THE IDENTITIES AND ANTI HERPES SIMPLEX VIRUS ACTIVITY OF *CLINACANTHUS NUTANS* AND *CLINACANTHUS SIAMENSIS*

Mr. Paween Kunsorn

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เอกลักษณ์และฤทธิ์ต้านเฮอร์ปีสซิมเพล็กซ์ไวรัสของต้นพญายอและต้นลิ้นงูเห่า

นายปวีณ คันศร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	THE IDENTITIES AND ANTI HERPES SIMPLEX VIRUS ACTIVITY OF *CLINACANTHUS NUTANS* AND *CLINACANTHUS SIAMENSIS*
By	Mr. Paween Kunsorn
Field of Study	Public Health Sciences
Thesis Advisor	Kanchana Rungsihirunrat, Ph.D.
Thesis Co-advisor	Associate Professor Vimolmas Lipipun, Ph.D.

Accepted by the College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the College of Public Health Sciences
(Professor Surasak Taneepanichskul, M.D.)

THESIS COMMITTEE

..... Chairman
(Naowarat Kanchanakhan, Ph.D.)

..... Thesis Advisor
(Kanchana Rungsihirunrat, Ph.D.)

..... Thesis Co-advisor
(Associate Professor Vimolmas Lipipun, Ph.D.)

..... Examiner
(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

..... External Examiner
(Assistant Professor Supawan Bunrathep, Ph.D.)

ปวีณ คันศร : เอกลักษณ์และฤทธิ์ต้านเฮอร์ปีสซิมเพล็กซ์ไวรัสของต้นพญายอและต้นลิ้นงูเห่า. (THE IDENTITIES AND ANTI HERPES SIMPLEX VIRUS ACTIVITY OF *CLINACANTHUS NUTANS* AND *CLINACANTHUS SIAMENSIS*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ. ดร. กาญจนา รังษีหิรัญรัตน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. วิมลมาศ ลิปิพันธ์, 108 หน้า.

พญายอและลิ้นงูเห่าเป็นพืชในจีนัส *Clinacanthus* ที่พบได้ในประเทศไทย พญายอมีชื่อเสียงและถูกใช้เป็นยาสมุนไพร โดยมีฤทธิ์แก้ผื่นคันที่ผิวหนัง แก้เริม,งูสวัด นอกจากนี้พญายอยังได้ถูกรับรองเป็นยาสมุนไพรตามประกาศบัญชียาจากสมุนไพร พ.ศ. 2549 โดยคณะกรรมการแห่งชาติด้านยา เนื่องจากพญายอและลิ้นงูเห่ามีสัณฐานวิทยาที่ใกล้เคียงกัน การจำแนกพืชทั้งสองชนิดด้วยวิธีการประเมินทางเภสัชเวทและอณูพันธุศาสตร์ ซึ่งรวมถึงการประเมินทางมหทรรศน์,การประเมินทางจุลทรรศน์ภาคตัดขวางลำต้นและเส้นกลางใบ และภาคขยายของผิวใบ,การวัดค่าจำนวนและดัชนีปากใบ,การวัดค่าอัตราส่วนเซลล์รั้ว ด้านอณูพันธุศาสตร์ ใช้เทคนิคการหาลำดับนิวคลีโอไทด์ในบริเวณ internal transcribed spacer (ITS) ผลการศึกษาพบว่า ภาคตัดขวางลำต้นและเส้นกลางใบและภาคขยายผิวใบของพืชทั้งสองชนิดมีองค์ประกอบที่คล้ายคลึงกัน ค่าจำนวนปากใบของพญายอและลิ้นงูเห่าเท่ากับ 168.32 ± 29.49 และ161.60 ± 18.04 ตามลำดับ,ค่าดัชนีปากใบของพญายอและลิ้นงูเห่าเท่ากับ 13.83 ± 0.86 และ 11.93 ± 0.81 ตามลำดับ และค่าอัตราส่วนเซลล์รั้วของพญายอและลิ้นงูเห่าเท่ากับ 6.84 ± 0.66 และ 3.37 ± 3.31 ตามลำดับ ส่วนลำดับนิวคลีโอไทด์บริเวณ ITS พบว่า เมื่อนำตัวอย่างพืชทั้งสองชนิด รวมทั้งสิ้น 6 ตัวอย่าง (ชนิดละ 3 ตัวอย่าง) มาเปรียบเทียบลำดับนิวคลีโอไทด์กันนั้น พบความแตกต่างของลำดับนิวคลีโอไทด์อยู่ 33 จุด ประกอบด้วยนิวคลีโอไทด์ไพลีมอร์ฟิซึม 23 ตำแหน่ง,การเพิ่มขึ้นหรือหายไปของนิวคลีโอไทด์ 11 ตำแหน่ง คิดเป็นความใกล้เคียง 97-99% นอกจากนี้ การทดสอบฤทธิ์ต้านเชื้อเฮอร์ปีสซิมเพล็กซ์ไวรัส ไทป์ 1 และ ไทป์ 2 (HSV-1 และ HSV-2) ของพืชทั้งสองชนิดด้วยวิธี Plaque reduction assay จากสารสกัดใบแห้งที่สกัดด้วยนอร์มอล-เฮ็กเซน,ไดคลอโรมีเทน และเมทานอล ตามลำดับ พบว่าค่า IC_{50} ต่ำสุดที่ยับยั้ง HSV-1 คือ 32.05 ± 3.63 ไมโครกรัม/มิลลิลิตร ค่าดัชนีความปลอดภัย (Selective index; SI) ซึ่งคำนวณจากค่าความเข้มข้นของสารสกัดที่เป็นพิษต่อเซลล์ร้อยละ 50 หารด้วยค่าความเข้มข้นของสารสกัดที่ยับยั้งเชื้อร้อยละ 50 มีค่ามากกว่า 50.36 จากสารสกัดนอร์มอล-เฮ็กเซนของต้นพญายอ ค่า IC_{50} ต่ำสุดที่ยับยั้ง HSV-2 คือ 46.52 ± 4.08 ไมโครกรัม/มิลลิลิตร มีค่า SI มากกว่า 34.53 จากสารสกัดนอร์มอล-เฮ็กเซนของต้นลิ้นงูเห่า การทดสอบค่าความเป็นพิษต่อเซลล์ด้วยวิธี MTT assay พบว่าสารสกัดด้วยนอร์-มอล-เฮ็กเซนและเมทานอลของพืชทั้งสองชนิดมีค่า CC_{50} มากกว่า 1,600 ไมโครกรัม/มิลลิลิตร ส่วนสารสกัดด้วยไดคลอโรมีเทนของพญายอและลิ้นงูเห่ามีค่า CC_{50} เท่ากับ 869 ± 141.93 และ194 ± 3.56 ไมโครกรัม/มิลลิลิตร ตามลำดับ จากข้อมูลดังกล่าวนี้จึงสรุปได้ว่าพืชทั้งสองชนิดนี้มีความใกล้เคียงกัน การใช้วิธีการประเมินทางเภสัชเวทพบว่าค่าดัชนีปากใบและค่าอัตราส่วนเซลล์รั้วสามารถใช้จำแนกพืชทั้ง 2 ชนิดนี้ได้ อย่างไรก็ตามการศึกษาลำดับนิวคลีโอไทด์บริเวณ ITS พบว่า ไม่สามารถใช้จำแนกได้ เนื่องจากพืชทั้ง 2 ชนิดมีความหลากหลายของบริเวณ ITS นอกจากนี้พืชเหล่านี้อาจนำไปพัฒนาหรือใช้เป็นแหล่งสำหรับสารต้านเชื้อเฮอร์ปีสซิมเพล็กซ์ไวรัสได้

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5379304653 : MAJOR PUBLIC HEALTH SCIENCES
KEYWORDS : *CLINACANTHUS NUTANS/CLINACANTHUS SIAMENSIS*/HERPES SIMPLEX VIRUS/BIOMOLECULAR/POLYMERASE CHAILN REACTION/INTERNAL TRANSCRIBED SPACER

PAWEEN KUNSORN : THE IDENTITIES AND ANTI HERPES SIMPLEX VIRUS ACTIVITY OF *CLINACANTHUS NUTANS* AND *CLINACANTHUS SIAMENSIS*. ADVISOR : KANCHANA RUNGSIHIRUNRAT, Ph.D., CO-ADVISOR : ASSOC. PROF. VIMOLMAS LIPIPUN, Ph.D., 108 pp.

Clinacanthus nutans and *Clinacanthus siamensis* (Thai name Phaya Yo and Lin Nguu Hao, respectively) are plants in genus *Clinacanthus* that can be found in Thailand. *C. nutans* has been used as a herbal drug to relief of skin rashes, herpes simplex and herpes zoster lesion, moreover, this plant was accepted to be a herbal drug in the list of herbal medicinal products A.D. 2006 by national drug committee. Since some of morphologies of *C. siamensis* are somewhat similar to *C. nutans*, distinguishing these related plants by pharmacognostic evaluation and biomolecular technique which are including of macroscopic, microscopic evaluation of stem, midrib cross section and components in leaf epidermis, measurement of stomatal number, stomatal index and palisade ratio, biomolecular technique by comparing the nucleotide sequence in internal transcribed spacer (ITS) region, the result revealed that stem, midrib cross section, components in leaf epidermis of *C. nutans* and *C. siamensis* are familiar. Stomatal number of *C. nutans* and *C. siamensis* are 168.32 ± 29.49 and 161.60 ± 18.04, respectively. Stomatal index of *C. nutans* and *C. siamensis* are 13.83 ± 0.86 and 11.93 ± 0.81, respectively. Palisade ratio of *C. nutans* and *C. siamensis* are 6.84 ± 0.66 and 3.37 ± 3.31, respectively. Comparison ITS region of all 6 samples of the two species (3 samples of each species), there are 33 polymorphisms which comprise of 23 nucleotide polymorphism and 11 Indels which indicated 97-99% similarity. Moreover, testing for the anti herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) by plaque reduction assay of dried leaves that extracted with n-hexane, dichloromethane and methanol, respectively. The result showed the lowest extract concentration that inhibited HSV-1 by 50% (IC_{50}) was 32.05 ± 3.63 µg/ml and selective index (SI) which was calculated from the extract concentration that causes death of tested cells by 50% divided by extract concentration that inhibited 50% of the virus was more than 50.36 from *C. nutans* n-hexane extract, whereas the lowest IC_{50} that inhibited HSV-2 was 46.52 ± 4.08 µg/ml, SI was 34.53 from *C. siamensis* n-hexane extract. Cytotoxicity was tested by MTT assay, CC_{50} of n-hexane and methanol extract of both plants was more than 1,600 µg/ml. CC_{50} of dichloromethane extract of *C. nutans* and *C. siamensis* were 869 ± 141.93 and 194 ± 3.56, respectively. According to these evidences, it could be concluded that these plants are closely related to each other. Pharmacognostic evaluation can distinguish these plants especially, stomatal index and palisade ratio. However, evolution of nucleotide sequence in ITS region cannot distinguish these closely related plants due to their sequence variation in the ITS region. Furthermore, these medicinal plants can be developed or used as a source for isolation of anti-HSV compounds.

Field of Study : Public Health Sciences Student's Signature Paween Kun

Academic Year : 2011 Advisor's Signature Kanchana Rungsihirunrat

Co-advisor's Signature Vimolmas Lipipun

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LIST OF SCHEME

LIST OF ABRIVIATIONS

1D NMR	=	1 Dimension nuclear magnetic resonance
2D NMR	=	2 Dimension nuclear magnetic resonance
AAPH	=	2, 2′-azobis (2-amidinopropane) hydrochloride
ACV	=	Acyclovir
bp	=	Base pairs
BW	=	Body weight
CC_{50}	=	50% Cytotoxicity concentration
CEFs	=	Chick embryo fibroblasts
cm	=	Centimeter
CMIR	=	Cell-mediated immune response
CTAB	=	Cetyl trimethylammonium bromide
^{o}C	=	Degree Celsius
DMSO	=	Dimethyl sulfoxide
dNTP	=	Deoxynucleoside triphosphates
DPPH	=	1,1-diphenyl-2 picrylhydrazyl
ED_{50}	=	50% Effective dose
EDTA	=	Ethylenediaminetetraacetic acid
FOS	=	Foscarnet
FRAP	=	Ferric reducing antioxidant power
g	=	Gram
GC	=	Gas chromatography
HA-test	=	Hemaglutination test
HPLC	=	High-performance liquid chromatography
HSV	=	Herpes simplex virus
IC_{50}	=	50% Inhibition concentration
IL-2	=	Interleukin-2
IL-4	=	Interleukin-4
ITS	=	Internal Transcribed Spacer
K_2HPO_4	=	Dipotassium hydrogen phosphate
kb	=	Kilobase

KCl	=	Potassium chloride
kg	=	Kilogram
KH_2PO_4	=	Potassium dihydrogen phosphate
KHV	=	Koi herpes virus
LD_{50}	=	Median lethal dose
M	=	Molar
m	=	Meter
MEM	=	Minimum essential medium
Mg^{2+}	=	Magnisium ion
mg	=	Milligram
$MgCl_2$	=	Magnesium chloride
MIC	=	Minimum inhibitory concentration
ml	=	Milliliter
mm	=	Millimeter
mm^2	=	Square millimeter
mM	=	Millimolar
MPO	=	Myeloperoxidase
MTS	=	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	=	(3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide)
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
Na_2HPO_4 anhydrous	=	Disodium hydrogen phosphate anhydrous
$NaHCO_3$	=	Sodium bicarbonate
NaCl	=	Sodium chloride
n-BuOH	=	Normal butanol
NDV	=	Newcastle disease virus
NK	=	Natural killer cells
NMR	=	Nuclear magnetic resonance

nm	=	Nanometer
nt	=	Nucleotide
OD	=	Optical density
PCR	=	Polymerase Chain Reaction
PCR/RFLP	=	polymerase chain reaction/restriction enzyme fragment length polymorphism
PFU	=	Plaque forming unit
PMA	=	Phorbol myristate acetate
ppm	=	Parts per million
RBL-1 cell	=	Rat basophilic leukemia cell
rDNA	=	Ribosomal deoxynucleic acid
RNA	=	Ribonucleic acid
rpm	=	Round per minute
S.D.	=	Standard deviation
SI	=	Selective Index
Taq	=	*Taq* DNA Polymerase
TBE buffer	=	Tris-boric EDTA buffer
TE buffer	=	Tris-EDTA buffer
TK	=	Enzyme thymidine kinase
TLC	=	Thin-layer chromatography
Tris-HCl	=	Tris-hydrochloric
VZV	=	Varicella zoster virus
w/v	=	Weight/volume
WST	=	Water soluble Tetrazolium salts
w/w	=	Weight by weight
XTT	=	(sodium 3'-[1- phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

CHAPTER I
INTRODUCTION

1. Background and significance of the study

Thailand is a country which is located in the tropical zone of the world [1]. This country is rich of many species of plant that can be used as therapeutic herbs and food [2]. Thai traditional medicine is wisdom of ancient Thai people. Due to the profusion of medicinal plants in the country, Thai people have been used them as a therapeutic herbal drugs to ease the diseases from the past until the present. Modern medicine becomes the mainstream health care system, and Thai traditional medicine becomes a branch of nonconventional or alternative medicine. Traditional medicinal plants play an important role to the world's population for human primary health care. Many species have been found to be economically and medicinally important [3].

There are 2 species of the plant in genus *Clinacanthus* which found in Thailand had been reported in 2001 by Tem Smitinand [4]. *Clinacanthus nutans* (Burm. f.) Lindau, Thai name Phaya yo (also known as Phaya plong dam, Phaya plong thong, Saled pang pon tua mia, etc.) is a medicinal plant used to relief of insect bites, skin rashes, antipyretic, anti inflammatory, anti snake venom, anti dysentery and treatment of aphthous ulcer [5] and was declared in the List of Herbal Medicinal Products A.D. 2006 by National Drug Committee [6]. While study on *Clinacanthus siamensis* Bremek., which is in the same genus and some closely related characters are limited. *C. nutans* has been subjected in many researches for their antiviral activity for treatment of herpes simplex virus (HSV) which is an important causative agent of pathogenic disease in human. The effective chemically synthesized antiviral drugs have been used to treat HSV infection. Acyclovir (ACV) was first approached for clinical used in the early 1980s for treatment of HSV [7]. However, high cost of synthetic drugs and development of drug resistant strains are clinical problems after long-term treatment. Screening of plant extracts for HSV treatment given interesting results for search of new antiviral drugs. Natural products have been reported for inhibition of HSV. Development of medicinal plants can play a role in drug discovery.

Since some morphology of *C. siamensis* somewhat similar to *C. nutans*, specific markers are needed for feasible and reliable identification of these plants. To study the nuclear genome, the internal transcribed spacer (ITS1 and ITS2) sequences which are clustered in an array of multiple repeated cistrons of 18S rRNA - ITS1 - 5.8S rRNA - ITS2 - 26S rRNA were employed in the study. These regions are frequently used to assess plant phylogenetic relationships because the highly conserved regions of 18S rRNA, 5.8S rRNA and 26S rRNA, the suitable for direct sequencing of PCR products due to their small size and high copy number, the detectable rapid concerted evolution and their higher amount of sequence divergence compared to that of their flanking coding regions. Several studies have indicated that the sequence of the ITS regions are used in phylogenetic reconstruction at the infrageneric and infrafamilial level [8-9]. Therefore, the comparison of their macroscopic and microscopic characters, nucleotide sequence and antiviral activity against herpes simplex virus type 1 and type 2 are evaluated.

2. Research questions

2.1 Are there the differences of leaf measurement: stomatal number, stomatal index and palisade ratio between *C. nutans* and *C. siamensis*, distinguished by macroscopic and microscopic evaluation?

2.2 Is there the difference of internal transcribe spacer (ITS) region sequence of the two species, distinguished by molecular evaluation?

2.3 Do both these two selected species show anti herpes simplex virus type 1 and type 2 activities?

3. Objectives of the study

The specific objectives of the present study were as follow:

3.1 To distinguish the differences of stomatal number, stomatal index and palisade ratio of *C. nutans* and *C. siamensis* by macroscopic and microscopic evaluation

3.2 To distinguish the differences of Internal Transcribe Spacer (ITS) region sequence between of *C. nutans* and *C. siamensis* for differentiation at DNA level

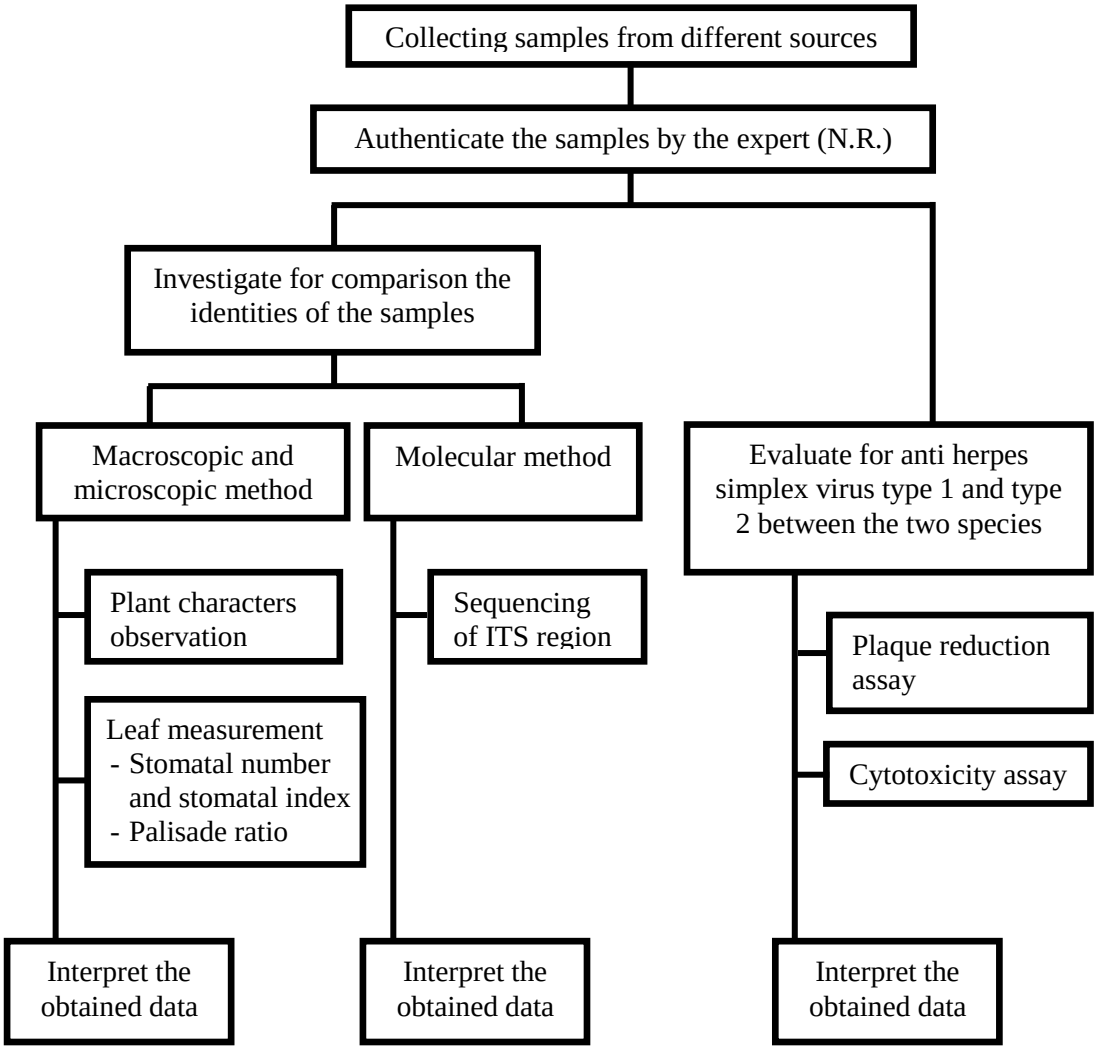
3.3 To evaluate the potential on antiviral activity of *C. nutans* and *C. siamensis* extracts against herpes simplex virus type 1 and type 2

4. Benefits of the study

4.1 This study provides the information regarding the distinguishing and comparing the differences between *C. nutans* and *C. siamensis* by macroscopic and microscopic methods and also by molecular method using the sequence of internal transcribed spacer (ITS) region.

4.2 This study provides the information regarding the comparison of antiviral activity against herpes simplex virus type 1 and type 2 between these two species.

5. Conceptual framework



CHAPTER II
LITERATURE REVIEW

1. Plants in family ACHANTHACEAE

The family Acanthaceae (or Acanthus family) is a taxon of dicotyledonous flowering plants containing 246 accepted genera according to Germplasm Resources Information Network (GRIN) and about 2500 species. Most are tropical herbs, shrubs, or twining vines; some are epiphytes. Only a few species are distributed in temperate regions mainly in four centers such as Indonesia and Malaysia, Africa, Brazil and Central America. The representatives of the family can be found in nearly every habitat, including dense or open forests, in scrublands, on wet fields and valleys, at the sea coast and in marine areas, and in swamps and as an element of mangrove woods. Plants in this family have simple, opposite, decussate leaves with entire (or sometimes toothed, lobed, or spiny) margins, and without stipules. The leaves may contain cystoliths, calcium carbonate concretions, seen as streaks on the surface. The flowers are perfect, zygomorphic to nearly actinomorphic, and arranged in an inflorescence that is either spike, raceme, or cyme. Typically there is a colorful bract subtending each flower; in some species the bract is large and showy. The calyx is usually 4-5 lobed; the corolla tubular, 2-lipped or 5-lobed; stamens either 2 or 4 arranged in pairs and inserted on the corolla; and the ovary superior, 2-carpellate, with axile placentation. The fruit is a two-celled capsule, dehiscing somewhat explosively. In most species, the seeds are attached to a small, hooked stalk (a modified funiculus called a jaculator) that ejects them from the capsule [10].

Genus *Clinacanthus*

*Clinacanthu*s Nees is a very small genus in the Acanthaceae. The plants in this genus are found distributing from southern China to Malaysia, young leaves are edible in Vietnam [11]. Flowers in dense cymes at the tops of the branches and their branchlets, resupinate; bracts narrow; calyx deeply 5-partite; segments narrow; corolla-tube long, curved, widened upwards, inside above the ovary with a whorl of hairs; limb bilabiate; upper lip forming a prolongation of the corolla-tube, turned downwards, shortly bilobate, innermost in bud, lower one slightly recurved, much

broader than the upper lip, of about the same length, 3-lobed; median lobe outermost in bud; stamens 2, inserted in the throat, subequalling the upper lip; anthers l-celled, ecalcarate; staminodes none; ovary compressed; ovary-cells 2-ovuled; style filiform, shortly bidentate; capsule basally contracted into a short, solid stalk, oblong, 4-seeded. Leaves opposite, ovate-oblong-lanceolate, with linear cystoliths. Shrub; branchlets erect-drooping or clambering over plants [12].

According to The Index Kewensis and its supplements, there are only 3 species of plant are in this genus as shown below.

- *Clinacanthus nutans* (Burm. f.) Lindau
- *Clinacanthus siamensis* Bremek.
- *Clinacanthus spirei* R. Ben.

1.1 *Clinacanthus nutans* (Burm. f.) Lindau (*Clinacanthus burmanni* Nees)

Clinacanthus nutans (Burm. f.) Lindau, Thai name Phaya yo (also known as Phaya plong dam, Phaya plong thong, Saled pang pon tua mia, etc.) is a medicinal plant that has been declared in the List of Herbal Medicinal Products A.D. 2006 by National Drug Committee that its properties are to relief of insect bites, skin rashes, relief of aphtous ulcer, herpes zoster and herpes simplex lesion, moreover, it reputed to relief of inflammatory, anti snake venom and anti dysentery.

