การผลิตพลัมบาจินในเซลล์แขวนลอยของเจตมูลเพลิงขาว
Plumbago zeylanica L.

นางสาวอรสา ชูสกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีทางชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2543
ISBN 974-13-0533-8

PLUMBAGIN PRODUCTION IN CELL SUSPENSION CULTURES OF *Plumbago zeylanica* L.

Miss Orasa Choosakul

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

Department of Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2000

ISBN 974-13-0533-8

Thesis Title	PLUMBAGIN PRODUCTION IN CELL SUSPENSION CULTURES OF *Plumbago zeylanica* Linn
By	Miss Orasa Choosakul
Field of study	Biotechnology
Thesis Advisor	Associate Professor Wichai Cherdshewasart, D. Sc.
Thesis Co-advisor	Associate Professor Wanchai De-Eknamkul, Ph. D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree.

……. Dean of Faculty of Science
(Associate Professor Wanchai Phothiphichitr, Ph.D.)

THESIS COMMITTEE

……. Chairman
(Professor Siriwat Wongsiri, Ph. D.)

……. Thesis Advisor
(Associate Professor Wichai Cherdshewasart, D. Sc.)

……. Thesis Co-advisor
(Associate Professor Wanchai De-Eknamkul, Ph. D.)

……. Member
(Associate Professor Chaiyo Chaichantipyuth, M. Sc. in Pharm.)

อรสา ชูสกุล : การผลิตพลัมบาจินในเซลล์แขวนลอยของเจตมูลเพลิงขาว (PLUMBAGIN PRODUCTION IN CELL SUSPENSION CULTURES OF *Plumbago zeylanica* L.) อ. ที่ปรึกษา : รศ.ดร.วิชัย เชิดชีวศาสตร์, อ. ที่ปรึกษาร่วม : รศ.ดร.วันชัย ดีเอกนามกูล, 76 หน้า. ISBN 974-13-0533-8

วิธีการของ TLC densitometry ได้ถูกพัฒนาขึ้นเพื่อใช้สำหรับการตรวจหาศักยภาพ การสร้างสารพลัมบาจินในต้นพืช และเซลล์แขวนลอยของเจตมูลเพลิงขาว การชักนำให้เกิดเนื้อเยื่อเพาะเลี้ยงและเซลล์แขวนลอยของเจตมูลเพลิงขาวทำได้โดยใช้ใบอ่อนของพืช ภายใต้สภาวะของอาหารสูตร LS ที่ประกอบด้วย NAA 0.2 มิลลิกรัมต่อลิตร, 2,4-D 0.2 มิลลิกรัมต่อลิตร และน้ำตาล 30 กรัมต่อลิตร ส่วนการทดลองหาสภาวะการเพาะเลี้ยงที่เหมาะสมที่สุดสำหรับการผลิตพลัมบาจินในเซลล์เพาะเลี้ยง ได้ดำเนินการโดยเปลี่ยนแปลงสูตรอาหารพื้นฐานต่างๆ รวมไปถึงชนิดและปริมาณของฮอร์โมน และแหล่งคาร์บอน ผลจากการศึกษาพบว่าเซลล์เพาะเลี้ยงสามารถผลิตพลัมบาจินได้มากในสูตรอาหาร MS ที่มี 2,4-D หรือ BA เป็นฮอร์โมนและแมนนิทอลเป็นแหล่งคาร์บอน สำหรับต้นเจตมูลเพลิงขาวซึ่งขึ้นที่เขตมีนบุรี กรุงเทพมหานคร เมื่อทำการวิเคราะห์พบว่า มีพลัมบาจินในทุกส่วนของต้นในปริมาณที่แตกต่างกัน โดยมีการสะสมในส่วนของรากมากที่สุดดังนี้ ปริมาณพลัมบาจินในส่วนราก เท่ากับ 0.226% ลำต้น 0.089% ใบแก่ 0.010-0.030% ใบอ่อน 0.014% และดอก 0.002% ต่อน้ำหนักแห้ง เนื่องจากยังไม่เคยมีรายงานเกี่ยวกับด้านนี้มาก่อน วิทยานิพนธ์นี้เป็นปฐมนิพนธ์ที่รายงานผลเกี่ยวกับการสร้างพลัมบาจินในเซลล์แขวนลอยของเจตมูลเพลิงขาว และการกระจายในเชิงปริมาณของพลัมบาจินในต้นเจตมูลเพลิงขาว

หลักสูตร…….. ลายมือชื่อนิสิต…….

สาขาวิชา…… ลายมือชื่ออาจารย์ที่ปรึกษา…….

ปีการศึกษา…… ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

4072456523 : MAJOR BIOTECHNOLOGY
KEYWORD : *Plumbago zeylanica* L./PLUMBAGINACEAE/PLUMBAGIN/QUANTITATIVE ANALYSIS/CELL SUSPENSION CULTURES
ORASA CHOOSAKUL : THESIS TITLE. PLUMBAGIN PRODUCTION IN CELL SUSPENSION CULTURES OF *Plumbago zeylanica* L. THESIS ADVISOR : ASSOCIATE PROFESSOR WICHAI CHERDSHEWASART, D. Sc., THESIS CO-ADVISOR : ASSOCIATE PROFESSOR WANCHAI DE-EKNAMKUL, Ph. D.
76 pp. ISBN 974-13-0533-8

TLC densitometry was developed for the detection of plant materials which potentially contain plumbagin such as the whole plants and cell suspension culture of *Plumbago zeylanica*. Callus and cell suspension cultures of *P. zeylanica* were successfully established from young leaf explants. The culture medium used for both callus induction and maintaining the cell suspension cultures was LS containing with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 30 g/l sucrose. To verify the optimum culture conditions for plumbagin production, cell suspension cultures were tested by changing the various basal media, plant growth regulators and carbon source. The cell suspension culture produced more plumbagin in MS medium, in the presence of 2,4-D or BA as a growth regulator and manitol as a carbon source. For the whole plants of *P. zeylanica* were obtained from Minburi Bangkok. The analysis revealed that plumbagin was present in every part of plant, especially in the roots. The plumbagin content in the roots were 0.226%, stem 0.089%, mature leaves 0.010-0.030%, young leaves 0.014% and flowers 0.002%, on the dry weight basis. Apparently, this is the first report on the formation of plumbagin in *P. zeylanica* cell suspension cultures and the quantitative distribution of plumbagin in the whole plant.

Programme……. Student's signature…….
Field of study……. Advisor's signature……..
Academic year……. Co-advisor's signature…….

ACKNOWLEDGEMENT

The author wishes to express her sincere gratitude to her advisor Associate Professor Dr. Wichai Cherdshewasart of the Department of Biology, Faculty of Sciences, Chulalongkorn University, for his helpful suggestions and keen interest.

The author wishes to express his deepest apprecitation and grateful thanks to her co-advisor, Associate Professor Dr. Wanchai De-Eknamkul of Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for his helpful guidances, invaluable advice and continual encouragment throughout the course of this work.

The author would like to thank the tissue culture unit, the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences Chulalongkorn University, for providing laboratory facilities during this research.

The author would also like to thank the Graduate School, Chulalongkorn University for granting her partial financial support to conduct this investigation.

The author would further like to extend her sincere thanks all members of the tissue culture unit for their friendship, encouragement suggestions kindnesses and helps.

The author would also like to acknowledge his appreciation to Mr. Phitsanu Soonthornrattanaruk for his helpful suggestions about using computer.

Finally, the author wishes to express her indebteness and grateful thanks to her family for their financial support, without whose love, understanding and cheerfulness this work would have not been successful.

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

%	=	percent (part per 100); percentage
δ	=	chemical shift
μg	=	microgram(s)
μl	=	microliter(s)
λ_{max}	=	maximum absorption wavelength
/	=	per
2,4-D	=	2,4-dichlorophenoxyacetic acid
BA	=	6-benzylaminopurine or N^6- Benzyladenine
ca.	=	about, approximately
cm	=	centimeter(s)
DW	=	dry weight
e.g.	=	for example
et.al	=	et alii
Fig	=	figure
g	=	gram(s)
HPLC	=	high performance liquid chromatography
hr	=	hour(s)
i.p.	=	intraperitoneal
IAA	=	indole-3-acetic acid
IBA	=	indole-3-butyric acid
in^2	=	square inch
kg	=	kilogram(s)
kinetin	=	kinetin-6-furfurylaminepurine
l	=	liter(s)
lb	=	pound(s)
LEV	=	leave(s)
LS	=	Linsmaier and Skoog (1965) medium
m	=	meter(s)
mg	=	milligram
min	=	minute(s)
ml	=	milliliter

mm = millimeter

NAA = α-naphthaleneacetic acid

nm = nanometer

no = number

°C = degree celsius

pH = the negative logarithm of the concentration of hydrogen ions

r.p.m. = revolution per minute

*R*f = distance spot moved/distance solvent moved (TLC)

RNA = ribonucleic acid

RP = reverse phase

RT = retention time

SD = standard deviation

ST = stem

TLC = thin layer chromatography

UV = ultraviolet light

w/v = weight/volume (concentration)

w/w = weight/weight (concentration)

CHAPTER I

INTRODUCTION

Plants produce more than 80,000 different compounds through their secondary metabolic pathways. Some are used as pharmaceuticals, agrochemicals, dyes, flavors, pesticides, fragrances, etc., and represent multibillion dollar industries. Enormous amounts of plant material are needed for the extraction of these metabolites. Many of these compounds are obtained by direct extraction from plants that are cultivated in the field or sometimes growing in their original habitats. Several factors can alter the yield of products of economic importance. The quality of the raw material can therefore vary widely, and some plants need to grow for several years before they are ready for harvesting. In addition, almost nothing is known about the control of pests and diseases of these plants, or of the postharvest procedures which are essential to preserve the active compounds until their extraction.

To overcome the inconvenience of manipulating plants, a great effort has been directed towards obtaining *in vitro* systems to produce compounds. During the past three decades, plant cell cultures have developed into useful experimental tools to study the metabolic pathways of higher plants, in particular those leading to natural products. There are several examples of scale up processes based on plant cell cultures which have led to the commercial production of bioactive plant metabolites (Table 1). Plant cell cultures have also permitted new insights into the physiology and biochemistry of plant secondary metabolism. However, the potential of plant cell cultures for the production of secondary metabolites is limited still by two major factors. The first factor is the low productivity of the desired compounds and the second is the genetic instability of plant cell lines. In order to manipulate plant secondary metabolism according to our needs, we have to understand the basic principles of biochemistry of product formation.

Table 1 Products already commercialized or under development (Petiard and Steck, 1987)

Product	Species	Application	Price ($/kg) With reservation	Company	Country
Arbutin	*Bergenia crassifolia*	Pharmacy	-	Mitsui, Shiseido	Japan
Berberine	*Coptis japonica*	Pharmacy	-	Mitsui	Japan
	Thalictrum minus				
Biomass	*Panax ginseng*	Dietetics	30	Nitto Denki Kogyo	Japan
Carthamin	*Carthamus tinctorius*	Cosmetic	-	Kibun	Japan
Digoxin	*Digitalis lanata*	Pharmacy	3000	Boehringer Mannheim	FRG
Geraniol	*Pelargonium zonale*	Perfumery	-	Kanebo	Japan
Citronellol	*Pelargonium zonale*	Perfumery	-		
Jasmine	*Jasminum sp.*	Flavour	5000-6000	-	Japan
Peroxidase	*Raphanus sp.*	Diagnosis	2000	Toyobo	Japan
Plaunotol	*Croton sublyratus*	Pharmacy	-	Sankyo	Japan
Rosmarinic acid	*Coleus blumei*	Pharmacy	-	Natterman	FRG
Shikonin	*Lithospermum*	Pharmacy, Cosmetic,	4000	Mitsui, Lion Co.	Japan
	erythrorhizon	Dyes			
Vincristine	*Catharanthus roseus*	Pharmacy	3000	Eli Lilly	USA
Vinblastine	*Catharanthus roseus*	Pharmacy	3000	Eli Lilly	USA

In this study, an emphasis is put on the production of naphthoquinones which are found naturally in *Plumbago zeylanica* L. plant. It is an important medicinal plant, distributed in the tropical regions of South Asia and Southeast Asia (Anomymous, 1989). The naphthoquinones include plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone), 3,3'-biplumbagin, 3,6'-biplumbagin, 3-chloroplumbagin, droserone, elliptinone, zeylanone and isozeylanone. (Sidhu and Sankaram, 1971; Sankaram et al., 1976; Gunaherath et al., 1983; Gunaherath and Gunatilak, 1984; Padhye and Kulkarni, 1973; Sankaram and Rao, 1979). For biological activities, plumbagin has been reported to have antiimplantation and abortifacient activity (Premakunari et al., 1977), antifertility (Bhargava, 1984), anticancer (Fujii et al., 1992; Parimala and Sachdanandam, 1993), antimicrobial (Mukharya and Dahia, 1977; Krishnaswamy and Purushothaman, 1980; Durga et al., 1990; Didry et al., 1994), antimalarial (Likhitwitayawuid et al., 1998), anticoagulant (Santhakumari and Rathinam, 1978). Plumbagin has also been reported to be an effective chitin-synthase inhibitor (Kubo et al., 1983; Gujar and Mehrotra, 1988; Mitchell and Smith, 1988; Fetterer et al., 1989; Joshi and Sehnal, 1989; Rao et al., 1996) and therefore, it can be utilized for insecticidal in agriculture. Plumbagin content has been reported to be 0.6500% (w/w) fresh weight in the roots, 0.1350% in the green stem and 0.0010% in the leaves.

Synthetically, it has been reported that plumbagin can be synthesized by the Retro-Diels-Alder reaction (Akitami et al., 1980). This involves the Diels-Alder reaction of juglone with cyclopentadiene which produces an adduct. This adduct is transformed to the triol by diisobutylaluminum hydride. The triol is then treated with dimethoxypropane in the presence of *p*-toluenesulfonic acid to acid an acetonide. Oxidation of the acetonide with chromium trioxide give a ketone. The overall yield from juglone to the ketone is approximately 30%. The smooth Retro-Diels-Alder reaction followed by oxidation gave plumbagin.

Biosynthetically, plumbagin and 7-methyljuglone are the first naphthoquinones in higher plants that were shown to be formed from the

polyketide pathway (Manito and Sammes, 1981). It has been demonstrated that the two naphthoquinones are biosynthesized by plants of the *Drosera* and *Plumbago* genera. Both arise from a hexaketide rather than the shikimic acid route as occurs for juglone and menadione (Durand and Zenk, 1971). Feeding experiments with [1-^{14}C, 2-^{14}C-acetate] and 2-^{14}C-malonate led to label both naphthoquinones heavily, suggesting that plumbagin is formed by the well-known polyketide pathway.

Since information on the enzyme level of plumbagin biosynthesis has not been clarified and the plants of *P. zeylanica* are widely available in Thailand, we aim to search for a suitable source of starting material used for the study. Since plant cell cultures rather than differentiated plants have been proved to be preferred material for studying secondary product formation and elucidating the enzyme pathway. We decided to producing cell culture of *P. zeylanica* and evaluate for their potential in synthesizing plumbagin.

CHAPTER II

HISTORICAL

1. Botanical aspects of *Plumbago zeylanica* Linn.

Plumbago zeylanica Linn. (Fig. 1) or เจตมูลเพลิงขาว (Jet-ta-moon-ploeng-Khaao) is in the family Plumbaginaceae (พเยาว์ เหมือนวงษ์ญาติ, 2529; วิทย์ เที่ยงบูรณธรรม, 2531; เสริมสิริ วินิจฉัยกุล และคณะ, 2539). The name "*Plumbago*" is derived from the Latin *plumbum*, meaning lead, which on account of the lead-coloured flowers some of these and "*zeylanica*" is a Latin adjective, meaning of Ceylon, and thus refers to Southern India or Ceylon as the country of origin of the species (Bor and Raizada, 1990).

