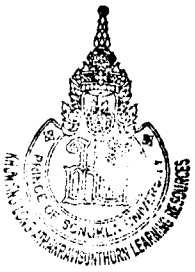

Rhinacanthin Production by *Agrobacterium rhizogenes* - induced Hairy Roots of *Rhinacanthus nasutus* (L.) Kurz

Songpol Hom-utai

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

Master of Science in Herb Sciences (International Program)

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Thesis Title	Rhinacanthin Production by *Agrobacterium rhizogenes* - induced Hairy Roots of *Rhinacanthus nasutus* (L.) Kurz
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ชื่อวิทยานิพนธ์ การสร้างสารไรนาแคนตินจากรากขนอ่อนของทองพันชั่งที่ชักนำด้วยอะโกรแบคทีเรียมไรโซจีเนส

ผู้เขียน นาย ทรงพล หอมอุทัย

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ปีการศึกษา 2551

บทคัดย่อ

ในการเหนี่ยวนำให้เกิดรากขนอ่อนของต้นทองพันชั่งจากชิ้นส่วนของใบโดยใช้อะโกรแบคทีเรียมไรโซจีเนส 4 สายพันธุ์ได้แก่ ATCC 11325, 13332, 13333 และ 15834 พบว่า ได้รากขนอ่อน 4 สายพันธุ์คือ รากขนอ่อน 11325, 13332, 13333, และ 15834 และพบว่าประสิทธิภาพของการเหนี่ยวนำให้เกิดรากมีค่า 55 %, 25 %, 70 %, และ 60 % ตามลำดับ วิธีการ Polymerase chain reaction แสดงชิ้นส่วนของยีน *rolB* และ *rolC* ในรากขนอ่อน ยืนยันการถ่ายชิ้นส่วนของ T-DNA จากอะโกรแบคทีเรียม Ri พลาสมิดไปยังใบทองพันชั่งแล้วเกิดเป็นรากขนอ่อน ผลการศึกษาปริมาณการสร้างสารไรนาแคนตินในรากขนอ่อนด้วยเทคนิค HPLC พบว่า รากขนอ่อนที่เหนี่ยวนำจากทั้งอะโกรแบคทีเรียมไรโซจีเนส 4 สายพันธุ์พบความแตกต่างกันของรูปแบบของการสร้างสาร โดยรากขนอ่อนที่เหนี่ยวนำด้วยสายพันธุ์ ATCC 11325 ผลิตไรนาแคนตินซี ไรนาแคนตินดี และไรนาแคนตินเอ็น ในขณะที่รากขนอ่อนที่เหนี่ยวนำด้วยสายพันธุ์อื่นผลิต ไรนาแคนตินซีและไรนาแคนตินดี และพบว่า รากขนอ่อนทองพันชั่ง 11325 สามารถสร้างสารไรนาแคนตินซีสะสมสูงสุด (2.16 ± 0.097 % w/w) ตามด้วย รากขนอ่อน 15834 (1.15 ± 0.054 % w/w), รากขนอ่อน 13332 (0.83 ± 0.172 % w/w) และ รากขนอ่อน 13333 (0.82 ± 0.121 % w/w) ตามลำดับ รากขนอ่อน 11325 มีรูปแบบกราฟการเจริญเติบโตเป็นแบบปกติ มีวงจรการเจริญเติบโตประมาณ 30 วัน โดยการเจริญเติบโตสูงสุดเกิดขึ้นในช่วง 25 วัน จากการศึกษาปริมาณการสร้างสารไรนาแคนติน พบว่ามีการเพิ่มขึ้นอย่างช้าๆควบคู่ไปกับการเจริญเติบโตของรากขนอ่อน

Thesis Title:	Rhinacanthin Production by *Agrobacterium rhizogenes* - induced Hairy Roots of *Rhinacanthus nasutus* (L.) Kurz
Author:	Mr. Songpol Hom-utai
Major program:	Herb Sciences (International Program)
Academic Year:	2008

ABSTRACT

Four strains of *Agrobacterium rhizogenes*, ATCC 11325, 13332, 13333, and 15834 were evaluated for induction of transformed hairy roots in *Rhinacanthus nasutus* using leaf explants. Transformed percentage of four hairy root lines, namely hairy root 11325, 13332, 13333 and 15834 were 55 %, 25 %, 70 %, and 60 %, respectively. The fragments of *rolB* and *rolC* genes were detected in all hairy root line using polymerase chain reaction technique, indicated the successful integration of the T-DNA fragment of *A. rhizogenes* Ri plasmid to the genome of hairy roots. Determination of rhinacanthin produced by the hairy roots was carried out by HPLC method. The hairy roots from 4 strains showed difference chemical patterns. Hairy roots 11325 produced rhinacanthin-C, -D, -N while other transformed hairy roots extract showed only rhinacanthin-C and rhinacanthin-D. Hairy root 11325 contained the highest amount of rhinacanthin-C (2.16 ± 0.097 % w/w) following by hairy root 15834 (1.15 ± 0.054 % w/w), hairy root 13332 (0.83 ± 0.172 % w/w), and hairy root 13333 (0.82 ± 0.121 % w/w). Growth kinetics of hairy root 11325 appeared to be a normal sigmoid curve with a growth period of 30 days and maximum growth occurred within 25 days. Time-course study of rhinacanthin production indicated gradually increased parallel with the growth of hairy roots cultures.

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Songpol Hom-utai

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LIST OF ABBREVIATIONS AND SYMBOLS

β	=	Beta
µL	=	Microliter
µm	=	Micrometre
bp	=	Base pair
°C	=	Degree Celsius
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleoside triphosphate
g	=	Gram
hr	=	Hours
Hairy root 11325	=	Hairy root transformed by *Agrobacterium rhizogenes* ATCC 11325
HPLC	=	High-performance liquid chromatography
lb/in^2	=	Pound per square inch
Kb	=	Kilobase
L	=	Liter
MeOH	=	Methanol
mg	=	Milligram
min	=	Minutes
mL	=	Milliliter
mM	=	Milli molar
MS	=	Murashige and Skoog (1962) media
OD	=	Optical density
pH	=	The negative logarithm of the concentration of hydrogen ions
rpm	=	Round per minute
s	=	Second
UV	=	Ultraviolet
w/w	=	Weight per weight
% w/w	=	Percent weight by weight
v/v	=	Volume by volume

CHAPTER 1

INTRODUCTION

1.1 General introduction

The technique in plant culture of cells, tissues and organ is an important tool in both basic and applied studies, e.g. in commercial applications and for the preservation of medicinal plant resources and efficient production of pharmaceutically important secondary metabolites (Bhojwani, 1990). Nowadays, plant cell culture method and biotechnological approach are attractively alternative ways for production of high-value secondary metabolites and for improvement of the productivity of plant cell cultured. When compared to traditional agricultural growth, medicinal plant tissue culture offers a number of year-round, continuous productions of plant medicinal compounds under highly controlled conditions. Natural production of secondary metabolites by plants can be highly influenced by plant growth environment factors such as climate, soil conditions, pathogen attack and herbivores (Wink, 2003).

Human depends on plants. In addition to basic nutrients such as protein, fats or carbohydrates, plants are a source of pharmaceuticals, cosmetics, food ingredients, wood, cellulose, agrochemicals, flavors, insecticides, and pigments. These compounds, which are not only essential for survival but also confer some advantages to plant cells, are called secondary metabolites (Vasconsuelo and Boland, 2007). Secondary metabolites were regarded for long time as waste products but now, most secondary metabolites are important for overall fitness of the plant that produces them. Major functions are defense against herbivores (insects, mollusks, vertebrate), and micro-organisms (viruses, bacteria, fungi) and display ecological functions (UV-protection, attraction of pollinator or seed-dispersing animals). The major groups of secondary metabolites used as medicines are terpenoids (mono-, sesqui-, di-, tri-, steroids, cardenolides), quinones, lignans, flavonoids, or alkaloids. (Charlwood and Rhodes, 1990).

Rhinacanthus nasutus that belongs to the family Acanthaceae and widely used as a "Thai traditional medicine". *R. nasutus* has long been used in Southeast Asia, South China and India for a treatment of dermatomycosis such as Tinea vesicolor and ringworm (Farnsworth and Bunyapraphatsara, 1992). It has been reported that *R. nasutus* possessed several interesting biological activities, e.g. antifungal (Wu *et al.*, 1998a; Panichayupakaranant *et al.*, 2003; Kongchai and Panichayupakaranant, 2002), antiviral (Kernan *et al.*, 1997), antitumor (Thirumurugan *et al.*, 2000), anti-platelet aggregation (Wu *et al.*, 1998b) and antibacterial (Sattar *et al.*, 2004) activities. Several chemical constituents were found from different parts of this plant. Rhinacanthins, naphthoquinones which posses several biological activities, are major constituents found in *R. nasutus*. Rhinacanthin contents in this plant are vary depending on several factors including varieties, cultivation, harvesting period, and distribution of compounds into several parts of plants. Leaves and roots of *R. nasutus* should be harvested in July to yield the highest amount of rhinacanthins (Panichayupakaranant *et al.*, 2006). However, there are little informations about *R. nasutus* tissue culture as an alternative sourse for rhinacanthin production. For this reason, plant tissue culture and strategies to improve the productivity of plant cell culture are important. The strategies for the improvement of secondary metabolite production such as medium optimization, cell line selection, cell immobilization, precursor addition, elicitation, genetic transformation, organ or hairy root cultures, metabolic engineering and integrated bioreactor engineering have successful for improvement of many plant products (Dornenburg and Knorr, 1995; Abdullah *et al.*, 2005).

For the pharmacologically active compounds, which storage sites are in the root, root harvesting is destructive for the whole plant (Flores *et al.*, 1999). Therefore, the hairy root culture may be a alternative source. With the hairy root culture technique, the production of secondary metabolites can be improved and applied to industrial scale. Transformation of plants using *Agrobacterium rhizogenes*, the gram negative soil bacterium which is causative agent of hairy root disease in several plants, has emerged as an important alternative to intact plants for the production of secondary metabolites. An advantage of using transformed hairy root culture is practically scaled up in the bioreactor for commercial purpose and used as an interesting plant material for the production of pharmacologically active secondary metabolites.

In this study, we aim to induce the hairy root culture of *R. nasutus* with 4 wild type strains of *Agrobacterium rhizogenes* ATCC 11325, 13332, 13333 and 15834. Rhinacanthins accumulated in *R. nasutus* hairy root was determined for their contents. The hairy root culture obtained from this study will be useful for the enhancement of secondary metabolites by plant tissue culture for medicinal development and will be good models for the study of rhinacanthin biosynthesis in the future.

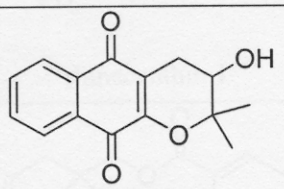
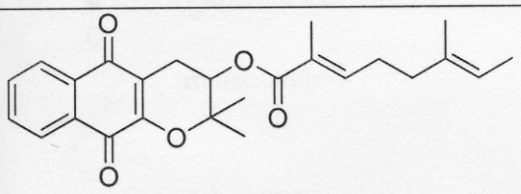
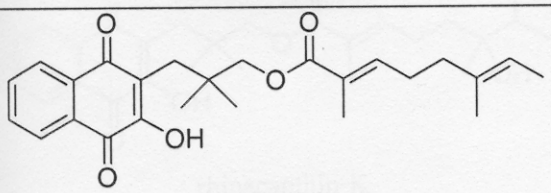
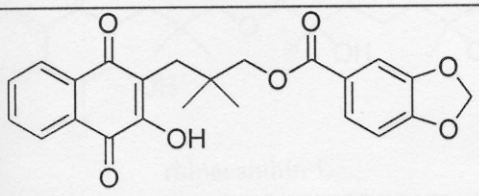
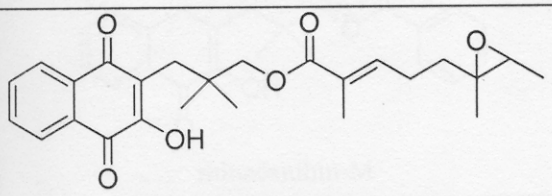
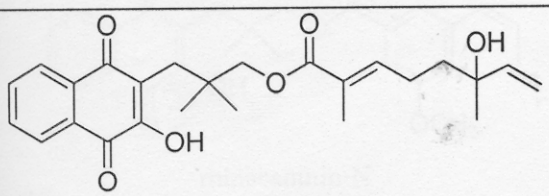
1.2 Objectives

The objectives of the present study were

1.2.1 To establish hairy root cultures of *Rhinacanthus nasutus* using 4 strains of *Agrobacterium rhizogenes*

1.2.2 To determine rhinacanthin formation in the 4 strains of *R. nasutus* hairy root cultures comparison with intact leaves and roots

Table 2.1 Chemical constituents of *Rhinacanthus nasutus*

Chemicals and Structures	Plant part	References
1. Naphthoquinones		
 rhinacanthin-A	Roots	Wu *et al.*, 1988; Wu *et al.*, 1998a; Wu *et al* 1998b; Singh *et al.*, 1992
 rhinacanthin-B	Roots	Wu *et al.*, 1988; Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-C	Whole plants	Sendl *et al.*, 1996; Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-D	Whole plants	Sendl *et al.*, 1996; Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-G	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-H	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b

CHAPTER 2

LITERATURE REVIEW

2.1 Botanical characteristics of *Rhinacanthus nasutus* (Linn.) Kurz

Rhinacanthus nasutus (Linn.) Kurz belongs to the family of Acanthaceae and widely distributed and cultivated in Southeast Asia, South China and India (Farnsworth and Bunyapraphatsara, 1992). It is known as "Thong phan chang" or "Yaa man kai" in Thailand.

The characteristic features of this plant (Figure 2.2) are described as follow. *R. nasutus* is a small shrub, up to 0.70 - 2 m height. The stems are erect and branched. When young, it is covered with fine up curved hairs (Fransworth and Bunyapraphatsara, 1992). The leaves are simple, opposite. The shape of the leaves is lanceolate with 2.5 - 5 cm wide and 6 - 10 cm long. The base of leaves is oblique. The leaves are glabrous yellowish green. Flowers are bisexual, zygomorphic petal and white color in short auxiliary clusters. The bract is small. The calyx is divided into 5 deeply acute parted, light green, 5 - 6 mm long. The corolla tube is bilabiate, upper lip erect, bifid, lower lip 3 lobed. The corolla has brownish purples spots at the throat of the tube. There are 4 stamens with didynamous. The ovary is superior with 2-loculed and ovule free placentation and the fruit is a capsule. Not only macroscopic character but also microscopic character revealed the presence of the upper epidemis, which the upper layer of surface view were composing of irregularly sharp cells with slightly wavy wall in surface view. The stoma is absent or very infrequent. The microscopy on the powder sample of the leaves revealed the presence of wavy-walled cells of the epidermis with numerous diacytic stomata and glandular trichome, multicellular uniseriate and collapsed trichome, mesophyll parenchyma, parenchyma containing reddish brown mass, lithocyst cells, fiber, and spiral vessel. Identification of the active principles of *R. nasutus* with Borntrager reaction which is specific test for quinone compounds, showed positive result, indicating the accumulation of quinone compounds in *R. nasutus*. The reaction with 20 % potassium hydroxide to produce a red color in ethyl acetate phase (Panichayupakaranant *et al.*, 2006).

2.2 Ecology and propagation

This plant is locally known and widely distributed in tropical countries. It scatters along the edges of evergreen forests. *R. nasutus* is usually grown as ornamentals and requires sandy and well-drained soil. It can be propagated by seeds or cutting.

2.3 Distribution of rhinacanthins

Total rhinacanthin content in the leaves, stems, and roots of *R. nasutus*, which were collected at a different period of times, has demonstrated that rhinacanthins markedly accumulated in the roots and leaves, but less in the stems of the plant. Regarding the effect of harvesting period, it was reported that the leaves and roots harvested in July yielded higher amounts of rhinacanthins. In July, *R. nasutus* is not yet in bloom. Thus, *R. nasutus* leaves and roots should be harvested before blossom (Panichayupakaranant *et al.*, 2006).

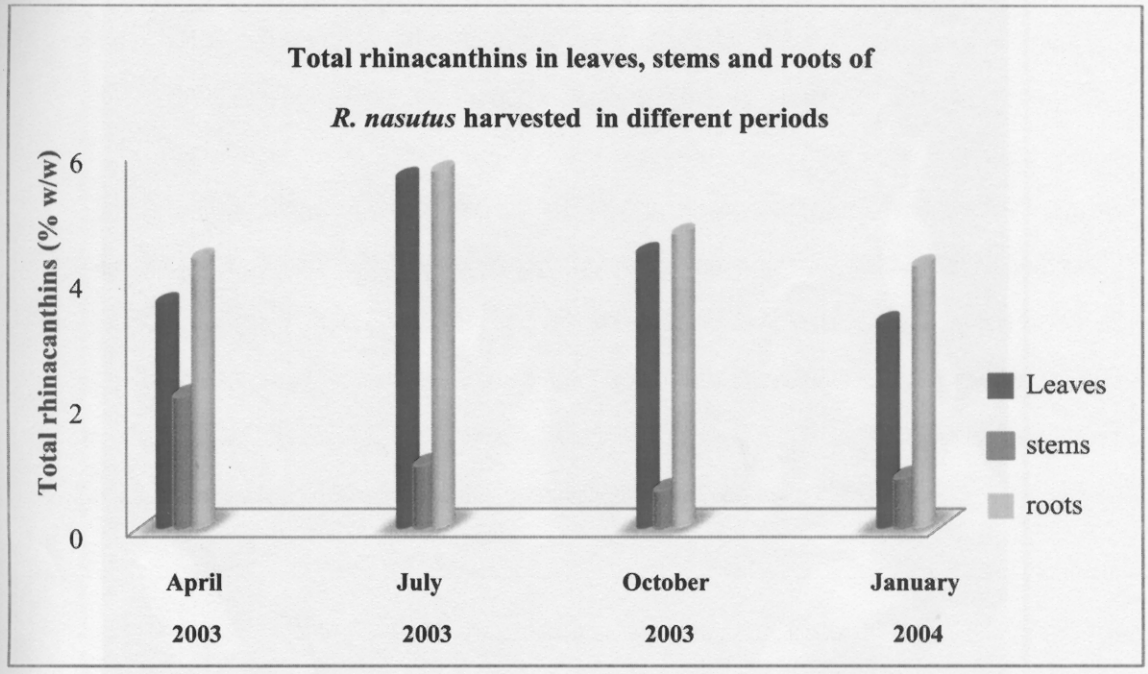


Figure 2.1 Total rhinacanthins in leaves, stems and roots of *R. nasutus* harvested in different periods

Stability evaluations of the rhinacanthin high-yielding *R. nasutus* leaf extract in several conditions in the period of 4 months found that the extract possessed a satisfactory stability at room temperature (30 ± 2°C). However, the extract should be stored in well-closed container, protected from light. The aqueous solutions of the extract are either stable in acid or base conditions (Charoonratana, 2007).

Figure 2.2 *Rhinacanthus nasutus* (Linn.) Kurz (Acanthaceae)

2.4 Ethnomedical uses

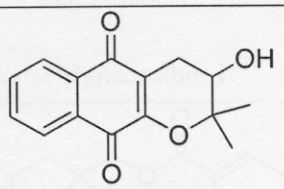
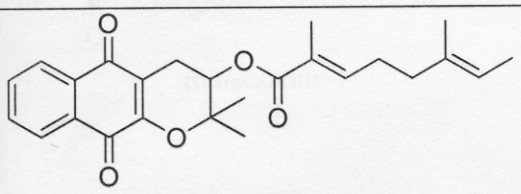
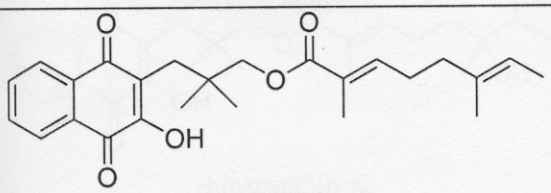
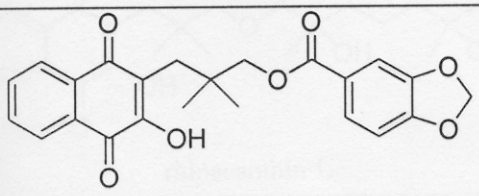
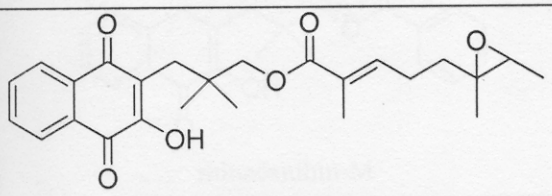
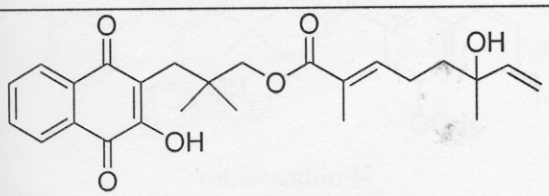
R. nasutus is a valuable plant which wildly distributed and cultivated in South China, Taiwan, India, and also in Thailand. Extracts of various parts of this plant are used for treatment of ringworm and other fungal-derived skin diseases as well as eczema, pulmonary tuberculosis, hepatitis, diabetes, hypertension and cancer (Siripong *et al.*, 2006a).

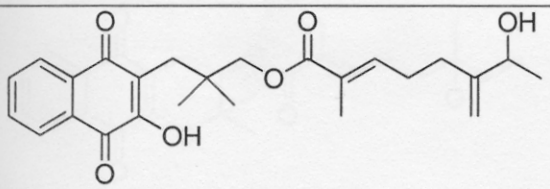
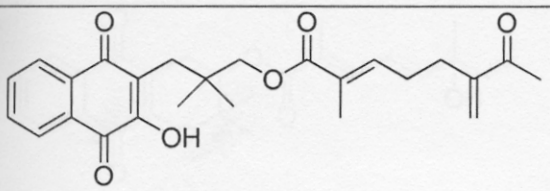
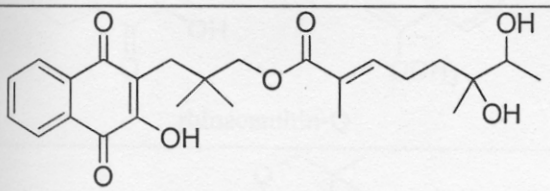
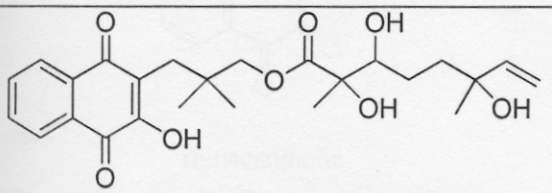
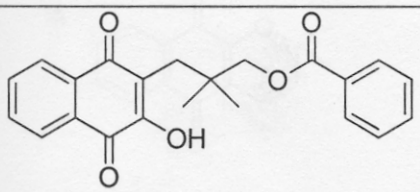
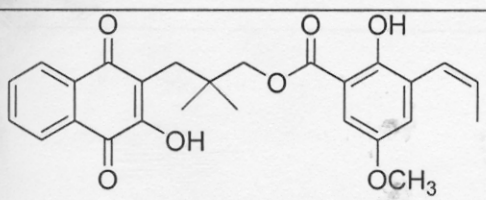
In traditional medicine preparation, leaves of this shrub are widely advocated for various skin problems. In Thai folk remedy, a tincture either with alcohol or vinegar is prepared to cure certain forms of ringworm (Farnsworth and Bunyapraphatsara, 1992). The recipe could be prepared by several ways. First, a tincture is prepared by soaking fresh leaves and roots in alcohol. The second, preparation is prepared by pounding the root with match tips and vaseline. In third preparation, the roots are pounded with lemon and tamarind juices. All three preparations were used by applying over the infected area (Farnsworth and Bunyapraphatsara, 1992). Pounded roots mix with vinegar or alcohol was applied on herpetic-like eruptions. For the same purpose, in Malaysia the leaves are applied with benzoin and sulfur. In Indonesia, the flowers and young leaves are rubbed with vinegar and lime to the skin (Wiart *et al.*, 2000). In India, this plant used in poultice for the treatment of eczema and ringworm. For people in Malay Peninsula, the leaves are pounded with benzoin and sulphur and the paste is applied to the area infected by ringworm. In Philippines it is traditionally used either as a sap or a decoction (Sattar, *et al.*, 2004). The decoction of its root or whole plant is used for treatment of some cancer e.g. cervical and liver cancer in Thai folk remedy (Siripong *et al.*, 2006a; Siripong *et al.*, 2006b).

