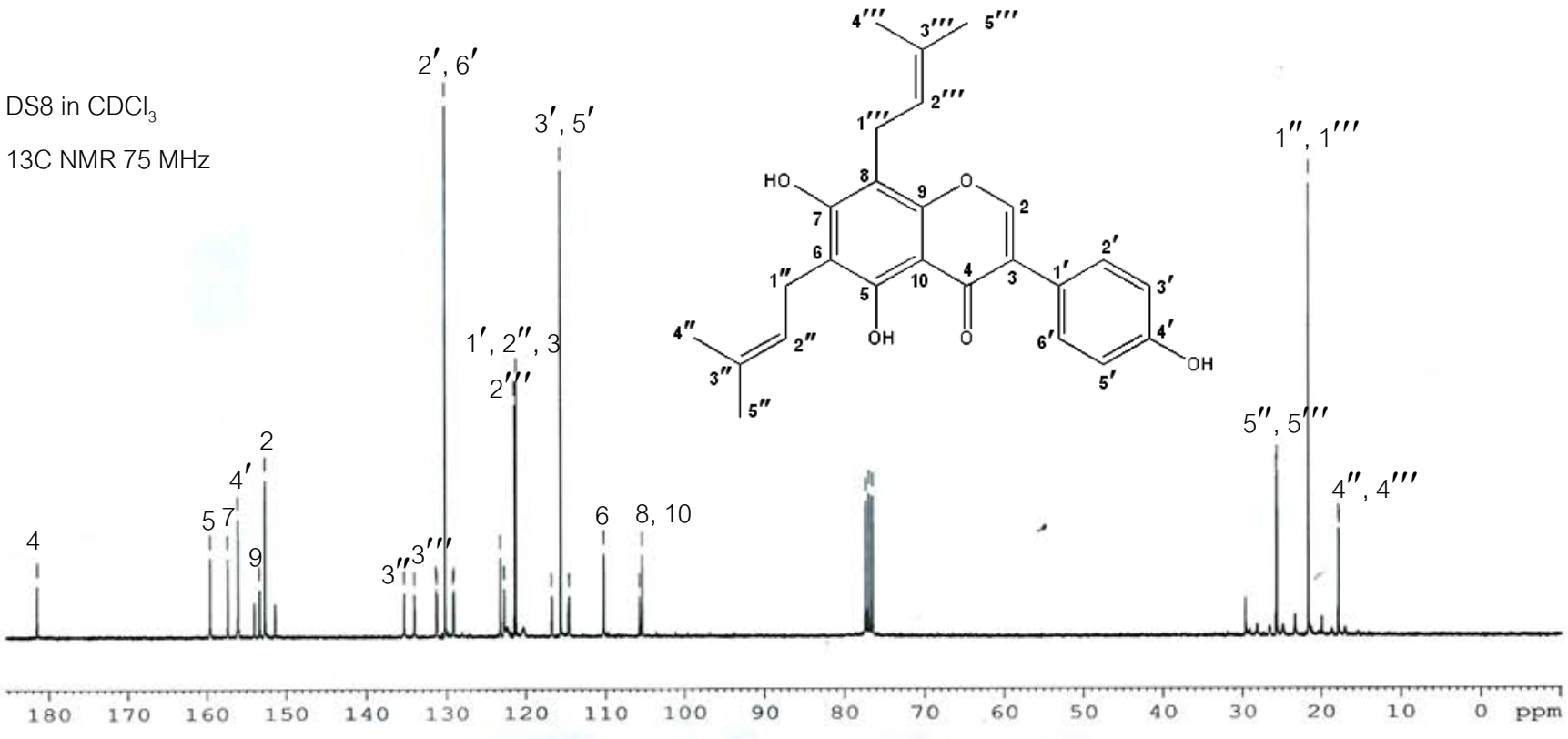


Figure C3 ^{13}C NMR (75 MHz) spectrum of compound DS8 ($CDCl_3$).