Botanical description

...The plant is a shrub with pubescent branches. Leaves are simple, opposite, narrowly elliptic oblong or lanceolate, 2.5-13 cm long, 0.5-1.5 cm wide. The leaves have apex acute or acuminate; margin exsculptate-dentate or subentire; base cuneate, obtuse, rounded or truncate often oblique; pubescent on the nerves; petiole 3-15 mm long. Flowers are in dense cymes at the top of the branches and their branchlets; cymes 5-∞ flowered, often terminating drooping horizontal branches but themselves erect, subsecund, combined into a large lax, leafy panicle. Each flower has calyx densely patently glandular-pubescent, about 1 cm long; corolla glandular-pubescent, about 3.5 cm, dull red with green base; lower lip (turned upwards) with yellow streaks, apically sordidly yellow or

greenish yellow; stamens 2, inserted in the throat, more or less appressed against the upper lip. Ovary is compressed, 2-celled, 2 ovules in each cell; having style filiform, shortly bidentate. Capsule is oblong, basally contracted into a short, solid stalk, 4-seeded [13].

Scandent shrub, erectdrooping, leave: opposite, ovate- or oblong-lanceolate, tapering or long acuminate, exsculptate-dentate or subentire, base often oblique, cuneate, obtuse or rounded, pubescent on the nerves, 2-3 cm wide and 7-9 cm long, yellowish green to dark green; petiole 0.5 mm long, inflorescence in dense cyme, terminal, flowers 3-4 cm long, bilabiate, calyx 5, green, united at the base, glandular-pubescent, 10-16 mm long; corolla tube long, dull red with a green base, lower lip 3 lobed, with yellow streaks, apically pale yellow or greenish yellow, turn upwards, much broader than the upper lip and about the same length, upper lip turn downwards, shortly bilobate; stamens 2, epipetalous, subequalling the upper lip; ovary 1, compressed, 2-celled; bracteol, 3.5-10 mm long, green: fruit a capsule, basally contracted into a short solid stalk, oblong, 4-seeded… **(Figure 1)** [14].

Figure 1 *Clinacanthus nutans* (Burm. f.) Lindau

Researches about *C. nutans* have been done continuously. Not only pharmaceutical or clinical perspective, but has also been investigated for the active chemical constituents and applicable for industry as exemplified:

Pharmacological activity

Anti snake venom

C. nutans is a herb reputed in Thailand to be snakebite antidote. It has been tested *in vitro* and *in vivo* for antivenin activity. It was concluded that the extract of *C. nutans* could not antagonize the action of cobra venom. The aqueous extract of *C. nutans* leaves was found to have no effect on the inhibition of neuromuscular transmission produced by purified *Naja naja siamensis* neurotoxin in isolated rat phrenic-nerve diaphragm preparations. When gave the extract orally or intraperitoneally to the experimental mice receiving lethal doses of *N.n. siamensis*, the results are ineffective in prolonging the survival time of crude venom. Oral administrations of the extract pretreated with alpha-amylase or beta-amylase also fail to protect the animal [15]. In 2005, Daduanga *et al*. screened 36 plants for the ability to antagonize activity to *Naja naja siamensis* cobra venom by modified Enzyme-linked immunosorbent assay (ELISA). *C. nutans* showed almost 35 per cent inhibitory activity in the ELISA test [16].

Antiviral activities

Ethanol extract of *C. nutans* leaves was tested for the ability of anti herpes simplex virus type 2 (HSV-2) compared to acyclovir by using plaque reduction assay in baby hamster kidney cell line. The ethanol extract of *C. nutans* leaves could inhibit plaque forming by HSV-2. After the test, the researcher further did clinical trial using *C. nutans* extract with patients who had genital herpes. The result revealed that the patients used *C. nutans* extract could develop crust lesion within 3 days and healing within 7 days which greater than those with placebo group. Beside of shortening the duration of infection and reducing of severity, *C. nutans* extract had no sticky, burning, stinging pain and any side effects [17].

The extracts of *C. nutans* (which were extracted in methanol then dissolved in acetone, solubilized by complexing with polyvinyl pyrrolidone) was

assessed for intracellular activities against standard herpes simplex virus type 2 strain G, and 5 clinical HSV-2 isolates along with *Barleria lupulina* determined by plaque inhibition assay. The result indicated that *B. lupulina* extract exhibited activity against all 5 isolates but not the standard strain while that of *C. nutans* did not show any activity against these viruses. However, the smaller plaque sizes observed on infected cell monolayers treated with *B. lupulina* and *C. nutans* extracts might indicate that both had affected intracellular activities of HSV-2. Contradict to those reported by Jayavasu *et al*., above. This might be due to differences in the extraction procedure so, the obtained constituents were different and the dose of extracts employed [18].

Not only testing with herpes simplex virus, *C. nutans* leaves extract was also evaluated with varicella zoster virus (VZV) using plaque reduction assay and DNA hybridization. The results showed the extract could inhibit plaque forming by VZV and it was recognized that the antiviral activity might be a direct interaction of *C. nutans* to the virus [19]. Fifty one herpes zoster patients were treated by applying 5% *C. nutans* extract cream 5 times daily for 7-14 days compared to the placebo group. The result revealed that the wounds were crusted within 3 days and the lesions were completely healed within 7 and 10 days which were significantly greater than the group with placebo. There were no side effect of treating with *C. nutans* extract and the pain score decreased significantly [20]. Moreover, patients with recurrent herpes simplex virus infection were treated with *C. nutans* extract compared to the acyclovir treated and placebo group. The result exhibited the *C. nutans* extract treated and acyclovir treated patients' lesion were crusted in 3-7 days which was significantly greater than those with placebo and there was no side effect in treating group during the treatment [21].

A double blind controlled trial was established to test for the efficacy of *C. nutans* orabase in treatment of recurrent aphthous stomatitis in 43 patients compared to triamcinolone acetonide in orabase and placebo. *C. nutans* extract in orabase provided better healing than the placebo but was less so when compared to triamcinolone acetonide in orabase [22].

The aqueous and ethanol extracts of *C. nutans* leaves were examined for the ability in inhibition of newcastle disease virus (NDV) which causes

Newcastle disease in chicken by testing in chick embryo fibroblasts (CEFs) using hemaglutination test (HA-test). The result exhibited both aqueous and ethanolic extracts of *C. nutans* leaves at every concentration had no ability to inhibit NDV to infected cells [23].

Activity on the immune system

Human immunocompetent cells were used to study the role of *C. nutans* extract in modulating cell-mediated immune response (CMIR) *in vitro*. The experiment was done by investigation of *C. nutans* extract affect to lymphocyte proliferation, function of natural killer (NK) cells, and production of interleukin-2 (IL-2) and interleukin-4 (IL-4). The results revealed that the plant extract increased lymphocyte proliferation, the activity of NK cells was decreased, the level of IL-2 released from *C. nutans* treated-mononuclear cells was undetectable, whereas that of IL-4 was shown to be induced by the extract. These data suggested that the effect of *C. nutans* on human CMIR *in vitro* may be partially due to the release of IL-4 from peripheral blood mononuclear cells [24].

Anti-inflammatory activities

Investigation for the anti-inflammatory activities of *C. nutans* extracts using *in vivo* inflammatory carrageenan-induced paw oedema and ethyl phenylpropiolate-induced ear oedema in rats model was examined. Myeloperoxidase (MPO) activity was assayed as an indicator of neutrophil migration. The result exhibited the extract induced powerful dose-dependent inhibitory effects in both edema models in rats. Importantly, there was a significant inhibition of MPO activity in the inflamed tissue indicating that the anti-inflammatory effect of the extracts was associated with reduced neutrophil migration. These findings suggested that the powerful anti-inflammatory properties of *C. nutans* extracts were mediated, in part, by inhibition of neutrophil responsiveness [25].

Antioxidant activity

Ethanolic extract of *C. nutans* dried leaves was examined for the antioxidant activity. The free radical (1,1-diphenyl-2 picrylhydrazyl; DPPH) scavenging activity, the ferric reducing antioxidant power (FRAP) and the intracellular antioxidant activity of the extract were determined. The protective effect of the extract against 2, 2′-azobis (2-amidinopropane) hydrochloride (AAPH)-induced rat red blood cell lysis was also evaluated. The results indicated that the extract could scavenge DPPH with the maximum scavenging activity of 67.65±6.59% and with an IC_{50} of 110.4±6.59 μg/ml. The extract demonstrated a significant inhibition of peroxide production in rat macrophages stimulated by phorbol myristate acetate (PMA) and protected red blood cell against AAPH-induced hemolysis [26].

Toxicity

Toxicity of ethanolic extract of *C. nutans* leaves was investigated in mice. The extract did not show any signs of toxicity in the treated mice even at the highest dose of 1.3 g/kg of body weight (g/kg BW) which is equivalent to 5.44 g/kg BW of dried powdered leaves given orally, subcutaneously or intraperitoneally. Subchronic toxicity study was also performed in rats. The extract was given daily for 90 days at the oral doses of 0.01, 0.1 and 1.0 g/kg BW, equivalent to dried leaves 0.042, 0.42 and 4.18 g/kg BW, respectively. It was found that the body weights of male rats receiving the highest dose of the extract were significantly lower than those with control group while food consumptions of the two groups were not different. Hematological and blood chemistry studies showed platelet counts of both sexes rats receiving the highest dose of the extract were significantly higher than the control group while creatinine levels of the treated groups were lower than the control group. Examination of internal organs histopathology did not show any abnormalities that could be due to the effect of the extract [27].

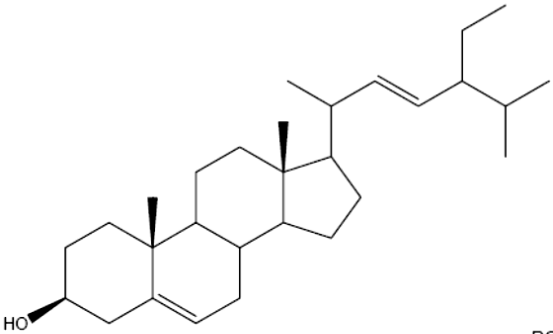
Cytotoxicity to Koi Fin cell line of *C. nutans* extract was tested. The experiment was done *in vitro* by directly exposed the various concentrations of plant extracts to the cell line compared to various concentrations of acyclovir. The plant extract at concentration not exceed 0.001% (w/v) did not damaged the

cell line. The result may be used as a minimum concentration for effective antiviral concentration against Koi herpes virus (KHV), which will be very useful for treating or preventing the disease [28].

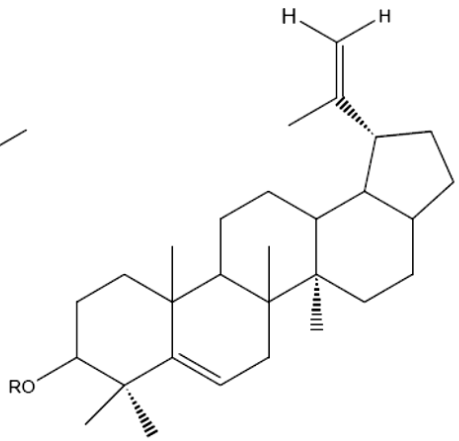
Chemical constituents and compound analysis

Compound analysis was also evaluated in this plant. Chemical constituents of *C. nutans* have been reported previously. Stigmasterol (1), lupeol (2) β-sitosterol (3) and betulin (4) were isolated from several parts of *C. nutans* [29-30].

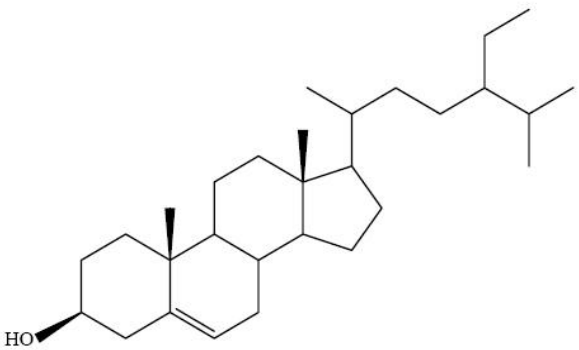
Six known C-glucocyl flavones were discovered. They were vitexin (5), isovitexin (6), shaftoside (7), isomollupentin-7-O-β-glucopyranocide (8), orientin (9) and isoorientin (10) from the methanol extract of stems and leaves of *C. nutans* [31].



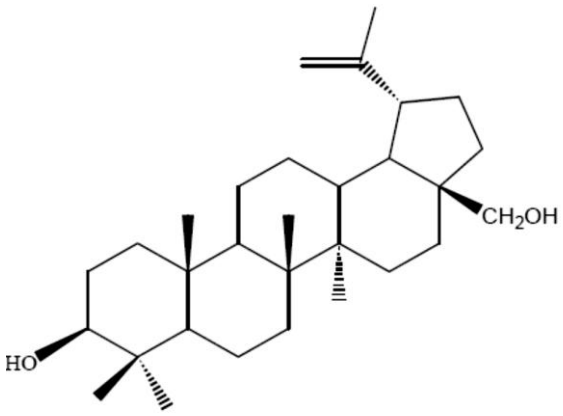
(1) Stigmasterol



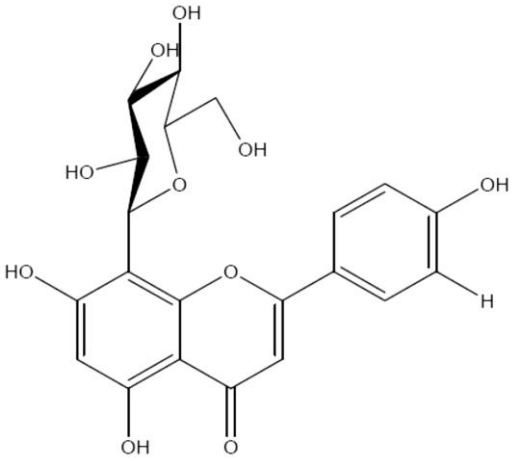
(2) Lupeol



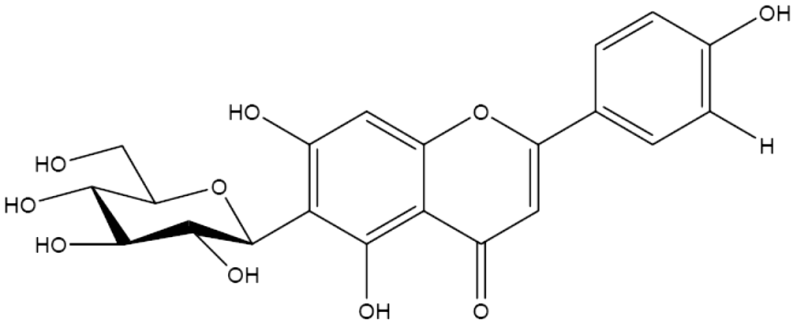
(3) β-sitosterol



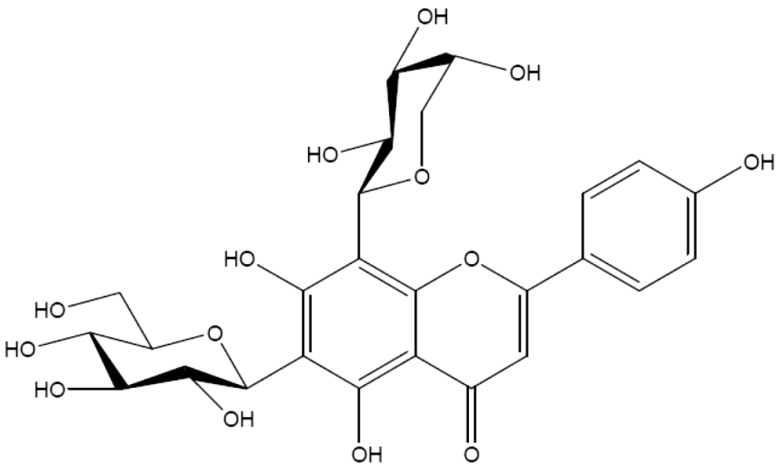
(4) Betulin



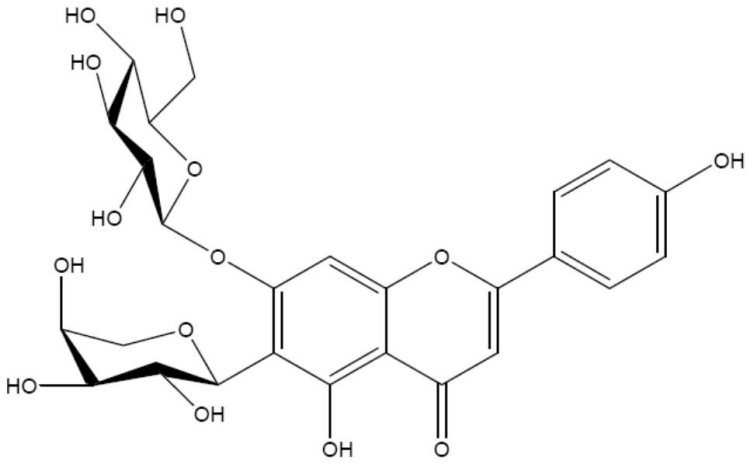
(5) Vitexin



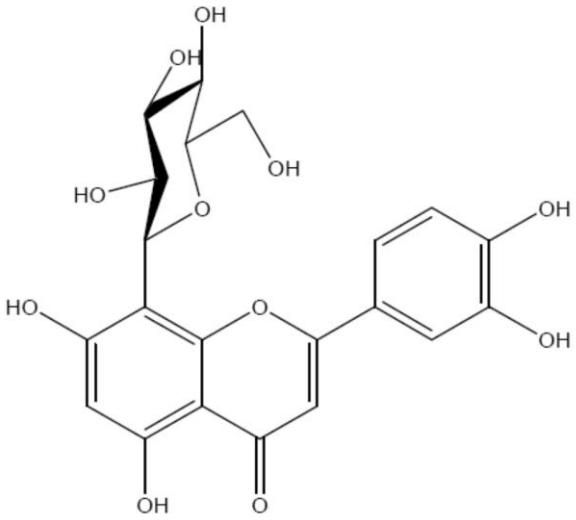
(6) Isovitexin



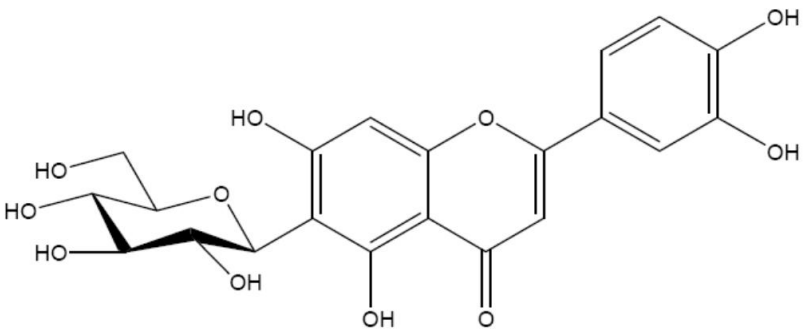
(7) Shaftoside



(8) Isomollupentin-7-O-β-glucopyranocide

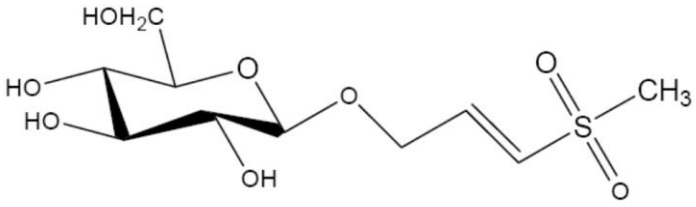


(9) Orientin

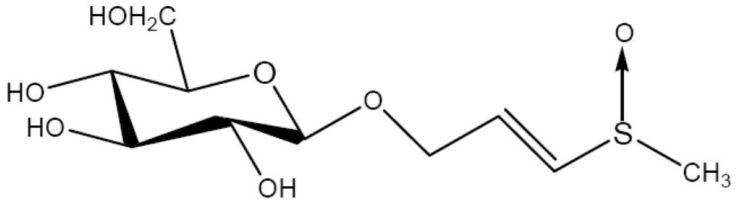


(10) Isoorientin

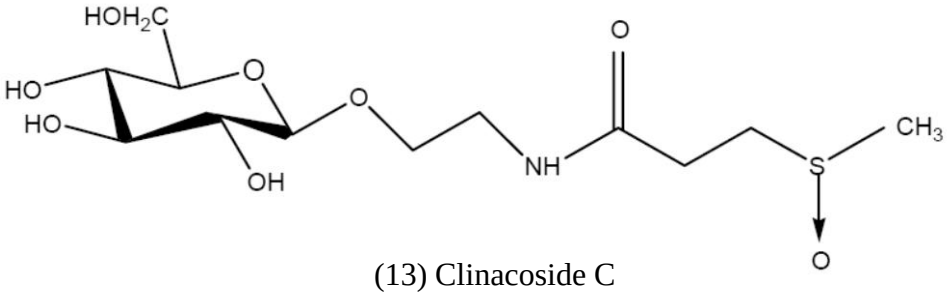
Five sulfur-containing glucoside were isolated. They were clinacoside A (11), clinacoside B (12), Clinacoside C (13), cycloclinacoside A1(14) and cycloclinacoside A2 (15) from methanol extract of stems and leaves of *C. nutans* by using the butanol and water soluble portion of the methanol extract [32].



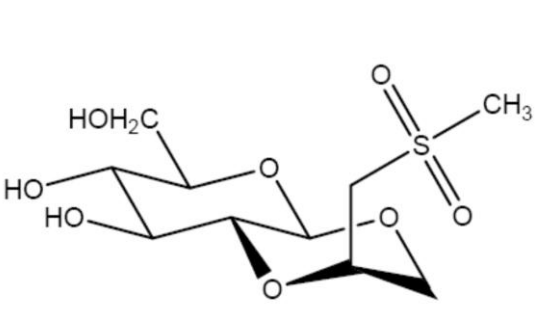
(11) Clinacoside A



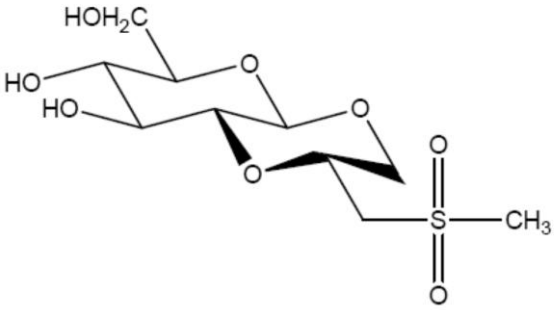
(12) Clinacoside B



(13) Clinacoside C

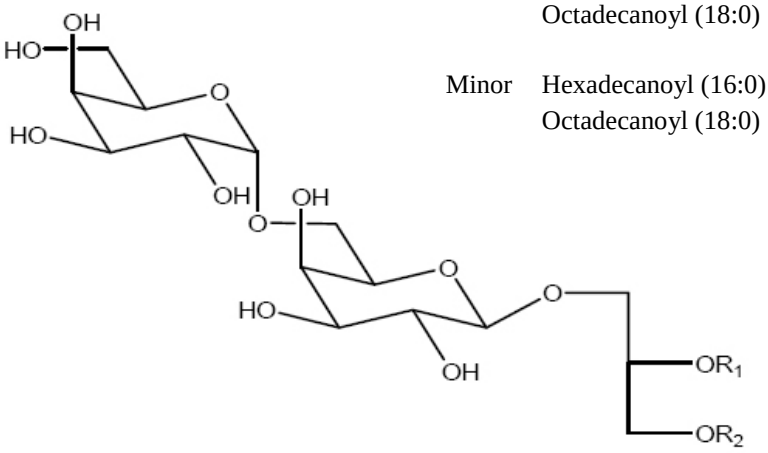


(14) Cycloclinacoside A1



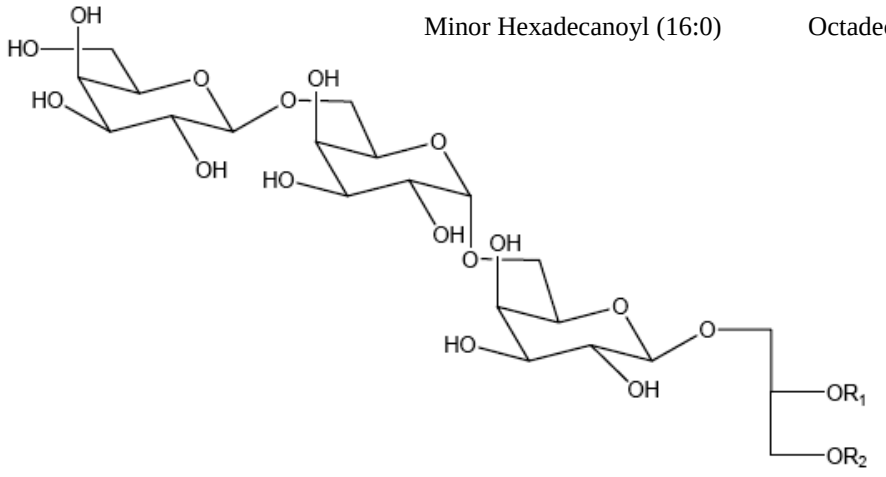
(15) Cycloclinacoside A2

Two glycoglycerolipids (1,2-O-dinolenoyl-3-O-β-D-galactopyranosyl glycerol (16) and 1-O-palmitoyl-2-Olinolenoyl-3-O-[α-D-galactopyranosyl-(1”→6’)-O-β-D-galactopyranosyl] glycerol (17)) were isolated from leaves of *C. nutans* and were further investigated and reported that the two mentioned glycoglycerolipids could inhibit the activity of herpes simplex virus [33].