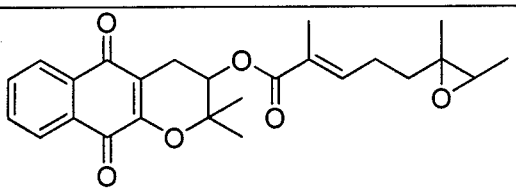
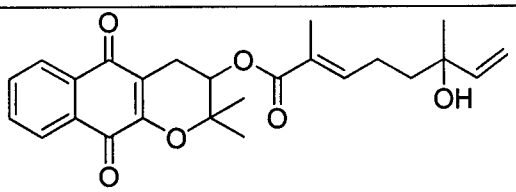
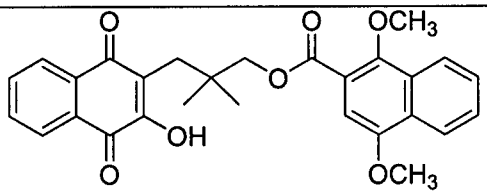
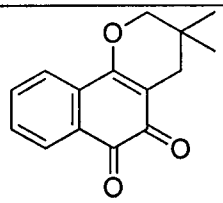
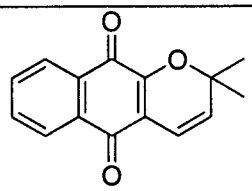
2.5 Chemical constituents of *R. nasutus*

Several compounds which, isolated from different parts of *R. nasutus* have been previously reported in many literatures. The lists of the compounds are shown in table 2.1

Table 2.1 Chemical constituents of *Rhinacanthus nasutus*

Chemicals and Structures	Plant part	References
1. Naphthoquinones		
 rhinacanthin-A	Roots	Wu *et al.*, 1988; Wu *et al.*, 1998a; Wu *et al* 1998b; Singh *et al.*, 1992
 rhinacanthin-B	Roots	Wu *et al.*, 1988; Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-C	Whole plants	Sendl *et al.*, 1996; Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-D	Whole plants	Sendl *et al.*, 1996; Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-G	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-H	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b

Chemicals and Structures	Plant part	References
 rhinacanthin-I	Leaves and Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-J	Leaves and Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-K	Roots	Wu *et al.*, 1998a
 rhinacanthin-L	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-M	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-N	Leaves and Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b

Chemicals and Structures	Plant part	References
 rhinacanthin-O	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-P	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b
 rhinacanthin-Q	Roots	Wu *et al.*, 1998b
 rhinacanthone	Leaves and Stems	Kodama *et al.*, 1993; Kuwahara *et al.*, 1995
 dehydro-α-lapachone	Roots	Wu *et al.*, 1998a; Wu *et al.*, 1998b

Chemicals and Structures	Plant part	References
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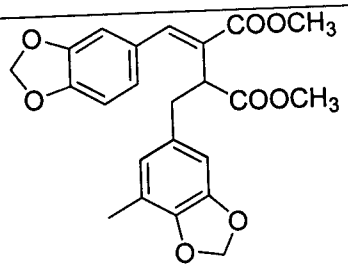
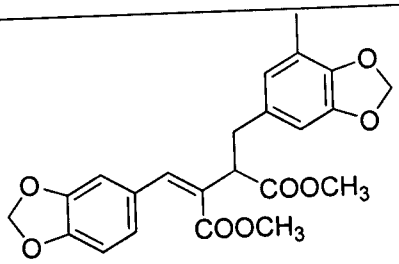
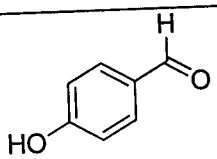
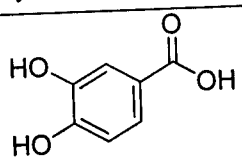
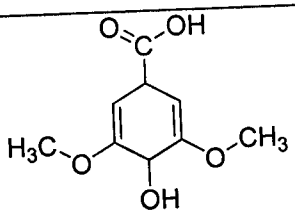
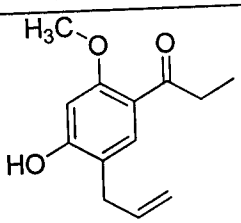
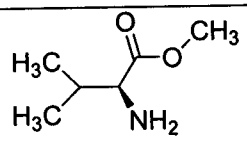
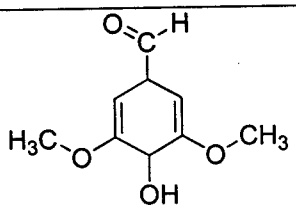
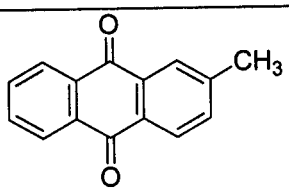
 rhinacanthin-E: Δ7E	Aerial parts	Kernan *et al.*, 1997
 rhinacanthin-F: Δ7Z	Aerial parts	Kernan *et al.*, 1997
3. Benzenoids		
 *p*-hydroxy-benzaldehyde	Roots	Wu *et al.*, 1998b
 vanillic acid	Leaves and Stems	Wu *et al.*, 1995
 syringic acid	Leaves and Stems	Wu *et al.*, 1995
 2-methoxy-4-propionylphenol	Leaves and Stems	Wu *et al.*, 1995

Chemicals and Structures	Plant part	References
 methyl valinate	Roots	Wu *et al.*, 1998b
 syringaldehyde	Roots	Wu *et al.*, 1998b
4. Anthraquinone		
 2-methyl anthraquinone	Leaves and Stems	Wu *et al.*, 1995
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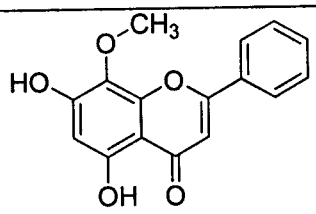
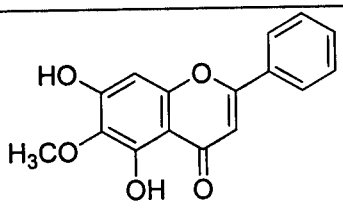
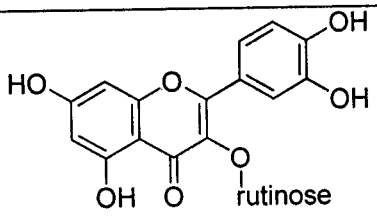
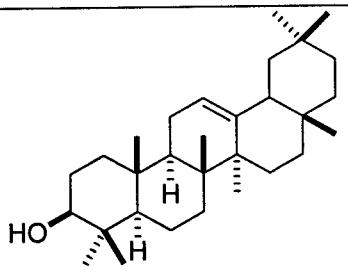
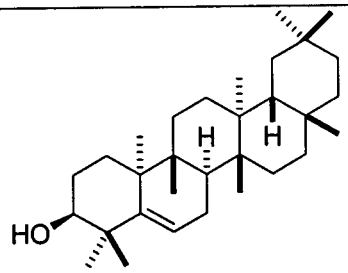
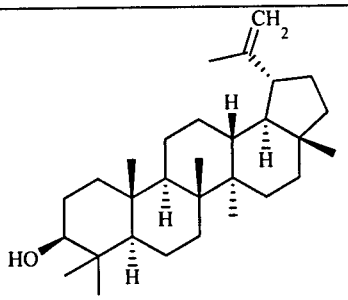
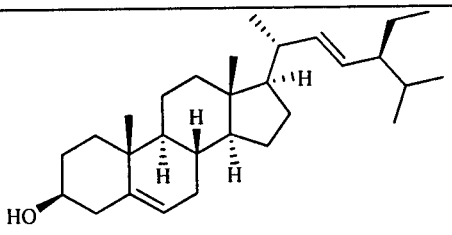
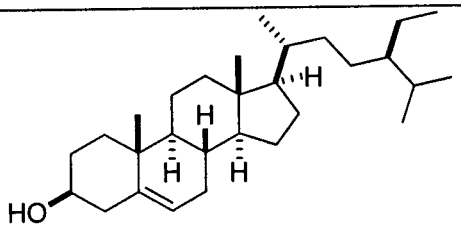
 wogonin	Roots	Wu *et al.*, 1998b
 oroxylin A	Roots	Wu *et al.*, 1998b
 rutin	Flowers	Subramanian *et al*, 1981

Chemicals and Structures	Plant part	References
6 Triterpenoid		
 *β*-amyrin	Roots	Wu *et al.*, 1995
 glutinol	Roots	Wu *et al.*, 1995
 lupeol	Roots	Wu *et al.*, 1988; Wu *et al.*, 1995; Wu *et al.*, 1998b
7. Sterol		
 stigmasterol	Roots	Wu *et al.*, 1988
 *β*-sitosterol	Roots	Wu *et al.*, 1988

Chemicals and Structures	Plant part	References
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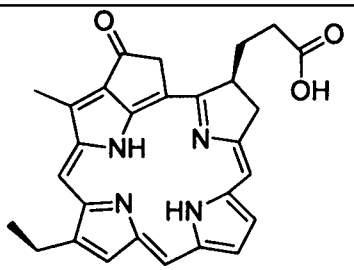
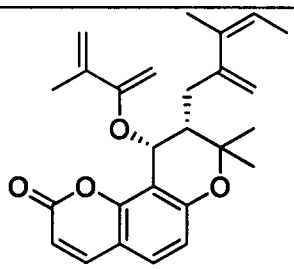
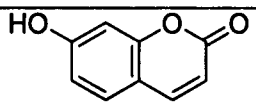
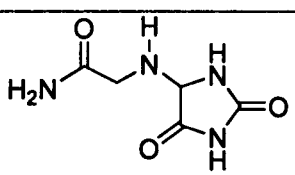
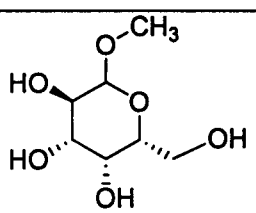
 methyl pheophorbide-A	Leaves and Stems	Wu *et al.*, 1995
9. Coumarin		
 (+)-praeruptorin	Roots	Wu *et al.*, 1998b
 umbelliferone	Leaves and Stems	Wu *et al.*, 1995
10. Amide		
 allantoin	Roots	Wu *et al.*, 1998b
11. Carbohydrate		
 methyl-α-D-galactopyranoside	Leaves and Stems	Wu *et al.*, 1995

Chemicals and Structures	Plant part	References
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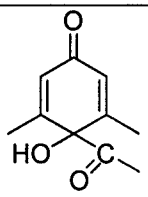
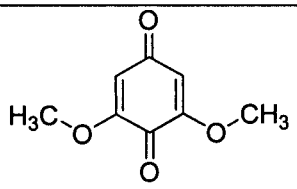
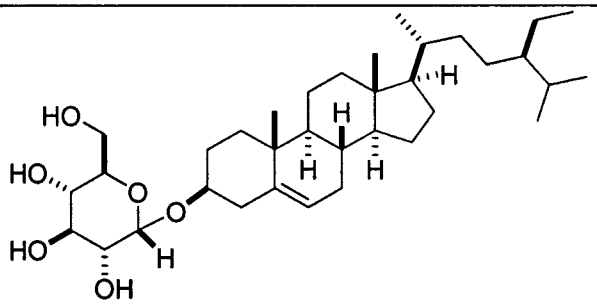
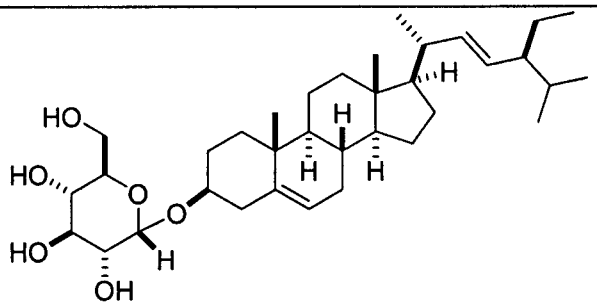
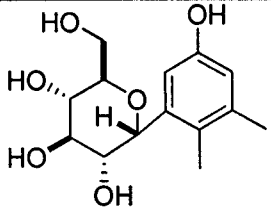
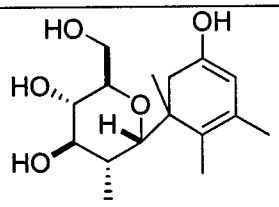
 4-acetonyl-3,5-dimethoxy-*p*-quinol	Leaves and Stems	Wu *et al.*, 1995
13. Benzoquinone		
 2,6-dimethoxy benzoquinone	Leaves and Stems	Wu *et al.*, 1995
14. Glycosides		
 sitosterol-β-D-glucopyranoside	Leaves and Stems	Wu *et al.*, 1995
 stigmasterol-β-D-glucopyranoside	Leaves and Stems	Wu *et al.*, 1995
 3,4-dimethylphenol-β-D-glucopyranoside	Leaves and Stems	Wu *et al.*, 1995

Chemicals and Structures	Plant part	References
 3,4,5-trimethylphenol-β-D-glucopyranoside	Leaves and Stems	Wu *et al.*, 1995

2.6 Biological activity of *R. nasutus* and rhinacanthins

It has been reported that the *R. nasutus* extract and compounds isolated from this plant exhibited interesting biological activities as follows:

Antifungal activity

Dermatophytes are fungi that infect skin, hair, and nails due to their ability to utilize keratin (Gupta *et al.*, 1998). The organisms colonize the keratin tissues. Inflammation is then caused by host response to metabolic by-products. The dermatophytes consist of three genera:

- *Epidermophyton* spp. The macroconidia are broadly clavate with typically smooth, thin to moderately thick walls and one to nine septa, with 20 - 60 x 4 - 13 μm in size. They are usually abundant and borne singly or in clusters. Microconidia are absent. This genus has only two known species to date, and only *E. floccosum* is pathogenic.
- *Microsporum* spp. Macroconidia is characterized by the presence of rough walls which may be asperulate, echinulate, or verrucose (Weitzman, 1995). There are 19 described species but only 9 are involved in human or animal infections.
- *Trichophyton* spp. Macroconidia, which have smooth, usually thin walls and one to 12 septa, are borne singly or in clusters. They may be elongate and pencil shaped, clavate, fusiform, or cylindrical. Their size is in the range of 8 - 86 x 4 - 14 μm. Microconidia, usually more abundant than macroconidia,

may be globose, pyriform or clavate, or sessile or stalked, and borne singly along the sides of the hyphae or in grape-like clusters (Weitzman, 1995). There are 22 species, most of them causing infections in humans or animals.

Dermatophytes invade the stratum corneum or keratinized structure derived from the epidermis, causing skin lesions, hair and nail infection (Duek *et al.*, 2004). Dermatophyte infections are commonly known as ringworm because the appearance of the lesions led to the erroneous belief that the infected skin harbored worms beneath its surface. Ringworm infections are named "tinea" followed by a second word that designates the infected site. For example, tinea capatis is ringworm of scalp; tinea corporis is ringworm of the body; tinea pedis is the disease popularly known as athlete's foot; tinea cruris is jock itch; and tinea unguium is ringworm of the nails (Mckanne *et al.*, 1996). Tinea pedis is the most common type of dermatophyte infection in the US and the rest of the world. Tinea capatis is one of the most common infections in children (Weinstein, 2002). *T. rubrum* is the most common cause of tinea corporis, tinea cruis, tinea pedis, and nail infection worldwide (Wilson, 2001; Weinstein, 2002).

R. nasutus extract possessed an antifungal activity against *Microsporum gypseum, Trichophyton rubrum, Tricophyton mentagrophytes, Epidermophyton floccosum, Candida albicans, Cryptococcus neoformans* and *Saccharomyces* spp. (Farnsworth and Bunyapraphatsara, 1992; Kodama *et al.*, 1993; Akatsuka *et al.*, 1994; Darah and Jain, 2001; Sattar *et al.*, 2004). It has been demonstrated that the water extract of *R. nasutus* leaves and stems exhibited the lowest antifungal activity, while the chloroform extract and 95% ethanol extract showed similar inhibitory activity against filamentous fungi (Farnsworth and Bunyapraphatsara, 1992).

R. nasutus extract showed moderate to potent (partial to complete inhibition of fungal growth) anti-fungal activity in relation to the dosages (0.0005, 0.005, 0.05, 0.5, and 5 mg/mL) applied by conventional agar plate-diffusion method. A spectrum of potent anti-fungal activity was observed in case of the highest dose (5 mg/mL) as it

caused a complete inhibition of growth of all the fungal strains tested (Sattar *et al.*, 2004).

The minimum inhibitory concentration (MIC) value of the leaf extract against *T. mentagrophytes* var. *mentagrophytes*, *T. mentagrophytes* var. *interdigitate*, *T. rubrum*, *M. gypseum* was reported at 13.6 mg/mL. *R. nasutus* leaf extract exhibited fungi static activity at lower concentration (<13.6 mg/mL) and fungicidal activity at higher concentration (>13.6 mg/mL). Moreover, it has been suggested that the *R. nasutus* leaf extract acted on the cell wall of dermatophytes which subsequently leading to the degeneration of cytoplasm and membrane structure and finally leading to cell lysis and death (Darah and Jain, 2001).

Rhinacanthone, has been also demonstrated as an antifungal active compound, which exhibited the inhibition on the spore germination of *Pyricularia oryzae* in *Oryza sativa* (Kuwahara *et al.*, 1995).

There is a report that show antifungal activity of rhinacanthins-C, -D and -N in *R. nasutus* leaf extract against *M. gypseum, T. rubrum, and T. mentagrophytes* causing Tinea in human. The MIC values were showed in the Table 2.2 (Kongchai and Panichayupakaranant, 2002).

In addition, there is a report that demonstrate the antifungal activity of rhinacanthin-C, -D and -N in *R. nasutus* leaf extract against *C. albicans* the fungi causing leucorrhea. All three naphthoquinones exhibited antifungal activity against *C. albicans* with minimal inhibitory concentration (MIC) of 512, 64 and 64 μg/mL, respectively (Panichayupakaranant *et al.*, 2003).

Table 2.2 Antifungal activity of naphthoquinones isolated from the leaves of *R. nasutus* (Kongchai and Panichayupakaranant, 2002)

Compounds	MIC (μg/mL)		
	T. rubrum	*T. mentagrophytes*	*M. gypseum*
rhinacanthin-C	31.2	31.2	125
rhinacanthin-D	62.5	62.5	250
rhinacanthin-N	125	125	250

Antibacterial activity

The leaf and stem extract of *R. nasutus* exhibited inhibitory activity against oral *Streptococcus* spp. (22 isolates strains), which were isolated from dental plaque of 25 patient cases. It was found that the MIC of the extract was 3.8 ng/mL (Apisariyakul *et al.*, 1991).

In addition, there are several studies in antibacterial activity of 75 % ethanolic *R. nasutus* leaves extract against gram positive bacteria such as *Bacillus cereus, B. globigii, B. subtilis*, and *Staphylococcus aureus* and gram negative bacteria such as *Proteus morgani, P. mirabilis, Salmonella typhi, Pseudomonas aeruginosa,* and *Escherichia coli* by conventional agar plate-diffusion method and the resulted showed that four different dosages (2.5, 5, 10, and 50 mg/mL) of the extract can inhibit the growth of *B. cereus, B. globigii, B. subtilis*c as clear zones. However, the highest dosages of the extract, 10 and 50 mg/mL could produce inhibitory effect on the growth of *S. aureus*. In case of gram - negative bacteria, none of the dosages of the extract could produce any inhibitory effect on their growth (Sattar *et al.*, 2004).

Antiviral activity

Sendl and his group had studied antiviral activity of the two naphthoquinone, rhinacanthin-C and rhinacanthin-D, against murine cytomegalovirus (mCMV) in mice and human cytomegalovirus (hCMV), influenza virus type A (FluA), herpes simplex virus type-2 (HSV-2) and respiratory syncytial virus (RSV) compared with gancyclovir, amantadine, acyclovir and, ribavirin. The result showed a good activity of rhinacanthin-C and rhinacanthin-D against hCMV with the EC_{50} values of 0.02 and 0.22 μg/mL, respectively and IC_{50} values of 0.56 and 0.75 μg/mL, respectively. For more data see Table 2.3. (Sendl *et al.*, 1996)

Table 2.3 *In vitro* antiviral activity of rhinacanthin-C and rhinacanthin-D (Sendl *et al.*, 1996)

Compounds	Virus	EC_{50} (μg/mL)	IC_{50} (μg/mL)
rhinacanthin-C	mCMV	0.57	2.6
	hCMV	0.02	0.56
	FluA	none	0.2 ± 0.2
	HSV-2	none	0.03
	RSV	none	0.3
rhinacanthin-D	mCMV	9.5	35
	hCMV	0.22	0.75
	FluA	none	0.78
	HSV-2	none	<0.8
Gancyclovir	mCMV	13.8 ± 5.2	>100
	hCMV	3.4 ± 1.1	>1000
Amantadine	FluA	0.054 ± 0.0004	12 ± 1.0
Acyclovir	HSV-2	2.3 ± 0.3	>10
Ribavirin	RSV	1.8 ± 0.2	35 ± 4.6

In addition, rhinacanthin-E and rhinacanthin-F, lignan compound, which were also isolated from the aerial parts of *R. nasutus*, showed significant antiviral activity

against influenza virus type A, with EC_{50} valued of 7.4 and 3.1 μg/mL, respectively in an anti influenza virus type A cytopathic effect (CPE) assay (Kernan *et al.*, 1997).

Cytotoxic activity

The methanolic extract and rhinacanthin-B isolated from the root of *R. nasutus* showed significant cytotoxicity against human KB tumor cell (human epidermoid carcinoma). For rhinacanthin-B, the ED_{50} was 3.0 μg/mL (Wu *et al.*, 1988).

