Enhancing Strategies of Naphthoquinone Production in *Impatiens balsamina* Root Cultures

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ชื่อวิทยานิพนธ์ กลยุทธการเพิ่มการสร้างสาร naphthoquinone ในรากเพาะเลี้ยงของเทียนบ้าน
ผู้เขียน นาย อธิป สกุลเผือก
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บทคัดย่อ

มีการสร้างรากเพาะเลี้ยงของเทียนบ้านเพื่อใช้ในการศึกษาการสร้าง lawsone, lawsone methyl ether และ 3,3′-methylenebislawsone เป็นสารกลุ่ม naphthoquinone ที่เป็นองค์ประกอบหลักในรากเพาะเลี้ยงของเทียนบ้าน และมีรายงานว่ามีฤทธิ์ทางเภสัชวิทยาที่หลากหลาย ในการศึกษาครั้งนี้ได้ใช้การเพาะเลี้ยงเนื้อเยื่อพืชเพื่อเพิ่มการสร้าง naphthoquinone ได้แก่ การคัดเลือกสายพันธุ์ การปรับเปลี่ยนปริมาณสารควบคุมการเจริญเติบโตของพืช การเติมสารตั้งต้นในวิถีชีวสังเคราะห์ และการเหนี่ยวนำให้เกิดรากขนอ่อนโดย *Agrobacterium rhizogenes* และ elicitation

วิธีวิเคราะห์สาร naphthoquinone ในสารสกัดจากรากเพาะเลี้ยงและใบของเทียนบ้านด้วยเทคนิค HPLC ได้ถูกพัฒนาขึ้นโดยใช้คอลัมน์ C18 และเฟสเคลื่อนที่เป็นเมธานอลกับ 2% กรดอซีติคในน้ำ โดยทำการชะ ในขั้นตอนการตรวจสอบความแม่นยำของวิธีวิเคราะห์พบว่า วิธีวิเคราะห์นี้มีค่าความแม่นยำที่ดี (%recovery อยู่ในช่วง 96-101%) ความสัมพันธ์ของสมการในช่วงที่วิเคราะห์นั้นมีความเป็นเส้นตรงดี ($r^2 > 0.9995$) นอกจากนี้ วิธีวิเคราะห์ยังมีค่าความเที่ยงตรงสูง ทั้งการวิเคราะห์ภายในวันเดียวกันและการวิเคราะห์ต่างวัน (%RSD < 5%)

ในการศึกษาสายพันธุ์ของต้นเทียนบ้านที่ให้สีของดอกต่างกันพบว่า ในใบของต้นเทียนบ้านดอกสีชมพูให้ค่าเฉลี่ยของปริมาณ naphthoquinone รวมสูงที่สุด ในขณะที่เทียนบ้านดอกสีแดงมีปริมาณสาร naphthoquinone รวมต่ำที่สุด จากนั้นได้มีการเหนี่ยวนำให้เกิดพรากเพาะเลี้ยงจากเทียนบ้านทั้งสี่สายพันธุ์เพื่อศึกษาผลของพืชต้นแม่ พบว่ารากเพาะเลี้ยงที่สร้างขึ้นจากใบของเทียนบ้านที่มีดอกสีชมพู มีปริมาณการสร้าง naphthoquinone รวมได้สูงที่สุดเมื่อเปรียบเทียบกับรากเพาะเลี้ยงที่ได้จากใบของเทียนบ้านสายพันธุ์อื่น เมื่อศึกษาผลของสารควบคุมการเจริญเติบโตของพืชพบว่า องค์ประกอบของสารควบคุมการเจริญเติบโตของพืชที่เหมาะสมต่อการสร้าง naphthoquinone คือ α-naphthalene acetic acid 0.1 mg/L, kinetin 1.0 mg/L และ 6-benzyladenine 2.0 mg/L ในสูตรอาหาร Gamborg's B5

ถึงแม้ว่ารากเพาะเลี้ยงของเทียนบ้านมีความสามารถในการสร้าง lawsone และ 3,3′-methylenebislawsone ได้ดีแต่มีปริมาณการสร้างของ lawsone methyl ether ที่ต่ำมาก จึงได้ใช้เทคนิคการเติม methionine เพื่อเป็นสารตั้งต้นในวิถีชีวสังเคราะห์ elicitation และการเหนี่ยวนำให้เกิดรากขนอ่อนโดย *Agrobacterium rhizogenes* เพื่อที่จะเพิ่มปริมาณ lawsone methyl ether ในรากเพาะเลี้ยงของเทียนบ้าน ในการศึกษาการเติม methionine เพื่อเป็นสารตั้งต้นในวิถีชีวสังเคราะห์ของ naphthoquinone พบว่า methionine สามารถเพิ่มปริมาณของ 3,3′-methylenebislawsone ได้ดี พบว่าการเติม methionine ในปริมาณ 300 mg/L ในรากเพาะเลี้ยงที่มีอายุ 21 วันพบว่าสามารถทำให้รากเพาะเลี้ยงสร้าง 3,3′-methylenebislawsone ได้สูงกว่ารากเพาะเลี้ยงปกติถึง 2.9 เท่า อย่างไรก็ตาม methionine ในความเข้มข้นสูงจะเป็นพิษต่อรากเพาะเลี้ยงของเทียนบ้าน

รากขนอ่อนที่เหนี่ยวนำด้วย *A. rhizogenes* จะมีการเจริญเติบโตที่รวดเร็ว และมีการสร้าง naphthoquinone ที่ดี รากขนอ่อนของเทียนบ้านสามารถสร้าง lawsone methyl ether ได้แม้จะเลี้ยงในอาหารที่ปราศจากฮอร์โมน อย่างไรก็ตามปริมาณของ lawsone และ lawsone methyl ether ในรากขนอ่อนนั้นไม่แตกต่างกับรากเพาะเลี้ยงของเทียนบ้านปกติ

การเพิ่มการสร้าง naphthoquinone ในรากเพาะเลี้ยงของต้นเทียนบ้านด้วยเทคนิค elicitation โดยใช้ elicitor ชนิดต่างๆ ได้แก่ yeast extract, *Candida albicans* homogenate, *Trichophyton rubrum* homogenate, chitosan และ methyl jasmonate พบว่าชนิดและความเข้มข้นของ elicitor ที่ใช้มีผลต่อการเพิ่มการสร้าง naphthoquinone โดยพบว่าการใช้ methyl jasmonate เป็น elicitor สามารถเพิ่มการสร้างสาร lawsone และ lawsone methyl ether ได้สูงกว่ารากเพาะเลี้ยงปกติถึง 8.6 และ 11.3 เท่า ตามลำดับ และจากการปรับสภาวะที่เหมาะสมในการใช้ methyl jasmonate เป็น elicitor พบว่าความเข้มข้นของ methyl jasmonate ที่เหมาะสมในการเพิ่มการสร้าง naphthoquinone คือ 300 μM อายุของรากเพาะเลี้ยงที่เหมาะสมในการเติม methyl jasmonate คือ 21 วัน และระยะเวลาที่รากเพาะเลี้ยงสัมผัสกับ methyl jasmonate ที่เหมาะสมคือ 36 ชั่วโมง ซึ่งในสภาวะดังกล่าวสามารถเพิ่มการสร้างสาร lawsone, lawsone methyl ether และ 3,3′-methylenebislawsone ได้สูงถึง 10.0, 0.78 and 0.23 mg/g DW ตามลำดับ

นอกจากนี้ยังสามารถแยก luteolin, 2,3-dihydroxy-1,4-naphthoquinone และ echinocystic acid ซึ่งเป็นสารในกลุ่ม flavonoid, napthoqunone และ triterpene ตามลำดับ จากรากเพาะเลี้ยงของเทียนบ้านซึ่งเป็นสารที่ไม่เคยมีรายงานว่าพบในเทียนบ้านมาก่อน และยังพบสารโมเลกุลเล็ก เช่น phthalic acid, phthalic anhydride และ *p*-hydroxybenzoic acid ซึ่งเป็นสารมัธยันตร์ (intermediate) ในวิถีชีวสังเคราะห์ของ naphthoquinone ในเทียนบ้าน

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ABSTRACT

Impatiens balsamina root cultures have been established in order to study on their naphthoquinone production. Lawsone, lawsone methyl ether and 3,3′-methylenebislawsone are main naphthoquinones found in *I. balsamina* root cultures which possess variety of pharmacological activities. In this study, many strategies were applied to the root cultures system in order to increase naphthoquinone production including effect of donor plant, hormonal manipulation, elicitation, feeding of biosynthetic precursor and using *Agrobacterium rhizogenes* to induce the transformed hairy root cultures.

A HPLC method for the simultaneous determination of three naphthoquinones has been developed and validated. The recovery of the method was 96–101% and linearity ($r^2 > 0.9995$) was obtained for all naphthoquinones. A high degree of specificity, as well as repeatability and reproducibility (%RSD < 5%), were also achieved.

After determination of the naphthoquinone content in 4 different varieties of *I. balsamina* leaves, found that the pink flower plant (IbP) produced the highest total naphthoquinone, while the red flower plant produced the lowest one. The effects of donor plants, and plant growth regulators on naphthoquinone production were evaluated. The leaf explants of four *I. balsamina* strains were used to establish the root cultures. The root cultures established from the IbP explants were capable of producing higher content of total naphthoquinones than

those established from the other strains. The results suggest that the tissue cultures initiated from the high-yielding donor plants are capable of producing higher content of secondary compounds than those initiated from low-yielding donor plants. In addition, plant growth regulator manipulation exhibited that a combination of 0.1 mg/L NAA, 1.0 mg/L kinetin and 2.0 mg/L BA in B5 medium was capable of increasing naphthoquinone production in *I. balsamina* root cultures.

Although the root cultures were capable of producing lawsone and 3,3′-methylenebislawsone in high amount, lawsone methyl ether production was very low. In order to increase of lawsone methyl ether, feeding of methionine as a precursor of lawsone methyl ether, elicitation and induction of hairy root cultures by *Agrobacterium rhizogenes* were investigated. Increased 3,3′-methylenebislawsone production instead of lawsone methyl ether production was achieved by feeding of methionine into *I. balsamina* root cultures. However, a high concentration of methionine may cause toxicity to the root cultures. Treatment 21-day old root cultures with 300 mg/L methionine was capable of increasing 3,3′-methylenebislawsone content up to 2.9 times higher than the untreated root cultures.

High growth rate and good productivity of naphthoquinones were a characteristic of transformed hairy root cultures induced by *A. rhizogenes*. The transformed hairy root cultures of *I. balsamina* were capable of producing lawsone methyl ether even in hormone-free B5 medium. However, lawsone and lawsone methyl ether levels in transformed hairy root cultures were not different from those of the ordinary root cultures.

I. balsamina root cultures were treated with yeast extract (YE), *Candida albicans* homogenate (CAH), *Trichophyton rubrum* homogenate (TRH), chitosan (CHI) and methyl jasmonate (MJ). MJ was the most effective elicitor and appeared to selectively increase production of lawsone and lawsone methyl ether. An investigation of the optimum concentration of MJ, period of elicitor contract and age of the root cultures for elicitation revealed that the

treatment of 21 day old root cultures with 300 μM MJ for 36 hr resulted in an increased production of these naphthoquinones. The production levels were 10.0, 0.78 and 0.23 mg/g DW, respectively for lawsone, lawsone methyl ether and 3,3′-methylenebislawsone, which were 10.4-, 26.0- and 1.3-fold higher than the levels for the controls.

In addition various secondary metabolites have been isolated from *I. balsamina* root cultures, and identified as luteolin, a flavonoid, 2,3-dihydroxy-1,4-naphthoquinone, a naphthoquinone, and echinocystic acid, a triterpenoid. This is the first report on occurrence of these compounds in this plant. The intermediate in lawsone biosynthesis such as phthalic acid, phthalic anhydride and *p*-hydroxybenzoic acid were found in the unorganized root cultures.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	=	Percent
% RSD	=	Percentage relative standard deviation
/	=	Per
µg	=	Micro gram
µL	=	Microliter
µm	=	Micrometer
2,4-D	=	2,4-Dichlorophenoxyacetic acid
B5	=	Gamborg's B5 medium
BA	=	6-Benzyladenine
CAH	=	*Candida albicans* homogenate
CC	=	Column chromatography
$CHCl_3$	=	Chloroform
CHI	=	Chitosan
cm	=	Centimeter
DW	=	Dry weight
EtOAc	=	Ethyl acetate
g	=	Gram
Hex	=	Hexane
HPLC	=	High performance liquid chromatography
hr	=	Hour
IAA	=	Indole-3-acetic acid
IbP	=	*Impatiens balsamina* pink flower plant
IbR	=	*I. balsamina* red flower plant
IbV	=	*I. balsamina* violet flower plant
IbW	=	*I. balsamina* white flower plant
IC_{50}	=	50% Inhibitory concentration
L	=	Liter
LD_{50}		50% Lethal dose

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

LOD	=	Limit of detection
LOQ	=	Limit of quantification
M	=	Molar
m.p.	=	Melting point
MeCN	=	Acetronitrile
MeOH	=	Methanol
mg/g DW	=	Milligram per gram dry weight
MIC	=	Minimum inhibitory concentration
MBC		Minimum bactericidal concentration
min	=	Minute
MJ	=	Methyl jasmonate
mL	=	Milliliter
Mm	=	millimeter
NAA	=	α-Naphthalene acetic acid
NaOH	=	Sodium hydroxide
nm	=	Nanometer
NMR	=	Nuclear magnetic resonance
ºC	=	Degree Celsius
OSB	=	*O*-Succinyl benzoic acid
PCR	=	Polymerase chain reaction
PDA	=	Photo diode-array
pH	=	Potential of Hydrogen
rpm	=	Round per minute
r^2	=	Correlation coefficient
SAM	=	*S*-adenosylmethionine
SE	=	Standard error of the mean
TFA	=	trifluoroacetic acid
TLC	=	Thin layer chromatography

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

t_R	=	Retention time
TRH	=	*Trichophyton rubrum* homogenate
UV	=	Ultra violet
w/v	=	Weight by volume
w/w	=	Weight by weight
YE	=	Yeast extract
δ	=	Chemical shift

CHAPTER 1

INTRODUCTION

1. Background and Rationale

Impatiens balsamina Linn. (or Thian-Ban in Thai) belongs to the plant family Balsaminaceae. It has long been used as Thai traditional medicine [1]. The leaves and roots have been used to treat thorn or glass-puncture wounds, felon, chronic ulcers caused by allergic reaction to detergent and the stems have been used for the treatment of abscesses and ingrown nail [1]. The groups of compounds commonly found in this plant are naphthoquinone [2], coumarin [2-3], phenolic acid, flavonoid [4] and anthocyanidin[3]. Lawsone (2-hydroxy-1,4-naphthoquinone) and lawsone methyl ether (2-methoxy-1,4-naphthoquinone) (Figure 1) are naphthoquinones found in *I. balsamina*, which posses antifungal activity [5]. Especially lawsone methyl ether exhibits potent antifungal activity against *Trichophyton rubrum, T. mentagrophytes, Microsporum gypseum, Candida albicans, Aspergillus niger, Cryptococcus neoformans, and Epidermophyton floccosum*[5].

In 1995 Panichayupakaranant *et al*. [6] had established the root cultures of *I. balsamina*. It was found that the root cultures of *I. balsamina* could produce a number of natural products, mostly naphthoquinone and coumarin derivatives. Two novel compounds, 3,3′-methylenebislawsone and 4,4′-biisofraxidin (Figure 1) were isolated from the root cultures of *I. balsamina* along with two known naphthoquinones (lawsone and lawsone methyl ether) and two known coumarin derivatives (scopoletin and isofraxidin) (Figure 1).

Although antimicrobial activities of 3,3′-methylenebislawsone have never been reported, *in vivo* antipruritic effect of this compound has been reported [7].

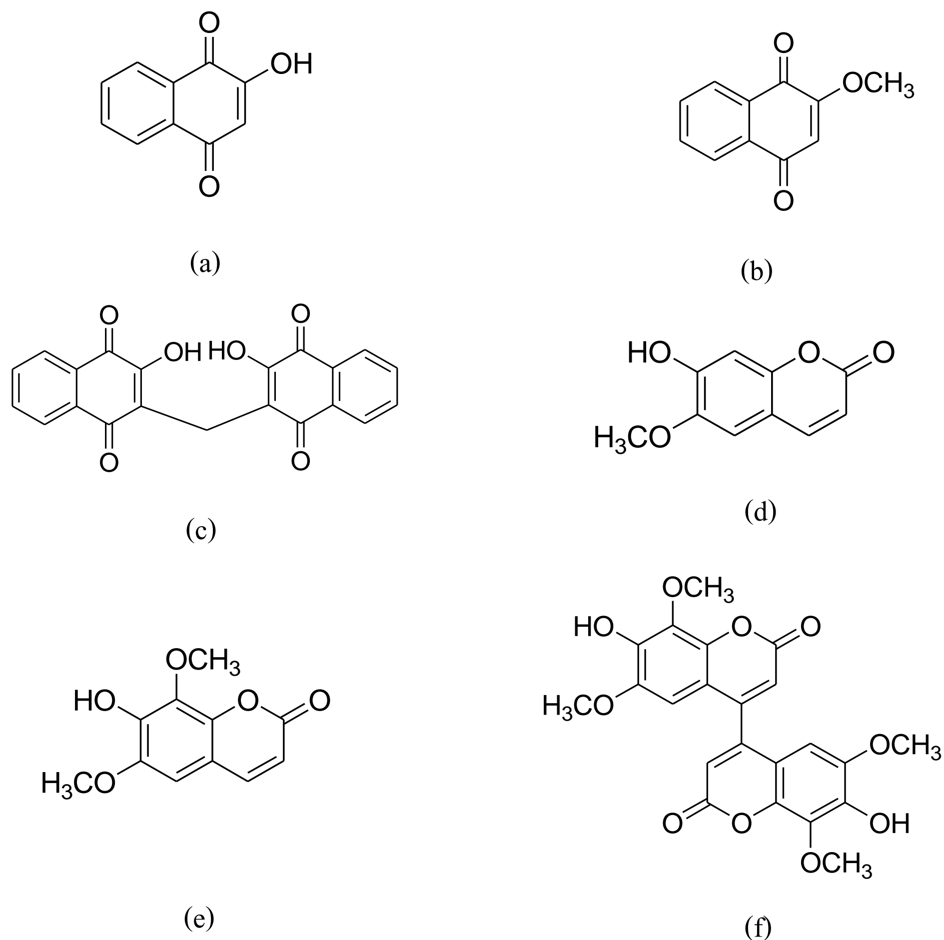


Figure 1 Chemical structures of (a) lawsone, (b) lawsone methyl ether, (c) 3,3′-methylenebislawsone, (d) isofraxidin, (e) scopoletin and (f) 4,4′-biisofraxidin

However, the content of lawsone methyl ether and 3,3′-methylenebislawsone accumulated in the root cultures were rather low. Plant cell and tissue cultures have been used as alternative sources to the whole plant for production of valuable phytochemical. Numerous strategies have been developed to improve the secondary metabolite production of plant tissue cultures, such as medium optimization, cell line selection, cell immobilization, precursor addition, elicitation, genetic transformation hairy root cultures, metabolic engineering and integrated bioreactor engineering [8].

Strain improvement begins with the choice of a parent plant with high contents of the desired products for callus induction to obtain high producing cell lines. Statistically high-producing plants give rise to high-producing cell lines [8]. In 2001 Panichayupakaranant have

studied on naphthoquinone formation in cell suspension of *I. balsamina* from 40 different parent plants and the result was supported this hypothesis [9]. Information concerning the factors regulating secondary metabolism is as important as the selection of high-producing cell lines in increasing the production of secondary metabolites.

A number of physical and chemical factors that could influence secondary metabolism in plant cell cultures have been found. Optimization of the hormone concentration and combinations are often effective; high auxin levels, although good for cell growth, are often deleterious to secondary metabolite production [8].

Transformed hairy root cultures are obtained after the successful transformation of a plant with *Agrobacterium rhizogenes*. Genetic transformation mediated by *Agrobacterium* sp. involves the transfer of a DNA molecule (T-DNA) from the bacterium to the eukaryotic host cell, and its integration into the host genome [10]. This T-DNA carries a set of genes that encode enzymes, which control auxin and cytokinin biosynthesis. These hormones induce the formation of hairy roots that emerges from the wounding site. Hairy root characters are high growth rate without exogenous hormone, absence of geotropism and high branching [11]. Furthermore, they often produce secondary metabolites for a long period of time, unlike natural roots [12]. This growth can be assimilated to an exponential model, when the number of generations of lateral roots becomes very large. In addition to their growth capacities, hairy roots display interesting properties regarding the production of secondary compounds. The metabolite pattern found in hairy roots is similar, but not always identical to that of plant roots. A major characteristic of hairy roots is that they are able to produce secondary metabolites concomitantly with growth. Hence, it is possible to get a continuous source of secondary compounds from actively growing hairy roots, unlike the usual results obtained with cell suspension cultures [13].

Several report suggested that the feeding of biosynthetic-precursor could improve secondary metabolite production. From the biosynthetic pathway of lawsone methyl ether, to enhance lawsone methyl ether production, feeding of methionine as a precursor of SAM, should be investigated.

Elicitation is the induction of secondary metabolite production by biotic (bacteria and fungi cell wall, yeast extract and microbial homogenate) or abiotic molecules (jasmonic acid and methyl jasmonate) or stress treatment (UV light, temperature or heavy metal) [14]. Many

studies have been successful in improving the secondary metabolite production by elicitation technique. Methyl jasmonate has been used for increasing of diterpene production such as paclitaxel [15]. The study on plumbagin production in *Plumbago rosea* cell suspension cultures showed that chitosan and yeast extract could enhance the plumbagin production up to 7-times higher than control [16]. Several studies reported that secondary metabolite production induced by using combination of two or more elicitors was higher than using one elicitor. For example, using combination of methyl jasmonate and yeast extract capable to elevate silymarin level in *Silybum marianum* cell suspension cultures higher than using methyl jasmonate or yeast extract alone [17], combination of methyl jasmonate, salicylic acid and fungal elicitor could significantly increase paclitaxel production in *Taxus baccata* cell cultures [18].

As part of our interest in increased production of the naphthoquinones in *I. balsamina* root cultures, several methods including selection of high yielding plant, medium manipulation, precursor feeding, transformed hairy root cultures and elicitation were investigated. Moreover, phytochemical study of the root cultures was carried out to make a chemical library of this plant.

2. Objectives

1. To study on naphthoquinone production in *I. balsamina* root cultures
2. To develop the suitable HPLC conditions for quantitative analysis of naphthoquinones in *I. balsamina* leaf and root culture extracts
3. To increase naphthoquinone production in *I. balsamina* root cultures through selection of high yield naphthoquinone strain, hormonal manipulation, precursor feeding, transformed hairy root cultures and elicitation
4. To isolate secondary metabolites from *I. balsamina* root cultures

CHAPTER 2

LITERATURES REVIEW

1. Botanical aspect of *Impatiens balsamina* L.

Impatiens balsamina Linn. belongs to the plant family Balsaminaceae. It has a number of synonyms such as *Balsamina hortensis* DC., *B. impatiens*, Hort. and *Impatiens coccinea* Sims. In Thailand it is known as Thian-ban, Thian-dok, Thian-Thai or Thian-suan. The other local names in various countries are Garden Balsam (English); Housenga (Japanese); Feng Hsien (China); Bond sun Hwa (Korean); Kamantigi (Tagalog); Saungga (Zulu); Suranga (Bikol, Bisaya).

I. balsamina is a native to southern Asia in India and Myanmar and now widely cultivated [19]. It is grown ornamentally for shaded the garden. The plants are erect, tender, succulent annual or perennial herb to 20-75 cm high; pubescent or nearly glabrous (Figure 2). *I. balsamina* leaves are alternate; narrowly or broadly lanceolate; 8-10 by 2-3 cm, tapering at tip and base, consisting of deeply serrate margin and have the glandular petiole. The flowers are red, pink, purple, or white. One of the sepals seems to have three and one long spurred. The sepals and petals are similar in the color and difficult to distinguish. The petals are apparently three, but two of them probably represent two units, thus making five petals. The fruits are explosive long woolly capsules. They have five carpels and very thin partitions, and seeds borne on axile placenta. When the capsules are ripe, a pinch will cause the valves to separate and contract. The seeds are then thrown with considerable force [19].

I. balsamina is easy to cultivate. They are propagated by seeds. The seeds are large and germinate quickly. The plants prefer a rich, sandy loam and must not suffer from moisture [19].

Figure 2 *Impatiens balsamina* (Balsaminaceae)

2. Chemical constituents of *Impatiens balsamina*

The groups of compounds found in *I. balsamina* are naphthoquinones, coumarins, phenolic acids, flavonoids, anthocyanidins, baccharanes and steroids. A list of the compounds in various parts of *I. balsamina* is shown in Table 1.

Table 1 Chemical constituents in various parts of *I. balsamina*

Plant part	Category	Chemical substance	References
Leaves	Naphthoquinone	lawsone	[2]
		lawsone methyl ether	[2]
	Phenolic acid	caffeic acid	[2]
		p-coumaric acid	[2]
		ferulic acid	[2]
		gentisic acid	[2]
		p-hydroxybenzoic acid	[2]
		sinapic acid	[2]
	Flavonoid	kaempferol	[20]
	Coumarin	scopoletin	[2]
Flower	Naphthoquinone	lawsone	[3]
		lawsone methyl ether	[21]
	Phenolic acid	*p*-coumaric acid	[22]
		ferulic acid	[22]
		Hydroxycinnamic acid	[22]
	Flavonoid	kaempferol	[3]
		myricetin	[3]
		quercetin	[3]
	Anthocyanidin	pelargonidin	[3]
		cyanidin	[3]
		malvidin	[3]
		peonidin	[3]

Table 1 (continued) Chemical constituents in various parts of *I. balsamina*

Plant part	Category	Chemical substance	References
Flower	Enzyme	*O*-methyltransferase	[23]
		flavonoid-3-β-glucosidase	[24]
Stem	Tetrahydronaph-thalenes	1α,2α-diol-4α-ethoxy-1,2,3, 4-tetrahydronaphthalene	[25]
		1α,2α,4β-triol-1,2,3,4-tetrahydronaphthalene	[25]
	Flavonoid	kaempferol	[25]
		kaempferol-3-*O*-β-D-glucoside	[25]
		quercetin	[25]
		quercetin-3-*O*-β-D-glucoside	[25]
Seed	Flavonoid	quercetin	[4]
	Fatty ester	ethyl palmitate	[26]
		ethyl stearate	[26]
		ethyl oleate	[26]
	Baccharane	hosenkol A	[27]
	Baccharane glycoside	hosenkoside A,B,C,D,E	[27]
		hosenkoside F,G,H,I,J,K	[28]
		Presapogenin I	[28]
	Steroid	β-sitosterol	[29]
		β-amyrin	[30]
		α-spinasetrol	[30]
	Monoglyceride	glycerol-1,9-octadecanoate	[26]
	Peptide	Ib-AMP 1,2,3,4	[31]
Pericarp	Naphthoquinone	2,2'-ethylidenebis(3-hydroxy-1,4-naphthoquinone)	[32]
		3,3′-methylenebislawsone	[32]
		2-methoxy-3-(2-hydroxyethyl)-1,4-naphthoquinone	[32]

Table 1 (continued) Chemical constituents of various parts of *I. balsamina*

Plant part	Category	Chemical substance	References
Root cultures	Naphthoquinone	lawsone	[6]
		lawsone methyl ether	[6]
		3,3′-methylenebislawsone	[6]
	Coumarin	isofraxidin	[6]
		scopoletin	[6]
		4,4′-biisofraxidin	[33]
	Steroid	spinasterol	[6]

3. Uses of *Impatiens balsamina*

I. balsamina has long been used as Thai traditional medicine [1]. The leaves and roots have been used to treat thorn or glass-puncture wounds, felon, chronic ulcers caused by allergic reaction to detergent and the stems have been used for the treatment of abscesses and ingrown nail [1].