	R_1	R_2
Major	Hexadecanoyl (16:0)	Hexadecatrienoyl (16:3)
	Octadecanoyl (18:0)	Hexadecatrienoyl (16:3)
Minor	Hexadecanoyl (16:0)	Octadecanoyl (18:0)
	Octadecanoyl (18:0)	Hexadecanoyl (16:0)

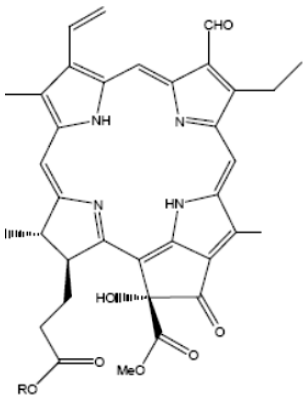
(16) 1,2-O-dinolenoyl-3-O-β-D-galactopyranosyl glycerol



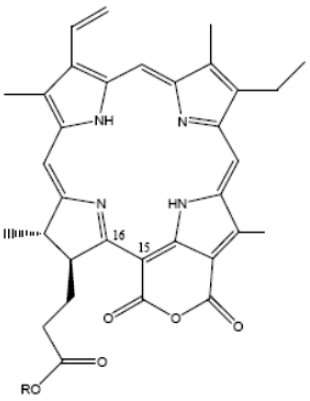
	R_1	R_2
Major	Octadecanoyl (18:0)	Octadecatetraenoyl (18:4)
	Octadecatetraenoyl (18: 4)	Octaadecanoyl (18: 0)
Minor	Hexadecanoyl (16:0)	Octadecatrienoyl (18:3)

(17) 1-O-palmitoyl-2-Olinolenoyl-3-O-[α-D-galactopyranosyl-(1”→6’)-O-β-D- galactopyranosyl] glycerol

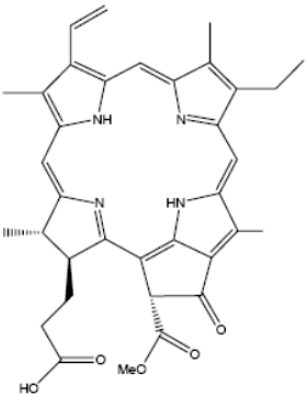
13^2-hydroxy-(13^2-*S*)-phaeophytin b (18), purpurin 18 phytylester (19) and phaeophorbide a (20) were isolated from chloroform extract of *C. nutans* leaves [34].



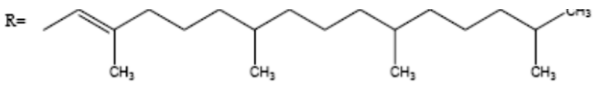
(18)
13^2-hydroxy-(13^2-*S*)-phaeophytin b



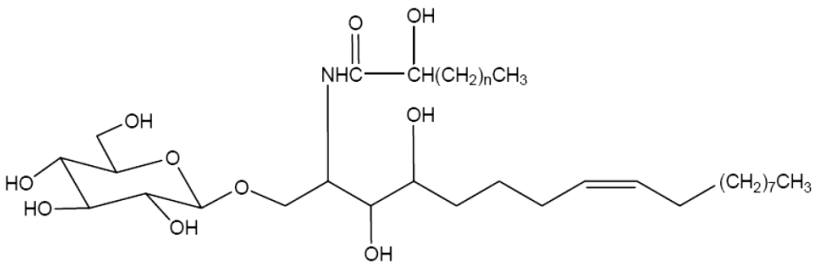
(19)
Purpurin 18 phytylester



(20)
Phaeophorbide a

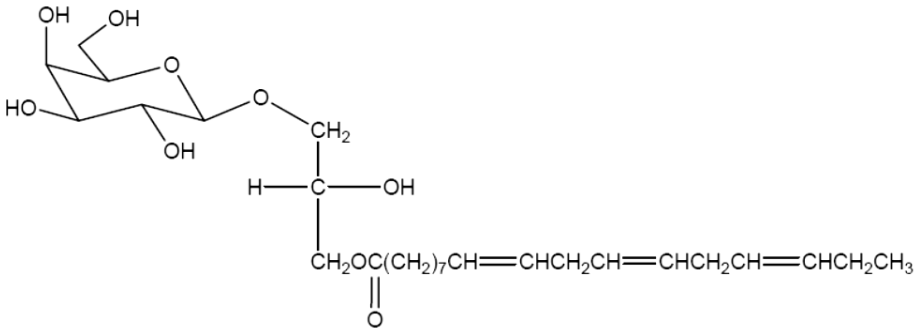


A mixture of nine cerebrosides (21) and a monoacylmonogalactosylglycerol (22) were isolated from the leaves of *C. nutans* and were separated by established on the basis of the spectroscopic data and chemical reaction. The structures of the cerebrosides were characterized as 1-O-beta-D-glucosides of phytosphingosines, which comprised a common long-chain base, (2*S*,3*S*,4*R*,8*Z*)-2-amino-8(*Z*)-octadecene-1,3,4-triol with nine 2-hydroxy fatty acids of varying chain lengths (C(16), C(18), C(20-26)) linked to the amino group. The glycosylglyceride was characterized as (2*S*)-1-O-linolenoyl-3-O-beta-D-galactopyranosylglycerol [35].



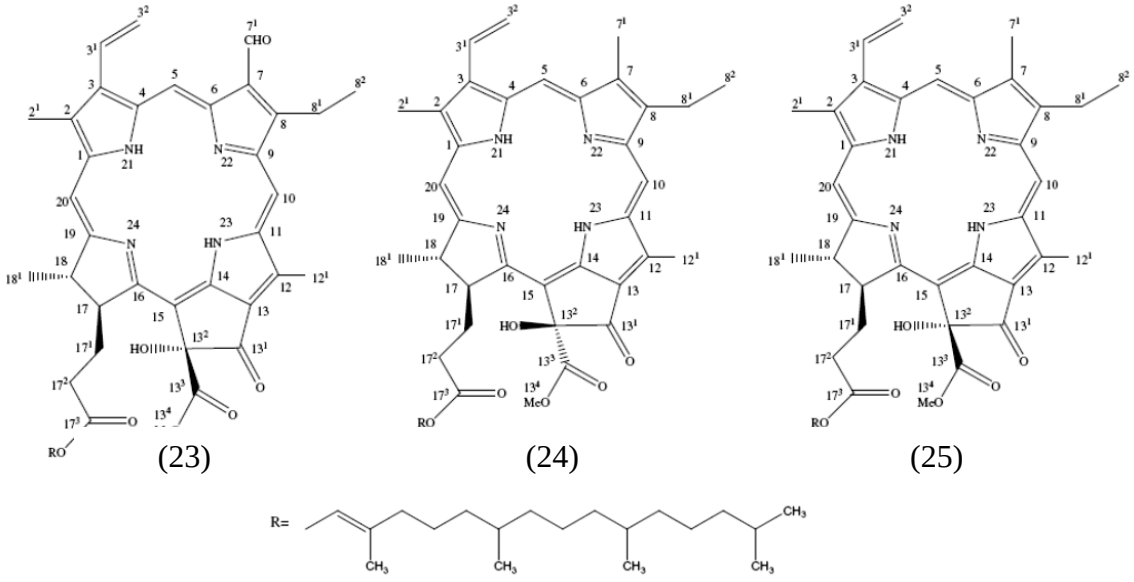
n= 13, 15, 17-23

(21) Cerebrosides



(22) (2*S*,3*S*,4*R*,8*Z*)-2-amino-8(*Z*)-octadecene-1,3,4-triol

Three chlorophyll derivatives were isolated from the chloroform extract of *C. nutans* leaves, they are chlorophyll b (13^2-hydroxy-(13^2-*R*)-phaeophytin b (23) and two isomers of chlorophyll a (13^2-hydroxy-(13^2-*S*)-phaeophytin a (24), 13^2-hydroxy-(13^2-*R*)-phaeophytin a (25). The structures of the isolated compounds were characterized on the basis of NMR spectra. The three compounds were also further investigated for anti herpes simplex virus type-1 activity. Their IC_{50} concentration exhibited to restrain HSV-1 for 100% in pre-viral entry step and about 30% in post-viral entry step [36].



(23) (24) (25)

(23) 13^2-hydroxy-(13^2-*R*)-phaeophytin b
(24) 13^2-hydroxy-(13^2-*S*)-phaeophytin a
(25) 13^2-hydroxy-(13^2-*R*) phaeophytin a

Research for the application to industry was also done. C-glycosyl flavones isolated from *C. nutans* leaves extract was tested for corrosion inhibition of AISI 1045 steel in 0.05 M sulfuric acid at 25^{o}C. The corrosion behavior of steel has been investigated using potentiodynamic polarization technique. Saturated calomel electrode was used as a reference electrode and a platinum plate was a counter electrode. It has been found that the extract from *C. nutans* the inhibition efficiency as high as 96% when the concentration of the extract was 3000 ppm. Moreover, the extract also exhibited the property of anodic inhibitor [37].

1.2 *Clinacanthus siamensis* Bremek.

Clinacanthus siamensis Bremek. (Thai name: Lin nguu hao, means cobra tongue) has been used in Thailand as a traditional medicine for treatment of insect bites and skin rashes [38].

Botanical description

…Caulis rami que an initio glabri, subteretes, leviter striati; internodia 1-4 cm. Longa et 2.5-3.0 mm. diam. Folia petiolo glabro plerumque 2-3 cm. Longo instructa; lamina lanceolata, plerumque 11-16 cm. Longa et 3-5 cm. Lata, apice acuminata, basi acuta vel subacuta, rarius paulum asymmetrica, costa subtus prominula, nervis utroque latere costae plerumque 5 vel 6. Inflorescentia corynbosa, nutans, rachidibus pedicellisque puberulis. Flores resupinati, bracteis linearibus 5-7 mm. longis suffulti. Pedicelli circ. 2 mm. longi. Calycis lobi lineares, 12 mm. longi, ut bracteae pilis capitatis puberulo-hirtelli. Corolla 5 cm. longa, extus pilis capitatis parce et vix notabile puberula, intus prope basin breviter pubescens, labiis 15 mm. longis, supero deltoideo, ad basin 6 mm. lato, apice obtusis, mediano conduplicato. Granula pollinis globosa, eis generis Pseuderanthemi similiora, plurima parva et sterilia, aliquae tamen normalia, 46 µ diam. Discus annularis glaber. Ovarium glabrum, 2.5 mm, altum; stylus glaber 4.5 cm. longus; stigma breviter bilobatum. Capsula nondum visa… [39]

Scandent shrub, 1.5-4 m high, branchlets erect-drooping climbing over other plants. Leaves simple, opposite, lanceolate or lanceolate-oblong, 2.5-4 cm

wide, 7-12 cm long. Inflorescence in terminal dense cyme; flowers dull red with green base, tubular, bilabiate. Fruit capsule [40].

Leaf lanceolate, oblong-lanceolate, ovate, subentire, oblique base, 2.5-4 cm wide and 7-12 cm long, color green to dark green (fresh leaves) and pale green to yellowish green (dried leaves) **(Figure 2)** [41].

Figure 2 *Clinacanthus siamensis* Bremek.

This plant was also investigated for its pharmaceutical properties and its chemical compounds, as exemplified;

Pharmacological activity

Antiviral activities

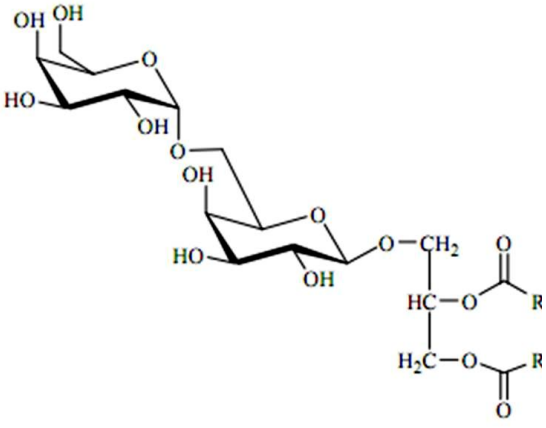
Some Thai medicinal plants including *C. siamensis* were determined for anti herpes simplex virus type 1 resistant to either acyclovir (ACV) or foscarnet (FOS) activity, the characteristics of one-step growth and antigenicity expressed by ACV- and FOS-resistant HSV-1 mutants. The results suggested that *Barleria lupulina*, *Clinacanthus siamensis* and *Nephelium lappaceum* showing better activities on the drug-resistant HSV. *C. siamensis* extract had effect to the herpes simplex virus ACV-resistant AR3, IC_{50} 62.5 µg/ml [42]. *C. siamensis* has a protective effect against infection by certain influenza viruses, evaluation of the

ethanolic extracts of *C. siamensis* leaves by treating 1 mg/kg per day, 5 times orally with BALB/C mice that had been induced to infect with influenza virus H3N2. The result exhibited the *C. siamensis* ethanolic extract had a great property to inhibit the specific influenza virus H3N2 [38].

Chemical constituents and compound analysis

Jamtaweekul and colleagues preliminarily screened for the phytochemistry in several extracts of *C. siamensis* leaves. The result revealed that *C. siamensis* leaves might contain triterpenes and steroids, reducing sugars and flavonoids [41].

Chemical constituents from the methanol extract of *C. siamensis* leaves were investigated which led to the isolation of glycoglycerolipids as the peracetyl derivatives. Structure elucidation and identification of the isolated compounds were mainly determined by spectroscopic data analysis especially 1D and 2D-NMR, comparison with previously reported data, together with analysis of gas chromatograms of fatty acid methyl esters from acid and enzymatic hydrolysis. The glycoglycerolipids (1) were one mixture of monogalactopyranosyl diglycerides and two mixtures of digalactopyranosyl diglycerides. The acyl groups of the glycoglycerolipids were hexadecanoic acid (16:0), hexadecaenoic acid (16:1), octadecanoic acid (18:0) and octadecatrienoic acid (18:3). In addition, a mixture of β-sitosterol-3-O-glucoside and β-stigmasterol-3-O-glucoside was also isolated [43].



Compound 1

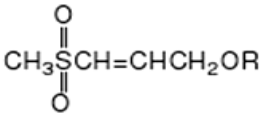
R_1	R_2
Hexadecanoyl (16:0)	Hexadecaenoyl (16:1)
Hexadecanoyl (16:0)	Octadecatrienoyl (18:3)

Compound 2

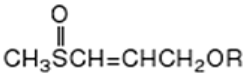
	R_1	R_2
Major	Hexadecanoyl (16:0)	Hexadecanoyl (18:0)
Minor	Hexadecanoyl (18:0)	Hexadecanoyl (16:0)
	Hexadecanoyl (16:0)	Octadecatrienoyl (18:3)
	Hexadecanoyl (18:0)	Octadecatrienoyl (18:3)
	Hexadecanoyl (16:0)	Hexadecanoyl (16:0)
	Hexadecanoyl (18:0)	Hexadecanoyl (18:0)

(1) glycoglycerolipids

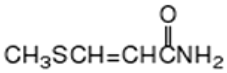
Two new sulfur-containing compounds, *trans*-3-methylsulfonyl-2-propenol (2) and *trans*-3-methylsulfinyl-2-propenol (3), have been isolated from the n-BuOH-soluble fraction of the ethanolic extract of the fresh leaves of *C. siamensis*, together with *trans*-3-methylthioacrylamide (4), entadamide A (5) and entadamide C (6). Subsequently, entadamide A has been shown to inhibit 5-lipoxygenase activity in RBL-1 cells, suggesting that it may have anti-inflammatory properties, and trans-3-methylthioacrylamide had anti mycobacterial activity against *Mycobacterium tuberculosis* H37Ra with a minimum inhibitory concentration (MIC) value of 200 μg/ml [44].



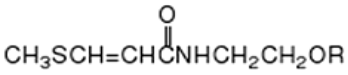
(2) *trans*-3-methylsulfonyl-2-propenol



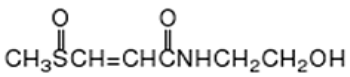
(3) *trans*-3-methylsulfinyl-2-propenol



(4) *trans*-3-methylthioacrylamide



(5) entadamide A



(6) entadamide C

1.3 *Clinacanthus spirei* R. Ben.

Botanical description

...Tiges cylindriques, striees longitudinalement, giabres. Feuilles brievement petiolees, lanceolees, arrondies a la base, acurninees au sommet, a bord entier, recourbe en dessous, glabres, mais un peu rudes sur les 2 faces, longues de 6-10 cm. sur 1.5-3 cm Inflorescence: fleurs en epis denses, terminaux, penches; bractees largement lane eo lees, a sommet arrondi, obtus, pubescentes-glanduleuses, longues de 20-22 mm. - Sepales 5, lineaires, pubescents-

glanduleux, libres des la base, longs de 10 mm. environ. Corolle longue de 35-40 mm., le tube legererement courbe et s elargissant graduellement jusqu a la gorge; Jevre superieure lanceolee, echancree au sommet; I inferieure brievement trilobee. Etamines 2, a filets poilus. Ovaire et base du style poilus… [45]

2. Method in plant authentication

The methods that are usually employed in the authentication of plant or herbal materials are macroscopic, microscopic and chromatography examination. These methods are rapid and inexpensive. In addition, chemical analysis is by far the best method for the detection of contaminants and can be an excellent method for plant identification. Unfortunately, each of these methodologies has limitations and more analytical methods are needed to assist in the authentication process. Thus, authentication of plant using biomolecular methods is widely applied nowadays and this method offers an assortment of techniques that can be very useful for authentication of medicinal plants [46].

2.1 Macroscopic evaluation

Macroscopic assessment is an effective tool for determining the identity of plant material. Macroscopic evaluation is an assessment of the plant material, either with the naked eye or with simple magnification such as with a hand lens or stereomicroscope. It typically includes gross morphological characteristics including colour, form, size, texture, and fracture (how the plant part breaks; usually associated with roots and barks) along with the plant's organoleptic characteristics (taste, aroma, quality). Similar species of plants can share similar morphological characteristics and so appropriate training is needed to acquire macroscopic identification skills.

2.2 Microscopic evaluation

Microscopy of medicinal plants focused on the observation of the cellular structures, and their content, of plant material by use of a microscope [47].

Stomatal number and stomatal Index

Stomata are openings (the stomatal pores or apertures) epidermis bounded by two specialized epidermal cells, the guard cells, which by changes in shape bring about the opening and closure of the aperture. The entire unit of pore and the two guard cells are convenient to apply in term "stomata".

For identification and characterization of leafy crude drugs, the stomatal number and the stomatal index were used. These values' criteria are very specific. Four different types of stomata are often available for matured leaves. They can be distinguished by their form and arrangements in the surrounding cells as shown in **Figure 3** [48].

- Anisocytic or Cruciferous stomata - These are unequal cell type, where the stomata is usually surrounded by three or four subsidiary cells one of which markedly small than the other.
- Anomocytic or Ranunculaceous stomata - These are irregular cell types. Here the stomata is surrounded by varying number of cells, which are generally not different from those of the epidermis.
- Paracytic or Rubiaceous stomata - These are parallel cell type. Here the stomata has two subsidiary cells with the parallel long axis of the stomata.
- Diacytic or Caryophyllaceous stomata - these are cross-celled type, the stomata is accompanied by two subsidiary cells, the common wall of which is at right angle to the stoma.

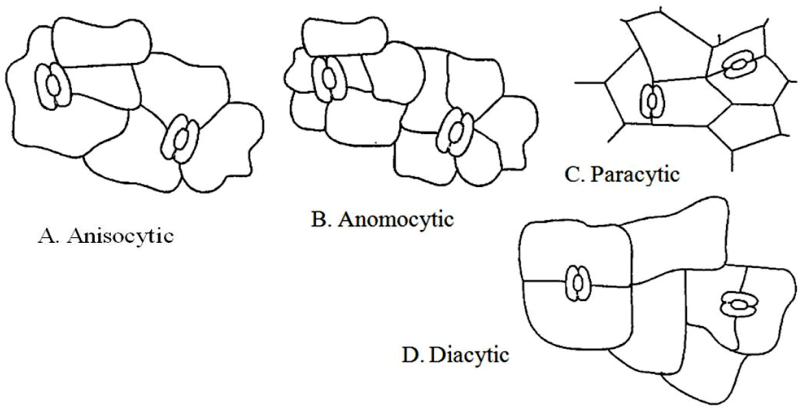


Figure 3 Surface view of epidermis illustrates 4 patterns of stomata where A. is Anisocytic B. is Anomocytic C. is Paracytic and D. is Diacytic

Stomatal number is the average number of stomata per square millimetre (mm^2) of epidermis and the number on each surface of a leaf. Each stoma consists of two guard cells and the spore is counted as a single unit. Though this has significance in determining the quality of crude drugs, this number varies unfortunately, depending on the environmental condition and geographical sources where the plants were grown. Stomatal index is one of the more distinguishing characteristics for herbal leafy drugs. It is the percentage proportion of stomata on one side and epidermal cells plus stomata on the other side. In other words, stomatal index is defined as the percentage of stomata from the total number of epidermal cells, which can be explained as:

$$\text{Stomatal index} = \frac{S}{E + S} \times 100$$

Where; S = The number of stomata in a given area of leaf;

E = Total number of epidermal cells including trichomes in the same area of leaf.

Palisade ratio

Palisade cells are a type of photosynthetic cells of the mesophyll of leaf occurring mostly just beneath the upper epidermal surface layer. The cells are elongated and more cylindrical and arranged in one or more rather regular, relatively compact layer near the ventral, or upper side of the leaf with the long axis of the cells perpendicular to the leaf surface **(Figure 4)**. This is another criterion for identification and evaluation of herbal drugs. It can be defined as the average number of palisade cells present beneath each upper epidermal cell. This value remains constant within a range for a given plant species and is of diagnostic value in differentiating the species. This value does not alter based on geographical variation and differs from species to species and that is why it is a very useful diagnostic feature for characterization and identification of different plant species. This determination is not applicable to monocot leaves, as the differentiation in mesophyll cell is not possible in monocot plants [48].

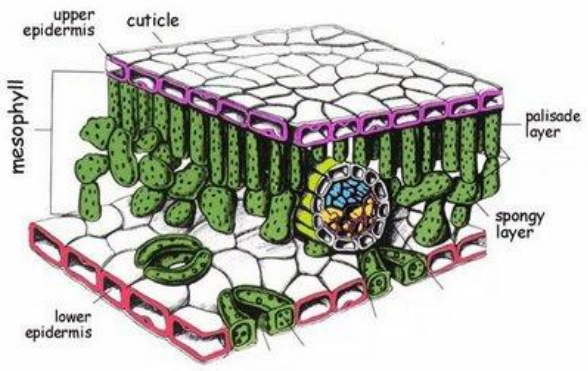


Figure 4 Palisade layer under upper epidermis.
Image from http://carmindydollheart.blogspot.com
(Retrieve 31 Jan 2012)

2.3 Chromatography

Chromatography is the separation of chemical compounds in a mixture. The mixture is dissolved in a fluid called the "mobile phase", which carries it through a structure holding another material called the "stationary phase". The various constituents of the mixture are separated due to the different speeds of the constituents travel. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation. There are various chromatographic techniques such as thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), etc [49].

2.4 Biomolecular evaluation

Investigation in biomolecular involved plant genomic DNA is important nowadays. Not only for generating the diversity of plants, it can be also applied for detecting of the adulteration in herbal drugs by the advantages of plants DNA identification. This method can be developed or used in various techniques. There are several DNA assessments reported such as evaluation of gene *atp*B nucleotide sequences for phylogenetic studies of ferns and other pteridophytes [50], the plants in genus *Ilex* (Aquifoliaceae) in southern South America were analyzed using AFLP (Amplified fragment length polymorphism) and ITS sequence data which the phylogeny tree was also generated from these evidences

[51]. Authentication of the valuable herbal drugs, for example, *Crocus sativus* was investigated for the patterns of the rDNA ITS sequence variation along with the mostly found adulterants - *Chrysanthemum chanetii*, *Nelumbo nucifera*, *Zea mays* and *Garthamus tinctorius*. The ITS sequence is an available molecular marker for identification of the *C. sativus* and can distinguish this plant from another misused substitutes [52].

Prior investigation the plants by biomolecular method, the genomic DNA is needed to isolate from the plants' cells. Besides the commercial instant DNA extraction kit, isolation DNA by CTAB method is considered to be a widely isolation method. Many different methods and technologies are available for the isolation of genomic DNA. The separation of DNA from cellular components can be divided into four stages: 1. Disruption, 2. Lysis, 3. Removal of proteins and contaminants and 4. Recovery of DNA. In some methods, stage 1 and 2 are combined [53]. In general, all methods involve disruption and lysis of the starting material followed by the removal of proteins and other contaminants and finally recovery of the DNA. Young fresh leaves are frozen rapidly in liquid nitrogen and grounded to powder then, lysed with the ionic detergent CTAB (cetyl trimethylammonium bromide), which forms an insoluble complex with nucleic acids in a low-salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away. Removal of proteins is typically achieved by organic solvent extraction. The DNA complex is solubilised by raising the salt concentration and precipitated with ethanol or isopropanol [54]. The obtained genomic DNA is then used as a DNA template for amplified the region. There are several regions in the DNA from various origins that used for studying the divergence or identity of plants, such as;

Nuclear genome

Nuclear genome is a linear DNA packed closely on the chromosome. It is the largest components in the nucleus. Nuclear genome is composed of information inherited equally from both parents, one male, and one female [55]. It is mostly used in forensic examinations. The regions of nuclear genome that commonly used in DNA fingerprint of herbal drug are;

Ribosomal DNA (rDNA)

Ribosomal DNA codes for ribosomal RNA. The ribosome is a macromolecule in the cell that is able to produce proteins or polypeptide chains. rDNA consists of a tandem repeat of a unit segment which comprises of non transcribed spacer (NTS), external transcribed spacer (ETS), 18S, ITS1, 5.8S, ITS2, and 28S tracts. In the large rDNA array, polymorphisms between rDNA repeat units are very low which means low rate of polymorphism among species, indicating that rDNA tandem arrays are evolving through concerted evolution, so, comparison of the rDNA segment including ITS region of the related species and phylogenetic analysis are accomplished **(Figure 5)** [56].