P. zeylanica is a perennial sub-scandent shrub, found wild in Peninsular India and West Bengal, and cultivated in gardens throughout India. (Dwarakanath, 1987). The leaves alternate, petiolate; petiole up to 2.5 cm. long, slightly auriculate at the base; blade up to 7.8 cm. long by 5 cm. broad, ovate, acute or obtuse, suddenly narrrowed into a cuneate base, dark green above, rather pale below, glabrous; margin entire. Flowers in elongate spikes; rachis glandular, striate; bracteoles ovate, acuminate, shorter than the calyx, glandular or not. Calyx 1-1.3 cm. long, narrowly tubular, persistent, densely covered with stalked glands; teeth small, with membranous margins. Corolla white, slender; tube 2-2.5 cm. long; lobes 8 mm. long, obvate-oblong, acute, apiculate. Stamens 5; filaments green, as long as the tube, slightly dilated at the base. Ovary shortly stipitate; style long, divided at the tip into 5 stigmas. Fruit a capsule contained in the viscid glandular persistent calyx. (Bor and Raizada, 1990; Kirtikar and Basu, 1935).

The propagation of *P. zeylanica* can be done by seedling, cutting and suckers although the method of seedling is unreliable due to poor germination and death of young seedlings under natural condition (Anonymous, 1989).

Figure 1 *Plumbago zeylanica* Linn. (Plumbaginaceae)

2. The uses of *P. zeylanica*

The root of *P. zeylanica* is known to be abortifacient and to have vesicant properties. It has been used as an appetizer and in skin diseases, diarrhoea, dyspepsia, piles and anasarca (Dwarakanath, 1987). In the form of a paste with vinegar, milk or salt and water it is applied externally in skin diseases including leprotic lesions. It is also used as a diuretic, caustic, expectorant and in rheumatism (Dwarakanath, 1987). The tincture of the root bark is a powerful sudorific and antiperiodic. The milky juice is used in the form of local application for scarbies and other unhealthy ulcers (Dwarakanath, 1987). The plant is used by the tribals for splenic complaints (Jain and Tarafder, 1970).

3. Chemical constituents of *P. zeylanica*

Since 1971, when Sidhu and Sankaram (1971) isolated and identified plumbagin from the roots of *P. zeylanica*, the research on isolation of the constituents from *P. zeylanica* has continued. The list of these compounds and their chemical structures is shown in Table 2.

Table 2 Chemical constituents and structure found in *P.zeylanica*

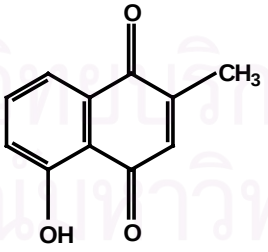
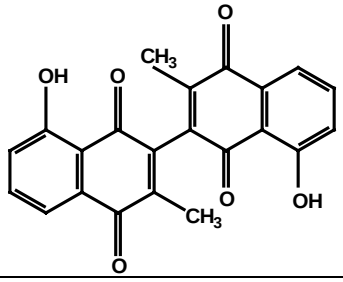
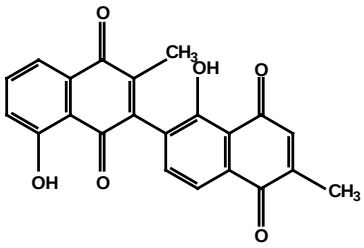
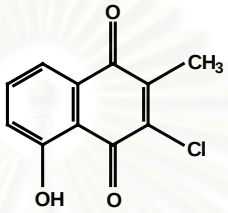
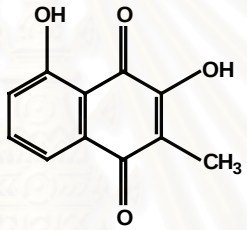
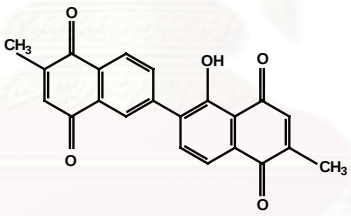
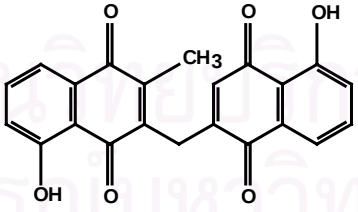
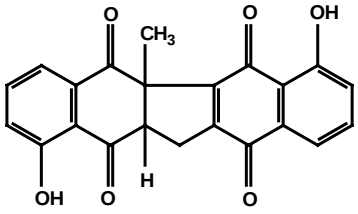
Chemical substance	Chemical structure	Reference
Plumbagin		Sidhu and Sankaram, 1971; Sankaram et al., 1976; Gunaherath et al., 1983; Gunaherath and Gunatilak, 1984
3,3'-biplumbagin		Padhye and Kulkarni, 1973; Sankaram et al., 1976; Gunaherath et al., 1983; Gunaherath and Gunatilak, 1984

Table 2 (continued)

Chemical substance	Chemical structure	Reference
3,6'-biplumbagin		Sankaram et al., 1976
3-chloroplumbagin		Sidhu and Sankaram, 1971; Padhye and Kulkarni, 1973; Sankaram et al., 1976; Gunaherath et al., 1983
Droserone		Sankaram et al., 1976; Gunaherath et al., 1983
Elliptinone		Sankaram et al., 1976; Gunaherath et al., 1983
Zeylanone		Sankaram and Rao, 1979; Gunaherath et al., 1983; Gunaherath and Gunatilaka, 1984.
Isozeylanone		Sankaram and Rao, 1979; Gunaherath et al., 1983; Gunaherath and Gunatilaka, 1984.

4. Plumbagin

4.1 Structure and chemical properties

Plumbagin is a natural naphthoquinone present in the roots of *P. zeylanica* (Iyengar and Pendse, 1962). Its chemical name is 5-hydroxy-2-methyl-1,4-naphthoquinone. It has a formular of $C_{11}H_8O_3$ with a molecular mass of 188.17.

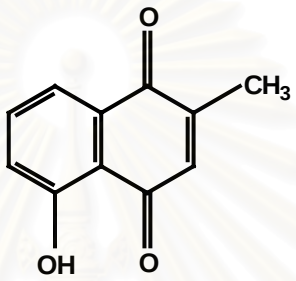


Figure 2 The chemical structure of plumbagin

Plumbagin occurs as yellow needles.Its melting point is 78-79 °C. It is slightly soluble in hot water and soluble well in alcohol, acetone, chloroform, benzene and acetic acid (The Merck Index, 1996).

For physicochemical properties of plumbagin, its ultraviolet spectrum shows λ_{max} (ethanol) at 212, 266, 410, 423 nm (log ε 4.35, 3.92, 3.39, 3.40). For NMR spectrum, plumbagin shows δ at 184.2 (s,c1), 149.2 (s,c2), 135.3 (d,c3), 189.8 (s,c4), 160.7 (s,c5), 123.8 (s,c6), 135.3 (d,c7), 188.8 (d,c8), 131.7 (s,c9), 114.7 (s,c10), 16.2 (s,c11) (Nahalka et al., 1996).

4.2 Occurance of plumbagin in the plant kingdom

In the literature, it has been reported plumbagin is report in a number of plant species belonging to many plant families (Table 3)

Table 3 Occurance of plumbagin in the plant kingdom

Plant	Thai name	Reference
Family Plumbaginaceae		
Plumbago capensis	เจตมูลเพลิงฝรั่ง	Goncalves et al., 1972; Kubo et al., 1983; Gujar and Mehrotra, 1988; Caniato et al., 1989; Crouch et al., 1990
Plumbago auriculata	เจตมูลเพลิงฝรั่ง	Crouch et al., 1990
Plumbago indica	เจตมูลเพลิงแดง	Chockalingam et al., 1990; Prasad et al., 1996; Ganasoundari et al., 1997; Kini et al., 1997; Devi et al., 1999; Jaya et al., 1999
Plumbago zeylanica	เจตมูลเพลิงขาว	Krishnaswamy and Purushothaman, 1980; Gunaherath et al., 1983; Bhargava, 1984; Gunaherath et al., 1984; Bhargava and Dixit, 1985; Durga et al., 1990; Saxena and Tikku, 1990; Tikku et al., 1992; Dhar and Rao, 1995; Gupta et al., 1995; Kavimani et al., 1996; Rao et al., 1996; Saxena et al., 1996;

Table 3 (continued)

Plant	Thai name	Reference
		Kini et al., 1997; Gupta et al., 1999
Plumbago scandens		Lima et al., 1968; Thiboldeaux et al., 1994
Plumbago europaea		Alnuri, et al., 1994; Kitanov Pashankov, 1994; Kini, et al., 1997
Plumbago rosea		Prasad et al., 1996; Ganasoundari et al., 1997; Kini et al., 1997; Devi et al., 1999
Plumbago pulchella		Villavicencio and Perez, 1992
Family Droseraceae *Drosera rotundifolia*		Goncalves et al., 1972; Canito et al., 1989; Bobak et al., 1995; Budzianowski, 1996; Krenn et al., 1998
Drosera intermedia		Budzianowski, 1996
Drosera binata		Goncalves et al., 1972; Caniato et al., 1989
Drosera peltata		Nari et al., 1990; Didry et al., 1998; Krenn et al., 1998
Drosera natalensis		Crouch et al., 1990
Drosera spatulata		Perica and Berljak, 1996
Drosera herba		Krenn, Blaeser and Havsknost, 1998
Drosera madagascariensis		Krenn, Digruber and Wawrosch, 1998
Drosera gigantea		Budzianowski, 2000
Drosophyllum lusitanicum		Nahalka et al., 1996; Nakalka et al., 1998

Table 3 (continued)

Plant	Thai name	Reference
Dionaea musciputa		Galek et al., 1990; Kreher et al., 1990; Pakulski and Budzianowski, 1996; Todorov et al., 1998
Family Ebenaceae *Diospyros mespiliformis*		Lajubutu et al., 1995
Diospyros usambarensis		Marston et al., 1984
Diospyros maritima		Kuo et al., 1997; Ogihara et al., 1997; Higa et al., 1998; Khan and Timi, 1999
Diospyros morrisiana		Ito et al., 1995; Kuke et al., 1998
Diospyros oleifera		Kuke et al., 1998
Diospyros variegata		Kuke et al., 1998
Diospyros japonica		Ito et al., 1995
Diospyros ferra		Ito et al., 1995
Diospyros lolin		Khan and Timi, 1999
Diospyros novoguinensis		Khan and Timi, 1999
Diospyros piscatoria		Adeniyi et al., 2000
Diospyros greeniwayi		Khan and Raekika, 1998
Diospyros olen		Evans et al., 1999
Family Ceratostigma *Ceratostigma minus*		Yue et al., 1994
Ceratostigma wilmottianum		Shcherbanovskii, 1981; Yue et al., 1997
Family Ancistrocladaceae *Ancistrocladus cochinchinensis*		Nguyen et al., 1997
Ancistrocladus heyneanus		Bringmann et al., 1999

Table 3 (continued)

Plant	Thai name	Reference
Family Peraceae *Pera benensis*		Fournet, Angelo, Munoz, Roblot et al., 1992; Fournet, Angelo, Munoz, Hocquemiller et al., 1992
Family Dentaria *Dentaria micrantha*		Shcherbanovskii, 1981
Family Solanaceae *Withania somnifera*		Ganasoundari et al., 1997
Family Nepenthaceae *Nepenthes thorelii*		Likhitwitayawuid et al., 1998
Family Caesalpiniaceae *Cassia obtusifolia*		Aboott et al., 1998
Family Triphyophyllum *Triphyophyllum peltatum*		Bringmann et al., 2000
Family Convolvulaceae *Cuscuta reflexa*	เครือเขาคำ	Bringmann et al., 1999

4.3 Extraction and isolation of plumbagin from *Plumbago* spp.

Extraction and isolation of plumbagin from *Plumbago* spp. have been reported previously (Gupta et al., 1993; Kitanov and Pashankov, 1994). The roots of *Plumbago europaea* L. have been extracted with petroleum ether and plumbagin isolated by high performance liquid chromatographic method. (Kitanov and Pashankov, 1994). Plumbagin has also been purified from the n-hexane extract of *P. zeylanica* roots by silica gel column chromatography employing n-hexane:ethyl acetate (95:5) as mobile phase (Gupta et al., 1993).

4.4 Detection and determination of plumbagin

Plumbagin has been detected and identified by thin layer chromatographic method (Zenk et al., 1969; Heble et al., 1974) and determined for its content either by spectrophotometric method (Nahalka et al., 1996) or high performance liquid chromatographic method (Gupta et al., 1993; Kitanov and Pashankov, 1994).

Thin layer chromatography (TLC) has been performed on silica gel 60 F254 plate using benzene and petroleum ether (2:1) (Zenk et al., 1969) or pure benzene as the solvent system (Heble et al., 1974). The identity of plumbagin was comfirmed by its mobility on TLC plates and by its UV spectrum.

Plumbagin was quantitatively determined by spectrophotometry in the toluene extract (Nahalka et al., 1996). For HPLC, a Spherogel column using n-hexane:chloroform:2-propanol (30:70:2,v/v/v) as the mobile phase and detector with wavelength 267 nm (Gupta et al., 1993) or a LiChrosorb RP-18 column using methanol:0.4% acetic acid (60:40,v/v) as the mobile phase (Kitanov and Pashankov, 1994).

4.5 Biological activities of plumbagin

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a natural naphthoquinone showing a broad range of pharmaceutical activities. It has been reported to manifest significant antiimplantation and abortifacient activity in the albino rats without any teratogenic effect after giving orally (1 and 2 mg/100 g) (Premakumari et al., 1977); Giving a dose of 10 mg/kg *i.p.* for 60 days has been shown to cause selective testicular lesions in dogs. In the latter case, the wet weight of tested and epididymides has been found to decrease. There has been shown to cause reduction in diameter of seminiferous tubules and leydig cells nuclei as well as drastic curtailment in cellular heights of epididymides. Significant reduction in protein RNA and sialic acid concentration has also been observed, while the intratesticular cholesterol and acid/alkaline phosphatase has been found to be raised after drug treatment (Bhargava, 1984). Plumbagin has been studied its effect on cell growth and mitosis in chick embryo fibroblast cultures (Santhakumari et al., 1980). It has been shown to behave, in lower concentration like a spindle poison by inhibiting cell mitosis in higher concentrations. However, it exhibited radio mimetic, nucleotoxic and cytotoxic effects (Santhakumari et al., 1980). It has been reported to exert various biological activies against microorganisms. It has been found to have antibacterial activities against *Bacillus mycoides, B. pumilus, B. subtilis, Salmonella.typi, S. paratyphi, Sarcina. lutea, Staphylococcus. aureus, Staph. citreus, Staph. albus* and *Staph. dublin.* (Mukharya and Dahia, 1977; Krishnaswamy and Purushothaman, 1980; Durga et al., 1990; Didry et al., 1994), It has also been found to have anticancer (Fujii et al., 1992; Parimala and Sachdanandam, 1993) and anticoagulant (Santhakumari and Rathinam, 1978). Plumbagin has also been shown to inhibit insect development, at least indirectly, presumably by interfering with hormonal processes of moulting (Kubo et al., 1983; Gujar and Mehrotra, 1988; Mitchell and Smith, 1988; Fetterer et al., 1989; Joshi and Sehnal, 1989; Rao et al., 1996) and therefore, it can be utilized for insect killing in agriculture.

4.6 Biosynthetic pathway of plumbagin

Plumbagin and 7-methyljuglone are the first naphthoquinones in higher plants were shown to be biosynthesized from the polyketide pathway (Manitto and Sammes, 1981). It has been demonstrated that the two naphthoquinones are formed by plants of the *Drosera* and *Plumbago* genera. Both arise from a hexaketide rather than the shikimic acid route as occurs for juglone and menadione (Durand and Zenk, 1971). Feeding experiments with [1-^{14}C, 2-^{14}C-acetate] and 2-^{14}C-malonate led to label both naphthoquinones heavily, suggesting that plumbagin is formed by the well-known polyketide pathway. Although the enzyme polyketide synthase in *Plumbago* spp. has not yet been reported, the possible biosynthetic pathway of plumbagin has been proposed as shown in Fig. 3 (Durand and Zenk, 1971).

