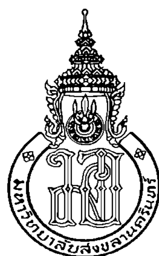

Study on anti-HIV-1 integrase activity of Thai medicinal plants

Kingkan Bunluepuech

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Herb Sciences (International Program)

Prince of Songkla University

2010

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Thesis Title Study on anti-HIV-1 integrase activity of Thai medicinal plants

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ชื่อวิทยานิพนธ์ การศึกษาฤทธิ์ต้านเอนไซม์ HIV-1 integrase ในสมุนไพรไทย

ผู้เขียน นางสาว กิ่งกาญจน์ บรรลือพืช

สาขา วิทยาศาสตร์สมุนไพร (นานาชาติ)

ปีการศึกษา 2553

บทคัดย่อ

สารสกัดหยาบจากชั้นเอทานอล และ ชั้นน้ำ ของสมุนไพรไทย ทั้ง 8 ชนิด ได้แก่ ท้าวยายม่อม (ทั้งต้น), ย่านาง (เถา), ชิงชี่ (เนื้อไม้), คนทา (เนื้อไม้), มะเดื่อชุมพร(เนื้อไม้), จันทน์ขาว (เนื้อไม้), จันทน์แดง (แก่น) และ บอระเพ็ด (เถา) ได้นำมาทดสอบฤทธิ์ต้านเอนไซม์ HIV-1 integrase โดยใช้ วิธี multiplate integration assay (MIA) จากสารสกัดพบว่า แก่นจันทน์แดง ชั้น เอทานอล ให้ % yield มากที่สุด คือ 39.9 % w/w ตามมาด้วย เถาบอระเพ็ด ชั้นน้ำ ได้% yield เท่ากับ 12.6 % w/w ส่วนพืชอื่นๆ นั้นทั้งชั้นเอทานอลและน้ำ ได้ % yield ในช่วง 1.2-6.8 % w/w การศึกษาฤทธิ์ต้านเอนไซม์ HIV-1 integrase พบว่า ชั้นเอทานอลของเนื้อไม้มะเดื่อชุมพร มีฤทธิ์ต้านเอนไซม์ HIV-1 integrase ได้มากที่สุดที่ IC_{50} เท่ากับ 7.8 μg/ml ในขณะที่ชั้นน้ำ พบว่าเนื้อไม้คนทา แสดงฤทธิ์ต้านเอนไซม์ HIV-1 integrase ได้มากที่สุดที่ IC_{50} เท่ากับ 2.3 μg/ml ซึ่งมีฤทธิ์ดีกว่า Suramin ที่ใช้เป็น positive control (IC_{50} เท่ากับ 3.4 μg/ml) ส่วนสารสกัดหยาบจากชั้นเอทานอล และชั้นน้ำของต้นอื่นๆ มีค่า IC_{50}ตั้งแต่ 22.1 ถึงมากกว่า 100 μg/ml

แม้ว่าสารสกัดหยาบจากชั้นน้ำของเนื้อไม้คนทาแสดงฤทธิ์ต้านเอนไซม์ HIV-1 integrase ได้ดีที่สุดก็ตาม แต่จากการทดลองศึกษาเบื้องต้น พบว่าสารสกัดหยาบจากชั้นน้ำของเนื้อไม้คนทายากต่อการแยกสารให้บริสุทธิ์ ดังนั้นสารสกัดหยาบจากชั้นเอทานอลของเนื้อไม้มะเดื่อชุมพร ซึ่งมีฤทธิ์ต้านเอนไซม์ HIV-1 integrase ดีที่สุดในชั้นเอทานอลได้ถูกนำมาแยกสารบริสุทธิ์ได้ทั้งหมด 5 สาร ได้แก่ β-sitosterol-D-glucoside **(1)**, aloe-emodin **(2)**, genistein **(3)**, 1, 3, 6-trihydroxy-8-methyl-anthraquinone **(4)**, และ 3-(1-C-β-D-glucopyranosyl)-2, 6-dihydroxy-5-methoxybenzoic acid **(5)** จากการทดสอบพบว่า aloe-emodin **(2)** มีฤทธิ์ต้านเอนไซม์ HIV-1 integrase ได้ 31.91 % และ 1, 3, 6-trihydroxy-8-methyl-anthraquinone **(4)** มีฤทธิ์ 19.59 % ที่ความเข้มข้น 100 μM ตามลำดับ ส่วนสารบริสุทธ์ที่เหลือ **(1, 3, 5)** ไม่แสดงฤทธิ์ต้านเอนไซม์ HIV-1 integrase นอกจากนี้สารบริสุทธิ์ **2-5** เป็นสารที่แยกได้ครั้งแรกจากจากต้นมะเดื่อชุมพร

Thesis Title Study on anti-HIV-1 integrase activity of Thai medicinal plants

Author Miss Kingkan Bunluepuech

Major Program Herb Sciences (International Program)

Academic Year 2010

Abstract

The aqueous and EtOH extracts of eight Thai plants including *Clerodendron indicum* (whole plant), *Tiliacora triandra* (stem), *Capparis micracantha* (wood), *Harrissonia perforata* (wood), *Ficus glomerata* (wood), *Diospyros decandra* (wood), *Dracaena loureiri* (heartwood) and *Tinospora crispa* (stem) were screened for their inhibitory activities against HIV-1 integrase (IN) using the multiplate integration assay (MIA). *Dracaena loureiri* (heartwood, EtOH) possessed high %yield with 39.9 %w/w, followed by *Tinospora crispa* (stem, water, 12.6 %w/w), whereas those of other plants were 1.2-6.8 % w/w. Among EtOH extracts, *Ficus glomerata* (wood) showed the highest activity against HIV-1 IN with an IC_{50} value of 7.8 µg/ml; whereas the water extract of *Harrisonia perforata* (wood) was the most potent for aqueous extracts (IC_{50} = 2.3 µg/ml). It was found that the aqueous extract of *Harissonia perforata* exhibited anti-HIV-1 IN activity higher than that of suramin, a positive control (IC_{50} = 3.4 µg/ml). Other plant extracts possessed moderate to weak activity with IC_{50} values ranging from 22.1- >100 µg/ml.

Although the water extract of *Harrisonia perforat*a (wood) showed the highest activity against HIV-1 IN, however from the preliminary study found that it is difficult to separate. Therefore, the EtOH extract of *F. glomerata* (wood) which showed the highest activity against HIV-1 IN of EtOH extracts was isolated to obtain five pure compounds: β-sitosterol-D-glucoside **(1)**, aloe-emodin **(2)**, genistein **(3)**, 1, 3, 6-trihydroxy-8-methyl-anthraquinone **(4)** and 3-(1-C-β-D-glucopyranosyl)-2, 6-dihydroxy-5-methoxybenzoic acid **(5).** From the result, it was found that compound **2** (aloe-emodin) showed activity against HIV-1 IN with % inhibition of 31.91, followed by compound **4** (1, 3, 6-trihydroxy-8-methyl-anthraquinone) with % inhibition of 19.59 at 100 µM; whereas other compounds (**1**, **3**, **5**) were inactive. Moreover, **2-5** compounds were isolated for the first time from *F. glomerata*.

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LIST OF ABBREVIATIONS AND SYMBOLS

AIDS	=	acquired immunodeficiency syndrome
AP	=	alkaline phosphatase
br	=	broad (for NMR spectra)
br d	=	broad doublet (for NMR spectra)
C	=	cysteine
°C	=	degree celsius
CA	=	cytosine, adenine
^{13}C-NMR	=	carbon-13 nuclear magnetic resonance
cm	=	centimeter
d	=	doublet (for NMR spectra)
D	=	aspartic acid
dd	=	doublet of doublet (for NMR spectra)
DIG	=	digoxigenin
DMSO-d_6	=	dimethyl sulphoxide
DNA	=	deoxyribonucleic acid
DTT	=	dithiothritol
E	=	glutamic acid
EDTA	=	ethylenediaminetetraacetic acid
EtOH	=	ethanol
EtOAc	=	ethyl acetate
fmol	=	femtomole, an SI unit of amount of substance equal to 10^{-15} moles
g	=	gram
GT	=	guanine, thymine
H	=	histidine
HCL	=	hydrochloric acid
HIV-1	=	human immunodeficiency virus type 1

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

HIV-2	=	human immunodeficiency virus type 2
HMBC	=	heteronuclear multiple bond correlation
HMQC	=	heteronuclear multiple-quantum correlation
^{1}H-NMR	=	proton nuclear magnetic resonance
H_2O	=	water
HPLC	=	high performance liquid chromatography
IC_{50}	=	inhibitory concentration at 50% of tested subject
IN	=	integrase enzyme
IR	=	infrared
J	=	nuclear spin-spin coupling constant (in Hz)
KCl	=	potassium chloride
KD	=	kilo dalton molecular weight
Kg	=	kilogram
LTR-D	=	long terminal repeat donor
M	=	molar (concentration)
m	=	multiplet (for NMR spectra)
m	=	meter
MeOH	=	methanol
mg	=	milligram
MHz	=	megahertz
MIA	=	multiplate integration assay
mM	=	millimolar
$MnCl_2$	=	manganese(II) chloride
mol	=	mole
MOPS	=	3-(*N*-morpholino) propane sulfonic acid
MS	=	mass spectrometry

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

MW	=	molecular weight
m/z	=	mass to charge ratio
µg	=	microgram
µM	=	micromolar
Na_2CO_3	=	sodium carbonate
NaCl	=	sodium chloride
NIH	=	the national institute of health
NMR	=	nuclear magnetic resonance
OD	=	optical density (absorbance)
P	=	phosphorus
PBS	=	phosphate buffer saline
pH	=	potential of hydrogen
pmol	=	picomole
*p*N	=	*p*-nitrophenol
p-NP	=	*p*-nitrophenyl phosphate
PR	=	protease enzyme
PTLC	=	preparative thin layer chromatography
RNA	=	ribonucleic acid
RT	=	reverse transcriptase
s	=	singlet (for NMR spectra)
S.E.M	=	standard error mean
t	=	triplet (for NMR spectra)
TB	=	tuberculosis
TLC	=	thin-layer chromatography
TS	=	target substrate
UN AIDS	=	joint united nations programme on HIV/AIDS

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

UV	=	ultraviolet
UV-vis	=	ultraviolet and visible (spectrometry)
WHO	=	world health organization
w/w	=	weight/weight
δ	=	chemical shift (in ppm, for NMR spectra)
λ_{max}	=	maximum wavelength
/	=	per
%	=	the percent; portion of a total of 100

CHAPTER 1
INTRODUCTION

1.1 Introduction

1.1.1 Rationale and background for investigation

An acquired immunodeficiency syndrome (AIDS) has been rapidly spreading in many countries and is worldwide public health problem. It is caused by human immunodeficiency virus type 1 or HIV-1. Three enzymes that are essential for the HIV-1 life cycle are HIV-1 protease (PR), reverse transcriptase (RT) and integrase (IN). HIV-1 IN has become an appealing target for AIDS treatment since only one HIV-1 IN inhibitor named raltegravir. It is now available in the market. HIV-1 IN functions as a dimer and the integration process is composed of two steps: 3′- processing and 3′- joining (strand transfer) which finally integrates viral DNA into host chromosome (Katz and Skalka, 1994; Lucia, 2007). Nowadays, there are several drugs used clinically as HIV-1 RT and HIV-1 PR inhibitors; however, they have some side effects such as nausea, headache and fever (Richman et al., 1987). Thus, searching for HIV-1 IN inhibitors from natural sources is become an interesting target for AIDS treatment.

Eight Thai plants used for treatment of blood-related disease in Thai traditional medicine were investigated for their HIV-1 IN inhibitory activity. These plants contain in the preparation given by Virotpanthai Clinic in Nakhonsrithamarat province [traditional medical clinic] for treatment of AIDS patients. The selected eight Thai plants are *Diospyros decandra* Lour., *Dracaena loureiri* Gagnep., *Clerodendron indicum* Kuntze., *Tiliacora triandra* Diels., *Harrisonia perforata* Merr., *Capparis micracantha* DC., *Ficus glomerata* Roxb. and *Tinospora crispa* Miers ex Hook. F &Thom. *Clerodendron indicum*, *Harrisonia perforata*, *Capparis micracantha*, *Ficus glomerata* and *Tiliacora triandra* have been used to decrease fever and detox. *Diospyros decandra* has been used to enrich working of brains and tonic. *Dracaena loureiri* has been used as heart tonic, antipyretic and wound healing. *Tinospora crispa* has been used as anti-pyretic and tonic (Wutthithamavet, 1997). From the previous studies, they were reported that *Dracaena loureiri* exhibited antinociceptive and anti-pyretic activities in rats (Reanmongkol et

al., 2003). The extract of *Dracaena loureiri* and *Myristica fragrans* significantly inhibited proliferation of leukemia cell line (Chirataworn et al., 2005). The extracts of *Tiliacora triandra* and *Harrisonia perforata* inhibited *Plasmodium falciparum* (Saiin and Markmee, 2003; Nguyen-Pouplin et al., 2007). A water extract of *Tinospora crispa* decreased blood glucose and increased insulin levels in diabetic rats (Noor and Ashscoff, 1989), decreased fever in male white rat (Kongsaktrakoon et al., 1994), had bitter tonic effect (Temsiririrkkul et al., 1986), and possessed antioxidant activity (Cavin et al., 1998). The extract of *Ficus glomerata* was found to exhibit gastroprotective effect in rats (Rao et al., 2008).

Since anti-HIV-IN activity of eight Thai plants have not been studied so far, we are interested in study on the activity of these plants which could be developed as natural anti-HIV-IN agents in the future.

1.1.2 Occurring of AIDS and epidemic

AIDS occurring from retrovirus called human immunodeficiency virus or "HIV". There are two types which are HIV-1 (Figure 1-1) and HIV-2. The most occurring type is HIV-1 and it is found that South Africa has the largest number of HIV patients in the world. UN AIDS and the WHO estimate the AIDS has killed more than 25 million people since it was first recognized in 1981. Thailand is the third worst affected and it has reported that there are about 500,000 AIDS patients in Thailand.

1.1.3 Immune system related to HIV

An immune system is a system of biological structures and processes within an organism that protects against disease by identifying and killing pathogens and tumor cells. It detects a wide variety of agents, from viruses to parasitic worms, and needs to distinguish them from the organism's own healthy cells and tissues in order to function properly. Detection is complicated since pathogens can evolve rapidly, producing adaptations that avoid the immune system and allow the pathogens to successfully infect their hosts. When HIV virus infects the host cell, they destroy an immune system. Then pathogens (bacteria, virus, fungi, or protozoa)

can infect AIDS patients easily. HIV leads to immunosuppression that allows opportunistic pathogens to cause disease and death in AIDS patients such as tuberculosis and pneumonia. In the previously reports found that the Western Cape region of South Africa has one of the highest recorded incidence rates of tuberculosis (TB) that it has related with the number of AIDS patients in rate 1600/100,000 (Rangaka et al., 2007).

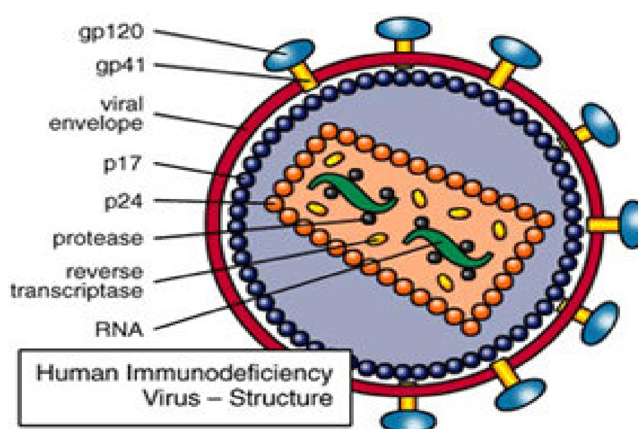


Figure 1-1 HIV-1 virus (Neolife Mission, 2009)

1.1.4 HIV-1 life cycle

HIV life cycle consists of five steps to infect T helper cells. Firstly, HIV binds and fuses to T helper cell and release its RNA to T-cell cytoplasm. Then, viral RNA converts to viral DNA using reverse transcriptase enzyme. After that, viral DNA enters host nucleus and integrates into host chromosomal DNA using HIV integrase. Next, HIV RNA is made and viral protease processes protein for viral assembly. Finally, newly made HIV virus is released and ready to infect other cells (Figure 1-2).