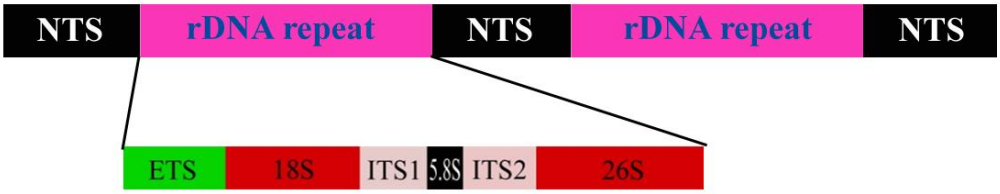


Figure 5 Gene sequence of eukaryotic rDNA

Internal transcribed spacers (ITS)

internal transcribed spacers (ITS) are sequences located in eukaryotic ribosomal DNA (rDNA) genes between the 18S and 5.8S rDNA coding regions (ITS1) and between the 5.8S and 26S rDNA coding regions (ITS2) [57]. It has been found as parts of repeat units that are arranged in tandem arrays. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary. Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 base pairs (bp)) enable easy amplification of ITS region due to high copy of rDNA repeats. This makes the ITS region an interesting subject for evolutionary/phylogenetic investigations [58]. The ITS region is typically been most useful for molecular systematics at the species level, and even within species (**Figure 6**) [59].

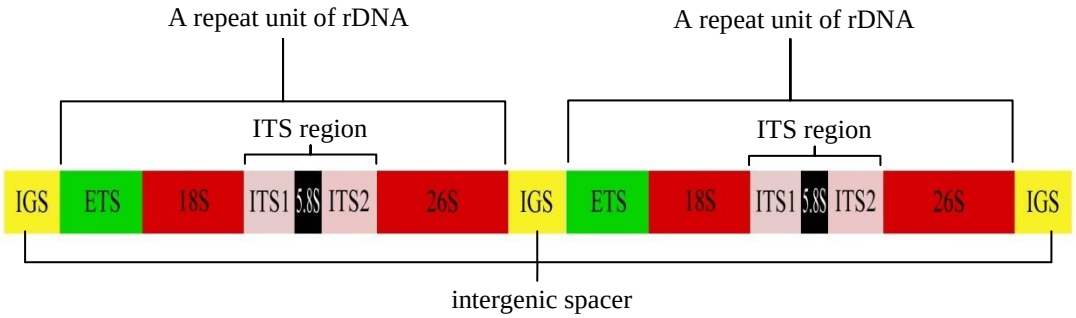


Figure 6 Structure of 2 repeat units of ribosomal DNA including ITS region which separated by intergenic spacer (IGS).

Other regions in nuclear genome that are used in evolution analysis of plants but was not generally used in DNA fingerprint in herbal drug such as *phy* gene (phytochrome), *gap*A gene (glyceraldehydes-3-phosphate dehydrogenase), *adh* gene (alcohol dehydrogenase) and *pgi* gene (phosphoglucose isomerase).

Chloroplast genome

Chloroplast genomes (cpDNA) are relatively large, usually approximately 140 kb in higher plants. Chloroplast genome codes for all the ribosomal RNA (rRNA) and transfer RNA (tRNA) species needed for protein synthesis [60]. It has been used extensively to infer plant phylogenies at different taxonomic levels. Direct sequencing of polymerase chain reaction (PCR) products is now becoming a rapidly expanding area of plant systematics and evolution [61]. Chloroplast DNA is uniparental inheritance, so it's pattern is homozygous which mean identical copies are present in the entire of a gene made sequencing easier. Chloroplast genome such as;

*mat*K Gene

The gene size approximatly 1500 base pairs, located within the intron of the chloroplast *trn*K gene. This gene can encode to enzyme maturase which presumably helps fold the intron RNA into the catalytically-active structure. The 3' end of the *mat*K was identified to contain a conserved region of about 100 amino acids and this region was named domain X. The *mat*K gene is

easy to amplify but, itself has fast evolution so, it is not possible to use the universal primer **(Figure 7**) [62].

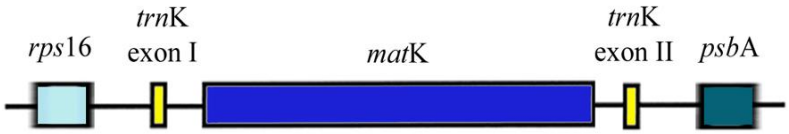


Figure 7 Structure of gene *mat*K which flanking between *trn*K exonI and *trn*K exon II.

*rbc*L Gene

*rbc*L gene or RuBisCO (Ribulose-1,5-bisphosphate carboxylase/ oxygenase) is a gene involved in catalyzing the primary chemical reaction by which inorganic carbon enters the biosphere which is first major step of carbon fixation. This gene has slow substitution rate and extensive database of sequences make *rbc*L sequence data well suited for phylogenetic studies at a variety of higher taxonomic levels, from interfamilial to subclass [63].

*atp*B Gene

*atp*B gene locates next to *rbc*L gene. It's common size is 1497 base pair in plants. This gene encoded β-subunit of ATP synthase which is an enzyme catalyzes ATP synthesis. Size, rate of evolution and lacking of intron, these are likewise to *rbc*L gene (**Figure 8**) [64].

Other chloroplast genomes are also used for investigating the plants such as gene *ndh*F, the region in the area of gene *trn*T, *trn*L and *trn*F, etc.

Figure 8 Structure of *rbc*L gene and *atp*B gene including the DNA flanking *atp*B-*rbc*L.

Mitochondrial genome

Mitochondrial genome is the DNA located in mitochondria which is involves in converting the chemical energy from food into adenosine triphosphate

(ATP), an energy form that cells can use. Mitochondrial genome is large and vary in size, moreover, substitute rate of the nucleotide in plants mitochondrial genome is slower than those of animals approximately 40-100 times and slower than those of nuclear genome and chloroplast genome around 12 and 3-4 times, respectively. Thus this genome is rarely used in authentication of herbal drugs [65].

To amplify the desire region, polymerase chain reaction (PCR) is commonly used.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a scientific technique in molecular biology developed in 1983 by Kary Mullis [66]. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. The principle is to amplify a single or a few copies of a piece of DNA and generating thousands to millions of copies of a particular DNA sequence [67]. A basic PCR set up requires several components and reagents such as, DNA template that contains the DNA region to be amplified, two primers that are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target, deoxynucleoside triphosphates (dNTPs; nucleotides containing triphosphate groups) which acts like the building-blocks from which the DNA polymerase synthesizes a new DNA strand, buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase, divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mg^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mg^{2+} concentration increases the error rate during DNA synthesis [68], monovalent cation potassium ions and *Taq* polymerase or another DNA polymerase with a temperature optimum at around 70^{o}C. *Taq* DNA Polymerase is a highly thermostable DNA polymerase of the thermophilic bacterium *Thermus aquaticus*. The enzyme catalyzes 5' to 3' synthesis of DNA, it has no proofreading activity which is no detectable 3' to 5' exonuclease and possesses low 5' to 3' exonuclease activity. In addition, *Taq* DNA Polymerase exhibits deoxynucleotidyl transferase activity, which frequently results in the addition of

extra adenines at the 3'-end of PCR products. Recombinant *Taq* DNA Polymerase is ideal for standard PCR of templates 5 Kilo base (kb) or shorter. The error rate of *Taq* DNA Polymerase in PCR is $2.2x10^{-5}$ errors per nucleotide (nt) per cycle, as determined by a modified method that was described [69]. Accordingly, the accuracy of PCR is $4.5x10^{4}$. Accuracy is an inverse of the error rate and shows an average number of correct nucleotides incorporated before an error occurs [70].

The PCR is commonly carried out in a reaction volume of 10–100 µl in small reaction tubes in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction [71]. A typical set of reactions might have a pre denaturation then, followed by 30-40 cycles of each comprising denaturation, annealing, extension and final extension. This would result in a theoretical amplification of over 10^{9}-fold (**Figure 9**). Then, evaluate the PCR product in 1.5% agarose gel electrophoresis which can separate nucleic acid molecules by size. Agarose gel that contains buffer is formed by a meshwork of molecules, and nucleic acids are driven through it by an electric field from charge negative to charge positive then visualize by staining the gel in ethidium bromide and observed under UV light [72]. There are some factors affect to the PCR exponential progression such as existing phenol or enzymes found in the sample which are inhibitors of the polymerase reaction, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product.

The advantage of PCR can lead to many applications such as sequencing, genetic engineering, cloning, forensic biology, etc [73].

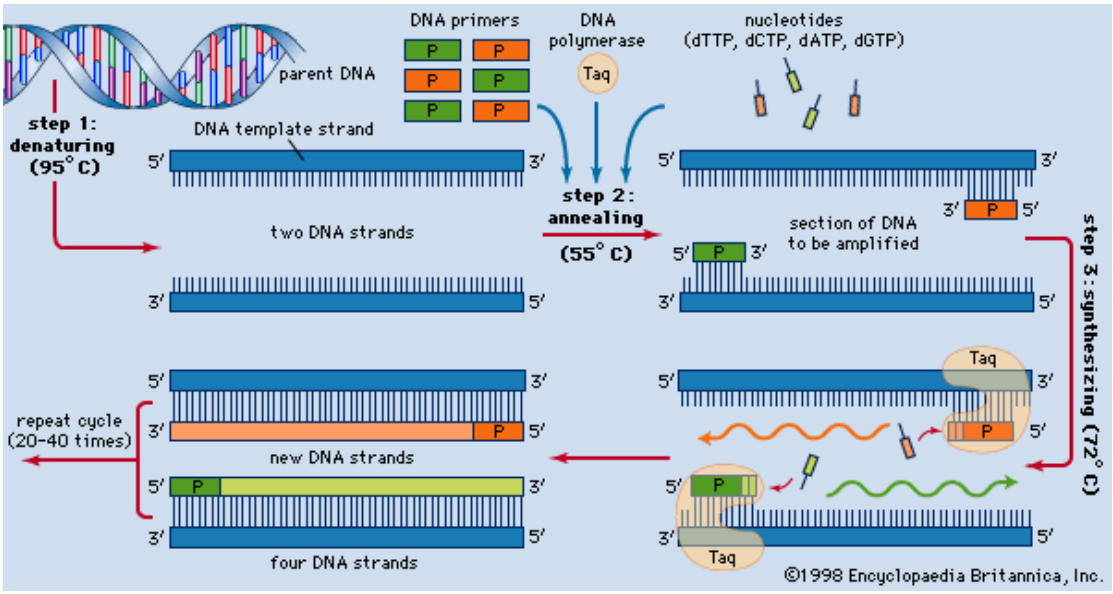


Figure 9 Process of PCR. Image from http://schoolworkhelper.net/2010/07/the-polymerase-chain-reaction-pcr-cloning-dna-in-the-test-tube/
(Retrieve 27 Apr 2012)

DNA sequencing

DNA sequencing is used for determining the order of the nucleotides which are adenine, guanine, cytosine, and thymine in a molecule of DNA. Knowledge of DNA sequences has become indispensable for basic biological research, other research branches utilizing DNA sequencing, and in numerous applied fields such as diagnostic, biotechnology, forensic biology and biological systematics. The advent of DNA sequencing has significantly accelerated biological research and discovery [74]. The methods can be categorized as 2 major methods as;

Chemical method

Chemical method may refer to Maxam-Gilbert sequencing. The method requires radioactive labeling at one 5' end of the DNA by a kinase reaction using gamma-32P ATP and purification of the DNA fragment. Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, C+T). For example, the purines (A+G) are depurinated using formic acid, the guanines (and to some extent the adenines) are methylated by dimethyl sulfate, and the pyrimidines

(C+T) are methylated using hydrazine. The addition of salt (sodium chloride) to the hydrazine reaction inhibits the methylation of thymine for the C-only reaction. The modified DNAs are then cleaved by hot piperidine at the position of the modified base. The concentration of the modifying chemicals is controlled to introduce on average one modification per DNA molecule. Thus a series of labeled fragments is generated, from the radiolabeled end to the first "cut" site in each molecule. The fragments in the four reactions are electrophoresed side by side in denaturing acrylamide gels for size separation. To visualize the fragments, the gel is exposed to X-ray film for autoradiography, yielding a series of dark bands each corresponding to a radiolabeled DNA fragment, from which the sequence may be inferred [75].

Chain termination method

The key principle of this method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. This method is better than chemical method because of the lower of toxic chemicals and lower amount of radioactivity is used. The method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleotidetriphosphates (dNTPs; dATP, dGTP, dCTP and dTTP), and modified nucleotides (dideoxyNTPs; (ddATP, ddGTP, ddCTP, or ddTTP), lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length. These ddNTPs will also be radioactively or fluorescently labelled for detection in automated sequencing machines. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides. The newly synthesized and labelled DNA fragments are heat denatured, and separated by size on a denaturing polyacrylamide-urea gel electrophoresis with each of the four reactions run in individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. In the image on the right, X-ray film was exposed to the gel, and the dark bands correspond to DNA fragments of different lengths. A dark band in a lane

indicates a DNA fragment that is the result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP). The relative positions of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence (**Figure 10**) [76].

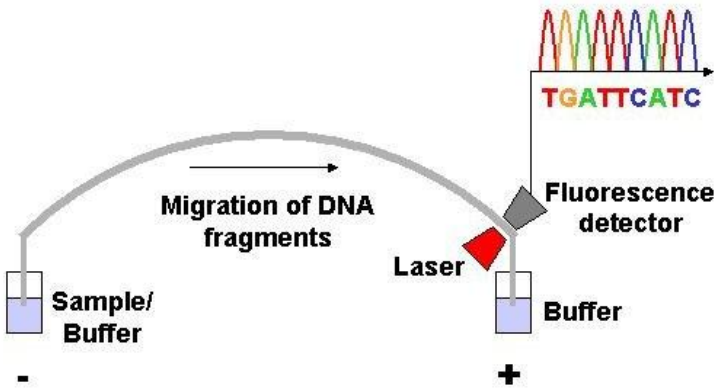


Figure 10 Image of the Chain Termination Sequence of DNA modeling. Image from http://www.dnasequencing.org/chain-method/49-dna-chain-termination (Retrieve 24 Apr 2012)

3. Antiviral activity

Virus is a small acellular microorganism that can infect to the host cell and causes diseases. It can only grow or reproduce only inside the host cells. According to the Baltimore classification, virus can be classified into 7 groups [77];

- Double-stranded DNA viruses such as Adenoviruses, Herpesviruses, Poxviruses.
- Single-stranded DNA viruses such as Parvoviruses.
- Double-stranded RNA viruses such as Reoviruses.
- Sense single-stranded RNA viruses such as Picornaviruses, Togaviruses.
- Antisense single-stranded RNA viruses such as Orthomyxoviruses, Rhabdoviruses.
- Sense single-stranded RNA - reverse transcriptase viruses such as Retroviruses.
- Double-stranded DNA - reverse transcriptase viruses such as Hepadnaviruses.

3.1 Herpes simplex virus

The herpes simplex virus is a DNA virus belongs in family Herpesviraldeae [78]. It is large, circular virus that is about 150-200 nm in diameter surrounded by an envelope and has a protein-bound caspid which holds the genetic information. The envelope that surrounds the virus is made up of glycoproteins which come from the host cell the virus is invading (**Figure 11**). This virus causes diseases in human, widely and frequently infect in human are herpes simplex virus type 1 and type 2. Herpes simplex virus type 1 causes cold sore which usually infect on oral area while those type 2 causes genital herpes which can infect via sexual intercourse. Herpes simplex virus can replicate itself by following steps; firstly, the HSV binds to the cell membrane of the host cell then the virus uncoat thus, the double strand DNA of the virus is injected to the host's DNA then, the important proteins which will be used for synthesizing nucleic acid will be synthesized, the next step, the HSV encodes for its own DNA then, structural protein is synthesized and assembled with the nucleic acid. The newly developed virus moves out of the nucleus and leaves the cell membrane (**Figure 12**) [79].

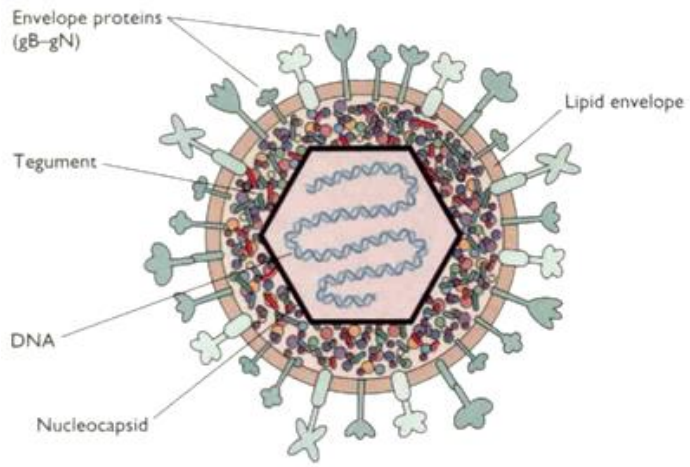


Figure 11 Structure of herpes simplex virus. Image from http://www.bio.davidson.edu/people/sosarafova/Assets/Bio307/jehodge/page01.html (Retrieve 12 Jan 2012)

The drug usually used against herpes viruses is acyclovir. It is a synthetic purine nucleoside analogue. The inhibitory activity of acyclovir is highly

selective due to its affinity for the enzyme thymidine kinase (TK) encoded by HSV. This viral enzyme converts acyclovir into acyclovir monophosphate, a nucleotide analogue. The monophosphate is further converted into diphosphate by cellular guanylate kinase and into triphosphate by a number of cellular enzymes. *In vitro*, acyclovir triphosphate stops replication of herpes viral DNA [80]. Besides acyclovir, there are other anti HSV agents for example, femicyclovir, foscarnet, etc.

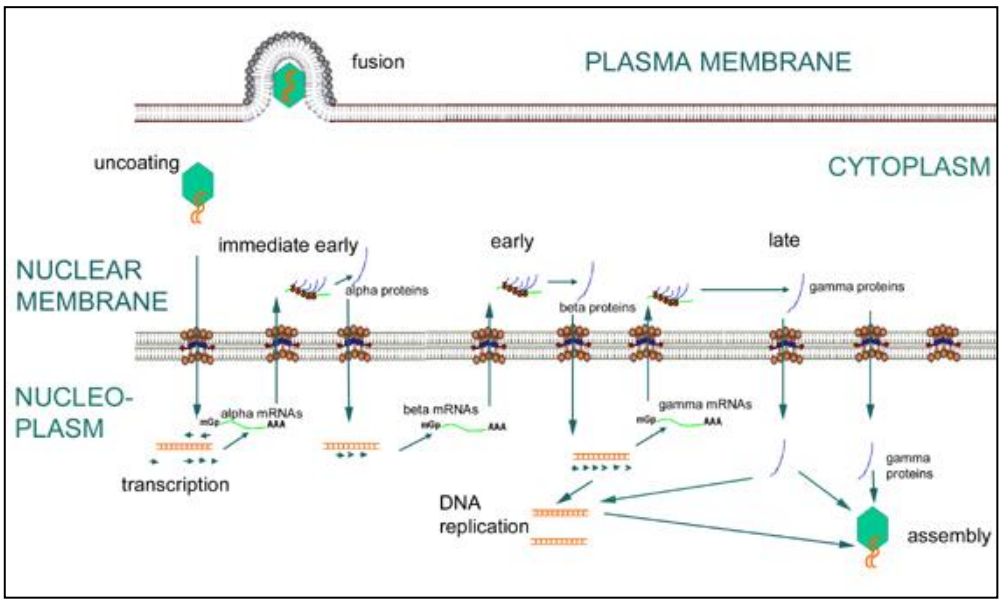


Figure 12 Replication of herpes simplex virus. Image from http://pathmicro.med.sc.edu/mhunt/dna1.htm (Retrieve 12 Jan 2012)

3.2 Cytotoxicity assay

Cytotoxicity is that toxic chemicals affect basic functions of cells which are common to all cells, and that the toxicity can be measured by assessing cellular damage [81]. Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as trypan blue or propidium iodide are normally excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components [82].

There are various methods to evaluate the cytotoxicity of the compound. The most frequently used of these are MTT (3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (sodium 3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate, and MTS. These are colorimetric assays for measuring the activity of mitrchondrial reductase enzyme that reduce MTT or close dyes (XTT, MTS, WSTs) (yellow tetrazole) to purple formazan dyes, giving a purple color. There are several disadvantages to the use tetrazolium salts. They are generally cytotoxic. MTT is not soluble in culture medium and the formazan crystals that are the product of the reduction of MTT must be solubilized with DMSO or HCl/isopropanol. This treatment destroys the cells under investigation. MTT is best suited for use with adherent cell lines. XTT has the advantage over MTT in that it is soluble in culture medium, and is therefore suitable for use with non-adherent as well as adherent cell lines. Because it is soluble, continuous monitoring can be performed using XTT. XTT requires the presence of PMS (phenozine methosulfate) for efficient reduction. The requirement of the presence of PMS has led to the questioning of the validity of results obtained with this method. MTS is a recently developed, alternative tetrazolium salt, which also has utility in cell proliferation assays. As with XTT, MTS has the advantage over MTT in that the formazan that results from its reduction is soluble in culture medium but also has the disadvantage of requiring the presence of PMS for effective reduction. WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is member of the tetrazolium salt family which when reduced produces a water soluble formazan product. In contrast to XTT and MTS, WST-1 is more stable, has a wider linear range and shows accelerated color development [83]. Trypan blue is a diazo dye - a vital stain used to selectively color dead tissues or cells blue. Live cells or tissues with intact cell membranes are not coloured. In a viable cell, trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color under a microscope [84].

3.3 Antiviral assay

There are some techniques employed for testing the antiviral activity of the antiviral agents such as;

Antiviral Assay by cytopathic effects (CPE)

This test is for initial screening of antiviral compounds. The antiviral activity of the compound is evaluated to observe the ability to prevent virus from causing viral cytopathic effects (CPE) in mammalian cell culture. The assay performed by the cell culture was incubated with the antiviral agent then add the virus and evaluated for the ability of the agent to protect the cell culture to infect the virus.

Virus Yield Reduction Assay

This test evaluates the ability of the compound to inhibit virus production in mammalian cell culture. It is a powerful technique for evaluating the efficacy of antiviral compounds. This is a two-step assay where virus is first produced in cultures containing the antiviral agent at varying dilutions, followed later by titration of the samples for virus titer by endpoint dilution in 96-well microplates.

Plaque reduction assay is a method of quantifying the number of infectious units by inoculating serial dilutions of a viral suspension on a cell culture monolayer, overlaying with a medium containing agarose and after several days incubation, counting the number of plaques formed; recorded as plaque forming units/ml compared back to the starting concentration of virus to determine the percent reduction in total virus infectivity [85].

Virucidal Assay

This assay shows a test compound inactivates virus outside of cells, in other words, the compound inactivates the virus before infection of the cells. The assay is performed by incubating virus with the compound, followed by determining virus titer by endpoint dilution in 96-well microplates of cells [86].

Using of the advantages of real-time PCR, some research group tested the susceptibilities of antiviral agents by exposed the culture of the virus to the antiviral agents then, determined by measuring the culture supernatants for reduction of the viral DNA levels using real-time PCR [87] .

3.4 Selective index

The selective index is a comparison of the amount of a testing agent that causes the desire positive effect to the amount that causes death or toxicity in the tested subject.

$$\text{Selective index} = \frac{CC_{50}}{IC_{50}}$$

Where; CC_{50} = 50% cytotoxicity concentration of the tested agent.

IC_{50} = 50% inhibitory concentration of the tested agent.

Quantitatively, it is the ratio given by the lethal or toxic dose divided by the therapeutic dose. In animal studies, the therapeutic index is the lethal dose of a drug for 50% of the population (LD_{50}) divided by the minimum effective dose for 50% of the population (ED_{50}), also to the *In vitro* evaluation, selective index given from 50% toxicity of the testing agent to the cells divided by 50% inhibitory concentration of the testing agent. A higher selective index is preferable to a lower one which means that using the higher dose of the extract to reach the lethal/toxic threshold than the dose taken to elicit the therapeutic effect [88].

CHAPTER III
MATERIALS AND METHODS

Part I Identities of *Clianacanthus nutans* (Burm. f.) Lindau and *Clianacanthus siamensis* (Bremek.)

1.1 Macroscopic evaluation

Plant sample

Fresh branches of *C. nutans* and *C. siamensis* which were obtained from botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University then, were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi, (N.R.), Department of Pharmacognocy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Apparatus

- 0.20 mm line width black micro pigment pen (Sakura Corp., Japan)
- Drawing board
- Drawing paper 100 gram (Master art, Thailand)
- HB pencil and eraser (Pentel, Thailand)

Procedure

A complete branch of each plant was subjected to thoroughly observed and compared for the differences. The drawing outline of the two plant samples was illustrated in the proportion size related to the original and approved by the expert (N.R.).