The naphthoquinones and flavonoid including rhinacanthin-A, -B, -C, -G, -H, -I, -K, -M, -N, -Q, and wogonin, isolated from the root of *R. nasutus* showed significant cytotoxic activity against murine leukemia (P-388), human lung carcinoma (A-549), human colon adenocarcinoma (HT-29), and leukemia (HL-60) cells with the ED_{50} values as shown in Table 2.4 (Wu *et al.*, 1998b).

Kongkathip *et al.*, (2003) have been reported that rhinacanthone, isolated from *R. nasutus* and two 1, 2-pyranonaphthoquinones, rhinacanthone derivatives, were synthesized to show very potent cytotoxicity against three cancer cell lines such as human epidermoid carcinoma (KB), human cervical carcinoma (HeLa) and human hepatocellular carcinoma (HepG2) with IC_{50} values of 0.92 - 9.63 mM (Kongkathip *et al.*, 2003).

Rhinacanthus nasutus, neither aqueous nor ethanolic extracts from leaf and stem had an effect on nitric oxide (NO). Moreover, the ethanolic of *R. nasutus* with lipopolysaccharide (LPS) exhibited an induction of NO and tumor necrosis factor-α production (TNF-α). These may augment macrophage function and thus contribute to cytotoxicity towards viruses, other pathogens and tumor cells (Punturee *et al.*, 2004). Moreover, studied on the influence of *R. nasutus* extracts on cell mediated and humoral immune response in human peripheral blood mononuclear cells (PBMC). The result showed that *R. nasutus* (water and ethanol extract) significantly increased proliferation

and the production of interleukin-2 (IL-2) and TNF-α production (Punturee *et al.*, 2005).

Ethanolic leaves and stem extracts of *R. nasutus* were partitioned with hexane and purified by gel filtration chromatography then used to investigate for cytotoxicity by MTT assay and inhibition of hepatitis B surface antigen secretion from human hepatocellular carcinoma cell line, PLC/PRF/5 cells, by ELISA. Result of ethanol extract showed CC_{50} (50 % cytotoxic concentration) was 541 μg/mL, whereas IC_{50} (50% inhibition concentration) was 140.48 μg/mL and SI (selectivity index) was 3.85 (Vachirayonstien *et al.*, 2006).

Table 2.4 Cytotoxic activity of naphthoquinones and flavonoid isolated from the roots of *R. nasutus* (Wu *et al.*, 1998b)

Compounds	Cell lines ED_{50} (μg/mL)				
	KB	**P-388**	**A-549**	**HT-29**	**HL-60**
rhinacanthin-A	6.75	0.72	3.06	2.17	1.16
rhinacanthin-B	8.01	0.35	6.50	3.01	2.57
rhinacanthin-C	6.26	0.26	0.35	0.68	0.68
rhinacanthin-D	25.0	3.79	8.26	8.89	11.8
rhinacanthin-G	4.45	0.14	0.75	0.57	1.14
rhinacanthin-H	23.8	6.43	9.97	11.5	8.87
rhinacanthin-I	13.2	4.88	7.18	6.30	5.12
rhinacanthin-K	17.3	3.17	16.4	7.75	6.81
rhinacanthin-N	4.80	0.71	1.97	2.67	1.38
rhinacanthin-Q	>50	0.61	3.61	7.60	8.90
Wogonin	4.46	1.70	4.14	3.35	4.66

The rhinacanthin-M, -N, and -Q isolated from *R. nasutus* root showed significant cytotoxic activity against oral human epidermoid carcinoma (KB), human cervical carcinoma (HeLa) and human hepatocellular carcinoma ($HepG_2$) cell lines with the ED_{50} values as shown in Table 2.5 (Kongkathip *et al.*, 2004).

Table 2.5 Cytotoxic activity of naphthoquinones isolated from the roots of *R. nasutus* (Kongkathip *et al.*, 2004)

Compounds	Cell lines ED_{50} (μg/mL)		
	KB	HeLa	$HepG_2$
rhinacanthin-M	1.53 ± 0.34	3.02 ± 0.58	4.85 ± 1.14
rhinacanthin-N	<0.22	0.30 ± 0.05	0.38 ± 0.06
rhinacanthin-Q	0.35 ± 0.16	1.09 ± 0.11	0.97 ± 0.10

Antitumour activity

The antitumour activity of rhinacanthone against Dalton's ascetic lymphoma (DAL) in mice has been reported a significant enhancement of mean survival time of tumor bearing mice and peritoneal cell count in normal mice was observed with respect to the control group (Thirumurugan *et al.*, 2000).

Gotoh and his group had studied on the antiproliferative activity of ethanol extract of root, aqueous extract of leaves of *R. nasutus*, and the supposed active moiety rhinacanthin-C against human cervix adenocarcinoma (HeLa), MDR1-overexpressing subline of human cervical carcinoma (Hvr100-6), human prostatic cancer cell (PC-3), and human bladder carcinoma (T24) cell lines. The antiproliferative activity of *R. nasutus* was also assessed *in vivo* using sarcoma 180-bearing mice. The result showed antiproliferative activities with the IC_{50} values as shown in Table 2.6 (Gotoh *et al.*, 2004).

The ethanol extract of root and aqueous extract of leaves of *R. nasutus* showed significant antiproliferative activity with inhibition rate (IR) of 52.5 % and 44.2 %, respectively comparison to mitomycin C activity. The data showed in Table 2.7 (Gotoh *et al.*, 2004).

Table 2.6 *In vitro* antiproliferative activities of the *R. nasutus* extract and rhinacanthin-C in tested cell lines (Gotoh *et al.*, 2004).

Compounds	IC_{50}			
	HeLa	**Hvr100-6**	**PC-3**	**T24**
root extract (μg/mL)	1,239 ± 257	977 ± 152	567 ± 135	373 ± 65.3
leaf extract (μg/mL)	1,499 ± 113	1,582 ± 101	359 ± 50.5	616 ± 17.9
rhinacanthin-C (μM)	26.2 ± 4.08	11.2 ± 4.21	1.92 ± 0.486	0.66 ± 0.188

Table 2.7 *In vivo* antiproliferative activities of the *R. nasutus* extract in sarcoma 180-bearing ICR mice (modified from Gotoh *et al.*, 2004)

Sample	Tumor weight (g)	IR (%)
Roots	1.74 ± 0.20	52.5
Leaves	2.04 ± 0.16	44.2
Mitomycin	1.73 ± 0.10	52.7
Control	3.66 ± 0.21	-

Bioassay-guided chromatographic fractionation of the active chloroform and methanolic extracts from the roots of *R. nasutus* led to the isolation of seven naphthoquinone: rhinacanthin-C, -D, -G, -O, -M, -N, -Q. All naphthoquinones showed apparent antiproliferative activity against cancer cells with the IC_{50} values of 0.29 - 54.4 μM, whereas they showed moderate activity against Vero cells (IC_{50} values of 4.2 - 41.1 μM). They suggested that the substitution of phenolic groups in quinine and quinolate parts of these compounds may be important for increasing their antiproliferative activity (Siripong *et al.*, 2006a).

In the previous study, rhinacanthin-C, -N, and -Q, three main naphthoquinone esters, isolated from the roots of *R. nasutus* are capable to inhibit proliferation and induce apoptosis in human cervical carcinoma cells (HeLaS3) in a dose- and time-dependent manners. However, these rhinacanthins showed limit solubility in aqueous medium. Therefore, they entrapped them into liposomal membrane that showed strong antiproliferative activity against HeLaS3 cells with the IC_{50} values of 32, 17, 70 μM; 19, 17, 52 μM and 2.7, 2.0, 5.0 μM for the exposure time of 24, 48, and 72 hrs, respectively (Siripong *et al.*, 2006b).

Hypotensive activity

The extract obtained from hot water maceration (decoction) was studied in anesthetized rats for their pharmacological action. The hypotensive activity of *R. nasutus* extract was found to increase with correlation to the amount of the extract (วรรณดี, 2528).

Antioxidant activity

Cosmetics, food, and pharmaceuticals containing *R. nasutus* extract have been reported for their antioxidant activity. The mechanism is to remove superoxide from the human body. Cosmetic containing the extract may be useful to reduce aging and hair loss (Wiart *et al.*, 2000).

Antiplatelet activity

The antiplatelet aggregation of naphthoquinones and one flavonoid, isolated from the roots of *R. nasutus* including rhinacanthin-A, -B, -C, -D, -G, -H, -I, -K, -M, -N, -Q, and wogonin has been reported. These compounds demonstrated 36 - 100 % inhibition of rabbit platelet aggregation induced by arachidonic acid (100 mM). Rhinacanthin-A, -B, -C and wogonin (10 μg/mL) showed 72 - 100 % inhibition of the

rabbit platelet aggregation induced by collagen, while rhinacanthin-B (2 ng/mL) inhibited platelet aggregation induced by platelet activation factor. The data showed in Table 2.8 (Wu *et al.*, 1998 b)

Table 2.8 The effects of *R. nasutus* chemical constituents on the aggregation of washed rabbit platelets induced by thrombin (Thr), arachidonic acid (AA), collagen (Col), and platelet activation factor (PAF) (Wu *et al.*, 1998 b)

	Induced inhibition (%)			
Compounds (mg/mL)	**Thr (0.1 U/mL)**	**AA (100 mM)**	**Col (10 mg/mL)**	**PAF (2 g/mL)**
rhinacanthin-A	2.30 ± 2.2	100 ± 1.1	100 ± 0.5	13.1 ± 3.3
rhinacanthin-B	0.88 ± 1.6	7.45 ± 6.7	100 ± 0.5	63.1 ± 8.5
rhinacanthin-C	1.75 ± 1.2	100 ± 1.1	75.2 ± 73	8.50 ± 2.2
rhinacanthin-G	0.22 ± 1.4	42.6 ± 8.9	13.8 ± 2.6	10.7 ± 2.1
rhinacanthin-H	0.11 ± 1.3	54.8 ± 4.4	31.0 ± 3.9	11.4 ± 2.1
rhinacanthin-I	-0.66 ± 1.5	54.9 ± 8.2	10.8 ± 1.8	22.2 ± 3.9
rhinacanthin-K	0.44 ± 1.7	36.8 ± 8.9	17.0 ± 1.6	12.0 ± 2.2
rhinacanthin-M	-0.55 ± 2.4	100 ±1.1	5.4 ± 1.3	9.4 ± 2.7
rhinacanthin-Q	0.22 ± 2.3	54.6 ± 1.1	20.4 ± 3.7	6.88 ± 2.3
wogonin	0.66 ± 2.3	100 ± 1.1	72.5 ± 3.9	8.60 ± 4.0

Insect attraction and signaling properties

They have been reported that the root extracts of *R. nasutus* exhibited the properties of an insect attractant and signaling to male Mediterranean fruit flies but showed equivocal results on *Aspiculurus tetraptera*, both male and female melon flies and oriental fruit flies (*Dacus dorsalis*) (Farnsworth and Bunyapraphatsara, 1992).

Juvenile hormone activity

An ether extract of *R. nasutus* roots, at a dose of 500 μg/animal exhibited juvenile hormone activity on *Oncopeltus fasciatus*, but no activity was observed at a dose of 250 μg/animal (Farnsworth and Bunyapraphatsara, 1992).

Larvicidal activity

Petroleum ether (PE) and methanol (MeOH) extracts of *R. nasutus* were tested for their larvicidal activity against 4 mosquito vector species. The PE extract of *R. nasutus* exhibited larvicidal effects against *Aedes aegypti, Culex quinquefasciatus, Anopheles dirus* and *Mansonia uniformis* with LC_{50} values between 3.9 and 11.5 mg/L, while the MeOH extract gave LC_{50} values of between 8.1 and 14.7 mg/L (Komalamisra *et al.*, 2005).

2.7 Toxicity test

There was a study on acute toxicity of *R. nasutus* by feeding the mice with 50 % ethanolic extract of *R. nasutus* and by injecting the mice with 10 g/kg (animal) *R. nasutus* extract but there was no toxicity (นันทวัน, 2530; นันทวัน, 2541).

2.8 Biosynthetic pathways of plant naphthoquinones

In vivo synthesizing of the naphthoquinones in higher plants, there are at least two different ways. Two routes involve direct incorporation of shikimic acid into the naphthoquinone nucleus, while the other route utilizes acetic acid, as in polyketide pathway (Figure 2.3).

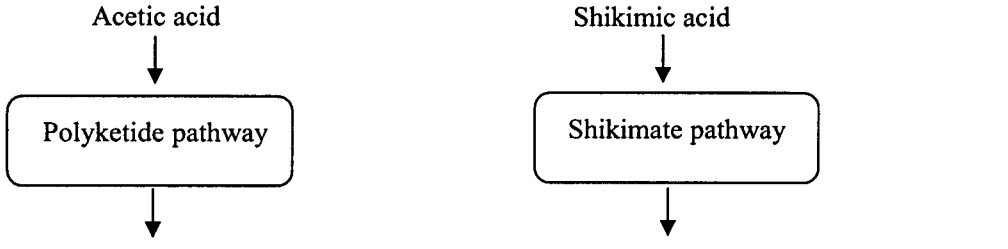
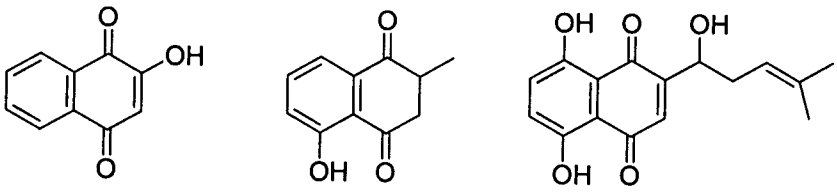


Figure 2.3 Biosynthetic routes of plant naphthoquinones

The wildly distributed natural quinines are formed by a diversity of routes: several are known for each of the benzoquinone, naphthoquinones, and anthraquinones. This can be attributed to the importance which the quinone moiety of these compounds has in the economy of living systems. Naphthoquinones are colored substances derived from phenylpropanoid and isoprenoid precursors (Gaisser and Heide, 1996) Natural naphthoquinone derivatives such as shikonin found in species of *Lithospermum erythrorhizon and Onosma paniculatum*, Plumbagin found in species *Plumbago rosea, Dionaea muscipula* and lawsone found in species *Lawsonia inermis* and *Impatiens balsamina* (Figure 2.4). The structure of naphthoquinone consists of two part of skeleton which are quinone (main part) and side-chain. In quinones can be formed phenolic systems generated by either the acetate or shikimate pathways. Recently, study on biosynthesis of naphthoquinone in *Rhinacanthus nasutus* has not been detected. There is some evidence to support biosynthesis of quinone structure in rhinacanthin, naphthoquinone derivatives. Dewick, (2001) suggested that naphthoquinones are biosynthesized through phylloquinones (vitmin K_1) and menaquinones (vitamin K_2). The phylloquinone structure (Figure 2.5) has a diterpenoid side-chain, whereas the range of menaquinone structures tends to be rather

wider 1-13 isoprene units (Figure 2.6). They are shikimate-derived naphthoquinone derivatives found in plants and algae (vitamin k_1) or bacteria and fungi (vitamin K_2). These quinones are derived from chorismic acid via its isomer isochorismic acid. Phylloquinones and menaquinones are formed by addition carbons for the naphthoquinone skeleton which provided by 2-oxoglutaric acid, incorporated by mechanism involving the coenzyme thiamine diphosphaste (TPP). 2-oxoglutaric acid is decarboxylated in the presence of TPP to give the TPP anion of succinic semialdehyde, which attacks isochorismic acid in a Michael-type reaction. Loss of the thiamine cofactor, elimination of pyruvic acid, and then dehydration yield the intermediate *o*-succinylbenzoic acid (OSB). This is activated by formation of a coenzyme A ester, and a Dieckmann-like condensation allows ring formation. The dihydroxynaphthoic acid is the more favored aromatic tautomer from the hydrolysis of the coenzyme A ester. This compound is now the substrate for alkylation and methylation as seen with ubiquinones and plastoquinones. However, the terpenoid fragment is found to replace the carboxyl group, and the decarboxylated analogue is not involved. The transformation of 1, 4-dihydroxynaphthoic acid to the isoprenylated naphthoquinone appears to be catalysed by a single enzyme, and can be rationalized by the mechanism. This involves alkylation using the diketo tautomer, decarboxylation of the resultant *β*-keto acid, and finally an oxidation to the *p*-quinone (Figure 2.7). OSB, and 1,4-dihydroxynaphthoic acid, or its diketo tautomer, have been implicated in the biosynthesis of a wide range of plant naphthoquinones and anthraquinones. There are parallels with the later stages of the menaquinone sequence shown in Figure 2.8.



Lawsone Plumbagin Shikonin

Figure 2.4 Several naphthoquinones found in higher plants

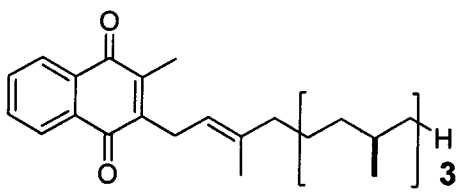


Figure 2.5 Phylloquinones (vitmin K_1) structure

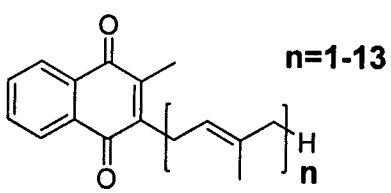


Figure 2.6 Menaquinones (vitamin K_2) structure

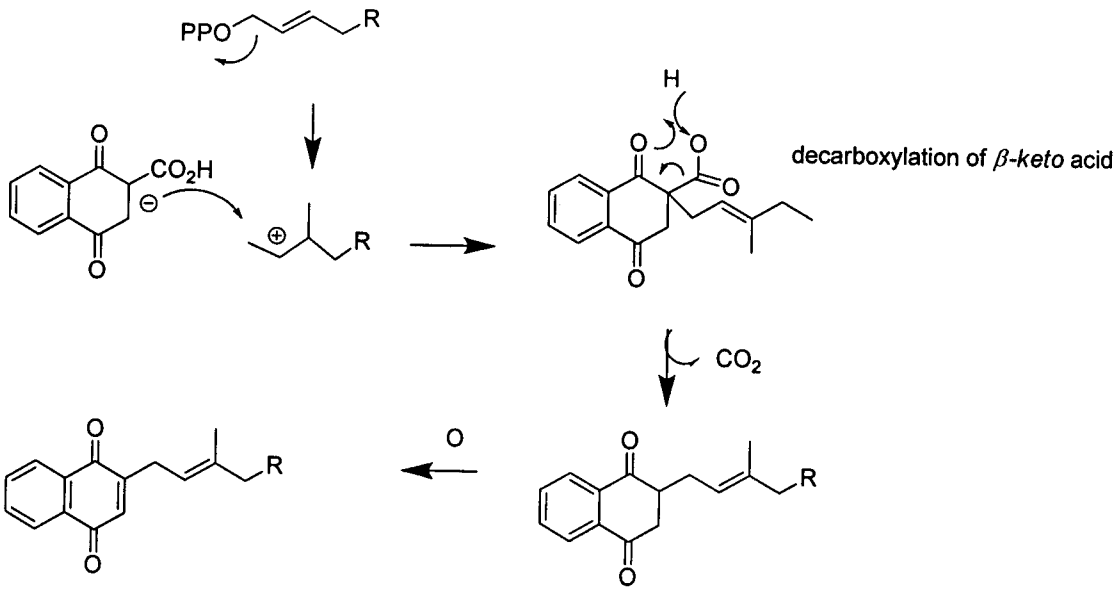


Figure 2.7 Transformation of 1,4-dihydroxynaphthoic acid to the isoprenylated naphthoquinone (Dewick, 2001)

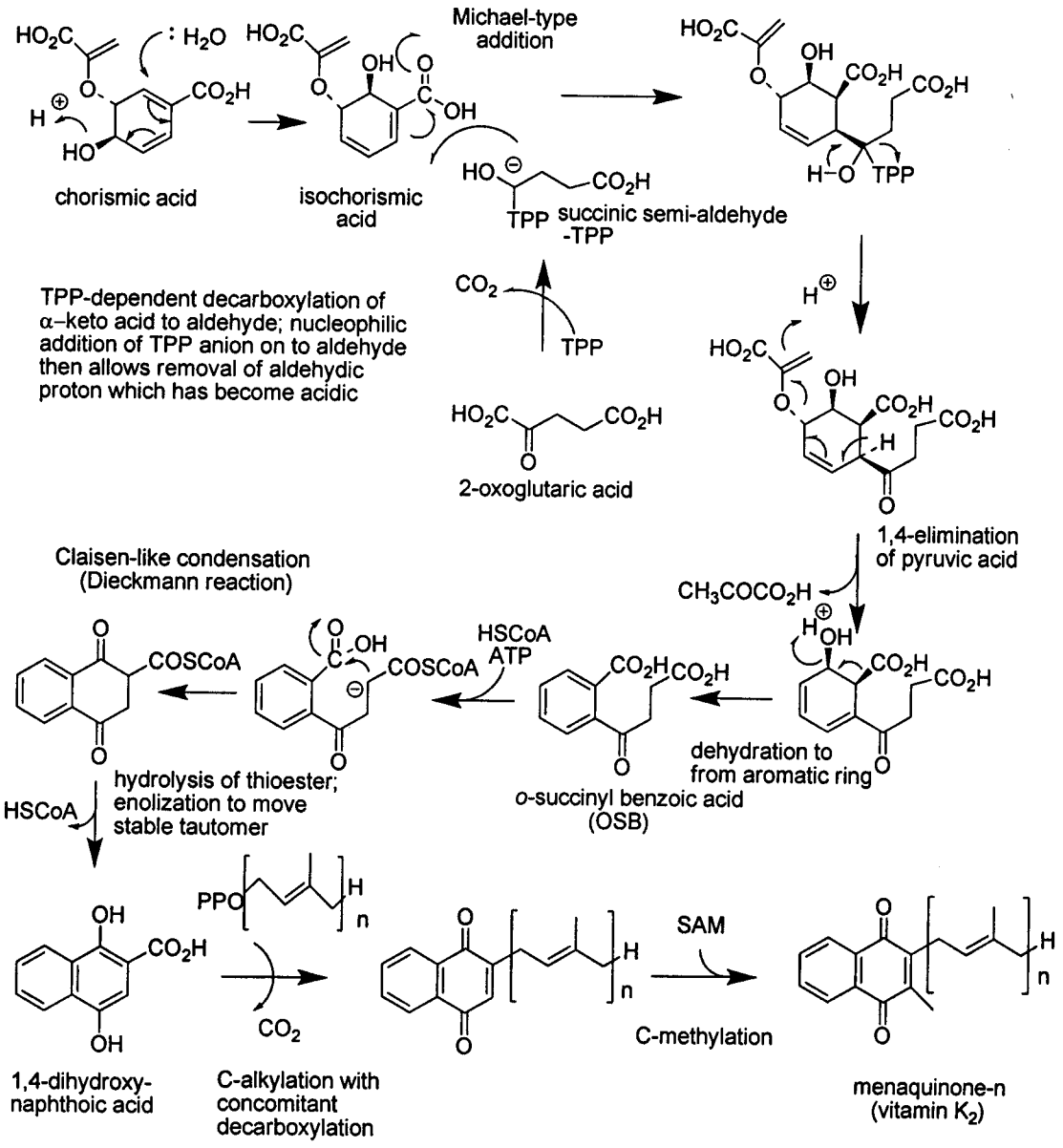


Figure 2.8 Biosynthesis of menaquinones (vitamin K_2) (Dewick, 2001)

Okamoto and his groups (1994) have been studied on biosynthesis of shikonin derivatives from L-phenylalanine via deoxyshikonin in *Lithospermum* cell culture using ^{14}C-labelled L-phenylalanine administered to shikonin-producing cell cultures of *Lithospermum erythrorhizon.* The present study has demonstrated that deoxyshikonin (Figure 2.9) is an intermediate in the biosynthesis of shikonin and its esters, suggesting that both hydroxylation of deoxyshikonin and esterification of shikonin are catalysed by membrane bound enzymes localized probably in vesicles. These data strongly suggested that shikonin derivatives are

biosynthesized from deoxyshikonin by hydroxylation and esterification at the C-1 position of its side chain both in cultured cells and in cell free extracts of *L. erythrorhizon* (Okamoto *et al.*, 1994).