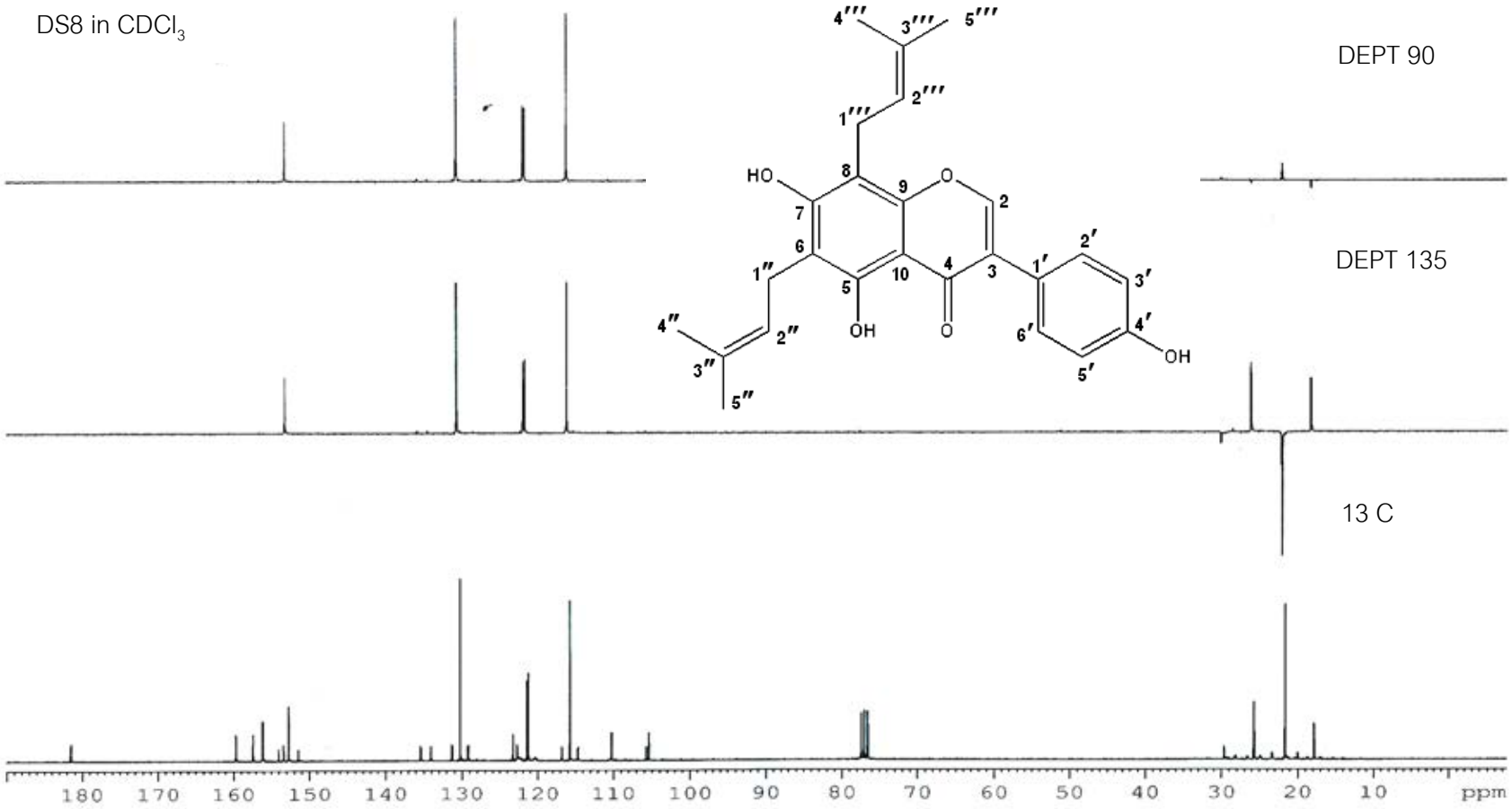


Figure C4 DEPT spectra of compound DS8 ($CDCl_3$).