In China, the powdered seeds of *I. balsamina* have been used to treat difficult labor, to suppress puerperal pain, to act as an emmenagogue, expectorant, and antidote for poison from fish. The seeds are also used to soften fish bone while cooking and to stop hiccups. When mixed the powdered seeds with arsenious acid and apply to dental caries, the teeth can be removed easily. The lower part of stem pounded to juice and added to rice liquor is used as a cure for contusions. It may also be used to wash abscesses and reduce swellings. The dried stems are used for improved circulation and to relieve pain in case of hard labor, leg cramps, and rheumatism. The flowers are mucilaginous and cooling, and are used for the treatment of snake bite, lumbago and intercostals neuralgia. They are thought to improve the circulation and to improve the circulation and to relieve stasis [34].

In Philippines, the leaves have been pounded and used in poultices to dissolve felons. As in Malaysia the leaves of *I. balsamina* have been used for poulting broken and torn nails [35].

In Indo-China, a decoction of leaves has been used to wash the hair and supposedly to promote its growth. The oil from *I. balsamina* seed may be used for cooking and for burning lamps. It is also suitable for the surface-coating industry [36].

In Korea the whole plant of *I. balsamina* has been used for treatment of scrofula, carbuncle and dysentery [5]. The dried herb is either boiled in water to make a tea used to treat systemic bacterial and fungal infections or applied directly on the skin or nails in a plaster form to treat local infections [37].

4. Pharmacological activities

4.1 Antimicrobial activity

Many studies have been reported that lawsone posses antifungal activity against *Alternaria, Absidia, Penicillium, Cladosporium, Trichophyton, Microsporum* [2, 38-39]. Furthermore a simple naphthoquinone derivative, lawsone methyl ether isolated from *I. balsamina* exhibited potent antifungal activity against *Trichophyton rubrum, T. mentagrophytes, Microsporum gypseum, Candida albicans, Aspergillus niger, A. fumigates, Cryptococcus neoformans, Fusarium oxysporum and Epidermophyton floccosum* [5, 37, 39-40]. Recently, lawsone methyl ether (0.025% w/v) mouthwash has been evaluated for treatment of oral candidiasis in AIDS patients and denture subjects. It was found that the lawsone methyl ether mouthwash was effective as 0.12% chlorhexidine mouthwash [41].

It has been reported that lawsone and lawsone methyl ether have antibacterial activities against *Bacillus subtilis, B. cereus, B. megaterium, Aeromonas salmonicida, Aquaspirillum serpens, Staphylococcus aureus, S. epidermidis Salmonella typhimurium, Pasteurella, Clostridium paraputrificum, C. perfringens, Escherichia coli, Brucella and Neisseria* [5, 37, 39-40, 42]. The activity of lawsone methyl ether on gram negative bacteria was lower than that of gram positive bacteria [5]. In the study of anti *Helicobacter pylori* activity of *I. balsamina* showed that all part (root/stem/leaf, seed, and pod) extracts of *I. balsamina* L. exhibited bactericidal *H. pylori* activity. The acetone and ethyl acetate pod extracts of *I. balsamina* exhibited very strong anti *H. pylori* activity [43]. Lawsone methyl ether and spinasterol were

isolated from *I. balsamina* and exhibited potent activities against antibiotic (clarithromycin, metronidazole and levofloxacin) resistant *H. pylori* especially lawsone methyl ether which showed the MICs and MBCs in the ranges of 0.156-0.625 and 0.313-0.625 mg/mL, respectively [44]. In contrast with lawsone methyl ether, a study various on naphthoquinones on *H. pylori* showed that lawsone exhibited mild activity against *H. pylori* [45].

Not only naphthoquinones that possess antimicrobial activity, Lim *et al*. [46] had isolated kaempferol and quercetin from flower of *I. balsamina* using anti-*Propionibacterium acnes* bioassay-guided isolation. These compounds exhibited strong inhibitory activity against *P. acnes* with MIC values of $\leq$ 32 µg/mL. They also showed synergistic activity when combined with erythromycin or clindamycin. Four antifungal peptides were isolated from *I. balsamina* seeds and identified as Ib-AMP1, Ib-AMP2, Ib-AMP3 and Ib-AMP4. These peptides exhibited potent antifungal avtivities against *Botrytis cinerea*, *Fusarium culmorum, Neurospora crassa, Saccharomyces cerevisiae and Pichia pastoris* without any toxicity to mouse myeloma cell or hemolytic to rabbit erythrocytes [47].

4.2 Anti-allergy activity

Ethanolic extract from the petals of *Impatiens balsamina* in mice showed that the extracts from the white flowers of *I. balsamina* had significant anti-anaphylactic activity. Bioassay-guided isolation showed that kaempferol-3-rutinoside and lawsone posses anti-anaphylactic activity, they were significantly inhibited the decrease of blood flow. Lawsone and 3,3′-methylenebislawsone were evaluated for *in vivo* antipruritic activities. These compounds significantly inhibited scratching behavior induced in mice by histamine-releasing agents, such as Dextran T40 and compound 48/80 (COM) [7].

4.3 Anti-tumor activity

Isolation of lawsone methyl ether from *I. balsamina* leaves showed an intensive *in vitro* anti-tumor activity against HepG2 cells with IC_{50} value of 6.08 ± 0.08 mg/L [48]. Lawsone was studied for *in vitro* anti-tumor promoting effect on Epstein-Barr virus early antigen activation produced by the tumor promoter. It was found that lawsone exhibited moderate anti-tumor promoting activity [49].

4.4 Anti-inflammatory activity

The study on inhibition of cyclooxygenase-2 (COX-2) showed that 2,2'-ethylidenebis(3-hydroxy-1,4-naphthoquinone) was a COX-2 selective inhibitor with IC_{50} value of 0.2 µM (selectivity rate = 505 times) [32].

4.5 Anthelmintic activity

Study on seed oil of *I. balsamina* revealed that the seed oil exhibited moderate to significant anthelmintic activity against *Pheritima posthuma* at the concentration of 10 mg/mL and caused the parasite death at the concentration of 100 mg/mL [50].

4.6 Antimalarial acivity

Lawsone exhibited mild antimalarial activity against *Plasmodium falciparum* with IC_{50} value of 18.0 µM with the mechanism of action by undergo enzyme-dependent single-electron reduction with subsequent redox cycling of their anion-radicals and formation of reactive oxygen species (ROS) and cause oxidative stress to the parasite [51].

5. Toxicity assessment of *I. balsamina* and its naphthoquinone

It has been reported that the chloroform extract of *I. balsamina* gave the LD_{50} value of 0.67 g/kg when given intraperitoneally in mice. The cream preparation, containing of 1% chloroform extract, potentiated irradiation caused by the cream base alone when tested on male rabbits. This effect is potentiated by UV exposure [52]. Lawsone methyl ether exhibited low acute toxicity with a LD_{50} value of 70.7 mg/kg upon intraperitoneal administration in mice [53]. Solution of lawsone methyl ether potassium salt caused skin irritation by produced erythema with some papulosquamous in the cumulative skin irritation test [53]. Toxicity of lawsone had been evaluated and found that lawsone was negative for induction of bone marrow micronuclei in mice up to 72 hr after administration in two different vehicles, and its *in vitro* clastogenicity was not due to oxidative damage. These results concluded that lawsone poses no or negligible genotoxic risk [54].

6. Biosynthetic pathway of *I. balsamina* naphthoquinones

O-Succinylbenzoate pathway has been demonstrated as a route for biosynthesis of bacterial menaquinones and some plant naphthoquinones and anthraquinones. It involves incorporation of shikimic acid and the intermediate, *O*-succinylbenzoic acid (OSB), into the naphthoquinones. Experiments with [1,6-$^{14}C_2$] and [3-^{3}H]-shikimic acid establish that lawsone is derived from shikimic acid 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 C-3 unit has been identified as having its origin from α-ketoglutaric acid [55]. OSB had been reported to arise from α-ketoglutaric acid, chorismic acid and thiamine diphosphate (TPP), presumably *via* the succinic semialdehyde thiamine diphosphate, derived from decarboxylation of α-ketoglutarate [55-57] (Figure 3). The substrate for biosynthesis of OSB is isochorismate, which derived from chorismate by the action of isochorismate synthase (isochorismate hydroxymutase) [58]. Succinic semialdehyde thiamine diphosphate attacks isochorismic acid in a Michael-type reaction by the action of thiamine phosphatase [59-60]. Loss of the thiamine cofactor, elimination of pyruvic acid, and then dehydration yield the intermediate OSB. This is activated by formation of a coenzyme A ester, and a Dieckmann-like condensation allows ring formation to yield 1,4-dihydroxy-2-naphthoic acid. 1,4-Dihydroxy-2-naphthoic acid, or its diketo tautomer, have been implicated in the biosynthesis of a wide range of plant naphthoquinones and anthraquinones (Figure 4). Lawsone is formed by an oxidative sequence in which hydroxyl replaces the carboxyl. A further interesting elaboration is the synthesis of an anthraquinone skeleton by effectively cyclizing a dimethylallyl substituent on to the naphthoquinone system. Rather little is known about how this process is achieved but many are known from the results of labeling studies [59]. The study on enzymic methylation of lawsone clarified that lawsone is further methylated at hydroxyl group by *O*-methyltransferase and use *S*-adenosylmethionine (SAM) as a methyl group donor for the enzymatic reaction [61] (Figure 5).

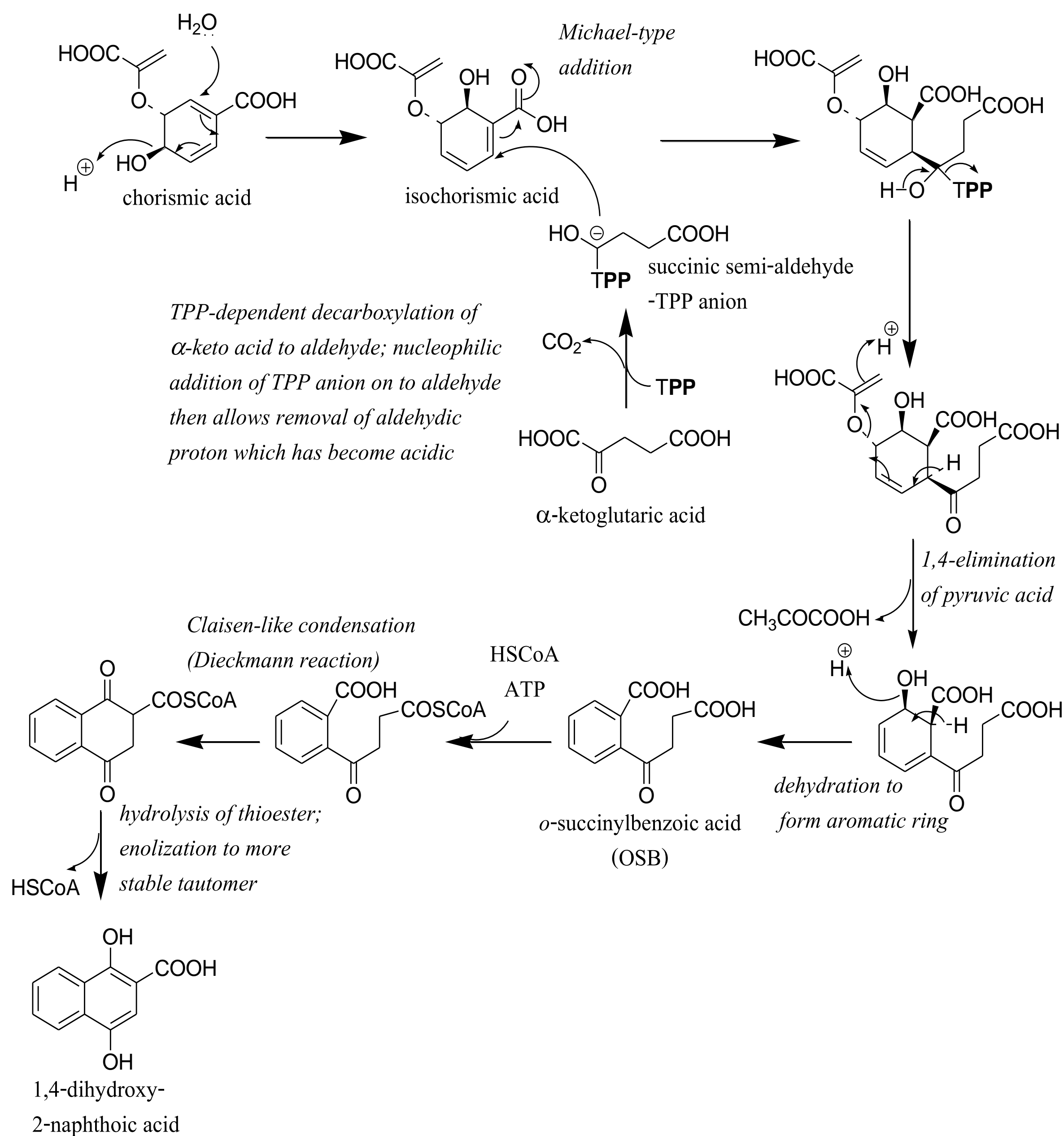


Figure 3 Biosynthetic pathway of lawsone in *I. balsamina* [59]

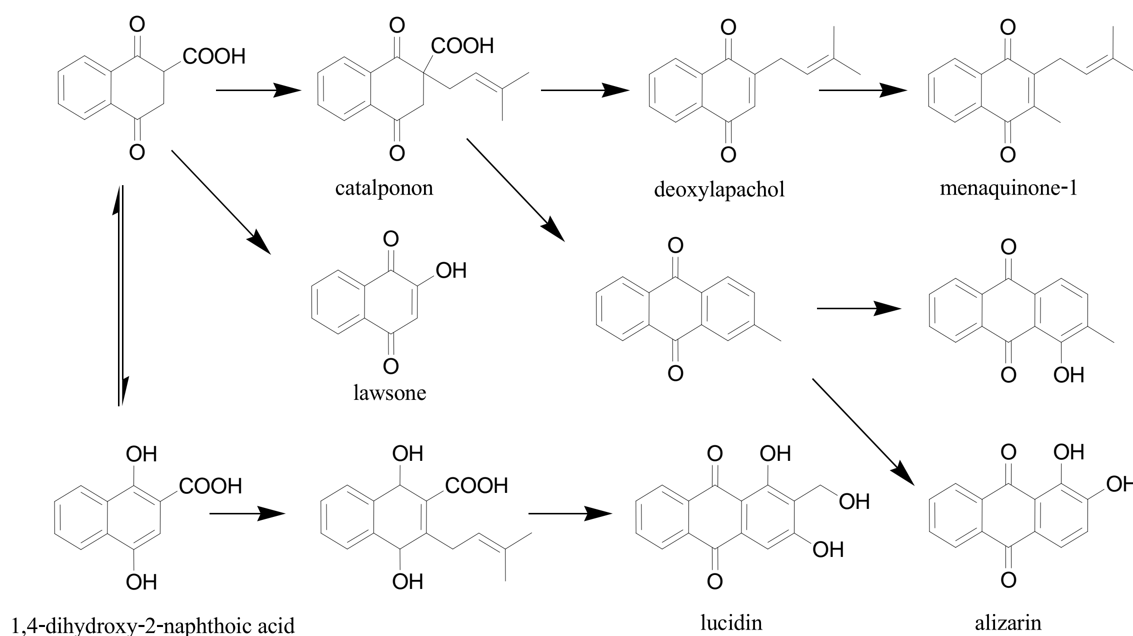


Figure 4 Transformation of 1,4-dihydroxy-2-naphthoic acid to some naphthoquinones and anthraquinones [59]

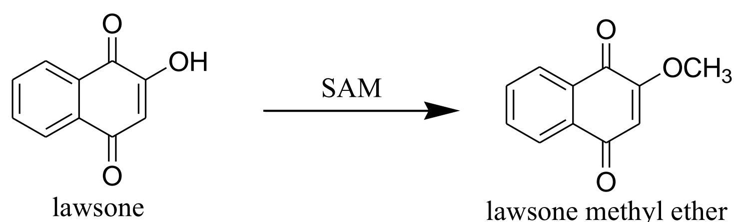


Figure 5 Methylation of lawsone by *O*-methyltransferase

7. Strategies to improve secondary metabolite production

Production of secondary metabolites using plant cells has been the subject of extended research. It was expected that the biosynthetic capacity of plants could be exploited *in vitro* using plant cells and cell tissue systems analogous to microbial cells in fermentation processes. Cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle. This is the most important advantage of plant cell cultures as model systems

for the study of biosynthetic pathways, as secondary metabolite formation can take place within a short cultivation time [8]. The general experience, however, is that plant cell cultures usually lose the ability to produce secondary metabolites that are characteristic of the intact plant [62]. In order to obtain yields in concentrations high enough for commercial exploitation, efforts have focused on the stimulating of biosynthesis activities of cultured cells using various methods [63-64]. Numerous strategies have been developed to improve the secondary metabolite production of plant tissue cultures, such as medium optimization, cell line selection, cell immobilization, precursor addition, elicitation, genetic transformation hairy root cultures, metabolic engineering and integrated bioreactor engineering [8].

8. Strain improvement

Strain improvement begins with the choice of a parent plant containing higher concentration of desire metabolitrs for callus induction to obtain high producing cell lines. Statistically high-producing plants give rise to high-producing cell lines [8, 64]. Cell cloning methods provide a promising way of selecting cell lines yielding increased levels of product. The heterogeneity in the biochemical activity existing within a population of cells has been exploited to obtain highly productive cell lines. Selection can be easily achieved if the product of interest is a pigment. Several products are accumulated in cultured cells at a higher level than found in intact plants. This is due to careful selection of productive cells and cultural conditions [62]. For example, in cultures of *Lithospermum erythrorhizon*, extensive screening of a number of clones resulted in a 13–20-fold increase in shikonin production [65]. A strain of *Euphorbia milli* also accumulated about 7-fold of anthocyanins produced to the parent culture after 24 times selections [66]. In 2001 Panichayupakaranant [9] have studied on naphthoquinone formation in cell suspension of *I. balsamina* from 40 different parent plants and the result supported this hypothesis. Increased secondary metabolite productions by plant tissue cultures were achieved through selection of high yielding plants (Table 2).

Table 2 Secondary metabolite production in high producing tissue [62]

Compound	Plant species	%Yield (% DW)	
		In vitro cultures	Intact plant
Shikonin	*Lithospermum erythrorhizon*	20	1.5
Ginsenoside	*Panax ginseng*	27	4.5
Anthraquinones	*Morinda citrifolia*	18	2.2
Ajmalicine	*Catharanthus roseus*	1.0	0.3
Rosmarinic acid	*Coleus blumeii*	15	3
Ubiquinone-10	*Nicotiana tabacum*	0.036	0.0003
Diosgenin	*Dioscorea deltoides*	2	2
Benzylisoquinoline alkaloids	*Coptis japonica*	11	5-10
Berberine	*Thalictrurn minor*	10	0.01
Berberine	*Coptis japonica*	10	2-4
Anthraquinones	*Galium verum*	5.4	1.2
Anthraquinones	*Galium aparbze*	3.8	0.2
Nicotine	*Nicotiana tabacum*	3.4	2.0
Bisoclaurine	*Stephania cepharantha*	2.3	0.8
Tripdiolide	*Tripterygium wilfordii*	0.05	0.001
Anthraquinones	*Senna alata*	0.95	0.17

9. Medium optimization

Information concerning the factors regulating secondary metabolism is as important as the selection of high-producing cell lines in increasing the production of secondary metabolites. A number of physical and chemical factors that could influence secondary metabolism in plant cell cultures have been found. Alterations in the environmental factors such as nutrient levels, light, and temperature may also be effective in increasing productivity (with the exception that reduced phosphate levels often stimulate product accumulation) [8]. Nitrogen

sources may also play an important role in product accumulation in plant cells. For example, in *Lithospermum erythorhizon***,** shikonin synthesis was inhibited by ammonium ions, but these ions promoted cell growth. It was necessary to change to a medium containing nitrate ions at the end of the growth phase [65]. Sucrose and glucose are the preferred carbon source for plant tissue cultures; although other carbohydrate sources are often used. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. In general, sucrose appears to be the preferred carbon source for cultured plant cell systems.

Plant growth regulators, auxin and kinetin levels have shown the most remarkable effects on growth and productivity of plant metabolites. High auxin levels, although good for cell growth, are often deleterious to secondary metabolite production [8, 67]. Increased auxin level such as 2,4-dichlorophenoxyacetic acid (2,4-D) in the medium promotes dedifferentiation of cells and diminishes secondary metabolites accumulation. Indeed, auxins stimulate callus induction, but for product synthesis it may be advisable to use low levels or remove auxins. For example, cytokinins stimulated alkaloid synthesis when auxin was removed from the medium of a cell line of *Catharanthus roseus.* However, some studies reported that high level of auxins may increased secondary metabolite accumulation such as l-DOPA in *Macuna pruriens* was stimulated by relatively high concentration of 2,4-D [62]. In such cases, elimination of 2,4-D or replacement of 2,4-D by naphthalene acetic acid (NAA) or indole acetic acid (IAA) has been shown to enhance the production of anthocyanins in suspensions of *Populus* and *D. carota*, of betacyanins in suspensions of *Portulaca*, of nicotine in suspensions of *N. tabacum*, of shikonin in suspensions of *L. erythrorhizon* and of anthraquinones in *M. citrifolia* [64]. Cytokinins showed variety of effects depending on the type of metabolites and species concerned. Thus, kinetin stimulated the production of anthocyanin in *Haplopappus gracilus* but inhibited the formation of anthocyanins in Populus cell cultures. Gibberellic acid and abscisic acid are reported to suppress production of anthocyanins in a number of cultures [64].

10. Elicitation

Higher plants induce various defense responses when they are attacked by microbial pathogens, such as fungi, bacteria or viruses. These defense responses include suicide of the attacked host cell (the so-called hypersensitive response); the production of antimicrobial secondary metabolites (called phytoalexins); the production of pathogenesis-related (PR) proteins, of which many exert antimicrobial properties; and the production and oxidative cross-linking of cell wall polymers [68]. The series of these defense responses have to be expressed in a synchronized fashion for its effectiveness [69]. Molecules that stimulate secondary metabolism are called elicitors. Elicitors may form inside or outside plant cells, and are distinguished as endogenously or exogenously inducers [8, 15]. In general, elicitors are classified on the basis of their origin and molecular structure [70] (Table 3).

Table 3 Classification of elicitors

Biotic elicitors		Abiotic elicitors	
Defined elicitor	Complex elicitor	Chemical	Physical
Chitosan	Fungal spore	Heavy metal salts	Thermal stress
Alginate	Yeast extract	Vanadyl sulphate	Osmotic stress
Pectin	Fungal homogenate	Sodium orthovanadate	UV irradiation
Chitin	Bacterial homogenate	Methyl jasmonate	Wounding
Elicitins	Coconut juice	Salicylic acid	

Each type of elicitor according to its characteristics can induce specific responses that depend on the interaction elicitor-plant culture. The biotic elicitors have biological origin, derived from the pathogen or from the plant itself (sometimes called endogenous elicitor). Biotic compounds can be of defined composition, when their molecular structures are known, or have a complex composition when they comprise several different molecular classes making impossible to define a unique chemical identity. On the other hand, abiotic elicitors have not a biological origin and are grouped in physical factors and chemical compounds. The classification described

above only takes into consideration the nature of the elicitor [70]. But these compounds may be also classified according to the interaction plant-elicitor, into two groups: 'general elicitors' which are able to trigger defense responses both in host and non-host plants and 'race specific elicitors' which induce responses leading to disease resistance only in specific host cultivars, depending on the simultaneous presence of a virulence and resistance genes in the pathogen and plant, respectively [71]. Some of successful experiments have been shown in Table 4.

Table 4 Comparison of secondary metabolite production after elicitation

Species	Products	Elicitors	Productivity (compare with control)	References
Ambrosia artemisiifolia	thiarubrine A	vanadyl sulfate	8 fold	[72]
Ammi majus	umbelliferone	bacterial homogenate (BH)	9.6 fold	[73]
Arachis hypogaea	resveratrol	sodium acetate	60 fold	[74]
Azadirachta indica	azadirachtin	jasmonic acid	6 fold	[75]
Azadirachta indica	azadirachtin	salicylic acid	9 fold	[75]
Brugmansia candida	tropane alkaloids	salicylic acid	6-10 fold	[76]
Brugmansia candida	tropane alkaloids	yeast extract	2-6 fold	[76]
Brugmansia candida	tropane alkaloids	$CuSO_4$	5 fold	[76]
Catharanthus roseus	ajmalicine	fungal homogenate (FH)	2.1 fold	[77]
Catharanthus roseus	indole alkaloids	FH	3 fold	[78]
Centella asiatica	triterpenes	methyl jasmonate	4-6 fold	[79]
Cichorium intybus	coumarin	FH	2.5 fold	[80]
Dioscorea deltoida	diosgenin	BH	1.7 fold	[81]
Dioscorea floribunda	diosgenin	2-chloroethylphosphonic acid	72 fold	[82]
Farsetia aegyptia	glucosinolate	MJ	5 fold	[83]
Galphimia glauca	galphimine-B	MJ	16 µg/g DW : not produce	[79]

Table 4 (continued) Comparison of secondary metabolite production after elicitation

Species	Products	Elicitors	Productivity (fold-compare with control)	References
Hyoscyamus muticus	sesquiterpenes	BH	0.1 mg/g FW : not produce	[84]
Lithospermum erythrorhizon	shikonin	MJ	6.2 fold	[85]
Lotus corniculatus	isoflavonoids	glutathione	160 µg/g FW : not produce	[86]
Morinda citrifolia	anthraquinones	chitin50	2.3 fold	[87]
Morinda elliptica	anthraquinones	jasmonic acid	2-4 fold	[63]
Nicotiana tabacum	capsidiol	FH	25 µg/mL : not produce	[88]
Panax ginseng	ginseng saponin	selenium	1.3 fold	[89]
Passiflora quadrangularis	flavonoids	UV-B radiation	5-12 fold	[90]
Plumbago rosea	plumbagin	chitosan	6.7 fold	[16]
Rubia cordifolia	anthraquinones	methyl jasmonate	2.1 fold	[91]
Rubia cordifolia	anthraquinones	salicylic acid	2.3 fold	[91]
Sanguinaria canadensis	sanguinarine	FH	4 fold	[92]
Saussurea medusa	syringin	yeast extract + chitosan + Ag^+	3.6 fold	[93]
Silybum marianum	silymarin	yeast extract + MJ	6 fold	[17]
Taxus baccata	paclitaxel	MJ + FH	6.8 fold	[18]
Taxus chinensis	taxuyunnanine C	trifluoroethyl salicylate	1.6 fold	[94]
Thalictrum rugosum	berberine	yeast elicitor	4 fold	[95]

10.1 Factors which influence elicitation

The effectiveness of elicitation as a tool to enhance the production of secondary metabolites depends on a complex interaction between the elicitor and the plant cell.