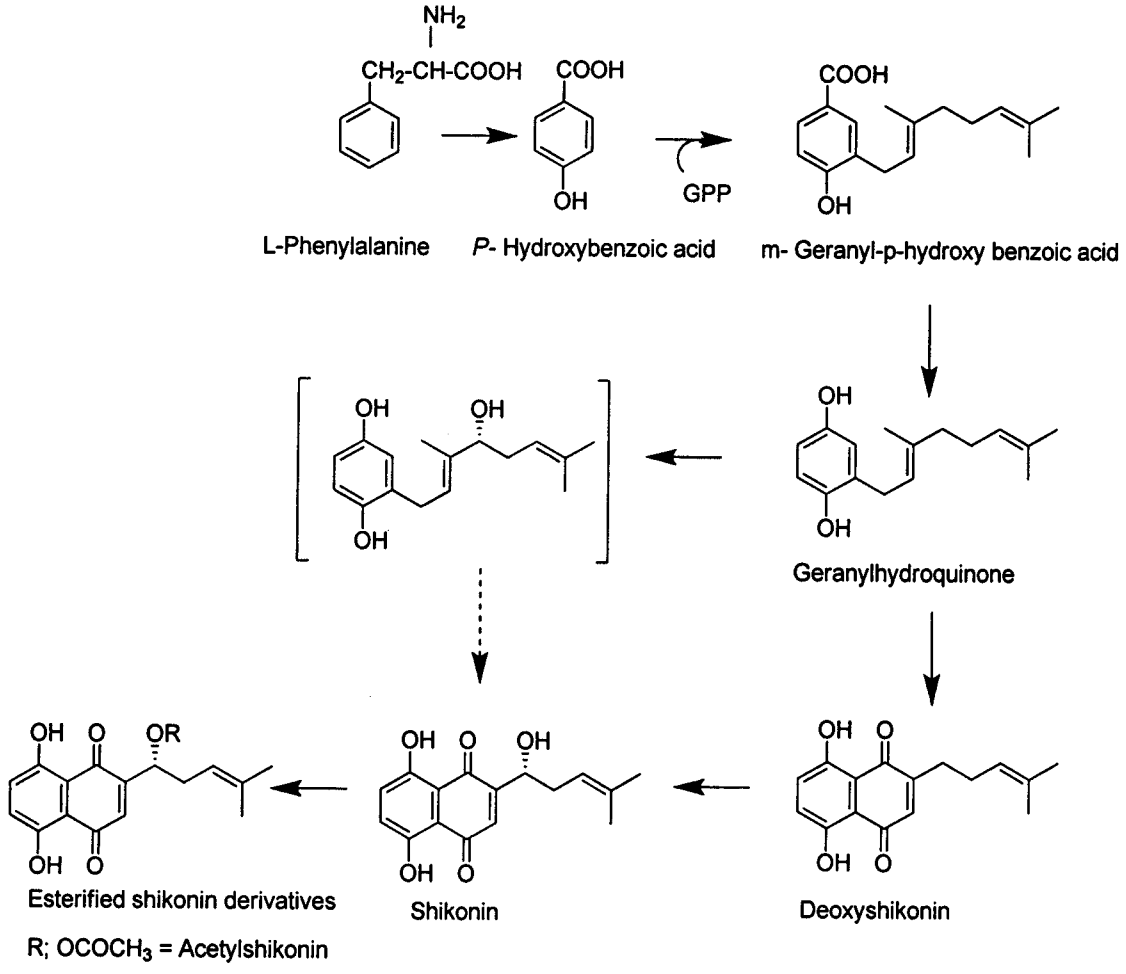


Figure 2.9 Proposed biosynthetic pathway leading to esterified shikonin derivatives via deoxyshikonin in cultured cells of *L. erythrorhizon* (modified from Okamoto *et al.*, 1994)

Plumbago indica is a well-studied example because it is used as medicinal plant in Southeast Asia. Plumbagin originates from acetate units, as shown already a long time ago (Durand and Zenk, 1974). This suggested that the biosynthesis is carried out by a polyketide synthase using acetyl-CoA as starter and performing five condensation reactions with malonyl-CoA. The biosynthesis of plumbagin includes loss of one carbon by decarboxylation and

presumably involves a reduction step (Figure 2.10). The details were not known, but, after the discovery that they can carry out more than three condensations, plant type III PKS type enzymes were certainly good candidates for such reaction. (Springob *et al.*, 2007).

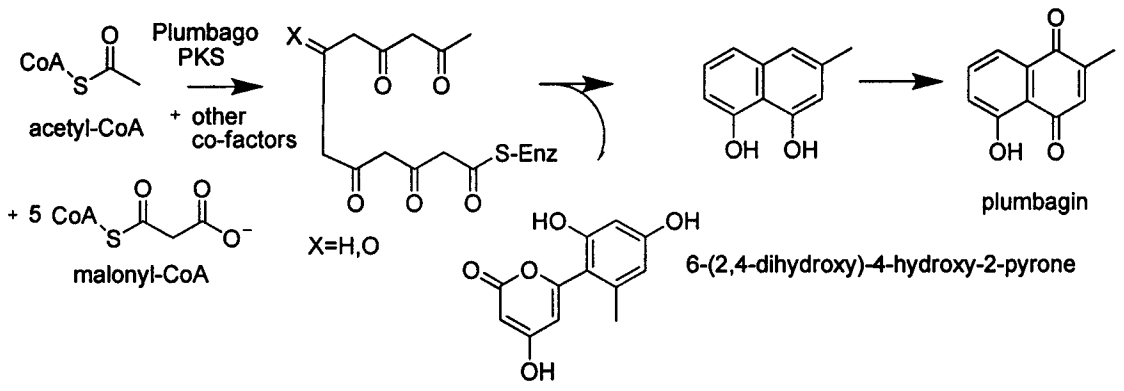


Figure 2.10 Putative reaction of naphthoquinone-forming PKS in *P. indica* (modified from Springob *et al.*, 2007)

Lawsone a naphthoquinone, presented in *Impatiens balsamina* has been demonstrated as same biosynthesis as of bacterial menaquinones. It involves incorporation of shikimic acid and the intermediated, *o*-succinylbenzoic acid, into the naphthoquinone (Figure 2.11). Experiments with [1,6-$^{14}C_2$] and [3-^{3}H]-shikimic acid establish that lawsone is derived from shikimate with C-1 and C-2 appearing at the naphthoquinone ring junction. The carboxy group of shikimic acid is retained on naphthoquinone formation, and thus accounts for seven of the ten nuclear carbon atoms in the molecule. The remaining C-3 unit has been identified as having its origin from glutamic acid or its transamination product, α-ketoglutaric acid (Champbell *et al.*, 1971; Grotzinger and Champbell, 1974).

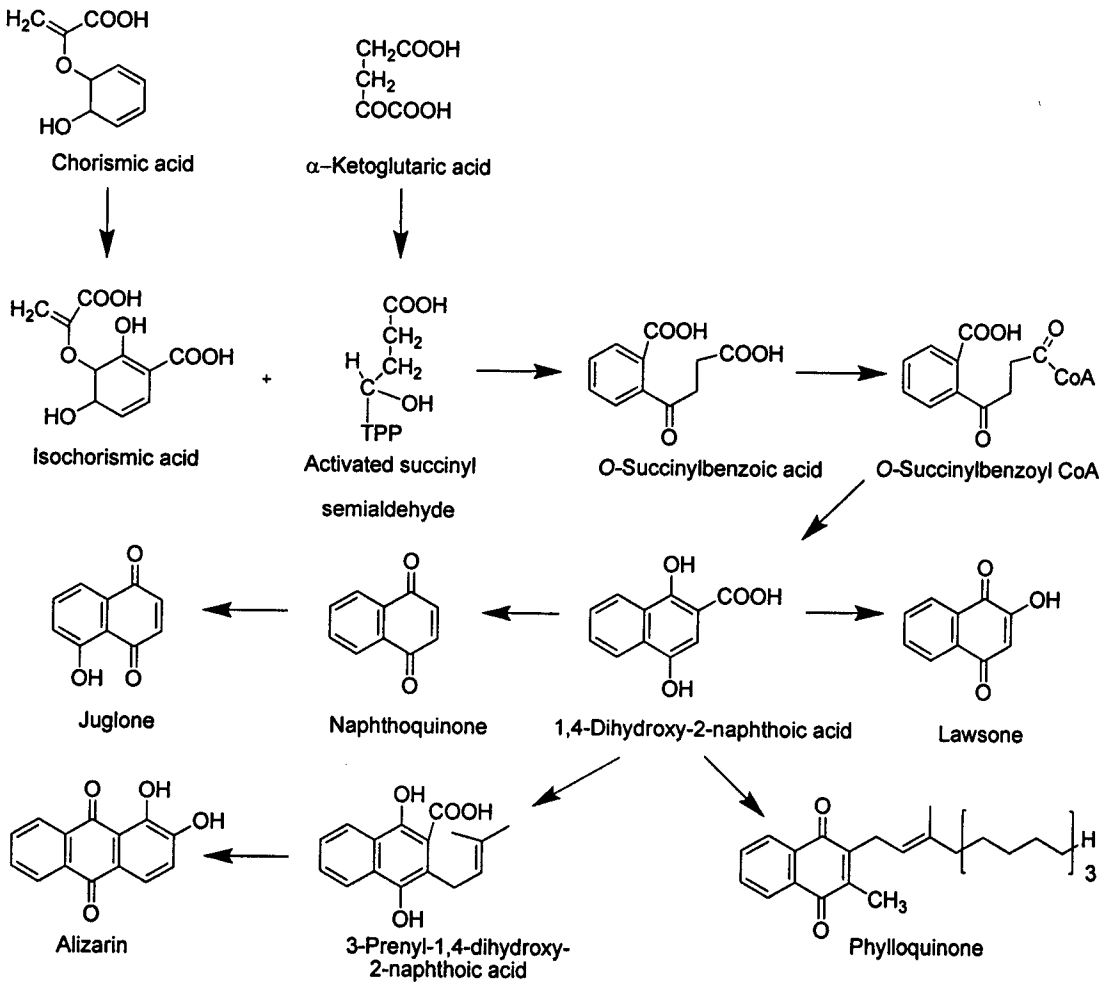


Figure 2.11 Biosynthetic pathway of lawsone and phylloquinone (modified from Panichayupakaranant, 1996)

2.9 Naphthoquinone production by tissue culture

Studies were undertaken for induction of resistance against acute ozone exposure in *R. nasutus*. Callus induced from leaf explants on Murashige and Skoog's (MS) medium supplemented with 3.4 μM of 2, 4-dichlorophenoxy acetic acid (2, 4-D) were treated with different concentrations of ozone. It was found that the concentration of ozone (1.0 μmol/mol) made the seedling developed more resistance to withstand acute ozone exposure by increased phenylalanine ammonia-lyase activity and possessed more chlorophyll pigments (Sudhakar *et al.*, 2007). The improvement of axillary shoot formation of *Lawsonia inermis* L. cultured *in vitro* was

depended on the iron concentration in the culture medium. Up to 4 new shoots were regenerated per explants in medium containing 0.25 μM of $FeSO_4$ which corresponding to one fourth of the required iron concentration in basal MS medium. The hairy root cultures were established by a co-culture method using leaf segments of *L. inermis* and *Agrobacterium rhizogenes* NCIB 8196 of several basal media tested (half strength MS, MS, B5, and half-strength LS). The production of lawsone was only observed in hairy roots tissues incubated in the dark and cultured in 1/2 MS or MS media with 0.13 % and 0.10 % dry weight, respectively (Bakkali *et al.,* 1997). Root cultures of *Plumbago rosea* L. were established from young leaf explants on solid Gamborg's B5 (B5) medium supplemented with 1.0 mg/L NAA and 0.1 mg/L kinetin. It was found to be best suited for growth promotion. Thus, the root cultures were maintained in liquid B5 medium supplemented with 1.0 mg/L NAA and 0.1 mg/L kinetin. The culture roots in liquid medium appeared as a small rosette aggregation form. The root biomass increased about 18 times of the inoculated root within a month. The production of plumbagin, determined by TLC-densitometry was 0.016 ± 0.0030 % dry weight in cultured roots obtained from B5 medium supplemented with 1.0 mg/L NAA and 0.1 mg/L kinetin (Panichayupakaranant and Tewtrakul 2002). Liu and his groups (2006) have studied on effect of light on shikonin formation in cultured of *Onosma paniculatum* cells. The cell culture in a production medium in the dark is capable of producing a large quantity of shikonin and its derivatives, which were completely inhibited when the cell cultures were irradiated with continuous white light and blue light and partially repressed under continuous red light (Liu *et al.,* 2006). Induction of naphthoquinone formation in *Impatiens balsamina* cell cultures was achieved by using parent plants yielding high levels of 2-methoxy-1,4-naphthoquinone as initiated explants. The cell culture was established in B5 medium supplemented with 0.1 mg/L 2, 4-D and 1.0 mg/L 6-benzylaminopurine (BA). The cell cultures were capable of producing lawsone production (Panichayupakaranant, 2001). Increasing of naphthoquinone production by elicitation technique was studied in *Drosera capensis* was investigated. *D. capensis* an important source of pharmacologically active 1, 4-naphthoquinones including 7-methyljuglone which has been shown to have significant antimicrobial and antifungal activity. *In vivo* conditions salicylic acid and jasmonic acid were used as elicitors to improve of 7-methyljuglone production. Elicitation of 7-methyljuglone in greenhouse grown plants using salicylic acid and jasmonic acid showed that the amount of 7-methyljuglone was the highest in

the shoots elicited with 50 μM of salicylic acid and jasmonic acid after 48 h and 3 h respectively. In roots, the highest amount of 7-methyljuglone was found in plants treated with 50 μM of salicylic acid and 100 μM of jasmonic acid after 1.5 hrs (Ziaratnia *et al.*, 2009). Moreover, elicitation technique were used for plumbagin production by cell cultures of *Plumbago rosea* L. (synonymous *P. indica*) by treating with several elicitors such as prepared from the fungi (*Aspergillus niger* and *Rhizopus oryzae*), bacteria (*Bacillus subtilis* and *Pseudomonas aeruginosa*), yeast extract and chitosan were used to for enhancement of the plumbagin production. Elicitation with chitosan to cells resulted in 6.71-fold of plumbagin production higher than that of control cells. The treatment of cells with *A. niger*, *R. oryzae* and yeast elicitors resulted in two- to three-fold more plumbagin over control cells. Bacterial elicitors did not show much (<two-fold) influence on plumbagin accumulation. Chitosan at 150 mg/L dose level enhanced permeability of plumbagin from the cell to the exterior and also elicited plumbagin synthesis significantly (Komaraiah *et al.*, 2002).

2.10 *Agrobacterium rhizogenes* genetic transformation

The bacteria in genus *Agrobacterium* includes both phytopathogenic (tumour- or hairy-roots inducing) and non-pathogenic soil-borne bacteria. The taxonomy of bacteria belonging to *Agrobacterium* has been unclear for along time. For many years, classification of species within this genus was based mostly on their phytopathogenic properties. Five species were distinguished (Figure 2.12) including *A. radiobacter*, a non-pathogenic bacteria; *A. tumefaciens*, a crown gall inducing bacteria; *A. rubi* which induce crown gall on genus *Rubus* of Rotaceae; *A. vitis*, which produce crown gall on *Vitis vinifera* (Vitaceae); and *A. rhizogenes*, a hairy root inducing bacteria (Young *et al.*, 2001; Pulawska *et al.*, 2006).

A new route for enhancing secondary metabolite production is producing a transgenic plant using the natural vector system of *Agrobacterium rhizogenes*. Genetically transformed hairy roots obtained by infection of plant with *A. rhizogenes*. Hairy root cultures are characterized by a high growth rate and their ability to synthesize root derived secondary metabolites. Moreover, these hairy roots are unique in their genetic and biosynthetic stability.

Hairy roots also offer a valuable source of root derived phytochemicals which are useful as pharmaceuticals, cosmetics, and food additives.

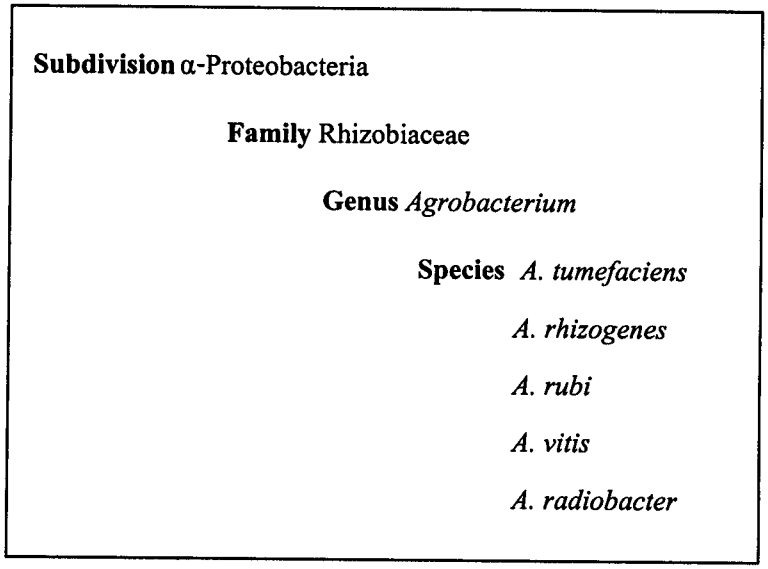
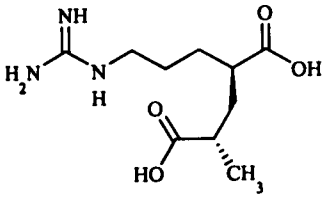


Figure 2.12 Systematic classification of *Agrobacterium* (Fischer, 2006)

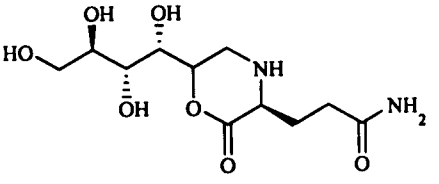
2.10.1 Strains of *Agrobacterium rhizogenes*

The strains of *A. rhizogenes* can be separated into several lines according to opine production such as octopine, agropine, nopaline, mannopine, cucumopine, (Charlwood and Rhodes, 1990; Hu and Du, 2006; Georgiev *et al.*, 2007; Jia *et al.*, 2008) and mikimopine (*Handayani, et al.*, 2005). They are varying in their chemical structures. (Figure 2.13) In agropine strains (such as LBA 9402, ATCC 15834), two sections of DNA are transferred, T_L and T_R. Agropine strains are most often used owing to their strongest induction ability. Mannopine and cucumopine strains transfer only one section of DNA transferred (Charlwood and Rhodes, 1990) (Table. 2.9). The different phenotypes of these several lines have not been clear. Source *A. rhizogenes* strains such as *A. rhizogenes* ATCC 11325, 13332 and 13333 have not been characterized for their constituents or plasmids. However, it has been reported on study of PCR primers for identification of opine types of *A. tumefaciens* (Tan *et al.*, 2003). Tan and his groups to designed two set of primers for identify the nopaline and octopine types of

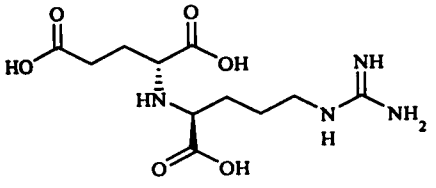
A. tumefaciens. Of 42 isolates determined to be *A. tumefaciens* and 7 were found to be octopine type; all the rest were R 225 type (Tan *et al.*, 2003). Maybe this technique is a model to classify opine type of *Agrobacterium* strain 11325, 13332, and 13333 in the future.