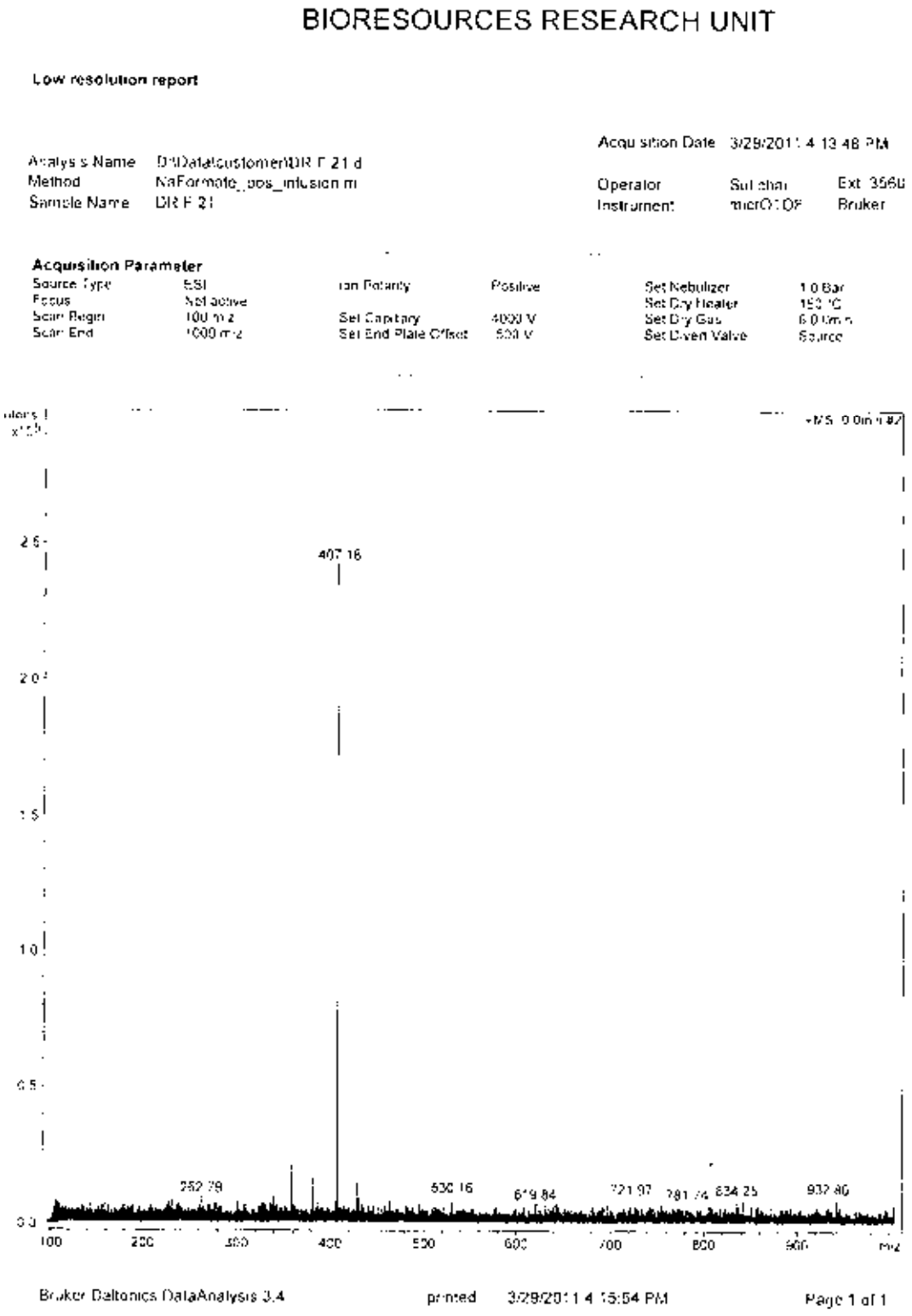


Figure C5 Mass spectrum of compound DS21.