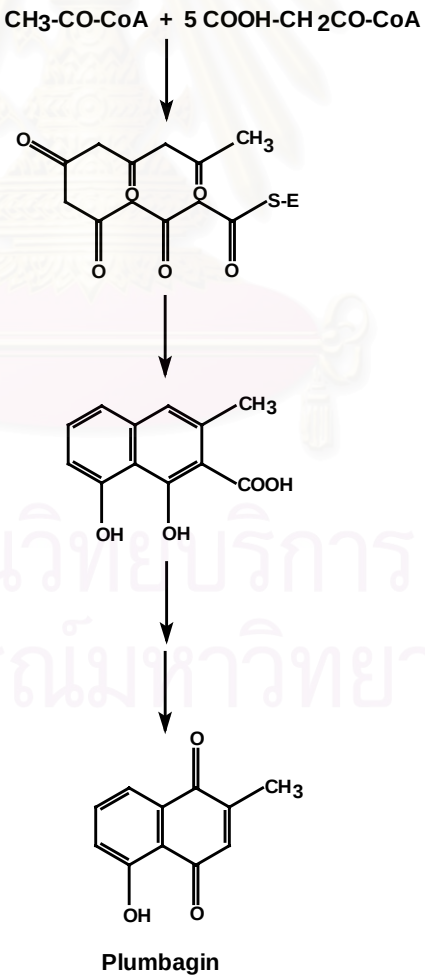


Figure 3 The proposed biosynthetic pathway of plumbagin

5 Source and type of plant tissue and cell culture

The techniques of plant tissue and cell culture are the art of growing isolated plant as explants on appropriate media under aseptic and heterotrophic conditions. These techniques can be considered to be extended from the nutritional methods of microbiology to higher plants. The term “tissue culture” embraces procedures and practices which may be applied to plant materials from source that may cover the entire morphological range. This range may be from young leaves, immature seeds, embryos, buds, apical shoots or roots and protoplasts. Since these plant parts can be grown without the requirement of the essential organization that supports life, the term “*in vitro*” culture was introduced. As there are many different building materials within a plant, there are many different types of *in vitro* culture Fig. 4.

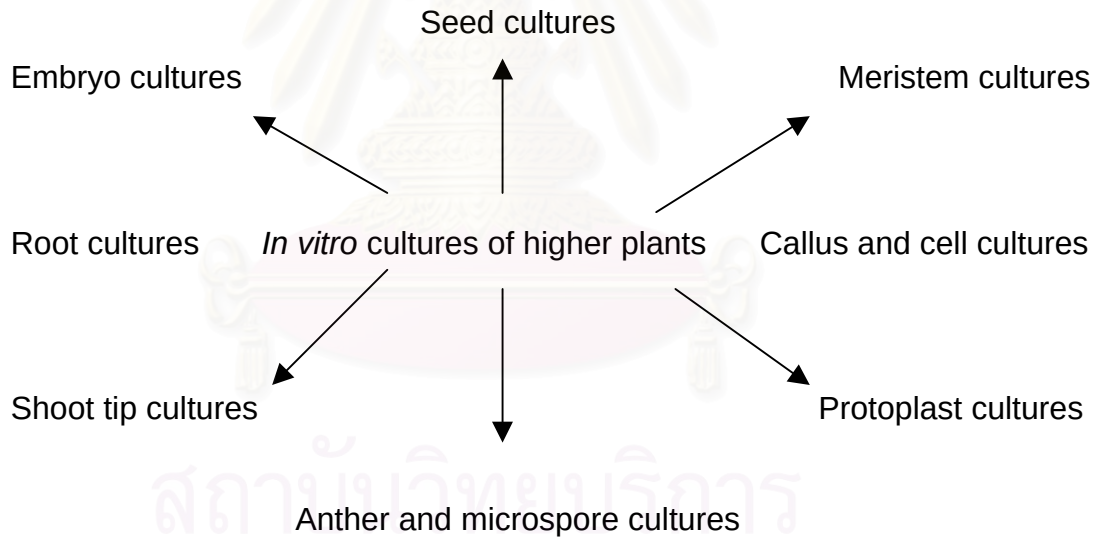


Figure 4 Schematic representation of *in vitro* cultures of higher plants

5.1 Callus cultures

Callus cultures may be obtained from a wide variety of plant organs (e.g., roots, shoots, leaves) or specific cell types (e.g., endosperm, pollen). Once the explant has been chosen, and a culture medium formulation decided upon, the worker is ready to initiate callus cultures. Firstly, ensure that the chosen explant is in a suitable “biological state” for callus initiation. Generally, young tissues are more suitable than mature ones. This is especially so in the case of leaves. Germination of sterilized seeds under aseptics conditions will often yield root, shoot and leaf material suitable for callus initiation because the production of callus by directly plating out whole seeds on agar culture medium plus growth regulators. The production, by germinating seeds on agar medium minus growth regulators, of sterile root, shoot and leaf material for use as explants Moreover excision of radicle and plumule tissue directly from the seeds for use as explants.

The size and shape of the initial explant is not, within limits, normally critical although there may be a critical minimum size below which explants will not proliferate. Before attempting to initiate a callus culture, it is first necessary to sterilize the plant organ from which an explant is to be taken. Callus initiation, aseptically transfer explants to the required semi-solid medium and gently press them into the agar so that good contact is made.

Assuming a suitable medium has been selected, most explants should have produced sufficient callus to allow for subculture within 3-8 weeks. Remove newly formed callus from the initial explant at this stage by cutting with a sterile scalpel. Take care to ensure that the new callus pieces to be transferred to fresh agar medium are not too small, as further growth may then be inhibited. Once well established, most callus culture lines will require regular subculture at approximately 4 weekly intervals. In general 25°C is a suitable temperature for incubating cultures and exposure to low light intensities is often beneficial.

The calli usually have a large number of morphological types. They vary according to external appearance, texture and cellular composition. Some calli consist of hard compact tissues with small closely packed cells, while others consist of soft tissues with minimal cellular contact. The pigmentation of callus is also variable, even among isolated from the same species.

5.2 Cell suspension cultures

Cell suspension cultures are obtained by transfer of friable callus clumps to agitated liquid medium of the same composition as that used for callus growth. It consist of isolated cell and very small cell aggregates remaining dispersed as they grow in agitated liquid medium. Agitation rates on orbital shakers should be in the range of 60-150 r.p.m. with an orbital motion stroke of 2-4 cm.

At the first subculture into fresh medium, remove large clumps of initial inoculum either by transferring material with a pipette or syringe of suitable orifice diameter to exclude large cell aggregates or alternatively by allowing the culture to settle for a short time and then transferring the cells from only the upper part of the culture. Some callus culture lines grow as compact, non-friable lumps and do not readily break up to form suspensions; however, in some cases callus friability increases following repeated subculture.

For each cell cultures line there is a minimum inoculum size below which the culture will not grow. The lag phase of the culture increase in length as the inoculum size decrease towards to minimum level.

Cell suspension cultures provide a relativety homogeneous population of cells, readily accessible to exogenously applied chemicals and growing under defined, aseptic conditions. Cell suspension cultures are widely used as model systems for studying pathways of secondary metabolism, enzyme induction and gene expression, degradation of xenobiotics and as a source of

material for enzyme purification. The lack of chlorophyll and carotenoid pigments in most plant cell suspension cultures is of great benefit for work involving isolation of enzyme or secondary products.

5.3 Plant cell cultures as metabolic model system

Plant tissue and cell cultures offer a number of advantages over intact plant for studies of metabolism. They are relatively easy to establish and maintain under strictly controlled nutritional and environmental conditions. They can be grown in either small containers (such as 250 ml Erlenmeyer flasks) or large fermentors and chemostats, and thus the amount of biomass are sufficiently available as needed.

From the mentioned advantages, the use of tissue cultures for metabolite studies has so far been limited, mainly because of the special metabolite characteristic impose by the usual culture environment. In the case of secondary metabolism, the culture of many species do not produce significant amount of the compound characteristic of intact plants. This may sometimes be due to the loss of genetic information during prolong culture, but in many cases it has been shown that even long-term cultures remain totipotent (Chaleff, 1983; Davey, 1983). It is more likely that the failure of culture cell to produce the pattern or level of secondary metabolites typical of the source plant is a consequence of the specific physiological and morphological state of cultured tissue.

CHAPTER III

MATERIALS AND METHODS

1. Chemicals

Standard plumbagin was purchased from Sigma (USA). Chemicals for culture media, all tissue culture grade, were also from Sigma (USA). Various plant growth regulators were purchased from Gibco Laboratories (New York, USA). Gelling agent (agar) was purchased from Difco Laboratories (Detroit Michigan, USA). Organic solvents for phytochemical study were all analytical grade (Labscan). TLC plates of silica gel 60 F254 0.2 mm thick on aluminium sheet were obtained from Merck (Damstadt, Germany) water was triple deionized and distilled in glass.

2. Plant material

The whole plants of *Plumbago zeylanica* Linn. were obtained from Minburi, Bangkok. Three mature plants with similar sizes were separated into flowers, leaves, stems and roots. The leaf and stem parts were further separated into smaller portions in which their position in the whole plants were recorded.

The leaves of *P. zeylanica* used for this tissue cultures work were collected the plant grown in the open field of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3. Distribution of plumbagin in *P. zeylanica* plant

The plant of *P. zeylanica* was separated into serveral organ parts (roots, stems, leaves and flowers). The separation was done in such a way that the connection of each part in the whole plant is still known and recorded. After separation, each part was dried by freeze drying and determined for

plumbagin content by TLC densitometric analysis (see the sections 5.1 and 5.2)

4. Plant tissue culture techniques

4.1 Nutrient media

Standard basal media used in this study was LS (Linsmaier and Skoog, 1965). The composition of these media and media preparation method were described in Appendix. For plant growth regulators, the following auxins and cytokinins were used :

Auxin : indole-3-acetic acid (IAA)
indole-3-butyric acid (IBA)
α -napthaleneacetic acid (NAA)
2,4-dichlorophenoxyacetic acid (2,4-D)
Cytokinin : 6-benzylaminopurine or N^6-Benzyladenine (BA)
kinetin-6-furfurylaminepurine (kinetin)

For solid media, the nutrient solutions were added with 0.8% (w/v) agar (Difo, Detroit Michigan, USA)

4.2 Cultures conditions

The *in vitro* cultures of *P. zeylanica* were maintained in a culture room with 16-hour photoperiod at illumination of 2,000 lux and 8-hour dark. The incubation temperature was maintained at 25$\pm$2 °C. The pH valve of all media under study was set to 5.6 before autoclaving.

4.3 Preparation of *P. zeylanica* explants

The leaves of *P. zeylanica* were used as explants or starting materials for plant tissue cultures studies. Before initialing the callus, the surface of the

explants were sterilized. Young leaves of *P. zeylanica* were cleaned with running tab water, dipped into 70% ethanol for a few minutes and surface sterilized in 15% Clorox® solution containing a few drops of Tween 80 and shaked gently for 15 minutes. After 15 minutes immersion remove young leaves and washed with sterile distilled water three times in laminar flow cabinet. Transferred to solid nutrient medium with aseptic technique, cut into small piece (approx. 0.5x0.5 cm^2) with a sharp scalpel.

4.4 Establishment of callus cultures

The surface sterilized leaf explants were placed on semi solid basal LS medium (Linsmaier and Skoog, 1965) supplemented with 0.2 mg/l NAA and 0.2 mg/l 2,4-D for callus induction. The cultures were maintained at 25±2 °C under controlled 16-hr photoperiod (2,000 lux) conditions. The formation of callus was observed after two weeks. After a month of callus induction, the callus cultures were maintained by regular subculture every three weeks on fresh medium with the same composition.

4.5 Establishment of cell suspension cultures

Cell suspension cultures of *P. zeylanica* were initiated from the established callus cultures. The callus tissues were placed in a 250 ml Erlenmeyer flask containing 50 ml of LS medium containing 30 g/l sucrose, 0.2 mg/l NAA and 0.2 mg/l 2,4-D. The suspension were incubated on rotary shaker rotated continuously at 120 r.p.m. at 25±2 °C. After obtaining stable *P. zeylanica* cell cultures, the cell suspensions were maintained under the same conditions and subcultures every two weeks by adding 10 ml of culture to 50 ml to fresh medium with the same composition.

4.6 The effect of basal media

The effect of basal media on growth and plumbagin production were tested with five different media. These LS (Linsmaier and Skoog,1965), MS

(Murashige and Skoog, 1962), B5 (Gamborg et al., 1970), N (Nitsch et al., 1968) and SH (Schenk and Hildebrandt, 1972) Experimentally, 10 ml of stock was transferred in triplicate onto 50 ml of each medium containing without plant growth regulators. The cultured were maintained at 25±2 °C under controlled 16-hr photoperiod. After 7 days, the cultures were harvested by suction filtration, and subjected to freeze drying using a lyophilizer. The resulting dry biomass of each cell cultures was weighed and recorded. All cell cultures were then kept in a sealed container under 4 °C subsequent analysis of plumbagin content.

4.7 The effect of plant growth regulators

The stock cultures of *P. zeylanica* (10 ml) were pretreated in a hormone-free MS medium about one week. After that transferred onto 50 ml of liquid MS medium (Murashige and Skoog, 1962) supplemented with different concentrations (0.00, 0.01, 0.10, 1.00 and 10.0 mg/l) of auxins (IAA, IBA, NAA and 2,4-D) or cytokinins (BA and kinetin) for testing the effect of these plant growth regulators on cultures growth and plumbagin production. After 7 days, the cultures grown in various conditions were harvested and prepared for dry weight and plumbagin determination as described. Each culture was analyzed triplicates for both parameter.

4.8 The effect of carbon source

This was carried out by using LS medium containing 3% (w/v) of various sugars (sucrose, glucose, sorbitol and manitol), 0.2 mg/l NAA and 0.2 mg/l 2,4-D.

4.9 Study on growth and plumbagin production in cell suspension cultures.

Duplicate 10 ml samples of a 7-day-old suspension were inoculated in liquid MS medium supplemented with 0.1 mg/l 2,4-D, 0.01 mg/l BA and 3%

(w/v) manitol. The cell suspension cultures were harvested every day for 10 days by suction filtration and then harvested every other day until day 16. The dry weight were recorded after freeze drying for 18 hours. The amounts of plumbagin was examined as described in the section of 5.3 and calculated in units of both percentages of dry weight and total content. These data were then plotted to obtain growth and plumbagin production curves.

4.10 Production of plumbagin in hairy root cultures of *Plumbago indica* and *P. zeylanica*

The hairy root cultures of both species were formed from the explants on solid Murashige and Skoog (MS) medium supplemented with 1.5 mg/l IBA and 0.5 mg/l NAA. The hairy root cultures were maintained in liquid MS medium under the same conditions and subcultured every 4 weeks.

5. Phytochemical techniques

5.1 Preparation of crude extracts of various *P. zeylanica* cultures and various plant parts

The dried samples of either cell suspension cultures or various plant parts of *P. zeylanica* were ground to fine powder in a grinder. Five hundred milligrams of each powdered sample was extracted with 20 ml methanol under reflux for one hour in a 20x2.5 cm tube connected with a 15 cm condenser. The crude extracts were then filtered (Whatman no.1) and evaporated *in vacuo* to dryness. One hundred microliters of petroleum ether were then added to each tube to dissolved the residue to obtain a solution for qualitative and quantitative analysis of plumbagin.

5.2 Identification of plumbagin in the sample extracts

Thin layer chromatographic (TLC) densitometric analysis was used as the method for identification of plumbagin in the crude extracts obtained from both the *in vitro* cultures and intact plants of *P. zeylanica*. Five microliters aliqout

of each extract was spotted on a TLC plate and separated under the following TLC conditions. Plumbagin in each sample in the TLC plate was quantitated by the developed TLC densitometric method and calculated based on its standard curve.