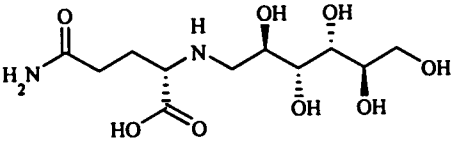
Octopine



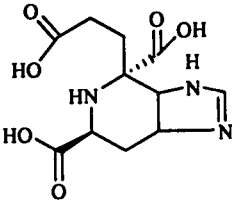
Agropine



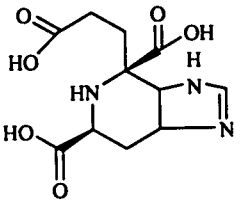
Nopaline



Mannopine



Cucumopine



Mikimopine

Figure 2.13 Chemical structures of opine production

Table 2.9 Strains of *Agrobacterium rhizogenes* (Charlwood and Rhodes, 1990)

Strain type / **Type of opine**	**Examples**	**DNA transferred**	
		T_L	T_R
Agropine	LBA9402	+	+
	A4	+	+
	15834	+	+
	HRI	+	+
Mannopine	8196	+	-
Cucumopine	2659	+	-

2.10.2 Ri plasmid

The induction of rhizogenesis and opine formation after infection is a consequence of the transfer of DNA from Ri a bacterial plasmid of *A. rhizogenes* into the host cell and its integration into the plant genome. The plasmid composes of three regions which are important for gene transfer: 1) The transfer DNA (T-DNA), which is integrated into the genome of the plant cells; 2) The border sequences of the T-DNA, including left border (LB) and right border (RB); 3) The virulence area (*vir*), which does not enter the plant cell but facilitates transferring of the T-DNA into plant genome. Ri plasmid is a large plasmid with the size varies from 200 to greater than 800 kb (Oksman-Caldentey *et al.*, 1996). The complete sequences of pRi 1724, a mikimopine-type Ri plasmid, has been determined and published for the first time in 2001 (Moriguchi *et al.*, 2001). This Ri plasmid is from *A. rhizogenes* MAFF 301724. The pRi 1724 plasmid is one of the most studied Ri plasmids because of the highly capacity of T-DNA transfer and root induction. (Huffman *et al.*, 1984). The pRi 1724 contains 217,594 nucleotides, 173 open reading frames (ORFs) shown in Figure 2.14

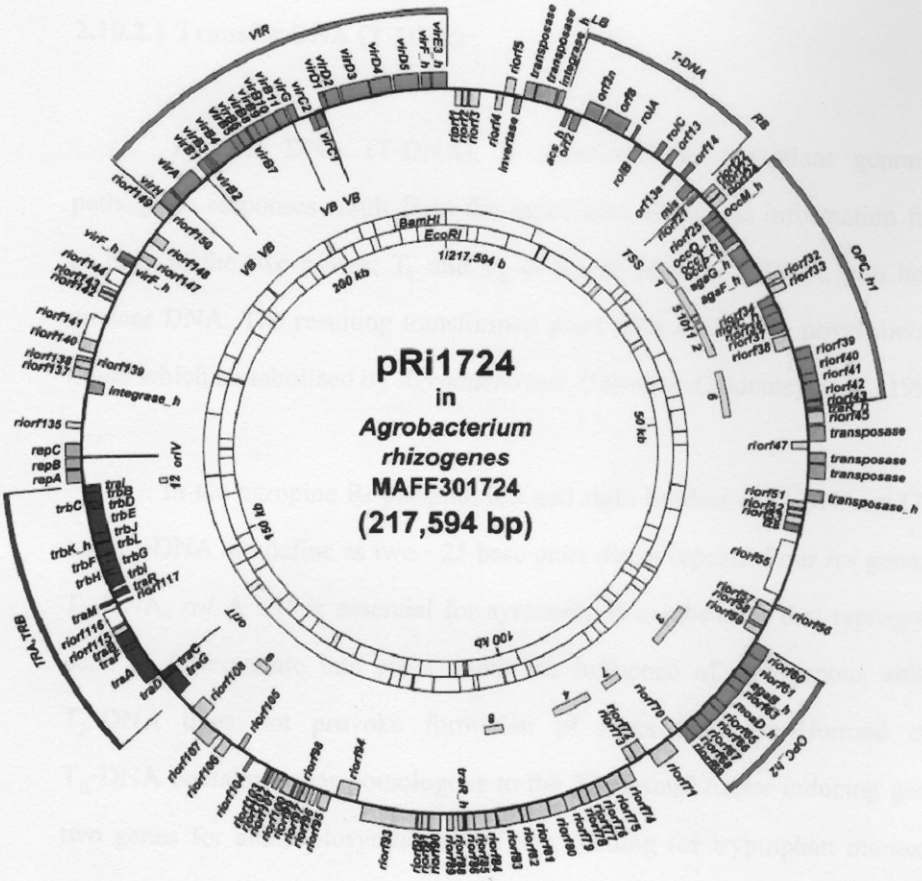


Figure 2.14 Circular gene map of pRi1724 Genes or ORFs are classified by color as follows: pink, virulence genes for plants; bright green, T-DNA-encoded genes including root-inducing genes (Moriguchi *et al.,* 2001).

2.10.2.1 Transfer DNA (T-DNA)

Transfer DNA (T-DNA), is transferred to the plant genome. The pathogenic responses result from the expression of genetic information from one or both of the two pieces; T_L and T_R of the Ri-plasmid T-DNA, into host plant nuclear DNA. The resulting transformed plant cells synthesize novel metabolites, opine which metabolized by *Agrobacterium.* (Oksman-Caldentey *et al.*, 1996).

In the agropine Ri plasmid, left and right borders of T-DNA as (T_L-DNA and T_R-DNA are define as two - 25 base pairs direct repeats. Four *rol* genes are on T_L-DNA, *rol* A - D is essential for synthesis of a substance that reprograms the cells to differentiate into roots under the influence of endogenous auxin, and T_R-DNA does not provoke formation of roots from transformed cultures. T_R-DNA contains genes homologous to the Ti plasmid tumor inducing genes and two genes for auxin biosynthesis: the tms_1, coding for tryptophan monoxygenes and tms_2, coding for indoylacetamide hydrolase. T-DNA is transferred to wounded plant cells and becomes stably integrated into the host genome. Transformants are selected by detecting the genes located in T-DNA such as *rolA, rolB, rolC* and *rolD* (Charlwood, and Rhodes, 1990; Giri, and Narasu, 2000; Li and Tao, 2006; Georgiev *et al.*, 2007).

2.10.2.2 Effect of *rol* genes on plant secondary metabolites

The *rolA*, *rolB*, and *rolC* genes, are plant oncogenes carried by plasmids of the plant pathogen *A. rhizogenes.* They are important for development, maintenance of the root phenotype, hairy root induction, and metabolite production. For instance, the production of secondary metabolites was increased in tobacco hairy root cultures (Palazon *et al.*, 1997). Ginsenoside production in hairy root of ginseng, *Panax ginseng*, transformed with *A. rhizogenes* was two fold higher than in the untransformed culture (Bulgakov *et al.*, 1998). It has been

demonstrated that *rolC*-transgenic roots produced three times more ginsenosides (Bulgakov *et al.*, 1998). Moreover, Bonhomme and his groups (2000) has established and examined the growth rate and alkaloid productivity of hairy roots of *Atropa belladonna.* The *rol* genes were evaluated the possible role in morphological differentiation and in tropane alkaloid formation of hairy root infected by a wild strain *A. rhizogenes* 15834 harboring the Ri-T_L-DNA comparison with hairy root infected by a disarmed *A. tumefaciens* strain harboring only a construction of *rolABC*. The result showed that the total alkaloid contents accumulated between 4 and 27 times more than the alkaloids from intact roots, respectively. The *rolABC* seemed to be sufficient parts to induce the "hairy root" phenotype, including fast growth rate and a high level of secondary metabolite accumulation (Bonhomme *et al.*, 2000).

2.10.2.3 Function of *rol* genes in plant secondary metabolite

A new function of the *rol* genes in plant-*Agrobacterium* interaction became apparent with the discovery that *rol* genes, which are potential activators of secondary metabolites production in transformed plant cells. Several available evidences show that genetic transformation by single *Agrobacterium rol* genes may be used as a powerful tool to manipulate secondary metabolites in cultured plant cell.

The *rolA* gene has been shown to have a stimulatory effect on nicotine production (Palazon *et al.*, 1997). The biochemical function of *rolA* protein is suggested to act as a transcription factor in the metabolism of gibberellins, as a reduction in their content has been found in *rolA*-transgenic tobacco plants (Dehio *et al.*, 1993).

The *rolB* gene is apparently the most powerful inducer of secondary metabolite, is also the most powerful suppressor of cell growth, and is absolutely

essential for the induction of hairy roots. *RolB* protein was suggested to be a β-glucosidase able to increase the levels of free active IAA (the natural endogenous auxin) by releasing it from inactive glucose (Estruch *et al.*, 1991).

The *rolC* gene, has been the most studied gene, provides a signal that activates secondary metabolic processes. It is likely that *rolC* may confer a wider spectrum of defense reactions in addition to secondary metabolite stimulation. The *rolC* are suggestive of cytokinin synthesis. Some of the morphological phenotypes of *rolC*-transformed plants, such as reduced apical dominance and enhanced lateral shoot development (Schmulling *et al.*, 1988; Zuker *et al.*, 2001).

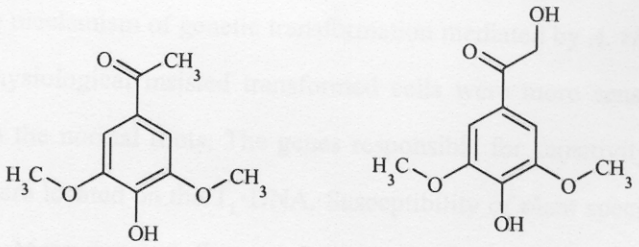
The function of *rolD* on secondary metabolite has never been investigated. The *rolD* was suggested to maintenance of hairy root growth and increase flowering through changes in the concentration of plant hormones in transformed plants (Mauro *et al.*, 1996; Casanova *et al.*, 2005; Bulgakov, 2008).

2.10.2.4 The virulence genes

The *vir* region, located on the *Agrobacterium* Ti or Ri plasmid, encodes most of the bacterial virulence (*Vir*) proteins used by the bacterium to produce its T-DNA and to deliver it into the plant cell. It is about 35 kb and encodes six transcriptional: *vir* A, B, C, D, E and G, which have important functions in gene transfer. The gene products of *virD* are attached to the border sequences and cause the formation of a single strand T-DNA molecule, the so-called T-strand. The T-DNA is linear and is covered with the protein coded by *vir*E. It is transferred into the plant cell as a protein-DNA complex (Oksman-Caldentey *et al.*, 1996; Tzfira and Citovšky, 2006).

2.10.2.5 Mechanism of *Agrobacterium* transformation

Mechanism of *Agrobacterium* plant cell interaction. Interaction between *Agrobacterium* and a plant is the attachment of the bacterium to the surface of the plant. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone and α-hydroxy-acetosyringone (Figure 2.15) which were found to radically affect the relative virulence of bacterial plasmid and reduction the time for induction of hairy roots. The virulence genes do not enter the plant cell, but they must be expressed in the bacterium for T-DNA transfer to the plant genome (Giri and Narasu 2000; Giri *et al.,* 2001; Sovon *et al.*, 2002). Tzfira and Citovsky reported that the model for the *Agrobacterium* mediated genetic transformation process comprises 10 majors steps and begins with recognition and attachment of *Agrobacterium* to the host cell (1) and the sensing of specific plant signals by the *Agrobacterium* *Vir*A/*Vir*G two component signal-transduction system (2). Following activation of the *vir* gene region (3) a mobile copy of the T-DNA is generated by the *Vir*D1/D2 protein complex (4) and delivered as a *Vir*D2-DNA complex (immature T-complex), together with several other *Vir* protein, into the host cell cytoplasm (5). Following the association of *Vir*E2 with the T-strand, the T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting protein (9) and integrated into the host genome (10) (Figure 2.16) (Tzfira and Citovsky, 2006).



(a) **(b)**

Figure 2.15 Chemical structures of acetosyringone (a) and α-hydroxy-acetosyringone (b)

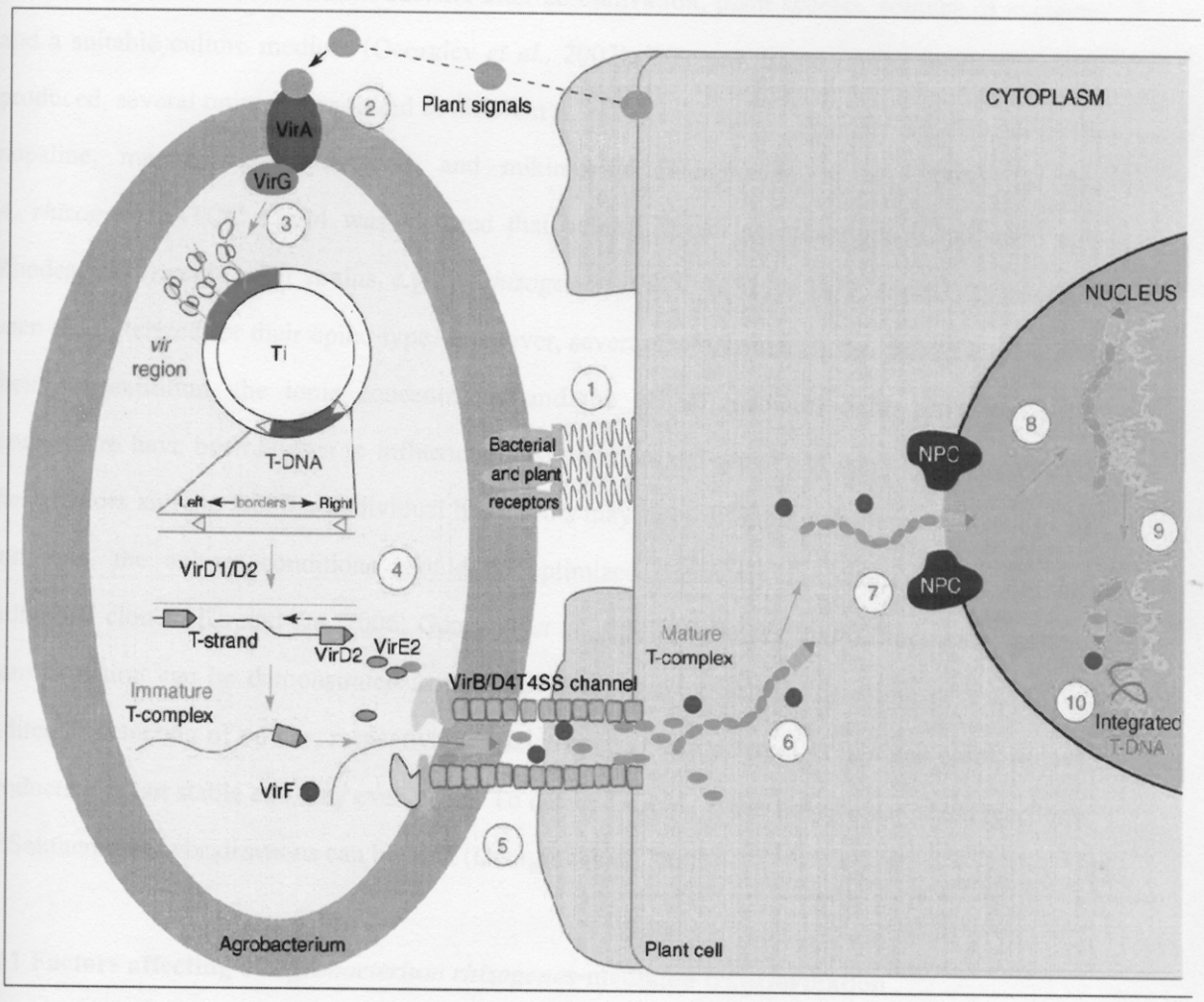


Figure 2.16 A model for the *Agrobacterium*-mediated genetic transformation (Tzfira and Citovsky, 2006)

The mechanism of genetic transformation mediated by *A. rhizogenes* is shown in Figure 2.16. The physiological insisted transformed cells were more sensitive to extracellular supplied auxins than the normal roots. The genes responsible for sensitivity increasing of hairy root cells to auxin were located on the T_L-DNA. Susceptibility of plant species to *Agrobacterium* strains varies greatly. Many factors influence of successful transformation of hairy root induction, are such as transformation ability of different strains of *Agrobacterium*, age and differentiation status of plant tissues, presence of phenolic compounds e.g. addition of acetosyringone, a proper antibiotic to eliminate redundant bacteria after co-cultivation, plant species, sources of explants, and a suitable culture medium (Georgiev *et al.*, 2007). Whereas, based on the types of opines-produced, several opines were found in different *A. rhizogenes* strains such as octopine, agropine, nopaline, mannopine, cucumopine, and mikimopine (*Handayani, et al.*, 2005). So far, *A. rhizogenes* ATCC 15834 was reported that belongs to an agropine-type (Charlwood and Rhodes, 1990) while other strains, e.g. (*A. rhizogenes*) ATCC 11325, 13332, and 13333 have not been characterized for their opine-type. Moreover, several factors such as, the carbon sources and their concentration, the ionic concentration and the pH of medium, light, phytohormones, temperature have been known to influence transformation and growth of hairy root. Because of these factors and the fact that individual hairy roots may have different requirements for nutrient condition, the culture conditions should be optimized separately for each species and for individual clones (Hu and Du, 2006; Georgiev *et al.*, 2007; Jia *et al.*, 2008). Successful genetic transformation can be demonstrated in either of two ways by directly detecting of T-DNA or indirectly detecting of opines, respectively. The direct way is preferred, as in some cases, opines production is not stable and may even cease. To detect T-DNA, either polymerase chain reactions or Southen blot hybridizations can be used (Georgiev *et al.*, 2007).

2.11 Factors affecting of *Agrobacterium rhizogenes*-mediated transformation

Many factors such as plant species, co-cultivation period, time of incubation explants in the bacterial suspension, present of acetosyringone, strains of bacteria, bacterial concentration, types of explants, have an effect on hairy roots induction. *Agrobacterium rhizogenes*-mediated genetic transformation of plant was investigates. Akramian and his groups

(2008) induced hairy roots from four Hyoscyamus species (*H. arachnoideus* Pojark., *H. kurdicus* Bornm., *H. reticulates* L., and *H. squarrosus* Griff.), which are rich sources of tropane alkaloid by *Agrobacrium rhizogenes* strain ATCC 15834. The result showed that the highest hairy roots induction was investigated in *H. arachnoideus* species (Akramian *et al.,* 2008). Kim and his groups (2007) were studied on the factors which effect on *Agrobacterium tumefaciens*-mediated transformation of *Panax ginseng.* The results showed that pH of the co-cultivation medium (pH 5.7), the bacterial growth phase (optical density; OD_{600}= 1), co-cultivation period (3 days), and acetosyringone concentration (100 μM) had positive effects on transformation (Kim *et al.,* 2007). Whereas, the co-cultivation period of 48 hr was suitable for inoculation of hypocotyls explants of *Arachis hypogaea* L. with *A. rhizogenes* 15834 (Karthikeyan *et al.,* 2007). Both strains of *Agrobacterium* and types of explants also have an influence of hairy roots induction. Three types of explants such as young leaf (2 weeks), mature leaf (2 months), and stem segments from 2-month-old *in vitro* grown plants of Gentiana macrophylla were inculated with different bacterial strains of *A. rhizogenes* (A_4GUS, R 1000, LBA 9402 and ATCC 11325) for hairy roots induction. Hairy roots appeared from wounded after 25-38 days of inoculation. Hairy roots could be observed in different explants inoculation with A_4GUS and R 1000 strains, whereas other strains (LBA 9402 and ATCC 11325), failed to induced to hairy roots from any of the explants. The highest transformation rate 32 % and 12 % were observed in mature leaf inoculated with strain R 1000 and strain A_4GUS, respectively (Tiwari *et al.,* 2007). Tao and Li (2006) studied on the effect of *A. rhizogenes* strains (R 1000, R 1601, A4, and R 1205), their concentrations, acetosyringone and co-cultivation pH on *Torenia fournieri* L. The results showed that strain R 1000, co-cultivation for 3 days, 30 μmol/L acetosyringone and pH 6.5 in the cultivation medium provided the optimal conditions with transformation frequency approached 90 % (Tao and Li, 2006). For hairy root culture of *Azadirachta indica* induced from stem and leaf explants, The efficiency of hairy roots induction by *A. rhizogenes* strain LBA 9402 were successfully 73 % from the leaf explants and 67 % from the stems of neem seedlings within 4 weeks of inoculation (Allan *et al.,* 2002). Moreover, Zhou and his groups (2007) had studied on parameters for hairy root induction from leaf explants of *Rhodiola sachalinensis* by *A. rhizogenes* A4. The results indicated that the best rate of infection (69.4 %) was achieved when the *Agrobacterium* concentration was at OD_{600} = 0.51 but when concentration was lower than 0.25, the efficiency of

infection was also low. Whereas when the *Agrobacterium* concentration was greater than 1, there was much severe harmful to the explants after infection. The time of submerging in bacterial suspension was achieved for 20 minutes and the optimal time of co-culture on solid medium was 3 days in the A4 strain (Zhou *et al.*, 2007).

2.12 Application of hairy root culture in production of secondary metabolites from higher plants

The technique in hairy root systems is advantageous for the production of secondary metabolites. This technique is a commonly used method to enhance the production of secondary metabolites. Thousands of species have been transformed with *A. rhizogenes* with the aim of achieving a transformed root induction reviewed by Matkowski in 2008. There are some research on hairy root cultures and naphthoquinone production. The production of lawsone, a naphthoquinone derivative, from hairy root of henna has been reported in significant quantities, whereas the accumulation of lawsone is restricted to the aerial parts and not found in the roots of wildtype henna (Bakkali *et al.*, 1997). Hairy root culture of *Lithospermum erythrorhizon*, a new brown benzoquinone derivative instead of red naphthoquinone (shikonin) derivatives as the main secondary metabolite (Fukui *et al.*, 1997). Recently, hairy root culture of *Sesamum indicum* established by the transformation of seedlings with *A. rhizogenes* ATCC 15834 also produced a naphthoquinone, 2-geranyl-1, 4-naphthoquinone (Furumoto *et al.*, 2007). Plumbagin was also produced from hairy root of *Plumbago indica* induced by *A. rhizogenes* strains K599 (Tatreerod *et al.*, 2003). Recently, *P. indica* were studied on hairy root culture with by infecting leaf explants with *A. rhizogenes* strain ATCC 15834. The highest plumbagin content was found to accumulate in roots at their exponential phase of growth (5.32 mg/g dry mass). Thereafter, a rise in biomass but a gradual decline in plumbagin content was noticed in cultures grown for 30 days (2.5 mg/g dry mass), 40 days (1.1 mg/g dry mass), and 50 days (0.5 mg/g dry mass) (Gangopadhyay *et al.*, 2008). Table 2.10 shows examples of some of the phytochemicals produced by hairy root cultures, according to reports published from 2000-2006.

There are more applications of hairy roots such as, functional analysis of genes, expressing foreign proteins, production of secondary metabolites, production of compounds which are not found in untransformed roots, changing composition of metabolites, and regeneration of whole plants (Guillon *et al.*, 2006). Therefore, hairy roots act as an attractive system to produce biomolecules. The cells of hairy root are differentiated cells. They are phenotypically and genetically stable. Hence the selected hairy root line will be exploited for a long time. In the near future, hairy roots will provide biotechnologists with powerful tools to reach the precious underground resources of plant kingdom.

Table 2.10 Examples of metabolites produced by transformed root cultures (Georgiev *et al.*, 2007)

Metabolites	Hairy root culture	Biological activity	References
Ajmalicine, ajmaline	*Rauvolfia micrantha*	Antihypertension	Sudha *et al.*, 2003
Artemisinin	*Artemisia annua*	Antimalaria	Weathers *et al.*, 2005
Benzylisoquinoline alkaloids	*Papaver somniferum; Eschscholzia californica*	Analgesic and antibiotic	Park and Facchini, 2000
Betalains	*Beta vulgaris*	Antioxidant and colorant	Pavlov and Bley, 2006
Camptothecin	*Ophiorrhiza pumila; Camptotheca acuminata*	Antitumor	Saito *et al.*, 2001
Iridoid glycosides	*Harpagophytum procumbens*	Anti-inflammatory, analgesic, and antidiabetic	Georgiev *et al.*, 2006
3,4-dihydroxy-*L*-phenylalanine	*Stizolobium hassjoo*	Therapeutic agent against Parkinson's disease	Sung and Huang, 2006
Rutin, hispidulin and syringin	*Saussurea involucrate*	Anti inflammatory and antifungal	Fu *et al.*, 2006

Table 2.10 (continued)

Metabolites	Hairy root culture	Biological activity	References
Scopolamine and hyoscyamine	*Datura innoxia*	Anticholinergic	Dechaux and Boitel-Conti, 2005
Taxol	*Taxus brevifolia*	Anticancer	Huang *et al.*, 1997
Thiarubrine A	*Ambrosia artemisiifolia*	Antifungal, antibacterial, and antiviral	Bhagwath and Hjortso, 2000
6-methoxy-podophyllotoxin	*Linum album;* *Linum persicum*	Anticancer	Wink *et al.*, 2005

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 Plant material

Young leaves of 4 years old of *R. nasutus* were collected from botanical Garden, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

3.1.2 Bacteria

Wild type *Agrobacterium rhizogenes* ATCC 11325, ATCC 13332, ATCC 13333, and ATCC 15834 were purchased from Microbiological Resources Centre, Pathumthani, Thailand. The *A. rhizogenes* strains were coded under Thailand Institute of Scientific and Technological Research (TISTR) number as TISTR 511, TISTR 509, TISTR 510, and TISTR 1450, respectively.