1.2 Microscopic evaluation

Plant sample

Mature leaves of *C. nutans* were collected from different locations which were;

- Muang District, Lampang Province
- Botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok
- Botanical garden of Swankhalok Hospital, Swankhalok District, Sukhothai Province
- Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya District, Nakhonpathom Province

C. siamensis were collected from different locations which were;

- Bang Yai District, Nonthaburi Province
- The Somdej Phra Thepraratanarajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province
- Botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok
- Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom Province

The samples were authenticated separately for each location by the expert (N.R.).

Chemical and reagent

- 95% ethanol
- Distilled water
- Sodium hypochlorite (Haiter Bleach, Kao industrial, Thailand)

Apparatus

- 0.20 mm line width black micro pigment pen (Sakura Corp., Japan)
- 250 ml beaker (Pyrex, Germany)
- Compound microscope (Axioskop, Germany)
- Digital Camera (Power Shot A640, Canon Inc., Japan)
- Drawing board
- Drawing paper 100 gram (Master art, Thailand)

- Forceps
- Glass slide and cover glass
- HB pencil and eraser (Pentel, Thailand)
- Hot plate
- Razors

Procedure

Stomatal number and stomatal index

In the process of bleaching and clearing the leaves for the experiments, sodium hypochlorite solution was used and method was modified from Dr. Pulok K. Mukherjee [48]. Briefly, sodium hypochlorite solution which consisted of sodium hypochlorite: water (1:1) was warmed on hot plate. Gently put the fractions of leaf which was cut off from the middle of the leaf. Let the fractions of leaf to boil in sodium hypochlorite solution for 20 minutes or until the samples were cleared. Then, the samples were rinsed in a beaker contained distilled water 2-3 times. After that, placed the samples on slide and covered with cover glass. The pieces were kept separately on a glass slide with its lower epidermal layer kept uppermost. A 20X magnification of objective lens of compound microscope was used and recorded the images. The images were recorded using digital camera and were scaled for the area of 0.5 mm^2 using program AxioVision version 4.1 prior counting the stomata and epidermal cells **(Figure 13)**. The number of stomata and epidermal cells was multiplied by 4 in order to give total number of stomata and epidermal cells in the area of 1 mm^2. The area of the sample was to be changed and recorded not less than 30 images from several fractions of leaves from one location.

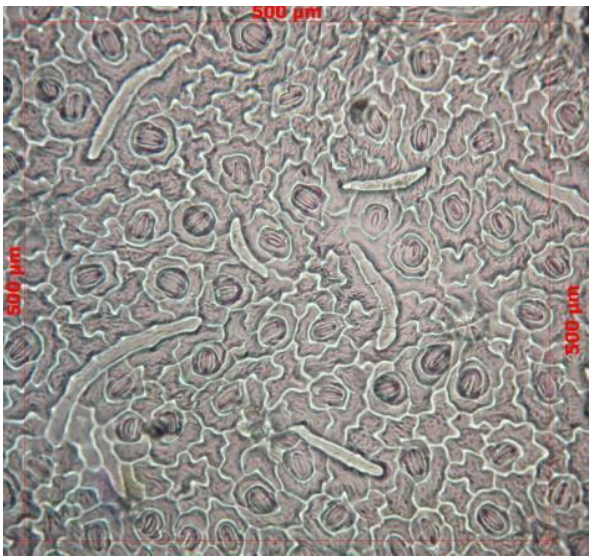


Figure 13 The lower epidermal layer of *Clinacanthus nutans* (Burm. f.) Lindau leaf in the area of 0.5 mm^2 (20X magnification)

Palisade ratio

The procedure was also modified from the method described in Dr. Pulok K. Mukherjee [48]. Briefly, sodium hypochlorite solution which consisted of sodium hypochlorite solution: water (1: 1) was warmed on hot plate. Gently put the middle fractions of leaf that had been soaked in 95% ethanol for at least 3 weeks prior the process went on. Let the fractions of leaf to boil in sodium hypochlorite solution for 20 minutes or until the samples were cleared. Then, the leaves were rinsed in a beaker contained distilled water 2-3 times. After that, placed the leaves on slide and covered with cover glass. The pieces were kept separately on a glass slide with its upper epidermal layer kept uppermost. A 40X magnification of objective lens of compound microscope was used and recorded. The image of 4 clear continuous epidermal cells was observed and then the fine adjustment knob of the compound microscope was turned down slowly to observe the round, closely packed palisade cells. The image was recorded using digital camera **(Figure 14)**. The area of the sample was to be changed and recorded not less than 30 images. The palisade cells inside the boundary and those that are 50% or more inside the outer boundary of 4 epidermal cells were taken into account.

The number of total palisade cells was divided by 4, which gave the average number of palisade cell under each epidermal cell.

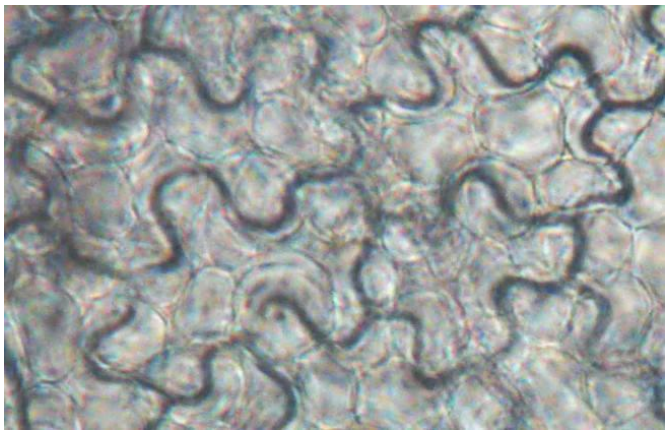


Figure 14 The round, closely packed palisade cells in the boundary of 4 clear continuous epidermal cells of *Clinacanthus nutans* (Burm. f.) Lindau leaf (40X magnification).

Stem and midrib cross section

Mature stem and midrib from mature leaf of *C. natans* and *C. siamensis* were thinly cross sectioned with razor by hand then, separately placed a complete piece on the glass slide and covered with a cover glass. The stem and midrib cross section were observed under microscope with magnification of 10X to 40X to evaluate the fine details and recorded the images **(Figure 15** and **Figure 16)**. The images were illustrated to evaluate the differences.

Figure 15 *Clinacanthus siamensis* Bremek. midrib cross section (10X magnification)

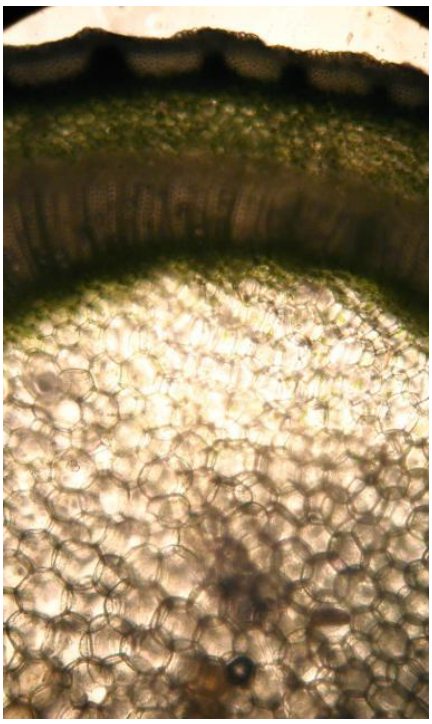


Figure 16 *Clinacanthus siamensis* Bremek. stem cross section (10X magnification)

1.3 Biomolecular evaluation

Plant sample

Fresh young leaves of *C. nutans* were collected from different locations which were;

- Botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok (CN_CU)
- Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya District, Nakhonpathom Province (CN_MU)
- Nakhonsawan Office of Provincial Public Health, Muang District, Nakhonsawan Province (CN_NS)

C. siamensis were collected from different locations which were;

- Botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok (CS_CU)

- Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya, Nakhonpathom Province (CS_MU)
- Khao Hin Son Botanical garden, Chacherngsao Province (CS_CS)

The samples were authenticated separately for each location by the expert (N.R.).

DNA extraction

Chemical and reagent

- 2-Mercaptoethanol
- 3M sodium acetate (pH 5)
- 70% ethanol
- Absolute ethanol
- CTAB (Cetyl trimethylammonium bromide) buffer
- Liquid Nitrogen
- Phenol:chloroform:isopropanol (25:24:1)
- Saturated phenol
- TE (Tris-EDTA) buffer

Apparatus

- 1.5 ml microcentrifuge tubes
- -20^{o}C refrigerator (Sharp, Japan)
- Auto pipette and tips
- Centrifugation machine (Sigma, Germany)
- Mortar and pestle
- Shaking waterbath (GFL 1086)
- Spatula
- Vortex mixer (Scientific Equipment, USA)

Procedure

Total genomic DNA was extracted from young leaves using modified CTAB method [89]. Briefly, young leaves were cleaned in water and dried then, were grounded by a mortar and pestle with liquid nitrogen. CTAB buffer was prepared and added 2-mercaptoethanol 2 µl per 500 µl of CTAB before use. Grounded leaves were placed in a microcentrifuge tube then add CTAB solution 500 µl straight away. After that, incubated the samples in shaking waterbath at 65^{o}C for 1 hour. Then, centrifuged the samples 10,000 round per minute (rpm) for 10 minutes. Transfered the aqueous phase to a new tube and added saturated phenol 500 µl to get rid of other phenolic compounds and proteins then, vortex to mix well. Centrifuged the samples 10,000 rpm for 10 minutes then, transfered the aqueous phase which was in the upper layer to a new tube and added 500 µl of phenol:chloroform:isopropanol (25:24:1) to get rid of the excessive phenol and proteins from the DNA then, mixed well by vortex mixer. Centrifuged the samples 10,000 rpm for 10 minutes then transfered the aqueous phase to a fresh tube. Added 1:10 volume of 3M sodium acetate (pH 5). Add 2 volume of cold absolute ethanol to precipitate DNA, invert tube and kept at -20^{o}C for 1 hour. Centrifuged the samples 10,000 rpm for 10 minutes then, discarded the supernatant. DNA pellet was washed with 1 ml of cold 70% ethanol and centrifuged 10,000 rpm for 10 minutes. Discarded the supernatant and dried DNA pellet at room temperature. 100 µl of TE buffer was added to the DNA pellet and left to dissolve homogeneously in 4^{o}C refrigerator. Five microliter of DNA solution was mixed with 1X loding dye and dispersed in 1.5% agarose gel electrophroresis compared to 1kb DNA ladder and then stained with ethidium bromide and visualized under UV transilluminator. The extracted DNA was stored at -20^{o}C for further use.

Amplification of Internal Transcribe Spacer (ITS) region by Polymerase Chain Reaction (PCR)

Chemical and reagent

- 10 mM dNTPs
- 10 µM Forward primer (ITS5)
- 10 µM Reverse primer (ITS4)
- 10X PCR Buffer (Fermentas, USA)
- 5 unit/µl *Taq* DNA polymerase (Recombinant) (Fermentas, USA)
- 25 mM $MgCl_2$ (Fermentas, USA)
- Distilled water
- DNA template

Apparatus

- 100 µl microcentrifuge tubes (Axygen, USA)
- Auto pipette and tips
- PCR tubes (Axygen, USA)
- Thermal cycler (Veriti, Applied Biosynthesis, USA)

Procedure

The internal transcribed spacer (ITS) including ITS1 - 5.8S rDNA - ITS2 region was amplified using a pair of universal primer (ITS5 and ITS4) as shown in **Table 1**.

Table 1 Details of the universal primers used in this PCR amplification [90].

Primer	Direction	Sequence (5'- 3')	Length (bp)	T_m (^oC)
ITS5	Forward	GGA AGT AAA AGT CGT AAC AAG G	22	55
ITS4	Reverse	TCC TCC GCT TAT TGA TAT GC	20	56

Amplifications were carried out in 20 µl reaction mixture containing of 1X PCR buffer (100mM Tris-HCl (pH 8.8), 500 mM KCl, 0.8% (v/v)

Nonidet P40), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 µM of each primer, 0.5 Unit/µl of *Taq* polymerase (Fermentas), 1 µl of DNA template. Placed the PCR mixture tubes in thermal cycler (Veriti, Applied Biosynthesis, Singapore). PCR amplification reaction conditions were as following: an initial denaturation for 5 min at 95°C, 30 cycles of denaturation at 95°C 30 second, annealing at 55°C 30 second and extension at 72°C 30 second, and then followed by a final 5 min extension at 72°C.

Figure 17 Internal Transcribed Spacers (ITS) including 18S, ITS1, 5.8S, ITS2 and 26S. Black arrows indicate the area on conserved sites that the primers (ITS4 and ITS5) will attach.

1.5% agarose gel electrophoresis

Chemical and Reagent

- 1 kb DNA Ladder 0.5 µg/µl (Fermentas)
- 1X loading dye
- 1X TBE (Tris-boric EDTA) buffer
- Agarose gel
- Ethidium bromide

Apparatus

- Auto pipette and tips
- Gel electrophoresis apparatus
- Gel tray and comb
- Microwave (Sharp, Japan)
- UV visualize gel documentation machine (Auto Chemi System, USA)

Procedure

One point five percent agarose gel was made by prepared from 1.5 g of agarose powder in 100 ml of 1X TBE buffer then, heated the mixing in microwave and swirled the bottle occasionally until completely melted. Let the agarose gel to cool down a bit. Add 10 µl of ethidium bromide, swirled to mix well and poured into the tray which had been attached with a comb then, let the agarose gel to set well. The PCR products were evaluated by mixing 5 µl of the PCR product with 1 µl of loading dye. Then, applied into the agarose well and ran electrophoresis at 100 volt until the loading dye went 2/3 of the agarose gel pad. Then, visualized and captured in UV visualize gel documentation machine.

DNA sequencing and sequence analysis

Chemical and Reagent

- PCR purification kit (QIAGEN, USA)

Apparatus

- Auto pipette and tips
- Centrifugation machine (Sigma, Germany)

Procedure

The PCR products were then purified by PCR purification kit (QIAGEN) according to the manufacturer's protocol prior sequencing. Sequencing reactions were carried out using genetic analyzer (Applied Biosynthesis, USA). The ITS region sequences were generated from both sense and antisense primer aligned to check for genetic polymorphism and compared the ITS sequences of *C. nutans* and *C. siamensis* using CLC DNA Workbench 6.0 program (limited mode, freeware) (www.clcbio.com).

Part II Antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) of *Clianacanthus nutans* (Brum. f.) Lindau and *Clianacanthus siamensis* Bremek.

In this study, evaluation of antiviral activity against herpes simplex virus type 1 and type 2 of crude extracts was performed using plaque reduction assay, the method was modified from Phrutivorapongkul, *et al*. (2003) [91].

Plant sample

The leaves of *C. nutans* were collected from Nakhonsawan Office of Provincial Public Health, Muang District, Nakhonsawan Province. The leaves of *C. siamensis* were collected from botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok. Both kinds of sample were collected in May 2011 and authenticated by the expert (N.R.).

Viruses and cells

Herpes simplex virus type 1 (KOS) (HSV-1 (KOS)) and type 2 (Baylor 186) (HSV-2 (Baylor 186)) and Vero cells (A continuous cell line of African green monkey (*Cercopithecus aethiops*) kidney) were obtained from Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Vero cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 1% antibiotics.

2.1 Plant extraction

Chemical and Reagent

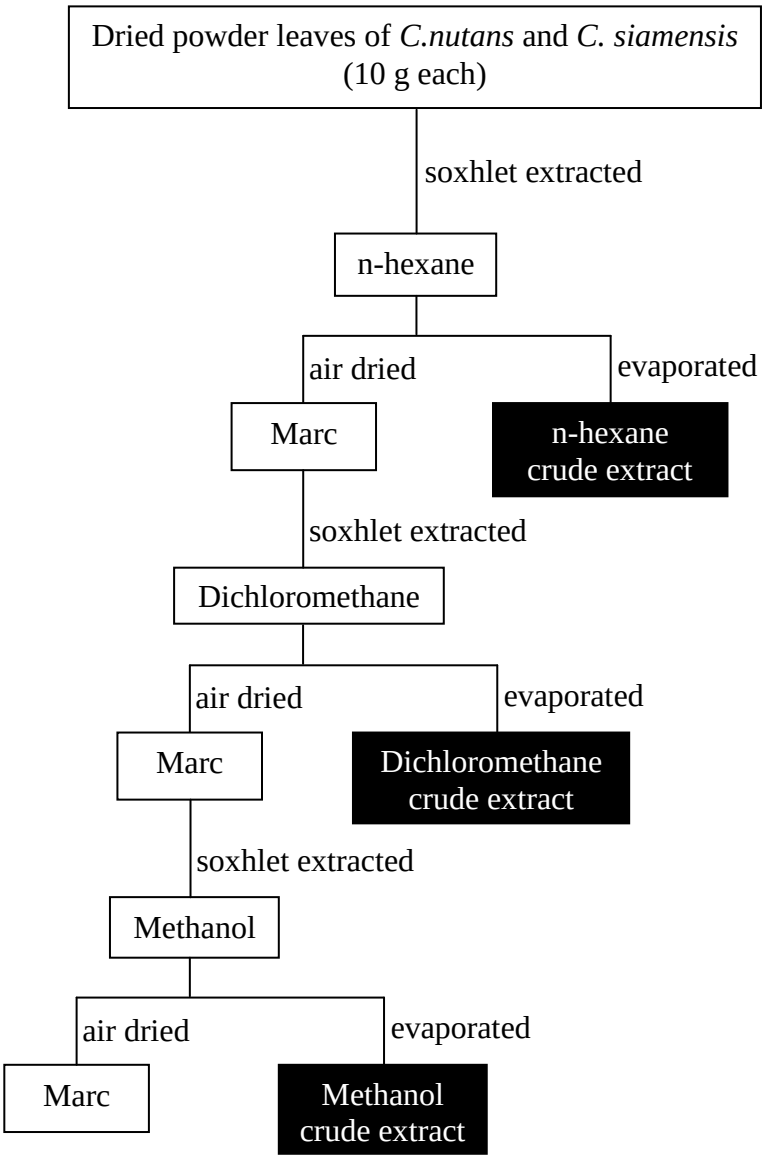
- Absolute Methanol (J.T. Baker Chemical, USA)
- Dichloromethane (RCI Labscan, Thailand)
- n-hexane (J.T. Baker Chemical, USA)

Apparatus

- Analytical balance (Satorious, Germany)
- Glass ware (Pyrex, USA)
- Rotary evaporator (BUCHI, Germany)
- Soxhlet extraction apparatus

Procedure

Ten grams of dried powder leaves of *C. nutans* and *C. siamensis* were individually extracted successively in 400 ml of n-hexane until exhausted in soxhlet extraction apparatus then, the n-hexane extract was evaporated under reduced pressure in a rotary evaporator to give n-hexane crude extract which was a dark brown gum. The residue and filter paper were air dried to prepare for further extraction in 400 ml of dichloromethane and methanol successively [92]. Percent yield of n-hexane, dichloromethane and methanol crude extract was calculated. The extraction is shown in **Scheme 1**.



Scheme 1 Extraction of the leaves of *C. nutans* and *C. siamensis*.

2.2 Plaque reduction assay

Chemical and reagent

- Acyclovir (Sigma, MO, USA)
- Dipotassium hydrogen phosphate GR (K_2HPO_4) (M & B , England)
- Disodium hydrogen phosphate AR (Na_2HPO_4 anhydrous) (M & B , England)
- DMSO (dimethyl sulfoxide) (Sigma, MO, USA)
- Fetal bovine serum (Gibco, USA)
- Formaldehyde 40% w/v AR (Carlo Erba, Milano, Italy)
- Methylene blue solution (Fluka, Switzerland)
- Minimum essential medium (MEM) (Gibco, USA)
- Potassium chloride (KCl) (M & B , England)
- Potassium dihydrogen phosphate GR (KH_2PO_4) (E. Merck, Darmstadt., Germany)
- Sodium bicarbonate ($NaHCO_3$) AR (E. Merck, Darmstadt., Germany)
- Sodium chloride (NaCl) (M&B, England)
- Tragacanth (Pharmaceutical chemicals, Denmark)
- Trypsin - EDTA (10X 0.5/0.2% w/v) (Seromed, Germany)

Apparatus

- Analytical balance (Satorious, Germany)
- Automatic pipette, P10-100 / P50-200 (Socorex, Switzerland)
- Centrifuge (Sigma , Germany)
- Cryotubes 1.8 ml (NUNC, Denmark)
- Glassware (Pyrex, USA)
- Hemocytometer (Spenser, USA)
- Humidified CO_2 incubator (Forma, USA)
- Inverted microscope (Olympus, Japan)
- Larminar Air Flow (Holten, USA)
- Microcentrifuge tubes 1.5 ml
- Millipore filters (0.2 µm) (Gel man Sciences, USA)

- Multichannel automatic pipette (8 channels) P10-100/ P50-200 (Socorex, Switzerland)
- Pipette tips for P10-100 / P50-200 (Treff Lab, Switzerland)
- pH Meter (Beckman, USA)
- Refrigerator 4°C (Sharp, Thailand)
- Freezer -20°C (Ariston, USA)
- Freezer -80°C (Forma Scientific, Ohio, USA)
- Tissue culture flasks (NUNC, Denmark)
- Tissue culture plates (NUNC, Denmark)
- Vortex Mixer (Scientific, NY, USA)
- Water bath (Thelco, USA)

Procedure

Vero cell line (ATCC CCL81) was grew and maintained in Eagle's minimum medium supplemented with 10% fetal bovine serum (Gibco, USA). Anti-HSV activity of the plants extracts were determined by plaque reduction assay modified from the previous reported method [89]. Briefly, Vero cell monolayer, in 96-well tissue culture plate, were infected with 30 plaque forming units (PFU) per well of HSV-1 (KOS) or HSV-2 (Baylor 186). After 1 h incubation at 37°C for virus adsorption, the overlay media containing various concentrations of the extracts was added. The infected cultures were incubated at 37°C for 2 days. The infected cells were fixed and stained with 5% formalin solution and were stained with 0.03% methylene blue solution, and then the number of plaques was counted comparing to the control (without active extracts). The 50% inhibition concentration of extract (IC_{50}, the concentration of the extract reducing the number of plaque by 50%) was determined from the curve relating the plaque number to the concentration of the extract. Acyclovir was used as a positive control.

2.3 Cytotoxicity assay

Chemical and reagent

- Dipotassium hydrogen phosphate GR (K_2HPO_4) (M & B , England)
- Disodium hydrogen phosphate AR (Na_2HPO_4 anhydrous) (M & B , England)
- DMSO (dimethyl sulfoxide) (Sigma, MO, USA)
- Fetal bovine serum (Gibco, USA)
- Minimum essential medium (MEM) (Gibco, USA)
- MTT (3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide)
- Potassium chloride (KCl) (M & B , England)
- Potassium dihydrogen phosphate GR (KH_2PO_4) (E. Merck, Darmstadt., Germany)
- Sodium bicarbonate ($NaHCO_3$) AR (E. Merck, Darmstadt., Germany)
- Trypsin - EDTA (10X 0.5/0.2% w/v) (Seromed, Germany)

Apparatus

- Analytical balance (Satorious, Germany)
- Automatic pipette, P10-100 / P50-200 (Socorex, Switzerland)
- Centrifuge (Sigma , Germany)
- Glassware (Pyrex, USA)
- Hemocytometer (Spenser, USA)
- Humidified CO_2 incubator (Forma, USA)
- Inverted microscope (Olympus, Japan)
- Larminar Air Flow (Holten, USA)
- Microcentrifuge tubes 1.5 ml
- Millipore filters (0.2 µm) (Gel man Sciences, USA)
- Multichannel automatic pipette (8 channels) P10-100/ P50-200 (Socorex, Switzerland)
- Pipette tips for P10-100 / P50-200 (Treff Lab, Switzerland)
- Tissue culture plates (NUNC, Denmark)
- Vortex Mixer (Scientific, NY, USA)

Procedure

All crude extracts were tested for cytotoxicity to Vero cell line. The crude extracts were solubilised in DMSO and serial diluted to 1600, 800, 400, 200,100, 50, 25 and 12.5 µg/ml with complete media (final concentration of DMSO was 1%).

Cytotoxicity of the extracts on Vero cells was evaluated by the MTT assay. Vero cell monolayers, 5 x 10^5 cells/ml, 50 µl were seeded in each well of 96-well tissue culture plate and incubated at 37^{o}C for 24 hours. Fifty microliter of various concentrations of the extracts were added. The cells were further incubated at 37°C for 2 days. After incubation 50 µl of a 1 mg/ml solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in media was added. Cells were incubated at 37 °C for 3 h, the supernate was removed and 50 µl of 0.04 N HCl in isopropanol was added to each well. The OD was read on a microplate reader using a test wavelength of 570 nm. Concentration of the extract reducing cell viability by 50% (CC_{50}) was determined from a plot of graph between cytotoxicity percentage and concentration of the extracts. DMSO, 1% was used as a reagent control and the wells containing the cells and medium (MEM) (triplicate) were used as a cell control [93].

CHAPTER IV
RESULTS

Part I Identities of *Clinacanthus nutans* (Burm. f.) Lindau and *Clinacanthus siamensis* Bremek.

1.1 Macroscopic evaluation

Although some apparent part of the two species such as flowers is quite similar, other visible parts including leaf, stem, plant size, color of leaves and character of the plant are different. From the observation of the areal part of the two plants, the drawing outline was done as shown in **Figure 18** and **Figure 19**.