10.1.1 Elicitor specificity

There is evidence that the same elicitor can stimulate secondary metabolism in different cell cultures and, on the other hand, that certain plant cultures are responsive to diverse elicitors. Treatments of a particular culture with different elicitors result in the accumulation of the same compounds, since there are specific of each plant culture. Although, the class of metabolite depends on the plant species, the kinetics of induction or accumulation levels varies with different elicitors. Curiously, if one assumes that the elicitor signal is detected by the plant specific receptor, the selectivity of the response would depend on the presence of such molecular entity and on the transduction pathways that each elicitor activates. Investigations have shown that in general the type of metabolites are specific for the plant cell culture and are not dependent on the elicitor class [70].

10.1.2 Elicitor concentration and treatment interval

The concentration of elicitor is a factor that strongly affects the intensity of the response and the effective dose, which varies according to the plant species, can only be found empirically. It has been demonstrated that elicitor levels, which exert stimulatory effects in certain plant systems when applied to other ones are devoid of activity, reflecting different sensibilities of the molecular components involved in elicitation. In general, two types of dose–response curves have been described [96-97], one which corresponds to a typical saturation profile where over dosage of the elicitor will not affect cell viability and the second type showing a sharp optimum [98] (Figure 6). Respect to the treatment interval, there are few data available. In general the elicitor is in contact with the system until harvest, but the time required for maximum secondary metabolite accumulation is a characteristic of each plant species and normally is preceded by an increase in activity of the metabolic enzymes involved. These facts point out the

importance of determining empirically the optimum conditions of elicitation time and elicitor concentration for each plant tissue culture in particular [70].

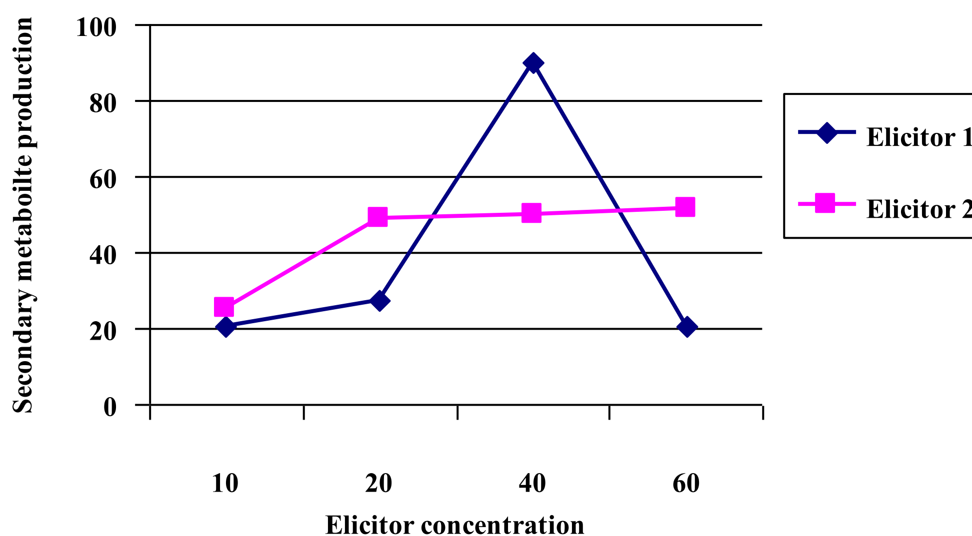


Figure 6 Dose-response curves between elicitor concentration and secondary metabolite production

10.1.3 Culture conditions: growth stage, medium composition, light

The literatures hold the view that the most appropriate moment to add the elicitor was during the exponential phase of growth [96, 99] when the enzymatic machinery was in the maximum operative status, the response to the elicitor being, in consequence, more efficiently achieved. Another factor is the presence of growth regulators in the medium, which can markedly affect the elicitation of secondary metabolism. For example, carrot cells cultured without auxin do not respond to elicitation [98]. The production of anthraquinone by cell suspensions of *Morinda citrifolia* is also affected by various growth regulators [67]. Likewise, culture light conditions may also play a significant role, as in jasmonic acid-induced hypericin production in *Hypericum perforatum* L. cells which exhibit higher cell growth and secondary metabolite production when incubated in the dark than under light [100], whereas other studies have reported light stimulation of secondary metabolite synthesis [101]. In view of the variability

in elicitation responses due to different factors like those described above, the optimization of medium composition and culture conditions represent an important aspect in elicitation protocols [70].

10.2 Mechanism of elicitor on plant cell

Signal perception is the first step of the elicitor signal transduction cascade and, for example, recognition of different stimuli is central to the ability of plants to respond through activation of kinases, generation of reactive oxygen species, ion fluxes and cytoplasm acidification. Numerous elicitor binding sites have been identified, and all these putative receptors were localized in plant plasma membranes [69-70]. The number of elicitors that have been found to activate phospholipase C (PLC) in plants causing polyphosphoinositide turnover and production of the second messengers; inositol trisphosphate (IP_3) and diacylglycerol (DAG) are increasing[69]. For example, in *Arabidopsis*, a gene (AtPLC1) was cloned that encodes a genuine PI-PLC. AtPLC1 is expressed at very low levels in plants under normal conditions but is induced to a significant extent under abiotic stimuli, such as dehydration, salinity, and low temperature [102]. Moreover, in *Vigna radiata* L. three distinct partial cDNAs (pVr-PLC1, pVr- PLC2 and pVr-PLC3) have been identified, which encode isoforms of putative PI-PLC, and, in agreement with the preceding observations, the Vr-PLC3 mRNA level was very low under standard growth conditions but was rapidly induced by environmental stress[103]. A role for IP_3 in releasing intracellular calcium is well established in plants. Moreover, in Arabidopsis, an accumulation of IP_3 that correlates with calcium mobilization was observed in response to abiotic stress. Likewise, IP_3 signaling is involved in biotic elicitor-induced accumulation of pisatin in pea, furanocoumarins in parsley, anthraquinones in *R. tinctorum*, medicarpin in lucerne cell cultures, scoparone in lemon seedlings and β-thujaplicin in the Mexican cypress [70].

Calcium is a ubiquitous signal in plants which mediates the regulation of many cellular processes by different stimuli [69]. There is evidence that the action of many elicitors involves changes in the intracellular calcium status. The process of elicitation activates various Ca^{2+}- and calmodulin-dependent protein kinases by increasing the level of free Ca^{2+} in the cytoplasm and somehow triggers the cellular responses, which may include alterations in gene expression. The growth-inhibitory effect of high calcium concentrations has been reported to be

due to an inhibition by calcium of the biochemical wall-loosening process[69]. In the case of treatment with oligosaccharides, a biphasic calcium increase was shown, the first peak induced by the influx of extracellular Ca^{2+}, whereas the second pulse was caused by PLC activation and IP_3-dependent Ca^{2+} release from intracellular calcium stores[104]. It is recognized that elicitor-induced calcium influx is an early response of plant cells, generally resulting in changes from the Ca^{2+} resting level of 50–100 nM to 1–5 mM, within 5 min after elicitor treatment [70].

11. Feeding of biosynthetic-precursor

Precursor feeding has been an obvious and popular approach to increase secondary metabolite production in plant cell cultures. The concept is based upon the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases [64]. For example, amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids and other products [62]. It is likely that the precursors may be either incorporated directly into the product, or precursors may enter a specific product indirectly through degradative metabolism and entry into interrelated pathways. Phenylalanine is a precursor of rosmarinic acid; addition of phenylalanine to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid and decreased the production time as well. Phenylalanine is also the precursor of the N-benzoylphenylisoserine side chain of taxol; supplementation of *Taxus cuspidata* cultures with phenylalanine resulted in increased yields of taxol [105-106]. Tyrosine and phenylalanine were fed to *Cistanche salsa* cell suspension cultures and these compounds capable of increasing total phenylethanoid glycosides production [107]. Solasodine level in *Solanum lyratum* cell suspension cultures was also increase after feeding with cholesterol and stigmasterol, which was about 10-fold higher than control [108]. The literature is replete with examples of the effects of precursor feeding on the production of secondary metabolites by cultured cells. The evidence clearly indicates that the timing of precursor addition is critical for an optimum effect. The effects of

feedback inhibition must surely be considered when adding products of a metabolic pathway to cultured cells [62].

12. Transformed hairy root cultures using *Agrobacterium rhizogenes*

Hairy roots disease is caused by the infection of wounded higher plants with *Agrobacterium rhizogenes*. The hairy root phenotype is characterized by fast hormone-independent growth, lack of geotropism, lateral branching and genetic stability. Owing to their stable and high productivity, hairy root cultures have been investigated for several decades for potential to produce the valuable metabolites that are present in wild type roots [10, 109]. *A. rhizogenes* transfer a DNA segment (T-DNA) from its large root-inducing Ri plasmid into the genome of the infected plant. This T-DNA carries a set of genes that encode enzymes which control auxin and cytokinin biosynthesis. The new hormonal balance induces the formation of proliferating roots, called hairy roots, which emerge at the wounding site [109-110]. In the agropine Ri plasmid T-DNA is referred to as left T-DNA (T_L-DNA) and right T-DNA (T_R-DNA). T_R-DNA contains genes homologous to Ti plasmid tumor inducing genes [111]. Genes involved in agropine synthesis are also located in the T_R-DNA region. 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 [111]. The *rol*A, *rol*B, *rol*C and *rol*D genes are plant oncogenes, and are carried on plasmids of the plant pathogen *A. rhizogenes* [110, 112].

The *rol*A gene has been shown to have a stimulatory effect on nicotine production [113]. *Rol*A-expressing calli from *Rubia cordifolia* produced a 2.8-fold higher level of anthraquinones (AQs) compared to control calli [114]. An interesting biotechnological peculiarity of *rol*A is that the gene expressed in *R. cordifolia* calli ensured remarkably stable levels of AQs, and *rol*A expression simultaneously provided conditions for vigorous callus growth [115].

The *rol*B gene is apparently the most powerful inducer of secondary metabolism and is also the most powerful suppressor of cell growth. In *R. cordifolia* transformed calli, *rol*B expression positively correlated with increased expression of a key gene for AQ biosynthesis, isochorismate synthase (ICS) gene and with anthraquinone production [114]. The most prominent example of the effectiveness of *rol*B transformation was recently demonstrated for *Vitis*

amurensis cells, where transformation led to more than a 100-fold increase in resveratrol production [116]. However, high levels of *rol*B expression inhibited callus growth in a manner dependent on the strength of expression [115].

*Rol*C provides a signal that activates secondary metabolic processes. It is likely that *rol*C may confer a wider spectrum of defense reactions in addition to secondary metabolite stimulation [115]. The *rol*C gene is capable of stimulating the production of tropane alkaloids, pyridine alkaloids, indole alkaloids, ginsenosides and anthraquinones in transformed plants and plant cell cultures [115]. In *R. cordifolia* transformed calli, expression of *rol*C led to a stable increase in anthraquinone content. Production of anthraquinones and the expression of the *ICS* gene were dependent on the strength of *rol*C expression: the higher the expression of the *rol*C gene, the higher the observed expression of the ICS gene and accumulation of anthraquinones [114].

The effects of *rol*D on secondary metabolism have never been investigated. *Rol*D is the only Rol protein which biochemical function is clearly determined. *Rol*D encodes ornithine cyclodeaminase, an enzyme that converts ornithine to proline. Decreasing the ornithine pool and increasing the proline pool in transformed cells is believed to contribute to the morphological effects of *rol*D, such as the maintenance of hairy root growth and abundant flowering of transformed plants [117].

CHAPTER 3

MATERIALS AND METHODS

1. Plant materials

Impatiens balsamina was grown in the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plants were divided into four groups according to their flower color: including white flower (IbW), pink flower (IbP), violet flower (IbV) and red flower (IbR). The voucher specimens (IbW specimen no. SKP 021 09 02.01 01; IbP specimen no. SKP 021 09 02.02 01; IbV specimen no. SKP 021 09 02.03 01; IbR specimen no. SKP 021 09 02.04 01) were deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University.

2. Bacterial strain

Wild type *Agrobacterium rhizogenes* ATCC 13333 were obtained from Microbiological Resources Centre, Pathumthani, Thailand, and used for hairy root induction.

3. Chemicals and reagent

- Lawsone (Sigma-Aldrich® Steinheim, Germany)
- Acetic acid, glacial AR grade (Lab-scan Asia, Thailand)
- Agar (Bacto) (Himedia laboratories, India)
- Beef extract (Himedia laboratories, India)
- Cefotaxime (M&H Manufacturing, Thailand)
- Chitosan (water soluble) (Wako, Japan)
- Chloroform, analytical grade (Lab-scan Asia, Thailand)
- Clorox® (Clorox, Malaysia)
- Ethanol (95 %v/v) (Lab-scan Asia, Thailand.)

- Ethyl acetate, commercial grade (Lab-scan Asia, Thailand)
- Diaion® HP-20 (Supelco USA)
- Methanol, analytical grade, HPLC grade (Lab-scan Asia, Thailand)
- Methionine (Sigma Aldrich, Germany)
- Methyl jasmonate (Sigma Aldrich, Germany)
- Peptone (Becton, Dickinson, France)
- Plant agar (Sigma, Germany)
- Sucrose (Mitrpol, Thailand)
- Silica gel (Merck, Germany)
- Yeast extract (Becton Dickinson and company, USA)

4. Preparation of lawsone methyl ether

Standard lawsone methyl ether was obtained by derivatized from lawsone as previously described by Panichayupakaranant and Reanmongkol [53]. Briefly, lawsone (1.0 g) was dissolved in absolute methanol (50 mL) and conc. hydrochloric acid (0.8 mL). The mixture was heated under reflux conditions for 4 hr. Then the mixture was cooled to room temperature, and the precipitate was separated by vacuum filtration. The resulting yellow precipitate was recrystallized in the mixture of ethyl acetate and methanol, to give the yellow needles of lawsone methyl ether.

5. Preparation of 3,3′-methylenebislawsone

Standard 3,3′-methylenebislawsone was prepared by Mannich reaction between lawsone and formaldehyde. Briefly, lawsone (1.74 g) and *p*-nitraniline (1.52 g) were dissolved in absolute ethanol (50 mL) at 25 $^{\circ}$C and 37% (v/v) formaldehyde (1 mL) was added in the solution. Yellow solid soon began to separate, and after 30 min the mixture was boiled for 5 min and filtered. The yellow residue of m.p. 248-250 $^{\circ}$C was identified as 3,3′-methylenebislawsone [118].

6. Equipments, instruments and kits

- Autoclave (Model HA-3D, Hirayama, Japan)
- Centrifuge (Kubota 5922, Japan)
- Electrophoresis chamber, Mupid α-2 plus (Takara Bio Inc, Japan)
- Gel documentation (Gel Doc model 1000, BIO-RAD, USA)
- Hot air oven (Memmert, Schwubuch, Germay)
- Hot plate and stirrer PC-101 (CORNING; Fisher Scientific, USA)
- HPLC (Agilent, U.S.A.; JASCO, Japan)
- HPLC analytical column (C18 Supelco 5 µm, 4.6 mm x 15 cm, USA)
- HPLC semi-preparative column (TSK-GEL ODS, 5 µm, 2 x 25 cm, Tosoh Chemicals Co., LTD; Capcell Pak ODS, 5 µM, 2 x 25 cm; Shiseido Fine Chemicals Co. LTD, and Ascentis Phenyl; 5 µm, 2.2 x 25 cm, Supelco)
- HPLC preparative column (Tosoh, TSK gel ODS, 5 µm, 6x60 cm x 2)
- Laminar air flow cabinet (Holten, Denmark)
- Microfilter 0.45 µM, 0.22 µM (Whatman, GE healthcare, UK)
- Micropipette (Gilson, USA)
- Microwave ovens (LG, Thailand)
- pH meter (ORION Research, USA)
- Refrigerator (4°C) Sanden Intercool, Thailand; (-20°C) Whirlpool, Thailand
- Rotary evaporator (Aspirator A-3S, EYELA, Japan)
- Shaker (Innova2300, New Brunswick Scientific, llinois, USA)
- Sonicator (Crest Ultrasonic Corporation, USA)
- Takara PCR Thermal Cycler Dice (Takara, Japan)
- Vacuum pump (Cacuubrannd, Wertheim, Germany)
- Vortex (Vortex-Genie 2TM, USA)
- NMR instrument (JEOL JNM-α 400 instrument)
- DNeasy Plant Mini Kit (Qiagen, Germany)
- GFX Micro Plasmid Prep Kit (GE Healthcare, UK)
- Gotaq® Flexi DNA Polymerase (Promega, USA)

7. Media and solution preparations

7.1 TAE buffer (50x)

TAE buffer was prepared as fifty times concentrated buffer (50X TAE) by dissolving 121 g Tris, EDTA·3Na 19.7 g and 35 mL glacial acetic acid in water to make 500 mL of buffer and pH was adjusted with conc. HCl to 8.0. The buffer was diluted 50 times by water prior to use.

7.2 YEB medium

YEB was prepared by mixing of 0.5 g beef extract, 0.1 g yeast extract, 0.5 g peptone, 0.5 g sucrose, 1.5 g agar (Bacto) and 0.5 mL 10% w/v $MgSO_4$. The volume was adjusted to 100 mL with distilled water, pH 7.2-7.4 and sterilized using autoclave.

7.3 Gamborg's B5 medium

To prepare B5 medium the stock solutions have been prepared as shown in Table 5. Stock 1a (50 mL), 1b (1 mL), 2 (1 mL), 3 (1 mL), 4 (5 mL) and 5 (1 mL) were combined together, the plant growh regulators were added as needed and adjusted the volume to 1000 mL. The pH of the medium was adjusted to 5.5 with 1N NaOH or 1N HCl. Plant agar (8 g/L) was used for solidified the medium.

Table 5 Stock solutions for Gamborg's B5 medium

Stock 1a Macronutrients (x20)	**g/500mL**
KNO_3	25.00
$MgSO_4.7H_2O$	2.50
$NaH_2PO_4.H_2O$	1.50
$(NH4)_2SO_4$	1.34
Stock 1b Macronutrients ($x10^3$)	**g/100mL**
$CaCl_2.2H_2O$	15.00
Stock 2 Micronutrients ($x10^3$)	**g/100mL**
H_2BO_3	0.30
$MnSO_4.H_2O$	1.00
$ZnSO_4.7H_2O$	0.20
$NaMo_4.2H_2O$	0.025
$CuSO_4.5H_2O$	0.0025
$CoCl_2.6H_2O$	0.0025
Stock 3 KI ($x10^3$)	**g/100mL**
KI	0.075
Stock 4 (Fe-EDTA)	**g/500mL**
$FeSO_4.7H_2O$	2.78
Na_2EDTA	3.72
Stock 5 Vitamins (x100)	**g/100mL**
Thaiamine hydrochloride	1.00
Pyridoxine hydrochloride	0.10
Nicotinic acid	0.10
Myo-Inositol	10.00
Plant growth regulators	**g/100mL**
NAA stock solution (100 mg/L)	0.01
Kinetin stock solution (100 mg/L)	0.01
BA Stock solution (100 mg/mL)	0.01

8. Development of HPLC quantitative analysis of naphthoquinones

HPLC analysis was carried out using an Agilent 1100 series equipped with photodiode-array detector (PDA) and autosampler. Data analysis was performed using Agilent 3D ChemStation software (Agilent, USA). Separation was achieved at 25°C on a Supelco® C18 column (5 µm, 150 mm × 4.6 mm i.d.). The mobile phase consisted of methanol-2% aqueous acetic acid with gradient elution as follows: 0-10 min, 25:75; 10-20 min, 32:68; 20-35 min, 55:45. The mobile phase flow rate was 1 mL/min. Sample injection volumes were 20 µL, and detection was by UV at wavelength 280 nm.

9. Validation of analytical method

For validation of the analytical method, the guidelines of International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use were followed [119]. Establishing parameters for linearity, accuracy, intra-day and inter-day precision, specificity, limit of detection (LOD) and limit of quantification (LOQ) were used for validating the HPLC method.

Linearity validation. Calibration curves were constructed on three consecutive days by analysis of mixtures containing each of reference standard compounds at five different concentrations and plotting peak area against the concentration of each reference standard. The linearity of the detector response for the standards was determined by means of linear regression.

Accuracy validation. Mixtures of laswsone, LME and 3,3′-methylenebislawsone were prepared at 3 different concentrations (50, 25 and 12.5 µg/mL) and mixed with the leaf extracts in a ratio of 1:1 (v/v). Three injections for each concentration were performed per day over 3 different days (3 injections × 3 concentrations × 3 days) and percentage recoveries of each naphthoquinone were then calculated.

Precision validation. The chromatographic method was tested by performing intra- and inter-day multiple injections of a standard solution. Six injections were performed each day for 3 consecutive days. The precision of the extraction procedure was validated by repeating the extraction procedure on the same sample of *I. balsamina*. An aliquot of each extract was then

injected and quantified. This parameter was evaluated by repeating the extraction in triplicate on 3 different days with freshly prepared mobile phase and samples.

Specificity validation. Peak identification was carried out by using authentic standards and scanning the UV spectrum of each peak using the photodiode-array detector. The UV spectra were taken at various points of the peaks to check peak homogeneity.

LOD and LOQ. Serial dilutions of reference standards were made with methanol and were then analyzed with the HPLC method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

10. Determination of solvent for extraction

To optimize the solvent for extraction, *I. balsamina* leaf powder (200 mg) was separately extracted with methanol, ethyl acetate and chloroform (20 mL) under reflux conditions for 1 hr. The extract was then filtered and the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in methanol, and volume adjusted to 10 mL and subjected to HPLC analysis. The experiments were performed in triplicate. To optimize the chloroform concentration in the extraction solvent, *I. balsamina* leaf powder (200 mg) was extracted with a solvent mixture (20 mL) consisting of methanol with increasing concentrations of chloroform (0, 30, 50 and 70%, v/v) under reflux conditions for 1 hr. The extract was then filtered and the solvent evaporated to dryness under reduced pressure. The residue was dissolved in methanol, and the volume adjusted to 10 mL and subjected to HPLC analysis. The experiments were performed in triplicate.

11. Establishment of *I. balsamina* root cultures

I. balsamina root cultures were initiated from the leaves of the plantlets, which were collected from 4 strains flowers of *I. balsamina* including red, violet, pink and white in B5 solid medium supplemented with 0.1 mg/L NAA, 0.1 mg/L kinetin and 1.0 mg/L BA and 0.2% w/v sucrose [6]. The 1 month old culture roots were transferred to liquid B5 medium supplemented with the same hormonal composition. The roots were cultured on a rotary shaker at

80 rpm, 25 °C with a 16-hr daily light period. The culture roots were maintained under these conditions and subcultured every 3 weeks.

12. Effect of the explants parts on naphthoquinone production in the root cultures

To study on the effect of the explants parts, the root cultures were initiated from 2 different plant parts (root or leaf) in the medium as described above. The culture roots were maintained and transferred to fresh medium every 3 weeks. The 1 month old culture roots were harvested and extracted as described below before subjected to quantitative analysis of naphthoquinone content using HPLC method as described above. The explants that can produce the highest yield naphthoquinone root cultures are selected for further studies.

13. Determination of naphthoquinone content in *I. balsamina* root cultures

In this study all experiment was extracted as described. Plant materials (root cultures or leaves) were harvested and dried at 50 ºC for 12 hr. The dried root cultures were ground with mortar and pestle to fine powder. The dried ground root was successfully extracted with 50% (v/v) chloroform in methanol under reflux conditions for an hr. The extract was then evaporated to dryness under reduced pressure (40 ºC). The residue was reconstituted with methanol and the volume adjusted to 10 mL. The solution was filtered through 0.45 µm nylon membrane filter before subjected to quantitative analysis of naphthoquinone content using HPLC method as described above.

14. Selection of naphthoquinone high yielding *I. balsamina* donor plant

To select the naphthoquinone high yielding *I. balsamina*, seeds of *I. balsamina* were separately collected from four *I. balsamina* donor plants (IbR, IbV, IbP and IbW) and sterilized by rinsing with 70% (v/v) ethanol for five seconds, immersion in 20% (v/v) Clorox® solution for 15 min and rinsing three times in sterile distilled water. Seeds were germinated on

hormone-free B5 solid medium with 20 g/L sucrose, at 25 ± 2°C under16 hr light / 8 hr dark. The one month old leaves were separately collected according to the plant strains and dried at 50°C. The dried leaves were extracted and subjected to HPLC quantitative analysis to monitor their naphthoquinone contents. *I. balsamina* root cultures were separately initiated from the leaves of the plantlets in solid B5 medium supplemented with 0.1 mg/L NAA, 0.1 mg/L kinetin, 1.0 mg/L BA, 20 g/L sucrose and 8 g/L agar. The root cultures were transferred into 250-mL Erlenmeyer flasks containing 50 mL of liquid B5 medium supplemented with the same hormonal composition. The cultures were incubated on a rotary shaker (80 rpm), at 25 ± 2 °C under16 hr light / 8 hr dark. Maintenance of the cultures was carried out by periodic subculture at three week intervals. After three successive subcultures the root cultures (1 month old) were harvested, extracted and subjected to HPLC quantitative analysis of naphthoquinones. The highest yielding plant strain was selected as explants for further study.

15. Study on effect of plant growth regulators on naphthoquinone production

15.1 Optimization of 6-benzylaminopurine (BA) concentration

The root cultures initiated from IbP strain (3-week old) were transferred to fresh B5 liquid medium supplemented with 0.1 mg/L NAA, 0.1 mg/L kinetin and different concentrations of BA as 0.1, 0.5, 1.0 and 2.0 mg/L. The 1-month old culture roots were harvested and subjected to determination of naphthoquinone content using HPLC method. The optimum concentration of BA in the medium was used for further experiments.