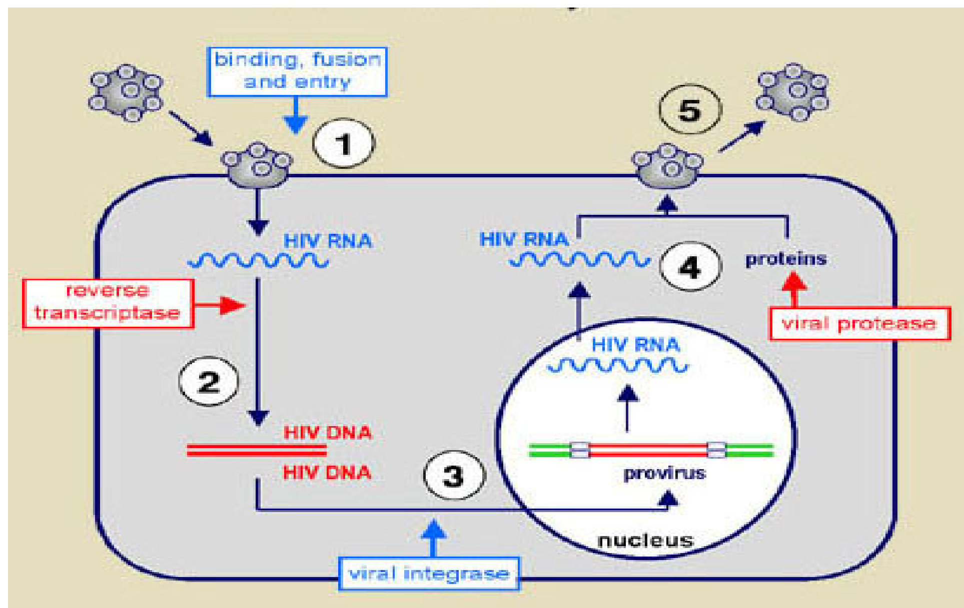


Figure 1- 2 HIV-1 life cycle (Harold, 2010)

1.1.5 HIV-1 IN structure

HIV-1 IN consists of 288 amino acids which is 32 KD of protein and it functions as a dimer. HIV-IN contains three domains that is composed of a N-terminal HH-CC zinc finger domain, a central catalytic domain and C-terminal domain (Figure 1-3). The active site is DD35E in a central catalytic domain (Katz and Skalka, 1994).

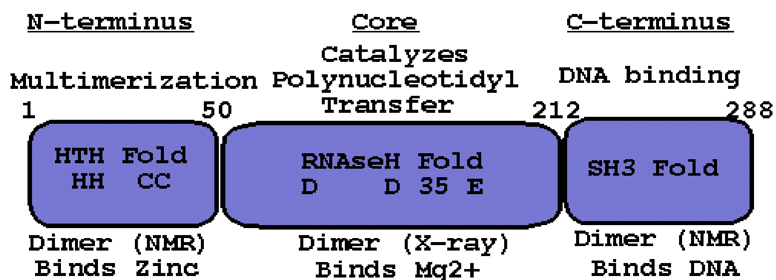


Figure 1-3 HIV-1 IN structure (Jame, 1998)

1.1.6 Function of HIV- 1 integrase enzyme

HIV-1 integrase acts to insert the proviral DNA into the host chromosomal DNA by catalyzing the excision of the last two nucleotides from each 3′end, leaving the terminal dinucleotide CA-3′OH at the recessed 3′ ends which is called 3′ processing. After transport to the nucleus as nucleoprotein complex, IN catalyzes a DNA strand transfer reaction involving the nucleophilic attack at these ends on the host DNA, which is called strand transfer or joining (Fujiwara and Mizuuchi, 1988; Katz and Skalka, 1994; Vink et al., 1994)(Figure 1- 4).

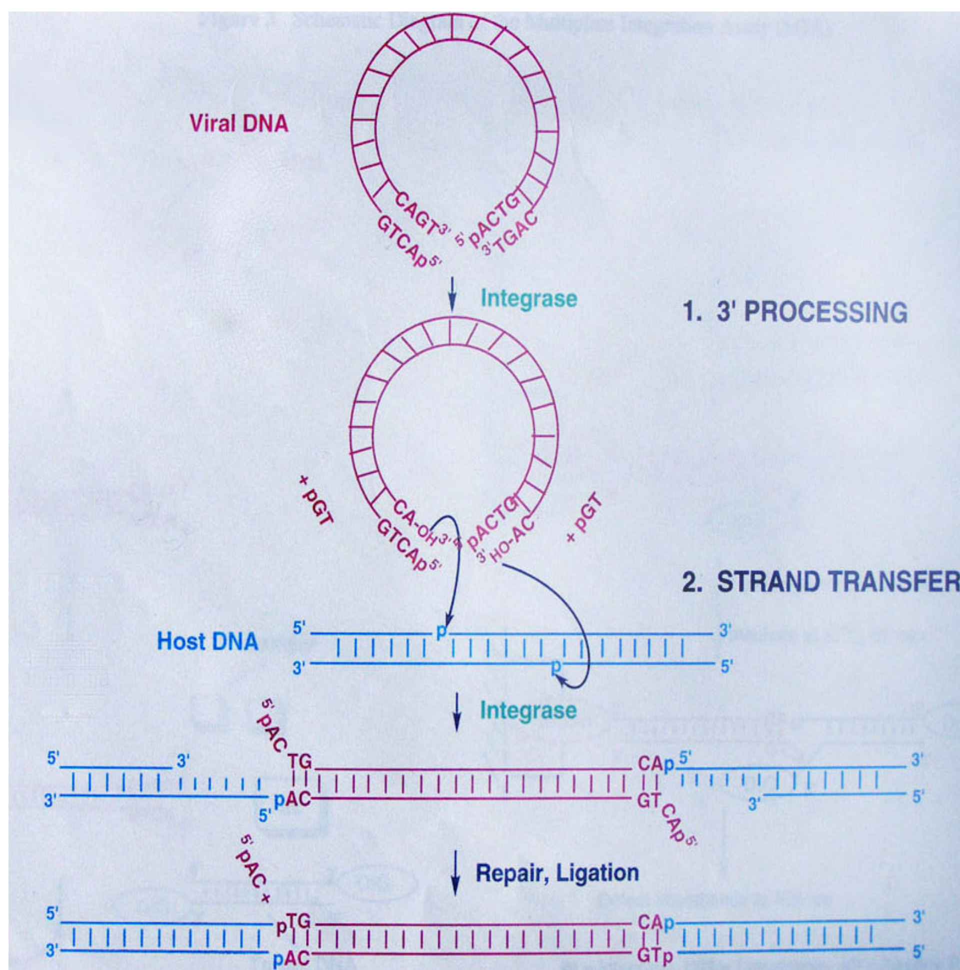


Figure 1-4 Function of HIV-1 integrase enzyme

1.1.7 Example of radio-labelled assay for integrase inhibitor screening

The radio-labelled assay used for screening integrase inhibitor is as follow; the 21-mer oligodeoxynucleotide is radio-labelled with ^{32}P at the 5′-terminus. Recombinant integrase catalyzes the last two oligonucleotide from 3′-end. Release of GT dinucleotide at the 3′-end of the radio-labelled strand generates 19-mer oligonucleotide that can be readily separated from the 21-mer substrate using gel electrophoresis. Figure 1-5 showed differential effect of 3′-processing and strand-transfer inhibitors (Figure 1-5 part I and II). This method is used for anti-HIV-1 integrase activity assay from *Salvia miltiorrhiza* (Ibrahim et al., 2002) and is used to detect both 3′ -processing and 3′-joining.

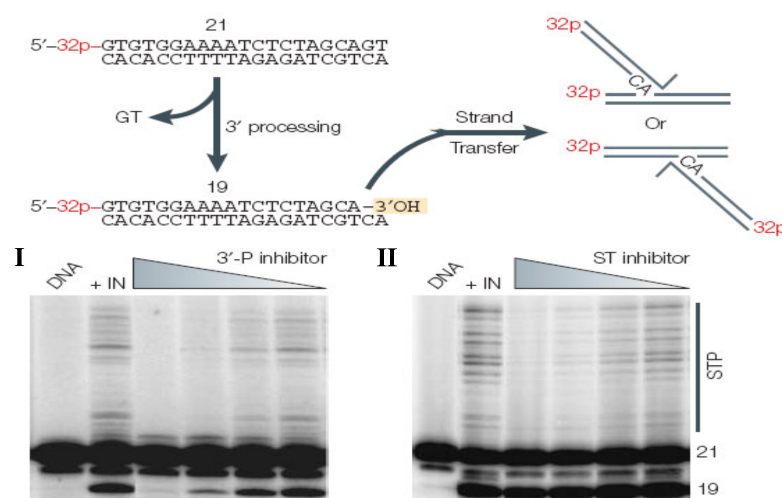


Figure 1-5 Radio-labelled assay for integrase inhibitor screening (Ibrahim et al., 2002)

Recently, there have been several reports on anti-HIV-1 IN assay using isotope-labelled substrate and denaturing gel separation of products (Ibrahim et al., 2002). However, they are inconvenient and time consuming, especially when screening inhibitors from many samples. Lately, an assay for HIV-1 IN activity using DNA-coated plates has been reported in a few reports (Chang et al., 1996; Hazuda et al., 1994; Vink et al., 1994). It is a non-radioisotopic technique and can be used for screening the inhibitory activity of plant extracts or any compounds against HIV-1 IN. In this method, 96 well plates were used for the screening test called a

multiplate integration assay (MIA). It is simple, convenient and accurate and doses not require the centrifugation, electrophoresis or other DNA denaturation steps. This assay screens for both 3′-processing and 3′- strand transfer and can be used without any exposure to radioisotopes. In this study, we therefore used this assay method for screening the HIV-1 IN inhibitory substances. MIA is the method to measure the incorporation of digoxigenin-labelled target DNA in to long terminal repeat (LTR) donor DNA. For this assay, a biotin-labelled donor DNA is added into each well, which strongly bind with a streptavidin coated- well plate, followed by addition of digoxiginin-labelled target DNA, integrase enzyme and sample solution. After integration process, the ligated two double-stranded DNA is immobilized on streptavidin-coated wells and subsequently bound with an alkaline phosphatase (AP)-labelled anti-digoxigenin antibody. Finally, it is colorized by adding *p*-nitrophenylphosphate as a substrate, In basic solution (pH 9.5), AP hydrolyzes *p*-nitrophenylphosphate to *p*-nitrophenol which exhibits a yellow color.

The screening of medicinal plants for HIV-1 IN inhibitory activity has been a promising approach to search for compounds that act as HIV-1 IN inhibitors.

1.2 Literature review

1.2.1 Thai medicinal plants used for AIDS treatment

There are several plants showing anti-HIV, anti-HIV- RT and anti-HIV- PR activities. They are listed in Table 1-1.

Table 1-1 Thai medicinal plants used for AIDS treatment (Project Herbs for AIDS, 2003)

Botanical name	Family	Part used / Extract	Anti-HIV			Anti-HIV-RT			Anti-HIV-PR		
			Conc. (μg/ml)	% In-hibition	IC_{50} (μg/ml)	Conc. (μg/ml)	% In-hibition	IC_{50} (μg/ml)	Conc. (μg/ml)	% In-hibition	IC_{50} (μg/ml)
จิงจ้อขน *Merremia vitifolia*	Convolvulaceae	Fresh stem /EtOH	62.5	93	30						
		Dry stem/ EtOH	166.7	96	71.5	ND			ND		
		Dry stem/ H_2O	60	96	17.6						
น้อยโหน่ง *Annona reticulate*	Annonaceae	Fresh leaf/ EtOH	ND			250	NA	ND	40	60	ND
ปัตตาเวีย *Jatropha integerrima*	Euphorbiaceae	Leaf/ EtOH	ND			250	NA	ND	66.6	100	ND
		Leaf/ H_2O				250	50.6	ND	200	NA	ND
พิทูเนีย *Petunia x hybrid*	Solanaceae	Fresh and dry aerial part/EtOH and H_2O	ND			250	NA		NA		

Table 1-1 Thai medicinal plants used for AIDS treatment (continued)

Botanical name	Family	Part used / Extract	Anti-HIV			Anti-HIV-RT			Anti-HIV-PR		
			Conc. (µg/ml)	% Inhibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% Inhibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% Inhibition	IC_{50} (µg/ml)
ขันทองพยาบาท *Suregada multiflorum*	Euphorbiaceae	Fresh seed and fruit/EtOH Fresh seed and fruit/ H_2O	ND			ND			ND		
ข่า *Alpinia galanga*	Zingiberaceae	Fresh and dry rhizome/EtOH and H_2O	ND			250	NA	ND	ND		
แคแสด *Spathodea campanulata*	Bignoniaceae	Fresh bark/ EtOH	ND			250	NA	ND	66.6	88.6	ND
		Dry bark/ H_2O				250	52.2	242	200	85	ND
		Fresh leaf / EtOH				250	NA	ND	66.6	88.3	ND
		Dry leaf/ H_2O				250	53.9	236	200	96	ND

Table 1-1 Thai medicinal plants used for AIDS treatment (continued)

Botanical name	Family	Part used / Extract	Anti-HIV			Anti-HIV-RT			Anti-HIV-PR		
			Conc. (μg/ml)	% In-hibition	IC_{50} (μg/ml)	Conc. (μg/ml)	% In-hibition	IC_{50} (μg/ml)	Conc. (μg/ml)	% In-hibition	IC_{50} (μg/ml)
มะรุม *Moringa oleifera*	Moringaceae	Fresh leaf /EtOH	166.7	NA	ND	250	NA	ND	66.6	66.6	ND
		Fresh young pod/EtOH	10.0	NA	ND	250	NA	ND	66.6	86.6	ND
		Dry leaf /50% EtOH	125	78	125	250	43.7	ND	100	33.3	ND
		Dry young pod / 50% EtOH	125	NA	ND	250	NA	ND	100	NA	ND
		Dry leaf / H_2O	125	NA	ND	250	41.8	ND	200	NA	ND
		Fresh old pod/EtOH	166.7	NA	ND	250	NA	ND	66.6	50	ND
		Dry old pod / 50% EtOH	125	78	125	250	41.3	ND	100	NA	ND
		Dry old pod / H_2O	250	78	250	250	41.3	ND	200	100	ND

Table 1-1 Thai medicinal plants used for AIDS treatment (continued)

Botanical name	Family	Part used / Extract	Anti-HIV			Anti-HIV-RT			Anti-HIV-PR		
			Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)
รำเพย *Thevetia peruviana*	Apocynaceae	Fresh leaf/ EtOH	7.5	NA	ND	50	70	ND	250	ND	ND
		Fresh leaf/ H_2O	30.0	NA	ND	200	100	ND	250	NA	ND
		Dry leaf/ EtOH	7.5	NA	ND	50	70	ND	250	ND	ND
		Dry leaf/ H_2O	30.0	NA	ND	200	NA	ND	250	ND	ND
ลั่นทมขาว *Plumeria obtusa*	Apocynaceae	Fresh and dry branch) /EtOH and H_2O	ND			NA			ND		
ลิ้นงูเห่า *Clinacanthus siamensis*	Acanthaceae	Fresh leaf/ EtOH	ND			250	NA	ND	18.1	65	ND
สบู่แดง *Jatropha gossypifolia*	Euphorbiaceae	Dry stem and leaf)/EtOH	ND			250	21.6	ND	200	16.6	ND
		Dry stem and leaf/ H_2O				250	NA	ND	200	22.2	ND

Table 1-1 Thai medicinal plants used for AIDS treatment (continued)

Botanical name	Family	Part used / Extract	Anti-HIV			Anti-HIV-RT			Anti-HIV-PR		
			Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)
สบู่เลือด *Stephania venosa*	Menispermaceae	Dry tuber orange /H_2O	250	87.4	ND	50	39.3	103.8	ND		
		Dry tuber yellow /H_2O	250	68.8	ND	50	20.6	170.3			
เสม็ด *Melaleuca cajuputi*	Myrtaceae	Oil from fresh leaf	250	14.3	NA	250	68.8	111.1	18.1	ND	ND
		Fresh leaf /EtOH	250	45.5	NA	250	48.7	ND	18.1	ND	ND
		Dry leaf/$CHCl_3$ from EtOH	250	ND	ND	250	75.6	74.6	18.1	ND	ND
		Fresh leaf/ $CHCl_3$	250	ND	ND	250	63	ND	18.1	55	ND
หญ้าคา *Imperata cylindrica*	Gramineae	Fresh rhizome /EtOH	ND			250	NA	ND	66.6	98	ND
		Fresh rhizome/ H_2O				250	NA	ND	100	60	ND
		Dry rhizome /EtOH				250	NA	ND	66.6	30	ND

Table 1-1 Thai medicinal plants used for AIDS treatment (continued)

Botanical name	Family	Part used / Extract	Anti-HIV			Anti-HIV-RT			Anti-HIV-PR		
			Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)	Conc. (µg/ml)	% In-hibition	IC_{50} (µg/ml)
หญ้าชันกาด *Panicum repens*	Gramineae	Dry rhizome /EtOH	ND			250	NA	ND	66.6	NA	ND
		Dry rhizome/ H_2O				250	NA	ND	200	50	ND
หญ้าเอ็นยืด *Plantago major*	Plantaginaceae	Whole plant /EtOH	ND			250	30	ND	40	0	ND

* ND = not determined

NA = negative

1.2.2 Description of *Ficus glomerata*

Ficus glomerata synonym; *Ficus racemosa* is a plant in the Moraceae family. Thai name is Ma duea chumphon. It is medium size to large evergreen or occasionally deciduous tree and found all over India and Southeast Asia (Rao et al., 2008). The tree is up 18 m high, leaves ovate, ovate-lanceolate or elliptic, subacute, entire and petiolate. Leaves are shed by December and replenished by January and April, when the tree becomes bare for a short period. *Ficus* spp. subglobose or pyriform, red when ripe, borne in large clusters, on short, leafless branched emerging from the trunk and the main branches. The tree is without aerial roots unlike its many family members. It naturally comes up in wasteland and forests in subtropical climate. It is seen dwelling in areas up to 1200 m altitude on hilltop. This requires well-drained medium to heavy soils for its successful cultivation and comes up in all kinds of soil except in water logged and

clay soil. The plant is propagated by using cuttings of stem and root suckers. Heartwood cutting 0.5 to 1.5 cm in diameter and about 30 cm long are taken from straight healthy 1-2 year old shoots and planted in December to February (Figure 1-6, 1-7). Seeds can also be used for propagation. Natural regeneration is very good from seeds dispersed by animals and birds (Paarakh, 2009; Atal et al., 1982).