3.1.3 Chemicals, kits and enzymes

- Acetic acid, Glacial AR grade (Lab-scan Asia, Thailand)
- Agar (Bacto) (Himedia laboratories, India)
- Beef extract (Himedia laboratories, India)
- Cefotaxime sodium (UTOPIAN CO, Thailand)
- Clorox® (Clorox, Malaysia)
- DNeasy Plant Mini Kit (Qiagen, Germany)
- Ethanol (95 %v/v) (Lab-scan Asia, Thailand.)
- Ethyl acetate, commercial grade (Lab-scan Asia, Thailand)
- GFX Micro Plasmid Prep Kit (GE Healthcare, UK)

- Gotaq® Flexi DNA Polymerase (Promega, USA)
- Methanol, HPLC grade (Lab-scan Asia, Thailand)
- Murashige-Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands)
- Peptone (Becton Dickinson and company, France)
- Plant agar (Sigma, Germany)
- Standard rhinacanthin-C, -D, and -N were kindly provides from Assoc. Prof. Dr. Pharkphoom Panichayupakaranant, Faculty of Pharmaceutical Sciences, Prince of Songkla University
- Sucrose (Mitrpol, Thailand)
- Yeast extract (Becton Dickinson and company, USA)

3.1.4 Equipments and Instruments

- Autoclave Model HA-3D (Hirayama, Japan)
- High-speed Centrifuge Kubota 5922 (Kubota, Japan)
- Electrophoresis chamber Mupid α-2 plus (Takara Bio Inc, Japan)
- UV illuminator (VilberLourmat, France)
- Takara PCR Thermal Cycler Dice (Takara, Japan)
- Hot air oven (Memmert, Germay)
- Hot plate and stirrer (Fisher Scientific, USA)
- HPLC (Agilent, U.S.A) equipped with 4 pumps, photodioadaray detector, recorder with agilent software
- HPLC column (150 mm x 4.6 mm i.d.) (TSK-GEL™; TOSOH, Japan)
- Laminar air flow cabinet (Holten, Denmark)
- Micropipette 0.1-2.0 μL, 2-20 μL, 20-200 μL, 100-1,000 μL (Socorex, Switzerland)
- Microwave ovens (LG, China)
- pH meter (ORION Research, USA)
- Rotary evaporator (EYELA, Japan)

- Shaker (Innova2300) (New Brunswick Scientific, USA)
- Sonicator (Crest Ultrasonic Corporation, USA)
- Vacuum pump (EYELA, Japan)
- Vortex mixer (Vortex-Genie 2TM, USA)

3.1.5 Media and solution preparations

3.1.5.1 Plant media

- Murashige and Skoog (MS) medium (1962)

MS medium powder (Duchefa biochemie, The Netherland) (4.4 g) and sucrose (30 g) were dissolved in distilled water and made up to 1 L. The pH was adjusted to 5.8 with 1 N KOH and sterilized by autoclaving at 121°C, 15 lb/in^2, for 15 min. The solid and semi-solid media were solidified with plant agar (Sigma, Germany) at the concentration of 0.8 % (w/v) and 0.4 % (w/v), respectively.

3.1.5.2 Bacterial media

- YEB medium

YEB (100 mL) was prepared by mixing the beef extract (0.5 g), yeast extract (0.1 g), peptone (0.5 g), sucrose (0.5 g), agar (Bacto) (1.5 g) and 10 % (w/v) magnesium sulfate (0.5 mL), The pH was adjusted to 7.2 - 7.4 and sterilized by autoclaving.

3.1.5.3 Solution

- **Tris acetate EDTA**

TAE buffer was prepared as fifty times concentrated buffer (50X TAE) by dissolving 121 g Tris-(hydroxymetyl)-aminomethane, ethylenediaminetetraacetic acid tetrasodium salts 19.7 g and 35 mL glacial acetic acid in water to make up to 500 mL of buffer and pH was adjusted with concentrated hydrochloric to 8.0. The buffer is diluted 50 times by water prior to use.

- **Cefotaxime stock solution**

Stock solution of cefotaxime was prepared by dissolved the 1 g cefotaxime with sterile distilled water up to 5 mL.

3.2 Experimental methods

3.2.1 Explants preparation

Young leaves of *Rhinacanthus nasutus* were washed with running tap water for 1 hr and surface sterilized by rinsing with 70 % (v/v) ethanol for 5 s, immersing in 20 % (v/v) Clorox® for 20 min and finally, rinsing 3 times with sterile distilled water.

3.2.2 Preparation of *Agrobacterium rhizogenes* culture

A. rhizogenes ATCC 11325, 13332, 13333, and 15834 were used for hairy root induction. The bacteria were cultured on YEB nutrient agar medium (peptone 5 g /L, beef extract 5 g/L, magnesium sulfate 5 g/L, yeast extract 1 g/L and agar 15 g/L) at room temperature for 3 days. A single bacterial colony was inoculated in 5 mL of YEB nutrient

broth medium and cultured on rotary shaker (220 rpm) at 25°C for 16 hours. Bacterial suspension culture was centrifuged at 3,000 rpm (1,730 *g*) for 10 min. The pellet was resuspended in 5 mL MS liquid medium. The OD_{600} was adjusted to 1 and used for co-cultivation of explants (Dhakulkar *et al.*, 2005).

3.2.3 Induction of hairy roots

Sterilized *R. nasutus* leaves were used as explants for hairy root induction. Whole leaves were wounded with sterile needle at each leaf vein then immersed in *A. rhizogenes* suspension culture as described in 3.2.2 for 20 min. Explants were blotted dry on sterile filter-paper and co-culture onto hormone-free MS solid medium at 25°C in the dark condition. After 3 days of co-cultivation, inculated explants were transferred to MS solid medium containing 500 mg/L cefotaxime so as to remove the residual *A. rhizogenes*. Concentration of cefotaxime was then reduced every week from 500, 300 to 100 mg/L. Finally, *A. rhizogenes*-free cultures were transferred to MS solid medium without plant growth regulator and cultured at 25°C in the dark condition. Percentage of leaves regenerating hairy roots by infection of 4 strains of *A. rhizogenes* was determined 30 days after inoculation. Data were examined in triplicate.

3.2.4 Hairy root culture

The hairy roots were cultured in hormone- free MS solid medium at 25°C in the dark condition for 4 weeks. To obtain sufficient amount of hairy root, the hairy roots were transferred to 50 mL of hormone-free MS liquid medium and incubated in the dark condition at 25°C on a rotary shaker (80 rpm) and subcultured at 4 weeks after intervals by cutting the fresh root and transferred to the new medium.

3.2.5 The cultured root for PCR negative control

The root cultures were established from the whole leaf explants of *R. nasutus* on MS solid media supplied with 3.0 mg/L of IBA. The cultures were incubated at 25°C under dark conditions (Meerungrueang, 2009).

3.2.6 Identification of transformed hairy roots

3.2.6.1 Plasmid isolation from *A. rhizogenes*

Plasmids of *A. rhizogenes* (a positive control) ATCC 11325, ATCC 13332, ATCC 13333, and ATCC 15834 were isolated from overnight culture using GFX Micro Plasmid Prep Kit. The overnight culture (1.5 mL) was centrifuged at 15,000 rpm (43,270 *g*) for 30 s. After removal of supernatant, the pellet was resuspended in 150 µL of resuspension buffer. The 150 µL lysis buffer was added and mixed by inverting tube for 10 - 15 times. The 300 µL of neutralization buffer was then added and mixed by inverting tube until the flocculent precipitate appeared. The mixture was then centrifuged for 5 min to pellet the cell debris. The supernatant was transferred to a GFX column and incubated for 1 min at room temperature and then centrifuged at full speed for 30 s. The flow-through was discarded and the column was washed with 400 µL of wash buffer before drying by centrifugation at full speed for 60 s. Finally, the GFX column was transferred to a new centrifuge tube and 100 µL of TE buffer was added, incubated at room temperature for 1 min and centrifuged at full speed for 1 min to recover the purified DNA. The purified DNA was kept at -20°C until used.

3.2.6.2 Genomic DNA isolation from transformed hairy root culture

Genomic DNA was isolated from the 4 types of transformed hairy roots which induced by 4 strains of *A. rhizogenes* (including strains ATCC 11325, 13332, 13333, and 15834) and cultured roots (a negative control) using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The 100 mg of fresh weight of plant tissues were ground into fine powder in the presence of liquid nitrogen. The powder was resuspended in 400 µL of extraction buffer AP1 plus 4 µL of RNase A stock solution (100 mg/mL) and vortexed vigorously. The suspension was incubated for 10 min at 65°C, mixed 2 - 3 times during incubation by inverting tube. Buffer AP2 (130 µL) was added to the lysate and incubated on ice for 5 min. The lysate was loaded into the QIA-shredder mini spin column in a 2 mL collection tube and centrifuged at 14,000 rpm (37,690 *g*) for 2 min. The flow-through fraction is transferred to a new tube. The 1.5 volumes of buffer AP3/E was added into the cleared lysate and mixed by pipetting. The 650 µL of mixture was applied to DNeasy mini spin column sitting in a 2-mL collection tube and centrifuged at 8,000 rpm (12,310 *g*) for 1 min. The flow-through was discarded. The 500 µL of buffer AW was added on DNeasy mini spin column and centrifuged for at 8,000 rpm 1 min. This step was performed in duplicate. Finally, the DNeasy mini spin column was placed in a 1.5 mL microcentrifuge tube and a 100 µL of buffer AE was added, and incubated for 5 min at room temperature. The DNA is eluted from the column by centrifugation at 8,000 rpm for 1 min. The DNA is stored at -20°C until use.

3.2.6.3 Verification of transformed DNA by polymerase chain reaction (PCR) technique

Four plasmids isolated from *A. rhizogenes* strains ATCC 11325, 13332, 13333 and 15834 (3.2.6.1) were used as templates. For control treatment and genomic DNA (3.2.6.2) were used as templates for DNA amplification by PCR

technique. Four specific primers (Table 3.1) designed from the nucleotide sequence of T-DNA region of *A. rhizogenes* (Oksman-Caldentey and Hiltunen, 1996). These primers were used for amplification of *rolB*, *rolC* fragments of transformed T-DNA from *A. rhizogenes*. The *rolB*-1 and *rolB*-2 primers were used for amplification of 780-bp fragment of *rolB* and *rolC*-1 and *rolC*-2 primers were used for amplification 545-bp fragment of *rolC*. The standard procedure for a PCR reaction was performed in a 50 μL mixture. (Table 3.2)

3.2.6.4 Preparation of agarose gel

Agarose gel electrophoresis was used for determination of DNA fragments. For 1 % of agarose gel, 1.2 g of agarose was resuspended in 120 mL distilled water supplemented with 2.4 mL of 50X TAE. The mixture was melt by heating in microwave oven, and then poured on a tray. After the agarose gel was completely set (30 - 45 min at room temperature), the gel was installed on the platform in the electrophoresis tank (Takara Bio Inc, Japan) containing TAE buffer. The PCR products (10 μL) were mixed with 1 μL 10X loading dye solution and loaded into the wells of agarose gel. The gel was run at 100 volts. The electrophoresis process was stopped when the loading dye has migrated to three fourth of the agarose gel. After the agarose gel was stained with SYBR® Safe DNA gel stain solution for 30 min, the DNA pattern was observed using UV light box (VilberLourmat, France) and the photograph was taken.

Table 3.1 Specific primers used for PCR amplification

Primer	Nucleotide sequences	T_H (°C)
rolB-1	ATGGATCCCAAATTGCTATTCCTTCCACGA	66.0
rolB-2	TTAGGCTTCTTTCTTCAGGTTTACTGCAGC	66.0
rolC-1	CATTAGCCGATTGCAAACTTG	58.6
rolC-2	ATGGCTGAAGACGACCTG	59.9

Table 3.2 Standard procedure for PCR

Reagents	Volume/reaction (μL)	Concentration per reaction
DNA Template	10	*ca.* 100 ng
$MgCl_2$	3	1.5 mM
dNTP mix, 2.5 mM	4	0.2 of each dNTP
primer 1	1	0.5μM
primer 2	1	0.5μM
Gotaq DNA polymerase	0.5	1.0 units/ reaction
Sterilize distilled water	30.5	
Total volume	50	

Table 3.3 PCR conditions for *rolB* and *rolC* amplification

Segment	Step	Temperature (°C)	Time (min)	cycles
1	Denaturing	94	5	1
2	Denaturing	94	1	30
	Annealing	42	1	
	Extension	72	2	
3	Extension	72	10	1
	Holding	4	∞	

3.2.7 Determination of rhinacanthin contents

3.2.7.1 Harvesting of explants

The transformed hairy roots, cultured roots, intact roots, and intact leaves of *R. nasutus* were harvested and dried in a hot air oven at 50°C overnight before ground into powder.

3.2.7.2 Hairy root extraction

The hairy roots 30 mg of samples were extracted with 20 mL ethyl acetate and sonicated for 1 hr. The extracts are filtered and evaporated to dryness, then dissolved in methanol and adjusted volume to 1 mL. The samples were filtered through 0.45-μm micro-filtration membrane. These sample preparations were then analysed for rhinacathin contents using HPLC method described by Charoonratana (2007).

3.2.7.3 HPLC analysis

3.2.7.3.1 Preparation of standard solution

Reference standard rhinacanthins-C, -D, and -N were previously purified (Panichayupakaranant, 2002). Stock solutions of the reference standards, rhinacanthins-C (10.30 mg), -D (2.10 mg), and -N (1.10 mg) were made by dissolving in methanol. Working solution of the combined standards was subsequently prepared in methanol and adjusted to 10 mL on volumetric flask. The stock solution was two-fold serial diluted to nine concentrations and filtered through the membrane filter (0.45 μm). Rhinacanthin-C ranging from 0.004 - 1.03 mg/mL, rhinacanthin-D ranging from 0.008 - 0.21 mg/mL and rhinacanthin-N

ranging from 0.0004 - 0.11 mg/mL, were used for construction of calibration curves

3.2.7.3.2 Calibration curve

Calibration curves were established by analysis of a mixture containing each of the standard compounds at nine concentrations and plotted peak area against the concentration of each reference standards. The linearity of the detector response for the standards was assessed by means of linear regression. The curves showed coefficient of correlation $r^2 \geq 0.9995$ which is acceptable criteria.

3.2.7.3.3 HPLC conditions

The content of rhinacanthins-C, -D, -N from each sample preparations was determined using HPLC. Peak retention times and UV absorption spectra of the corresponding peaks were compared with the standard compounds. The areas under the peaks were converted to concentration by using their calibration curves (as mentioned in 3.2.7.3.2). HPLC analysis was carried out by using Agilent 1100 series equipped with photodiode-arrays detector (PDA) and autosampler. Data analysis was performed using Agilent software (Agilent, U.S.A). HPLC condition was performed using the previous studies (Charoonratana, 2007). Separation was achieved isocratically on a TSK-gel ODS-80Tm column with (150 mm x 4.6 mm i.d.) The mobile phase consists of methanol and 5 % acetic acid in water in ratio of 80:20 (v/v). The flow rate was 1 mL/min and the injection volume was 20 µL. The quantitative wavelength was set at 254 nm. The analysis of all samples was carried out in triplicate.

3.2.8 Time-course of growth and rhinacanthin production

Hairy root infected by 4 strains of *A. rhizogenes* were harvested for determination of rhinacanthin contents. Thirty mg of hairy roots were cultured in hormone-free MS liquid medium on rotary shaker (80 rpm) at 25°C in the dark condition. The analysis of all samples was in triplicate. On day 30, hairy roots were harvested for determination of rhinacanthin content by HPLC method. The hairy root 11325 with the highest rhinacanthin production was selected for studying on time-course of growth and rhinacanthin production.

The 50 mg of hairy root 11325 were inoculated in hormone-free MS semi-solid medium and cultured on rotary shaker (80 rpm) at 25°C in the dark condition. The hairy roots were harvested every 5 day by vacuum filtration until day 30. The dry weight was determined after drying at 50°C for 24 hrs. The amount of rhinacanthins were examined and calculated in the unit of both percentages of dry weight (mg) and total content (mg/dry weight). These data are plotted to obtain growth and rhinacanthin production curves. The analysis of all samples was done in triplicate.

3.2.9 Statistical methods

The one way ANOVA program is used for statistical analyzing of rhinacanthin content in (hairy roots, root cultures, natural roots, and natural leaves). The data is presented as mean ± S.D.

CHAPTER 4

RESULTS

4.1 Establishment of transformed hairy root culture

The transformed hairy root cultures of *R. nasutus* were established by infection the leaf explant with four strains of *A. rhizogenes* (ATCC 11325, ATCC 13332, ATCC 13333 and ATCC 15834). The hairy roots were formed around 14 days after infection (Figure 4.1). Hairy root transformed by *A. rhizogenes* ATCC 13333 (hairy root 13333) showed highest percent hairy root induction (70 %) following by hairy root 15834 (60 %), hairy root 11325 (55 %) and hairy root 13332 (25 %) However, number of explants generating hairy root not significant in strains 11325, 13333, and 15834.

After four weeks, hairy root in MS solid media were transferred to MS liquid media. Unfortunately, the growth of hairy root induced by ATCC 13333 was not stable after several subcultures. The hairy roots which were induced by 4 strains of *A. rhizogenes* showed different in the morphology. Hairy root 11325 and hairy root 15834 were high branching and grew very well after subcultures. Hairy root 13332 showed the lowest of percent hairy root with is small single root and grew very slowly in MS liquid medium. (Table. 4.1)

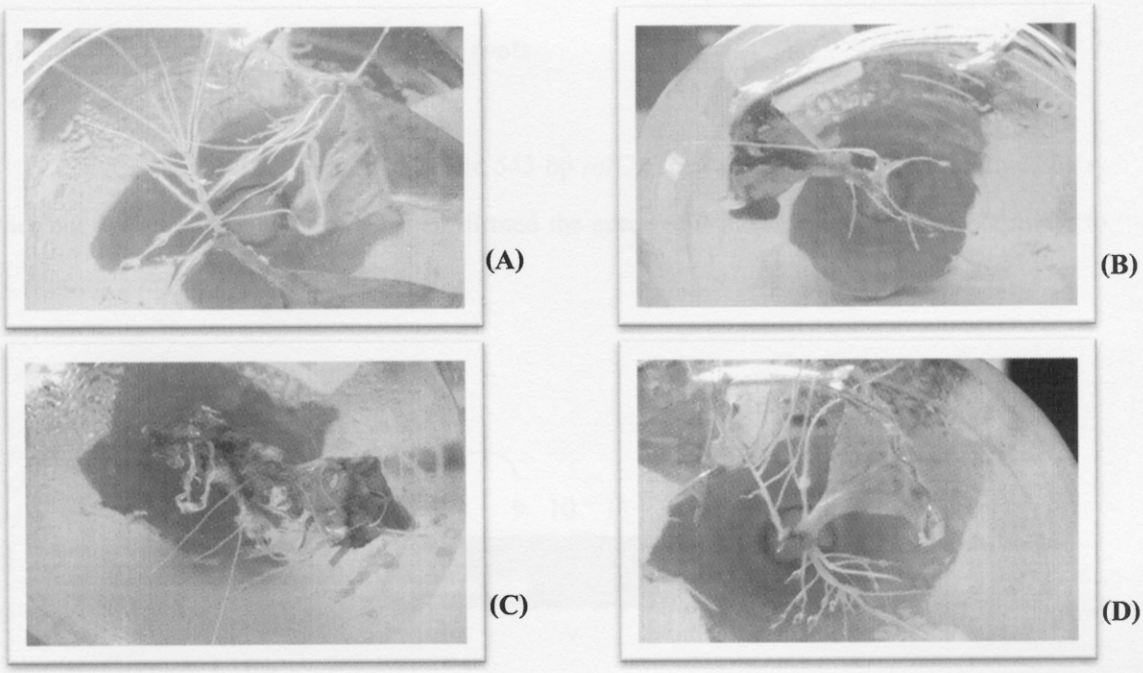


Figure 4.1 Hairy roots regeneration from leaf explants inoculated with *A. rhizogenes* 11325 (A), 13332 (B), 13333 (C), and 15834 (D) (30 days after infection)

Table 4.1 Induction of hairy roots from *R. nasutus* leaf explants by several strains of *A. rhizogenes*

***A. rhizogenes* strains**	**Number of explants**	**Number of explants generating hairy root**	**% Hairy root**
11325	20	11^{b}	55 %
13332	20	$\mathbf{5}^{a}$	25 %
13333	20	14^{b}	70 %
15834	20	12^{b}	60 %

*Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. (n = 20)

4.2 Confirmation of transformed hairy roots

The 780-bp *rolB* gene and 545-bp *rolC* gene were observed in transformed hairy root but absent in normal root. This confirmed the successful transformation of Ri plasmid into *R. nasutus* hairy root. (Figure 4.2)

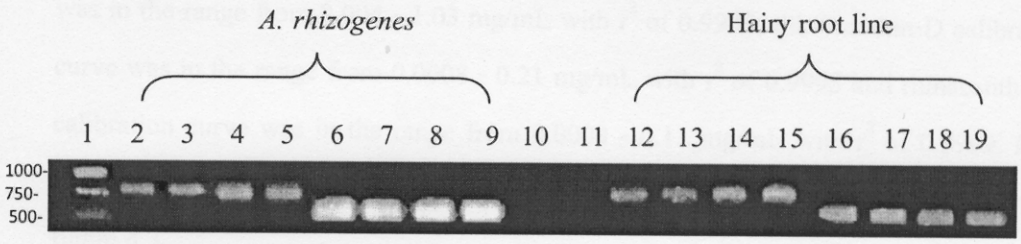


Figure 4.2 PCR product analysis of the *rol*B and *rol*C gene fragments in hairy roots using 1 % (w/v) agarose gel, PCR products were detected under UV illuminator (312 nm) after SYBR Safe staining

Lane 1 = Marker (10 kb DNA ladder)

Lane 2-5 = *A. rhizogenes* strains of ATCC 11325, 13332, 13333 and 15834, detection for *rolB* (positive control)

Lane 6-9 = *A. rhizogenes* strains of ATCC 11325, 13332, 13333 and 15834, detection for *rolC* (positive control)

Lane 10-11 = root cultures (negative control), detection for *rolB* and *rolC*, respectively

Lane 12-15 = hairy root line of hairy root 11325, hairy root 13332, hairy root 13333 and hairy root 15834, detection for *rolB*

Lane 16-19 = hairy root line of hairy root 11325, hairy root 13332, hairy root 13333 and hairy root 15834, detection for *rolC*

4.3 Determination of rhinacanthin production

4.3.1 Construction of rhinacanthin calibration curve

The calibration curve of the standard rhinacanthin was established according to the method as described in 3.2.7.3.2. The linearity of the rhinacanthin-C calibration curve was in the range from 0.004 - 1.03 mg/mL with r^2 of 0.9999, rhinacanthin-D calibration curve was in the range from 0.0008 - 0.21 mg/mL with r^2 of 0.9998 and rhinacanthin-N calibration curve was in the range from 0.0004 - 0.11 mg/mL with r^2 of 0.9999. Each calibration point was carried out in triplicates. The calibration curves were shown in figure 4.3.