15.2 Optimization of kinetin concentration

The selected culture roots of *I. balsamina* (3-week old) were transferred to fresh B5 liquid medium supplemented with 0.1 mg/L NAA, optimum concentration of BA (result from 15.1) and different concentrations of kinetin at 0.1, 0.5, 1.0 and 2.0 mg/L. The 1-month old culture roots were harvested and subjected to determination of naphthoquinone content using HPLC method. The optimum concentration of kinetin in the medium was used for further experiments.

15.3 Optimization of naphthaleneacetic acid (NAA) concentration

The culture roots of *I. balsamina* (3-week old) were transferred to fresh B5 liquid medium supplemented with optimum concentration of kinetin and BA (result from 15.1 & 15.2) and different concentrations of NAA at 0.1, 0.5, 1.0 and 2.0 mg/L. The 1-month old culture roots were harvested and subjected to determination of naphthoquinone content using HPLC method. The optimum concentration of NAA, kinetin and BA in the medium were used for further experiments.

16. Time-courses of growth and naphthoquinone production

The root cultures (1.0 g fresh weight) were transferred to B5 liquid medium supplemented with 0.1 mg/L NAA 1.0 mg/L kinetin and 2.0 mg/L BA. The roots were harvested every three days for the period of 30 days. The dry weights of the roots were recorded after drying at 50°C for 24 hr. The dried roots were extracted and subjected to quantitative analysis of naphthoquinones using the methods as described above. These data were plotted to produce growth and naphthoquinone production curves.

17. Effect of methionine feeding on naphthoquinone production

The culture roots of *I. balsamina* (3-week old) were transferred to the fresh B5 liquid medium. Various concentrations of methionine were added to the beginning of the cycle (day 0) to produce the final concentrations of 50, 100, 300, 500 and 1000 mg/L, respectively. The 1-month old culture roots were harvested and subjected to HPLC to determine naphthoquinone content.

High concentration of methionine (300, 500 and 1000 mg/L) were added to 21 day old root cultures. The 1-month old culture roots were harvested and subjected to HPLC to determine naphthoquinone content. The culture media were collected and extracted as described in **19.6** prior to determine the naphthoquinone content by HPLC.

18. Increasing of naphthoquinone production by transformed hairy root cultures using *Agrobacterium rhizogenes*

18.1 Preparation of *A. rhizogenes*

The preparation method of *A. rhizogenes* was modified from that of Dhakulkar [120]. *A. rhizogenes* ATCC 13333 were used for hairy root induction. The bacteria were cultured on YEB nutrient agar medium. A single bacterial colony was inoculated in 5 mL of YEB nutrient broth medium and cultured on rotary shaker (220 rpm) at 25°C. The bacterial suspension was centrifuged at 3,000 rpm for 10 min. The pellet was resuspended in 5 mL B5 liquid medium and used for co-cultivation with explants.

18.2 Induction of the transformed hairy roots

The sterile leaves of *I. balsamina* were inoculated with sterile needle at the leaves vein. The leaf explants were immerged in bacterial suspension and transferred to B5 solid medium. After 3 days of infection, those explants were transferred to B5 solid medium containing 500 mg/L cefotaxime to eliminate the residual *A. rhizogenes*. Cefotaxime concentration was then reduced every week from 500, 300 and 100 mg/L, respectively. Finally the cultures explants, which were free from bacteria were transferred to hormone free and antibiotic B5 solid medium. The transformed hairy root cultures were maintained in hormone free B5 liquid medium for 1 month before harvest, then they were extracted and subjected to HPLC for quantitative analysis of naphthoquinone content.

18.3 Plasmid isolation from *A. rhizogenes* ATCC 13333

Plasmid of *A. rhizogenes* (positive control) was isolated from overnight culture using GFX Micro Plasmid Prep Kit (GE Healthcare, Life Sciences) according to its manual's instruction. The overnight culture of *A. rhizogenes* (1.5 mL) was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 14,000 rpm for 30 seconds to precipitate the cells. The supernatant was removed. The pellet was resuspended in 150 µL of solution I and vortex vigorously. The pellet was added with 150 µL of solution II and mixed. Solution III (300 µL) was added and mixed by inverting the tube until a flocculent precipitate appeared and was centrifuged

at 14,000 rpm for 5 min at room temperature to precipitate cell debris. The supernatant was transferred to a GFX column and incubated for 1 min at the room temperature and centrifuged at 14,000 rpm for 30 seconds. The flow-through was discarded. The column was added with 300 µL solution III and centrifuged at 14,000 rpm for 30 seconds and added 400 µL of wash buffer and then centrifuged at 14,000 rpm for 60 seconds. Finally the GFX column was transferred to a new tube and added with 100 µL of Tris-EDTA buffer and incubated at room temperature for 1 min. The incubation mixture was centrifuged at 14,000 rpm for 1 min to recover the purified DNA. The purified DNA was kept at -20°C until use.

18.4 Genomic DNA isolation from transformed hairy root cultures

Total DNA was isolated from transformed hairy root cultures as well as from control non-transformed roots using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The plant tissues were ground into fine powder in the presence of liquid N_2. The powder was resuspended in 400 µL 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 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 for 2 min. The flow-through fraction was transferred to a new tube. The 1.5 volumes of buffer AP3/E was added into the cleared lysate and mixed by pipette. The mixture 650 µL were applied to DNeasy mini spin column sitting in a 2 mL collection tube and centrifuged at 8,000 rpm for 1 min. The flow-through was discarded. Buffer AW (500 µL) was added on DNeasy mini spin column and centrifuged at 8,000 rpm for 1 min. This step was performed in duplicate. Finally, the DNeasy mini spin column was placed in a 1.5 mL microcentrifuge tube and adding 100 µL of buffer AE, incubates for 5 min at room temperature (15-25°C). The plant DNA was eluted from column by centrifugation at 8,000 rpm for 1 min. The plant DNA was stored at -20°C until use.

18.5 DNA amplification by polymerase chain reaction (PCR)

Transformed hairy root cultures was identified the virulence genes for *rol*B and *rol*C using polymerase chain reaction (PCR) analysis. Genomic DNA was used as template for

identification with PCR technique, which PCR primers were used for amplifications of a 750 base pairs fragments of the *rol*B gene and 545 base pairs fragment of the *rol*C gene. For control, treatment the plasmid *of A. rhizogenes* ATCC 13333 was used to compare. Primers used in this study were shown in Table 6. The *rol*B-1 and *rol*B-2 primers were used for amplification of 780-bp fragment of *rol*B and *rol*C-1 and *rol*C-2 primers for amplification 545-bp fragment of *rol*C respectively. For control treatment, four plasmids isolated from *A. rhizogenes* strain ATCC 13333 were used as templates. The standard procedure for a PCR reaction was performed in a 50 µL mixture (Table 7 and 8).

Table 6 Primers used in this study

Primer	**Nucleotide sequences (5′→3′)**
*rol*B-1	ATGGATCCCAAATTGCTATTCCTTCCACGA
*rol*B-2	TTAGGCTTCTTTCTTCAGGTTTACTGCAGC
*rol*C-1	CATTAGCCGATTGCAAACTTG
*rol*C-2	ATGGCTGAAGACGACCTG

Table 7 Standard procedure for PCR

Reagents	**Volume/reaction (µL)**
DNA Template	10
$MgCl_2$	3
dNTP mix, 2.5 mM	4
primer 1	1
primer 2	1
Gotaq DNA polymerase	0.5
Sterilize distilled water	30.5
Total volume	50

Table 8 PCR conditions for *rol*B and *rol*C amplification

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

18.6 Agarose gel electrophoresis

Agarose gel (1% w/v) was prepared. Gel electrophoresis is used for determining the size of the inserted DNA. Agarose (1.2 g) was added into sterile distilled water (120 mL) and 2,400 µl of TAE (50X) was added and heated up in the microwave until clear solution and poured on a plastic tray. After the agarose gel had completely set (30-45 min at room temperature), the comb was carefully removed and the gel was installed on the platform in the electrophoresis tank containing 1X TAE buffer. The 10 µL PCR products were loaded into the wells of 1% w/v agarose gel using micropipette. The electrophoresis was run at 100 volts. The electrophoresis process was stopped until the loading dye has migrated to three fourth of the agarose gel. The developed agarose gel was then stained with SYBR® Safe DNA gel stain solution for 30 min. After that the DNA pattern was observed using a UV light box (Vilber Lourmet) and photographed.

19. Increasing of naphthoquinone production by elicitation

19.1 Elicitor preparation

CAH was prepared by culturing *Candida albicans* in conical flasks containing 250 mL Saboraud dextrose broth at 37°C for 3 days as described previously by Rajendran *et al.* [121]. The turbidity of the suspension broth was adjusted with 0.85% NaCl solution to obtain an

absorbance reading of 1.8-2.0 at 530 nm. The culture was then homogenized in an ultrasonic bath for 1 hr, and centrifuged at 8000 rpm for 30 min. The supernatant was autoclaved at 121°C for 15 min and diluted with distilled water to various concentrations as shown in Table 6.

TRH was prepared by culturing *Trichophyton rubrum* in Saboroud dextrose agar for 10-14 days. Fungal mycelia were collected and dried at 50°C overnight. Dried mycelia (150 mg) were then finely pulverized, and suspended in 20 ml of B5 medium. The suspension of the pulverized mycelia was autoclaved at 121°C for 15 min and diluted with distilled water to various concentrations (Table 6) prior to use [83].

YE was suspended in B5 medium at pH 5.5 ± 0.1, to afford a stock solution of 0.3 g/ml and sterilized by autoclaving at 121°C for 15 min and adjusted to various elicitor concentrations (Table 6) prior to use [83].

CHI was dissolved in distilled water. The solution was adjusted to pH 5.5 with 1 N NaOH, and the final concentration, adjusted to 10 mg/mL. Aliquot was autoclaved for 15 min at 121°C prior to use.

MJ was dissolved in 95% (v/v) ethanol. The solution (30 mM) was filtered through a microfilter (0.22 μm) and diluted with 95% (v/v) ethanol to various concentrations (Table 6) prior to use [122].

19.2 Effect of elicitor types used at different concentrations

The root cultures of *I. balsamina* (3 week old, 1 g) were transferred to fresh B5 liquid media (50 mL). Various types of elicitors at different concentrations (Table 9) were added to the 21 day old root cultures and incubated for 72 hr. Five individual root cultures were used for each elicitor types/concentrations. Control root cultures were treated with distilled water (5 mL) or ethanol (1 mL). After 72 hr, the cultured roots were harvested by vacuum filtration, dried at 50°C and powdered, and then extracted and subjected to quantitative HPLC analysis for the three naphthoquinones. In the case of MJ only, the liquid medium after filtration was subjected to liquid-liquid extraction (see section **19.6**) and quantitative HPLC analysis for the three naphthoquinones

Table 9 Types of elicitors and concentrations used in this study

Elicitors	Unit	Concentrations			
C. albicans homogenate (CAH)	% (v/v)	1.0	2.0	5.0	10.0
T. rubrum homogenate (TRH)	% (v/v)	1.0	2.0	5.0	10.0
Yeast extract (YE)	mg/L	10	50	100	1000
Chitosan (CHI)	mg/L	10	50	100	250
Methyl jasmonate (MJ)	µM	100	200	400	600

19.3 Determination of the optimum concentration of MJ and elicitor contact period

The cultured roots of *I. balsamina* (3 week old) were transferred to fresh B5 liquid media. Various amount of the MJ solution were added (final concentrations in incubation media were 100, 200, 300 and 400 µM) to the 21 day old root cultures. The incubation periods at each concentration were varied (24, 36, 48 and 72 hr). The root cultures were harvested, dried at 50°C and powdered, then extracted and subjected to quantitative HPLC analysis for the three naphthoquinones.

19.4 Effect of age of root cultures for maximal MJ elicitation

After the most appropriate elicitor (MJ) was selected from earlier experiments, the concentration (300 µM), and incubation period (36 hr) were optimized, an appropriate age of root cultures for maximal elicitation was investigated. MJ (300 µM) was added to 14, 18, 21 and 25 day old root cultures at a final concentration of 300 µM. After 36 hr of incubation with the elicitor, the root cultures were harvested, dried at 50°C and powdered, then extracted and subjected to quantitative HPLC analysis for the three naphthoquinones.

19.5 Extraction of naphthoquinones from the root cultures

The root cultures were dried at 50°C and ground to a fine powder by mortar and pestle. The root powder (0.2 g) was extracted with 50% chloroform in methanol (20 mL) under

reflux conditions for 1 hr and then filtered. The filtrates were evaporated to dryness under reduced pressure (40°C). The residue was dissolved with methanol and the volume adjusted to 10 mL.

19.6 Extraction of naphthoquinones from liquid media

Culture medium (50 mL) was acidified with 1N HCl to pH 2.0, and then partitioned with ethyl acetate (20 mL × 3). The pooled ethyl acetate fractions were then evaporated to dryness under reduced pressure (40°C). The residue was dissolved with methanol and the volume adjusted to 10 mL.

20. Isolation of secondary metabolites from *I. balsamina* root cultures

I. balsamina root cultures (150 g dry weight) were extracted with 50% $CHCl_3$/MeOH (2 L x 3) under reflux conditions for 1 hr. The extracts were combined and concentrated under reduced pressure to afford a crude extract (15.4 g). The crude extract was then partitioned between EtOAc and 10% NaOH solution (Figure 7). The pooled fractions of ethyl acetate were evaporated to produce E-1 fraction (7.2 g). The aqueous phase was subsequently adjusted to pH 2.0 with conc. HCl and again partitioned with ethyl acetate to produce E-2 fraction (6.0 g). E-1 fraction was subjected to isolation by a preparative HPLC [column: Tosoh, TSK gel ODS, 5 μm, 6 x 60 cm x 2; solvent: MeOH-0.05% aqueous trifluoroacetic acid (TFA) (40:60⟶60:40); detector: UV 205 nm] to afford 15 fractions. Fraction no. 9 (12.6 mg) was then subjected to purification by a semi-preparative HPLC [column: Tosoh® TSK gel ODS 100V, 2 x 25 cm; solvent: MeOH-0.05% TFA in water 35:65; detector: 205 nm] to afford a pure compound **1** (2 mg; t_R 37 min). Fraction no. 6 (50 mg) was subjected to purification by a semi-preparative HPLC [column: Supelco, Ascentis™ Phenyl, 2.2 x 25 cm; solvent: acetronitrile-water 10:90; detector: 205 nm] to yield compound **2** (5 mg; t_R 144 min [recycle mode]) and compound **3** (4 mg; t_R 163 min [recycle mode]). Fraction no. 15 (838 mg) was chromatographed by a silica gel column chromatography and eluted (50 mL for each fraction) using a hexane-$CHCl_3$ (95:5) : MeOH gradient solvent system (9:1, 7:3, 1:1, 1:4, 1 L each). Fractions were collected and pooled by TLC analysis to afford 7 fractions. From these fractions, fraction I [120 mg: eluted with hexane-$CHCl_3$ (95:5) : MeOH 9:1] was subjected to purification by a semi-preparative HPLC

[column: Shiseido® Capcell Pak ODS, 5 μm, 2 x 25 cm; solvent: acetronitrile-water 90:10; detector: 205 nm] to give compound **4** (28 mg; t_R 28 min). Fraction no.V [85 mg: eluted with hexane-$CHCl_3$ (95:5) : MeOH 1:1] was recrystalized using $CHCl_3$ and MeOH to obtain pure compound **5 (**13 mg**)**.

E-2 fraction (6 g) was subjected to isolation by HP20 column chromatography using 25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH and EtoAc as eluents (2 L each) to yield 25% MeOH (1.77 g), 50% MeOH (0.56 g), 75% MeOH (1.04 g), 100% MeOH (1.78 g), and EtOAc fractions (0.47 g). The 25% MeOH fraction (100 mg) was subjected to purification by a semi-preparative HPLC [column: Supelco, Ascentis™ Phenyl, 2.2 x 25 cm; solvent: acetronitrile-0.05% TFA in water 10:90; detector: 205 nm] to give compound **6** (7 mg; t_R 26 min). The 50% MeOH fraction (500 mg) was subjected to purification by a semi-preparative HPLC [column: Tosoh®, TSK gel ODS 100V, 2 x 25 cm; detector: 205 nm] with various solvent system to afford pure compounds as follows: MeOH-0.05% TFA in water (20:80) solvent system gave compound **7** (8 mg; t_R 36 min) and compound **8** (10 mg; t_R 42 min); acetronitrile-0.05% TFA in water (10:90) yielded compound **9** (15 mg t_R 37 min). The 100% MeOH fraction (500 mg) were subjected to purification by a semi-preparative HPLC [column: Shiseido® Capcell Pak ODS, 5 μm, 2 x 25 cm; solvent: acetronitrile-water 85:15; detector: 205 nm] to afford pure compound **10** (19 mg; t_R 49 min).

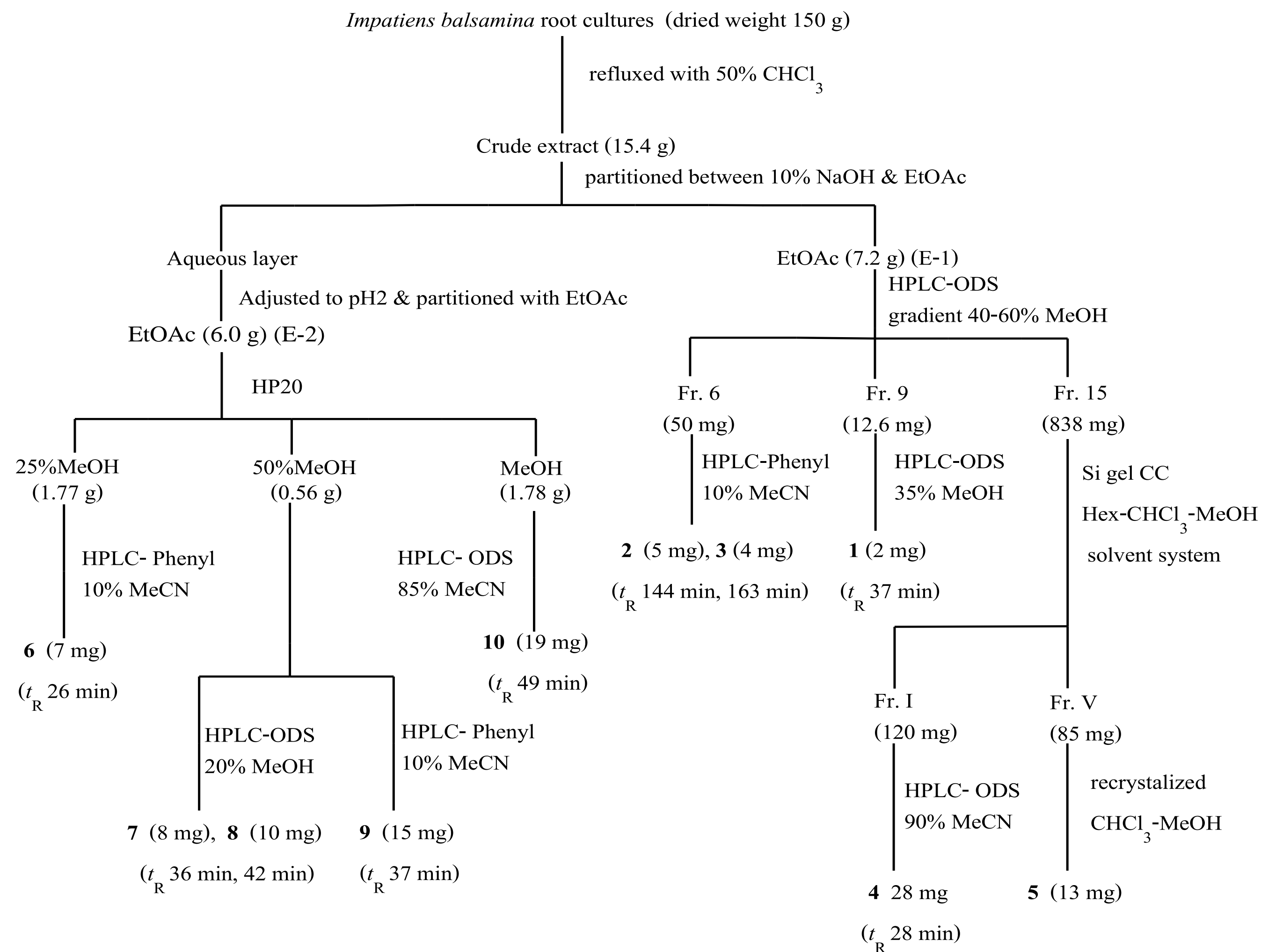


Figure 7 Isolation of secondary metabolites from *Impatiens balsamina* root cultures

21. Statistical analysis

All experiments were repeated three times. The data [mean ± standard error of mean (SE)] obtained were statistically analyzed by SPSS version 15.0. Data were submitted to analysis of variance, and mean were then compared with one-way ANOVA, using Tukey's test for multiple comparison. The term significant has been used to denote the differences for which $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

1. HPLC method development and optimization

Appropriate conditions for the simultaneous quantitative determination of lawsone, lawsone methyl ether and 3,3′-methylenebislawsone in *I. balsamina* leaf extracts required the use of gradient reversed-phase HPLC. Since all three compounds have a high UV absorption at 280 nm, this wavelength was used for quantification. Mixtures of methanol and 2% aqueous acetic acid were examined as the mobile phase, and the ratios as well as gradient elution system were altered until all three compounds were satisfactorily resolved at the baseline in 35 min (Figure 8). This HPLC method was required shorter period of analysis time than those of the HPLC method previously described by Lobstein *et al.* [123], and was capable of simultaneous quantification of lawsone, lawsone methyl ether and 3,3′-methylenebislawsone. The method is simple, selective and sensitive. In addition, the method does not require many steps for sample preparation, and therefore it is possible to analyze 30–35 samples within a 24 hr period. This HPLC method readily allowed identifying of all the naphthoquinones in *I. balsamina* leaf extracts at the same time. These three naphthoquinones were detected as the major naphthoquinones in *I. basamina* leaves. This is in contrast to a previous report which failed to find 3,3′-methylenebislawsone in natural *I. balsamina* plants, but only identified 3,3′-methylenebislawsone in the root cultures of *I. balsamina*. The sources of plant material, age of the plant and growing conditions may affect secondary metabolite production to account for these different observations.

2. Validation of HPLC method

Validation of analytical method for naphthoquinone from *I. balsamina* was examined for linearity, precision, accuracy, specificity, LOD and LOQ. Linearity was evaluated

using standard samples over five calibration points (3.12, 6.25, 12.5, 25.0 and 50.0 mg/mL). On a given day, the measurement was carried out with each concentration in triplicate, with a total of nine measurements for each calibration point. Three separate calibration curves were obtained on three different days by plotting the mean peak area against concentration. Excellent linearity was observed for the analytes over the ranges 3.12–50 µg/mL, with correlation coefficients 0.9995, 0.9998 and 0.9998 for lawsone, lawsone methyl ether and 3,3′-methylenebislawsone, respectively (Table 10). The results of LOD and LOQ analysis for these naphthoquinones (Table 10) indicate that the established HPLC method is sufficiently sensitive for determination of these three naphthoquinones in *I. balsamina* leaf extracts.

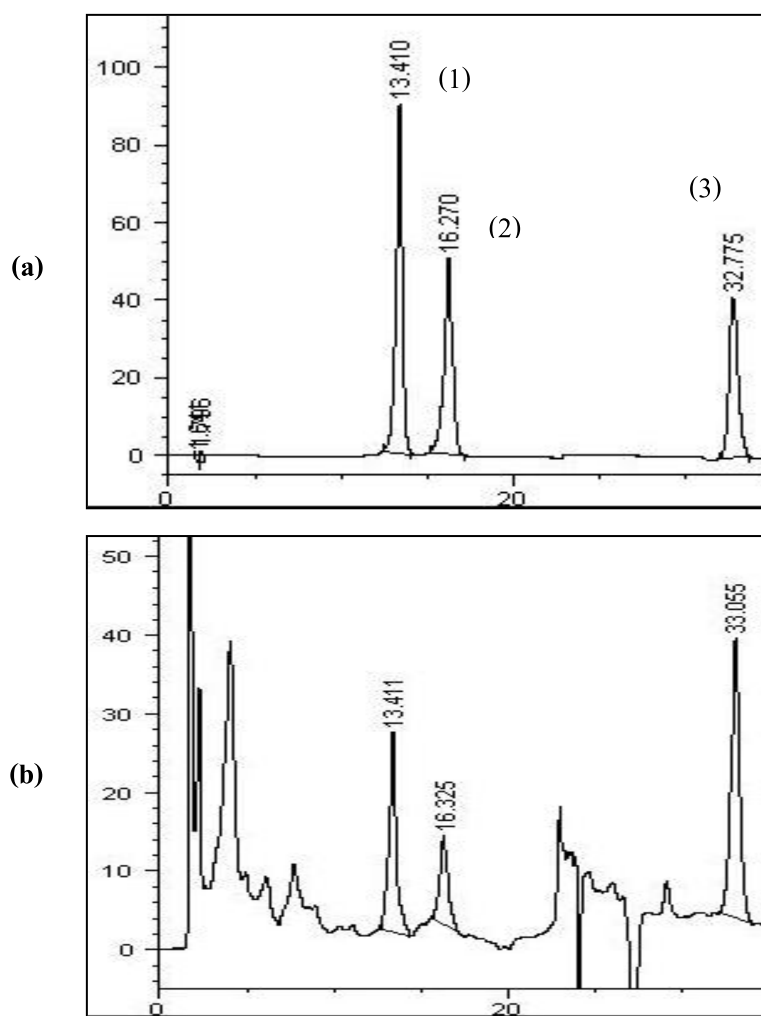


Figure 8 HPLC-chromatograms of (**a**) authentics: lawsone (**1**), lawsone methyl ether (**2**) and 3,3′-methylenebislawsone (**3**); (**b**) methanol extract of *I. balsamina* leaves.

Table 10 HPLC-PDA characteristics of analyzed naphthoquinones

Compound	Linear range (µg/ml)	t_R (min)	Equation[a]	Linearity (r^2)	LOD[b]/LOQ[c] (µg/ml)
1	3.12 – 50.0	13.1	Y = 79.239X - 60.38	0.9995	0.04/0.10
2	3.12 – 50.0	15.8	Y = 79.678X - 54.18	0.9998	0.04/0.10
3	3.12 – 50.0	32.4	Y = 73.712X - 75.96	0.9998	0.16/0.43

* 1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

[a] Y = AX + B, where Y is peak area, X is the concentration of the analyzed material.