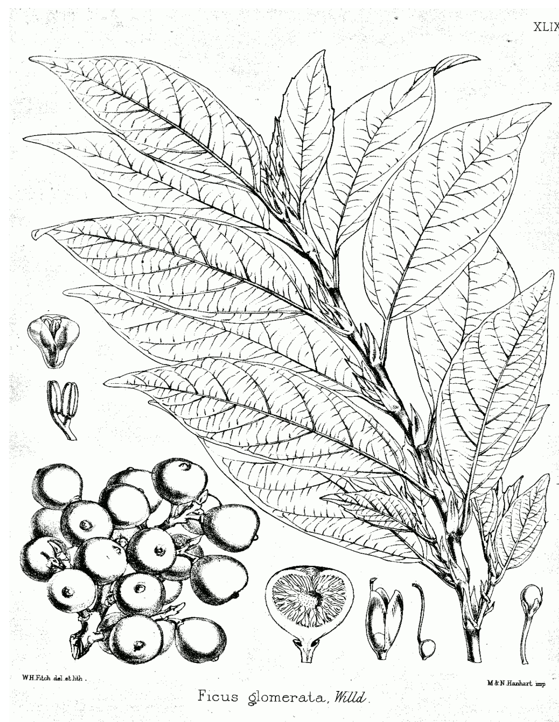


Figure 1- 6 *Ficus glomerata* (Dietrich, 1874)

Figure 1-7 *Ficus glomerata*

1.2.3 Review of compounds containing in *Ficus* spp. in Thailand

There have been several reports on chemical constituents isolated from *Ficus* spp. that have been used in Thai traditional medicine in Thailand. The identified compounds are presented in Tables 1-2 and Figures 5-8.

Table 1-2 Compounds isolated from *Ficus* spp. that have been used in Thai traditional medicine.

Compounds isolated from *Ficus benjamina*	References
Acetylglucosaminidase	
α- amyrin	
Baurenol	
benzoic acid	
bergapten	
β-carotene	
Catecholase	
Cerebroside	
Dopamine	
Ficin	
Furocoumarin	Bunyapraphatsara, 1999
germacrene D	
hematin	
heptulose	
4-hexanolide	
Imperatorin	
Linoleic	
Linolenic	
Lutein	
Neoxanthin	

Table 1-2 Compounds isolated from *Ficus* spp. that have been used in Thai traditional medicine (continued).

Compounds isolated from *Ficus benjamina*	References
Noradrenaline	Bunyapraphatsara, 1999
oleic acid	
palmitic acid	
serotonin	
β-sitosterol	
sorbic acid	
violaxanthin	
(9,11), (18,19)-disecoolean-12-en-28-oic acid	
serrat-3-one	Parveen et al., 2009
friedelin	
benjaminamide	Simo et al., 2008
psoralen	
β-amyrin acetate	
betulinic acid	
platanic acid	
β-amyrone	Farag, 2005
β-friedelanol	
taraxerol	
stigmasterol	
stigmasterol 3-*O*-β-D-glucopyranoside	
kaempferol	
kaempferol 3-*O*-β-D-glucopyranoside	
kaempferol 3-*O*-α-L-rhamnopyranosyl-(1$\rightarrow$6)-β-D-glucopyranoside	
kaempferol 3-*O*-α-L-rhamnopyranosyl-(1$\rightarrow$6)-β-D-galactopyranoside	

Table 1-2 Compounds isolated from *Ficus* spp. that have been used in Thai traditional medicine (continued).

Compounds isolated from *Ficus religiosa*	References
α- amyrin	Bunyapraphatsara, 1999
β- amyrin	
bergapten	
bergaptol	
campesterol	
fucosterol	
n-hentriacontane	
hexacosan -1-ol	
oleanolic acid methyl ester	
pelargonidin-5,7-dimethyl ether 3- *O* -α-L-rhamnoside	
β-sitosterol	
solanesol	
stigmasterol	
megastigmane glycoside	Cam et al., 2009
n-octacosanol	Swami et al., 1989
Me oleanolate	
lanosterol	
lupen-3-one	

Table 1-2 Compounds isolated from *Ficus* spp. that have been used in Thai traditional medicine (continued).

Compounds isolated from *Ficus hispida*	References
β-amyrin	Bunyapraphatsara, 1999
β-amyrin acetate	
bergapten	
hispidine	
oleanolic acid acetate	
pergularinine	
β-sitosterol	
triacontan-1- ol acetate	
tylophorinidine-*O*-methyl	
3',4',5',5,7-pentamethoxy-4-acetyldel phinidin-3-*O*-α-L-rhamnoside	Asem et al., 2008
4',5,7-trimethoxy pelargonidin-6-C-glucopyranosyl-3-*O*-α-L-rhamnoside	
3',4',5',5,7-pentamethoxy delphinidin-3-*O*-α-L-rhamnoside	
ketoester, 24-ketopentacosyl-γ-hydroxypentanoate	
leucocyanidin-3-*O*-α-D-glycopyranosyl-(1$\rightarrow$4)-*O*-β-D-arabinopyranoside	Yadava, 1990
gluanol acetate	Acharya and Kumer, 1984
norisoprenoid	Peraza et al., 2002
O-methyltylophorinidine	
postpollinated	Song Q et al., 2001
postparasitized	
linalool	
palmitic oil	
9,12-octadecadienoic acid	

Table 1-2 Compounds isolated from *Ficus* spp. that have been used in Thai traditional medicine (continued).

Compounds isolated from *Ficus hirta*	References
5-methoxyl-4,2'-epoxy-3-(4',5'-dihydroxyphenyl)-linear pyranocoumarin	Ya et al., 2010
3-acetyl-3,5,4'-trihydroxy-7-methoxylflavone	
psoralen	Ya et al., 2008
umbelliferon	
5,3',4'-trihydroxy-3,7-dimethoxyflavone	
norartocarpetin	
5-hydroxy-3,7,4'-trimethoxyflavone	
kaempferol	
astragalin	
acacetin 7-*O*-β-D-glucopyranoside	
luteolin 7-*O*-β-D-glucopyranoside	
narigenin	
daucosterol	Li et al., 2006
β-sitosterol	
stigmasterol	
psoralene	
3β-hydroxy-stigmast-5-en-7-one	
5-hydroxy-4',6,7,8-tetramethoxy flavone	
4',5,6,7,8-pentamethoxy flavone	
4',5,7-trihydroxy-flavone	
3β-acetoxy-β-amyrin	
3α-acetoxy-α-amyrin	
hesperidin	

1.3 Biological activities of *Ficus glomerata*

There are several reports on biological effects of *Ficus glomerata*. A green fruit of *Ficus glomerata* has been reported for anti-oxidant activity (Verma et al., 2010). *Ficus glomerata* extract showed good activity against chemically induced renal carcinogenesis and oxidative damage response in Wistar rats (Khan et al., 2005), reduced the blood sugar level in rats (Rahman, et al., 1994), antidiuretic (Rastnasooriya et al., 2003), antitussive (Bhaskara et al., 2003), hepatoprotective (Mandal et al., 1999), radio protective effect (Veerapur et al., 2007), antiulcer (Rao et al., 2008 ; Patel et al., 1985), wound healing (Biswas et al., 2003), anti-inflammatory (Mandal et al., 2000 ; Forestieri et al., 1996 ; Li et al., 2003), anthelmintic (Chandrashekhar et al., 2008), antifilarial (Mishra et al., 2005), antidiarrhoeal (Mukherjee et al., 2006), analgesic (Malairajan et al., 2006), antipyretic (Rao et al., 2002), antifungal (Vonshak et al., 2003 ; Deraniyagala et al., 1998) and antibacterial activities (Mandal et al., 2000).

The fruits of *Ficus glomerata* are effective against leprosy, blood diseases, fatigue, bleeding nose and cough. Its bark is helpful against asthma and its leaves are used against bronchitis. It is used as vermifuge and an anti-dysentery drug. The extract of fruit is used in diabetes. The plant is used locally to relieve inflammation of skin wounds. The alcoholic extract of the stem bark possessed antiprotozoal activity against *Entamoeba histolytica*. It is used in the treatment of mumps, smallpox and inflammatory conditions (Khan et al., 2005).

1.4 Chemical constituents of *Ficus glomerata*

Regarding constituents of *Ficus glomerata*, the aerial part of this plant contains β-sitosterol, lupeol and quercetin (Verma et al., 2010). The stem bark showed the presence of two leucoanthocyanins: leucocyanidin-3-*O*-β-glucopyranoside, leucopelarogonidin-3-*O*-α-L-rhamno-pyranoside, β-sitosterol, cerylbehnate, lupeol and α-amyrin acetate. From trunk bark, lupeol, β-sitosterol and stigmasterol were isolated. The fruit contains gluanol, hentriacontane, β-sitosterol, gluanol acetate, glucose, tiglic acid, ester of taraxasterol, lupeol acetate and friedelin. A tetracyclic triterpene glauanol aetate which is characterized as 13α, 14β, 17 βH, 20 αH-lanosta-8, 22-diene-3 β-acetate and racemosic acid were isolated from the leaves (Figure 1-9).

An unusual thermostable aspartic protease was isolated from latex of the plant (Paarakh, 2009). The structure of HIV-1 IN inhibitor, raltegravir, is shown in Figure 1-9.

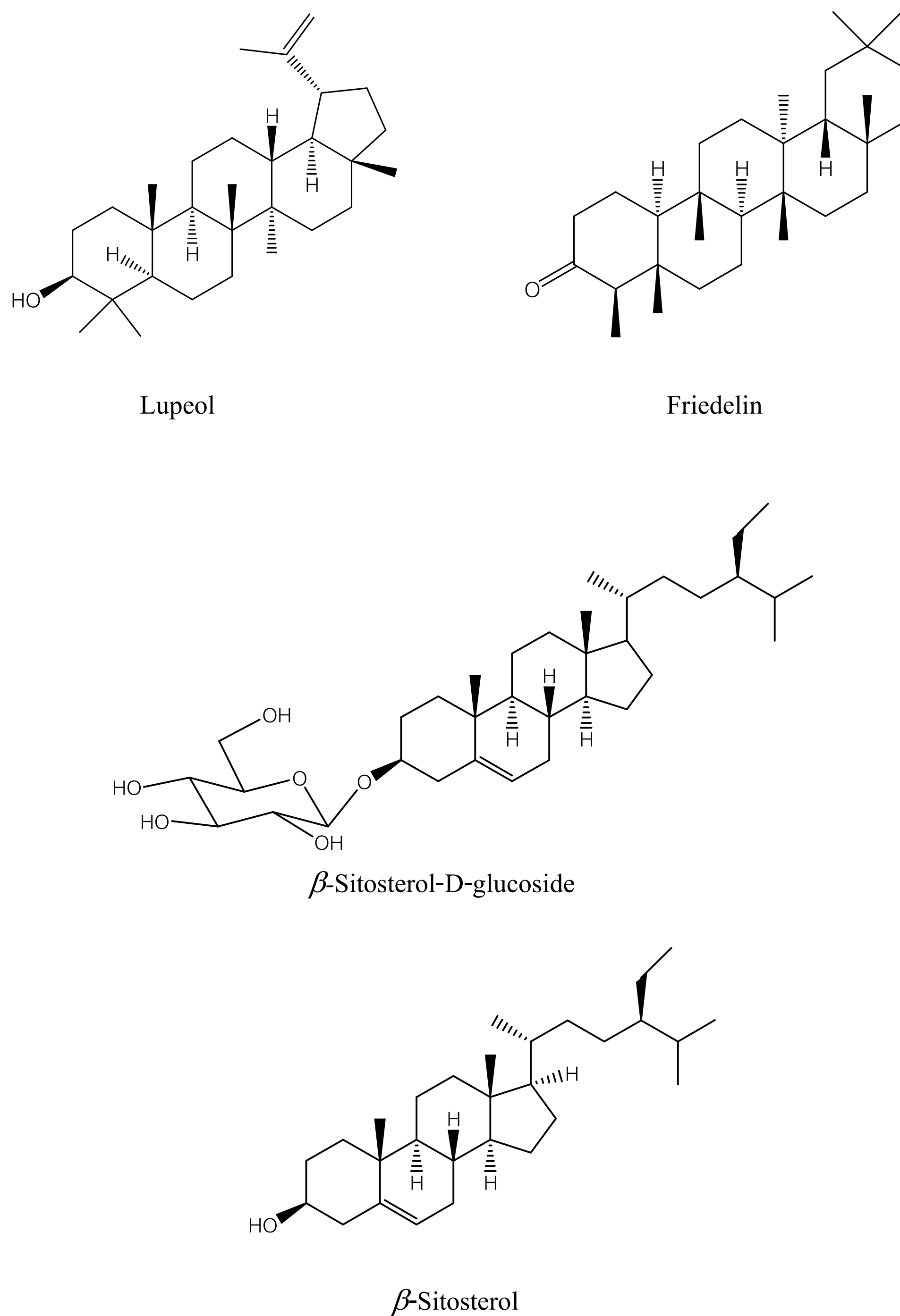
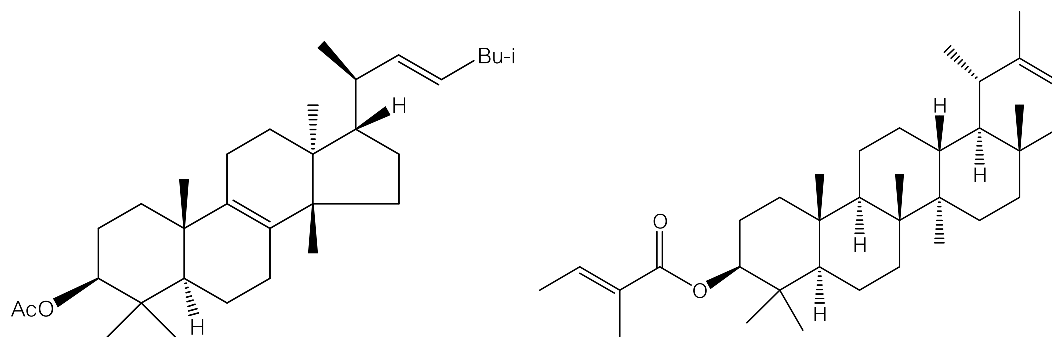
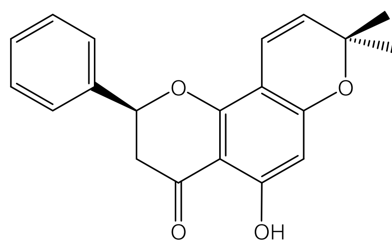


Figure 1-8 Chemical structures of compounds isolated from *Ficus glomerata*

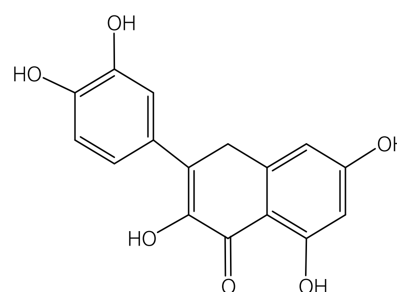


Gluanol acetate

Ester of taraxasterol



Racemosic acid



Quercetin

Figure 1-8 Chemical structures of compounds isolated from *Ficus glomerata* (continued)

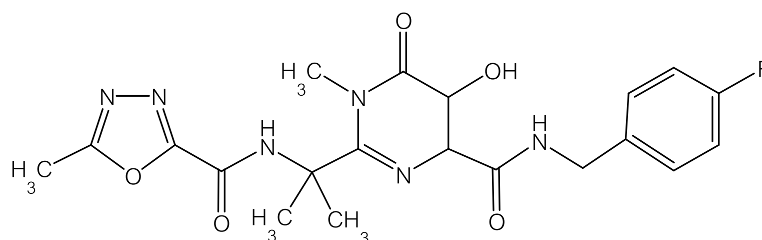


Figure 1-9 Chemical structure of raltegravir

1.5 Thai plants showing anti-HIV-1 IN activity

Some Thai plants have been reported for their HIV-1 IN inhibitory activity on both ethanol and water extracts as shown in Table 1-3 (Tewtrakul et al., 2003).