Thin layer chromatographic conditions for plumbagin separation.

Technique	:	one dimension, ascending,simgle development.
Stationary phase	:	aluminium sheet silica gel 60 F254 (precoated, Merck)
Plate size	:	10X20 cm^2
Layer thickness	:	0.2 mm
Solvent system	:	toluene:formic acid (9.9:0.1)
Sample size	:	5 μl
Distance	:	8 cm
Temperature	:	25-30 °C
Detection	:	ultraviolet light at 254 nm

5.3 Quantitative analysis of plumbagin

Plumbagin in each sample on the TLC plate was quantitated by the densitometric method at 270 nm and calculated based on the standard curve of plumbagin (peak area-concentration relationship) The conditions for densitometric analysis were described below.

5.3.1 TLC densitometric analysis

Plumbagin spot, obtained after thin layer chromatography, which had the same *R*f valves as standard plumbagin, was studied for their ultraviolet absorption spectra and was quantitated by using TLC densitometer. The TLC densitometric conditions are as follows:

Instrumental model	:	Shimadzu Dual-wavelengthTLC-scanner Model CS-930
Lamp	:	Deuterium (D_2)
Determination mode	:	absorption
Scan width	:	X = 6.0 mm
		Y = 0.2 mm
Sensitivity	:	medium
Slit width	:	1.2X1.2 mm^2
Wavelength scanned range	:	270 nm

5.3.2 High performance liquid chromatographic analysis

The accuracy and precision of the TLC densitometric method was comfirmed by high performance liquid chromatography (HPLC)

High performance liquid chromatograpic conditions.

Instrument model	:	Shimadzu C-R6A Chromatopac
		SPD-10A Shimadzu UV-VIS Detector
		LC-10AD Liquid Chromatograph
Chromatographic column	:	Merck 50943 LiChroCART®
		125-4 LiChrospher® 100 RP-18 (μm)
Mobile phase	:	CH_3OH:0.4% CH_3COOH (60:40,v/v)
Injection volume	:	10 μl
Flow rate	:	1.0 ml/min
Temperature	:	25-30 °C
Detector	:	ultraviolet 270 nm

5.4 Preparation of standard for calibration curve

Plumbagin 0.2 mg were dissolved in 0.2 ml petroleum ether to give 1 mg plumbagin per ml stock solution. The stock solution was diluted to the concentration range of 0.0125-0.1 mg/ml for constructing their calibration

curve of plumbagin by TLC densitometric analysis. For high performance liquid chromatography, the stock solution was diluted by half-dilution technique and the concentration range of 0.001-0.05 mg/ml was used for constructing the calibration curve of plumbagin.

CHAPTER IV

RESULTS

1. Development of TLC densitometric method for the determination of plumbagin in various *P. zeylanica* parts

In order to examine for plumbagin content in various plant parts and *in vitro* cultures of *P. zeylanica*, it is necessary to have an assay method which is simple, rapid and accurate. TLC densitometry is a method for separation on a TLC plate of components in a plant crude extract without prior product purification and followed by scanning the plate using densitometer to produce a chromatogram. This technique has not been reported to determine plumbagin content in any plant material.

So far, there has been only a report on the determination of plumbagin in other plant growth by using the spectrophotometric method (Nahalka, 1996).

1.1 TLC separation of plumbagin in various *P. zeylanica* parts crude extracts

In this study, the development of TLC densitometry for plumbagin determination was started with finding solvent system suitable for separation of various compounds in the crude methanolic extracts. It was found that the solvent system of toluene : formic acid, 9.9 : 0.1 could separate a yellow spot (which was co-chromatographed with authentic plumbagin) form other components present in various crude methanolic extracts for *P. zeylanica*. These included the crude extracts prepared from the plant parts of flowers, leaves, stems and roots. No significant interference from other components in these extracts was observed under this TLC condition. When the TLC plate was scanned at the wavelength of 270 nm by a TLC densitometer, various chromatograms of the crude methanolic extracts were obtained. As shown in

Fig. 5, all plant parts of *P. zeylanica* appeared to contain plumbagin which well peak separated from other peaks under the established conditions.

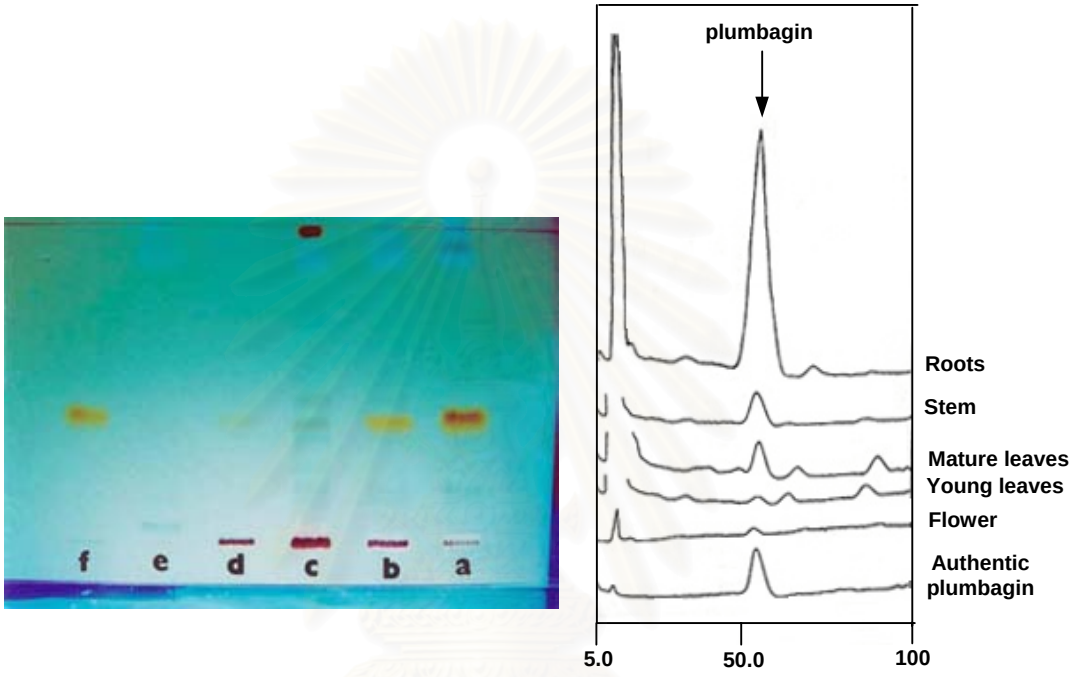


Figure 5A) TLC patterns of methanolic extracts obtained from various plant parts of *P. zeylanica* under 254 UV light.

Samples : a) roots b) stems c) mature leaves d) young leaves e) flowers

Standard : f) plumbagin

Solvent system : toluene: formic acid (9.9:0.1)

B) TLC densitometric chromatograms of the methanolic extracts of various plant parts. The chromatograms were obtained from TLC plate of A (from a to e)

1.2 Identification of plumbagin on TLC plate

To confirm that the peak of plumbagin in the TLC densitometric chromatogram was absolutely contributed by pure plumbagin, the spot on the TLC plate corresponded to the *R*f value of standard plumbagin was scanned to produce a UV absorption spectrum. The obtained UV absorption spectrum was then compared with that of authentic plumbagin. As shown in Fig. 6, it was found that plumbagin in the methanolic extract and standard plumbagin had identical absorption spectrum with their λ_{max} at 270 nm. Therefore, it was clear that the peak with the *R*f value of 0.6 on the TLC plate was contributed by only pure plumbagin present in the methanolic extract of *P. zeylanica*. Furthermore, based on the UV absorption spectrum, the wavelength of 270 nm appeared to be suitable for performing quantitative analysis of plumbagin in *P. zeylanica* by TLC densitometric method.

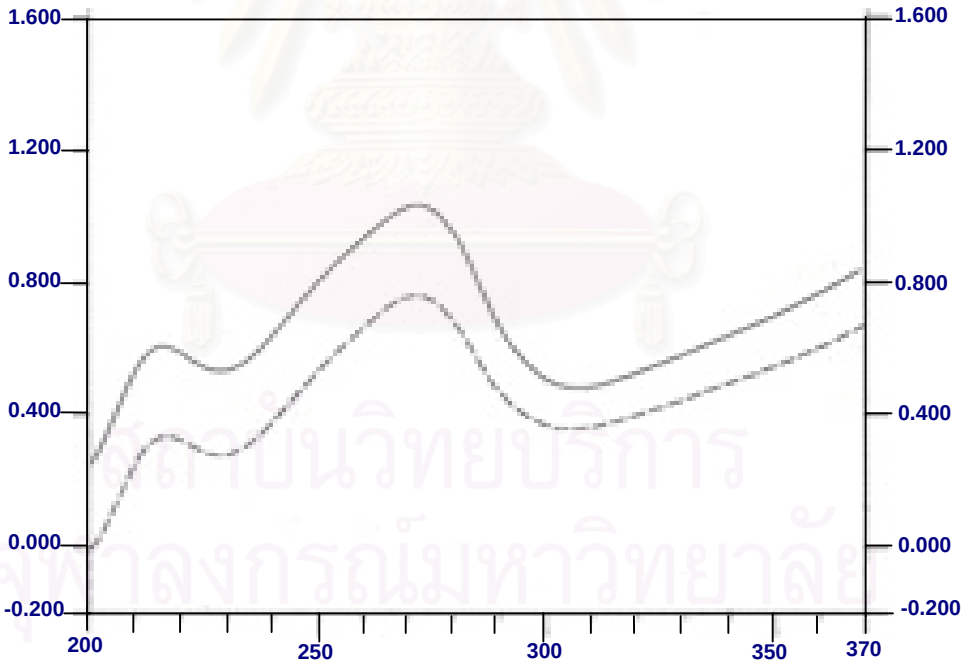


Figure 6 UV absorption spectra of authentic plumbagin (----) and the compound of similar *R*f value to plumbagin which was separated from the methanolic extract of *P. zeylanica* roots (—)

1.3 Standard calibration curve

The complete separation of plumbagin from other components by one development of TLC plate allowed the compound be quantitated by the method of densitometry which generated a chromatogram. The area under the peak of plumbagin could be used for calculation of its content if a calibration curve of authentic plumbagin is available. The calibration curve of standard plumbagin was obtained by plotting the peak areas against plumbagin concentration is shown in Fig. 7. This graph showed linearity relationship between 0.31 to 2.50 μg per 5 μl application volume which are equivalent to the concentration of 0.0125 to 0.1 mg plumbagin per ml. The regression analysis and the correlation coefficient was found to be 0.995.

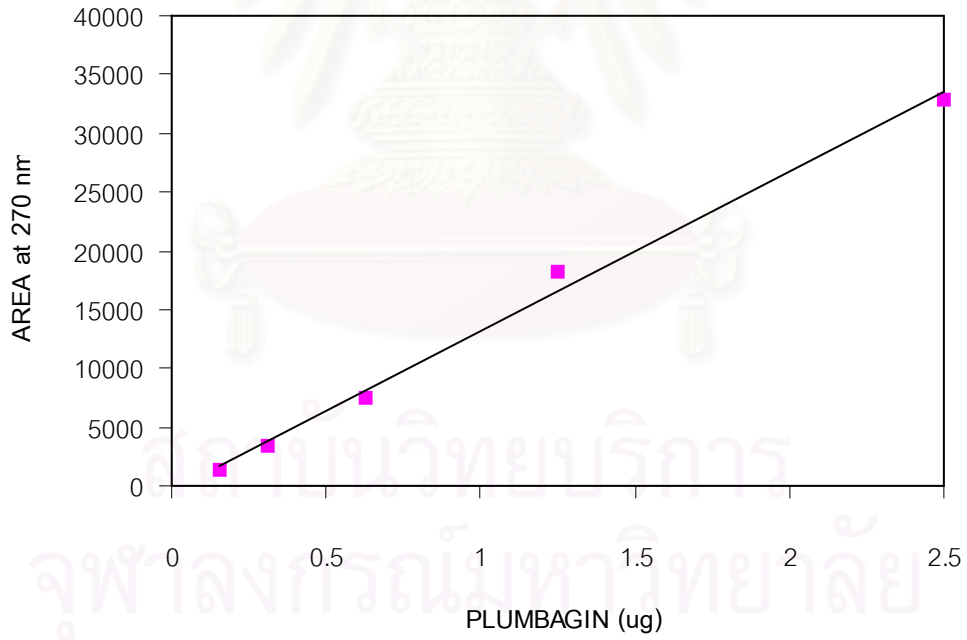


Figure 7 Calibration curve of plumbagin by TLC densitometric method

1.4 Accuracy and precision

The accuracy of TLC densitometry was confirmed by HPLC method. The separation of root plumbagin by HPLC is shown in Fig. 8. Its retention time appeared in the chromatogram was found to be approximate 8.8 min. The calibration curve of plumbagin obtained by the HPLC method (Fig. 9) showed linearity of the relationship from 0.001 to 0.05 mg/ml and the correlation coefficient was found to be 0.9997. In this study, *P. zeylanica* was analyzed for plumbagin content using the two methods and results were compared. It can be seen in Table 4 and Fig. 10 that the values of plumbagin content determined by both methods were very closed from one another. In terms of precision, the three separate determinations of sample showed a very narrow value of standard deviation of its plumbagin content. These results indicated that the accuracy and precision of the TLC densitometric method was reliable.

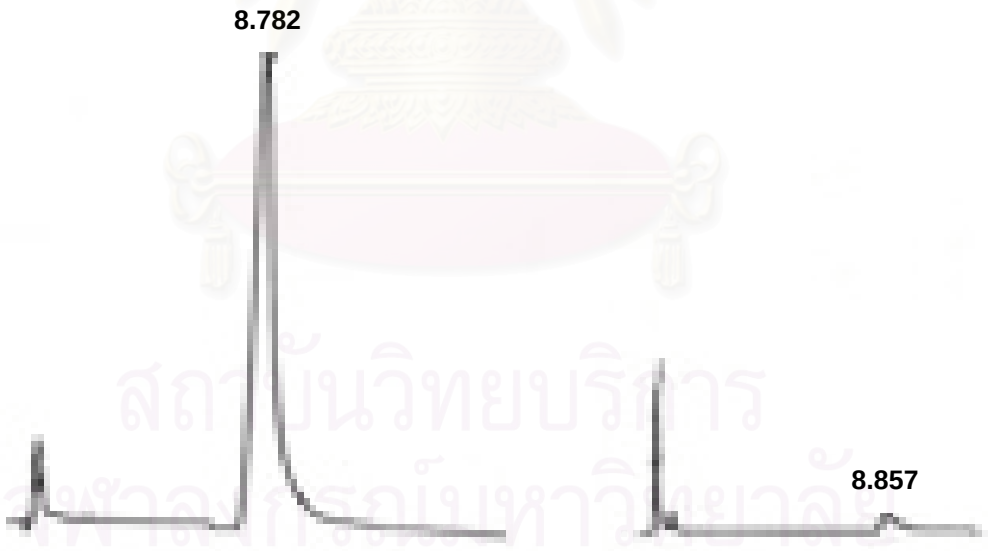


Figure 8 HPLC chromatogram of authentic plumbagin (A,RT=8.782) min and plumbagin in root extract (B,RT=8.857)

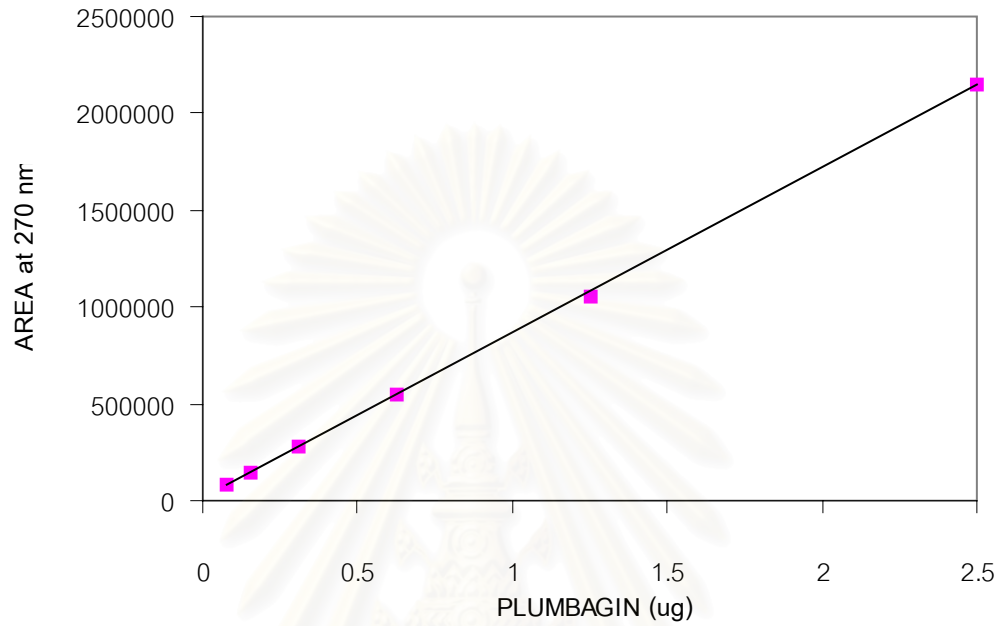


Figure 9 Calibration curve of plumbagin by HPLC method

Table 4 Percentage of plumbagin content obtained from both TLC densitometric and HPLC methods

Sample	Plumbagin content (mg/g DW)	
	TLC densitometric method	HPLC method
1	0.710±0.005	0.735±0.002
2	2.413±0.030	2.490±0.014
3	3.486±0.052	3.503±0.026

Each value represented the mean±SD of plumbagin in triplicate.