[b] Limit of detection (LOD): signal to noise ratio = 3

[c] Limit of quantification (LOQ): signal to noise ratio = 10

The precision of the chromatographic method was tested by performing intra- and inter-day multiple injections of a standard solution containing lawsone, lawsone methyl ether and 3,3′-methylenebislawsone. The percentage relative standard deviation (%RSD) of the retention times and peak areas were calculated, and the values of intra- and inter-day for lawsone, lawsone methyl ether and 3,3′-methylenebislawsone were each less than 1% for both retention times and peak areas (Table 11), indicating high precision of the developed HPLC method. The precision of the extraction procedure was similarly evaluated by determining intra- and inter-day %RSD data from repeated extraction of the same batch of leaves. The results (Table 12) suggest that the method is capable of quantification of *I. balsamina* naphthoquinones with high precision.

The accuracy of the analytical method was studied by spiking solutions of lawsone, lawsone methyl ether and 3,3′-methylenebislawsone at concentrations of 50, 25 and 12.5 µg/mL into *I. balsamina* leaf extracts to evaluate recoveries of naphthoquinones for this method. The recoveries near to 100% (Table 13) indicate a good accuracy of this method.

Table 11 Validation of precision of analytical method of naphthoquinones

Compound	Intra-day (n=3)						Inter-day (n=9)	
	Day 1		Day 2		Day 3			
	$t_{R\ (min)}$	RSD (%)	$t_{R\ (min)}$	RSD (%)	$t_{R\ (min)}$	RSD (%)	$t_{R\ (min)}$	RSD (%)
1	13.4	0.42	13.3	0.22	13.3	0.25	13.3	0.43
2	16.3	0.36	16.2	0.14	16.1	0.09	16.2	0.62
3	32.7	0.15	32.9	0.11	33.1	0.13	32.9	0.61
	mAU	**RSD (%)**	**mAU**	**RSD (%)**	**mAU**	**RSD (%)**	**mAU**	**RSD (%)**
1	2211	0.44	2181	0.65	2213	0.42	2202	0.81
2	2065	0.32	2091	0.29	2092	0.66	2083	0.73
3	1821	0.54	1829	0.23	1841	0.43	1830	0.56

* 1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

Table 12 Validation of precision of the extraction of naphthoquinones

Compound	Intra-day (n=3)						Inter-day (n=9)	
	Day 1		Day 2		Day 3			
	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)
1	0.95	1.27	0.97	1.13	1.01	1.08	0.98	3.25
2	0.50	2.47	0.46	1.77	0.51	2.21	0.50	4.43
3	0.75	1.68	0.78	1.52	0.77	1.44	0.77	2.41

* 1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

Table 13 Validation of the accuracy of the analytical method of naphthoquinones

Compound	Spiked level (μg/mL)	Recovery (%)[a] (n = 9)			Mean (%)[b]
		1	2	3	
1	50	100.19 ± 1.15	101.76 ± 1.66	100.39 ± 1.07	100.78 ± 0.85
	25	102.30 ± 1.09	101.38 ± 1.22	99.72 ± 1.35	101.13 ± 1.30
	12.5	99.25 ± 2.65	103.54 ± 1.11	99.66 ± 1.51	100.82 ± 2.37
2	50	96.27 ± 1.04	98.84 ± 1.10	95.08 ± 1.04	96.73 ± 1.92
	25	97.14 ± 2.30	100.37 ± 2.04	97.72 ± 2.12	98.41 ± 1.72
	12.5	98.56 ± 1.98	98.25 ± 1.09	96.79 ± 1.12	97.87 ± 0.94
3	50	100.52 ± 1.30	99.45 ± 0.98	100.27 ± 1.74	100.08 ± 0.56
	25	101.14 ± 1.81	98.85 ± 1.02	103.23 ± 1.10	101.07 ± 2.19
	12.5	98.47 ± 1.21	101.76 ± 1.55	99.45 ± 1.34	99.89 ± 1.69

*Compound 1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

[a] All values were mean ± S.D. obtained by triplicate analyzes.

[b] Result expressed as a mean ± S.E.

The specificity of the method was evaluated using UV absorption spectra at three points of each of the three peaks. Comparison of these spectra with those for authentic samples revealed that each peak was homogeneous and not overlapping with any impurity peaks (Figure 9).

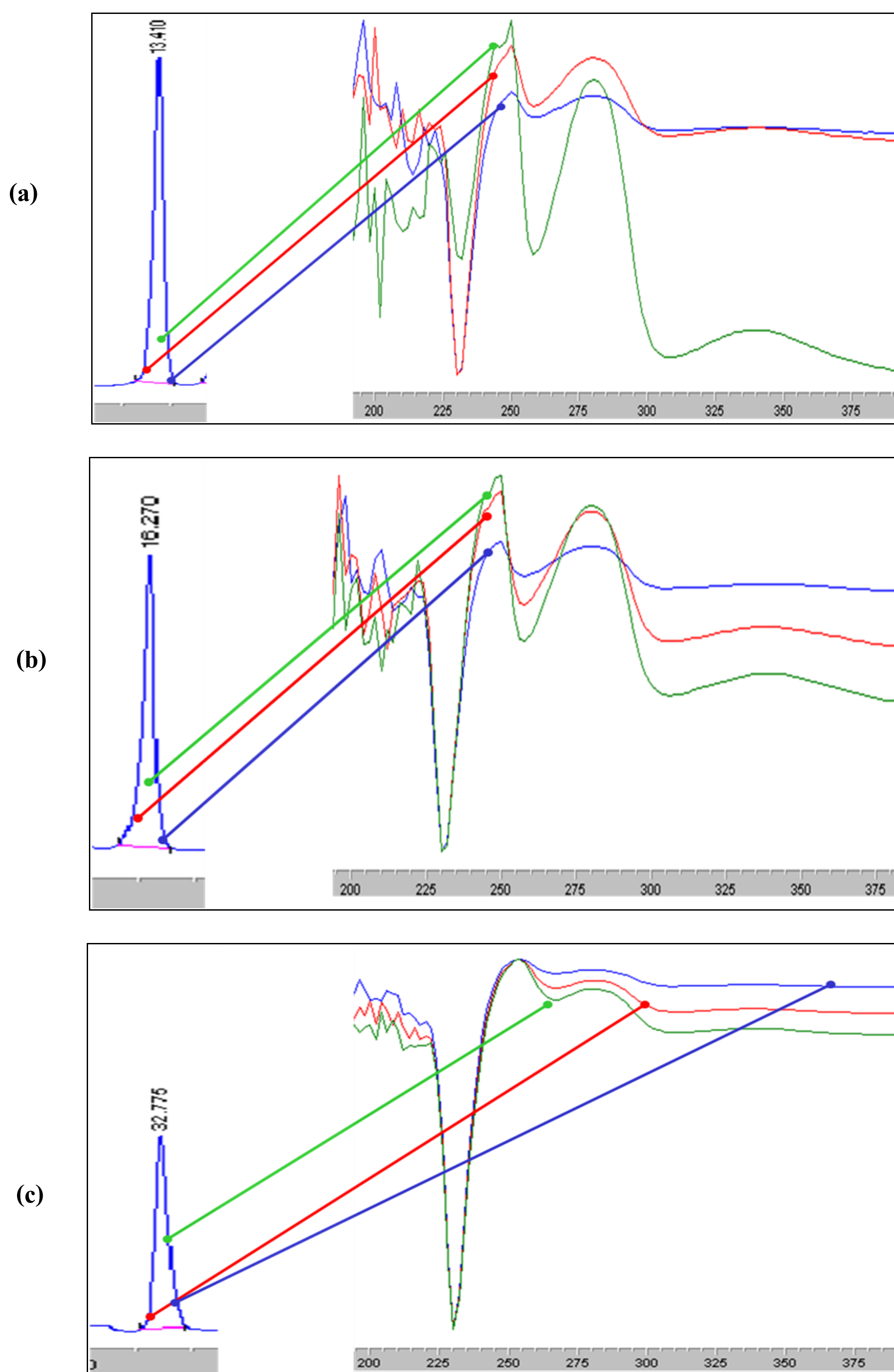


Figure 9 Absorption spectra of (a) lawsone, (b) lawsone methyl ether and (c) 3,3′-methylenebislawsone in *I. balsamina* leaf extract detected by PDA

3. Optimization of solvent for extraction of naphthoquinones

A few extraction solvents with different polarities were examined to maximize naphthoquinone content in *I. balsamina* leaf extracts. The three naphthoquinones in *I. balsamina* leaves possessed quite different polarities. Our preliminary study suggested that extraction by refluxing gave a higher yield of naphthoquinones, rather than those of extraction with ultrasonication (data not show). Methanol, ethyl acetate and chloroform were therefore examined as possible extracting solvents. As shown in Figure 10, methanol gave significantly higher total naphthoquinone content (0.26 ± 0.016% w/w) than those of ethyl acetate (0.19 ± 0.015% w/w). Methanol was efficient for the extraction of three naphthoquinones, whereas chloroform alone failed to extract 3,3′-methylenebislawsone but suitable for lawsone methyl ether extraction. Although ethyl acetate was as efficient as methanol at extracting 3,3′-methylenebislawsone, it showed a lower capacity for extracting lawsone and lawsone methyl ether. This suggested that polarity of the solvent used for extraction was affecting the naphthoquinone content of the extracts. Mixed extraction solvents containing methanol and chloroform were therefore further examined to optimize naphthoquinone content of *I. balsamina* leaf extracts. Increasing chloroform concentration up to 50% v/v in methanol resulted in an increased extraction of all naphthoquinones. However, the naphthoquinone content was decreased when chloroform concentration was further increased to 70% v/v (Figure 10). An extraction solvent mixture of 50% v/v chloroform in methanol was capable of increasing the naphthoquinone content in leaf extracts up to 0.33 ± 0.022% w/w, and this solvent mixture was judged to be the ideal solvent from among the mixtures studied.

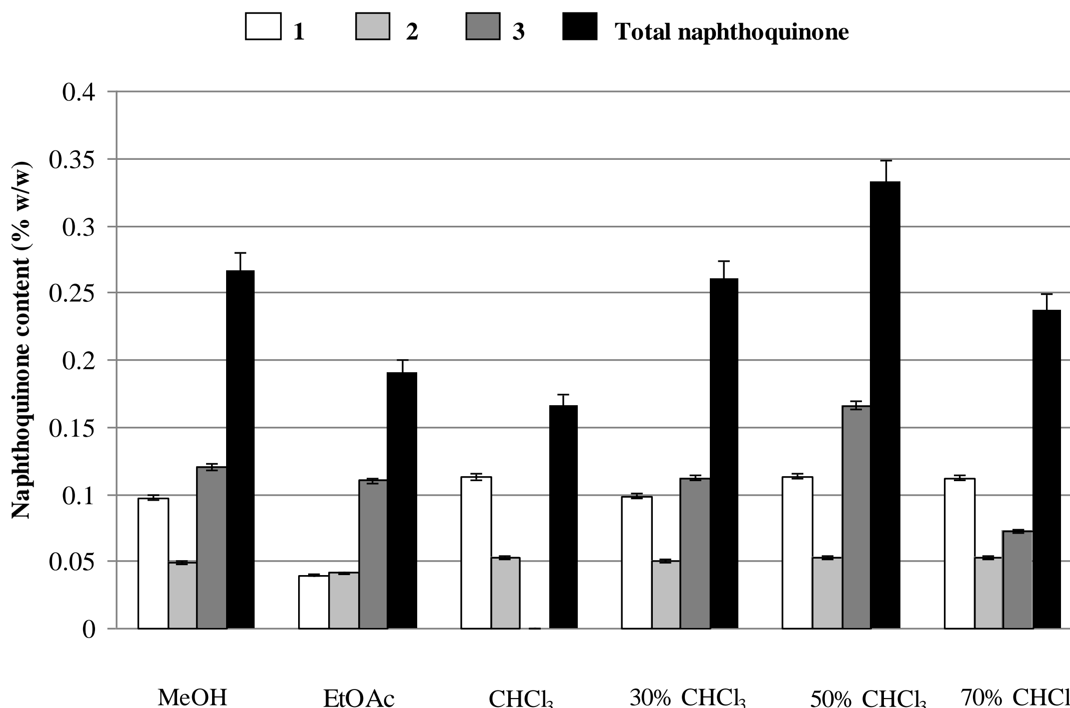


Figure 10 Effect of solvent type on the extraction of naphthoquinones (1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone) from *I. balsamina*

4. Effect of source of explant on naphthoquinone production

It has been demonstrated that the leaves of *I. balsamina* produce higher naphthoquinones content than the roots [124]. In this study, we investigated the effect of explants that possesses different potential of naphthoquinone production in *I. balsamina* root cultures. Our finding indicated that the root cultures that initiated from different explant exhibited different naphthoquinone production. The leaf explant could provide the root cultures that produced significantly higher naphthoquinone content than those initiated from the root explant (Table 14). This result suggests that a starting plant material that possesses higher potential of secondary metabolite production may provide a higher secondary metabolite producing plant tissue culture.

Table 14 Naphthoquinone contents in *I. balsamina* root culture established from the leaf and root explants of IbR

Explants	Naphthoquinone content (mg/g DW) Mean ± SE (n=6)			
	1	**2**	**3**	**Total naphthoquinone**
Leaves	0.52 ± 0.032*	0.14 ± 0.001*	0.35 ± 0.007*	1.01 ± 0.046*
Roots	0.29 ± 0.011	0.11 ± 0.002	0.22 ± 0.003	0.62 ± 0.023

1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

*Significant difference (P<0.05) when compared within the same column according to independent T-test.

5. Effect of donor plants on naphthoquinone production

A variation of naphthoquinone content in *I. balsamina* leaves was assessed in four strains of *I. balsamina*. The plant strains were categorized by their flower colors. HPLC analysis of naphthoquinones in the leaves of *I. balsamina* plantlets revealed that all 4 strains produced lawsone, lawsone methyl ether and 3,3′-methylenebislawsone. In addition, lawsone and lawsone methyl ether are found as the main naphthoquinones in the leaves of *I. balsamina* (Figure 7). A variation of naphthoquinone production among the plant strains was observed. The content of naphthoquinones in IbP was significantly higher than those of the other strains (Table 15). Among these, IbR produced the least content of naphthoquinones. All four strains of plantlets were used to establish the root cultures of *I. balsamina* in order to investigate the effect of the donor plants on naphthoquinone production of the root cultures. After several subcultures, the root cultures of *I. balsamina* were examined for their potential of naphthoquinone production using HPLC. The cultured root extracts contained lawsone and 3,3′-methylenebislawsone as the major naphthoquinones, while the intact leaf extracts contained lawsone and lawsone methyl ether as the major naphthoquinones (Figure 11). Although the root cultures produced higher amount of lawsone and 3,3′-methylenebislawsone, it produced lower amount of lawsone methyl ether than the leaves of the intact plant (Tables 15 and 16). This may be due to a lack of *O*-methyltransferase enzyme, a key enzyme in the final step of lawsone methyl ether biosynthesis

[61]. The obtained naphthoquinone high-producing root cultures can be used as a material of choice for biosynthetic studies of naphthoquinones in *I. balsamina*. They can be used for detection of *o*-succinylbenzoyl-CoA ligase activity which converts *o*-succinylbenzoic acid (OSB) to be its activated form, OSB-CoA ester in the biogenesis of lawsone (Figure 12). However, the strategies to increase lawsone methyl ether production in the root cultures should be further investigated. Regarding the effect of the donor plants, the root cultures established from naphthoquinone high-yielding strain (IbP) significantly produced higher total naphthoquinone content than those established from naphthoquinone low-yielding strains (Table 16). In addition, the root culture established from IbR produced the lowest amount of total naphthoquinones. These results support our hypothesis that the root cultures initiated from the naphthoquinone high-yielding donor plants are capable of producing higher amount of naphthoquinones than those initiated from the naphthoquinone low-yielding strains.

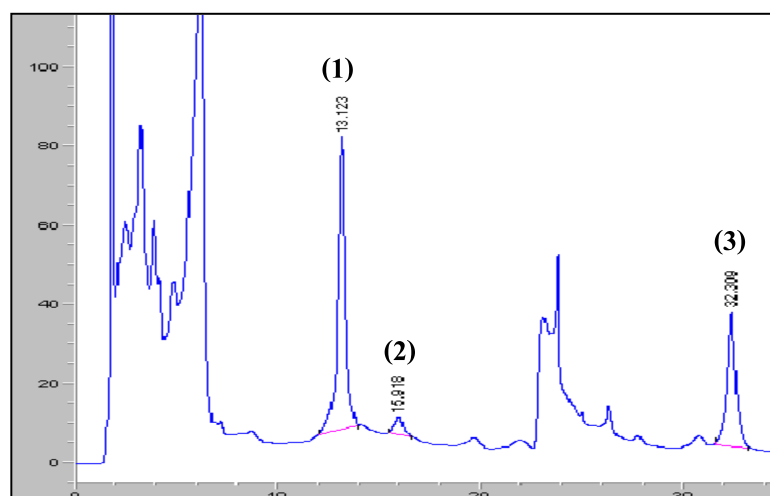


Figure 11 HPLC chromatogram of 1-month old *I. balsamina* root culture extract

(1) = lawsone, **(2)** = lawsone methyl ether, **(3)** = 3,3′-methylenebislawsone

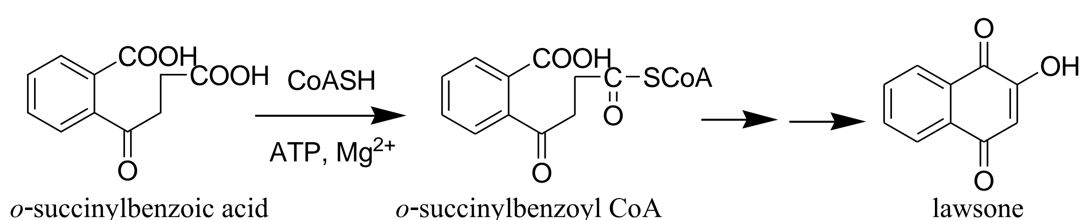


Figure 12 Biogenesis of lawsone from OSB *via* it activated form, OSB-CoA ester

Table 15 Naphthoquinone contents in the leaves of various *I. balsamina* strains

Plant strains	Naphthoquinone content (mg/g DW) Mean ± SE (n=16)			
	1*	**2***	**3***	**Total naphthoquinone***
IbR	0.50 ± 0.027d	0.89 ± 0.057c	0.06 ± 0.003b	1.45 ± 0.055c
IbV	1.01 ± 0.027b	1.27 ± 0.071b	0.06 ± 0.002b	2.34 ± 0.061b
IbP	1.50 ± 0.064a	1.81 ± 0.079a	0.09 ± 0.003a	3.39 ± 0.072a
IbW	0.84 ± 0.039c	1.37 ± 0.068b	0.07 ± 0.002b	2.28 ± 0.052b

1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

* Significant difference (P<0.05) when compared within the same column. Means followed the same letter within column are not significantly different.

Table 16 Naphthoquinone contents in root cultures of *I. balsamina* established from the various plant strains

Plant strains	Naphthoquinone content* (mg/g DW) Mean ± SE (n=16)			
	1*	**2***	**3***	**Total naphthoquinone***
IBR	0.75 ± 0.027d	0.11 ± 0.004b	0.10 ± 0.005b	0.96 ± 0.031d
IBV	2.04 ± 0.067b	0.14 ± 0.003a	0.14 ± 0.016a	2.32 ± 0.071b
IBP	2.49 ± 0.081a	0.12 ± 0.003b	0.16 ± 0.005a	2.76 ± 0.093a
IBW	1.70 ± 0.019c	0.09 ± 0.007c	0.16 ± 0.007a	1.95 ± 0.024c

1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

* Significant difference (P<0.05) when compared within the same column. Means followed the same letter within column are not significantly different.

6. Effect of plant growth regulators on naphthoquinone production

Our previous report has demonstrated the production of lawsone, LME and bilawone in *I. balsamina* root cultures that were cultured in B5 medium supplemented with 0.1 mg/L NAA, 0.1 mg/L kinetin and 1.0 mg/L BA [6]. However, the content of naphthoquinones produced by the root cultures as well as a medium manipulation to improve naphthoquinone production in the root cultures have not been investigated yet. In this study, we investigated the effect of the plant growth regulator concentration on the naphthoquinone production in *I. balsamina* root cultures. Variation of BA concentrations in the culture medium (supplemented with 0.1 mg/L NAA and 0.1 mg/L kinetin) demonstrated that the naphthoquinone production in the root cultures was gradually increased by the increasing of BA concentration (Table 17). The optimum BA concentration that produced the highest naphthoquinone content in the root cultures was 2.0 mg/L. Thus, the further determination of kinetin concentration was performed by fixing of BA and NAA at 2.0 and 0.1 mg/L, respectively. Variation of kinetin concentrations indicated that naphthoquinone production was reached to the highest content when kinetin concentration was 1.0 mg/L (Table 17). An increasing of kinetin concentration up to 2.0 mg/L resulted in a decreasing of naphthoquinone production. Thus, the further determination of NAA concentration was performed by fixing of BA and kinetin concentrations at 2.0 and 1.0 mg/L, respectively. In contrast to cytokinin, increasing of NAA concentration caused decrease in both naphthoquinone production and growth. In addition, at high concentration of NAA, the root cultures were dedifferentiated to be callus (Figure 13). This dedifferentiation of the root cultures may cause a functional impairment of naphthoquinone biosynthetic pathway. Our finding on the effect of high concentration of NAA that suppressed naphthoquinone production seems to agree with the previous reports on the inhibitory effect of auxin on naphthoquinone production of *Echium lycopsis* callus cultures [125] and *Drosophyllum lusitanicum* cell suspension cultures [126] as well as anthraquinone production of *Morinda citrifolia* cell cultures [127]. These finding thus suggest B5 supplemented with 0.1 mg/L NAA, 1.0 mg/L kinetin and 2.0 mg/L BA as an appropriate medium for naphthoquinone production of *I. balsamina* root cultures.

(a)

(b)

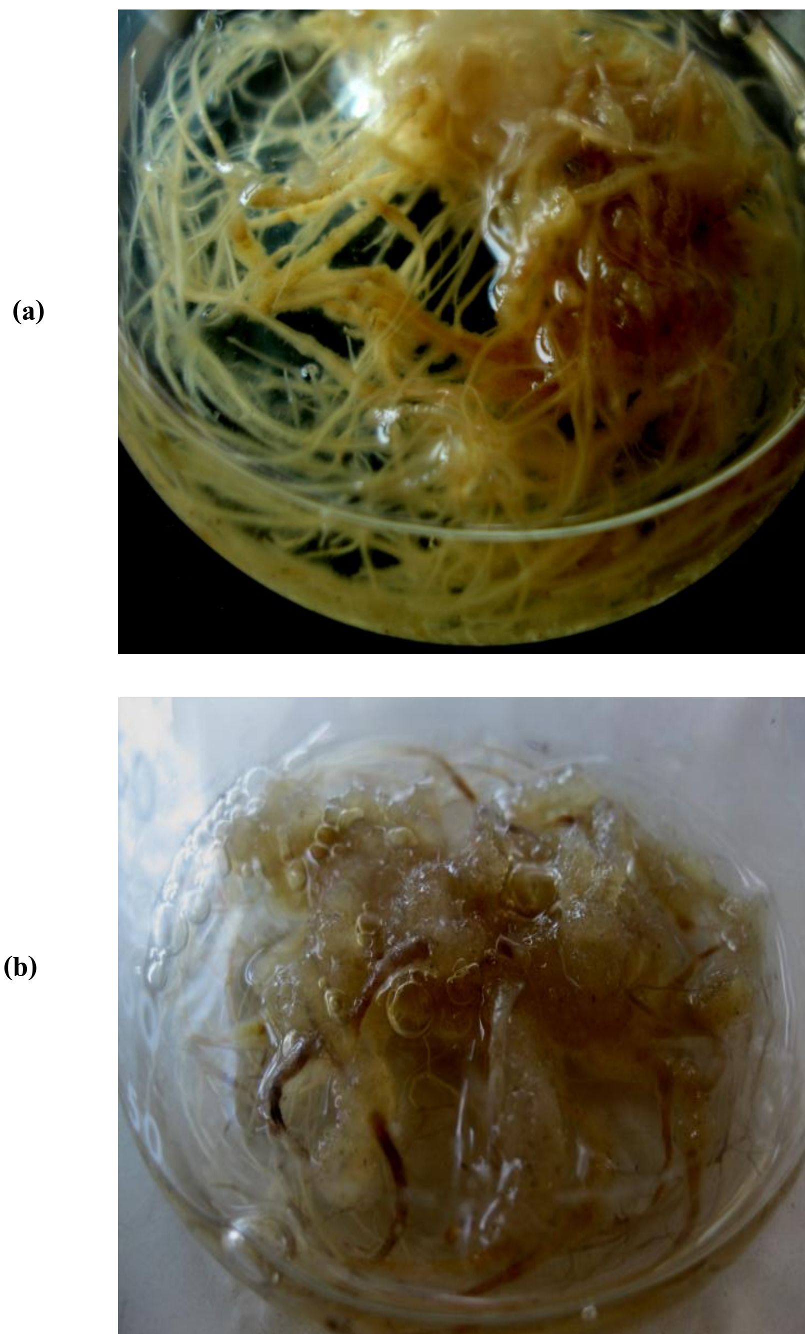


Figure 13 *I. balsamina* root cultures in (a) low auxin supplemented B5 medium (b) high auxin supplemented B5 medium

Table 17 Effect of plant growth regulator on naphthoquinone contents in *I. balsamina* root cultures

Phytohormone (mg/L)			**Naphthoquinone content (mg/g DW) Mean ± SE**			
NAA	**Kn**	**BA**	**1**	**2**	**3**	**Total naphthoquinone**
0.1	0.1	0.1	0.22 ± 0.006*d	0.11 ± 0.004*b	0.17 ± 0.008*c	0.49 ± 0.010*d
0.1	0.1	0.5	0.35 ± 0.006*c	0.09 ± 0.008*b	0.19 ± 0.016*c	0.63 ± 0.031*c
0.1	0.1	1.0	0.55 ± 0.012*b	0.15 ± 0.006*a	0.33 ± 0.022*b	1.03 ± 0.037*b
0.1	0.1	2.0	1.60 ± 0.027*a	0.15 ± 0.004*a	0.50 ± 0.024*a	2.25 ± 0.029*a
0.1	0.1	2.0	1.72 ± 0.081#b	0.15 ± 0.005#b	0.47 ± 0.012#c	2.34 ± 0.093#b
0.1	0.5	2.0	1.82 ± 0.032#b	0.17 ± 0.011#a	0.41 ± 0.020#c	2.40 ± 0.077#b
0.1	1.0	2.0	2.14 ± 0.070#a	0.17 ± 0.005#a	0.62 ± 0.009#a	2.93 ± 0.101#a
0.1	2.0	2.0	1.88 ± 0.056#b	0.16 ± 0.003#b	0.57 ± 0.003#b	2.61 ± 0.088#b
0.1	1.0	2.0	2.12 ± 0.081†a	0.18 ± 0.015†a	0.67 ± 0.042†a	2.97 ± 0.072†a
0.5	1.0	2.0	1.33 ± 0.026†b	0.11 ± 0.009†b	0.35 ± 0.020†b	1.79 ± 0.063†b
1.0	1.0	2.0	0.94 ± 0.070†c	n.d.	0.32 ± 0.040†b	1.26 ± 0.126†c
2.0	1.0	2.0	0.19 ± 0.038†d	n.d.	0.17 ± 0.003†c	0.36 ± 0.044†d

n.d. = Not detect, Kn = kinetin

1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

*, #, † significant difference (P<0.05) when compared within the same column. Means followed the same letter within the same column are not significantly different.