Table 1-3 Thai medicinal plants showing anti HIV-1 IN activity

Botanical name	Family	Part- used	Extract	IC_{50} (μg/ml)
Acacia concinna DC.	Mimosaceae	Leaf	Ethanol	3.8±0.4
Adhatoda vasica Nees.	Acanthaceae	Leaf	Ethanol	12.0±2.1
Andrographis paniculata	Acanthaceae	Leaf	Ethanol	12.0±2.9
Wall ex. Ness.			Water	1.5±0.3
Baleria lupulina Lindl.	Acanthaceae	Leaf	Ethanol	10.0±2.0
			Water	10.0±1.8
Bixa orellana L.	Bixaceae	Leaf	Ethanol	2.2±0.4
			Water	0.7±0.1
Bixa orellana L.	Bixaceae	Seed	Ethanol	3.0±0.6
			Water	0.3±0.1
Calophyllum inophyllum L.	Guttiferae	Leaf	Ethanol	4.5±0.8
			Water	4.0±0.5
Cassia angustifolia Vahl.	Caesalpiniaceae	Leaf	Ethanol	4.9±1.4
Cassia fistula L.	Caesalpiniaceae	Fruit	Ethanol	10.0 ±2.0
			Water	2.8±0.5
Clinacanthus nutans Lindau.	Acanthaceae	Leaf	Ethanol	2.8±0.2
			Water	2.5±0.3
Coleus parvifolius Benth.	Labiatae	Arial parts	Ethanol	9.2±2.9
			Water	2.0±0.6
Combretum quadrangulare	Combretaceae	Leaf	Ethanol	2.5±0.2
Kurz.			Water	2.9±0.6
Croton sublyratus Kurz.	Euphorbiaceae	Leaf	Ethanol	3.0±0.4
Derris scandens Benth.	Papilionaceae	Leaf	Ethanol	3.9±1.2

Table 1-3 Thai medicinal plants Thai medicinal plants showing anti HIV-1 IN activity (continued)

Botanical name	Family	Part-used	Extract	IC_{50} (μg/ml)
Hibiscus sabdariffa L.	Malvaceae	Flower	Water	1.4±0.2
Lawsonia inermis L.	Lythraceae	Leaf	Ethanol	2.1±0.4
			Water	3.3±0.4
Morinda citrifolia L.	Rubiaceae	Leaf	Ethanol	1.2±0.3
			Water	6.0±1.2
Myristica fragrans L.	Myristicaceae	Leaf	Ethanol	3.0±0.4
			Water	2.3±0.3
Ocimum basilicum L.	Labiatae	Leaf	Water	6.0±2.0
Ocimum canum Sims.	Labiatae	Leaf	Ethanol	1.6±0.3
Piper betle L.	Piperaceae	Leaf	Ethanol	4.0±0.4
Piper nigrum L.	Piperaceae	Fruit	Water	8.0±1.2
Piper ribesoides Wall. (A*)	Piperaceae	Stem	Water	0.9±0.2
Piper ribesoides Wall. (A*)	Piperaceae	Leaf	Ethanol	0.6±0.3
			Water	0.5±0.1
Piper ribesoides. (B*)	Piperaceae	Stem	Water	0.4±0.2
Piper ribesoides. (B*)	Piperaceae	Leaf	Ethanol	0.1±0.2
			Water	4.1±0.5
Piper sarmentosum Roxb.	Piperaceae	Leaf	Ethanol	1.2±0.4
Plumbago indica L.	Plumbaginaceae	Leaf	Ethanol	6.0±1.2
			Water	2.9±0.4
Psidium guajava L.	Myrtaceae	Leaf	Ethanol	2.5±0.5
			Water	1.7±0.3
Quisqualis indica L.	Combretaceae	Leaf	Ethanol	2.0±0.2
			Water	1.2±0.2
Rhinacanthus nasutus Kurz	Acanthaceae	Leaf	Ethanol	0.8±0.1
			Water	0.7±0.1

Table 1-3 Thai medicinal plants showing anti HIV-1 IN activity (continued)

Botanical name	Family	Part-used	Extract	IC_{50} (μg/ml)
Terminalia citrine Roxb. Ex. Flemming	Combretaceae	Fruit	Ethanol	2.7±0.5
			Water	0.3±0.1
Theobroma cacao L.	Sterculiaceae	Leaf	Ethanol	8.0±1.0
			Water	2.5±0.6
Thevetia peruviana Schum.	Apocynaceae	Leaf	Water	8.8±1.0
Thunbergia laurifolia L.	Thunbergiaceae	Arial parts	Ethanol	3.0±0.4
			Water	2.8±0.3
Tribulus terristris L.	Zygophyllaceae	Arial parts	Ethanol	8.0±1.4
Zingiber officinale Roscoe	Zingiberaceae	Rhizome	Ethanol	4.0±0.8
			Water	1.8±0.3
Zingiber zerumbet Smith	Zingiberaceae	Rhizome	Water	2.8±0.4

The result are the mean ± S.D. (n=4)

*A= lanceolate shaped leaf and ** B= cordate shaped leaf, IC_{50} = 50% inhibitory concentration on HIV-1 integrase

CHAPTER 2

RESEARCH METHODOLOGY

2.1 General

2.1.1 Equipments

Equipments	Company, Country
Balance	Explorer, OHAUS Corp, USA
Hot air oven	Memmert, Germany
IR spectrophotometer,	JASCO IR-810, Japan Spectroscopic, Japan
Mass spectrometer	MAT95 XL MS, Thermofinigan
Microplate reader	Biotek Power-x, BioTek Instruments, Inc, USA
Micropipette	Socorex, Switzerland; Pipetman, France
Rotary evaporator	Aspirator A-3S, EYELA, Japan
TLC cabinet	CN-6, Vilber Lourmat, France
TLC-plate silica gel GF_{254}	Merck, Germany
TLC-plate RP-18 F_{254s}	Merck, Germany
UV-VIS spectrophotometer	Genesis-6, Thermo scientific, USA
Water bath	Memmert, Germany

2.1.2 Chemicals

Chemicals	Company, Country
Acetic acid, glacial	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Anisaldehyde	Fluka, Switzerland
Chloroform, analytical grade	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Dichloromethane, analytical grade	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Ethanol (95%v/v)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Ethyl acetate, analytical grade	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Hexane, analytical grade	Lab-scan Asia Co., Ltd., Bangkok, Thailand
TLC-plate silica gel GF_{254}	Merck, Germany
TLC-plate RP-18 F_{254s}	Merck, Germany
Methanol	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Silica gel 60 (SiO_2 60, 230-400 mesh)	Merck, Germany
Sulfuric acid	J.T. Baker, USA

2.2 Plant materials

The plants were bought from traditional drug store in Nakhonsrithamarat province in 2008, and they were identified by Thai traditional doctor. They were *Clerodendron indicum* (whole plant), *Tiliacora triandra* (stem), *Capparis micracantha* (wood), *Harrissonia perforate* (wood), *Ficus glomerata* (wood), *Diospyros decandra* (wood), *Dracaena loureiri* (heartwood) and *Tinospora crispa* (stem). For *Ficus glomerata*, it was checked by microscopic technique comparing with standard samples (Sorlalum and Bunplang, 2007).

2.3 Screening for HIV-1-IN inhibitory activity of eight Thai plants

Twenty grams of each dried plant were extracted two times with water and ethanol separately (150 ml each) under reflux for 3 h. The solvents were removed under reduced pressure

to give the respective dry extracts and dissolved in 50% DMSO for bioassay. Sample solutions of these extracts were prepared in the concentration ranging from 3-100 µg/ml.

2.4 Preparation of the plant extract

Ten kilograms dried weight of *Ficus glomerata* wood were ground and macerated with ethanol at room temperature, four times. The ethanolic (EtOH) extract was concentrated and partitioned between water and hexane, and successively partitioned with chloroform and water. After that, the water layer was partitioned with ethyl acetate (EtOAc). Each partition was evaporated to dryness *in vacuo* to give residues of hexane, chloroform, EtOAc and water fractions, respectively (Figure 2-1).

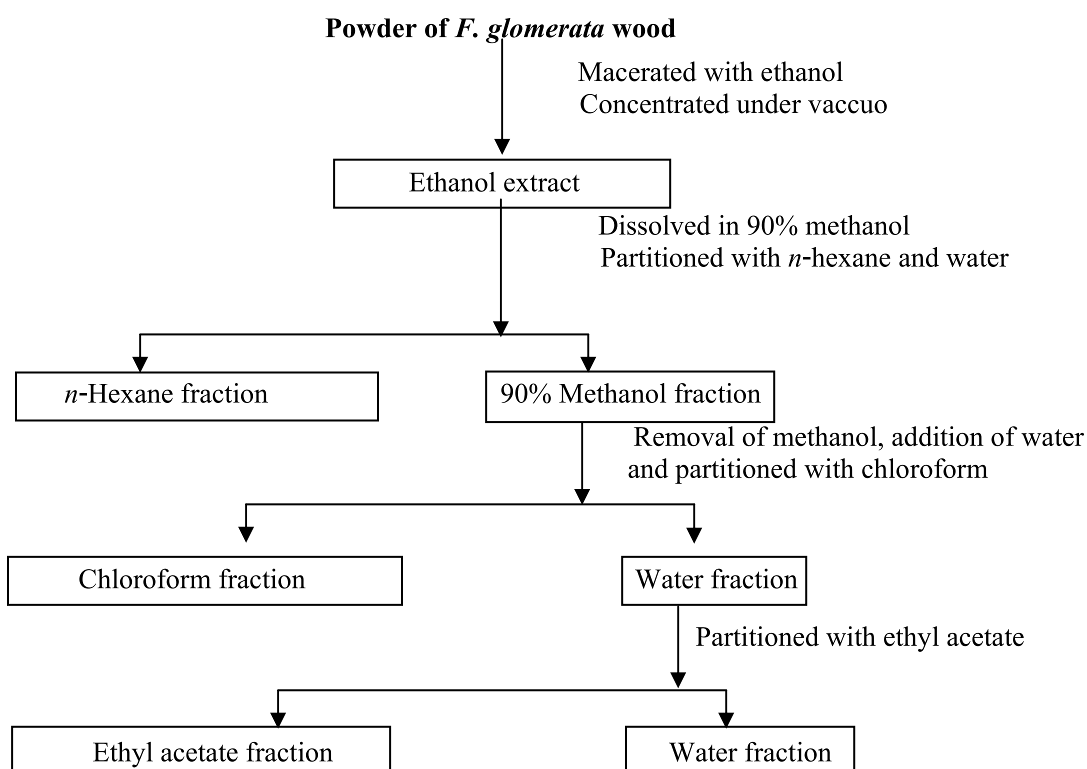


Figure 2-1 Flow chart of separation and partition of *Ficus glomerata*

2.5 Purification of compounds

Fractions of *Ficus glomerata* were purified using chromatography techniques such as classical column chromatography (silica gel, Sephadex LH-20), preparative thin layer chromatography (PTLC), followed by high performance liquid chromatography (HPLC). After that, compounds were tested for their purification using thin layer chromatography (TLC), and the structures were interpreted using spectroscopic techniques.

2.6 Structure elucidation

Structure elucidation of compounds was interpreted using spectroscopic techniques such as ultraviolet visible spectroscopy (UV-Vis spectroscopy), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry (MS).

2.7 Multiplate integration assay (MIA) procedure

2.7.1 Principle of MIA

MIA is the method to measure the incorporation of digoxigenin-labelled target DNA into long terminal repeat (LTR) donor DNA. For this assay, a biotin-labelled donor DNA is added into each well, which strongly bind with a streptavidin coated-well plate, followed by addition of digoxigenin-labelled target DNA, integrase enzyme and sample solution. After integration process, the ligated two double-stranded DNA is immobilized on streptavidin-coated wells and subsequently bound with an alkaline phosphatase (AP)-labelled anti-digoxigenin antibody. Finally, it is colorized by adding *p*-nitrophenyl phosphate as a substrate. In basic solution (pH 9.5), AP hydrolyzes *p*-nitrophenyl phosphate to *p*-nitrophenol which exhibits a yellow color.

2.7.2 Enzyme

HIV-1 IN protein was kindly provided by Dr. Robert Craigie, the National Institute of Health (NIH), Bethesda, Maryland, USA. This enzyme was expressed in *Escherichia coli* and purified according to a previous method (Goldgur et al., 1999), and stored at -80 °C before use.

2.7.3 Oligonucleotide substrates

Oligonucleotides of long terminal repeat donor DNA (LTR-D) and target substrate (TS) DNA were purchased from QIAGEN Operon, USA and stored at -25°C before use. The sequence of biotinylated LTR donor DNA and its unlabelled complement were 5'-biotin-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3' (LTR-D1) and 3'-GAAAATCAGTC-ACACCTTTTAGAGATCGTCA-5' (LTR-D2), respectively. Those of the target substrate DNA (digoxigenin-labelled target DNA, TS-1) and its 3'-labelled complement were 5'-TGACCAA-GGGCTAATTCACT-digoxigenin and digoxigenin-ACTGGTTCCCGATTAAGTGA-5' (TS-2), respectively.

2.7.4 Annealing of the substrate DNA

The anti-HIV-1 IN assay was carried out following the procedure in a previous report (Tewtrakul et al., 2001). Two separate solutions, the first containing LTR-D1 and LTR-D2 and the second containing TS-1 and TS-2 were made to concentrations of 2 pmol/µl and 5 pmol/µl, respectively by dilution with a buffer solution [containing 10 mM Tris-HCl (pH 8.0), 1mM EDTA and 100 mM KCl]. The LTR- and TS solutions were heated at 85 °C for 15 min in an incubator. After heating, each solution was gradually cooled to room temperature. Both solutions were then stored at -20 °C until use.

2.7.5 Pretreatment of the multiplate (Microplate)

A 96 well plate was coated with 50 µl of streptavidin solution containing 40 µg/ml streptavidin, 90 mM Na_2CO_3 and 10 mM KCl. After discarding streptavidin coating solution, the coated plate was washed with sterilized water (270 µl) two times and PBS solution (270 µl) two times. Then the blocking buffer (270 µl) containing 1% skim milk in PBS was added into each well, and the plate was kept gently at roomtemperature for 30 min. After discarding the blocking buffer, each well was washed with PBS solution (270 µl) three times and then the PBS solution was removed completely. A biotinylated -LTR donor DNA (50 µl) solution containing 10 mM Tris-HCl (pH 8.0), 1mM NaCl and 40 fmol/ µl of LTR donor DNA was added into each well and kept gently at room temperature for 60 min. After discarding the LTR donor solution, the microplate was washed with PBS solution (270 µl) three times and then each well was filled with 270 µl of PBS solution. Just before the integration reaction, the PBS solution of each well was discarded and rinsed with 270 µl of distilled water three times, and then the distilled water was removed completely.

2.7.6 Integration reaction

A mixture (45 µl) composed of 12 µl of IN buffer [containing 150 mM 3-*(N*-morpholino) propane sulfonic acid, pH 7.2 (MOPS), 75 mM $MnCl_2$ 5 mM dithiothritol (DTT), 25% glycerol and 500 µg/ml bovine serum albumin], 1 µl of 5 pmol/µl digoxigenin-labelled target DNA and 32 µl of sterilized water were added into each well of a 96-well plate. Subsequently, 6 µl of sample solution and 9 µl of 1/5 dilution of integrase enzyme was added to the plate and incubated at 37 ^{o}C for 80 min. After wells were washed with PBS three times, 100 µl of 500 mU/ml alkaline phosphatase (AP) labelled anti-digoxigenin antibody were added and incubated at 37 ^{o}C for 1 h. The plate was washed again with washing buffer containing 0.05% Tween 20 in PBS three times and with PBS three times. Then, AP buffer (150 µl) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ and 10 mM *p*-nitrophenyl phosphate was added to each well and incubated at 37 ^{o}C for 1 h. Finally, the plate was measured with a microplate reader at a wavelength of 405 nm (Figure 2-2). A control composed of a reaction

mixture, 50 %DMSO and integrase enzyme, while a blank was buffer-E containing 20 mM MOPS (pH 7.2), 400 mM potassium glutamate, 1mM ethylenediaminetetraacetate disodium salt (EDTA. 2Na) 0.1% Nonidet-P 40 (NP-40), 20% glycerol, 1mM DTT and 4 M urea without the integrase enzyme (Tewtrakul et al., 2001). Suramin, a polyanionic HIV-1 IN inhibitor was used as a positive control. The % inhibition against HIV-1 IN was calculated as follows:

$$\% \text{ Inhibition against HIV-1 IN} = [(\text{OD control} - \text{OD sample})/\text{OD control}] \times 100$$

Where OD is the absorbance detected from each well at 405 nm.

2.8 Statistics

For statistical analysis, the values are expressed as mean ± S.E.M of four determinations. The IC_{50} values were calculated using the microsoft excel programme.

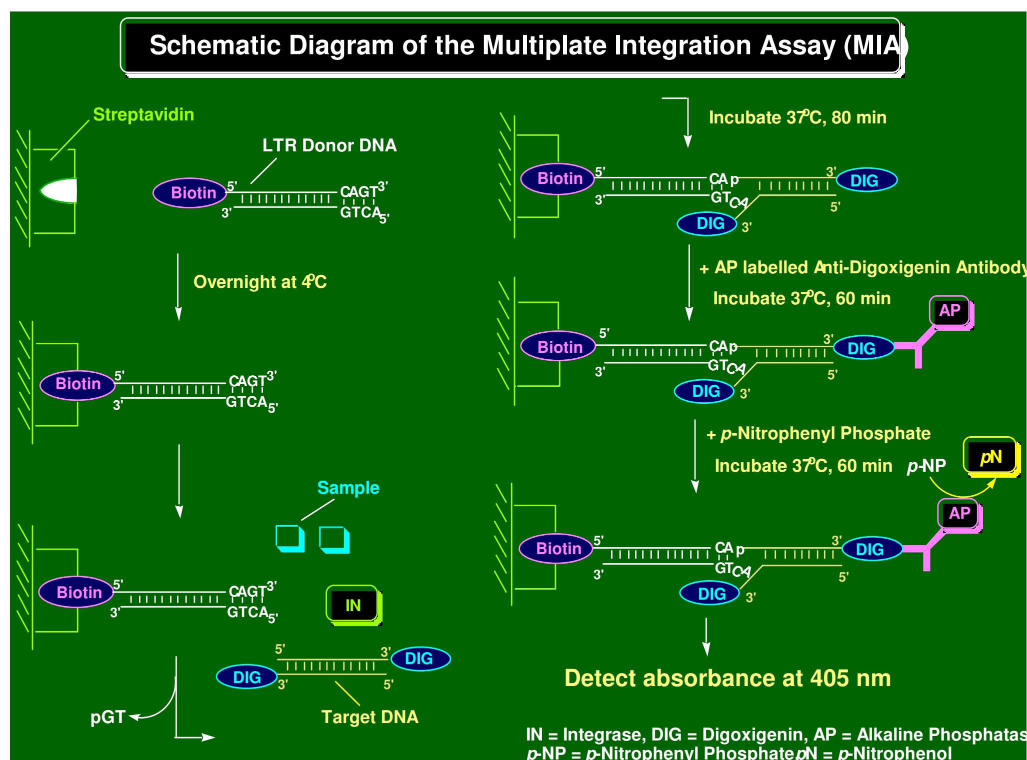


Figure 2-2 Diagram of the multiplate integration assay using the 96-well plate

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Screening for biological activities of eight Thai plants

The aqueous and EtOH extracts of eight Thai plants including *Clerodendron indicum* (whole plant), *Tiliacora triandra* (stem), *Capparis micracantha* (wood), *Harrissonia perforata* (wood), *Ficus glomerata* (wood), *Diospyros decandra* (wood), *Dracaena loureiri* (heartwood) and *Tinospora crispa* (stem) were screened for their inhibitory activities against HIV-1 integrase (IN) using the multiplate integration assay (MIA). From these plant extracts, *Dracaena loureiri* (heartwood, EtOH) possessed high %yield with 39.9 %w/w, followed by *Tinospora crispa* (stem, water, 12.6 %w/w), whereas those of other plants were 1.2-6.8 %w/w (Table 3-1). Of the EtOH extracts, *Ficus glomerata* (wood) showed the highest activity against HIV-1 IN with an IC_{50} value of 7.8 µg/ml; whereas the water extract of *Harrisonia perforata* (wood) was the most potent for aqueous extracts (IC_{50} = 2.3 µg/ml). It was found that the aqueous extract of *Harissonia perforata* exhibited anti-HIV-1 IN activity higher than that of suramin, a positive control (IC_{50} = 3.4 µg/ml). Other plant extracts possessed moderate to weak activity with IC_{50} values ranging from 22.1- >100 µg/ml (Table 3-2 and Figure 3-1).