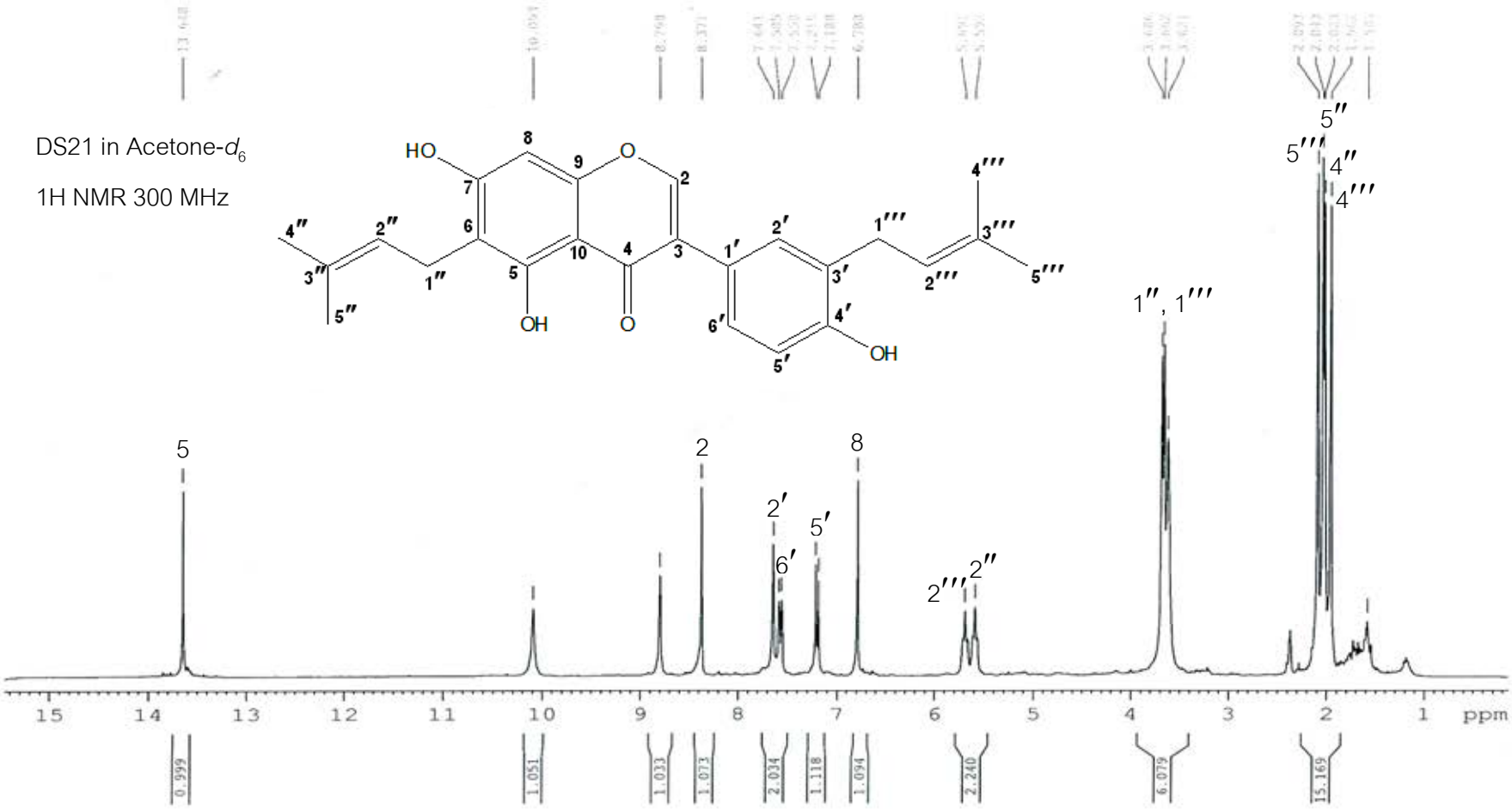


Figure C6 ^{1}H NMR (300 MHz) spectrum of compound DS21 (Acetone-d_6).