7. Time-courses of growth and naphthoquinone production

The growth cycle of *I. balsamina* root cultures during a period of 30 days demonstrated that there was a very short lag phase of growth followed by a rapid growth of exponential phase (9 days) and linear phase (15 days). This resulted in a continuous increase of biomass throughout the period of 24 days. Thereafter, the dried biomass weights became constant and then gradually decreased, indicating that the root cultures had reached the stationary and decline phases, respectively (Figure 14). The root cultures attained their highest dry biomass weight of 507.8 mg/250-mL flask at day 24, equivalent to about nine times of the inoculated dry biomass. The formation of all naphthoquinones in the root cultures seemed to begin in the late exponential phase or early linear phase (day 9) of the growth. Lawsone was actively biosynthesized throughout the linear and stationary phases. The highest level of lawsone was observed at day 27 and then began to decline at the end of the growth cycle. In addition, 3,3′-methylenebislawsone was initially accumulated in small amount throughout the linear phase. However, it was actively biosynthesized when the root cultures reached the stationery and decline phases. This may be due to a need of high lawsone accumulation to trigger the biosynthesis of 3,3′-methylenebislawsone. In contrast, lawsone methyl ether was initially biosynthesized in the late exponential phase with a constant rate of production and a small amount of accumulation throughout the growth cycle. These results suggested that the biosynthesis of lawsone is operational in the early stage of growth and throughout the growth cycle. Subsequently, the biosynthesis of 3,3′-methylenebislawsone is functional after the biosynthesis of lawsone is at the maximum. In contrast, the biosynthesis of lawsone methyl ether is not working in the root cultures even if they produce high amount of lawsone, a precursor of lawsone methyl ether biosynthesis. This could be due to a limit of *O*-methyltransferase enzyme expression in the root cultures of *I. balsamina*. Since cytotoxic activity of lawsone methyl ether had been reported, lawsone methyl ether might be toxic to the root cell, and excreted out of the root cultures, therefore lawsone methyl ether content was constant throughout of growth.

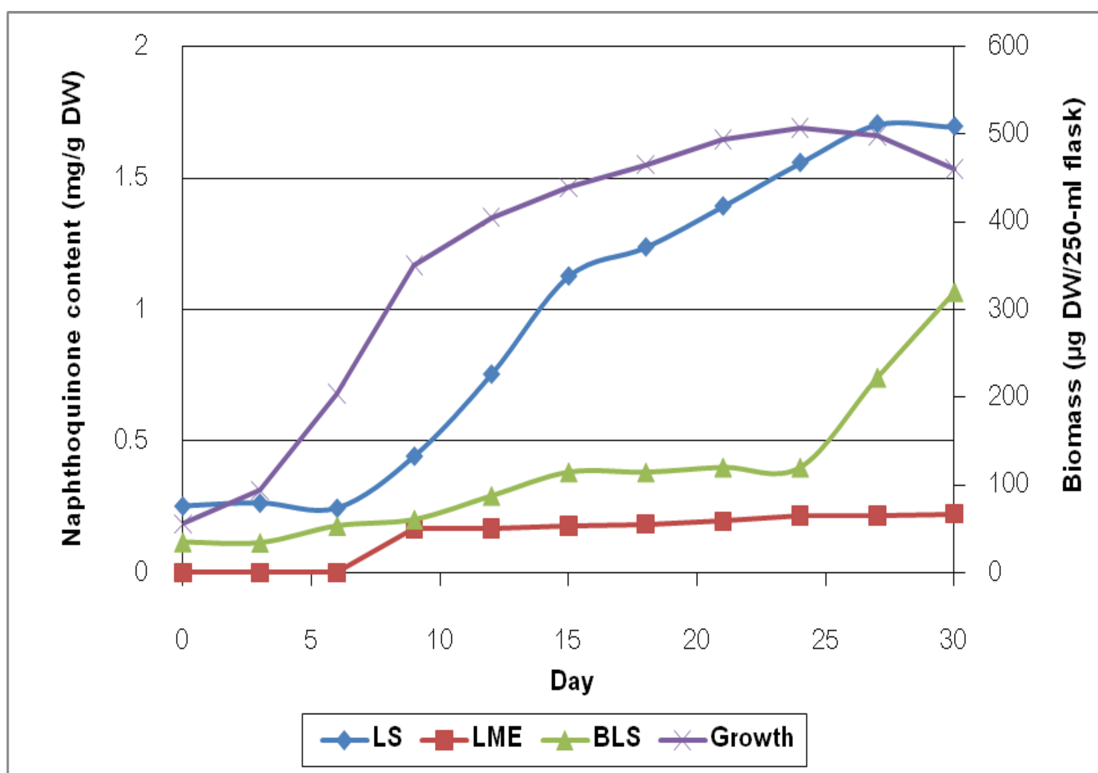


Figure 14 Time-course of growth and naphthoquinone production of *I. balsamina* root cultures (LS = lawsone, LME = lawsone methyl ether, BLS = 3,3'-methylnebislawsone)

8. Effect of methionine-feeding on naphthoquinone production

Methionine was added to *I. balsamina* root cultures at the beginning of the growth cycle (day 0). It was found that high concentrations of methionine (500 and 1000 mg/L) caused cell death, while at low concentrations (50, 100 and 300 mg/L), methionine inhibited cell growth significantly when compared to the untreated group. Naphthoquinone production in the root cultures after feeding with methionine was determined and compared with the control group. It was found that low concentration of methionine was not affect on lawsone and lawsone methyl ether production but slightly increased 3,3′-methylenebislawsone production (Table 18). Methionine at 300 mg/L led to the highest 3,3'-methylenebislasone production up to 0.63 ± 0.103 mg/g DW, which was 1.95 times higher than in the control group (0.32 ± 0.045 mg/g DW). From the result indicated that high concentration of methionine might cause cell toxicity. Although its

toxicity has never been report in plant, methionine imbalance and toxicity affect in bodyweight loss and decreased gain/feed and *N*-utilization efficiency in Holstein bull calves [128]. Moreover in infants, methionine intakes of 2-5 times normal resulted in impaired growth and extremely high plasma methionine levels [129]. From these reports high concentration of methionine might be affecting on cell growth and eventually caused the root cultures death. In this study feeding of methionine was more appropriate for increased 3,3′-methylenebislawsone production than lawsone methyl ether. This implies that the methylene bridge in the structure of 3,3′-methylenebislawsone might be concerning with methylation of lawsone using *S*-adenosylmethionine. In case of lawsone methyl ether, even though methionine was fed to the root cultures, lawsone methyl ether was not increase. This might be due to the root cultures system probably does not express the *O*-methyltransferase for lawsone methyl ether properly.

Because the toxicity of methionine had been observed at high concentration, feeding of methionine at late linear phase to stimulate the plant defensive respond was further investigated. Methionine was feeding to 21-day old root cultures to make final concentrations range of 300 – 1000 mg/L. Like previous experiment, methionine at 500 and 1000 mg/L caused cell death and releasing of naphthoquinones into the culture medium. Lawsone and lawsone methyl ether were secreted while 3,3′-methylenebislawsone was still accumulated in the root cells. However, methionine feeding at 500 mg/L was capable of stimulating lawsone methyl ether production up to 0.17 mg/g DW. In contrast, all methionine concentrations were not effect on lawsone production (Figure 15). At the concentration of 300 mg/L methionine, the cultured roots were still healthy, and lawsone methyl ether level was comparable with control. Interestingly, 3,3′-methylenebislawsone level was moderately increased to 0.86 ± 0.020 mg/g DW which was 2.87 times higher than in the control group. The result agrees with many reports that the timing of precursor addition is critical for an optimum effect [62].

Table 18 Naphthoquinone production in *I. balsamina* root cultures after feeding with methionine in various concentrations at day 0 of cultures

Dose (mg/L)	Naphthoquinone content (mg/g DW) Mean ± SE (n=6)			
	1*	2*	3*	Total naphthoquinone
CTRL	1.45 ± 0.393a	0.07 ± 0.037a	0.32 ± 0.045b	1.85 ± 0.455a
50	1.58 ± 0.241a	0.10 ± 0.040a	0.46± 0.094b	2.14 ± 0.311a
100	1.36 ± 0.374a	0.09 ± 0.032a	0.51± 0.100a	1.96 ± 0.402a
300	1.49 ± 0.298a	0.06 ± 0.026a	0.63 ± 0.103a	2.26 ± 0.365a
500	n/a	n/a	n/a	n/a
1000	n/a	n/a	n/a	n/a

1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

* Significant difference ($P<0.05$) when compared within the same column. Means followed the same letter within column are not significantly different.

n/a = data not available

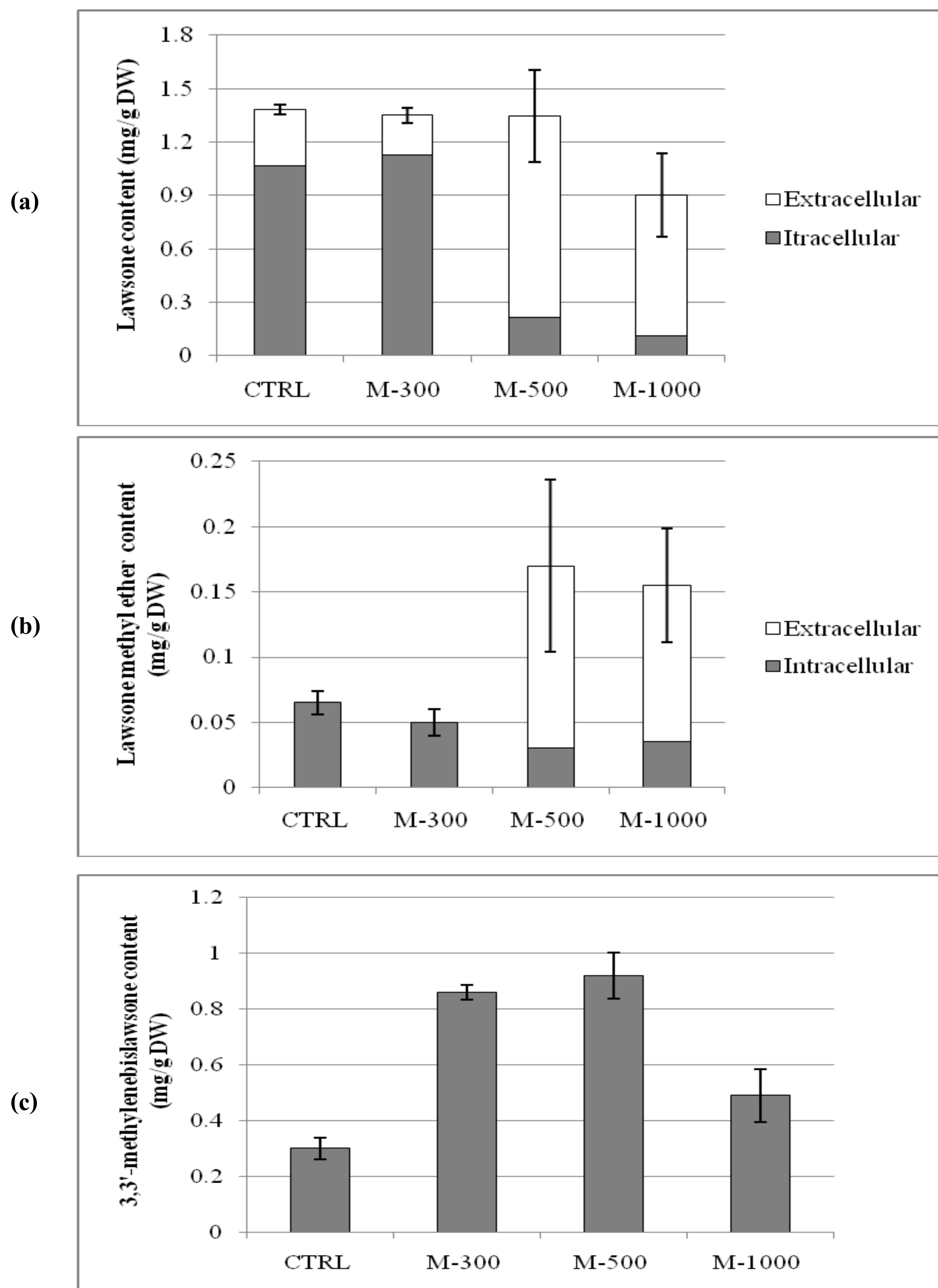


Figure 15 Intracellular and extracellular content of (a) lawsone, (b) lawsone methyl ether and (c) 3,3′-methylenebislawsone after feeding with various concentration of methionine at 21-day old root culture. Data are mean ± SE of n = 5 (M-300, M-500 and M-1000 = methionine concentration at 300, 500 and 1000 mg/L, respectively)

9. Establishment of transformed hairy roots cultures

Transformed hairy root cultures of *I. balsamina* were initiated by infection of *A. rhizogenes* ATCC 13333 to the young leaf explants. The transformed roots emerged from the young leaf within 1 week after infection, and subcultured to cefotaxime (500 mg/L) containing B5 medium and consequently transferred to lower concentration of cefotaxime until the bacteria were eliminated. The sterile transformed hairy root cultures were maintained in hormone-free B5 liquid medium (Figure 16). The transformed hairy root cultures were further detected for *rol*B and *rol*C gene in the genomic DNA in order to confirm that the root cultures were indeed obtained by the infection. The 780-bp *rol*B gene and 545-bp *rol*C gene were amplified in both transformed hairy root cultures and ordinary root cultures genomic DNA. The *rol*B region was not observed in both cultures. At *rol*C region was observed in both ordinary root cultures and transformed hairy root cultures (Figure 16). Normally, ordinary plant cell should not showed the region which amplified by *rol*C primers. This might be due to the plant has been transformed by *A. rhizogenes* for long time. However identification of this region by DNA sequencing technique must be further investigated.

Figure 16 Transformed hairy root cultures of *I. balsamina* (1 month old)

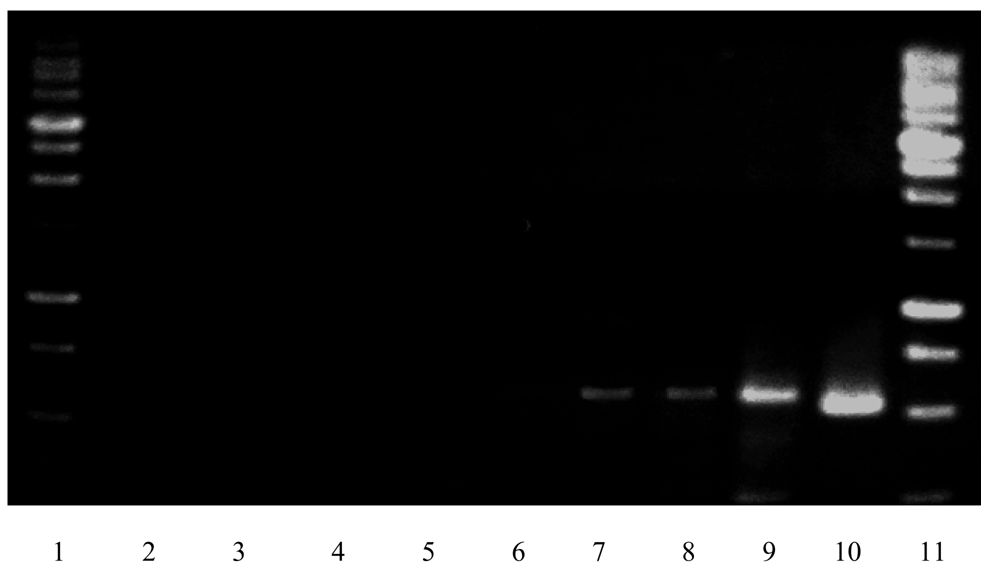


Figure 17 PCR product analysis of the *rol*C gene fragments in hairy roots (PCR products were detected under UV illuminator (312 nm) after SYBR® Safe staining)

Lane 1, 11 = Marker (10 kb DNA ladder)

Lane 2 = Ordinary root cultures, detection for *rol*B

Lane 3 = Transformed hairy root cultures, detection for *rol*B

Lane 4 = *A. rhizogenes* ATCC 13333, detection for *rol*B

Lane 7 = Transformed hairy root cultures, detection for *rol*C

Lane 8 = Ordinary root cultures, detection for *rol*C

Lane 9 = *A. rhizogenes* ATCC 13333, detection for *rol*C

10. Naphthoquinone production by transformed hairy root cultures

Growth parameter and naphthoquinone production in the transformed hairy root cultures were monitored and compared with the ordinary root cultures, which maintained in B5 medium supplemented with 0.1 mg/L NAA, 1.0 mg/L kinetin and 2.0 mg/L BA, and hormone-free B5 liquid medium (Table 19). The growth rate of transformed hairy root cultures was considerably high when compare with the others. The result agrees with previous reports that transformed hairy root cultures are unique in their genetic and biosynthetic stability and their fast growth [10-11, 113]. Although the *rol*C region has been observed in both ordinary root cultures and transformed hairy root cultures, the naphthoquinone productions in these root cultures were in

different pattern. Lawsone production in transformed hairy root cultures was extremely high when compared with the ordinary root cultures that grown in hormone-free B5 medium and slightly higher than the ordinary root cultures in B5 supplemented with 0.1 mg/L NAA, 1.0 mg/L kinetin and 2.0 mg/L BA. Likewise 3,3′-methylenebislawsone production in transformed hairy root cultures was also high. Normally, the root cultures could not produce lawsone methyl ether when cultured in hormone free medium. They only produced when the appropriate combination of plant growth regulators were supplemented in the culture medium. However, the transformed hairy root cultures were capable of producing lawsone methyl ether, even though the amount was slightly less than that of the ordinary root cultures which cultured in B5 medium supplemented with 0.1 mg/L NAA, 1.0 mg/L kinetin and 2.0 mg/L BA. Since the integrated *rol* genes were function in modulating of phytohormone balance in transformed plant [115], the new balance of phytohormone may appropriate for naphthoquinone production. From the results we concluded that the transformed hairy root cultures were suitable for further enhancement of naphthoquinone production due to their high productivity.

Table 19 Naphthoquinone production in various types of *I. balsamina* root cultures

Types	**Growth (mg DW)**	**Naphthoquinone content mg/g DW Mean ± SE (n=5)**			
		1	**2**	**3**	**Total naphthoquinone**
Transformed root[#]	710	2.63 ± 0.166	0.09 ± 0.003	0.35 ± 0.019	3.07 ± 0.174
Ordinary root[#]	620	1.03 ± 0.055	Not detected	0.05 ± 0.002	1.08 ± 0.06
Ordinary root[†]	517	2.24 ± 0.181	0.12 ± 0.031	0.66 ± 0.029	3.02 ± 0.215

* 1 = lawsone, 2 = lawsone methyl ether, 3 = 3,3′-methylenebislawsone

[#] Cultured in hormone-free B5

[†] Cultured in B5 + 0.1 mg/L NAA + 1.0 mg/L kinetin + 2.0 mg/L BA

11. Increased production of naphthoquinone by elicitation

11.1 Effect of types and concentrations of elicitors

The optimal type and concentration of elicitors was determined based on their effect on productivity, and the data used for subsequent experiments. Addition of elicitors on day 21 of the root cultures, which corresponded to the late linear or early stationary growth phase. There was almost no significantly difference in growth patterns between the control and all the treatment groups. This may be due to the fact that treatment with elicitors was during the early stationary growth phase. Measurement of production of naphthoquinones in the untreated root cultures revealed that lawsone, and 3,3′-methylenebislawsone were the major naphthoquinones (0.79 mg/g DW and 0.15 mg/g DW), while lawsone methyl ether was a minor product (0.03 mg/g DW). Different elicitor types and concentrations exerted different effects on naphthoquinone production by the root cultures. The effect of all elicitors was positive and dose dependent on the production of naphthoquinones (Figure 18). However, the specificities and optimum concentrations of the elicitors to were different. Biotic and abiotic elicitors demonstrated different effects, MJ (abiotic elicitor) increased lawsone and lawsone methyl ether, whereas the biotic elicitors increased 3,3′-methylenebislawsone.

Treatment with YE, CAH and TRH resulted in only slight, but statistically significantly, increased production of lawsone, and lawsone methyl ether (Figure 18a and 18b). In contrast, YE, CAH and TRH as well as CHI strongly stimulated production of 3,3′-methylenebislawsone, particularly at higher concentrations (Figure 18c). Among these elicitors, TRH (5% v/v) and CAH (10% v/v) were the most successful in increased production of 3,3′-methylenebislawsone, which was almost 1.8-fold higher than in the untreated root cultures. MJ was specific for the production of lawsone and lawsone methyl ether but not 3,3′-methylenebislawsone. The content of lawsone and lawsone methyl ether were increased by MJ treatment in a dose-dependent manner.

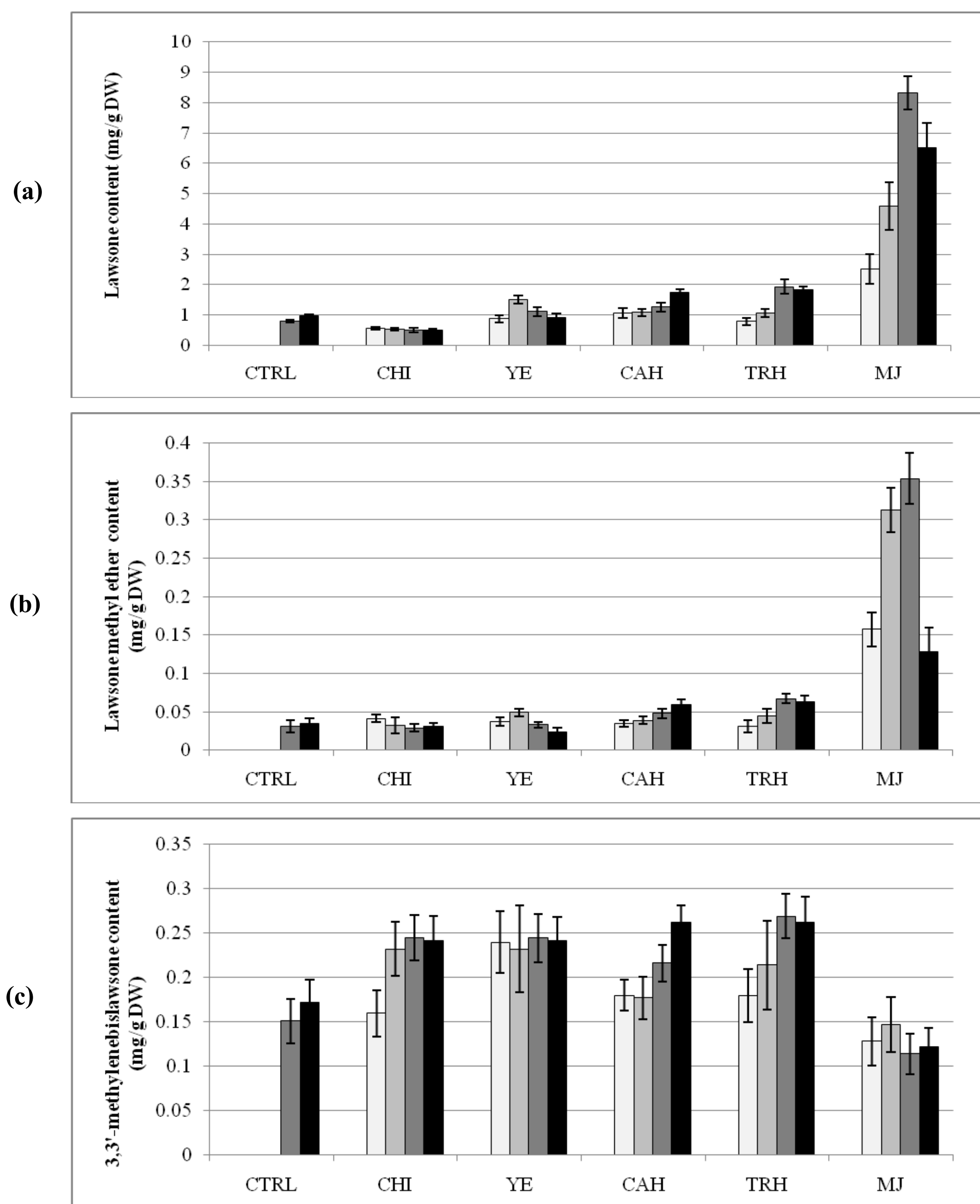


Figure 18 Effects of different elicitors on (a) lawsone, (b) lawsone methyl ether and (c) 3,3′-methylenebislawsone production in *I. balsamina* root cultures with elicitor treatment on 21 day old root cultures and exposed for 72 hr. Colors of the bar indicate the lowest concentration (white) to the highest concentration (black). The concentrations of elicitors are shown in Table 6. CTRL is control culture; gray bar is control with water and black bar is control with ethanol. Data of MJ are total naphthoquinones (intra- and intercellular). Data are mean ± SE of n = 5.

The optimum concentration of MJ was found to be 400 µM, which increased production of lawsone and lawsone methyl ether up to 8.6- and 11.3-fold higher, respectively, than the control groups. MJ treatment at the concentration of 200 µM or higher resulted in the root cultures secreting naphthoquinones into the liquid media (Figure 19 and 20). This observation is in agreement with a previous report on induction of anthraquinone in *Morinda elliptica* cell suspension cultures treated with 200 µM jasmonic acid [63]. It is noteworthy that, only lawsone and lawsone methyl ether were secreted into the liquid media (Figure 20). This may be due to the large molecular size of 3,3′-methylenebislawsone, making it release difficultly through the cell membrane. MJ treatment at 600 µM markedly diminished growth of the root cultures, as well as production levels of lawsone and lawsone methyl ether. At this high concentration of MJ browning of the cultures and cell death was extensive. An increase of naphthoquinone content in the cultured media leads to the low viability of the root cultures, possibly due to a cytotoxic effect of lawsone methyl ether that is released into the media [48]. This browning effect and cell growth retardation has also been reported in *Panax ginseng* cultures treated with high concentrations of MJ [130]. It has been suggested that browning coloration could also be due to other phenolic compounds as a response to elicitor-induced stress [131]. A two-phased culture, or *in situ* extraction, may have to be introduced in further studies to improve the cell viability and naphthoquinone production from the root cultures. It has been reported that the simultaneous use of *in situ* extraction and elicitation cell treatment had a synergistic effect on the stimulation of shikonin production in suspension cultures of *Arnebia euchroma* [132].