Table 3-1 Part used and %yield of aqueous and ethanolic extracts of eight Thai plants

Botanical name	Family	Part used	Extract	Yield (% w/w)
Clerodendron indicum	Verbenaceae	whole plant	Ethanol	1.3
			Water	4.4
Tiliacora triandra	Menispermaceae	stem	Ethanol	2.0
			Water	3.5

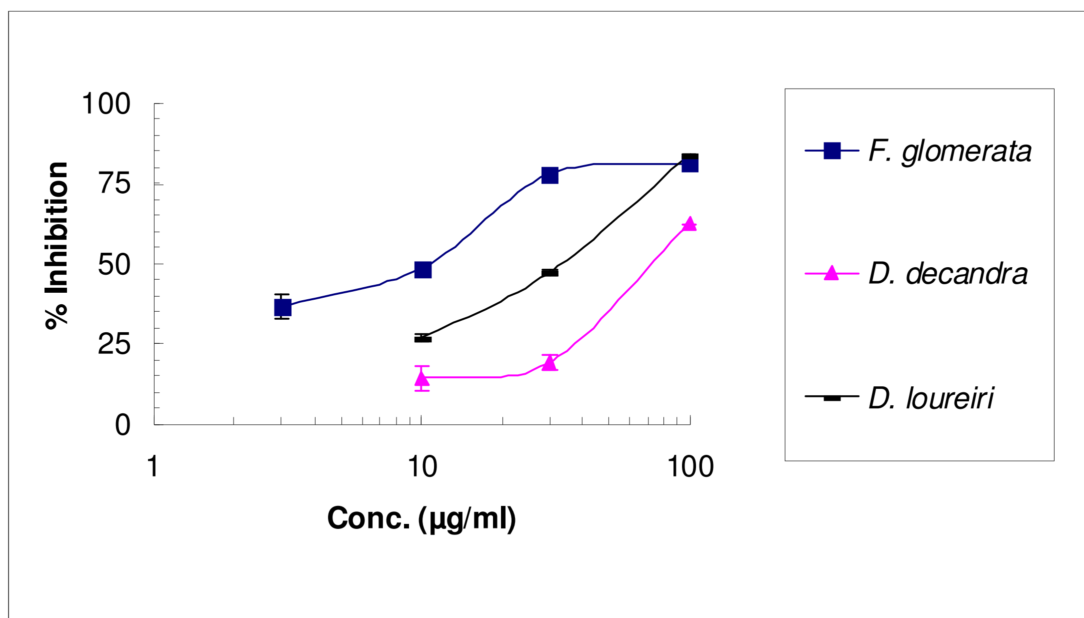
Table 3-1 Part used and %yield of aqueous and ethanolic extracts of eight Thai plants (continued)

Botanical name	**Family**	**Part used**	**Extract**	**Yield (% w/w)**
Capparis micracantha	Capparidaceae	wood	Ethanol	2.8
			Water	5.4
Harrisonia perforata	Simaroubaceae	wood	Ethanol	1.6
			Water	6.8
Ficus glomerata	Moraceae	wood	Ethanol	1.2
			Water	4.4
Diospyros decandra	Ebenaceae	wood	Ethanol	3.0
			Water	5.4
Dracaena loureiri	Agavaceae	heart wood	Ethanol	39.9
			Water	3.6
Tinospora crispa	Menispermaceae	stem	Ethanol	6.4
			Water	12.6

Table 3-2 % Inhibition and IC_{50} values of aqueous and ethanolic extracts of eight Thai plants against HIV-1 IN activity

Botanical name	% Inhibition at various concentrations (μg/ml)			Extract	IC_{50} (μg/ml)
	10	30	100		
Clerodendron indicum	-	-	27.94±3.27	Ethanol	>100
	23.58±2.29	34.30±1.86	71.06±1.73	Water	43.5
Tiliacora triandra	-	-	25.35±1.40	Ethanol	>100
	-	-	30.50±3.35	Water	>100
Capparis micracantha	-	-	13.37±3.20	Ethanol	>100
	-	-	17.08±3.11	Water	>100
Harrisonia perforata	0.31±1.02	7.93±1.12	32.59±0.71	Ethanol	>100
	64.48±2.55	92.25±0.72	95.13±0.42	Water	2.3
Ficus glomerata	48.56±3.62	78.07±1.20	81.12±1.43	Ethanol	7.8
	23.81±0.39	42.85±1.66	87.25±1.96	Water	29.5
Diospyros decandra	14.51±1.63	19.30±3.8	62.61±2.15	Ethanol	69.9
	19.91±0.88	55.34±1.99	83.91±1.30	Water	27.8
Dracaena loureiri	26.69±1.98	47.20±1.07	83.94±1.14	Ethanol	28.0
	22.31±2.25	64.92±1.45	94.55±1.52	Water	22.1
Tinospora crispa	-	-	11.39±3.15	Ethanol	>100
	-	-	9.54±1.57	Water	>100
Suramin	59.72±0.54	59.45±0.73	99.89±0.45	-	3.4

A



B

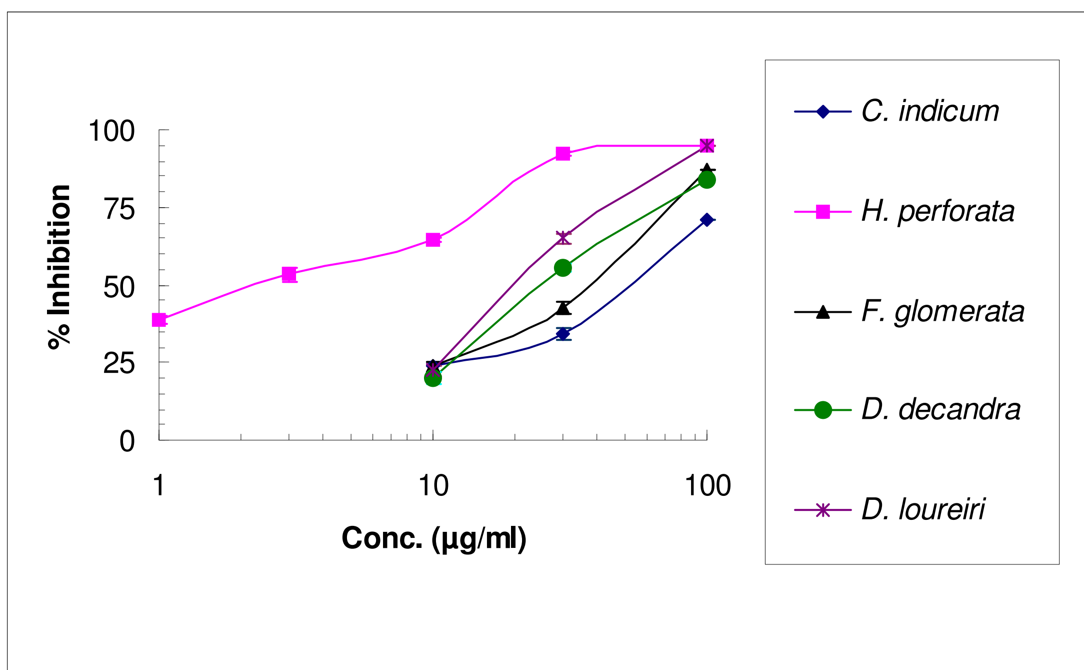


Figure 3-1 Dose-response curves of EtOH (A) and aqueous extracts (B) of Thai plants against HIV-1 IN

3.2 Screening on anti-HIV-1 IN activity of ethanolic extract and fractions from *Ficus glomerata*

From screening of eight Thai plants, the water extract of *Harrisonia perforata* (wood) showed the highest activity against HIV-1 IN with an IC_{50} value of 2.3 μg/ml followed by the EtOH extract of *Ficus glomerata* (wood) IC_{50} value = 7.8 μg/ml). However from the preliminary study of TLC, it was found that *Harrisonia perfarata* is difficult to separate. Therefore, *Ficus glomerata* was then selected for this study. We investigated the inhibitory activity of compounds isolated from this plant against HIV-1 IN.

Ten kilogram of dry wood of *Ficus glomerata* were cleaned, cut into small pieces and ground to powder. The powder (10 kg) was extracted four times with ethanol at room temperature. The solvent were removed under reduced pressure to give 192.9 g of crude extract and then partitioned between 90% methanol and hexane, removed of methanol, added of water and partitioned with chloroform. After that the water layer was partitioned with ethyl acetate. Each partition was evaporated to dryness in *vacuo* to give residues of hexane (39.6 g), chloroform (25.4 g), ethyl acetate (9.8 g) and water fraction (38.2 g) (Figure 3-2), respectively. After that, each fractions was tested against HIV-1 IN activity at various concentrations (10-100 μg/ml) (Table 3-3).

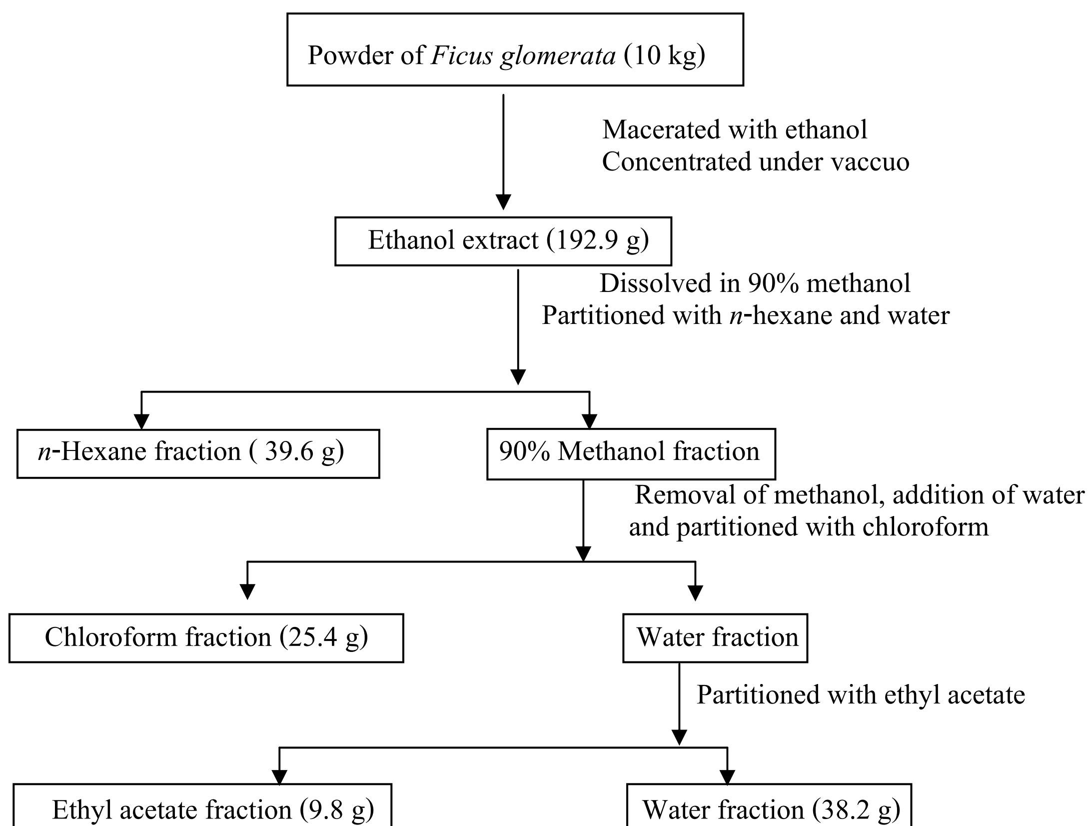


Figure 3-2 The procedure of *Ficus glomerata*

Table 3-3 IC_{50} values of the extract and fractions of *Ficus glomerata* against HIV-1 IN activity

Sample	% inhibition at various concentrations (µg/ml)			IC_{50}(µg/ml)
	10	30	100	
Ethanol extract	14.30±2.36	37.52±3.11	61.23±1.75	49.1
Hexane fraction	-12.65±1.76	5.51±1.45	17.68±0.98	>100
Chloroform fraction	4.55±0.84	8.62±0.67	29.04±1.83	>100
Ethyl acetate fraction	57.25±1.94	84.15±1.49	90.96±0.79	4.6
Water fraction	32.68±1.61	66.25±0.76	88.19±1.61	18.5
Precipitate Chloroform:Water	20.87±1.36	74.08±2.35	93.30±1.15	20.6
Suramin	59.72±0.54	59.45±0.73	99.89±0.45	3.4

The results showed that the ethyl acetate fraction exhibited the highest anti-HIV-1 IN activity with an IC_{50} value of 4.6 µg/ml. The ethyl acetate fraction was therefore isolated to obtain the compounds which further tested for HIV-1 IN inhibitory activity.

3.3 Isolation of compounds from ethyl acetate fraction

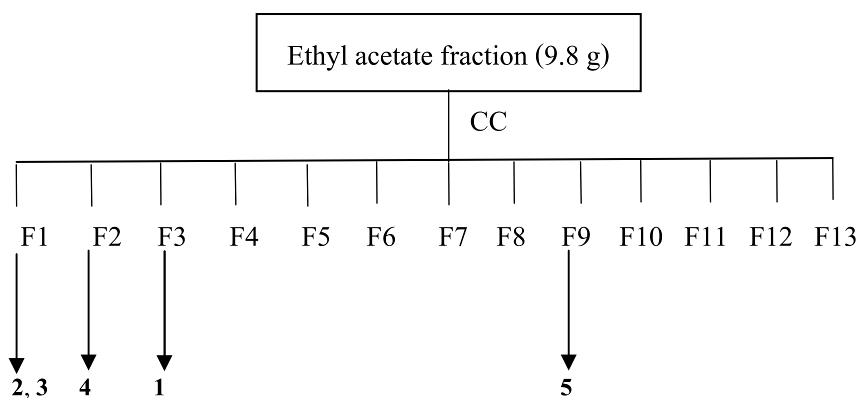


Figure 3-3 Isolation of compounds **1-5**

The crude ethyl acetate extract of *Ficus glomerata* (192.9 g) as dark greenish brown color was purified by column chromatography using silica gel and eluted with gradient elution of chloroform and methanol. On the basis of their TLC characteristic, the collected fraction which contained major components were combined to fractions F1-F13 (Figure 3-3).

Fraction F1 (5.0 g) was separated by silica gel column chromatography using 31 % ethyl acetate in hexane to give thirteen subfractions (F1/1a – F1/13a).

Subfraction F1/11a (60 mg) was purified by recrytallization with 95% methanol in hexane to give compound **2** (17 mg).

Subfraction F1/13a was purified by column chromatography on sephadex LH-20 with 50 % water in methanol and recrystallization to obtain compound **3** (12 mg).

Fraction F2 (5.5 g) was purified by column chromatography on silica gel using 2% methanol in chloroform to give six subfractions (F2/1a-F2/6a).

Subfraction F2/5a (11.0 mg) was purified by column chromatography on silica gel using the mixture of 80 % hexane, 20 % ethyl acetate and 10% acetonitrile, after that it was repurified by silica gel column chromatography using 60% ethyl acetate in hexane to obtain compound **4** (1.0 mg).

Fraction F3 (9.1 g) was separated by column chromatography on silica gel using 20% methanol in chloroform to yield compound **1** (4.9 mg).

Fraction F9 (50 mg) was separated using ethyl acetate, methanol and water (98:1:1) to give compound **5** (18.0 mg).

From the present study, the isolated compounds **2-5** were isolated for the first time from *Ficus glomerata*.