The morphological evaluation for the identification of *C. nutans* and *C. siamensis* was illustrated and described. *C. nutans* is a shrub with short hair branches. Leaves are light green, simple, opposite, narrowly elliptic oblong with acute apex, size 2.5-13 cm long and 0.5-1.5 cm wide. There are 6-7 pairs of side veins and quite prominent below. The stem is straight green with white internodes and vertical strips throughout the entire stem. Flowers are in dense cymes at the top of the branches, often terminating drooping horizontal branches but themselves erect. Each flower has glandular-pubescent calyx. Corolla glandular-pubescent, bilabiate, about 3.5 cm long, dull red with green base and yellow streaks on lower lip. There are 2 stamens appressed against the upper lip.

C. siamensis is shrub, 1.5-4 m high, branchlets erect-drooping. Leaves are glossy green to dark green, simple, opposite, lanceolate-oblong with acute apex, size 11-16 cm long and 3-5 cm wide. There are 5-6 pairs of side veins and prominent below. Stem is dark green or dark purple with white internodes along the entire stem. Flowers are in terminal dense cyme. They are tubular pubescent, bilabiate, about 3.5-4.5 cm long, color dull red with green base. There is a yellow streak on lower lip. There are 2 stamens appressed against the upper lip.

Figure 18 *Clinacanthus nutans* (Burm. f.) Lindau
a: branch
b: flowers

Figure 19 *Clinacanthus siamensis* Bremek.
a: branch
b: flower

1.2 Microscopic evaluation

The stomata of both plants could be found only on the lower epidermis of the leaf with other components (glandular trichomes and lithocysts). The plants' stomata were diacytic type which the stomata were accompanied by two subsidiary cells at right angle to it **(Figure 20** and **Figure 21)**.

The constant number of leaf measurements which consists of stomatal number, stomatal index and palisade ratio were analyzed by microscopic assessment as described in chapter III. The average stomatal number, stomatal index in the area of 1 mm^2 and palisade ratio of *C. nutans* was 168.32 ± 29.49, 13.83 ± 0.86 and 6.84 ± 0.66, respectively whereas *C. siamensis* was 161.60 ± 18.04, 11.93 ± 0.81 and 3.37 ± 0.31, respectively **(Table 2)**.

Table 2 The average leaf measurement values of *Clinacanthus nutans* (Burm. f.) Lindau and *Clinacanthus siamensis* Bremek. (mean ± S.D., n = 30)

	Average stomatal number	**Stomatal index**	**Palisade ratio**
C. nutans	168.32 ± 29.49	13.83 ± 0.86	6.84 ± 0.66
C. siamensis	161.60 ± 18.04	11.93 ± 0.81	3.37 ± 3.31

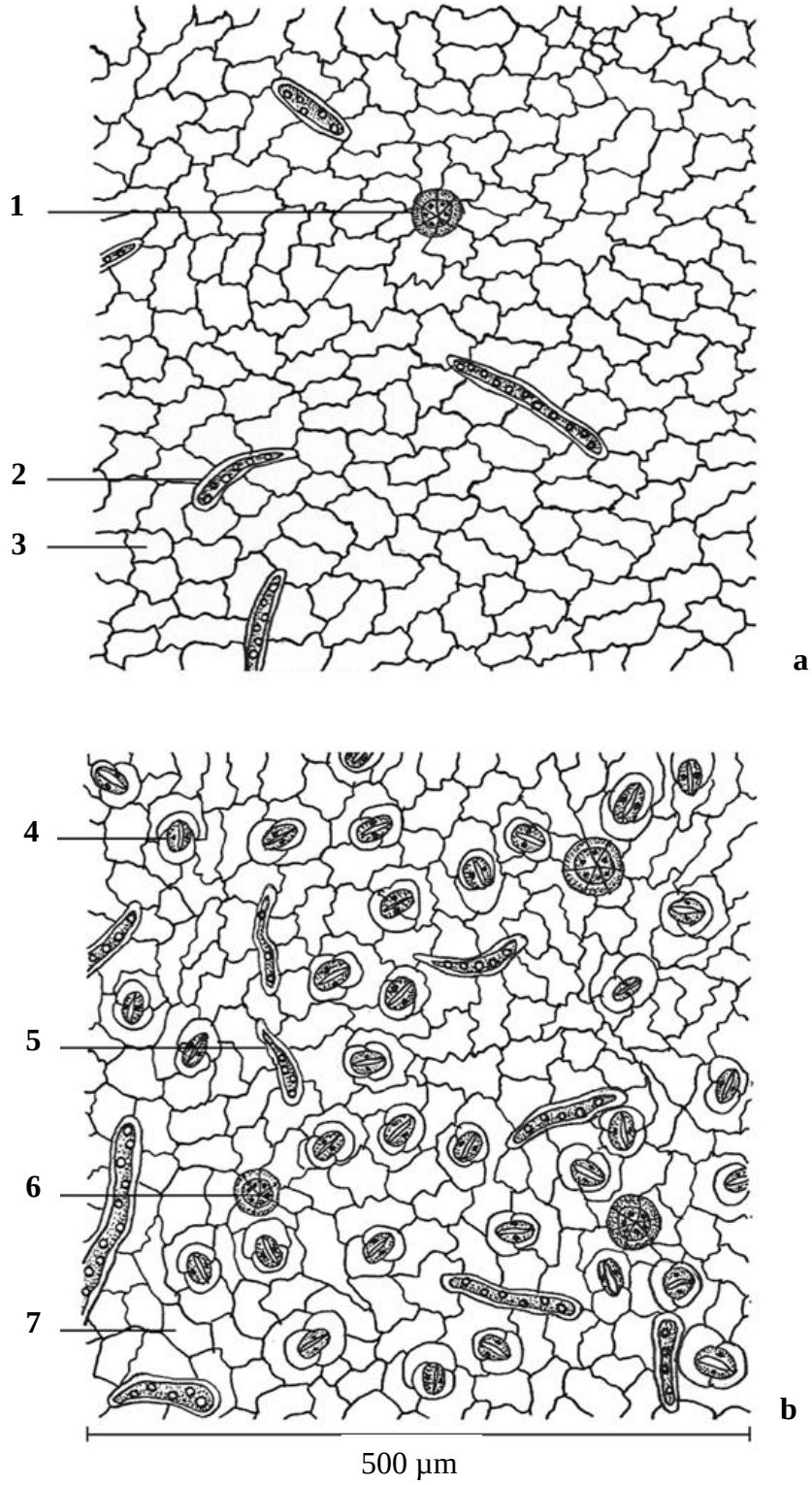


Figure 20 Epidermis of the leaf of *Clinacanthus nutans* (Burm. f.) Lindau with 20x magnification

a: upper epidermis, glandular trichome (1), lithocyst (2) and epidermal cell (3).

b: lower epidermis, Diaccytic type stoma (4), lithocyst (5), glandular trichome (6) and epidermal cell (7).

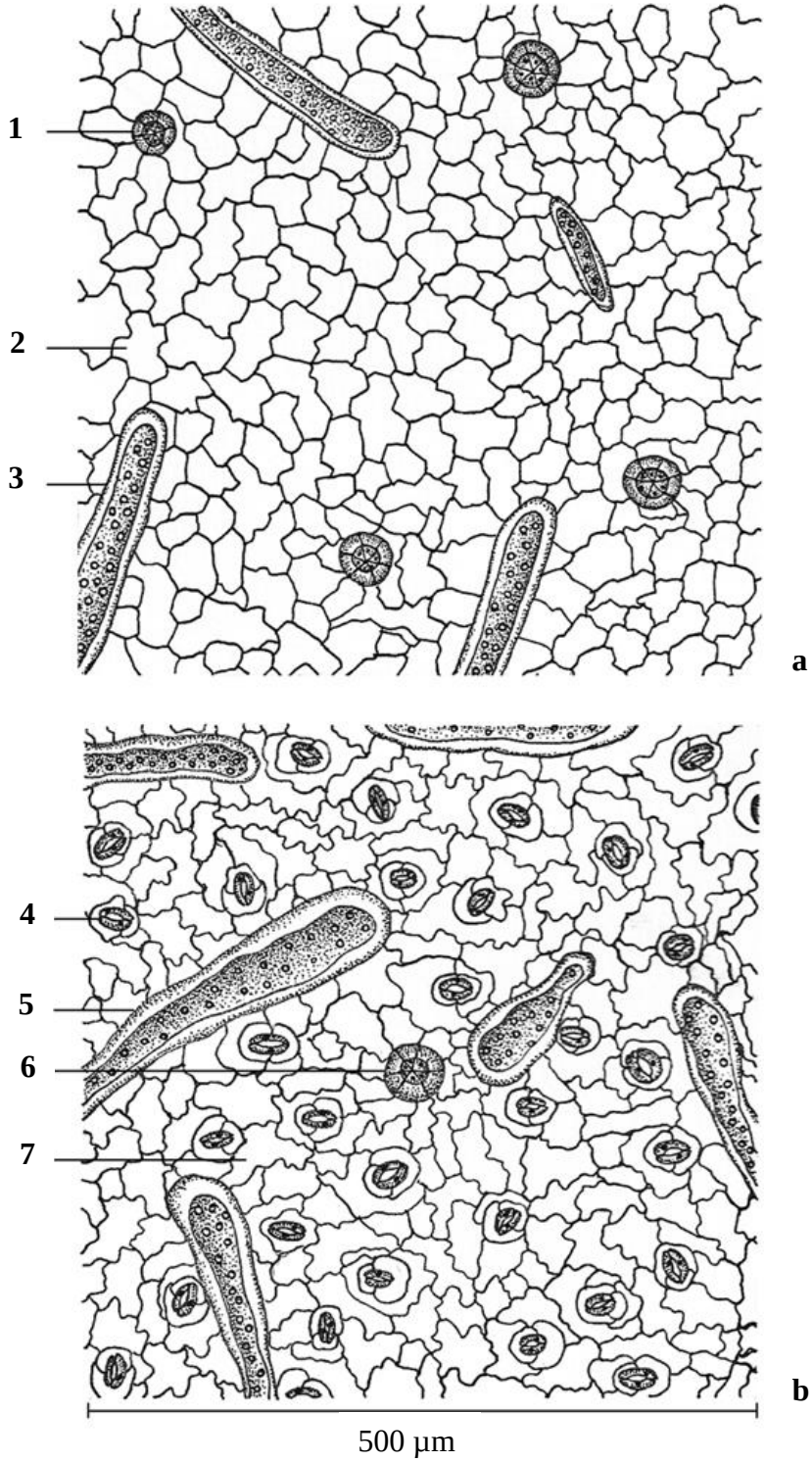


Figure 21 Epidermis of the leaf of *Clinacanthus siamensis* Bremek. with 20x magnification

a: upper epidermis, glandular trichome (1), epidermal cell (2) and lithocyst (3).

b: lower epidermis, Diaccytic type stoma (4), lithocyst (5), glandular trichome (6) and epidermal cell (7).

The stem cross section of *C. nutans* revealed that there were lithocysts interposed in the cortical parenchyma which lay beneath epidermis. Groups of collenchyma were located above parenchyma of cortex and pisiform parenchymal cell layer that located above the phloem tissues. The xylem fibers align gather in group and were interposed longitudinally with parenchyma ray. There were xylem vessels with a wide lumen interposed sparsely in the xylem fibers. The cells in the ground tissue were polygonal shape in various sizes. The stem cross section of *C. siamensis* showed that the epidermis was interposed with lithocysts. The cortical parenchyma was located beneath the epidermis and also interposed with various sizes of lithocysts. Groups of collenchyma were located between the cortical parenchyma and pisiform parenchymal cell layer. The phloem tissue lie above the xylem fibers which were arrange longitudinally flanked with the parenchyma rays. The xylem fibers were interposed sparsely with wide lumen xylem vessels. The parenchyma cells in the ground tissue are polygonal shape, various in size packed closely **(Figure 22** and **Figure 23)**.

Midrib cross section of *C. nutans* and *C. siamensis* revealed multicellular and glandular trichomes on the epidermis which was interposed sparsely with lithocysts. Collenchyma located next to the epidermis. Palisade mesophyll and chlorenchyma lied next to the collenchyma. Xylem vessels arranged longitudinally and were interposed by parenchyma ray in the central portion of the polygonal shape parenchyma. Phloem tissue lied semicircular next to the xylem vessels in the central portion of the parenchyma **(Figure 24** and **Figure 25)**.

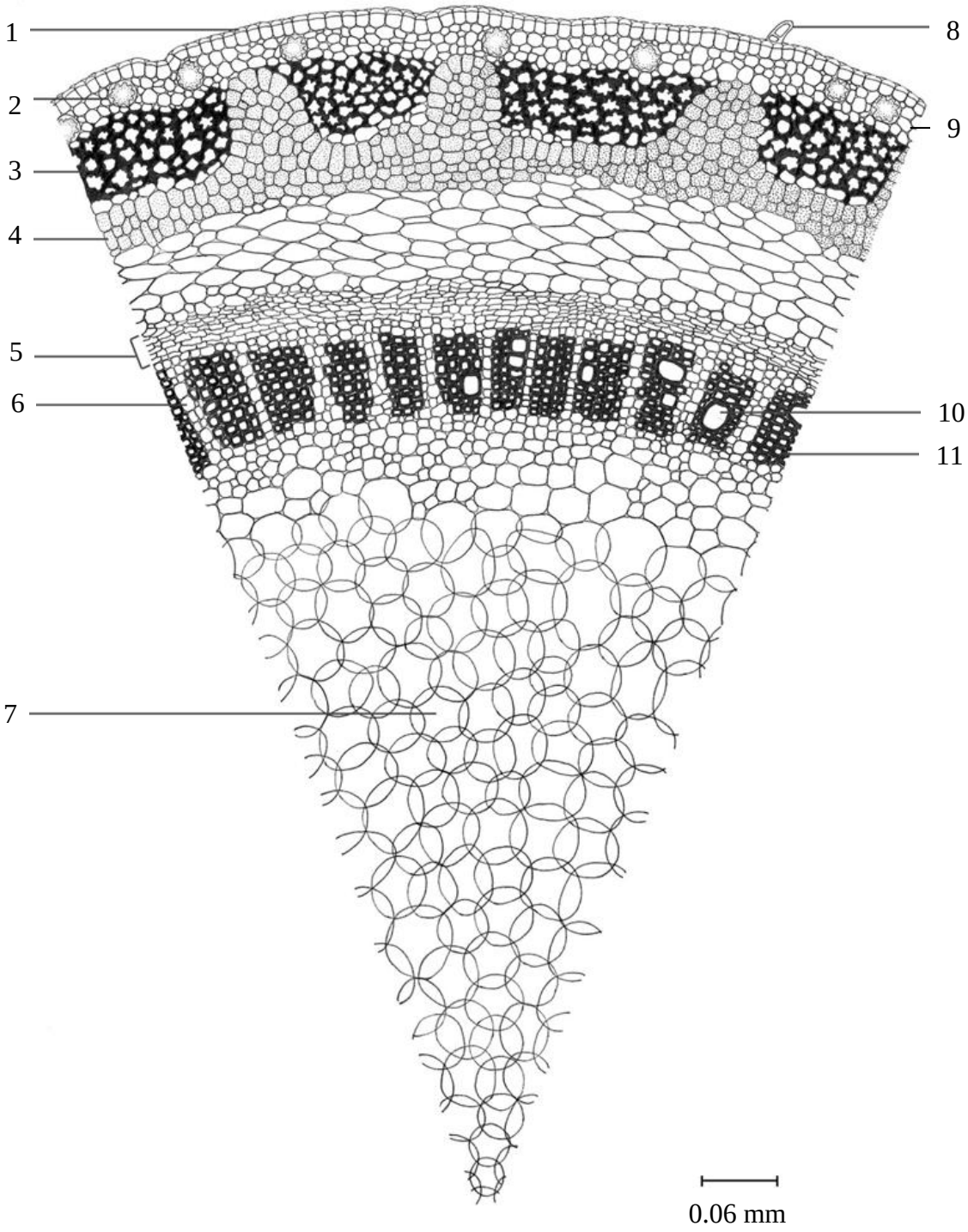


Figure 22 Stem cross section of *Clinacanthus nutans* (Burm. f.) Lindau
epidermis (1), lithocyst (2), group of collenchyma (3), parenchyma of cortex (4), phloem tissues (5), parenchyma ray (6), ground tissue (7), multicellular trichome (8), cortical parenchyma (9), xylem vessel (10) and xylem fiber (11).

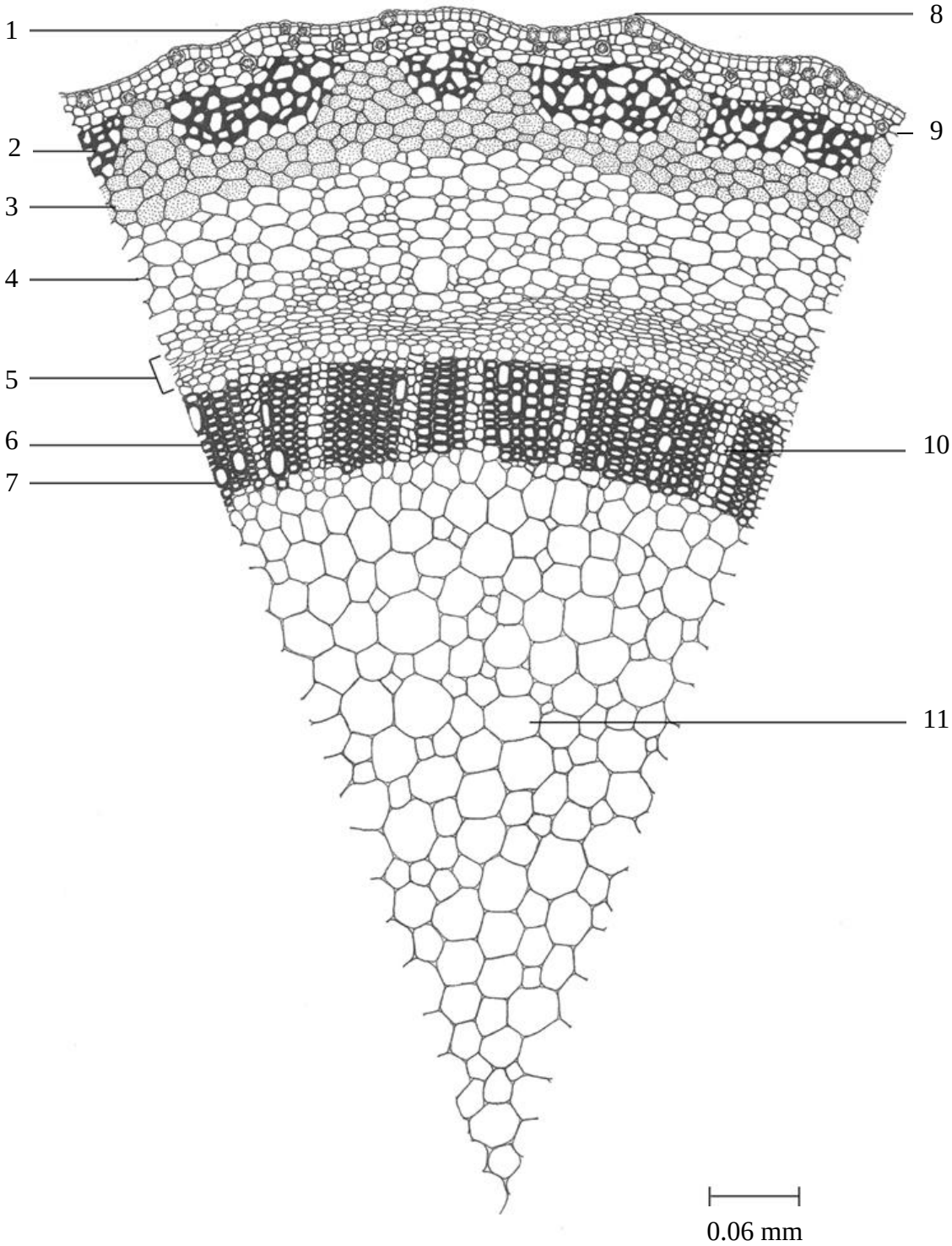


Figure 23 Stem cross section of *Clinacanthus siamensis* Bremek.
epidermis (1), group of collenchyma (2), parenchyma of cortex (3), parenchyma (4), phloem tissues (5), xylem fiber (6), xylem vessel (7), lithocyst (8), cortical parenchyma (9), parenchyma ray (10) and ground tissue (11).

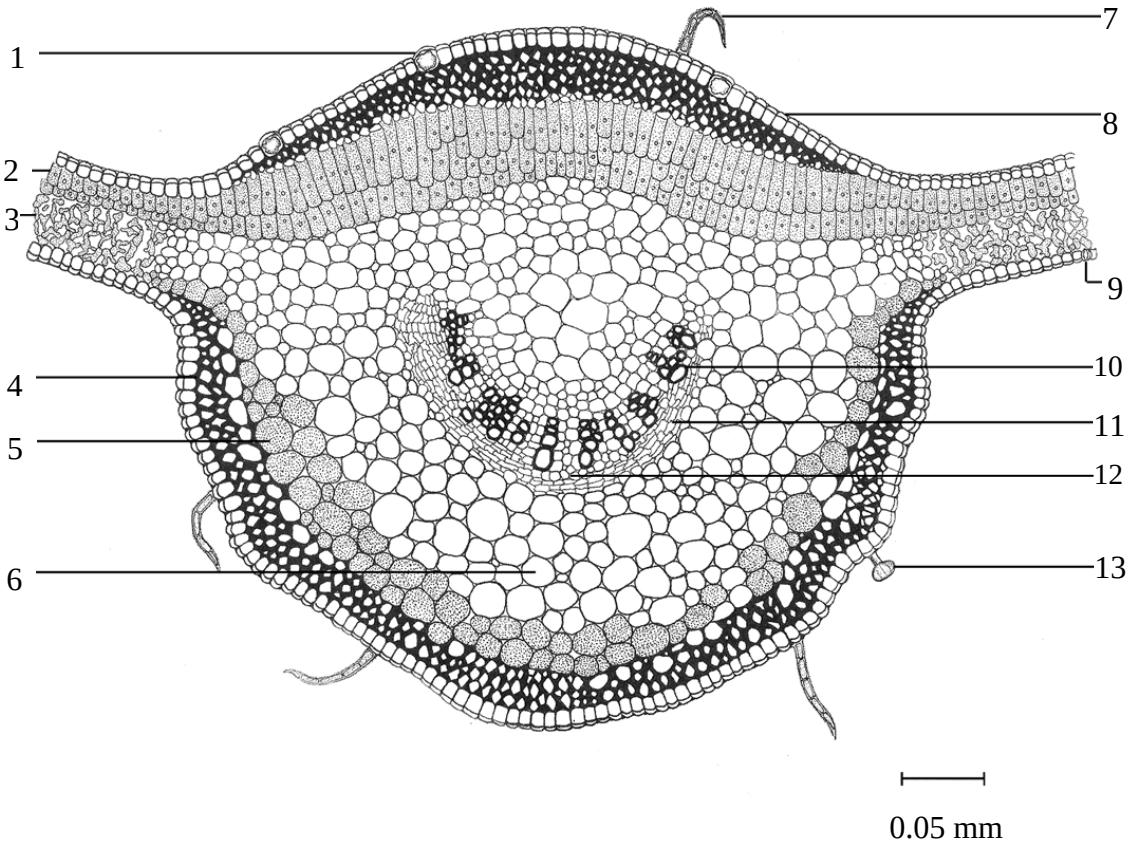


Figure 24 Midrib cross section of *Clinacanthus nutans* (Burm. f.) Lindau
Lithocyst (1), palisade mesophyll (2), spongy mesophyll (3), collenchyma (4), chlorenchyma (5), parenchyma (6), multicellular trichome (7), epidermis (8), stoma (9), xylem vessel (10), phloem tissue (11), parenchyma ray (12) and glandular trichome (13)

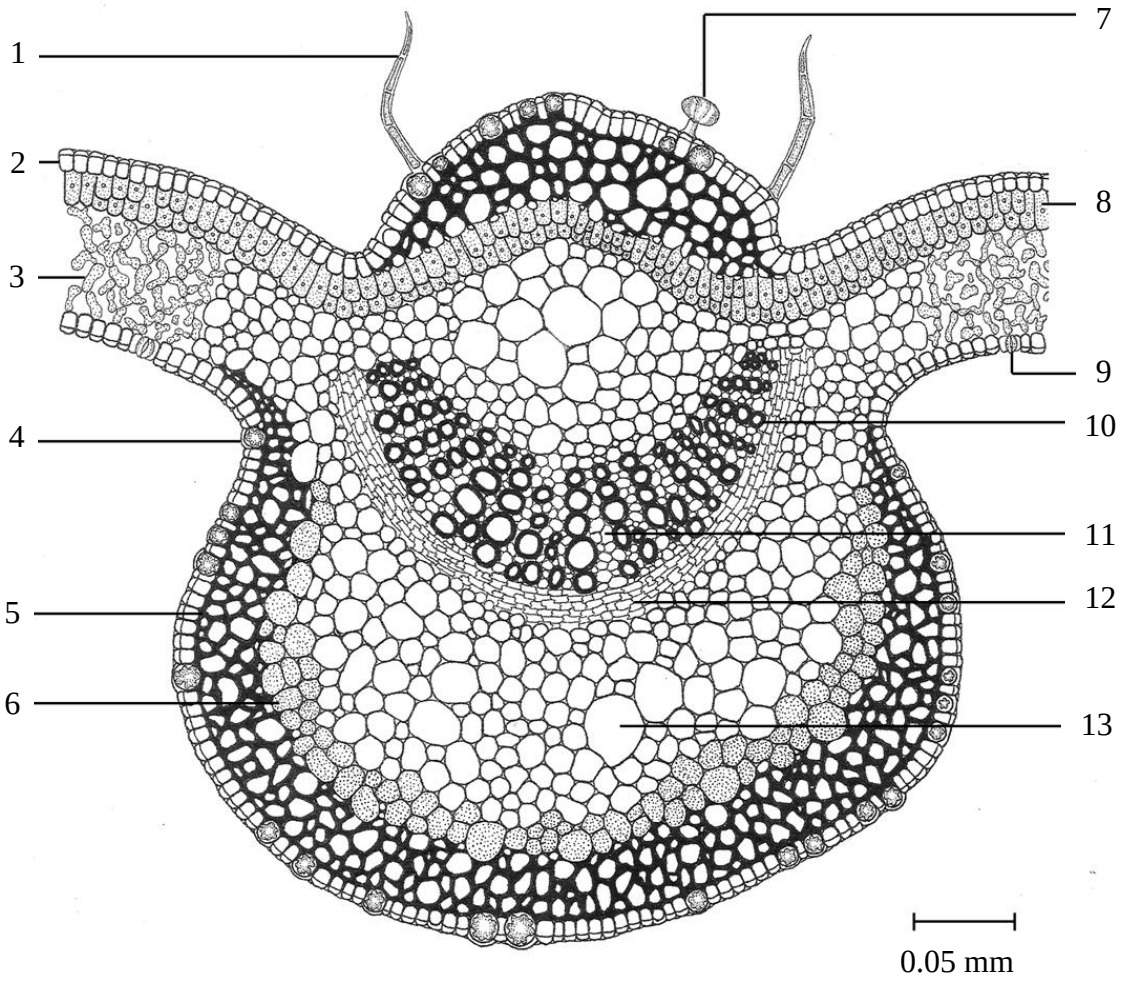


Figure 25 Midrib cross section of *Clinacanthus siamensis* Bremek. Multicellular trichome (1), epidermis (2), spongy mesophyll (3), lithocyst (4), collenchyma (5), chlorenchyma (6), glandular trichome (7), palisade mesophyll(8), stoma (9), xylem vessel (10), parenchyma ray (11), phloem tissue (12) and parenchyma (13).