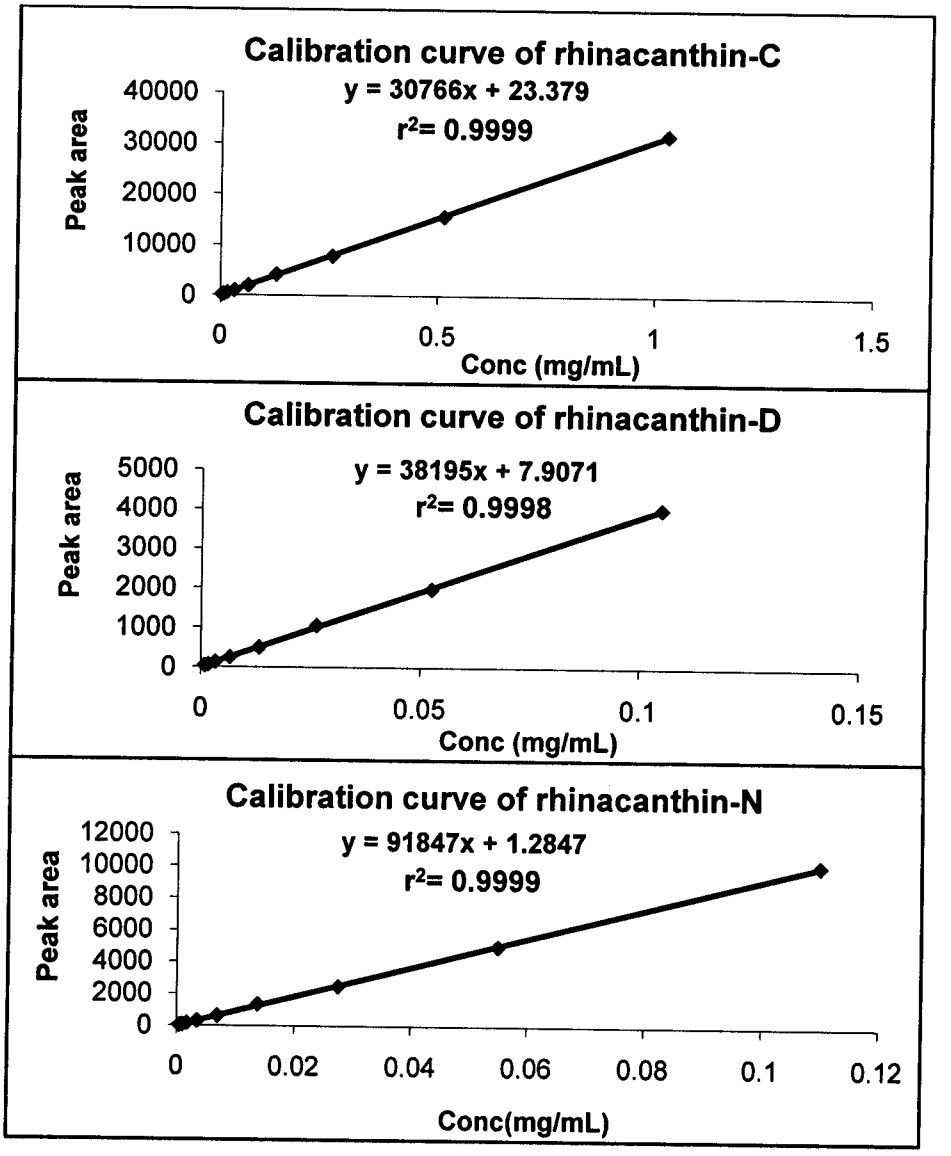


Figure 4.3 Calibration curves of rhinacanthins-C, -D, -N

4.3.2 Rhinacanthin production in *R. nasutus* hairy roots

Dry hairy roots of *R. nasutus* (10 mg each) which infected by 4 strains of *A. rhizogenes* were extracted and analyzed for rhinacanthin contents, including rhinacanthin-C, -D, -N using HPLC coupled with a photodiode array detector with a condition mentioned in section 3.2.7.3.3. Under these conditions, a well separation of the analyses was observed in both standard solutions and in the hairy root extracts. The HPLC- chromatograms showed the peaks for rhinacanthin-D which was eluted at 5.697 min, rhinacanthin-C at 14.306 min and rhinacanthin-N at 19.307 min (Figure 4.4). Areas under peaks were converted to rhinacanthin content using the calibration curve. Samples were investigated in triplicates.

HPLC chromatograms of the *R. nasutus* hairy root 11325, 13332, 13333 and 15834 extract showed difference in the chemical constituents. Only chromatogram of hairy root 11325 exhibited the peaks of rhinacanthin-C, -D, -N while other hairy roots extract showed the peaks of rhinacanthin-C and rhinacanthin-D (Figure 4.5). All hairy roots produced and accumulated the rhinacanthin-C as major component.

The rhinacanthin production of *R. nasutus* hairy root 11325 gave the highest contents of rhinacanthin-C (2.16 ± 0.097 % w/w) as a major product and followed by hairy root 15834 (1.15 ± 0.054 % w/w), hairy root 13332 (0.83 ± 0.172 % w/w) and hairy root 13333 (0.82 ± 0.121 % w/w), respectively (Table. 4.2). Therefore, the suitable *A. rhizogenes* strain for induction of *R. nasutus* hairy root was ATCC 11325.

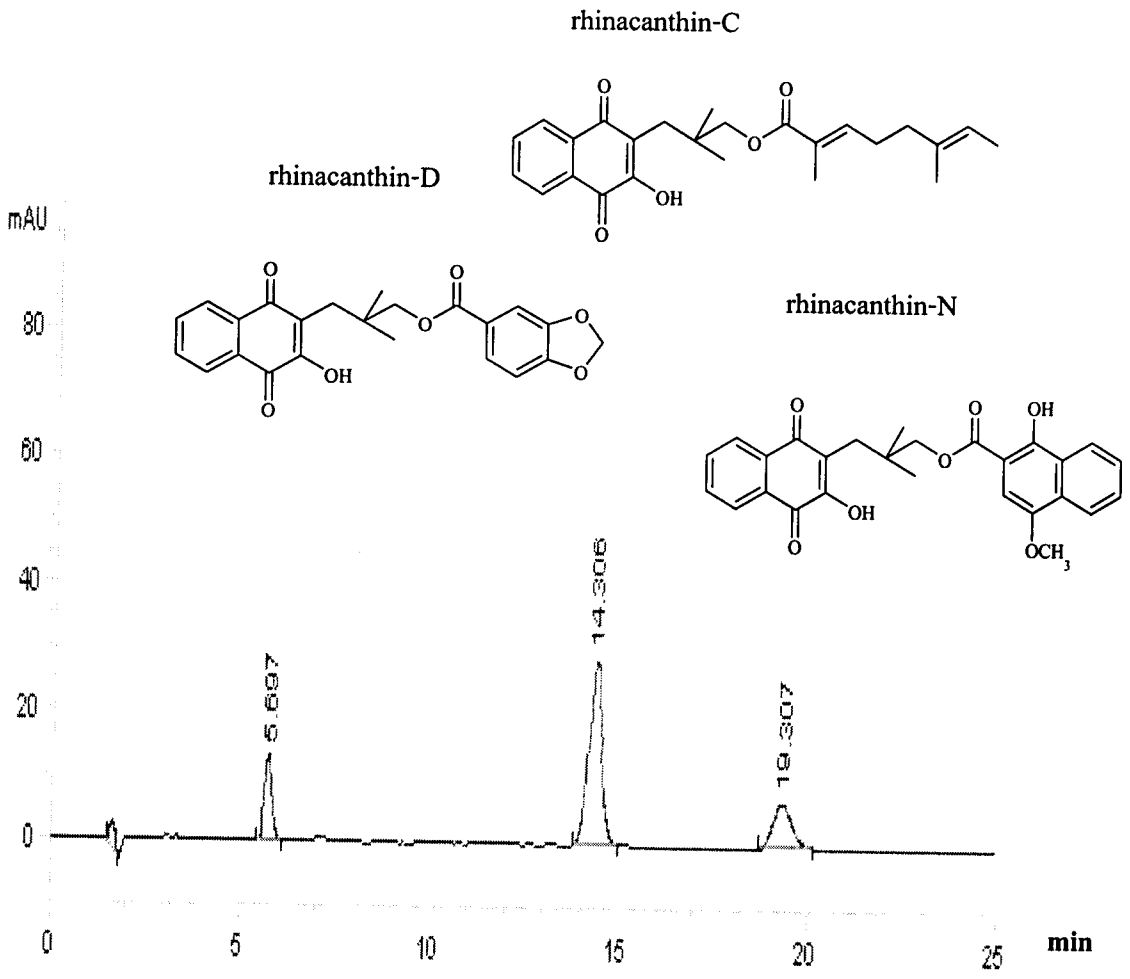


Figure 4.4 HPLC chromatogram of standard rhinacanthin-C, -D, -N

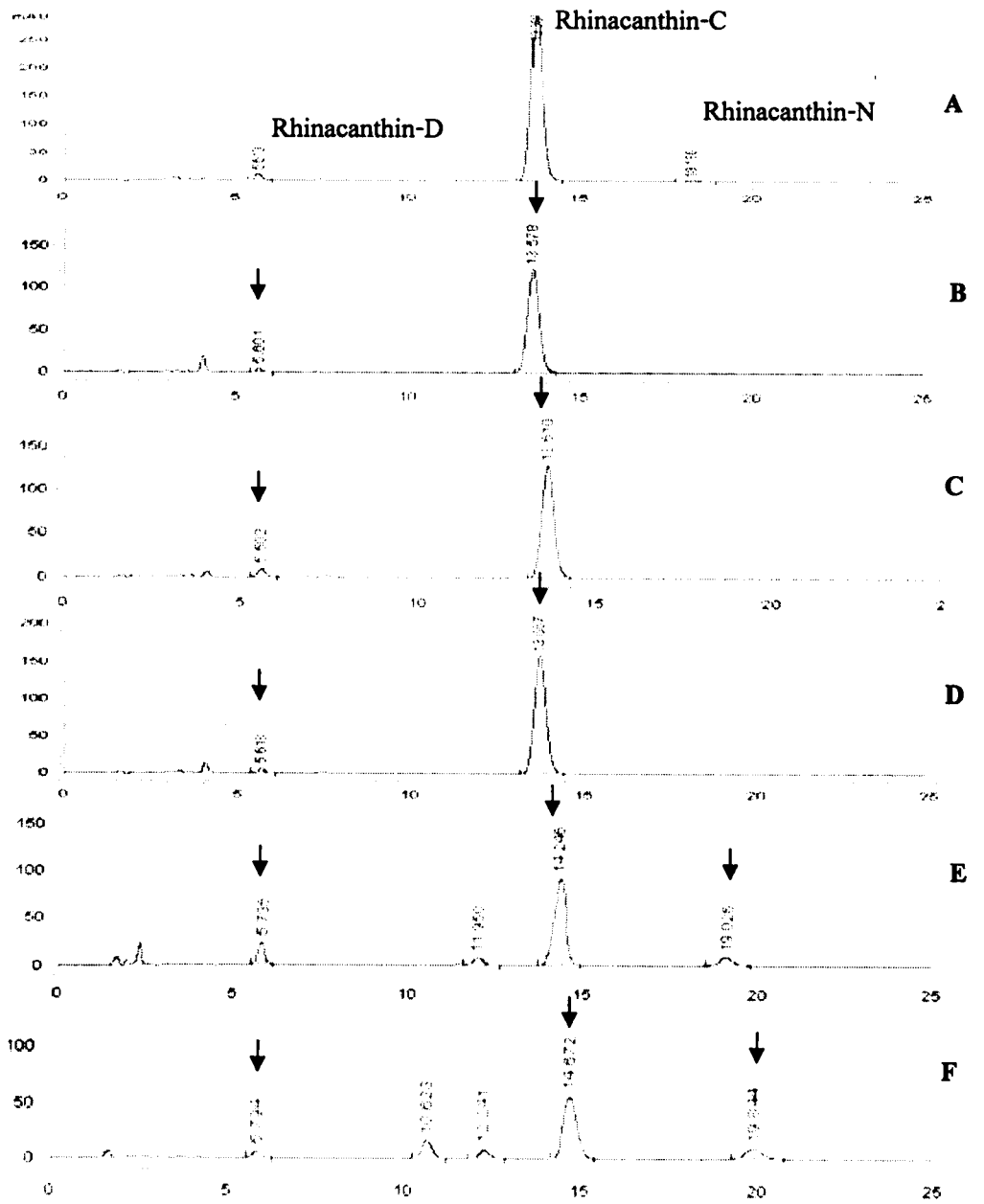


Figure 4.5 HPLC chromatograms of extracts of *R. nasutus* hairy root 11325 (A), hairy root 13332 (B), hairy root 13333 (C), hairy root 15834 (D), natural leaves (E), and natural roots (F)

The rhinacanthin production by intact roots and intact leaves were shown in Table 4.2. Among three rhinacanthins, rhinacanthin-C was formed as major chemicals at 2.83 ± 0.015 % w/w in intact roots and 2.19 ± 0.031 % w/w in intact leaves. Rhinacanthin-C from hairy root 11325 was 2.16 ± 0.097 % w/w which is quite same

amount with the amount of intact leaves and a bit smaller than that of intact roots. Rhinacanthin-D and -N production of hairy root 11325 were significantly lower in their amount than intact leaves and intact roots.

Table 4.2 Total rhinacanthin content in *R. nasutus* hairy roots infected by 4 strains of *A. rhizogenes*

Materials	Rhinacanthins (% w/w ± S.D.)		
	rhinacanthin-C	rhinacanthin-D	rhinacanthin-N
Hairy root 11325	2.16 ± 0.097^{b}	0.04 ± 0.032	0.01 ± 0.026
Hairy root 13332	0.83 ± 0.172^{d}	0.04 ± 0.042	-
Hairy root 13333	0.82 ± 0.121^{d}	0.01 ± 0.012	-
Hairy root 15834	1.15 ± 0.054^{c}	0.02 ± 0.081	-
Natural roots	2.83 ± 0.015^{a}	0.30 ± 0.163	0.16 ± 0.019
Natural leaves	2.19 ± 0.031^{b}	0.12 ± 0.023	0.14 ± 0.017
Root culture	0.25 ± 0.343^{e}	n.d.	n.d.

n.d. : can not calculated due to the area under the peak is under the lower limit of detection (n = 3)

* Statistical analyses were carried out using One-way ANOVA. By convention, results are considered statistically significant when $P < 0.05$. (n = 3)

4.4 Time courses of growth and rhinacanthin production of *R. nasutus* hairy root cultures

At the first period of study, hairy root were cultured in hormone-free MS solid medium before transferred into hormone-free MS liquid medium. Although the cultures could not be consistency growth, several attempts to increase hairy root growth were done. Observation that hairy root could better grow in solid medium than liquid medium. Moreover, in liquid medium hairy root culture died during cultivation and the roots turned to brown color at the end of culturing, these reasons lead to the idea of culturing hairy roots in semi-solid medium. MS semi-solid medium was prepared by adding 0.4 % (w/w) of agar into normal MS liquid medium. The culture condition was same as mentioned in 3.2.4.

During the 30-day period of the culture, culture growth and rhinacanthin production of *R. nasutus* hairy roots 11325 were also determined in MS semi-solid medium (Figure 4.6). The dry weight of the harvested hairy root mass was used as a parameter for representation of the culture growth (Table 4.3). The growth pattern of *R. nasutus* hairy roots appeared to be a normal sigmoid curve. The result showed 5 days of lag phase for cell adaptation and showed long linear phase (20 days) before entering to the stationary phase. (Figure 4.7) The highest amount of the obtained biomasss was 25.6 mg on day 25.

For rhinacanthin content accumulated in *R. nasutus* hairy roots. The highest rhinacanthin-C content, major compound was 20.8 mg/g dried weight at day 20 (Table 4.4). Time-course of rhinacanthin production gradually increased parallel with the growth of hairy roots cultures (Figure 4.8). Although, the rhinacanthin-C production during the growth cycle showed a fluctuation on day 25, the accumulation of rhinacanthin-C per culture flask still increased in paralleled pattern with the growth. (Figure 4.9) The identities of rhinacanthin-C, -D, and -N were confirmed by UV absorption spectra comparing with the standard rhinacanthin. (Figure 4.10, 4.11, and 4.12)

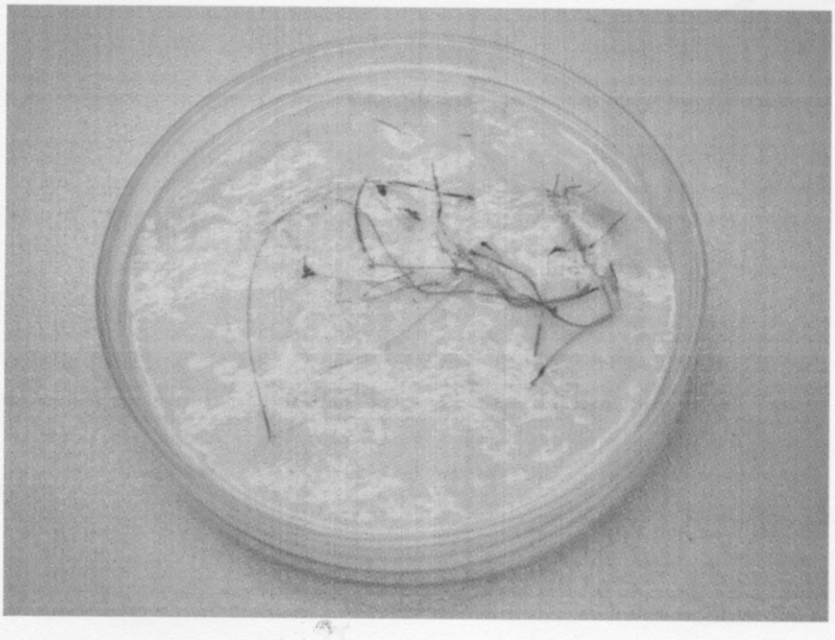


Figure 4.6 Hairy root 11325 in hormone-free MS semi-solid medium

Table 4.3 Biomass production of *R. nasutus* hairy root cultures cultured on MS semi-solid media (n=3)

Day	Biomass (mg/g dry weight ± S.D.)
0	2.4 ± 0.40
5	3.5 ± 0.45
10	7.2 ± 0.30
15	12.6 ± 0.60
20	17.9 ± 0.30
25	25.6 ± 0.77
30	25.4 ± 0.66

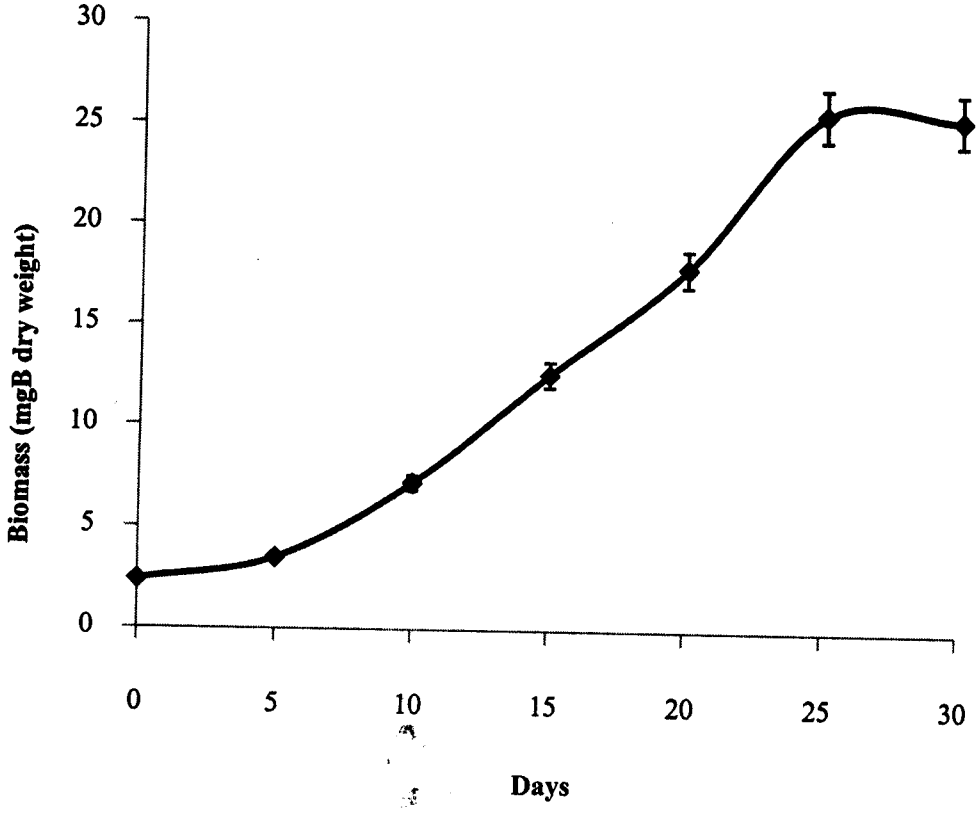


Figure 4.7 Time course of growth of *R. nasutus* hairy root in MS semi-solid medium

Table 4.4 Rhinacanthin content accumulated in *R. nasutus* hairy root

Day	Rhinacanthin content (mg/g dry weight)		
	rhinacanthin-C	rhinacanthin-D	rhinacanthin-N
0	11.6 ± 0.42	0.29 ± 0.284	0.17 ± 0.450
5	7.6 ± 0.23	0.45 ± 0.035	0.13 ± 0.346
10	17.6 ± 0.14	0.55 ± 0.353	0.24 ± 0.485
15	19.7 ± 0.07	0.82 ± 0.794	0.32 ± 0.654
20	20.8 ± 0.30	1.07 ± 0.035	0.45 ± 0.326
25	17.2 ± 0.25	1.02 ± 0.086	0.47 ± 0.225
30	19.2 ± 0.14	1.07 ± 0.438	0.48 ± 0.676