3.4 **Structure elucidation of the isolated compounds**

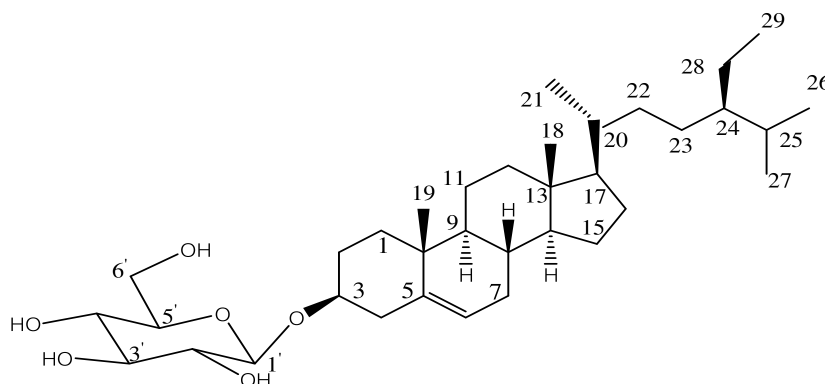


Figure 3-4 Compound 1; *β*-Sitosterol-D-glucoside

Compound **1** was obtained as a white solid (4.9 mg): mp 275-277 °C. The molecular formula of **1** was proposed to be $C_{35}H_{60}O_6$ as observable in the EI mass spectrum, which showed C_{29} sterol peak at *m/z* 397.0. IR spectrum showed absorption band for hydroxyl (3414 cm^{-1}). The ^{13}C NMR spectral data (Table 3-4) recorded in DMSO-d_6 showed the existence of 35 signals for 35 carbon atoms in the molecule. This compound suggested the presence of six methyl (δ 11.8, 11.9, 18.7, 19.0, 19.2 and 19.8), twelve methylene (δ20.7, 22.7, 24.0, 25.6, 27.9, 28.8, 31.5, 33.5, 36.3, 36.9, 38.4 and 61.2), fourteen methine (δ29.4, 31.5, 35.6, 45.3, 49.7, 55.5, 56.3, 70.3, 73.6, 76.9, 76.9, 77.0, 121.3), including one anomeric corbon at δ 100.0 and three quaternary carbons (δ36.3, 42.0, and 140.0).

The ^{1}H NMR spectral data (Table 3-4) recorded in DMSO-d_6 displayed a characteristic signal of sitosterol and a sugar unit. The sitosterol unit was shown as two methyl singlet signals at δ0.64 (3H-18) and 0.94 (3H-19), three methyl doublets at δ0.89 (*d*, J = 6.6 Hz, 3H-21), 0.80 (3H-26) and 0.78 (3H-27) [each *d*, J = 6.8 Hz], one methyl triplet at δ 0.81 (*t*, J = 6.9 Hz, 3H-29), one olefinic proton at δ 5.36 (*br d*, J = 5.0 Hz, H-6) and one oxymethine proton at δ 3.44 (1H, *m*, H-3). The four methine protons in the sugar unit were shown as multiplet signals at δ 2.88 (H-2′), 3.00 (H-5′), 3.05 (H-3′) and 3.11 (H-4′), one anomeric proton at δ4.20 (*d*, J = 7.8 Hz, H-1′) and the oxymethylene protons were shown at δ3.38 (*dd*, J = 11.8, 1.0 Hz) and 3.63 (*dd*, J = 12.0, 4.5 Hz) which were assigned to H-6′. Thus on the basis of its spectroscopic data and comparison with the previously reported data (Jayaprakasha et al., 2010/ Table 3-4), compound **1** was assigned as *β*-sitosterol-D-glucoside (Figure 3-4).

Table 3-4 Spectral data of compound **1** (DMSO-d_6; 500 MHz for ^1H NMR, DMSO-d_6; 125 MHz for ^{13}C NMR) comparing with the reference compound **R** (DMSO-d_6; 400 MHz for ^1H NMR, DMSO-d_6; 100 MHz for ^{13}C NMR)

Position	**Type of C**	δ_C**/ppm**		δ_H**/ppm**	
		Compound 1 DMSO-d_6	**R** DMSO-d_6	**Compound 1** DMSO-d_6	**R** DMSO-d_6
1	CH_2	36.3	36.8	-	-
2	CH_2	28.8	28.7	-	-
3	CH	77.0	76.9	3.44 (*m*)	3.46
4	CH_2	36.9	36.8	2.62 (m), 2.40 (*m*)	-
5	C	140.0	140.4	-	-
6	CH	121.3	121.2	5.36 (*br d*, 5.0)	5.32
7	CH_2	31.5	31.4	-	-
8	CH	31.5	31.4	-	-
9	CH	49.7	49.6	-	-
10	C	36.3	36.2	-	-
11	CH_2	20.7	20.6	-	-
12	CH_2	38.4	39.1	-	-
13	C	42.0	41.8	-	-
14	CH	56.3	56.2	-	-
15	CH_2	24.0	23.8	-	-
16	CH_2	27.9	27.8	-	-
17	CH	55.5	55. 4	-	-
18	CH_3	11.8	11.7	0.64 (*s*)	0.64
19	CH_3	19.2	19.1	0.94 (*s*)	0.95
20	CH	35.6	35.5	-	-
21	CH_3	18.7	18.6	0.89 (*d*, 6.6)	0.89
22	CH_2	33.5	33.3	-	-
23	CH_2	25.6	25.4	-	-

Table 3-4 Spectral data of compound **1** (DMSO-d_6; 500 MHz for ^{1}H NMR, DMSO-d_6; 125 MHz for ^{13}C NMR) comparing with the reference compound **R** (DMSO-d_6; 400 MHz for ^{1}H NMR, DMSO-d_6; 100 MHz for ^{13}C NMR)

Position	**Type of C**	δ_C**/ppm**		δ_H**/ppm**	
		Compound 1 DMSO-d_6	**R** DMSO-d_6	**Compound 1** DMSO-d_6	**R** DMSO-d_6
24	CH	45.3	45.1	-	-
25	CH	29.4	28.7	-	-
26	CH_3	19.8	18.9	0.80 (*d*, 6.8)	0.81
27	CH_3	19.0	19.7	0.78 (*d*, 6.8)	0.81
28	CH_2	22.7	22.6	-	-
29	CH_3	11.9	11.8	0.81 (*t*, 6.9)	0.82
1′	CH	100.0	100.8	4.20 (*d*, 7.8)	4.21
2′	CH	73.6	73.3	2.88 (*m*)	2.88
3′	CH	76.9	76.7	3.05 (*m*)	3.11
4′	CH	70.2	70.0	3.11 (*m*)	3.01
5′	CH	76.9	76.6	3.0 (*m*)	3.05
6_a′	CH_2	61.2	61.0	3.63 (*dd*, 11.2, 1.0)	3.63
6_b′	-	-	-	3.38 (*dd*, 11.8, 1.0)	3.39

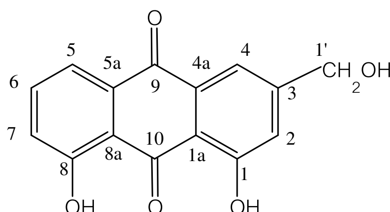


Figure 3-5 Compound 2; Aloe-emodin

Compound **2** was isolated as an orange solid (17 mg): mp 224-225 °C. The molecular formula of **2** was proposed to be $C_{15}H_{10}O_5$ as observable in the EI mass spectrum, which showed a molecular peak at *m/z* 270.8. IR spectrum showed absorption band for the O-H stretching at 3412 cm^{-1} , aromatic C-H stretching at 2923 cm^{-1}, methylene C-H stretching at 2851 cm^{-1}, overtone of aromatic at 2302 cm^{-1}, aromatic C=C stretching at 1454 cm^{-1} and ketone C=O stretching at 1624 cm^{-1}. The UV spectrum showed absorption bands at λ_{max}: 290, 330 nm.

The ^{13}C NMR spectral data (Table3-5) recorded in DMSO-d_6 showed 15 signals for 15 carbons. This compound presented five aromatic methine at δ 117.2, 119.4, 120.9, 124.6, 137.4, five quaternary carbons at δ 114.6, 116.1, 133.3, 133.5, 153.7, a signal of benzylic methylene group at δ 62.2, two signals characteristic of phenolic carbons at δ 161.5, 161.8 and two ketones (δ 191.7, 181.7) were also observed.

The ^{1}H NMR spectral data (Table 3-5) consisted of five proton signals in aromatic region at δ 7.30 (*s*, 1H-2), 7.70 (*s*, 1H-4), 7.72 (*d*, *J*=7.5,1H-5), 7.80 (*t*, *J*=7.5, 8.5, 1H-6) 7.38 (*d*, *J*=8.5, 1H-7), one methylene proton at δ 4.62 (*d*, *J*=4.1, 2H-1′) and three phenolic hydroxyl group at δ 11.98 (*br*, *s*, 1H-1) and 11.98 (*br*, *s*, 1H-8), 5.57 (*br*, *t*, 1H-2′).

Therefore on the basis of its spectroscopic data and comparison with the previous report (Kametani et al., 2007/Table 3-5), compound **2** was assigned to be aloe-emodin (Figure 3-5).

Table 3-5 Spectral data of compound **2** (DMSO-d_6; 500 MHz for ^{1}H NMR, DMSO-d_6; 125 MHz for ^{13}C NMR) comparing with the reference compound **R** (DMSO-d_6; 500 MHz for ^{1}H NMR, DMSO-d_6; 125 MHz for ^{13}C NMR)

Position	Type of C	δ_C/ppm		δ_H/ppm	
		Compound 2 DMSO-d_6	**R** DMSO-d_6	**Compound 2** DMSO-d_6	**R** DMSO-d_6
1	C(OH)	161.8	161.5	11.98 (1H, *br, s*)	11.90 (1H, *br, s*)
2	CH	120.9	120.6	7.30 (1H, *s*)	7.30 (1H, *s*)
3	C	153.7	153.6	-	-
4	CH	117.2	117.0	7.70 (1H, *s*)	7.71 (1H, *d*, 1.7)
5	CH	119.4	119.2	7.72 (1H, *d*, 7.5)	7.73 (1H, *dd*, 1.2, 8.5)
6	CH	137.4	137.2	7.80 (1H, *dd*, 7.5, 8.5)	7.81(1H, *dd*, 7.6, 8.3)
7	CH	124.6	124.2	7.38 (1H, *d*, 8.5)	7.38 (1H, *dd*, 1.2, 8.5)
8	C(OH)	161.5	161.2	11.98 (1H, *br, s*)	11.96 (1H, *br, s*)
9	C=O	191.7	191.5	-	-
10	C=O	181.7	181.4	-	-
C-1a	C	114.6	114.4	-	-
C-4a	C	133.5	133.1	-	-
C-5a	C	133.3	133.3	-	-
C-8a	C	116.1	116.8	-	-
1′	CH_2	62.2	62.0	4.62 (2H, *d*, 4.1)	4.63 (2H, *br, s*)
	CH_2(OH)			5.57 (1H, *br, t*)	5.52 (1H, *br, t*)

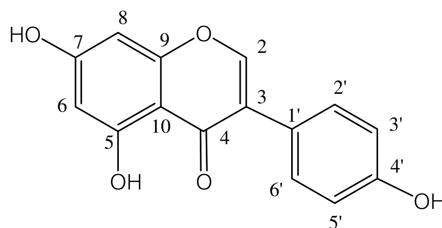


Figure 3-6 Compound 3; Genistein

Compound **3** was isolated as a white solid (12 mg): mp 297-298 °C. The molecular formula of **3** was proposed to be $C_{15}H_{10}O_5$ as observable in the EI mass spectrum, which showed a molecular peak at *m/z* 270.8. IR spectrum absorption band for the hydroxyl group at 3434 cm^{-1} and carbonyl group at 1630 cm^{-1}. The UV spectrum showed absorption band at λ_{max} : 290 nm.

The ^{13}C NMR spectral data (Table 3-6) recorded in DMSO-d_6 showed 15 signals for 15 carbons. This compound suggested the presence of five quaternary aromatic carbons at δ 104.5, 121.4, 122.4, 157.5, 180.3, eight aromatic methine carbons at δ 99.2, 93.8, 115.2, 115.2, 130.2, 130.2, 154.0. Three signals characteristic of phenolic carbons at δ 162.1, 164.8, 157.7.

The ^{1}H NMR spectral data (Table 3-6) recorded in DMSO-d_6 showed seven aromatic protons at δ 6.20 (*d*, *J*=2.0, 1H-6), 6.36 (*d*, *J*=2.0, 1H-8), 6.80 (*dd*, *J*=6.6, 2.0, 1H-3′) 6.90 (*dd*, *J*=6.6, 2.0, 1H-5′), 7.36 (*dd*, *J*=6.6, 2.0, 1H-6′), 7.37 (*dd*, *J*=6.6, 2.0, 1H-2′), 8.29 (*s*,1H-2), one hydroxyl group at δ 12.94 (*s*, 1H-5).

Based on the basis of its spectroscopic data and comparison with previously reported data (Durango et al., 2002/ Table 3-6), compound **3** was identified to be genistein (Figure 3-6).

Table 3-6 Spectral data of compound **3** (DMSO-d_6; 500 MHz for ^{1}H NMR, DMSO-d_6; 125 MHz ^{13}C NMR) comparing with reference compound **R** (MeOD ; 300 MHz for ^{1}H NMR , MeOD; 125 MHz ^{13}C NMR)

Position	**Type of C**	δ_C/ppm		δ_H/ppm	
		Compound 3 DMSO-d_6	**R** MeOD	**Compound 3** DMSO-d_6	**R** MeOD
1	-	-	-	-	-
2	CH	154.0	153.5	8.29 (*s*)	8.17 (*s*)
3	C	122.4	122.2	-	-
4	C	180.3	180.0	-	-
5	C(OH)	162.1	162.0	12.94 (*s*)	13.03 (*s*)
6	CH	99.2	98.5	6.20 (*d*, 2.0)	6.29 (*d*, 2.3)
7	C(OH)	164.8	164.0	-	-
8	CH	93.8	94.1	6.36 (*d*, 2.0)	6.42 (*d*, 2.3)
9	C	157.5	157.8	-	-
10	C	104.5	104.5	-	-
1′	C	121.4	121.5	-	-
2′	CH	130.2	130.0	7.37 (*dd*, 6.6, 2.0)	7.46 (*d*, 8.9)
3′	CH	115.2	115.2	6.80 (*dd*, 6.6, 2.0)	6.90 (*d*, 8.9)
4′	C(OH)	157.7	158.0	-	-
5′	CH	115.2	115.2	6.90 (*dd*, 6.6, 2.0)	6.90 (*d*, 8.9)
6′	CH	130.2	130.0	7.36 (*dd*, 6.6, 2.0)	7.46 (*d*, 8.9)

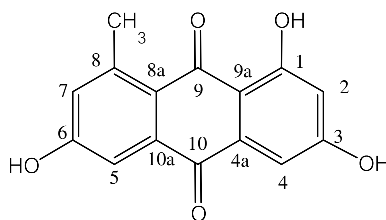


Figure 3-7 Compound 4; 1, 3, 6-Trihydroxy-8-methyl-anthraquinone

Compound **4** was isolated as an orange microcrystalline solid (1 mg). The molecular formula of **4** was proposed to be $C_{15}H_{10}O_5$ as observable in the FAB mass spectrum, which showed a molecular peak (M+ 277.1 glycerol) at *m/z* 547.7. The UV spectrum showed λ_{max} (methanol containing): 290 sh, 339, 431 nm.

The ^{1}H NMR spectral data (Table 3-7) recorded in DMSO-d_6 showed one methyl aromatic protons at δ 2.70 (*s*, 3H-8), four aromatic protons at δ 6.56 (*d*, *J*=2.65, 1H-2), 7.05 (*d*, *J*=2.65, 1H-4), 7.43 (*d*, *J*=2.4, 1H-5) and 7.02 (*d*, *J*=2.4, 1H-7), and one hydroxyl group at δ 13.25. Because compound **4** was isolated about 1.0 mg that was not enough to run ^{13}C NMR. However this compound can be identified by comparison with ^{1}H NMR spectral data and the molecular formula from observable mass spectrum in the previous reports (Ngamga et al., 2007/ Table 3-7), compound **4** was assigned as 1, 3, 6-trihydroxy-8-methyl-anthraquinone (Figure3-7).

Table 3-7 Spectral data of compound **4** (DMSO-d_6; 500 MHz for ^{1}H NMR) comparing with the reference compound **R** (CD_3COCD_3; 300 MHz for ^{1}H NMR)

Position	**Type of C**	δ_H/ppm	
		Compound 4 DMSO-d_6	**R** CD_3COCD_3
1	C	-	-
2	CH	6.56 (*d*, 2.65)	6.64 (*d*, 2.4)
3	C	-	-
4	CH	7.05 (*d*, 2.65)	7.19 (*d*, 2.4)
4a	C	-	-
5	CH	7.43 (*d*, 2.4)	7.57 (*d*, 2.5)
6	C	-	-
7	CH	7.02 (*d*, 2.4)	7.09 (*d*, 2.4)
8	C	-	-
8a	C	-	-
9	C	-	-
9a	C	-	-
10	C	-	-
10a	C	-	-
1-OH		13.25	13.30
8-Me		2.70 (s)	2.80 (s)

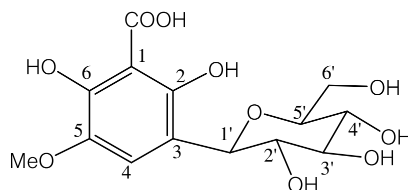


Figure 3-8 Compound 5; 3-(1-C-*β*-D-Glucopyranosyl)-2, 6-dihydroxy-5-methoxybenzoic acid

Compound **5** was isolated as a white solid (18 mg). The molecular formula of **5** was proposed to be $C_{14}H_{18}O_{10}$ as observable in the EI mass spectrum, which showed a molecular peak ($M–H_2O$) at *m/z* 328.7. IR spectrum absorption band for the hydroxyl group at 3434 cm^{-1} and carbonyl group at 1650 cm^{-1}. The UV spectrum showed absorption bands at λ_{max}: 320 nm.