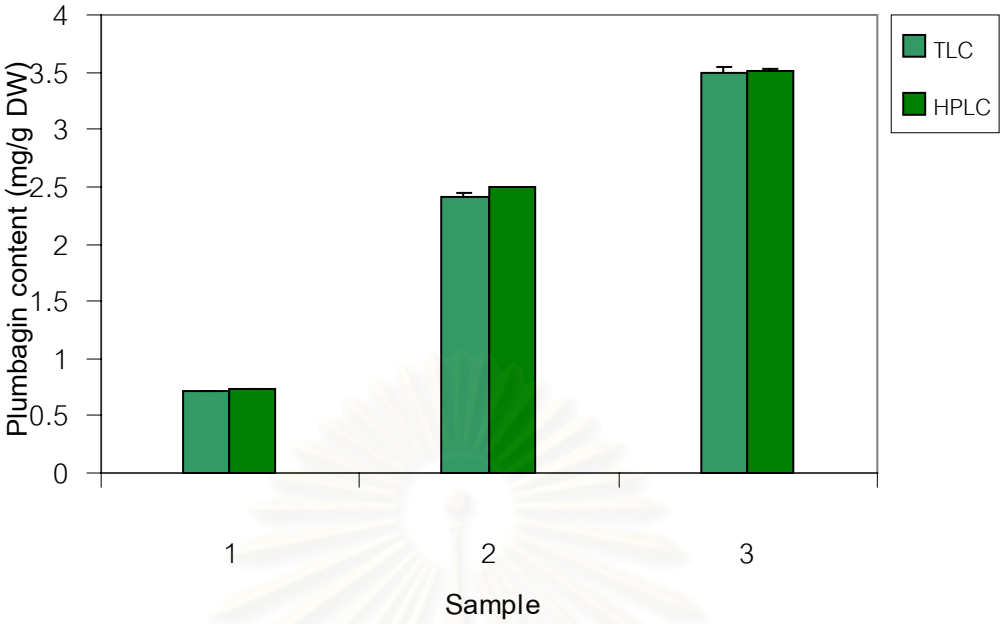


Figure 10 Bar graphs of plumbagin in *P. zeylanica* roots determined by TLC densitometric method and HPLC method. The values of plumbagin content were obtained from Table 4

2. Plumbagin content in various parts of *P. zeylanica* plant

After dryness, each sample was ground to powder and determined for its plumbagin content by TLC densitometry established as described in Section 1.1. The results of plumbagin content in *P. zeylanica* are summerized in Table 5 and Fig. 11. It can be seen that various parts of *P. zeylanica* contained highly variable plumbagin content ranging from 0.002 to 0.266% (w/w) dry weight (Fig. 12). When considering high plumbagin producing in various part *P. zeylanica* more than 0.08% (w/w), it was found that samples ST1 and root contained plumbagin in the level of 0.089% and 0.226%. For low plumbagin producing flowers, ST7 and ST6 were found to contain less than 0.007% (w/w) plumbagin.

The content of plumbagin in the roots appeared to be the highest (0.226%)followed by the stems, mature leaves (0.02-0.03%), young leaves (0.015%) and flowers (0.002%), respectively. Moreover, the distribution of plumbagin along the stem and leaves was carried out. It seems that the plumbagin content in the middle part of the stem was slightly higher than that in the bottom and the top parts (Fig. 12).

These results clearly indicate that the stems (0.089%) of *P. zeylanica* contains lower content of plumbagin than the roots (0.226%) and, therefore, the roots are a potential source of plumbagin.

Table 5 Plumbagin content in various plant of *P. zeylanica*

Various part of *P.zeylanica*	Plumbagin content (% w/w dry weight)
Roots	0.226±0.0231
Stems	
ST 1	0.089±0.0103
2	0.022±0.0032
3	0.015±0.0008
4	0.007±0.0006
5	0.007±0.0004
6	0.005±0.0010
7	0.002±0.0011
Leaves	
LEV 1	0.013±0.0008
2	0.024±0.0032
3	0.032±0.0032
4	0.014±0.0013
5	0.013±0.0005
Flowers	0.002±0.0014

The value of each represents the mean±SD of triplicate analysis.

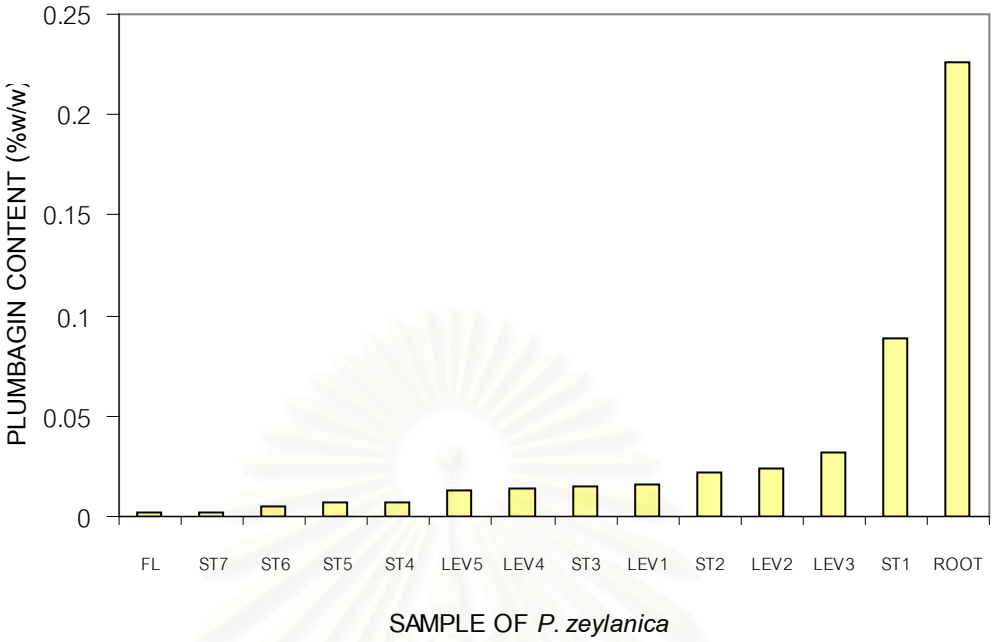


Figure 11 Variation of plumbagin content in various part of *P. zeylanica*

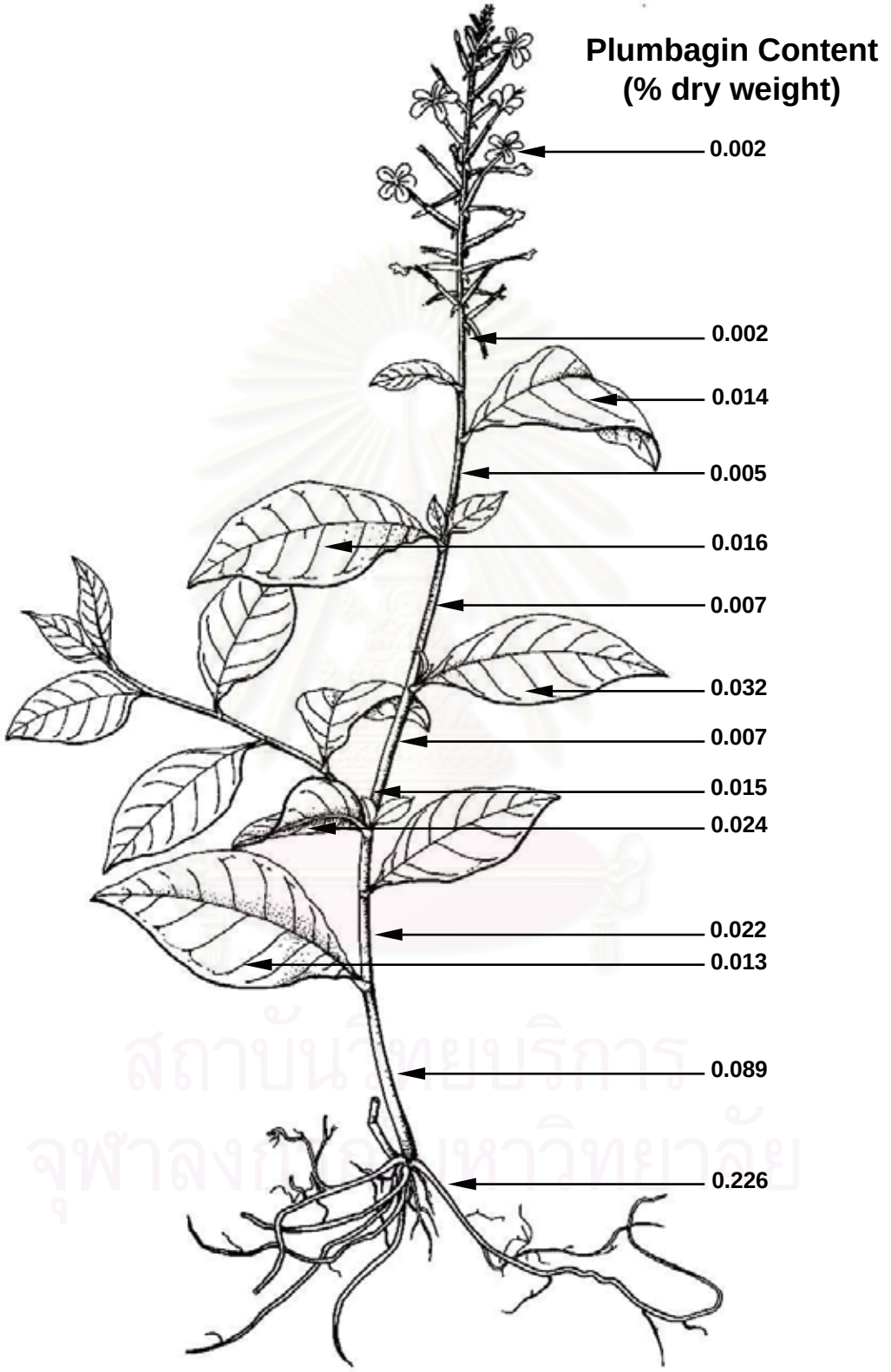
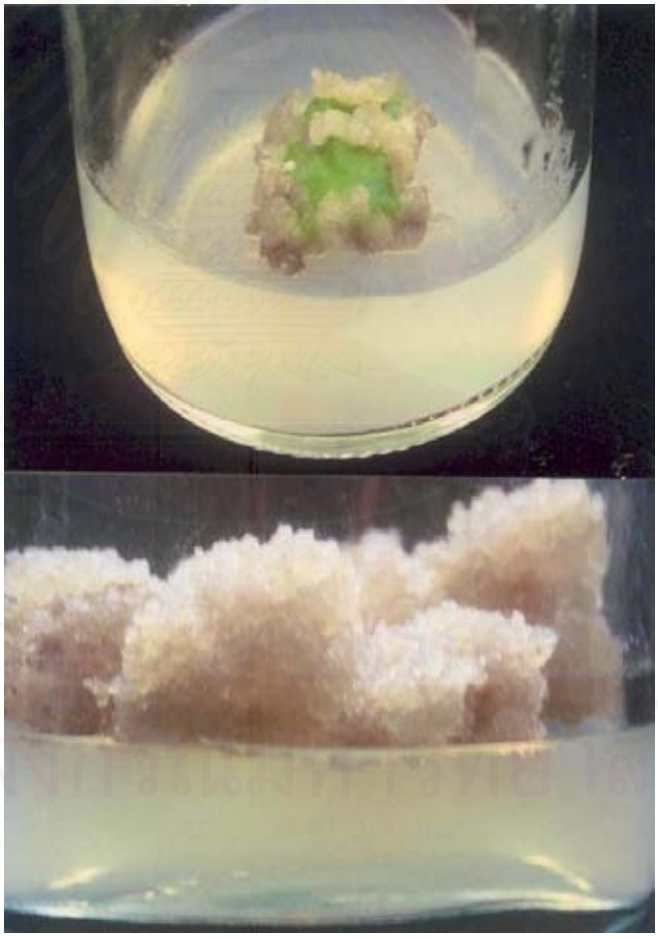


Figure 12 Distribution of plumbagin in the mature *P. zeylanica* plant

3.Tissue cultures of *P. zeylanica*

3.1 Establishment of callus cultures

Callus formation was induced successfully on LS medium supplemented with the combination of 30 g/l sucrose, 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.8% (w/v) agar. As shown in Fig. 13A, the calli were initiated at the top edge of leaf segments before enlarging to the bottom and formed as friable with pale gray color. These callus cultures could be maintained by using the same medium with regular subculturing for every three weeks. In this medium the callus had a high growth rate resulting in friable, soft and grayish tissues (Fig. 13B).



A

B

Figure 13A) Induction of callus from the leaf explants of *P. zeylanica* on LS agar medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D

B)The apparent friable, soft and grayish callus cultures in LS agar medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D

3.2 Establishment of cell suspension cultures

Cell suspension cultures of *P. zeylanica* were obtained from the friable grayish callus which were maintained by a regular subculturing as described earlier. The callus tissues were separated into small aggregates before transferring into LS liquid medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D and rotated at 120 r.p.m. on a rotary shaker. Under these conditions, however, the suspension cultures appeared a fast growth rate and formed small grayish aggregates (Fig. 14). After stable cell suspension cultures were obtained, it was maintained in the same medium by subculturing every two weeks.