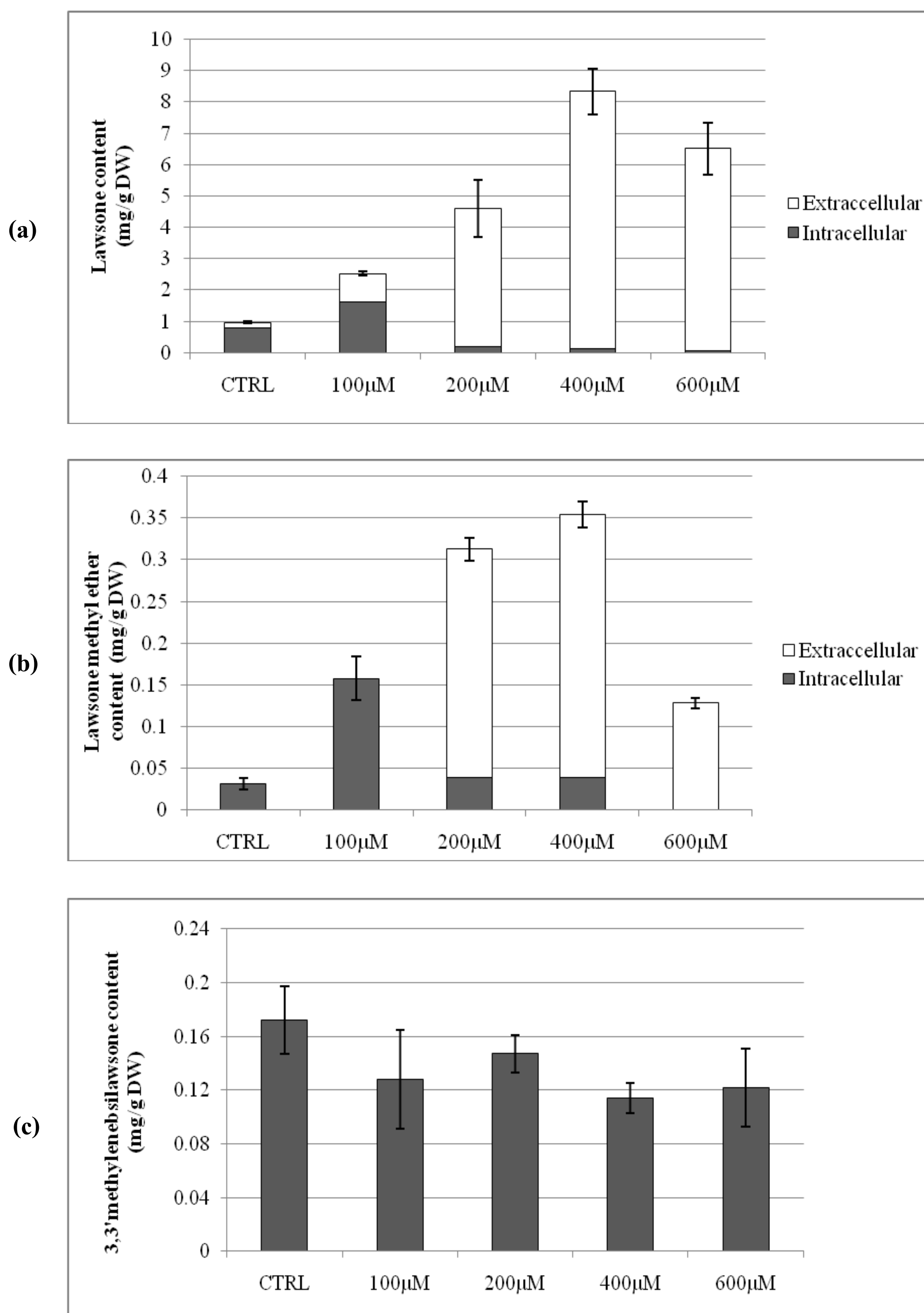
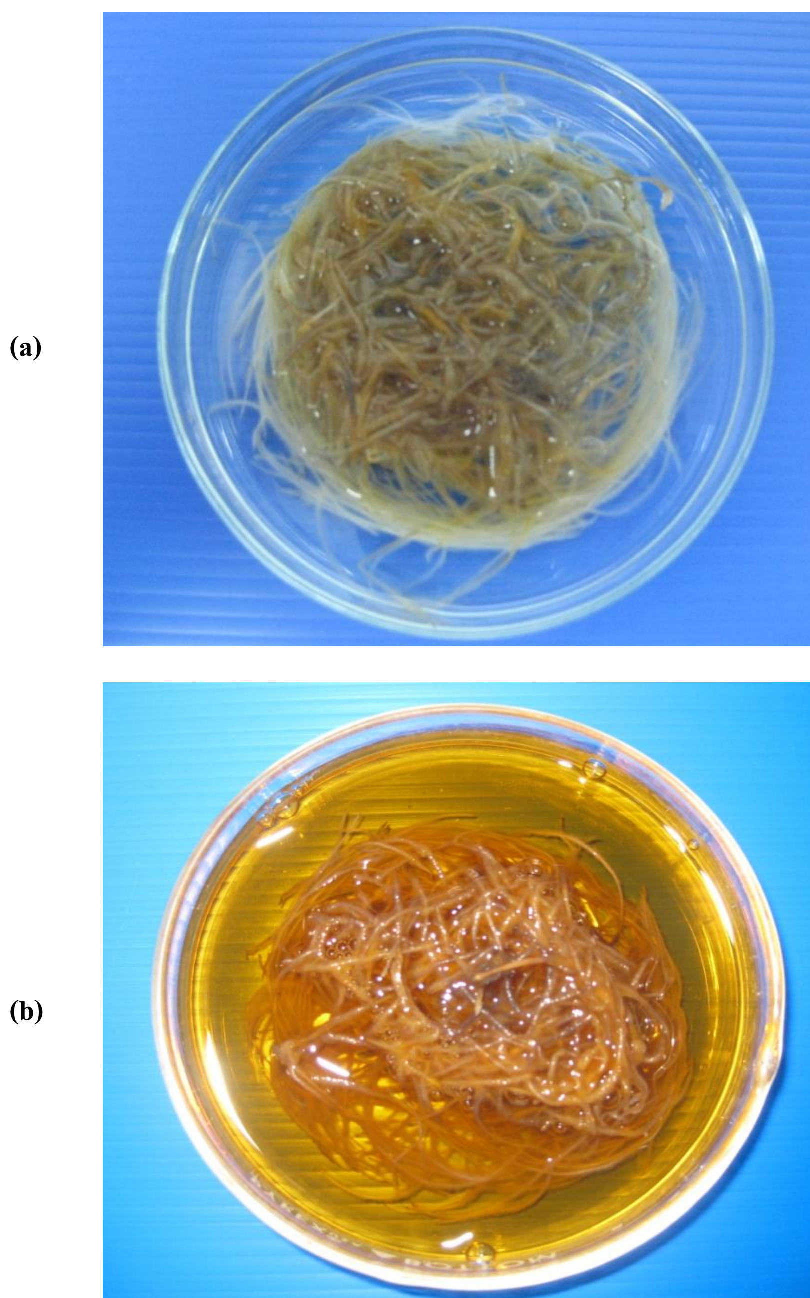


Figure 19 Intracellular and extracellular content of (a) lawsone, (b) lawsone methyl ether and (c) 3,3′-methylenebislawsone after treatment of the 21 day old root cultures with various concentration of MJ for 72 hr. Data are mean ± SE of n = 5.

(a)



(b)

Figure 20 Comparison between (a) control root cultures and (b) MJ-treatment root cultures

Effect of MJ on isochorismate synthase activity had been studied in cell suspension cultures of *Galium mollugo*. The enzyme activity of isochorismate synthase, which catalyzed chorismic acid to isochorismic acid was extremely increased (7-fold) after treated the *G. mollugo* cell cultures with MJ within 15-18 hr after the treatment as well as the increasing of anthraquinone production in the cell cultures [133]. A study on effect of MJ on *O*-

methyltransferase enzyme activity in *Eschscholtzia californica* suspension cultures reported that 3′-hydroxy-(S)-*N*-methylcoclaurine-4′-*O*-methyltransferase was rapidly induced with high levels of expression at 6–12 h after MJ treatment [122]. From these evidences MJ might be involved in isochrismate synthase and *O*-methyltransferase activities in *I. balsamina* root cultures, and resulted in the increasing of lawsone and lawsone methyl ether production. Since MJ was capable of increasing total naphthoquinone to the highest extent. It was used to elicitate *I. balsamina* root cultures to optimize elicitor conditions, including optimum concentration, period of contract and age of root cultures for elicitation.

11.2 Effect of MJ concentrations and contact periods

The concentration and contact period of MJ were further optimized, having previously demonstrated that treatment 21 day old cultures for 72 hr with 600 µM resulted in browning coloration and diminished growth of the root cultures. The highest concentration of MJ in subsequent studies with 21 day old cultures was therefore limited at 400 µM. The contact periods were also varied between 24 to 72 hr. The results revealed that naphthoquinones were secreted into the media after treatment with 200 µM and higher concentrations. The optimum concentration of MJ for increasing naphthoquinone production was judged to be 300 µM (Figure 21). The elicitor contact period was 36 hr, since elicitor contact longer than this time led to decreased culture growth and sometimes naphthoquinone production after subculture. Concentrations of MJ higher than 200 µM resulted in decrease of root viability when contaction periods were longer than 48 hr. The highest content of lawsone, lawsone methyl ether and 3,3′-methylenebislawsone were 10.16 ± 0.92, 0.63 ± 0.031 and 0.19 ± 0.031 mg/g DW, respectively, after treatment with 300 µM MJ for 36 hr. Under these conditions production of lawsone and lawsone methyl ether were 12.2- and 15.7-fold higher, respectively, than in the untreated root cultures (see Figure 22).

(a)

(b)

(c)

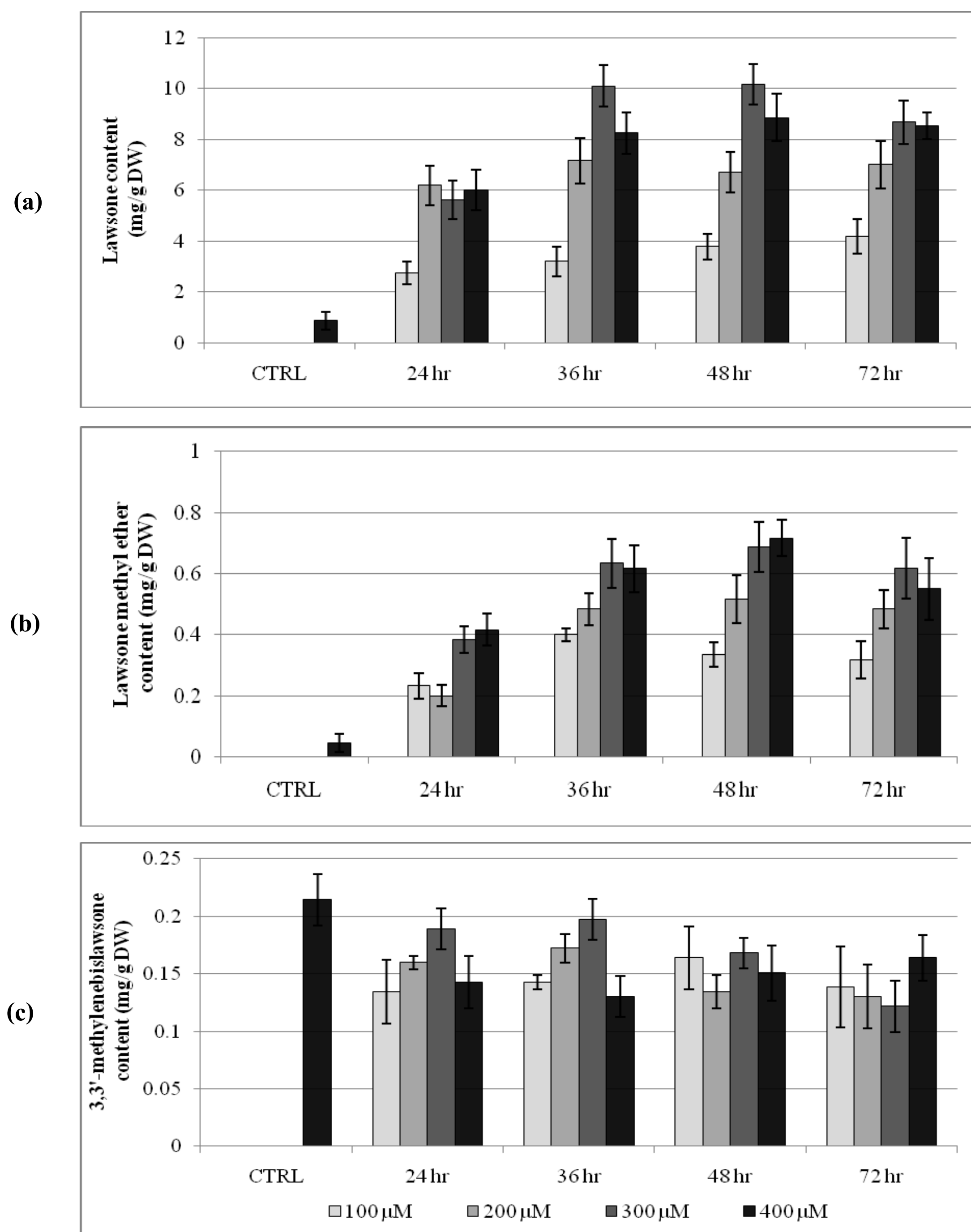


Figure 21 Effect of various concentration of MJ and elicitor contact periods on (a) lawsone, (b) lawsone methyl ether and (c) 3,3′-methylenebislawsone production. Data are mean ± SE of total naphthoquinones (intra- and extracellular), n = 5.

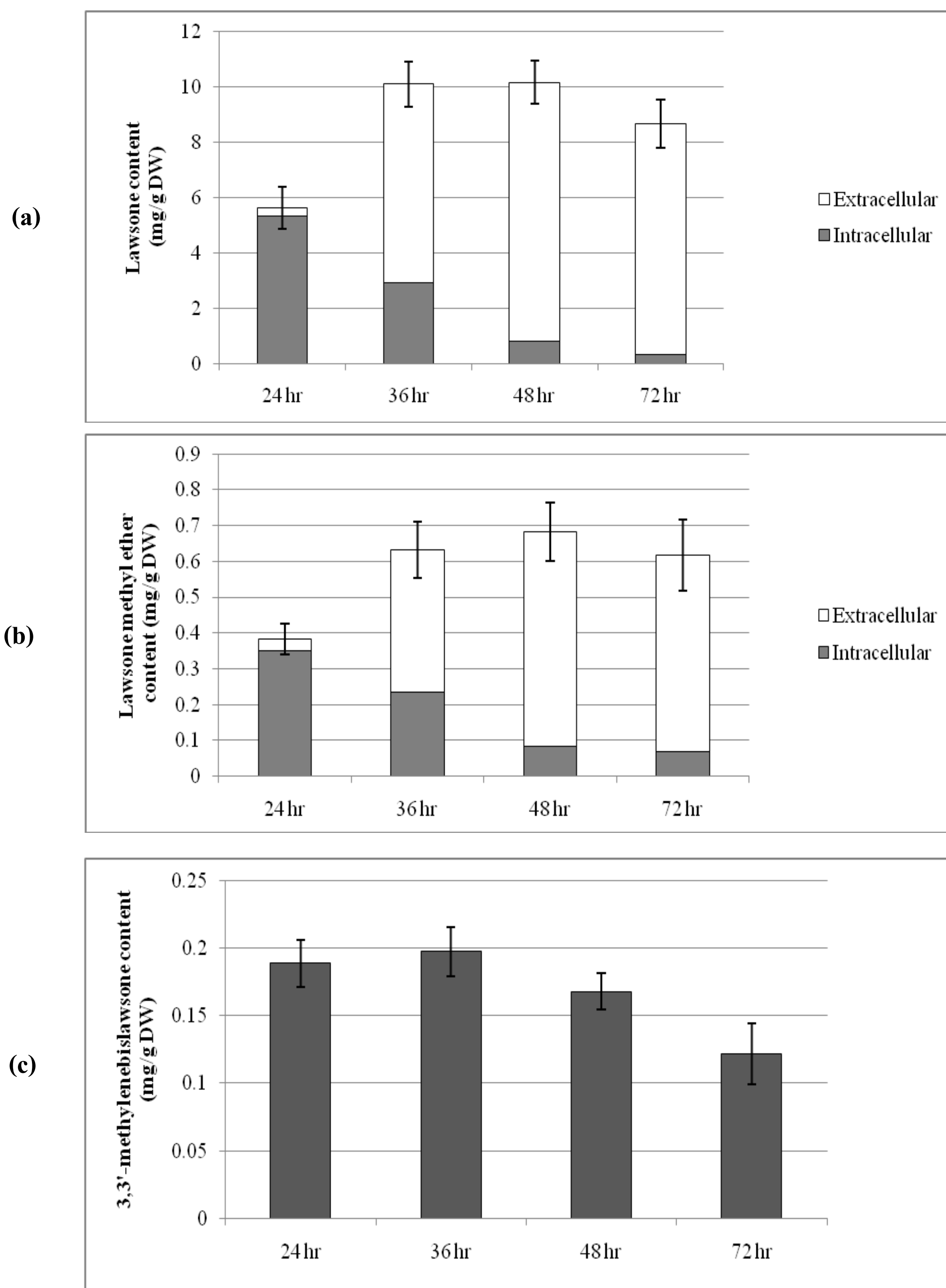
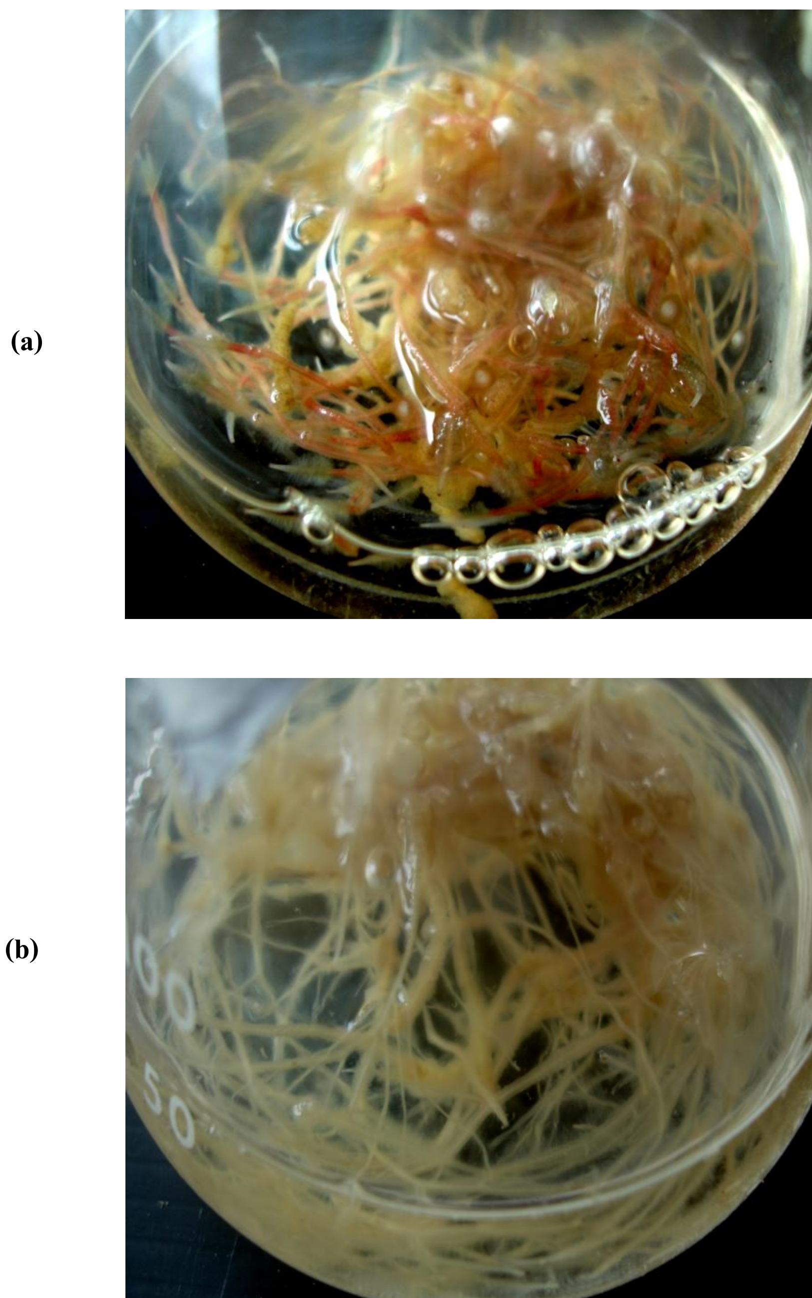


Figure 22 Intracellular and extracellular content of (a) lawsone, (b) lawsone methyl ether and (c) 3,3′-methylenebislawsone after treatment of the 21 day old root cultures with 300 µM MJ for different time periods. Data are mean ± SE of n = 5.

11.3 Effect of culture age

Cells at different stages of growth have different levels of mRNA and proteins, and elicitor treatment on different growth phases may yield varied responses in terms of cell growth and secondary metabolite production [63]. In this final study, the age of the root cultures was varied between 14 to 25 days. The root cultures were exposed to the elicitor (300 µM) for 36 hr, the roots then harvested and subjected to naphthoquinone content determination. After subculture the elicited root to fresh medium, the cultured root has become redness while the control group was still white (Figure 23). The redness of the root culture might be use to indicate the high production rate root cultures. An increase of content of lawsone and lawsone methyl ether in the cultured media was observed as the age of root cultures increased (Figure 24). The appropriate age of root cultures for maximum production of naphthoquinones was found to be 21 days. This may be due to the biosynthesis of both lawsone and lawsone methyl ether being more active at the late linear growth phase [134]. Treatment of 21 day old root cultures with 300 µM MJ for 36 hr led to the highest production of lawsone, lawsone methyl ether and 3,3′-methylenebislawsone, up to 10.0 ± 0.7, 0.8 ± 0.07 and 0.23 ± 0.04 mg/g DW, respectively, which were 10.4-, 26.0- and 1.3-fold higher than the levels in the controls. In contrast, treatment of the root cultures in the stationary growth phase (day 25) with 300 µM MJ resulted in a decreased production of lawsone and lawsone methyl ether but not 3,3′-methylenebislawsone. This may be because the biosynthesis of 3,3′-methylenebislawsone is operational in the stationary growth phase [134].

(a)



(b)

Figure 23 Second generation of *I. balsamina* root cultures after (a) treated with MJ or (b) ethanol (control)

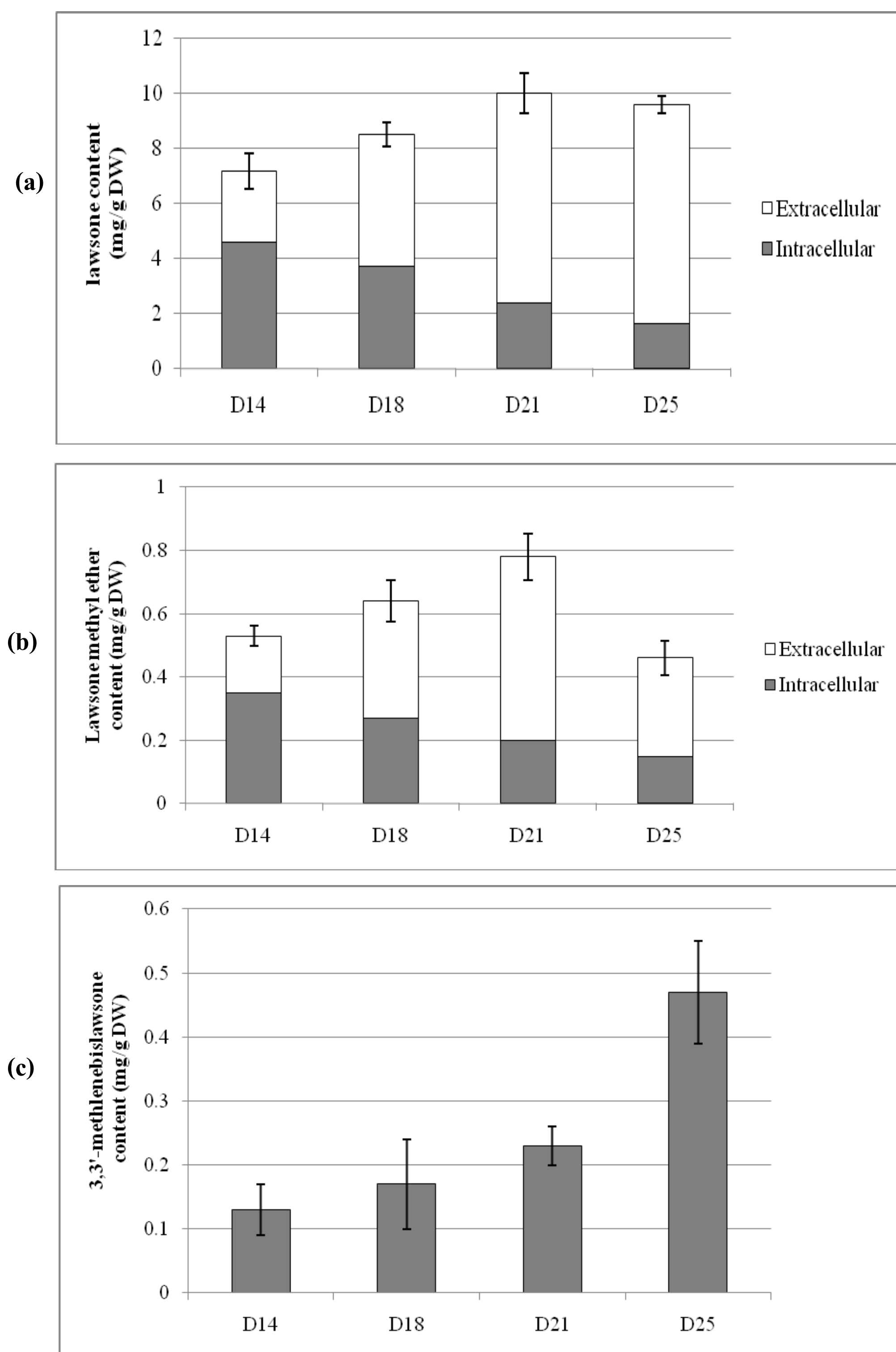


Figure 24 Intracellular and extracellular content of (a) lawsone, (b) lawsone methyl ether and (c) 3,3′-methylenebislawsone after treatment of root cultures of various ages with 300 μM MJ for 36 hr. Data are mean ± SE of n = 5.