The ^{13}C NMR spectral data (Table 3-8) recorded in DMSO-d_6 showed 14 signals for 14 carbons. This compound suggested the presence of one carbonyl group at δ 163.4, five quaternary aromatic carbons at δ 118.1, 148.1, 116.0, 140.7, 151.0, one aromatic methine carbon at δ 109.6, five methine of sugar unit (δ 73.8, 79.9, 70.8, 72.2, 81.8), one methylene of sugar unit (δ 61.2) and one methoxyl group at δ 59.9.

The ^{1}H NMR spectral data (Table 3-8) recorded in pyridine-d_5 displayed a sharp singlet integrating for 1H at δ 7.78 (*s*, 1H-4) and its position was confirmed by HMBC spectrum. Two broad signals at δ 9.38 and 12.00 were attributed to the phenolic OH group, while a singlet at δ 3.99 (*s*, 3H) was to MeO group. Its ^{13}C NMR spectrum (Table 3-8) exhibited the presence of 14 C-atom, while the DEPT spectrum (page 92) showed one methyl, one methylene, six methine moieties, and six quatrernary C-atoms. A signal at δ 163.4 in the ^{13}C NMR spectrum could be assigned to the C=O group of the acid. The ^{1}H-NMR spectrum showed a doublet at δ 5.26 (*J*= 10.5, 1H-1′) confirmed the presence of an anomeric H of the sugar moiety, which was found linked to the C-atom appearing in the upfield region at δ 73.8 in the HMQC spectrum. In the HMBC spectrum the H at δ 7.78 (*s*, 1H-4) showed strong correlation with C3, C1, C1′ and C5 and correlated weakly with C2, C6 and COOH. The linkage of the anomeric C-atom (C1′) to the aglycone (C3) was also confirmed by HMBC correlations.

Thus on the basis of its spectroscopic data and comparison with previously reported literature (Rana et al., 2005/Table 3-8), compound **5** was assigned to be 3-(1-C-*β*-D-glucopyranosyl)-2,6-dihydroxy-5-methoxybenzoic acid (Figure 3-8).

Table 3-8 Spectral data of compound **5** (pyridine-d_5 ; 300 MHz for ^{1}H-NMR, DMSO-d_6 ; 125 MHz for ^{13}C- NMR) comparing with the reference compound **R** (pyridine-d_5 ; 500 MHz for ^{1}H-NMR, pyridine-d_5 ; 125 MHz for ^{13}C- NMR)

Position	**Type of C**	δ_C**/ppm**		δ_H**/ppm**	
		Compound 5 DMSO	**R** pyridine-d_5	**Compound 5** pyridine-d_5	**R** pyridine-d_5
1	C	118.1	119.5	-	-
2	C	148.1	149.4	-	-
3	C	116.0	116.6	-	-
4	CH	109.6	111.1	7.78 (*s*)	7.75 (*s*)
5	C	140.7	141.9	-	-
6	C	151.0	152.7	-	-
C=O	C	163.4	164.4	-	-
MeO	OCH_3	59.9	60.2	3.99 (*s*)	3.92 (*s*)
1′	CH	73.8	73.9	5.26 (*d*, *J*=10.2)	5.20 (*d*, *J*=10.5)
2′	CH	79.9	75.5	4.65 (*t*, *J*=9.9, 9.9)	4.43 (*t*, *J*=10.2, 8.5)
3′	CH	70.8	71.3	4.49 (*t*, *J*=8.7, 8.7)	4.42 (*t*, *J*=10.2, 9.0)
4′	CH	72.2	72.1	4.17 (*d*, *J*=8.7)	4.17 (*d*, *J*=9.0)
5′	CH	81.8	83.5	4.21 (*d*, *J*=8.4)	4.20 (*d*, *J*=8.3)
$6'_a$	CH	61.2	62.6	4.71 (*d*, *J*=9.9)	4.64 (*d*, *J*=10.2)
$6'_b$	CH	-	-	4.29 (t, *J*=9.3, 7.5)	4.22 (*t*, *J*=10.2, 7.3,)

3.5 Effect of isolated compounds on anti-HIV-1 IN activity

Compounds **1- 5** were isolated from the ethanolic extract of *Ficus glomerata*. They were carried out for testing on anti-HIV-1 IN activity. The % inhibition and IC_{50} values are shown in Table 3-9. The result indicated that compound **2** (aloe-emodin) showed activity against HIV-1 IN with % inhibition of 31.91 at 100 μM, followed by compound **4** (1, 3, 6-trihydroxy-8-methyl-anthraquinone) with % inhibition of 19.59; whereas other compounds (**1**, **3**, **5**) were inactive. Moreover, from the present study, compounds **2-5** were isolated for the first time in *Ficus glomerata*. It was reported that β-sitosterol-D-glucoside **(1)** and genistein **(3)** were also found in *Ficus septica* (Lanka et al, 2008). It was found that aloe-emodin **(2)** was isolated from *Rheum rhabarbarum* (Xia et at., 2006), *Aloe excelsa* (Coopoosamy et al., 2006) and *Cassia alata* (Fernand et al., 2008); 1, 3, 6-trihydroxy-8-methyl-anthraquinone **(4)** from *Gladiolus psittascinus* (Ngamag et al., 2007), *Rheum palmatum* (Wang et al., 2010) and *Rheum rhabarbarum* (Lai et al., 2009); whereas 3-(1-C-β-D-glucopyranosyl)-2, 6-dihydroxy-5-methoxybenzoic acid **(5)** from *Mallotus roxburghianus* (Rana et al., 2005).

Regarding biological activities of the isolated compounds, β-sitosterol-D-glucoside **(1)** has been reported for antibacterial activity (Bayor et al., 2009), uv-radiation protection, anti-oxidant, moisture holding (Fan, 2010), antimicrobial (Chung et al., 2005), antiatherogenic (Zhao et al., 1990) and gastroprotective activities (Navarrete et al., 2002). Aloe-emodin **(2)** has been reported for antibacterial (Wang et al., 2010), anti-cancer in human colon carcinoma (Lin et al., 2010; Zheng et al., 2010), migration and invasion inhibitory effect in human tongue cancer (Chen et al., 2010; Chiu et al., 2009), antitumor (Li et al., 2009), anti cancer in human nasopharyngeal carcinoma (Lin et al., 2010), anti-gastric cancer (Zhang et al., 2009) antipigmentation (Lee et al., 2010), anticancer and antioxidant activities (El-Shemy et al., 2010), anti-tuberculosis (Camacho-Corona et al., 2009), hypoglycemic activity (Naqishbandi et al., 2009), anti-inflammatory activity (Pake et al., 2009), anti-angiogenic activity (He et al., 2009) and antiviral activity against Japanese encephalitis virus and enterovirus (Lin et al., 2008). Genistein **(3)** has been reported for antioxidant activity (Park et al., 2010), anti-rheumatoid arthritis (Gao and Zhang, 2008), anti-oxidative stress (Kim and Kim, 2007) and anti-osteoporosis (Wang et al., 2007). Whereas 1, 3, 6-

trihydroxy-8-methyl-anthraquinone **(4)** has been reported for anti-lung cancer activity (Su et al., 2010).

From the previous report on anti-HIV-1 IN activity from *Eclipta prostrata* (Tewtrakul et al., 2007), it was found that orobol has similar structure to that of genistein (Figure 4-1). However, genistein had no activity while orobol showed potent anti-HIV-1 IN effect (IC_{50}= 8.1 μM). Thus, this may imply that vicinal hydroxyl groups at C-3′ and C-4′ positions are required for this type of activity.

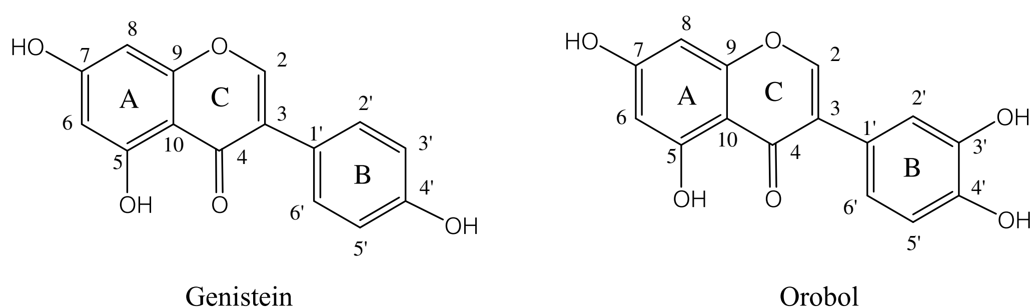


Figure 3-9 Structures of genistein and orobol

Table 3-9 % inhibition and IC_{50} values of isolated compounds from ethyl acetate fraction against HIV-1 IN activity

Compound	**% Inhibition at various concentrations (μM)**			
	10	**30**	**100**	**IC_{50}(μM)**
1) *β*-Sitosterol-D-glucoside	-	-	-3.54±0.58	>100
2) Aloe-emodin	-16.36±4.85	-1.44±2.83	31.91±3.99	>100
3) Genistein	-	-	-10.71±4.11	>100
4) 1, 3, 6-Trihydroxy-8-methyl-anthraquinone	-7.64±3.95	1.79±4.67	19.59±1.29	>100
5) 3-(1-C-*β*-D-Glucopyranosyl)-2, 6-dihydroxy-5-methoxybenzoic acid	-	-	-43.16±1.76	>100
Suramin	59.72±0.54	59.45±0.73	99.89±0.45	3.4

1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione = aloe-emodin

5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one = genistein

CHAPTER 4

CONCLUSION

The aqueous and EtOH extracts of eight Thai plants including *Clerodendron indicum* (whole plant), *Tiliacora triandra* (stem), *Capparis micracantha* (wood), *Harrissonia perforata* (wood), *Ficus glomerata* (wood), *Diospyros decandra* (wood), *Dracaena loureiri* (heartwood) and *Tinospora crispa* (stem) were screened for their inhibitory activities against HIV-1 integrase (IN) using the multiplate integration assay (MIA). From these plant extracts, *Dracaena loureiri* (heartwood, EtOH) possessed high %yield with 39.9 %w/w, followed by *Tinospora crispa* (stem, water, 12.6 %w/w), whereas those of other plants were 1.2-6.8 % w/w. Of the EtOH extracts, *Ficus glomerata* (wood) showed the highest activity against HIV-1 IN with an IC_{50} value of 7.8 µg/ml; whereas the water extract of *Harrisonia perforata* (wood) was the most potent for aqueous extracts (IC_{50} = 2.3 µg/ml). It was found that the aqueous extract of *Harissonia perforata* exhibited anti-HIV-1 IN activity higher than that of suramin, a positive control (IC_{50} = 3.4 µg/ml). Other plant extracts possessed moderate to weak activity with IC_{50} values ranging from 22.1->100 µg/ml.

Since the EtOH extract of *Ficus glomerata* (wood) showed the highest activity against HIV-1 IN, this extract was further partitioned to four fractions of hexane (39.6 g), chloroform (25.4 g), ethyl acetate (9.8 g) and water fractions (38.2 g), respectively. The IC_{50} value of each fraction was found to be > 100, > 100, 4.6 and 18.5 µg/ml, respectively. The EtOAc fraction was then separated to give five pure compounds which are β-sitosterol-D-glucoside **(1)**, aloe-emodin **(2)**, genistein **(3)**, 1, 3, 6-trihydroxy-8-methyl-anthraquinone **(4)** and 3-(1-C-β-D-glucopyranosyl)-2, 6-dihydroxy-5-methoxybenzoic acid **(5).** From the result, it was found that compound **2** (aloe-emodin) showed activity against HIV-1 IN with % inhibition of 31.91 at 100 µM, followed by compound **4** (1, 3, 6-trihydroxy-8-methyl-anthraquinone) with % inhibition of 19.59; whereas other compounds (**1**, **3**, **5**) were inactive. Moreover, from the present study compounds **2-5** were isolated for the first time in *Ficus glomerata*. It is concluded that the isolated compounds from *Ficus glomerata* may have a synergistic effect on anti-HIV-1 IN

activity since these pure compounds showed less activity than the EtOH extract (81.12% inhibition at 100 μg/ml).

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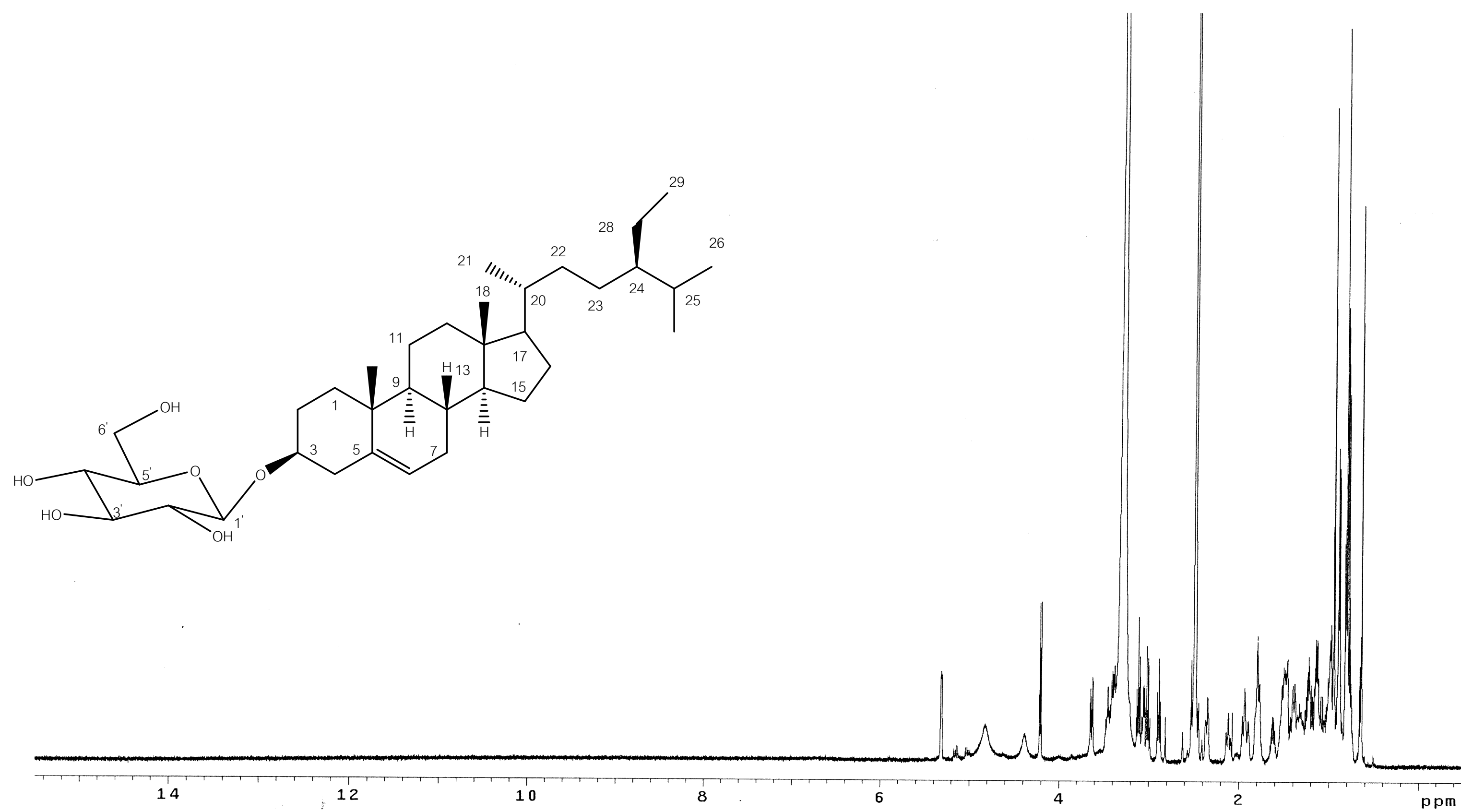
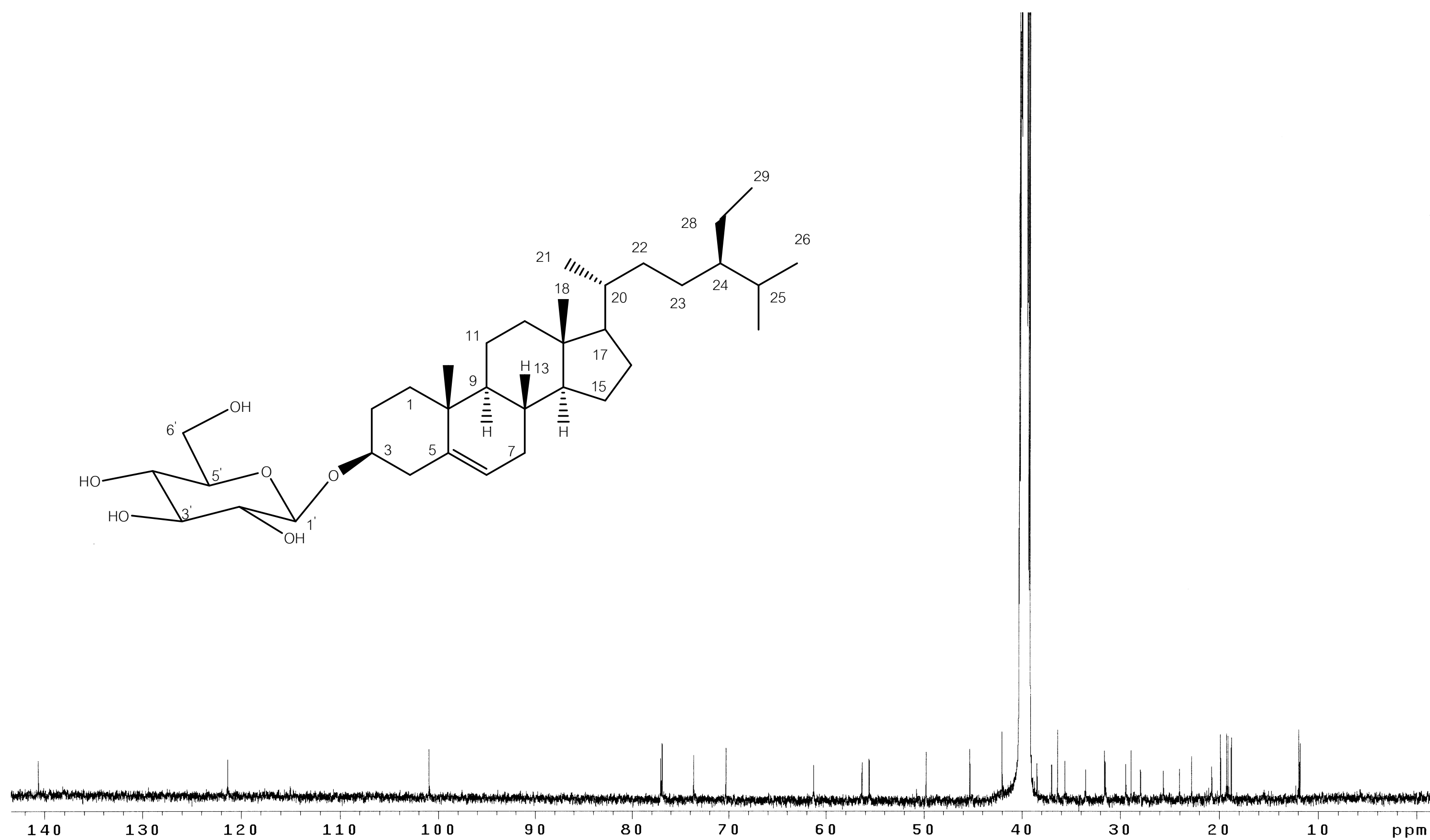
APPENDEIX