1.3 Biomolecular evaluation

The total genomic DNA was isolated from young leaves of all 6 samples (3 samples of each plant species) using modified CTAB method as described in chapter III. The genomic DNA was then dispersed in 1.5% agarose gel electrophoresis compared to 1kb DNA ladder and then strained with ethidium bromide as shown in **Figure 26**. The total genomic DNA was then stored at -20^{o}C for the further use in PCR amplification.

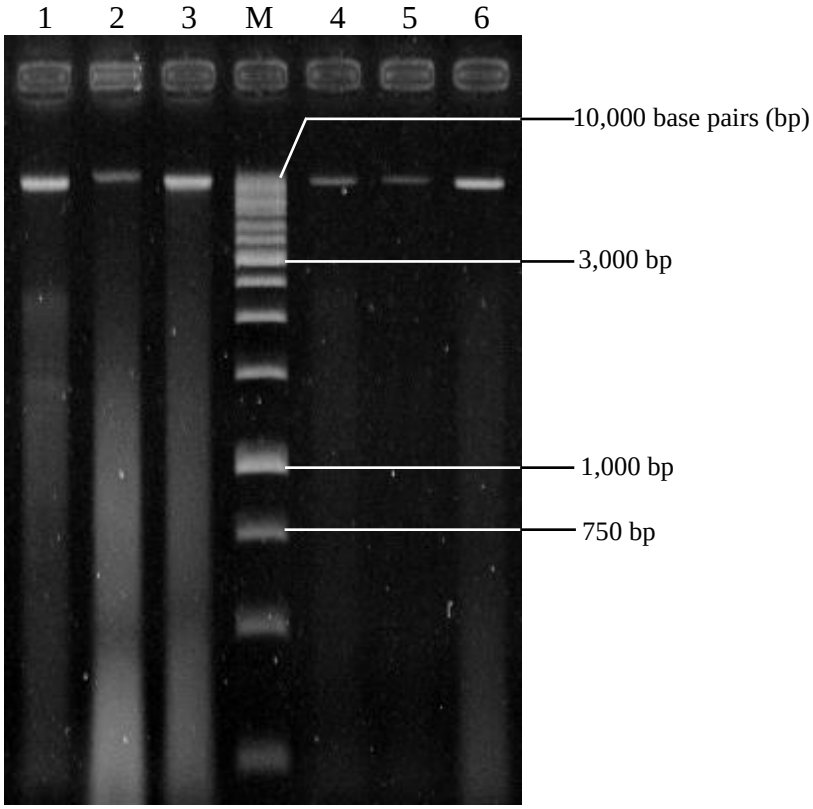


Figure 26 Total genomic DNA which dispersed in 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transillumination.

Lane 1: *C. nutans* from Chulalongkorn University, Bangkok.
Lane 2: *C. nutans* from Mahidol University, Nakhonpathom Province.
Lane 3: *C. nutans* from Nakhonsawan Office of Provincial Public Health, Nakhonsawan Province.
Lane M: 1kb DNA ladder (Fermentas).
Lane 4: *C. siamensis* from Chulalongkorn University, Bangkok.
Lane 5: *C. siamensis* from Mahidol University, Nakhonpathom Province.
Lane 6: *C. siamensis* from Khao Hin Son Botanical garden, Chacherngsao Province.

A pair of universal PCR primers (ITS5 and ITS4) designed from highly conserved regions flanking the Internal transcribe spacer (ITS) region and the total genomic DNA were used for PCR amplification. The PCR products were dispersed in 1.5% agarose gel electrophoresis then strained with ethidium bromide and visualized in UV transilluminator. When compared to 1 kb DNA ladder (Fermentas, USA), the PCR products were approximately 700 base pairs (bp) in size as shown in **Figure 27**.

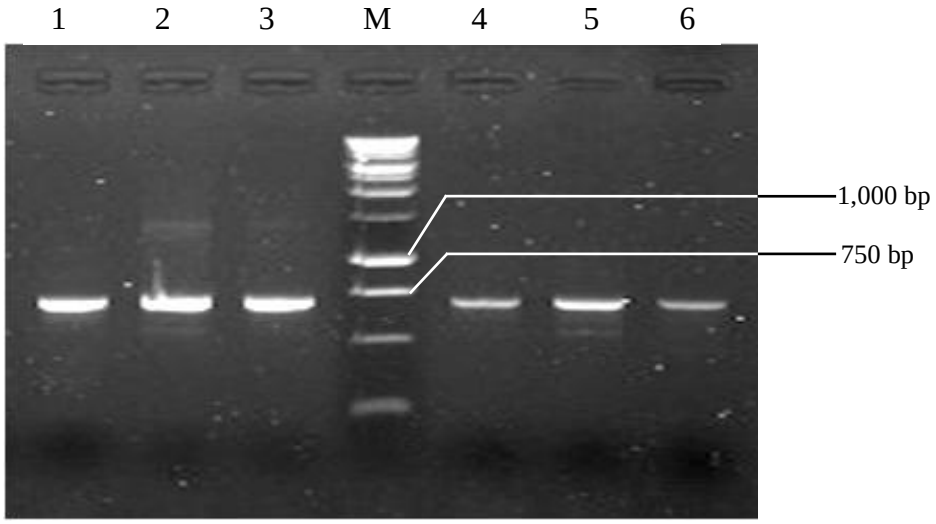


Figure 27 PCR products which dispersed in 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transillumination.

Lane 1: *C. nutans* from Chulalongkorn University, Bangkok.
Lane 2: *C. nutans* from Mahidol University, Nakhonpathom Province.
Lane 3: *C. nutans* from Nakhonsawan Office of Provincial Public Health, Nakhonsawan Province.
Lane M: 1kb DNA ladder (Fermentas).
Lane 4: *C. siamensis* from Chulalongkorn University, Bangkok.
Lane 5: *C. siamensis* from Mahidol University, Nakhonpathom Province.
Lane 6: *C. siamensis* from Khao Hin Son Botanical garden, Chacherngsao Province.

The ITS sequence of three samples of *C. nutans* were aligned using CLC DNA Workbench 6 program. The result showed 97-98% similarity. There were 20 polymorphisms consisted of 3 indels and 17 nucleotide substitutions; 7 polymorphisms in ITS 1, 3 polymorphisms in 5.8S and 10 polymorphisms in ITS2 region (**Figure 28**).

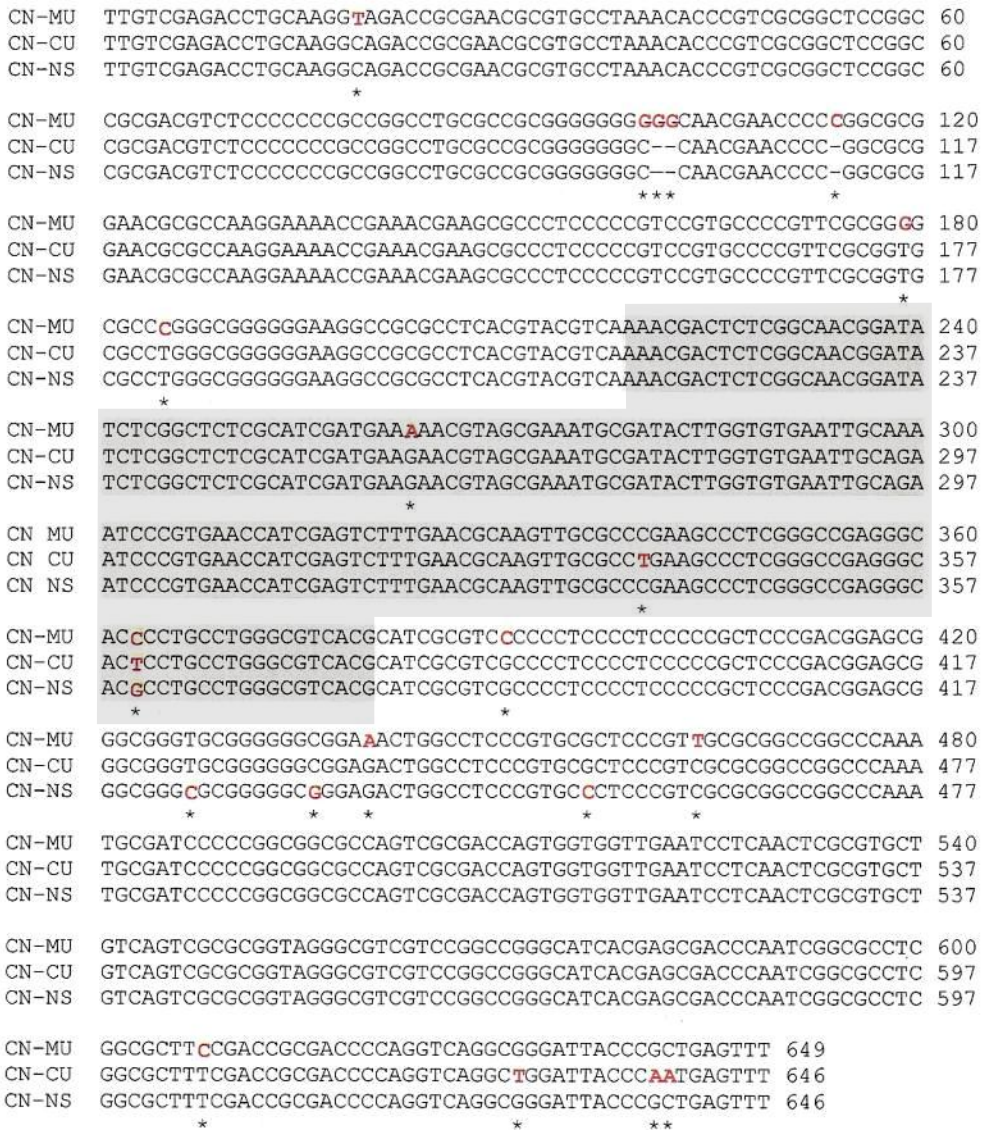


Figure 28 Sequence alignment of three samples of *Clinacanthus nutans* (Burm. f.) Lindau. Highlight indicate 5.8S region, * indicate polymorphism, - indicate indels.

CN_MU: *C.nutans* from Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya District, Nakhonpathom Province.

CN_CU: *C.nutans* from Botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

CN_NS: *C.nutans* from Nakhonsawan Office of Provincial Public Health, Muang District, Nakhonsawan Province.

Three samples of *C. siamensis* were aligned. The alignment showed 96-99% similarity. There were 23 polymorphisms consisted of 10 indels and 13 nucleotide substitutions; 7 polymorphisms in ITS 1, 2 polymorphisms in 5.8S and 14 polymorphisms in ITS2 region (**Figure 29**).

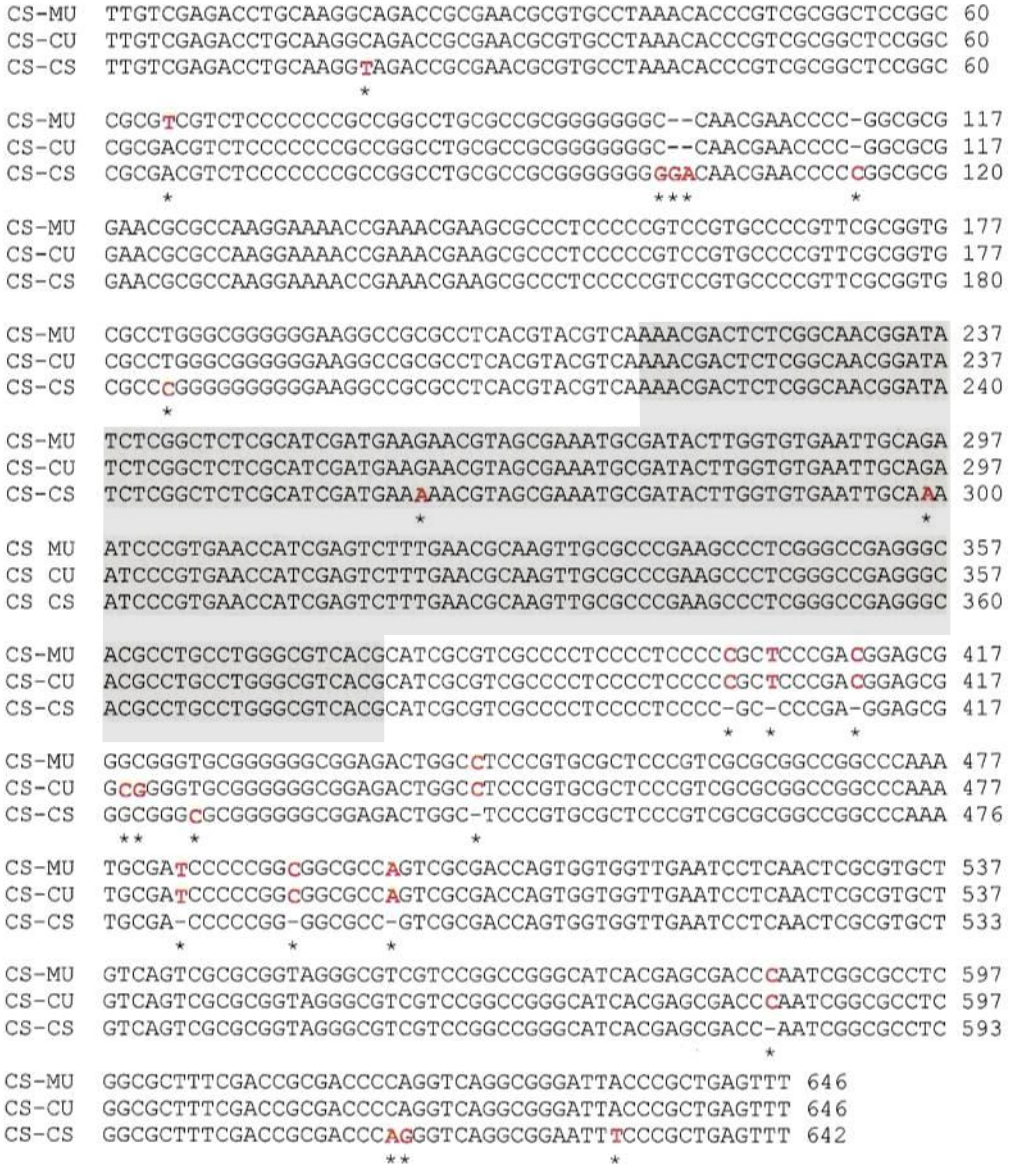


Figure 29 Sequence alignment of three samples of *Clinacanthus siamensis* Bremek. Highlight indicate 5.8S region, * indicate polymorphism, - indicate indels.

CS_MU: *C. siamensis* from Sirirukkachat garden, Faculty of Pharmacy, Mahidol University, Salaya District, Nakhonpathom Province.

CS_CU: *C. siamensis* from Botanical garden of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

CS_CS: *C. siamensis* Khao Hin Son Botanical garden, Chacherngsao Province.

Comparison of nucleotide sequence of ITS region between *C. nutans* and *C. siamensis* was showed in **Figure 30**. Sequence analysis of *C. nutans* and *C. siamensis* in ITS sequence showed high degree of sequence homology with 96-99% similarity and 68% GC content. There were 33 polymorphisms consisted of 11 indels and 22 nucleotide substitutions; 9 polymorphisms in ITS 1, 4 polymorphisms in 5.8S and 20 polymorphisms in ITS2 region.

Table 3 Sequence summary of *C. nutans* and *C. siamensis*

Summary	*C. nutans*	*C. siamensis*
Sequence length	646-649 base pairs	642-646 base pairs
% similarity (intraspecies)	97-99%	98-99%
Polymorphisms (intraspecies)	20 positions (3 indels, 17 nucleotide substitutions)	23 positions (10 indels, 13 nucleotide substitutions)
	ITS1 : 7 polymorphisms	**ITS1** : 7 polymorphisms
	5.8S : 3 polymorphisms	**5.8S** : 2 polymorphisms
	ITS2 : 10 polymorphisms	**ITS2** : 14 polymorphisms
% similarity (interspecies)	97-99%	
Polymorphisms (interspecies)	33 positions (11 indels, 22 nucleotide substitutions)	
	ITS1 : 9 polymorphisms	
	5.8S : 4 polymorphisms	
	ITS2 : 20 polymorphisms	
% GC	68%	

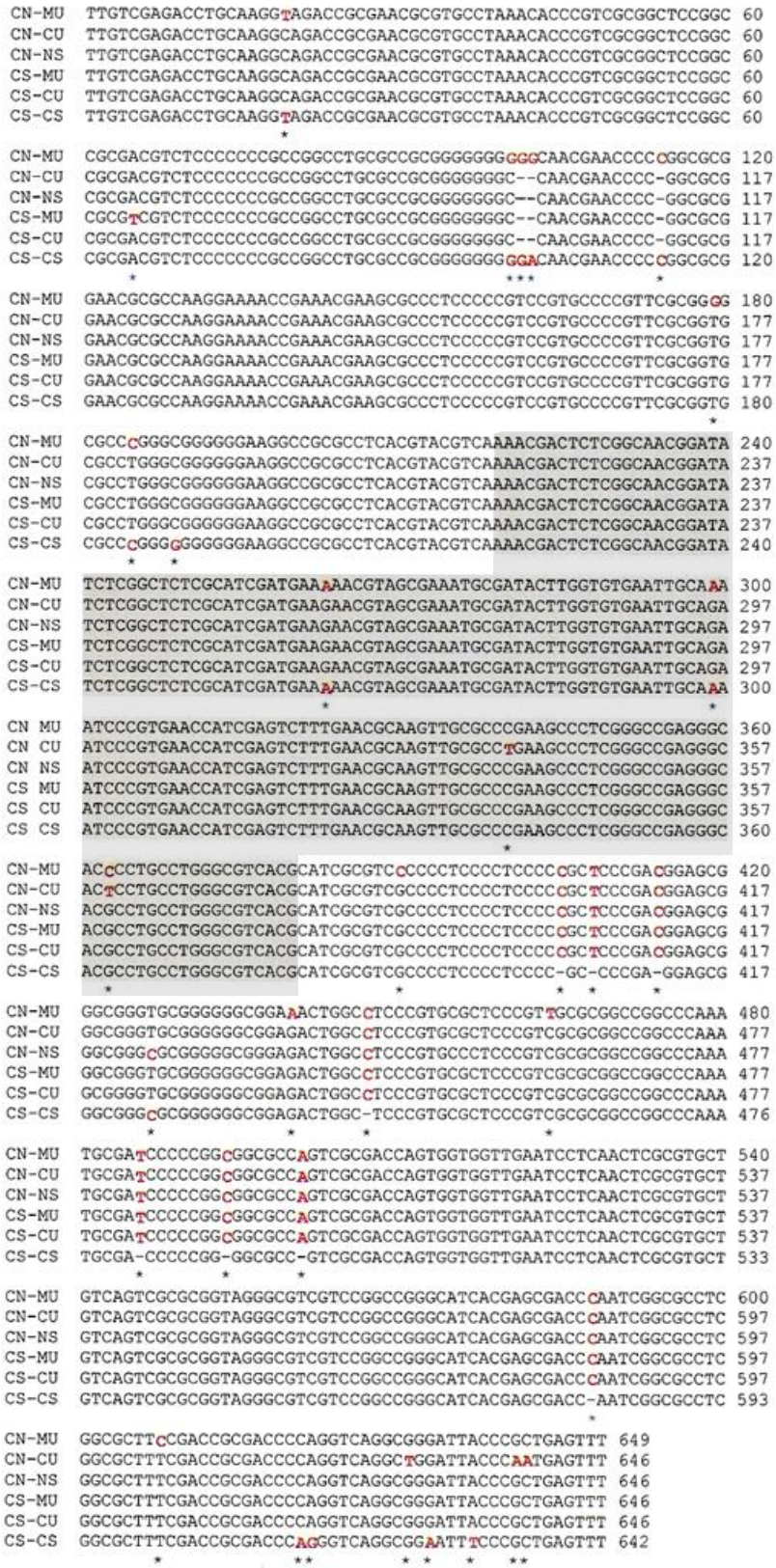


Figure 30 Comparison of nucleotide sequence of ITS (ITS1-5.8S- ITS2) region of rDNA gene of *Clinacanthus nutans* (Burm. f.) Lindau and *Clinacanthus siamensis* Bremek. Highlight indicate 5.8S region, * indicate polymorphism, - indicate indels.

Part II Antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) of *Clianacanthus nutans* (Burm. f.) Lindau and *Clianacanthus siamensis* Bremek.

Anti viral activity against herpes simplex virus type 1 and type 2

Three extracts were prepared successively from the leaves of *C. nutans* and *C. siamensis* ranging from the least polar to the most polar (n-hexane, dichloromethane and methanol, respectively). The crude extracts yield of *C. nutans* and *C. siamensis* obtained from n-hexane were 0.36 g (3.58% w/w) and 0.16 g (1.59 w/w) respectively, dichloromethane crude extract of *C. nutans* and *C. siamensis* were 0.16 g (1.59% w/w) and 0.24 g (2.39 % w/w), respectively and methanol crude extract of *C. nutans* and *C. siamensis* were 1.72g (17.15% w/w) and 1.70 g (16.95 % w/w) dry weight of plant material, respectively. To determine the anti viral activity against HSV-1 and HSV-2, percent inhibition of plaque formation was determined compared to the untreated cells**.** The IC_{50} value of each extract was determined. *C. nutans* leaves extracts showed values of IC_{50} against HSV-1, which were 32.05 ± 3.63 µg/ml for n-hexane extract, 44.50 ± 2.66 µg/ml for dichloromethane extract and 64.93 ± 7.00 µg/ml for methanol extract. The IC_{50} against HSV-2 of *C. nutans* leaves extracts were 72.62 ± 12.60 µg/ml for n-hexane extract, 65.19 ± 21.45 µg/ml for dichloromethane extract and 65.13 ± 2.22 µg/ml for methanol extract. The three extracts of *C. siamensis* leaves showed values of IC_{50} against HSV-1, which were 60.00 ± 11.61 µg/ml for n-hexane extract, 55.69 ± 4.41 µg/ml for dichloromethane extract and 37.39 ± 5.85 µg/ml for methanol extract. The IC_{50} against HSV-2 of *C. siamensis* leaves extracts were 46.52 ± 4.08 µg/ml for n-hexane extract, 49.63 ± 2.59 µg/ml for dichloromethane extract and 72.64 ± 6.52 µg/ml for methanol extract. Compared to acyclovir, which its IC_{50} was 0.09 ± 0.02 µg/ml against HSV-1 and 0.43 ± 0.04 µg/ml against HSV-2. Fifty per cent cytotoxicity concentration (CC_{50}) of these extracts was more than 1600 µg/ml for n-hexane and methanol extract of the both plants whereas the CC_{50} of dichloromethane extracts were 869 ± 141.93 and 194 ± 3.56 µg/ml of *C. nutans* and *C. siamensis*, respectively. Selective index was determined from the 50% cytotoxicity concentration (CC_{50}) divided by the 50% inhibitory concentration (IC_{50}) of each extract to find out an effective concentration of the extract against HSV-1 and

HSV-2 without any dangerous toxic action [94]. A higher selective index is preferable to a lower one which means that using the higher dose of the extract to reach the lethal/toxic threshold than the dose taken to elicit the therapeutic effect **(Table 4** and **Table 5)**.