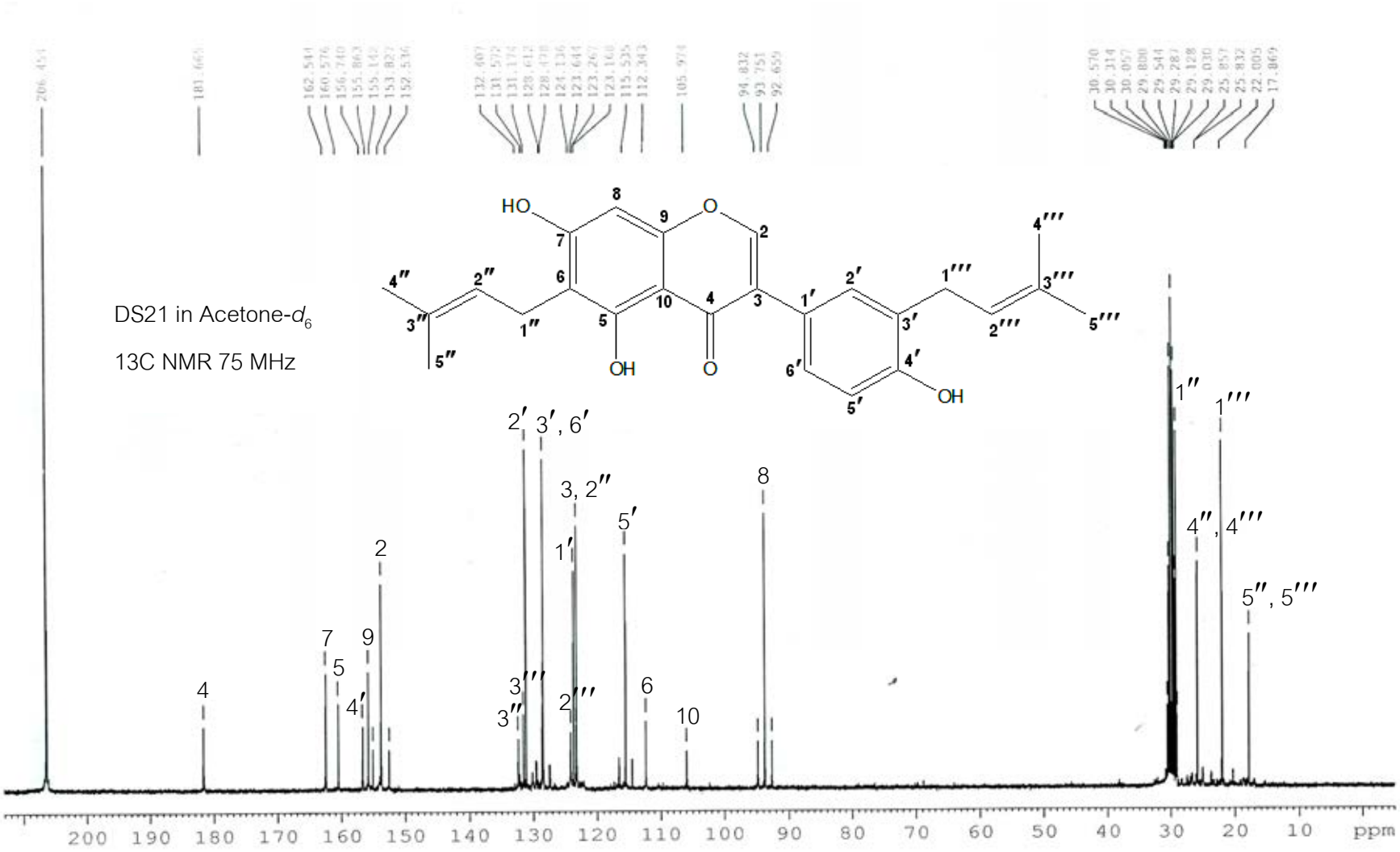


Figure C7 ^{13}C NMR (75 MHz) spectrum of compound DS21 (Acetone-d_6).