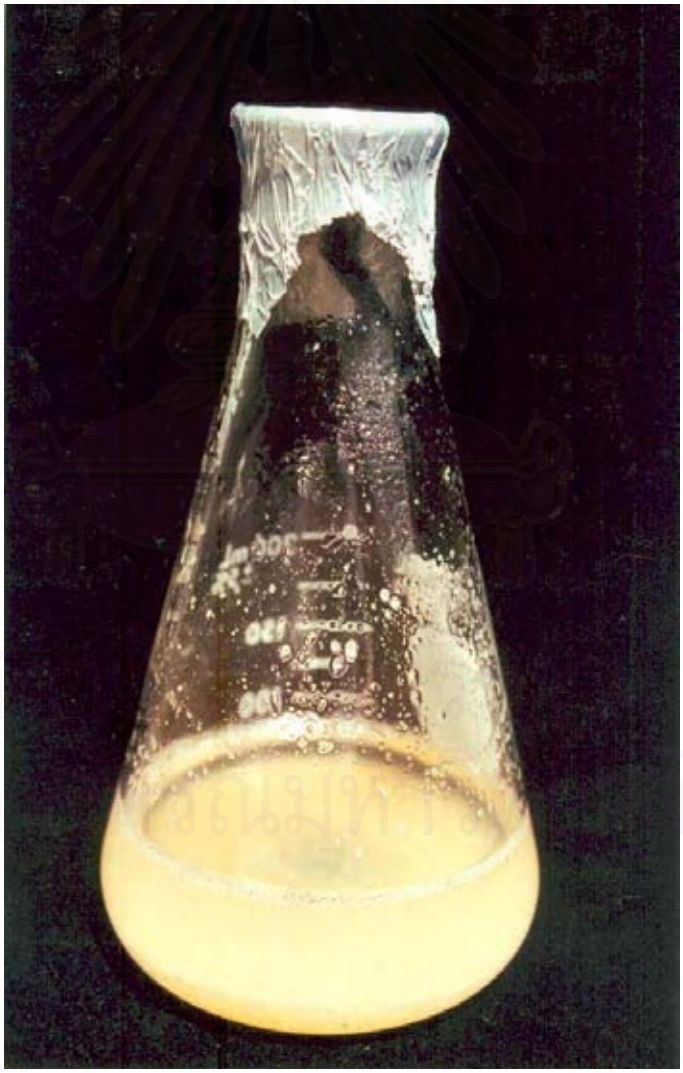


Figure 14 Cell suspension cultures of *P. zeylanica* maintained in LS liquid medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D

3.3 Effect of basal media and plumbagin production in cell suspension cultures

The effect of various culture media containing 30 g/l sucrose without growth regulators on growth and plumbagin content was examined after a culture period of 7days under light conditions. The media tested were LS, MS, B5, N and SH. The cell suspension cultures showed the fastest growth in MS medium, while slowest growth followed by SH, LS, N and B5 medium, respectively (Table 6). Plumbagin was detected in cell suspension cultures in all the culture media. The highest yield was found in MS medium (20.600 μg/g DW), while N medium showed the lowest plumbagin content (13.400 μg/g DW). On the other hand, MS medium was effective in promoting both cell growth and plumbagin content. Hence in this study, MS medium was chosen as the medium to under further investigations.

Table 6 Dry weight and plumbagin content obtained from various cultures media

Medium	Dry weight (g/flask)	Plumbagin (μg/g DW)
LS	1.090±0.045	19.600±0.400
MS	1.200±0.037	20.600±1.000
B5	0.883±0.048	13.800±0.600
N	0.907±0.015	13.400±0.200
SH	1.203±0.029	15.000±0.800

Each value represented the mean±SD of plumbagin in triplicate.

3.4 Effect of auxins

This experiment aimed to study four types of auxins, namely IAA,IBA, NAA and 2,4-D on the growth and plumbagin formation in *P.zeylanica* cell cultures. These auxins were examined at various concentrations (0.01-10.0 mg/l) in MS medium. As shown in Fig. 15, NAA and IBA at a wide range of concentrations gave higher culture growth than IAA and 2,4-D. The formers, on the other hand, resulted to low contents of plumbagin at all concentrations tested. Whereas both 2,4-D and IAA appeared to induce plumbagin formation with slightly superior for 2,4-D. The concentrations of 2,4-D from 0.01 to 1.0 showed no difference on the product formation although at 10.0 mg/l 2,4-D, plumbagin content was reduce significantly.

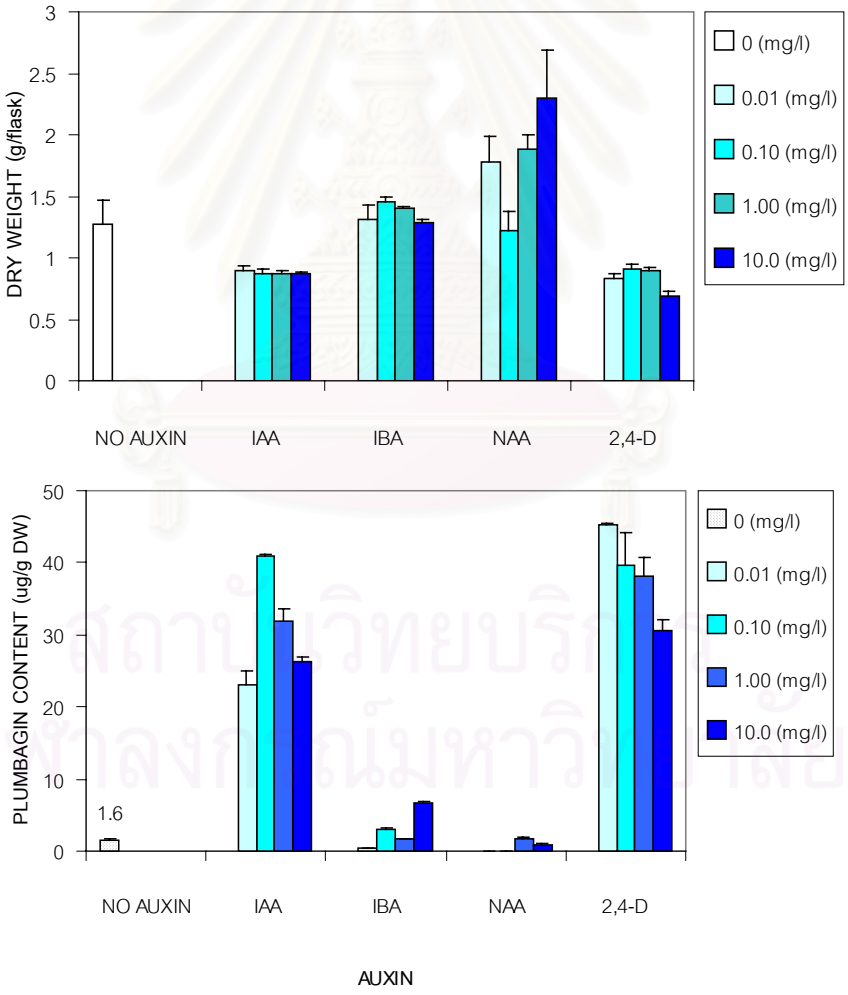


Figure 15 The effect of auxins on plumbagin production in cell suspension cultures

3.5 Effect of cytokinins

The effect of cytokinins on culture growth and plumbagin formation were also investigated. The two cytokinins chosen for this study were the commonly used BA and kinetin. The concentration range from 0.01 to 10 mg/l was also used. It was found that both cytokinins had no difference on inducing culture growth. For the plumbagin formation, BA showed slightly better although its concentrations gave variable values (Fig. 16). However, comparing to the auxin supplement, it was found that the degree of plumbagin induction by BA was about 10 fold lower than 2,4-D.

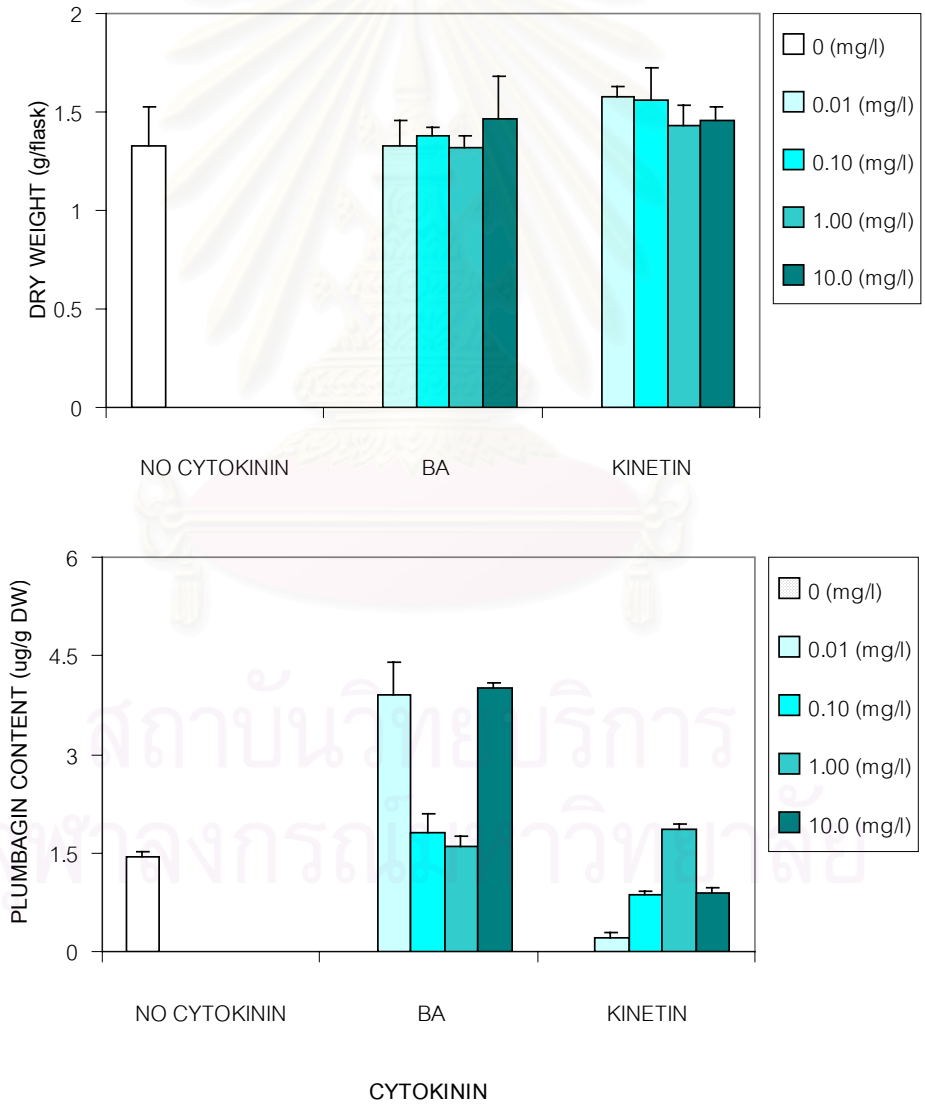


Figure 16 The effect of cytokinins on plumbagin production in cell suspension cultures.

3.6 Effect of carbon source

The effect of different carbon source at 3% concentration on growth and plumbagin formation in *P. zeylanica* is shown in Table 7. Among the four types tested of sucrose, glucose, sorbitol and manitol. Sucrose was found to give good growth followed by glucose, sorbitol and manitol. On the other hand, manitol affected the cell culture to form highest plumbagin content. Glucose showed slightly lower plumbagin content than manitol.

Table 7 The effect of carbon source on growth and plumbagin production in *P. zeylanica*

Carbon source (3%)	Dry weight (g/flask)	Plumbagin (μg/g DW)
Sucrose	1.45±0.09	10.00±3.00
Glucose	1.26±0.15	20.00±1.00
Sorbitol	0.96±0.02	8.00±0.00
Manitol	0.96±0.13	31.00±2.00

3.7 Time-courses of culture growth and plumbagin content in *P. zeylanica* cell suspension

Cell suspension cultures of *P. zeylanica* were regularly maintained in MS medium containing 0.01 mg/l BA, 0.1 mg/l 2,4-D and 30 g/l manitol. Monitoring of the growth and plumbagin content of the suspension culture under these conditions was performed. As shown in Fig. 17, it was found that *P. zeylanica* cell culture (0.35 g/flask) had a short lag phase of only one day after subculturing. After that the culture started to growth and increased in dry weight rapidly until day 9 when the maximum cell mass was observed (1.27 g/flask). Subsequently, the growth appeared to decline continuously until day 16 (0.7 g/flask) of the culture cycle. Thus the cell suspension showed a *ca.* four-fold increase in dry biomass during the culture cycle.

For the formation of plumbagin, the cell culture also appeared to start producing plumbagin after one day of subculturing. The content also

increased rapidly until day 6. After that the level of plumbagin was declined also rapidly until the end (day 16) on the culture cycle.

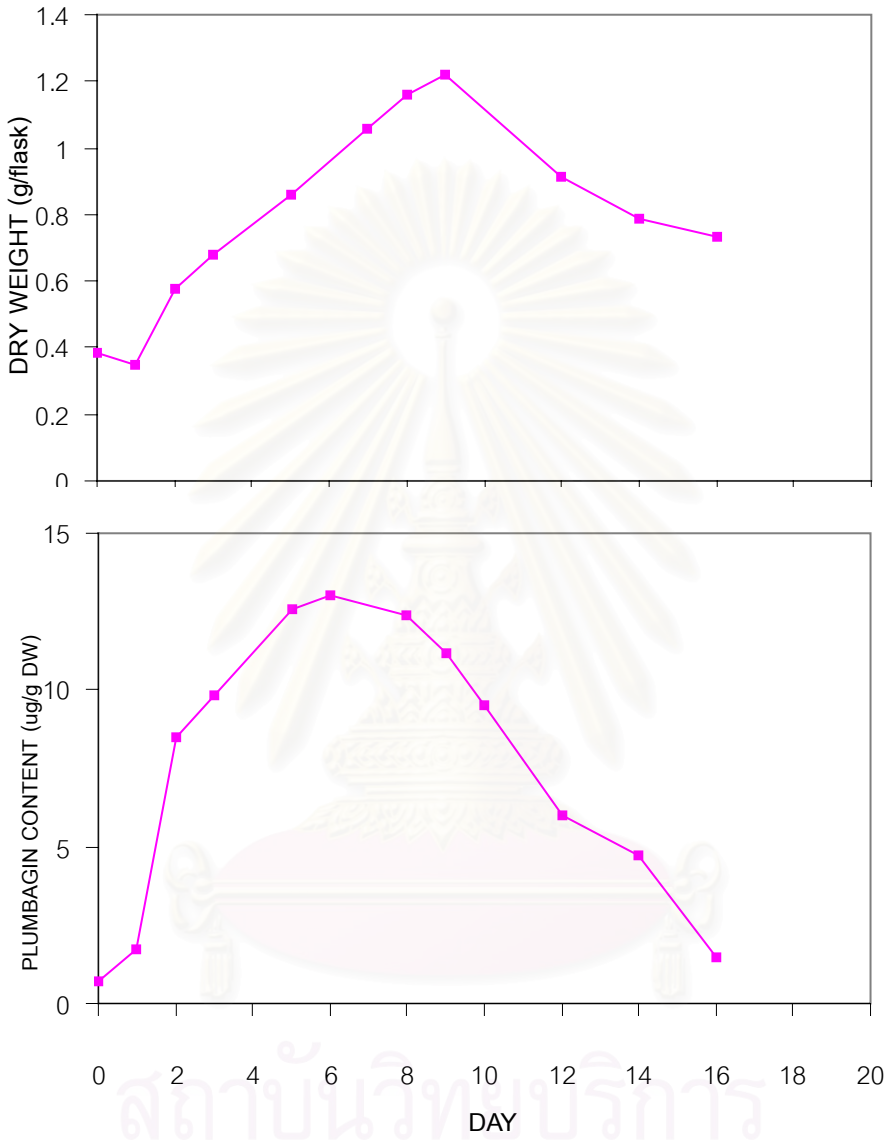


Figure 17 Time-courses of culture growth and plumbagin content in *P. zeylanica* cell suspension cultures

3.8 Production of plumbagin in hairy root cultures of *Plumbago indica* and *P. zeylanica* is shown in Table 8.

Table 8 Quantitative TLC densitometric analysis of plumbagin in hairy root cultures of *Plumbago indica* and *P. zeylanica*

Species	Plumbagin content (% w/w dry weight)
P. indica	0.170
P. zeylanica	0.090

CHAPTER V

DISCUSSION

1. Development of TLC densitometric method for the determination of plumbagin in various plant parts of *P. zeylanica*.

In principle, the screening for the plants with a desired characteristic from a plant population requires an analytical technique which is simple and allows a large number of samples be analyzed. For effectively evaluation a large number of various parts of *P. zeylanica*, it is necessary to have a simple, accurate and rapid method for estimation of their plumbagin content. So far, several methods for quantitative analysis of plumbagin has been reported to be determined by using high performance liquid chromatographic method (Gupta et al., 1993; Kitanov and Pashankov, 1994). This HPLC method is accurate and precise but usually requires steps of complicated sample preparation and partial purification of the crude extracts before quantitation. As a consequence, the overall procedure is still time consuming. In contrast, our method developed for the determination of plumbagin content is much simpler and time-saving TLC densitometric method for the quantitative analysis of plumbagin in various part of the *P. zeylanica*. The process of methanolic extraction allowed complete separation of plumbagin from other impurities present in the crude extract (Fig. 5A and Fig. 5B). In terms of precision and accuracy, the developed method was compared with HPLC method. The results showed that the TLC densitometry and the HPLC gave very similar value of plumbagin content in the same samples (Table 4 and Fig. 10) and also with narrow range in their of standard deviation (SD) (Table 4). For sensitivity, the calibration curve obtained from this method can quantitate plumbagin even in the concentration of as low as 0.0125 mg/ml and in a wide concentration range from 0.0125-0.1 mg/ml (Fig. 7). The developed TLC densitometric method was therefore used throughout this study for the determination of plumbagin in various plant parts and in callus of *P. zeylanica.*

2. Plumbagin content in various parts of *P. zeylanica* plant

In the analysis of various parts (roots, stems, leaves and flowers) of *P. zeylanica*, it could be concluded that the plant contained highly variable plumbagin content in various plant parts ranging from 0.002 to 0.226% (w/w) dry weight (Table 5, Fig.11 and Fig.12). These results appear to be similar to the distribution of colchicine in the mature *Gloriosa superba* L. (Kitcharoen and De-Eknamkul, 1993). The content of plumbagin in the roots appeared to be the highest followed by the stems, mature leaves, young leaves and flowers, respectively. These results clearly confirm previous report (Iyengar and Pendse, 1962) that the root is the main plant containing high content of plumbagin which is up to 0.23% (w/w) dry weight.