12. Study on secondary metabolite production in *I. balsamina* root cultures

I. balsamina root cultures obtained from pink flower plants and maintained in B5 medium supplemented with 0.1 mg/L NAA, 1.0 mg/L kinetin and 2.0 mg/L BA have been extracted and isolated to study their secondary metabolite production. Ten known compounds were isolated and identified as luteolin (**1**) [135], phthalic acid (**2**), phthalic anhydride (**3**), methyl linolenate (**4**) [136], spinasterol (**5**) [137], *p*-hydroxybenzoic acid (**6**) isofraxidin (**7**) [6], scopoletin (**8**) [138], 2,3-dihydroxy-1,4-naphthoquinone (**9**) [139] and echinocystic acid (**10**) [140] (Figure 25), by comparison of their NMR data with the reported values in the literatures. Isofraxidin and scopoletin are coumarins found in *I. balsamina* root cultures, which have been previously reported [6]. Small molecules such as phthalic acid, phthalic anhydride and *p*-hydroxybenzoic acid were found in *I. balsamina*. These compounds might be involved in naphthoquinone biosynthesis via shikimate pathway. Although phthalic acid and phthalate anhydride have never been reported about their function in shikimate pathway, phthalic acid had been used as a precursor in a chemical synthesis of OSB. Thus, phthalic acid and phthalic anhydride might be the intermediates in shikimate pathway. It has been reported that *p*-hydroxybenzoic acid was an important intermediate in the biosynthetic pathway of ubiquinone (Figure 26) [59]. Shikonin, a naphthoquinone in *Lithospermum erythorhizon*, is biosynthesized through the prenylation of *p*-hydroxybenzoic acid derived from L-phenylalanine with geranylpyrophosphate by *p*-hydroxybenzoic geranyltransferase (Figure 27) [141]. The finding of *p*-hydroxybenzoic acid in the root cultures system suggested that other type of naphthoquinones which derived from *p*-hydroxybenzoic acid might be found in *I. balsamina* root cultures.

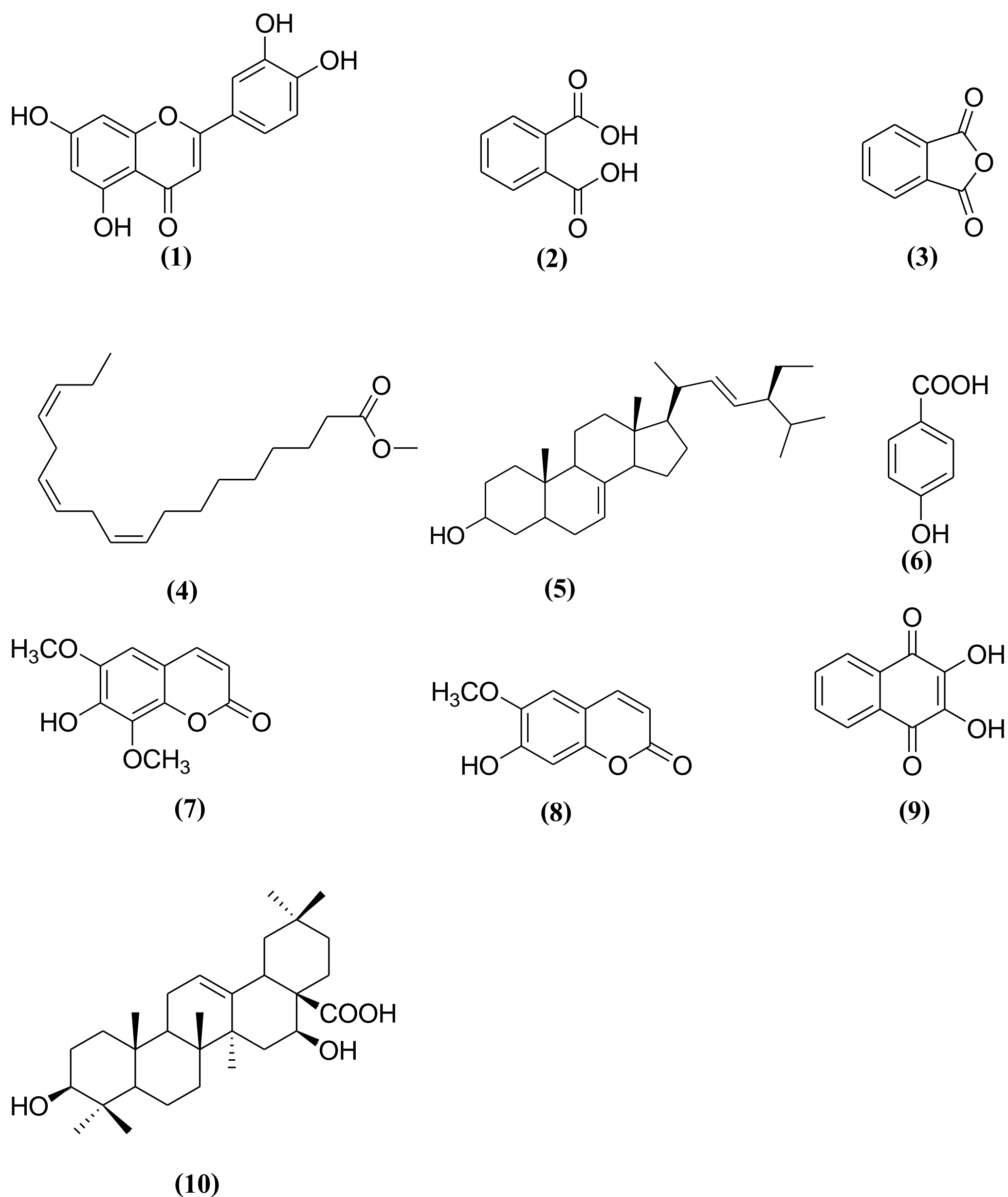


Figure 25 Structures of (**1**) luteolin, (**2**) phthalic acid, (**3**) phthalic anhydride, (**4**) methyl linolenate, (**5**) spinasterol, (**6**) *p*-hydroxybenzoic acid, (**7**) isofraxidin, (**8**) scopoletin, (**9**) 2,3-dihydroxy-1,4-naphthoquinone and (**10**) echinocystic acid

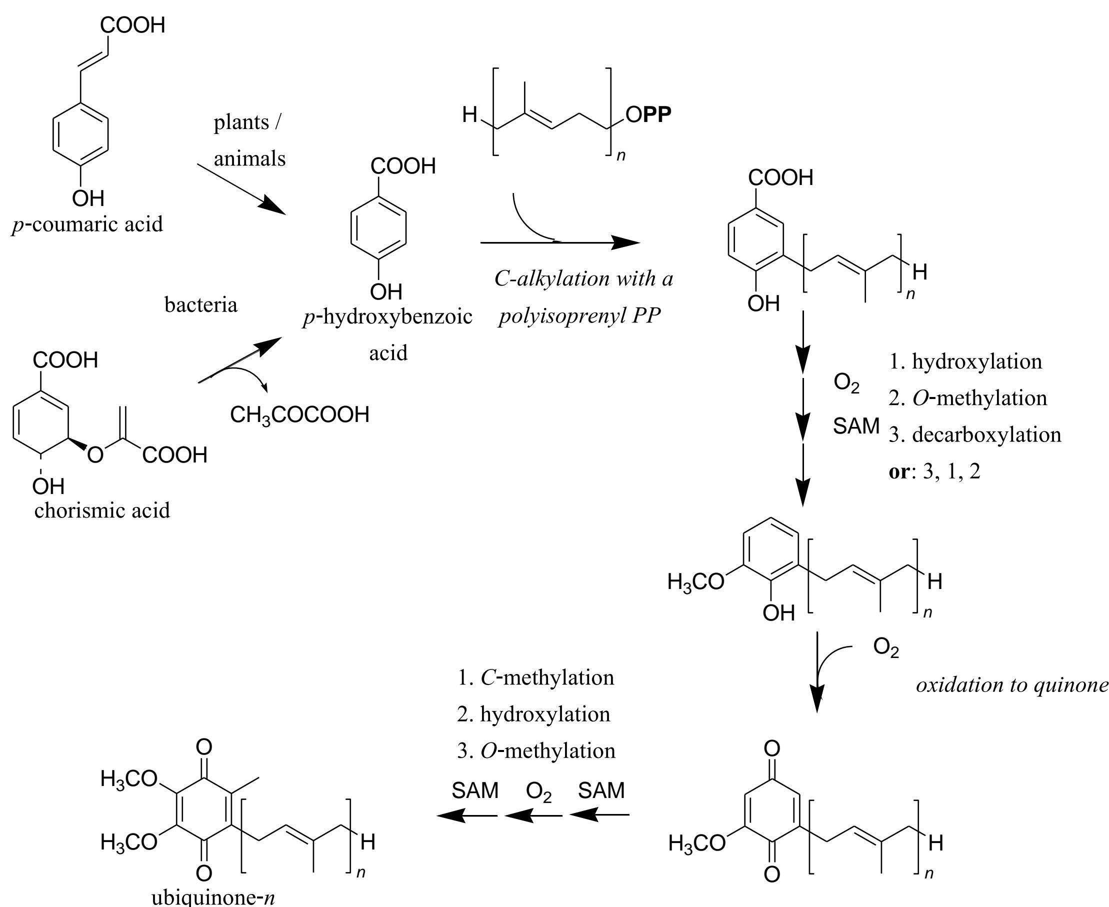


Figure 26 Involvement of *p*-hydroxybenzoic acid in ubiquinone production [59].

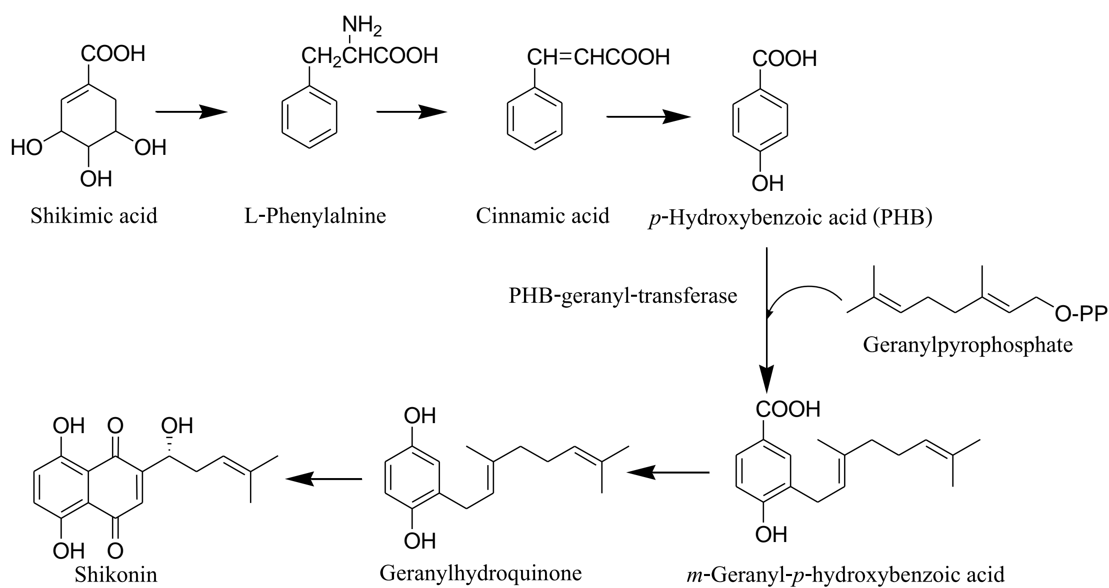


Figure 27 Biosynthesis of shikonin in *Lithospermum erythorhizon*

Luteolin (Figure 28) was found as pale yellow amorphous powder. Its ^{1}H-NMR data showed a characteristic of flavones (Table 20). It has been reported that this compound possessed good antioxidant activity [142]. Luteolin has been reported as anti mutagenic agent. It had suppressive effects on *umu* gene expression of the SOS response against many antimutagens. Normally, flavonoids which found in *I. balsamina* are kaempferol, quercetin and myriticin. This is the first report of luteolin in *I. balsamina*.

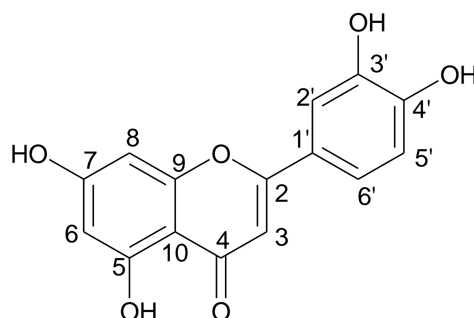


Figure 28 Structure of luteolin

Table 20 ^{1}H-NMR (400MHz) data of luteolin (DMSO-d_6)

Position	δ H (*J* in Hz)	Position	δ H (*J* in Hz)
1	-	9	-
2	-	10	-
3	6.72, s	1’	-
4	-	2’	7.41, d (2.2)
5	-	3’	-
6	6.44, d (2.0)	4’	-
7	-	5’	6.91, d (9.0)
8	6.78, d (2.0)	6’	7.43, dd (2.2, 9.0)

2,3-Dihydroxy-1,4-naphthoquinone (Figure 29) was found as red crystal. The ^{13}C-NMR spectral data indicated 5 symmetry ^{13}C signals of naphthoquinone (Table 21). This compound has never been report in *I. balsamina* but it has been obtained by chemically synthesized and known to form complex with lanthanide [139]. It is the first report of 2,3-dihydroxy-1,4-naphthoquinone as a natural occurring naphthoquinone. Thus, *I. balsamina* root cultures can be consider as a new source of this compound.

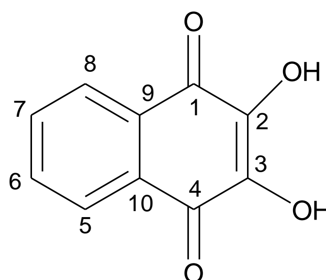


Figure 29 Structure of 2,3-dihydroxy-1,4-naphthoquinone

Table 21 ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100MHz) data of 2,3-dihydroxy-1,4-naphthoquinone (CD_3OD)

Position	δ H (*J* in Hz)	δ C
1	-	182.6
2	-	141.6
3	-	141.6
4	-	182.6
5	7.98, dd (5.8, 3.5)	126.7
6	7.70, dd (5.8, 3.5)	134.6
7	7.70, dd (5.8, 3.5)	134.6
8	7.98, dd (5.8, 3.5)	126.7
9	-	131.8
10	-	131.8

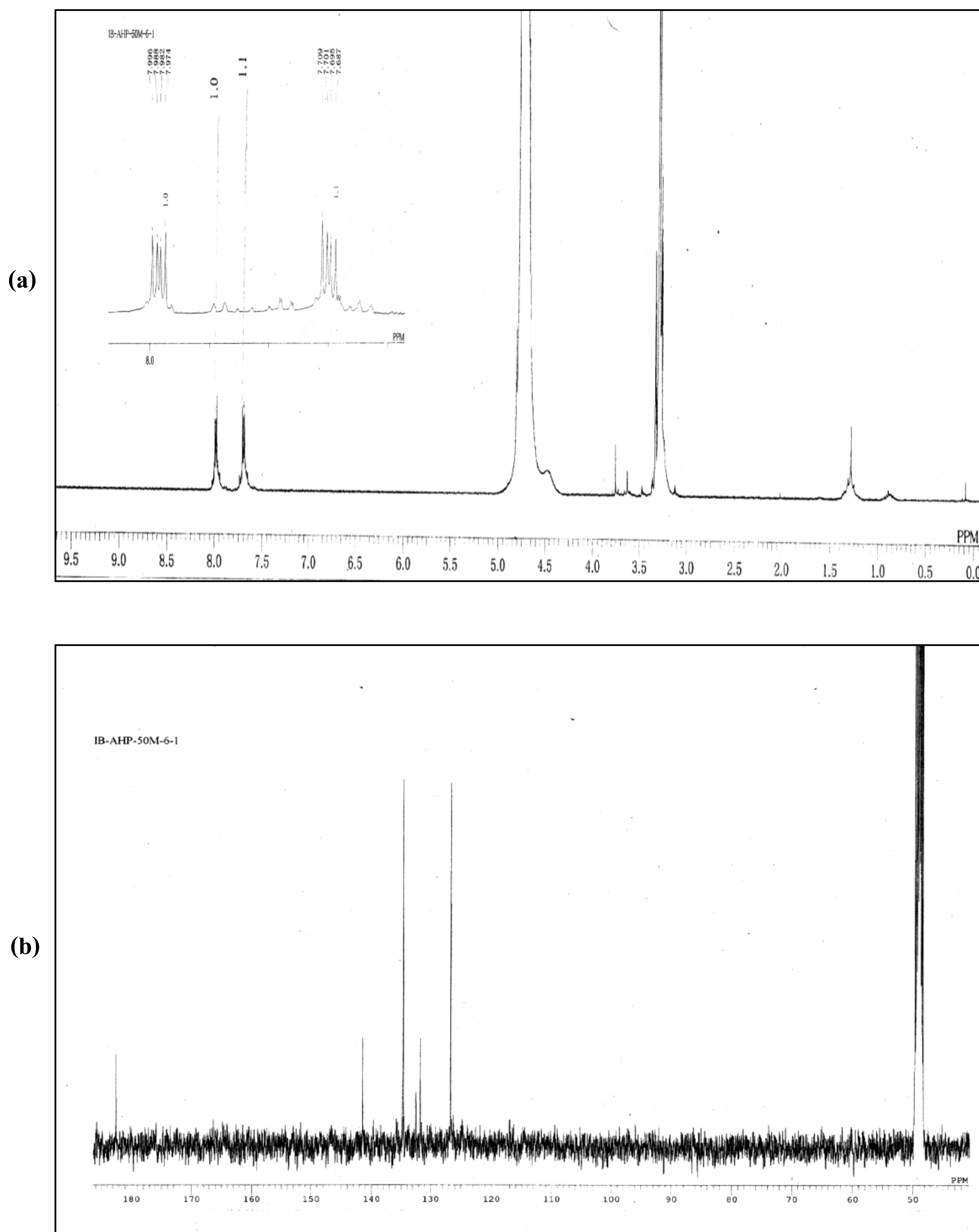


Figure 30 ^{1}H-NMR (400 MHz) spectrum (a) and ^{13}C-NMR (100 MHz) spectrum (b) of 2,3-dihydroxy-1,4-naphthoquinone (in CD_3OD)

Echinocystic acid (Figure 31) was found as white amorphous powder. Its ^{13}C-NMR and ^{1}H-NMR spectral data (Table 22) showed a characteristic of pentacyclic triterpene, which has 7 singlet methyl signals [δ 1.84 (C27), 1.23 (C23), 1.19 (C30), 1.07 (C26), 1.06 (C29), 1.03 (C24), 0.95 (C25)]. Echinocystic acid possessed many pharmacological activities such as anti-HIV activity, anti-inflammatory activity and cytotoxicity against in HL-60 cells through ROS-independent mitochondrial dysfunction pathway [143].

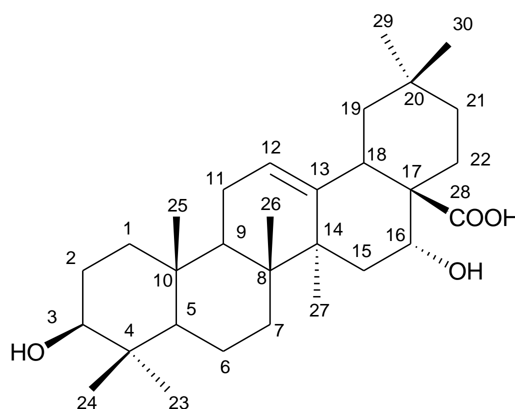


Figure 31 Structure of echinocystic acid

Table 22 ^{13}C-NMR (100 MHz) data of echinocystic acid ($CDCl_3$)

Position	δ C	Position	δ C	Position	δ C
1	39.1	11	23.9	21	36.2
2	28.2	12	122.5	22	32.9
3	78.2	13	145.2	23	28.8
4	39.4	14	42.2	24	16.6
5	56.0	15	36.3	25	15.7
6	18.9	16	74.8	26	17.6
7	33.7	17	49.0	27	27.3
8	40.0	18	41.6	28	180.0
9	47.4	19	47.4	29	33.4
10	37.5	20	31.1	30	24.8

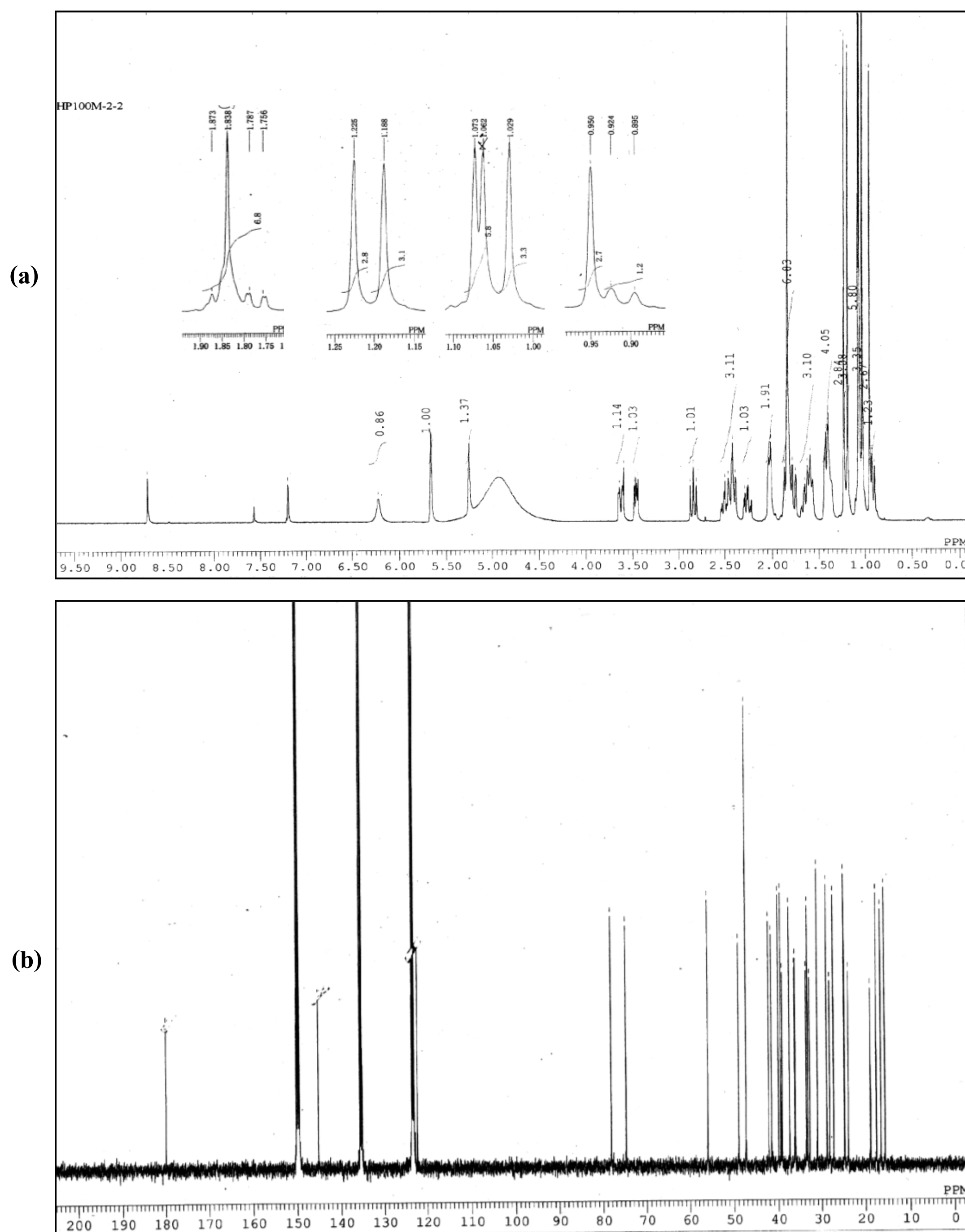


Figure 32 ^{1}H-NMR (400 MHz) spectrum (a) and ^{13}C-NMR (100 MHz) spectrum (b) of echinocystic acid (in $CDCl_3$)

CHAPTER 5

CONCLUSIONS

1. A simple, specific, precise, accurate and reproducible HPLC method has been developed to quantify the active principles in *I. balsamina* leaves. The simultaneous quantitative determination of lawsone, lawsone methyl ether and 3,3′-methylenebislawsone provides useful marker information for the quality control of *I. balsamina* leaf extracts. This study suggests that a mixture of chloroform and methanol (50% v/v) is a suitable solvent for the extraction of naphthoquinones from *I. balsamina* leaves. The pink strain of *I. balsamina* appears to give the highest amount of naphthoquinones and should be considered as a source of plant materials for further study of this medicinal plant.

2. Strain improvement and selection plays an important role in secondary metabolite production. Regarding the effect of the donor plants, the root cultures established from naphthoquinone high-yielding strain (IbP) significantly produce higher total naphthoquinone content than those established from naphthoquinone low-yielding strains. These results support our hypothesis that the root cultures initiated from the high-yielding donor plants were capable of producing higher amount of secondary metabolite than those initiated from the naphthoquinone low-yielding strains

3. Variety of plant hormone affected on naphthoquinone production in *I. balsamina*. The high amount of BA and kinetin were suitable to increase naphthoquinone production, while NAA showed negative effect on naphthoquinone production. The result suggests that B5 supplemented with 0.1 mg/L NAA, 1.0 mg/L kinetin and 2.0 mg/L BA is an appropriate culture medium for increased naphthoquinone production in *I. balsamina* root cultures

4. Elicitation technique is a useful method to stimulate production of naphthoquinones in *I. balsamina* root cultures. The type and concentration of elicitor, elicitor contract period and age of the root cultures played an important role on stimulation of naphthoquinone production. This study reveals that MJ (300 μM) is a specific elicitor for

increased production of lawsone and lawsone methyl ether, while TRH (5% v/v) is a specific elicitor for increased production of 3,3′-methylenebislawsone. The appropriate age of the root cultures and contact period for treatment with MJ (300 μM) are 21 days and 36 hr, respectively. However, simultaneous treatment with combined MJ and TRH may be considered in future studies for increased production of all the three naphthoquinones in the root cultures.

5. Feeding of methionine is suitable for 3,3′-methylenebislawsone but not for lawsone and lawsone methyl ether production. However, methionine toxicity has been observed when administrated in high concentrations. Feeding of 300 mg/L methionine to 21-day old root cultures is appropriate conditions to stimulate 3,3′-methylenebislawsone production.

6. The transformed hairy root cultures of *I. balsamina* were capable of producing lawsone methyl ether even they were grown in hormone-free B5 medium. However, the lawsone and lawsone methyl ether levels in the transformed hairy root cultures are not different from the ordinary root cultures. A medium manipulation may be used for increased naphthoquinone production in the transformed hairy root cultures.

7. A flavonoid, luteolin, a naphthoquinone, 2,3-dihydroxy-1,4-naphthoquinone, and a triterpenoid, echinocystic acid have been isolated from *I. balsamina* root cultures. This is the first report on occurrence of these compounds in this plant.

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