^{1}H NMR spectrum of compound **1** (DMSO-d_6; 500 MHz)



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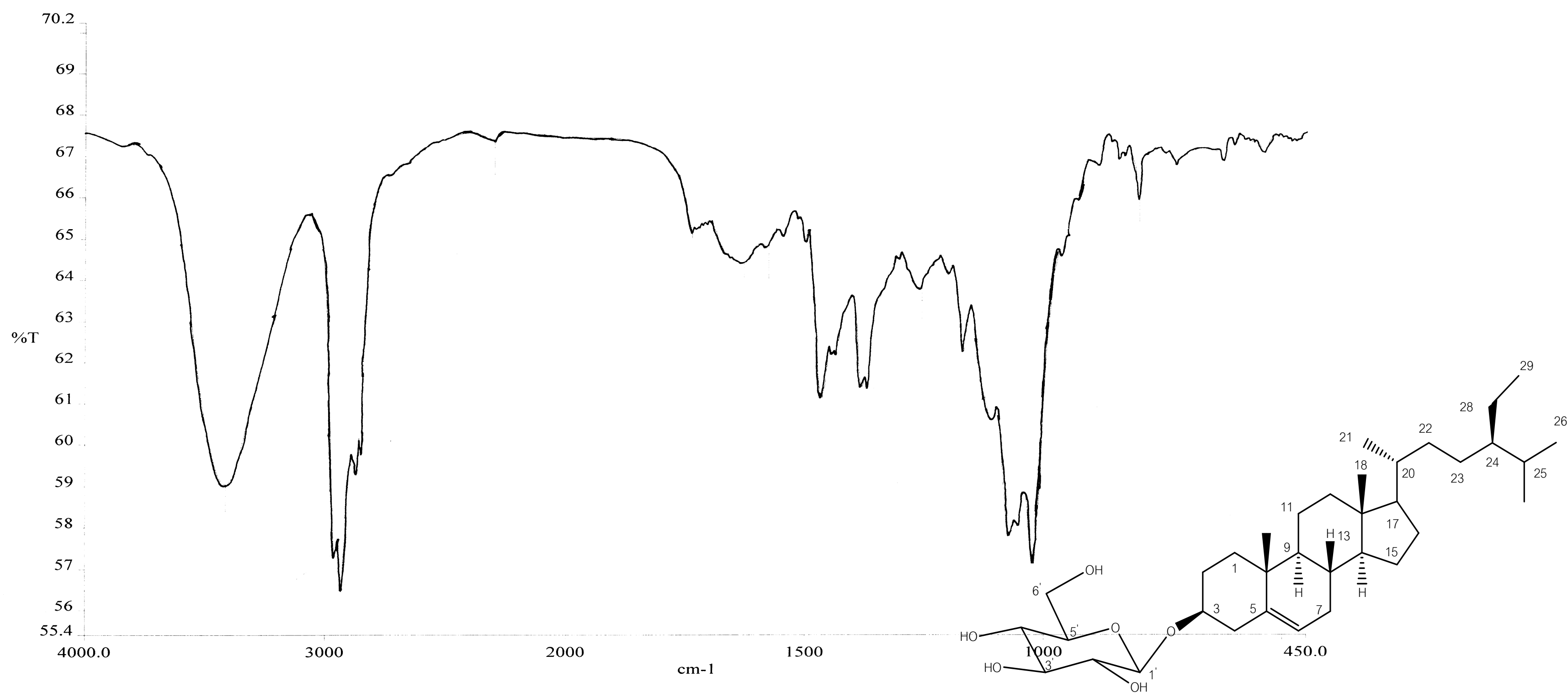
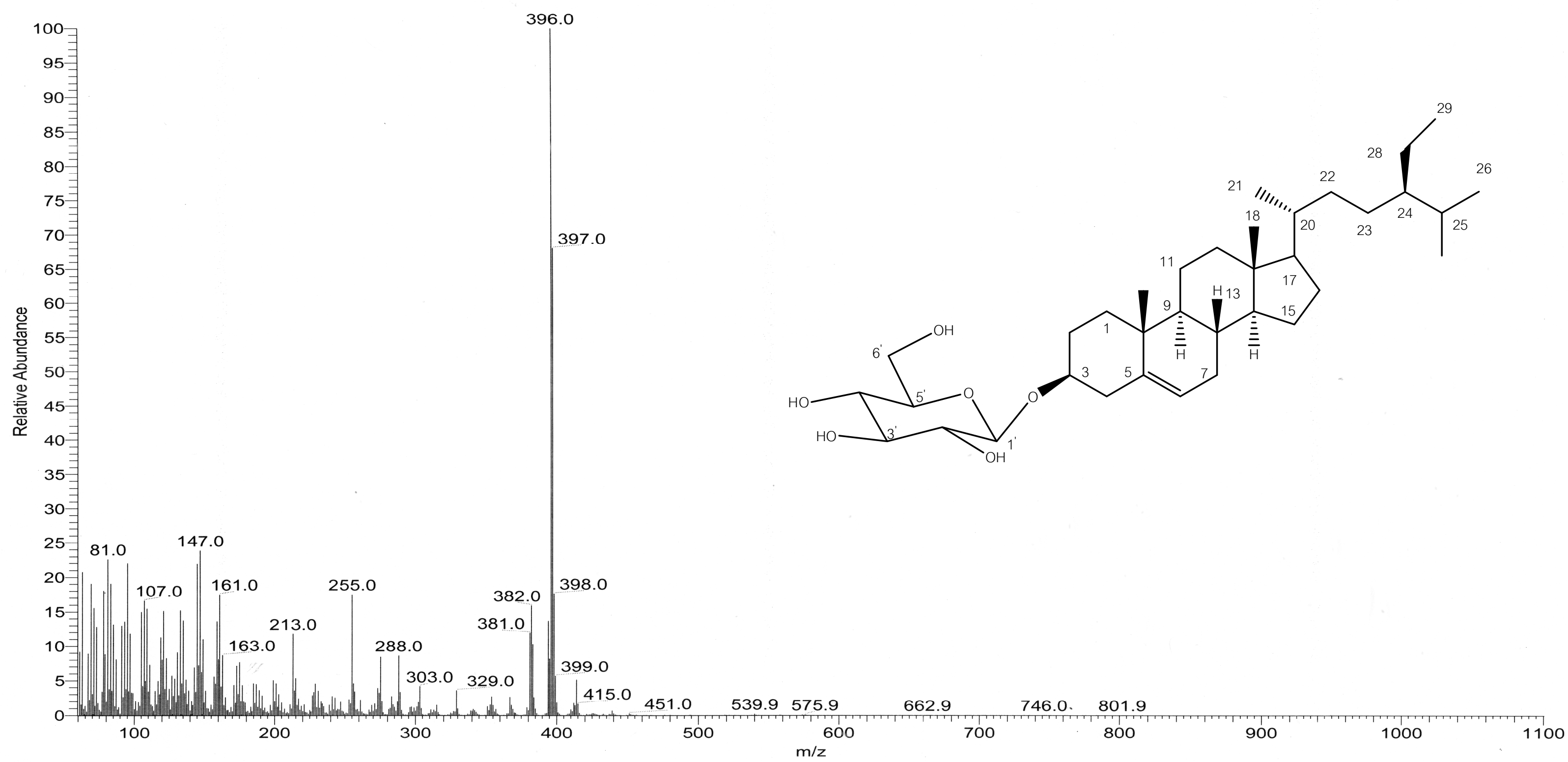


R spectrum of compound **1** (! r)



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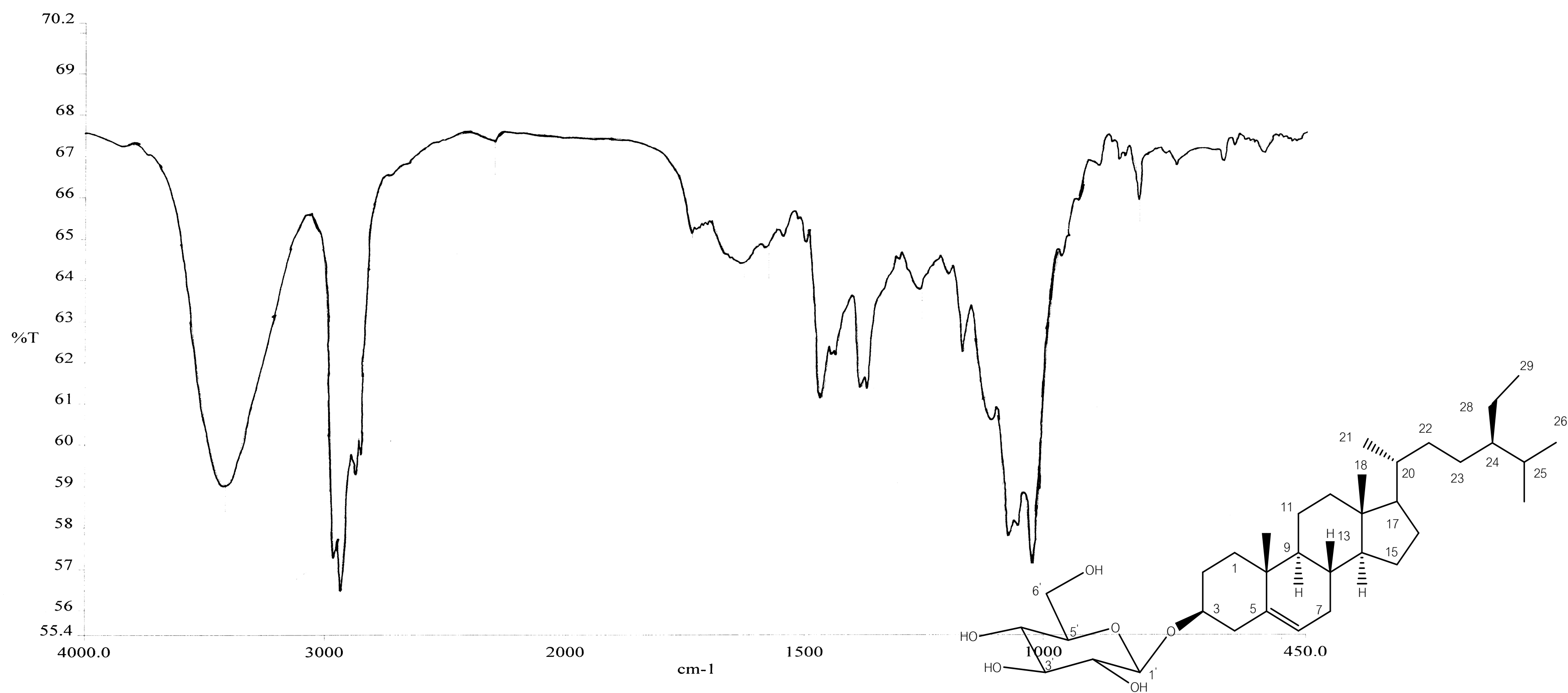
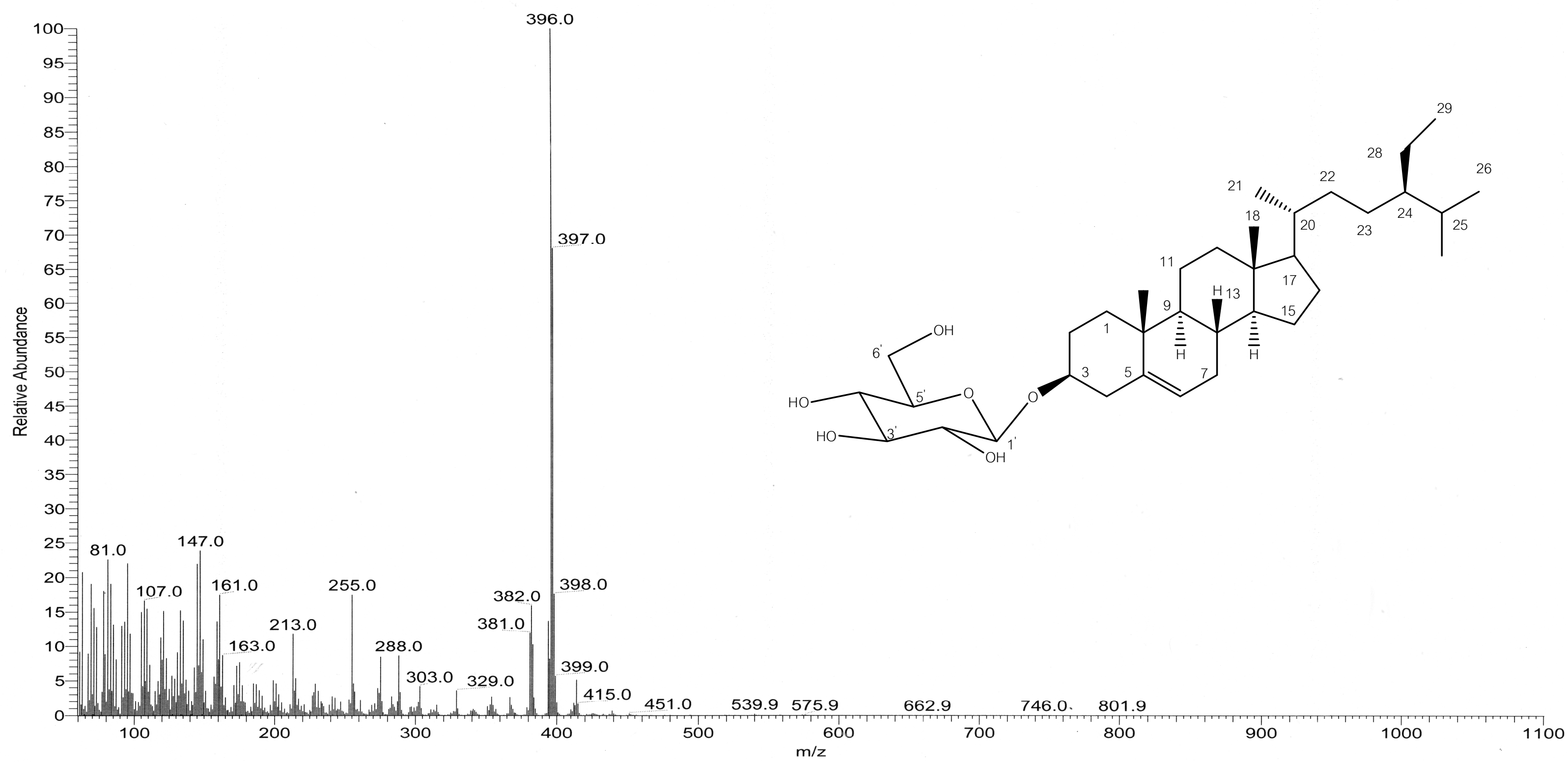


R spectrum of compound **1** (! r)



" m#ss spectrum of compound **1**

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