Table 4 Inhibitory effect of extracts of *Clinacanthus nutans* (Burm. f.) Lindau against HSV-1 and HSV-2.

Extracts	**IC_{50}* (µg/ml)** (mean ± S.D.)		**SI****		**CC_{50} *****
	HSV-1	**HSV-2**	**HSV-1**	**HSV-2**	**(µg/ml)**
n-Hexane	32.05 ± 3.63	72.62 ± 12.60	>50.36	>22.50	>1600
Dichloromethane	44.50 ± 2.66	68.25 ± 16.07	19.57	14.09	869±141.93
Methanol	64.93 ± 7.00	65.13 ± 2.22	>24.84	>24.59	>1600

*50% inhibitory concentration (IC_{50}) determined from these independent experiments
**Selective Index
***50% cytotoxic concentration to Vero cell

Table 5 Inhibitory effect of extracts of *Clinacanthus siamensis* Bremek against HSV-1 and HSV-2.

Extracts	**IC_{50}* (µg/ml)** (mean ± S.D.)		**SI****		**CC_{50} *****
	HSV-1	**HSV-2**	**HSV-1**	**HSV-2**	**(µg/ml)**
n-Hexane	60.00 ± 11.61	46.52 ± 4.08	>27.31	>34.53	>1600
Dichloromethane	55.69 ± 4.41	49.63 ± 2.59	3.50	3.92	194±3.56
Methanol	37.39 ± 5.85	72.64 ± 6.52	>43.52	>22.14	>1600

*50% inhibitory concentration (IC_{50}) determined from these independent experiments
**Selective Index
***50% cytotoxic concentration to Vero cell

CHAPTER V
DICUSSION AND CONCLUSION

According to Thai plant name by Tem Smittinad [4], not only the plant names Lin Nguu Hao refered to *Clinacanthus siamensis* Bremek., it may also refered to *Blepharis maderaspatensis* Heyne ex Roth (ACANTHACEAE) or *Limnophila balsamea* (Benth.) Benth. (SCROPHULARIACEAE), so naming the plant by local name may lead to misunderstanding and using the wrong plant thus, identification the related or similar morphology plants is important.

Macroscopic and microscopic examinations can be used as rapid and inexpensive identification techniques. *Kirganelia reticulata* Baill. (Euphorbiaceae), a medicinal plant uses in Indian ethnomedicine was investigated by macroscopic and microscopic assessment. The result of this investigation could be used as a definitive of the plant's identification which its application can distinguish it from the substitutes or adulterants [95].

Each of these mentioned methodologies has limitations and more analytical methods are required to assist in the authentication process. biomolecular activity offers an assortment of techniques that can be very useful for authentication of medicinal plants. The four species of plants in genus *Morinda* existing in Thailand were distinguished by combination of macroscopic, microscopic and biomolecular technique by PCR/RFLP, the result indicated that using these assessments together could give the identity of each species even they are in the same genus [96]. Using the various techniques of biomolecular evaluation may also give the identity and can distinguish between Korean and foreign ginseng which can be developed and applied for detecting of the adulterants of the rare or expensive herbal drug [97].

Also to the researches exemplified above, this present study deals with the distinguishing between the two closely related plants in genus *Clinacanthus* by macroscopic, microscopic and biomolecular technique. Macroscopic assessment is an effective tool for determining identity of plant material. Although the overall appearance of the whole plant of these two related species are different, but the flowers are similar. However, it is subjective and depends on the experience of the

examiner when the closely related plants are involved. Microscopic assessment, the cross section of stem, midrib and the presence/absence of the components in leaves epidermis of the two species are similar and measuring of stomatal number may not give the exact value of each species due to the affection of environmental conditions and geographical sources where the plants grow, interestingly, stomatal index and palisade ratio which are important property for species identification showed different values. By the way, counting the stomatal number, stomatal index and palisade ratio must be counted for a lot of image fields from various plant sources which is laborious and some expensive instruments, reagents and skills are needed. Thus, DNA sequencing is used in addition as a definitive means for identifying species [49]. This method can detect even such a single nucleotide polymorphism. The ITS region is widely used in plant taxonomy and molecular phylogeny because it has a high degree of variation even between closely related species [98]. According to the result, there are intraspecies polymorphisms of ITS sequences of *C. siamensis* and *C. nutans* which showed considerable variation. The sequence of the ITS region of the two plants were almost identical with 97-99% similarity, indicated closely related species. Due to their sequence variation, ITS region may not suitable molecular marker for differentiation between *C. nutans* and *C. siamensis*. Thus, other coding and spacer regions of nuclear, chloroplast and mitochondrial genes such as *rbc*L, *mat*K and *trn*K gene should be explored and used as molecular markers for authentication and it is generally accepted that multi-locus combinations will be required for species discrimination [99]. There are many interesting further studies should be performed in these plants such as using PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism) or using the new conserved region in genomic DNA as a marker to distinguish the plants which may be further applied to the DNA barcode.

Chemical constituents and the efficiency of antiviral activity may be used as a tool for distinguishing between *C. nutans* and *C. siamensis*. Due to the reputation of these two plants that can relief of herpes simplex lesion, to determine the anti HSV-1 and HSV-2 effects and its safety, plaque reduction and MTT assay were employed in this study. The result indicates that n-hexane, dichloromethane and methanol extracts of *C. nutans* and *C. siamensis* dry leaves can inhibit the plaque forming of both HSV-1 and HSV-2. The obtained results revealed that methanolic extract of *C.*

nutans exhibit only a slight anti-HSV-1 activity (IC_{50}=64.93 µg/ml) compare to the n-hexane (IC_{50}=32.05 µg/ml) and the selective index (SI), determined by the ratio of concentration causing 50% cellular cytotoxicity (CC_{50}) to IC_{50} against HSV-1 was >50.36. By contrast, methanolic extract of *C. siamensis* exhibited the strongest activity against HSV-1 among its three extracts with IC_{50}= 37.39 µg/ml, SI value >43.52 while the n-hexane exhibit only a slight anti-HSV-1 activity (IC_{50}=60.00 µg/ml). Comparison to the anti-HSV-2 activity, n-hexane, dichloromethane and methanol extracts of *C. nutans* exhibited slight activity (72.62, 65.19, 65.13 µg/ml, respectively) whereas n-hexane and dichloromethane exhibited better activity (46.52 and 49.63 µg/ml) than methanolic extract (72.64 µg/ml) of *C. siamensis*. Acyclovir which was used as a positive control in plaque reduction assay which expressed the IC_{50} value at 0.09 ± 0.02 µg/ml for HSV-1 and 0.43 ± 0.04 µg/ml for HSV-2.

According to the previous investigation, the bioactive constituents of the extracts of *C. nutans* and *C. siamensis* leaves were reported by various research groups. Methanol extract of *C. siamensis* leaves was examined to inactivate of HSV-1 and HSV-2. The extract was further purified to pure compounds for 4 fractions and then the pure compounds were individually tested to inactivate HSV-1 and HSV-2. These four pure compounds were a mixture of β-sitosterol-3-O-glucoside and β-stigmasterol-3-O-glucoside which showed no activity to inactivated the virus, monogalactosyl diaglycerol (1,2-*O*-diacyl-3-*O*-(β-galactopyranosyl)-*sn*-glycerol) which showed weak activity, digalactosyl diacylglycerol (1,2-*O*-diacyl-3-*O*-(α-galactopyranosyl)-(1″→6′)-*O*-(β-galactopyranosyl)-*sn*-glycerol) and a mixture of glycoglycerolipids (1,2-*O*-diacyl-3-*O*-(α-galactopyranosyl)-(1″→6′)-*O*-(β-galactopyranosyl)-*sn*-glycerol) which showed moderate activity [43]. Chloroform extract of *C. nutans* leaves was investigated. Three known pure compounds namely 13^2-hydroxy-(13^2-*R*)-phaeophytin b, 13^2-hydroxy-(13^2-*S*)-phaeophytin a and 13^2-hydroxy-(13^2-*R*)-phaeophytin a. these compounds were individually tested for anti-herpes simplex virus type 1 (HSV-1F) activity at maximal subtoxic concentrations which exhibited 100% inhibition activity in pre-viral entry step and about 30% in post-viral entry step. The results indicated that the mechanisms of these compounds affected on HSV-1F may be binding of the compounds to viral glycoproteins involved in host cell adsorption and penetration, whereas the mechanism of ACV is

terminate the replication of herpes viral DNA [36]. Compared between crude extracts and pure compounds of the 2 plants from these evidences, the crude extracts showed better inhibitory effect against HSV-1 and HSV-2 than the effects of pure compounds. Therefore, these compounds should be used in a synergistic treatment of HSV infection.

From the evidences, these plants have a potential to be an anti HSV-1 and HSV-2 agent. Though the lowest IC_{50} value of the extracts that inhibit HSV-1 and HSV-2 are higher than that of acyclovir, but these plants show their interesting efficacy and safety that can be further developed to be the antiviral agents which will decrease the expenditure for the modern antiviral medicine. We suggest that the extracts derived from various solvents should be studied and compared for the activity against other viruses and their toxicity both *in vitro* and *in vivo* which can be developed to the clinical trial in human.

Conclusion

Macroscopic, microscopic especially stomatal index and palisade ratio are able to authenticate the closely related plants; *C. nutans* and *C. siamensis*. Comparison of the ITS sequence may not give reliable discrimination due to its intraspecies and interspecies variation. Accordingly, these medicinal plants can be a source for isolation of anti-HSV compounds and the extraction of these medicinal plants exhibit antiviral activity against both HSV-1 and 2, suggesting that these Thai medicinal plants have a potential to be an antiviral agent.

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APPENDIX

Table 6 Stomatal number and stomatal index of *Clinacanthus nutans* (Burm. f.) Lindau.

Location: Muang District, Lampang Province.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	104	736	12.38
2	114	792	12.58
3	110	700	13.58
4	116	748	13.43
5	120	744	13.89
6	112	736	13.21
7	112	748	13.02
8	174	1008	14.72
9	158	968	14.03
10	196	1184	14.20
11	168	960	14.89
12	180	1040	14.75
13	188	1076	14.87
14	182	1064	14.61
15	174	1028	14.48
16	160	956	14.34
17	156	916	14.55
18	140	848	14.17
19	152	904	14.39
20	168	984	14.58
21	164	964	14.54
22	158	936	14.44
23	154	864	15.13
24	126	808	13.49
25	140	848	14.17
26	144	828	14.81
27	122	868	12.32
28	136	880	13.39
29	134	832	13.87
30	154	936	14.13
Mean	147.2	896.8	14.03
S.D.	25.96	119.43	0.76

Table 7 Stomatal number and stomatal index of *Clinacanthus nutans* (Burm. f.) Lindau.

Location: Botanical garden, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	144	924	13.48
2	138	804	14.65
3	134	776	14.73
4	134	756	15.06
5	132	784	14.41
6	138	728	15.94
7	128	736	14.81
8	134	752	15.12
9	144	904	13.74
10	134	908	12.86
11	150	892	14.40
12	130	856	13.18
13	148	884	14.34
14	162	972	14.29
15	142	920	13.37
16	168	1000	14.38
17	182	1120	13.98
18	176	1112	13.66
19	162	984	14.14
20	140	924	13.16
21	146	936	13.49
22	160	1020	13.56
23	178	1080	14.15
24	154	1020	13.12
25	134	888	13.11
26	140	832	14.40
27	186	1088	14.60
28	158	988	13.79
29	144	948	13.19
30	186	1084	14.65
Mean	150.2	920.67	14.06
S.D.	17.65	116.02	0.73

Table 8 Stomatal number and stomatal index of *Clinacanthus nutans* (Burm. f.) Lindau.

Location: Botanical garden, Sawankhalok Hospital, Sawankhalok District, Sukhothai Province.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	240	1272	15.87
2	236	1276	15.61
3	240	1324	15.35
4	208	1292	13.87
5	180	1088	14.20
6	192	1156	14.24
7	196	1212	13.92
8	224	1344	14.29
9	232	1232	15.85
10	236	1432	14.15
11	224	1280	14.89
12	152	1084	12.30
13	168	1096	13.29
14	160	1116	12.54
15	188	1248	13.09
16	176	1124	13.54
17	196	1168	14.37
18	188	1228	13.28
19	128	948	11.90
20	144	1068	11.88
21	124	1016	10.88
22	204	1180	14.74
23	204	1224	14.29
24	184	1048	14.94
25	184	1052	14.89
26	172	988	14.83
27	196	1072	15.46
28	200	1108	15.29
29	180	1080	14.29
30	228	1328	14.65
Mean	192.80	1169.47	14.09
S.D.	31.83	118.32	1.25

Table 9 Stomatal number and stomatal index of *Clinacanthus nutans* (Burm. f.) Lindau.

Location: Sirirukkachat garden, Faculty of Pharmacy, Mahidol University Salaya Campus, Nakhonpathom Province.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	200	1308	13.26
2	184	1216	13.14
3	196	1260	13.46
4	204	1328	13.32
5	212	1368	13.42
6	200	1344	12.95
7	188	1220	13.35
8	160	1032	13.42
9	160	1140	12.31
10	176	1124	13.54
11	156	1044	13.00
12	192	1160	14.20
13	160	1068	13.03
14	168	1124	13.00
15	204	1224	14.29
16	192	1284	13.01
17	180	1204	13.01
18	208	1208	14.69
19	200	1144	14.88
20	168	1172	12.54
21	172	1208	12.46
22	172	1252	12.08
23	156	1100	12.42
24	156	1108	12.34
25	160	1140	12.31
26	128	856	13.01
27	136	984	12.14
28	196	1320	12.93
29	292	1784	14.07
30	216	1424	13.17
Mean	183.07	1204.93	13.16
S.D.	30.51	163.41	0.72

Table 10 Palisade ratio of *Clinacanthus nutans* (Burm. f.) Lindau.

Location: Muang District, Lampang Province.

Position	Number of Palisade cell*	Palisade ratio
1	26	6.50
2	25	6.25
3	29	7.25
4	31	7.75
5	28	7.00
6	32	8.00
7	27	6.75
8	29	7.25
9	32	8.00
10	27	6.75
11	35	8.75
12	26	6.50
13	28	7.00
14	25	6.25
15	33	8.25
16	29	7.25
17	24	6.00
18	28	7.00
19	29	7.25
20	27	6.75
21	28	7.00
22	28	7.00
23	29	7.25
24	26	6.50
25	23	5.75
26	26	6.50
27	27	6.75
28	25	6.25
29	26	6.50
30	27	6.75
Mean	27.83	6.96
S.D.	2.70	0.68

*Number of Palisade cell beneath 4 epidermal cells

Table 11 Palisade ratio of *Clinacanthus nutans* (Burm. f.) Lindau.
Location: Botanical garden, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

Position	Number of Palisade cell*	Palisade ratio
1	25	6.25
2	23	5.75
3	27	6.75
4	29	7.25
5	30	7.50
6	26	6.50
7	32	8.00
8	28	7.00
9	27	6.75
10	25	6.25
11	26	6.50
12	26	6.50
13	26	6.50
14	24	6.00
15	24	6.00
16	23	5.75
17	24	6.00
18	24	6.00
19	25	6.25
20	24	6.00
21	24	6.00
22	24	6.00
23	31	7.75
24	24	6.00
25	25	6.25
26	25	6.25
27	23	5.75
28	24	6.00
29	25	6.25
30	24	6.00
Mean	25.57	6.39
S.D.	2.34	0.59

*Number of Palisade cell beneath 4 epidermal cells

Table 12 Palisade ratio of *Clinacanthus nutans* (Burm. f.) Lindau.
Location: Botanical garden, Sawankhalok Hospital, Sawankhalok District, Sukhothai Province.

Position	Number of Palisade cell*	Palisade ratio
1	28	7.00
2	30	7.50
3	31	7.75
4	30	7.50
5	24	6.00
6	26	6.50
7	24	6.00
8	29	7.25
9	28	7.00
10	29	7.25
11	29	7.25
12	31	7.75
13	32	8.00
14	32	8.00
15	29	7.25
16	30	7.50
17	25	6.25
18	30	7.50
19	26	6.50
20	27	6.75
21	24	6.00
22	31	7.75
23	29	7.25
24	24	6.00
25	28	7.00
26	26	6.50
27	25	6.25
28	25	6.25
29	29	7.25
30	24	6.00
Mean	27.83	6.96
S.D.	2.63	0.66

*Number of Palisade cell beneath 4 epidermal cells

Table 13 Palisade ratio of *Clinacanthus nutans* (Burm. f.) Lindau.
Location: Sirirukkachat garden, Faculty of Pharmacy, Mahidol University Salaya Campus, Nakhonpathom Province.

Position	Number of Palisade cell*	Palisade ratio
1	27	6.75
2	29	7.25
3	29	7.25
4	26	6.50
5	31	7.75
6	32	8.00
7	32	8.00
8	28	7.00
9	28	7.00
10	31	7.75
11	26	6.50
12	33	8.25
13	33	8.25
14	32	8.00
15	24	6.00
16	29	7.25
17	27	6.75
18	24	6.00
19	29	7.25
20	25	6.25
21	30	7.50
22	24	6.00
23	25	6.25
24	26	6.50
25	25	6.25
26	28	7.00
27	25	6.25
28	27	6.75
29	29	7.25
30	29	7.25
Mean	28.10	7.03
S.D.	2.78	0.70

*Number of Palisade cell beneath 4 epidermal cells

Table 14 Stomatal number and stomatal index of *Clinacanthus siamensis* Bremek. Location: Bang Yai District, Nonthaburi Province.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	192	1164	14.16
2	140	1044	11.82
3	148	1104	11.82
4	152	1116	11.99
5	172	1140	13.11
6	164	928	15.02
7	160	1176	11.98
8	160	940	14.55
9	136	1056	11.41
10	128	972	11.64
11	132	1000	11.66
12	160	1040	13.33
13	168	1096	13.29
14	136	1048	11.49
15	164	1140	12.58
16	156	1152	11.93
17	140	1112	11.18
18	176	1180	12.98
19	136	1100	11.00
20	128	972	11.64
21	140	1056	11.71
22	128	988	11.47
23	152	1120	11.95
24	156	1156	11.89
25	140	1044	11.82
26	152	1132	11.84
27	168	1236	11.97
28	132	1032	11.34
29	168	1064	13.64
30	152	1152	11.66
Mean	151.20	1082.00	12.26
S.D.	16.32	76.90	1.03

Table 15 Stomatal number and stomatal index of *Clinacanthus siamensis* Bremek. Location: The Somdej Phra Thepraratanarajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	160	1060	13.11
2	144	1012	12.46
3	152	1096	12.18
4	164	1132	12.65
5	140	1128	11.04
6	140	1112	11.18
7	140	1060	11.67
8	112	940	10.65
9	144	1076	11.80
10	160	1172	12.01
11	132	1016	11.50
12	148	1084	12.01
13	164	1096	13.02
14	148	1068	12.17
15	156	1164	11.82
16	112	904	11.02
17	152	1076	12.38
18	144	1016	12.41
19	148	1044	12.42
20	144	1136	11.25
21	120	924	11.49
22	152	1076	12.38
23	112	892	11.16
24	124	940	11.65
25	116	984	10.55
26	164	1088	13.10
27	128	1012	11.23
28	136	1068	11.30
29	172	1160	12.91
30	184	1196	13.33
Mean	143.73	1128.00	11.93
S.D.	18.40	96.17	0.76

Table 16 Stomatal number and stomatal index of *Clinacanthus siamensis* Bremek. Location: Botanical garden, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	180	1184	13.20
2	180	1264	12.47
3	116	936	11.03
4	176	1288	12.02
5	164	1228	11.78
6	152	1104	12.10
7	148	1084	12.01
8	160	1132	12.38
9	160	1080	12.90
10	140	1112	11.18
11	128	996	11.39
12	140	1060	11.67
13	120	1064	10.14
14	152	1144	11.73
15	128	1020	11.15
16	152	1176	11.45
17	180	1244	12.64
18	148	1128	11.60
19	152	1072	12.42
20	120	956	11.15
21	188	1236	13.20
22	152	1196	11.28
23	160	1256	11.30
24	160	1276	11.14
25	140	1084	11.44
26	140	1176	10.64
27	132	1036	11.30
28	140	1008	12.20
29	132	1160	10.22
30	140	1092	11.36
Mean	149.33	1126.40	11.68
S.D.	19.12	96.43	0.77

Table 17 Stomatal number and stomatal index of *Clinacanthus siamensis* Bremek. Location: Sirirukkachat garden, Faculty of Pharmacy, Mahidol University Salaya Campus, Nakhonpathom Province.

Position	Number of stomata (1 sq.mm.)	Number of epidermal cell (1 sq.mm.)	Stomatal index
1	184	1368	11.86
2	200	1512	11.68
3	192	1520	11.21
4	156	1260	11.02
5	160	1324	10.78
6	208	1528	11.98
7	224	1428	13.56
8	200	1384	12.63
9	180	1456	11.00
10	200	1432	12.25
11	192	1428	11.85
12	192	1364	12.34
13	188	1420	11.69
14	196	1440	11.98
15	188	1368	12.08
16	212	1492	12.44
17	212	1480	12.53
18	216	1680	11.39
19	196	1520	11.42
20	220	1744	11.20
21	220	1948	10.15
22	200	1468	11.99
23	204	1580	11.43
24	224	1548	12.64
25	224	1616	12.17
26	224	1632	12.07
27	200	1508	11.71
28	220	1640	11.83
29	200	1472	11.96
30	232	1576	12.83
Mean	202.13	1504.53	11.86
S.D.	18.31	136.88	0.68

Table 18 Palisade ratio of *Clinacanthus siamensis* Bremek.

Location: Bang Yai District, Nonthaburi Province.

Position	Number of Palisade cell*	Palisade ratio
1	16	4.00
2	13	3.25
3	13	3.25
4	15	3.75
5	11	2.75
6	12	3.00
7	14	3.50
8	12	3.00
9	14	3.50
10	13	3.25
11	15	3.75
12	13	3.25
13	15	3.75
14	12	3.00
15	13	3.25
16	13	3.25
17	11	2.75
18	12	3.00
19	14	3.50
20	14	3.50
21	14	3.50
22	13	3.25
23	13	3.25
24	12	3.00
25	13	3.25
26	14	3.50
27	13	3.25
28	14	3.50
29	14	3.50
30	15	3.75
Mean	13.33	3.33
S.D.	1.21	0.30

*Number of Palisade cell beneath 4 epidermal cells

Table 19 Palisade ratio of *Clinacanthus siamensis* Bremek.

Location: The Somdej Phra Thepraratanarajsuda Medicinal Plants Garden, Petroleum Authority of Thailand, Rayong Province.

Position	Number of Palisade cell*	Palisade ratio
1	14	3.50
2	12	3.00
3	12	3.00
4	13	3.25
5	12	3.00
6	12	3.00
7	14	3.50
8	13	3.25
9	12	3.00
10	13	3.25
11	15	3.75
12	12	3.00
13	13	3.25
14	13	3.25
15	12	3.00
16	13	3.25
17	14	3.50
18	12	3.00
19	15	3.75
20	16	4.00
21	13	3.25
22	14	3.50
23	13	3.25
24	14	3.50
25	11	2.75
26	16	4.00
27	12	3.00
28	12	3.00
29	12	3.00
30	12	3.00
Mean	13.03	3.26
S.D.	1.27	0.32

*Number of Palisade cell beneath 4 epidermal cells

Table 20 Palisade ratio of *Clinacanthus siamensis* Bremek.

Location: Botanical garden, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

Position	Number of Palisade cell*	Palisade ratio
1	12	3.00
2	15	3.75
3	13	3.25
4	14	3.50
5	13	3.25
6	14	3.50
7	15	3.75
8	13	3.25
9	14	3.50
10	15	3.75
11	12	3.00
12	12	3.00
13	12	3.00
14	13	3.25
15	14	3.50
16	13	3.25
17	12	3.00
18	14	3.50
19	13	3.25
20	15	3.75
21	14	3.50
22	12	3.00
23	14	3.50
24	12	3.00
25	14	3.50
26	12	3.00
27	16	4.00
28	14	3.50
29	16	4.00
30	12	3.00
Mean	13.47	3.37
S.D.	1.25	0.31

*Number of Palisade cell beneath 4 epidermal cells

Table 21 Palisade ratio of *Clinacanthus nutans* (Burm. f.) Lindau.
Location: Sirirukkachat garden, Faculty of Pharmacy, Mahidol University Salaya Campus, Nakhonpathom Province.

Position	Number of Palisade cell*	Palisade ratio
1	14	3.50
2	17	4.25
3	16	4.00
4	13	3.25
5	15	3.75
6	14	3.50
7	14	3.50
8	13	3.25
9	14	3.50
10	14	3.50
11	14	3.50
12	15	3.75
13	15	3.75
14	14	3.50
15	14	3.50
16	12	3.00
17	13	3.25
18	15	3.75
19	12	3.00
20	13	3.25
21	12	3.00
22	16	4.00
23	16	4.00
24	12	3.00
25	14	3.50
26	15	3.75
27	12	3.00
28	15	3.75
29	14	3.50
30	15	3.75
Mean	14.07	3.52
S.D.	1.34	0.33

*Number of Palisade cell beneath 4 epidermal cells

VITA

Mr. Paween Kunsorn was born on Octerber 12, 1986 in Bangkok, Thailand . He received his Bachelor's degree of Sciences (Applied Thai Traditional Medicine) from Faculty of Public Health, Naresuan University, Thailand in 2008.

Publication

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