3. Tissue culture of *P. zeylanica*

The major aim of the establishment of *P. zeylanica* cell cultures is to study its potential in producing plumbagin. Plumbagin is a plant naphthoquinone presumably biosynthesized by the polyketide pathway. So far, very little is known about the enzyme polyketide synthase in this plant. Therefore, a successful induction of *P. zeylanica* cell culture to form plumbagin would lead to a possibility to study such an enzyme and to understand the biosynthetic pathway of plumbagin eventually. In this study, *in vitro* cultures of *P. zeylanica* can be established successfully in LS medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D. (Fig. 13A and Fig. 13B). The friable calli were subsequently used for establishing cell suspension cultures by transferring the tissues to the same medium. The cell cultures showed small aggregates with gray color (Fig. 14). Both callus and cell suspension cultures were maintained by regular subculturing. For this case of *P. zeylanica* cell cultures, we have shown that both hormonal and nutritional factors have an effect on plumbagin formation. The culture grows well and produce plumbagin in MS medium. On the other hand, NAA appears to clearly stimulate growth but diminish product formation whereas 2,4-D seems to have opposite effect. For the cytokinins, the effect of BA and kinetin also seems to have different degrees on plumbagin formation although the product

level is much lower than the 2,4-D containing medium. Therefore, as shown in many cases, the effects of these hormonal factors are usually unpredictable and cannot be generalized. It has been reported that plumbagin accumulation is observed in callus cultures of *Drosophyllum lusitanicum* growth on MS medium containing with 1.0 mg/l IBA and 0.5 mg/l NAA. (Nahalka et al., 1996). Charlwood and Rhodes (1990) explained that the dedifferentiation of plant tissue *in vitro* to produce callus is usually accompanied by a apparent loss of ability to accumulate secondary compound.

Nutrient media are based on the metabolites within the plant which are sufficient for the growth of explant tissues. The general composition of nutrient media consist of a salt mixture, an energy source (usually sucrose), certain amino acids, vitamins and growth factors, together with any supplementary compounds that are necessary for a particular species. (Sakuta and Komamine, 1987). Not only various plant species but also different cell types of the same species require a specified compositions of the nutrient medium. Therefore, concentrations of the medium components have to be frequently tested for each new system. We used other basal media for screening employed in plant cell cultivation. These media were compared from the standpoints of culture growth (DW) and production of plumbagin after a culture period of 7 days. The screening results that the cell suspension cultures showed that grows well and produce plumbagin in MS medium, with up 1.203 g/flask and 20.600 μg/g DW respectively (Table 6).

Comparing with the whole plant of *P. zeylanica*, it is obvious that plumbagin content in the cell cultures is still much lower than that found in the plant. Base on this work, plumbagin can be found in every part of the plant. The root part contains highest level which is up to 2.26 mg/g (or 0.226% dry weight).

The reason may be:

1) the lack of expression in non-specialized cells or genes that control the essential steps in the biosynthetic pathway.
2) the non-availability of storage sites in which secondary metabolites would normally be sequestered.
3) the non-operation of transport mechanisms by which potentially toxic end-products may be removed from the biosynthetic site.
4) the diversion of substrate away from secondary product formation
5) the unregulated catabolism of synthesized product.

For the ability to grow root cultures and the cell cultures of *P. zeylanica* produces plumbagin in relatively low amount, the biosynthetic pathway of plumbagin is still expressed. This allow us to use the cell culture to study the biosynthetic enzyme of this pathway. Therefore, it is likely that the cell culture of *P. zeylanica* producing plumbagin can be used for enzymological study of its biosynthetic pathway. This aspect of study should be continued.

CONCLUSION

From this research work of "Plumbagin production in cell suspension cultures of *Plumbago zeylanica* L." The following conclusions can be drawn :

1. Two types of the *in vitro* cultures of *P. zeylanica*, including callus and cell suspension cultures, have been obtained from this study.
2. Callus cultures of *P. zeylanica* can be established from the young leaf explants on LS medium containing 30 g/l sucrose, 0.2 mg/l NAA 0.2 mg/l 2,4-D and 0.8% (w/v) agar and subcultured on this medium.
3. Cell suspensions cultures of *P. zeylanica* can be established from the callus cultures. The suspension culture can be maintained on LS liquid medium containing with 30 g/l sucrose, 0.2 mg/l NAA and 0.2 mg/l 2,4-D on shaker at 120 r.p.m. The cell suspension culture can produce plumbagin.
4. MS medium appear to be effective in promoting both cell growth and plumbagin content.
5. NAA and IBA at a wide range of concentrations give higher culture growth than IAA and 2,4-D.
6. IAA appear to induce plumbagin formation with slightly superior for 2,4-D.
7. BA and kinetin show no difference on including culture growth.
8. The plumbagin formation, BA shows slightly better than kinetin.
9. Manitol appears to affect the cell culture to form highest plumbagin content.
10. The production of the plumbagin occurs mainly during the lag phase of the culture cycle.
11. The cell suspensions cultures can be used as a source for biosynthetic studies.
12. Various parts of *P. zeylanica* all contain plumbagin but in variable plumbagin content ranging from 0.002 to 0.226% (w/w) dry weight.
13. The content of plumbagin in the roots appears to be the highest (0.226%) followed by the stems (0.089%), mature leaves (0.010-0.030%), young leaves (0.014%) and flowers (0.002%) respectively.

REFERENCES

ภาษาไทย

กำจร มนุญปิจุ. 2526. **จำนวนผลงานวิจัยพรรณไม้ในประเทศไทย เล่ม** 1. กรุงเทพมหานคร: ภาควิชาเคมี วิทยาศาสตร์ มหาวิทยาลัยมหิดล

พเยาว์ เหมือนวงษ์ญาติ. 2529. **ตำราวิทยาศาสตร์สมุนไพร**. กรุงเทพมหานคร: สำนักพิมพ์ดอกหญ้า

วิทย์ เที่ยงบูรณธรรม. 2531. **พจนานุกรมสมุนไพรไทย**. กรุงเทพมหานคร: โอ. เอส. พริ้นติ้งเฮ้าส์

เสริมสิริ วินิจฉัยกุล. 2539. สมุนไพร... **ไม้พื้นบ้าน** (1). กรุงเทพมหานคร: บริษัทประชาชน จำกัด

ภาษาอังกฤษ

Abbott, T. P., Vaughn, S. F., Dowd, P. F., Mojtahedi, H., and Wilson, R. F. 1998. Potential uses of sicklepod (*Cassia obtusifolia*). **Industrial crops and products.** 8: 77-82.

Adeniyi, B. A., Fong, H. S., Pezzuto, J. M., Luyengi, L., and Odelola, H. A. 2000. Antibacterial activity of diospyrin, isodiospyrin and bisisodiospyrin from the root of *Diospyros piscatora* (Gurke) (Ebenaceae). **Phytotherapy research.** 14: 112-117.

Akitami, I., Makoto, U., and Sadao, S. 1980. Synthesis of plumbagin by the Retro-Diels-Alder reaction. **Agric. Biol. Chem.** 44: 211-213.

Alnuri, M. A., Hannoun, M. A., Zatar, N. A., Abueid, M. A., Aljondi, W. J., Hussein, A. I., and Alishtayeh, M. S. 1994. Plumbagin, a natrually-occuring naphthoquinone-its isolation spectrophotometric determination in roots, stems, and leaves in *Plumbago europaea* L. **Spectroscopy letters.** 27: 409-416.

Anonymous. 1989. **The wealth of india: A dictionary of indian raw materials and industrial products. Vol. II.** New Delhi: CSIR press.

Bhargava, S. K. 1984. Effect of plumbagin on reproductive function of male dog. **Indian journal of experimental biology.** 22: 153-156.

Bhargava, S. K., and Dixit, V. P. 1985. Antifertility effects of embelin and plumbagin in female rats. **Plantes medicinales et phytotherapie.** 19: 29-34.

Bobak, M., Blehova, A., Kristin, J., Ovecka, M., and Samaj, J. 1995. Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured in vitro. **Plant cell tissue and organ culture.** 43: 43-49.

Bor, N. L., and Raizada, M. B. 1990. **Some beautiful indian climbers and shrubs.** Bombay delhi calculta madras: Bombay natural history society oxford university press.

Bringmann, G., Rischer, H., Wohlfarth, M., Schlauer, J., and Assi, L. A. 2000. Droserone from cell cultures of *Triphyophyllum peltatum* (Dioncophyllaceae) and its biosynthetic origin. **Phytochemistry.** 53: 339-343.

Bringmann, G., Schlauer, J., Ruckert, M., Wiesen, B., Ehrenfeld, K., Proksch, P., and Czygan, F. C. 1999. Host-derived acetogenins involved in the incompatible parasitic relationship between *Cuscuta reflexa* (Convolvulaceae) and (*Ancistrocladus heyneanus*) (Ancistrocladaceae). **Plant biology.** 1: 581-584.

Budzianowski, J. 1996. Naphthohydroquinone glucosides of *Drosera rotundifolia* and *D. intermedia* from in vitro cultures. **Phytochemistry.** 42: 1145-1147.

Budzianowski, J. 2000. Naphthoquinone glucosides of *Drosera gigantea* from in vitro cultures. **Planta medica.** 66: 667-669.

Caniato, R., Filippini, R., and Cappelletti, E. M. 1989. Naphthoquinone contents of cultivated *Drosera* species *Drosera binata*, *D. binata* var. dichotoma, and *D. capensis*. **International journal of crude drug research.** 27: 129-136.

Chaleff, R. S. 1983. Isolation of agonomically useful mutants from plant cell cultures. **Science.** 219: 676-682.

Charlwood, V. B., and Rhodes, M. J. C. 1990. **Physiology of secondary product formation in plants.** Oxford: Charendon press.

Chockalingam, S., Thenmozhi, S., Nalina, S., and Sundari, M. 1990. Larvicidal activity of different products against mosquito larvae. **Journal of environmental biology.** 11: 101-104.

Crouch, I. J., Jinnie, J. F., and Staden, J. V. 1990. Studies on the isolation of plumbagin from in vitro and in vivo grown Drosera species. **Plant cell tissue and organ culture.** 21: 79-82.

Culham, A., and Gornall, R. J. 1994. The taxonomic significance of naphthoquinones in the Droseraceae. **Biochem. Sys. Ecol.** 22: 507-515.

Davey, M. R. 1983. Recent development in the culture and regeneration of plant protoplast. **Experientia Suppl.** 46: 19-20.

Devi, P. U., Solomon, F. E., and Sharada, A. C. 1999. Plumbagin, a plant naphthoquinone with antitumour and radiomodifying properties. **Pharmaceutical biology.** 37: 231-236.

Dhar, S. K., and Rao, P. G. 1995. Hormonal profile of plumbagin. **Fitoterapia.** 66: 442-446.

Didry, N., Dubreuil, L., Trotin, F., and Pinkas, M. 1998. Antimicrobial activity of aerial parts of *Drosera peltata* Smith on oral bacteria. **Journal of ethopharmacology.** 60: 91-96.

Didry, N., Dubrevil, L., and Pinkas, M. 1994. Activity of anthraquinonic and naphthoquinonic compounds on oral bacteria. **Pharmazie.** 49: 681-683.

Durand, R., and Zenk, M. H. 1971. Biosynthesis of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) via the acetate pathway in higher plants. **Tetrahedron Letters.** 32: 3009-3012.

Durga, R., Sridhar, P., and Polara, H. 1990. Effects of plumbagin on antibiotic resistance in bacteria. **Indian. J. Med. Res.** 91: 18-20.

Dwarakanath, C. 1987. **Medicinal plants of india. Vol. 2.** New Delhi: Cambridge printing.

Evans, P. H., Bowers, W. S., Litaudon, M., and Sevent, T. 1999. Plumbagin from *Diospyros olen*. **Molecules.** 4: 93.

Farnsworth, N. R. 1977. **The current importance of plants as a source of drugs.** In: Seigler Ds (ed) Crop resources. Academic press: New York.

Fetterer, R. H., Urban, J. F. Jr., and Miller, R. W. 1989. Effects of chitin synthase inhibitor diflubezuron on development of *Ascaris suum* and *Haemonchus contortus*. **Vet. Parasit.** 32: 181-189.

Fournet, A., Angelo, A., Munoz, V., Roblot, F., Hocquemiller, R., and Cave, A. 1992. Biological and chemical studies of *Pera benesis*, a Bolivan plant used in folk medicine as a treatment of cutaneous leishmaniasis. **J. Ethno. Pharmacol.** 37: 159-164.

Fournet, A., Angelo, B. A., Munoz, V., Hocquemiller, R., and Cave, A. 1992. Effect of natural naphthoquinones in BALB/C mice infected with *Leishmania amazonensis* and L. **Tropical medicine and parasitology.** 43: 219-222.

Francis, F. J. 1987. Lesser known food colorants. **Food Technol.** 41:62.

Fujii, N., Yamashita, Y., Arima, Y., Nagashima, M., and Nakano, H. 1992. Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. **Antimicrobial agents and chemotherapy.** 36: 2589-2594.

Galek, H., Osswald, W. F., and Elstner, E. F. 1990. Oxidative protein modification as predigestive mechanism of the carnivorous plant *Dionaea muscipula*: an hypothesis based on in vitro experiments. **Free. Radic. Biol. Med.** 9: 427-434.

Ganasoundari, A., Zare, S. M., Uma, D. P., and Devi, P. U. 1997. Modification of bone marrow radiosensitivity by medicinal plant extracts. **British journal of radiology.** 70: 834, 599-602.

Goncalves, O., Maciel, G. M., Oliveira, L. L., Lacerda, A. L., Moreira, L. C., Martins, D. G. 1972. Antimicrobial subtances from higher plants. **Revista do instituto de antibioticos.** 12: 1-2, 3-12.

Gujar, G. T., and Mehrotra, K. N. 1988. Toxicity and morphogenetic effects of plumbagin on *Dysdercus koenigii* F. (Het., Pyrrhocoridae). **Journal of applied entomology.** 105: 466-470.