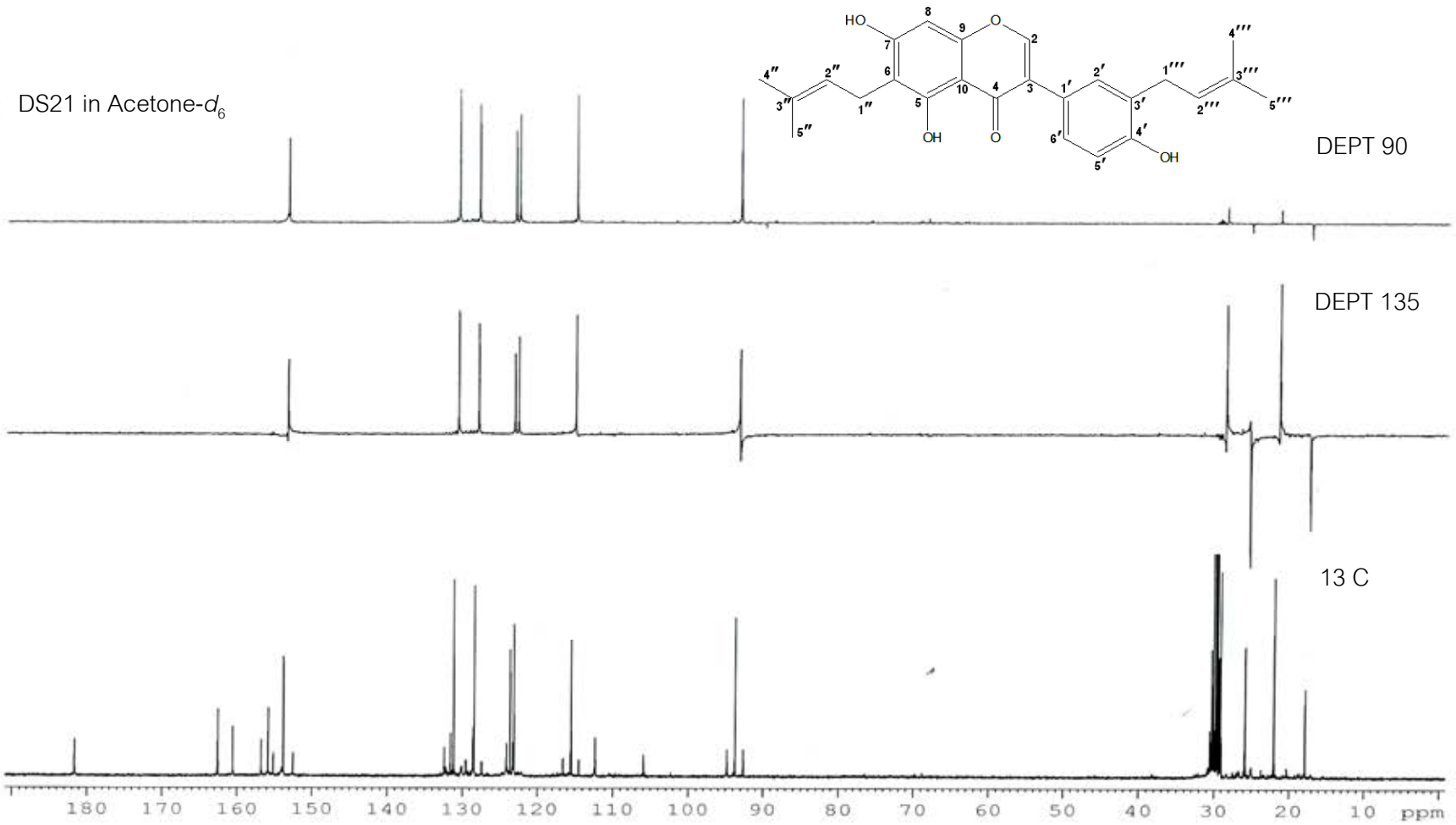


Figure C8 DEPT spectra of compound DS21 (Acetone-d_6).

VITA

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