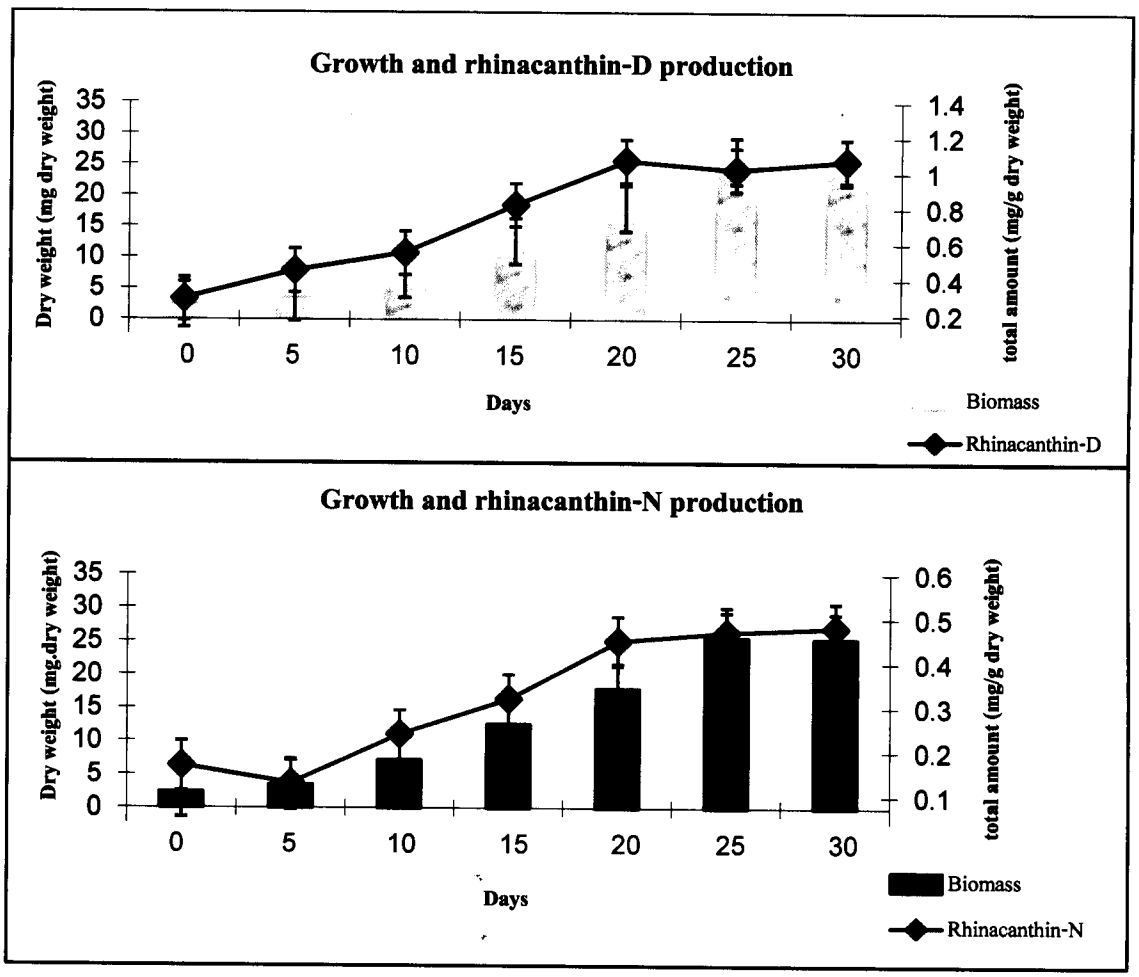


Figure 4.8 Time courses study of root biomass (dried mass) and rhinacanthin-D,-N content (mg/g dry weight) in hairy root culture of *R. nasutus*

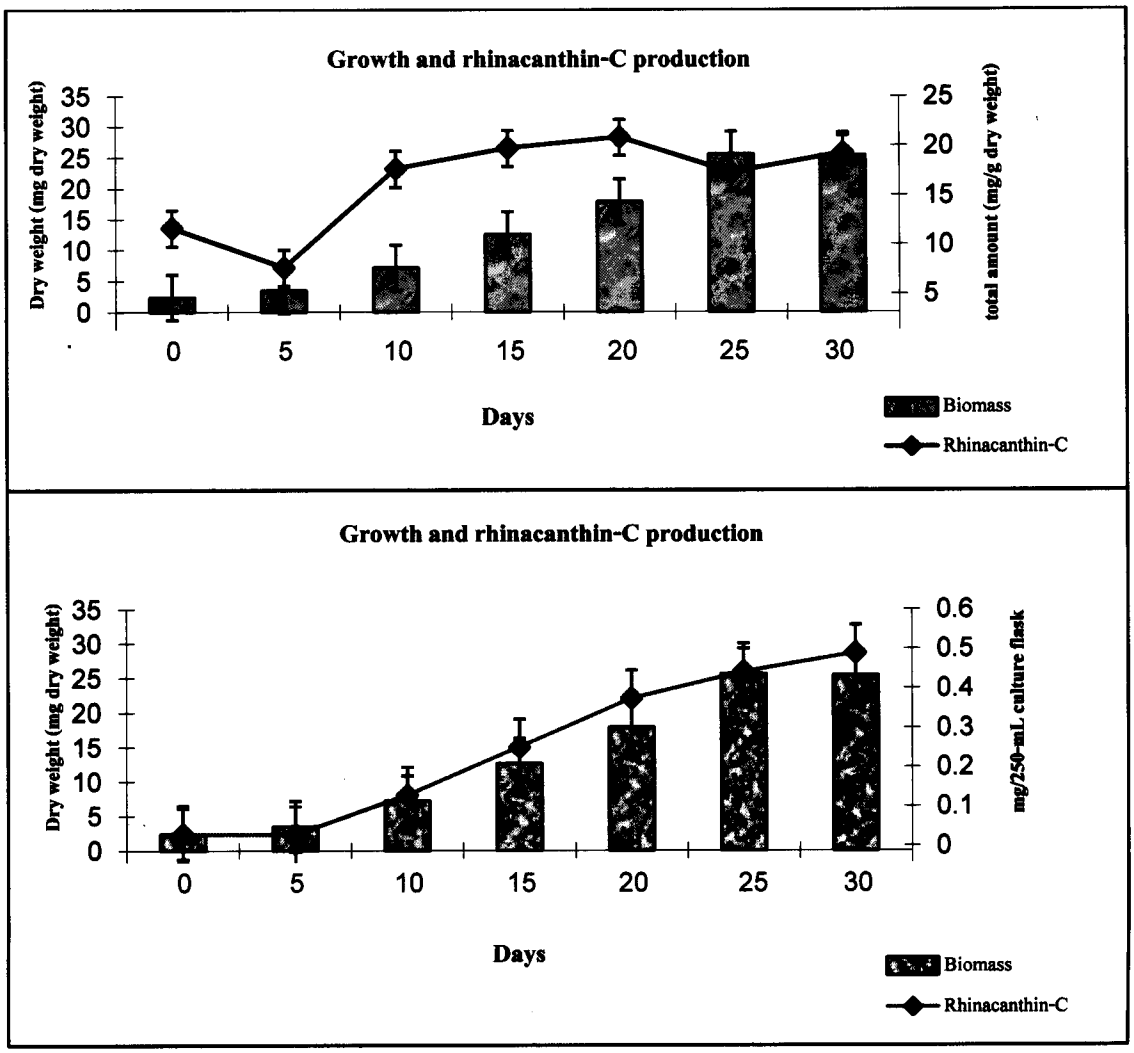


Figure 4.9 Time course study of root biomass (dried mass) with rhinacanthin-C content (mg/g dry weight) in hairy root culture of *R. nasutus* (A) and rhinacanthin-C yield per 250-mL culture flask in hairy root culture of *R. nasutus* (B)

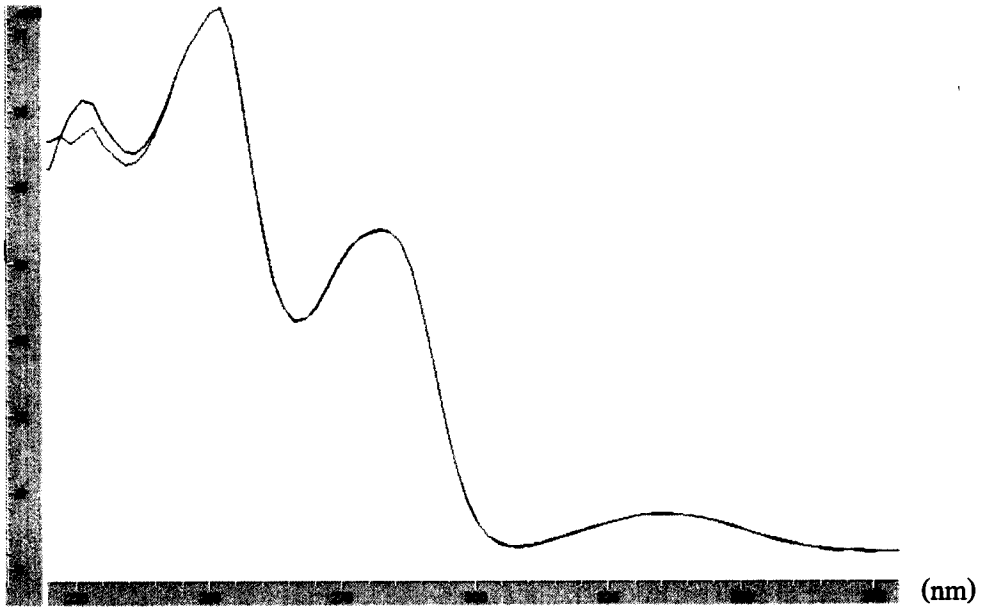


Figure 4.10 UV-absorption spectra of standard rhinacanthin-C (red) and the rhinacanthin-C obtained from *R. nasutus* hairy root cultures (blue).

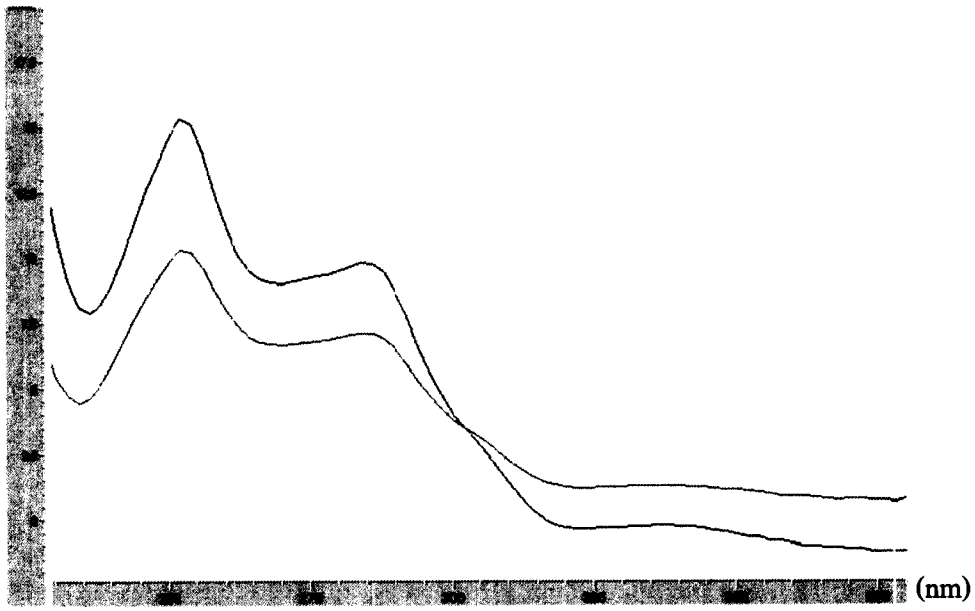


Figure 4.11 UV-absorption spectra of standard rhinacanthin-D (red) and the rhinacanthin-D obtained from *R. nasutus* hairy root cultures (blue).

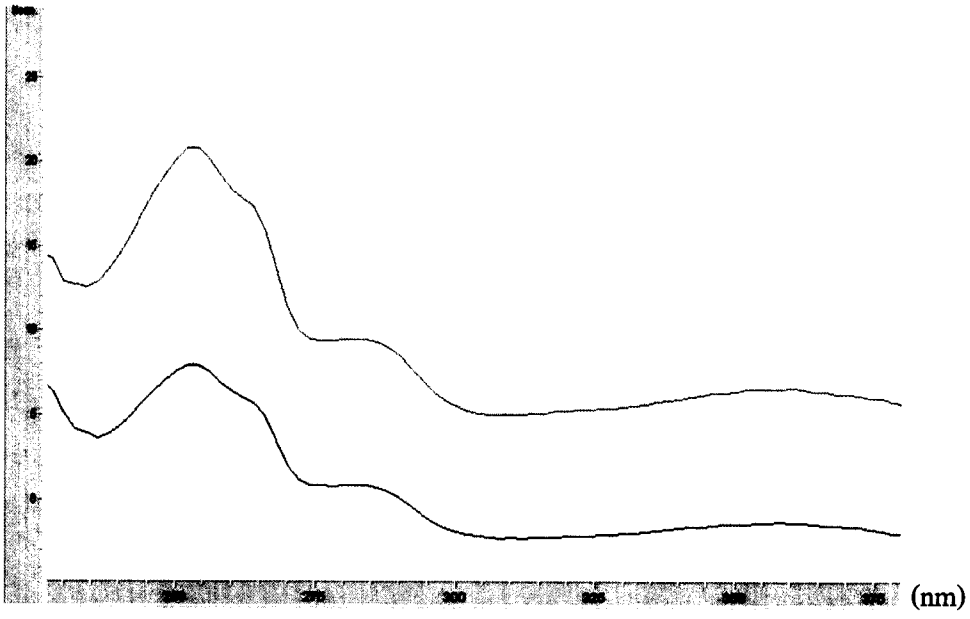


Figure. 4.12 UV-absorption spectra of standard rhinacanthin-N (red) and the rhinacanthin-N obtained from *R. nasutus* hairy root cultures (blue).

CHAPTER 5

DISCUSSION

In the present study, we have succeeded in the production of transformed hairy root of *R. nasutus* mediated by 4 strains of *A. rhizogenes*. These strains were not equally efficient in hairy root induction. The highest hairy roots induction efficiency was 70 percent, achieved by infected leaf explants with strain ATCC 13333. However, it is not significant in statistical analysis, when comparison in term of number of explants generating hairy root. In agropine strains, such as LBA 9402, ATCC 15834 two sections of DNA are transferred, T_L and T_R. Agropine strains are most often used owing to their strongest induction ability. (Charlwood and Rhodes, 1990). The hairy roots of *Ammi majus* were inoculated with seven different *A. rhizogenes* strains (A4, LBA 9402, ATCC 15834, ICPB TR 7, NCPPB 8196, ATCC 11325, and ICPB TR 107). The hairy root lines were established only after inoculation with the two agropine strains; A4 and LBA 9402 (Krolicka *et al.*, 2001). The hairy root induction capacity might be related to the effect of the different plasmids contained by these strains, in which genes of Ri T_L-DNA direct the synthesis of a substance that induces the cell to differentiate into root formation under the influence of endogenous auxin/cytokinin ratio or the sensitivity to the previous hormonal balance of each genotype. In addition strain virulence, susceptibility of the species may also play an important role in determining the frequency of hairy root induction. Co-cultivation is very important in the transformation process. Bacteria attachment, T-DNA transfer and integration are occurred during this stage (Fu *et al.*, 2005; Satdive *et al.*, 2007). Tao and Li (2006) studied on the effect of bacterial strains (R 1000, R 1601, A4, and R 1205), bacterial concentration, acetosyringone, and co-cultivation pH on *Torenia fournieri* L. The results showed that Strain R 1000, Co-cultivation for 3 days, 30 μmol/L acetosyringone and pH 6.5 in the cultivation medium provided the optimal conditions under which transformation frequency approached 90 %. The suggestion, if the co-cultivation time was too short, these transformation processes can not be completed. However, long co-cultivation time could negatively affect transformation by reducing bacterial affinity to the plant cell or by competitive inhibition (Tao and Li, 2006). Moreover, bacterial concentration plays important role in the production of transformed roots.

Kim and his groups (2007) were studied on the effect of the co-cultivation period, bacterial concentration, pH, and the presented of acetocyringone on *Agrobacterium tumefaciens*-mediated transformation of *Panax ginseng*. The results showed that pH of the co-cultivation medium (5.7), the bacterial growth phase (optical density; OD_{600}= 1), co-cultivation period (3 days), and acetosyringone concentration (100 μM) had positive effects on transformation (Kim *et al.*, 2007). Suboptimal concentrations resulted in low availability of bacteria for transforming the plant cell while high concentrations decrease their potential by competitive inhibition. The optimum bacterial concentration was 1.0 of OD_{600} on hairy root formation. However, transformation efficiency depended on plant species and sources of explants (Jia *et al.*, 2008).

By the end of a 4-week culture period, rhinacanthin-C, major compounds, accumulation in hairy roots 11325, 13332, 13333, and 15834 had reached 2.16 ± 0.097 % w/w, 0.83 ± 0.172 % w/w, 0.82 ± 0.121 % w/w, and 1.15 ± 0.054 % w/w, respectively. The content of rhinacanthin-C in hairy roots was lower than that of intact roots but did not different in statistical analysis. However, the hairy root cultures in this studied were a good model to improvement the rhinacanthin production. Because rhinacanthin content in hairy roots 11325 (2.16 ± 0.097 % w/w) higher estimate 2 times than previous studied of rhinacanthin production by *R. nasutus* tissue culture (0.0992 % w/w) (ภาคภูมิ, 2540). Wang and his groups (2006) indicated that hairy root of *Echinacea purpurea* produced less phenolic compound than the nature root did. Possibly, that is because hairy root were cultured for a comparatively short time, resulting in the less metabolite accumulation (Wang *et al.*, 2006). In the contrary, *P. indica* were studied on hairy root culture with by infecting leaf explants with *A. rhizogenes* strain ATCC 15834. The highest plumbagin content (naphthoquinone derivative) was found as 5.32 mg/g dry mass. Thereafter, a rise in biomass but a gradual decline in plumbagin content was noticed in cultures grown for 30 days (2.5 mg/g dry mass), 40 days (1.1 mg/g dry mass), and 50 days (0.5 mg/g dry mass) (Gangopadhyay *et al.*, 2008). In addition, the *rol* genes have a significant effect on the secondary metabolites in plants by affecting hormone contents and response of transgenic plants. Accumulation of secondary metabolites could be improved by physical and chemical means in hairy roots culture. For example, chemical techniques refer to changing nutritious ingredients of culture, adding hormone or precursor (Inoue *et al.*, 2003; Jia *et al.*, 2008). Satdive and his groups

(2007) have studied the influence of different culture media and elicitation on growth and production of azadirachtin by hairy root culture of *Azadirachta indica*. They found that the hairy root cultured on Ohyama and Nitsch' basal medium produced maximum yield of azadirachtin (0.0166 mg/g dry weight). The production of azadirachtin was enhanced by ~ 5-fold (0.074 mg/g dry weight) after addition of the biotic elicitor (Satdive *et al.*, 2007). Culturing hairy root in bioreactor was also effective to increase the content of secondary metabolites of hairy roots (Wang *et al.*, 2006).

To approach to the comparison of rhinacanthins production abilities, we also analyzed rhinacanthin production from the different hairy root lines which infection by 4 strains of *A. rhizogenes*. The hairy root lines infected by bacterium strain ATCC 11325 were selected. This hairy root contained the highest amount of as a mojor compound, rhinacanthin-C. The growth cycle of *R. nasutus* hairy root cultures appeared to be a normal sigmoid curve, with maximum growth occurring on 25 days. Time-course study of rhinacanthin production indicated gradually increase with the growth of hairy roots cultures during a growth period of 30 days.

In this study we have been successful in the selection of the hairy root lines infected by *A. rhizogenes* strain ATCC 11325. The hairy root 11325 could be cultured in MS semi-solid medium and incubated in the dark condition at 25°C on rotary shaker (80 rpm) and cultured for 30 days after inoculation for the highest hairy roots and highest accumulation of rhinacanthin-C (2.16 ± 0.097 % w/w). This result showed that *R. nasutus* hairy root 11325 could synthesize rhinacanthin-C in a nearly same amount with that of intact leaves. Further studies are necessary to establish a more productive hairy root system by manipulating the nutrient elements. Particularly, lowering the potassium level leaded to increasing of tropane alkaloid synthesis in *Atropa acuminate* (Khan and Harborne, 1991). Satdive and his groups (2007) have studied the influence of different culture media on growth and production of azadirachtin by hairy root cultures of *Azadirachta indica*. They reported that hairy roots cultured on Ohyama and Nitsch basal medium produced maximum yield of azadiractin (0.0166 % dried weight) (Satdive *et al.*, 2007). Moreover, Report on elicitation technique of *Drosera capensis* was investigated. Elicitation of 7-methyljuglone (naphthoquinone derivative) in greenhouse grown plants using salicylic acid and jasmonic acid showed that the amount of 7-methyljuglone was the highest in

the shoots of plants elicited with 50 μM of salicylic acid and jasmonic acid after 48 hrs and 3 hrs respectively. In roots, the highest amount of 7-methyljuglone was found in plants treated with 50 μM of salicylic acid and 100 μM of jasmonic acid after 1.5 hrs (Ziaratnia *et al.*, 2009). Therefore, the elicitation technique using several elicitors such as jasmonic acid and salicylic acid upon rhinacanthin production is also noteworthy.

CHAPTER 6

CONCLUSIONS

The conclusion could be drawn that

The induction of hairy roots was performed from leaf explants of the *R. nasutus* by infection with 4 strains of *A. rhizogenes* ATCC 11325, ATCC 13332, ATCC 13333, and ATCC 15834. The highest hairy root induction was observed in hairy root 13333 following by hairy root 15834, hairy root 11325 and hairy root 13332 with the percent hairy root induction of 70 %, 60 %, 55 %, and 25 %, respectively.

The presence of *rolB* and *rolC* genes in hairy root 11325, 13332, 13333, and 15834 showed *Agrobacterium*'s genes have successfully transferred to plant genome.

The rhinacanthin production was detected by using HPLC analysis. The hairy roots infection from 4 strains showed difference chemical patterns. The extract of hairy root 11325 exhibited rhinacanthins-C, -D, -N production while those of other strains exhibited only rhinacanthins-C and -D.

Rhinacanthin production of *R. nasutus* hairy root infected by *A. rhizogenes* strains 11325 contained the highest amount of rhinacanthin-C (2.16 ± 0.097 % w/w) as a major compound and followed by *A. rhizogenes* strains 15834 (1.15 ± 0.054 % w/w), 13332 (0.83 ± 0.172 % w/w) and 13333 (0.82 ± 0.121 % w/w).

Study of time-course of growth in the hairy roots cultures in semi-solid media infected by *A. rhizogenes* strain 11325. The growth pattern showed growth cycle spent 5 days lag phase and showed long linear phase (20 days) before entering to the stationary phase. The highest amount of the biomasss obtained was 25.6 mg dry weight on day 25. Time-course study of

rhinacanthin production indicated gradually increased parallel with the growth of hairy roots cultures during a growth period of 30 days.

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APPENDIX

Table A-1 Inorganic salt and vitamin composition of Murashige & skoog medium (MS) (DUCHEFA BIOCHEMIE B.V.) (Murashige and Skoog, 1962)

Constituent	Concentration (mg/liter)
Macronutrients:	
$CaCl_2$	332.02
KH_2PO_4	170.00
KNO_3	1,900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Micronutrients:	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_2BO_3	6.20
KI	0.83
$MnSO_4.H_2O$	16.90
$NaMoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
Sucrose (g)	30.00
Vitamins:	
Glycine	2.00
myo-Inositol	100.00
Nicotinic acid	0.50
Pyridoxine hydrochloride	0.50
Thiamine hydrochloride	0.10
pH	5.8

To prepare the MS medium using the mixed powder 4.4 g/L

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Proceedings

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