Gunaherath G. K., Gunatilaka, A. L., and Thomson, R. H. 1984. Structure of plumbazeylanone: a novel trimer of plumbagin from *Plumbago zeylanica*. **Tetrahedrom letters.** 25: 4801-4804.

Gunaherath, G. K., Gunatilaka, A. L., Sultanbawa, M. S., and Balasubramaniam, S. 1983. 1,2(3)-Tetrahydro-3-3'-biplumbagin: a naphthalenone and constituents from *Plumbago zeylanica*. **Phytochemistry.** 22: 1245-1247.

Gunaherath, G. M., and Gunatilaka, A. A. L. 1984. Structure of plumbazeylanone: A novel trimer of plumbagin from *Plumbago zeylanica*. **Tetrahedron Letters.** 25: 4801-4804.

Gupta, A. P., Verma, R. K., Gupta, M. M., Sushi, K., and Kumar, S. 1999. Estimation of plumbagin using high performance thin layer chromatography. **Journal of medicinal and aromatic plant sciences.** 21: 661-663.

Gupta, A., and Singh, J. 1999. New naphthoquinone from *Plumbago zeylanica*. **Pharmaceutical biology.** 37: 321-323.

Gupta, A., Rai, R., Siddiqui, I. R., and Singh, J. 1998. Two new triterpenoids from *Plumbago zeylanica*. **Fitoterapia.** 69: 420-422.

Gupta, M. M., Verma, R. K., and Gupta, A. P. 1995. A chemical investigation of *Plumbago zeylanica*. **Current research on medicinal and aromatic plants.** 17: 161-164.

Gupta, M. M., Verma, R. K., Uniyal, G. C., and Jain, S. P. 1993. Determination of plumbagin by normal-phase high-performance liquid chromatography. **Journal of chromatography.** 637: 209-212.

Harborne, J. B. 1982. **Introduction of ecological biochemistry.** Second edition. New York: Academic press.

Heble, M. R., Narayanaswamy, S., and Chadha, M. S. 1974. Tissue differentiation and plumbagin synthesis in variant cell strains of *Plumbago zeylanicai* L. *in vitro*. **Plant science letters.** 2: 405-409.

Higa, M., Ogihara, K., and Yogi, S. 1998. Bioactive naphthoquinone derivatives from *Diospyros maritima* BLUME. **Chemical and pharmaceutical bulletin.** 46: 1189-1193.

Ito, Y., Hayashi, Y., and Kato, A. 1995. Antifungal compounds from trees of the genus Diospyros with complete assignment of nuclear magnetic resonance data. **Journal of the Japan wood research society.** 41: 694-698.

Iyengar, M. A., and Pendse, G. S. 1962. Pharmacognosy of the root of *Plumbago zeylanica* Linn. **Indian. J. Pharm.** 24: 290.

Jain, S. K., and arafder, C. R. 1970. Medicinal plant-lore of the *Santals*. **Econ. Bot.** 24: 241.

Jaya, S., Ramakrishna, S. V., and Kishor, P. K. 1999. Development of hairy root cultures of *Plumbago indica*. **Plant tissue culture and biotechnology.** 273-274.

Joshi, N. K., and Sehnal, F. 1989. Inhibition of ecdysteroid production by plumbagin in *Dydercus cingulatus*. **J. Insect. Physiol.** 35: 737-741.

Kavimani, S., Iiango, R., Madheswaran, M., Jayakar, B., Malaya, G., Majumdar, U. K., and Gupta, M. 1996. Antitumour activity of plumbagin against Dalton's ascitic lymphoma. **Indian journal of pharmaceutical sciences.** 58: 194-196.

Khan, R. M., and Timi, D. 1999. Constituents of *Diospyros lolin*, *D. maritima* and *D. novoguinensis*. **Fitoterapia.** 70: 194-196.

Khan, R. M., Rwekika, E. 1998. A binaphthoquinone from *Diospyros greeniwayi*. **Phytochemistry.** 49: 2501-2503.

Kini, D. P., Pandey, S., Shenoy, B. D., Singh, U. V., Udupa, N., Umadevi, P., Kamath, R., Ramanarayan, K. 1997. Antitumor and antifertility activities of plumbagin controlled release formulations. **Indian journal of experimental biology.** 35: 374-379.

Kirtikar, K. R., and Basu, B. D. 1935. **Indian medicinal plants. Vol. II.** New Delhi: Jayyed press.

Kitanov, G. M., and Pashankov, P. P. 1994. Quantitative investigation on the dynamics of plumbagin in *Plumbago europaea* L. roots and herb by HPLC. **Pharmazie.** 49: 462.

Kreher, B., Neszmelyi, A., and Wagner, H. 1990. Naphthoquinones from *Dionaea muscipula*. **Phytochemistry.** 29: 605-606.

Krenn, L., Blaeser, U., Hausknost, C. N. 1998. Determination of naphthoquinones in *Droserae herba* by reversed-phase high performance liquid chromatography. **Journal of liquid chromatography and related technologies.** 21: 3149-3160.

Krenn, L., Digruber, B., and Wawrosch, C. 1998. Influence of drying and storage on the naphthoquinone content of sundew herb. **Zeitschrift fur arznei and gewurzpflanzen.** 3: 3-4, 162-165.

Krishnaswamy, M., and Purushothaman, K. K. 1980. Plumbagin: a study of its anticancer, antibacterial and antifungal properties. **Indian journal of experimental biology.** 18: 876-877.

Kubo, I., Uchida, M., and Klocke, J. A. 1983. An insect ecdysis inhibitor from the African medicinal plant, *Plumbago capensis* (Plumbaginaceae); a naturally occurring chitin synthetase inhibitor. **Agricultural and biological chemistry.** 47: 911-913.

Kuke, C., Williamson, E. M., Roberts, M. F., Watt, R., Banasri, H., Lajubutu, B. A., Yang, S. L., Hara, B., and Yang, S. L. 1998. Antiinflammatory activity of binaphthoquinones from *Diospyros* species. **Phytotherapy research.** 12: 155-158.

Kuo, Y. H., Chang, C. I., Li, S. Y., Chou, C. J., Chen, C. F., Kuo, Y. H., and Lee, K. H. 1997. Cytotoxic constituents from the stems of *Diospyros maritima*. **Planta medica.** 63: 363-365.

Lajubutu, B. A., Pinney, R. J., Roberts, M. F., Odelola, H. A., and Oso, B. A. 1995. Antibacterial activity of diosquinone and plumbagin from the root *Diospyros mespiliformis* (Hostch). **Phytother. Res.** 9: 346-350.

Likhitwitayawuid, K., Kaewamatawong, R., Ruangrungsi, N., and rungkrai, J. 1998. Antimalarial naphthoquinones from *Nepenthes thorelii*. **Planta medica.** 64: 237-241.

Lima, O. G., Albuquerque, I. L., Maciel, G. M., and Maciel, M. C. 1968. Antimicrobial substances of superior plants. XXVII. Isolation of plumbagin from *Plumbago scandens* L. Rev. **Inst. Antibiot. Recife.** 8: 95-97.

Luckner, M., and Nover, L. 1977. **Expression of secondary metabolism. An aspect of cell specialzation of microorganisms, higher plants, and animals.** Secondary metabolism and cell differentiation. Berlin: Springer-Verlag.

Manitto, P., and Sammes, P. G. 1981. **Biosynthesis of natural products.** New York: Halsted press.

Marini-Bettolo, G. B. 1980. Present aspects of the use of plants in traditional medicine. **J. Ethnopharmacol.** 2: 5.

Marston, A., Msonthi, J. D., and Hostettmann, K. 1984. Naphthoquinones of *Diospyros usambarensis*; their molluscicidal and fungicidal activities. **Planta medica.** 50: 279-280.

Maryadele, J., and Smith, A. 1996. **The merck index: an encyclopedia of chemicals, drugs, and biologicals.** Whitehouse Siation, N. J. press.

Mitchell, M. J., and Smith, S. L. 1988. Effects of the chitin synthease inhibitor plumbagin and its 2-methyl derivative juglone on insect ecdysone 20-monooxygenase activity. **Experentia.** 44: 990-991.

Moshy, R. J. 1985. Impact of biotechnology on food product development. **Food Technol.** 39: 113.

Mukharya, D., and Dahia, M. S. 1977. Antimicrobial activity of some plant extracts. **Indian drugs.** 14: 160.

Nahalka, J., Blanarik, P., Gemeiner, P., Matusova, E., and Partlova, I. 1996. The chemical/osmotic conditions for growth and plumbagin accumulation of *Drodophyllum lusitanicum* Link. suspension cultures. **Biotechnology letters.** 18: 1453-1458.

Nahalka, J., Nahalkova, J., Gemeiner, P., and Blanarik, P. 1998. Elicitation of plumbagin by chitin and its release into the medium in *Drosophyllum lusitanicum* Link. **Biotechnology letters.** 20: 841-845.

Nair, A. G. R., Shanmugasundaram, P., and Madhusudhanan, K. P. 1990. Naphthaquinones and flavonols from leaves of *Drosera peltata*. **Fitoterapia.** 61: 85-86.

Nguyen, H. A., Ripperger, H., Porzel, A., Tran, V. S., and Adam, G. 1997. Tetralones from *Ancistrocladus cochinchinensis*. **Phytochemistry.** 44: 549-551.

Ogihara, K., Yamashiro, R., Higa, M., and Yogi, S. 1997. Preparation of naphthoquinone derivatives from plumbagin and their ichthytoxicity. **Chemical and pharmaceutical bullentin.** 45: 437-445.

Padhye, S. B., and Kulkarni, B. A. 1973. Studies on the root constituents of *Plumbago zeylanica*. **J. Univ. Poona.** 44: 27.

Pakulski, G., and Budzianowski, J. 1996. Ellagic acid derivatives and naphthoquinones of *Dionaea muscipula* from in vitro cultures. **Phytochemistry.** 41: 775-778.

Parimala, R. and Sachdanandam, P. 1993. Effects of plumbagin on some glucose metabolizing enzymes studied in rats in experimental hepatoma. **Mol. Cell. Biochem.** 125: 59-63.

Perica, M. C., and Berljak, J. 1996. In vitro growth and regeneration of *Drosera spatulata* Labill. on various media. **Hortscience.** 31: 1033-1034.

Petiard, V., and Steck, P. 1987. **Industrial application of plant cell culture metabolic production.** Berlin Heidelberg New York press.

Prasad, V. S., Devi, P. U., Rao, B. S., Ravindra, K., Kamath, R., Uma, D. P., and Bisht, K. S. 1996. Radiosensitizing effect of plumbagin on mouse melanoma cells grown in vitro. **Indian journal of experimental biology.** 34: 857-858.

Premakumari, P., Rathinam, K., and Santhakumari, G. 1977. Antifertility activity of plumbagin. **Indian. J. Med. Res.** 65: 829.

Rao, J. V., Sreenivasan, C., and Makkapati, A. K. 1996. Plumbagin effect on growth and metamorphis of housefly *Musca domestica* L. (Diptera: Muscidae). **International pest control.** 1: 24-27.

Rao, J. V., Sreenivasan, C., and Makkapati, A. K. 1996. Plumbagin effect on growth and metamorphosis of housefly *Musca domestica* L. (Diptera: Muscidae). **International pest control.** 38: 24-25.

Sakuta, W. D., and Komamine, A. 1987. **Cell growth and accumulation of secondary metabolites.** In F. Constabel and I. K. Vasil (eds.), Cell culture and somatic cell genetics of plants, pp. 97-110. New York: Academic press.

Sankaram, A. V. B., and Rao, A. S. 1979. Zeylanone and isozeylanone, two novel quinones from *Plumbago zeylanica*. **Phytochemistry.** 15: 237-238.

Sankaram, A. V. B., Srinivasarao, A., and Sidhu, G. S. 1976. Chitranone-a new binaphthaquinone from *plumbago zeylanica*. **Tetrahedron.** 35: 1777-1782.

Santhakumari, G., and Rathinam, K. 1978. Anticoagulant activity of plumbagin. **Indian. J. Exp. Biol.** 16: 485-487.

Santhakumari, G., Saralamma, P. G., and Radhakrishnan, N. 1980. Effect of plumbagin on cell growth and mitosis. **Indian. J. Exp. Biol.** 18: 215.

Saxena, B. P., and Tikku, K. 1990. Effect of plumbagin on haemocytes of *Dysdercus koenigii* F. **Proceedings of the indian academy of sciences animal sciences.** 99: 119-124.

Saxena, B. P., Thappa, R. K., Tikku, K., Sharma, A., and Suri, O. P. 1996. Effect of plumbagin on gonadotrophic cycle of the housefly, *Musca domestica* L. **Indian journal of experimental biology.** 34: 739-744.

Shcherbanovskii, L. R. 1985. Plumbagin- a new antimicrobial substance of plant origin. **Trudy gosudarstvennogo nikitskogo botanicheskogo sada.** 83: 7-23.

Sidhu, G. S., and Sankaram, A. V. B. 1971. A new biplumbagin and 3-chloroplumbagin from *Plumbago zeylanica*. **Tetrahedron Letters.** 26: 2385-2388.

Spears, K. 1988. Developments in food colourings: the natural alternatives. **TIBTECH.** 6: 283.

Thiboldeaux, R. L., Lindroth, R. L., and Tracy, J. W. 1994. Differential toxicity of juglone (5-hydroxy-1,4-naphthoquinone) and related naphthoquinones to saturniid. **Journal of chemical ecology.** 20: 1631-1641.

Tikku, K., Saxena, B. P., Satti, N. K., and Suri, K. A. 1992. Plumbagin induced ultrastructural haemocytic response of *Dysdercus koenigii* F. **Insect science and its application.** 13: 787-791.

Todorov, D. K., Iiarionova, M. V., Timcheva, K. B., and Pajeva, I. K. 1998. Antitumor activity of *Dionaea musciputa* E. preparation carnivora (R) new in vitro and in vivo, on animal and human tumors, sensitive and resistant to antitumor drugs. **Biotechnology and biotechnology equipment.** 12: 61-66.

Villavicencio, M. A., and Perez, B. E. 1992. Activity of plumbagin (from *Plumbago putchiella* Boiss: Plumbaginaceae) as an antifeedant in three species of Orthoptera. **Folia entomologica mexicana.** 86: 191-198.

Yue, J. M., Xu, J., Zhao, Y., Sun, H. D., and Lin, Z. W. 1997. Chemical components from *Ceratostigma willmottianum*. **Journal of natural products.** 60: 1031-1033.

Yue, J., Lin, Z., Wang, D., Feng, Y., and Sun, H. 1994. Plumbasides A-C three naphthoquinone derivatives from *Ceratostigma minus*. **Phytochemistry.** 35: 1023-1025.

Zenk, M. H., Furbringer, M., and Steglich, W. 1969. Occurrence and distribution of 7-methyljuglone and plumbagin in the droseraceae. **Phytochemistry.** 8: 2199-2200.

APPENDIX

PLANT TISSUE CULTURES TERMS

Apical dominance. The specific inhibition of axillary bud development excerted by the terminal (or apical) bud.

Aseptic. Absence of proliferating microorganisms such as fungi, bacteria, viruses in some cases, mycoplasms or other microorganisms in cultures.

Callus. An unorganized, proliferative mass of undifferentiated plant cells; a wound response (plural-calluses or calli).

Cell culture. This term is used to denote the growing of cells *in vitro* including the culture of single cells. In cell cultures, the cells are no longer organized into tissues.

Cell line. A cell line arises from a primary culture at the time of the first successful subculture. The term cell line implies that cultures from it consist of numerous lineages of cells originally present in the primary culture. The terms "finite" or "continuous" are used as prefixes if the status of the cultures is known. If not, the term "line" will suffice.

Chemically defined medium. A nutritive solution for culturing cells in which each component is of know chemical structure. Although it is recognized that even the "purest" chemical compounds may have some contaminants, high quality "Analar" chemicals should be used accompanied my their analytic data.

Clonal propagation. Asexual reproduction of plants that are considered to be genetically uniform and to have originated from a single mother or a single explant.

Differentiated. Cells existing in a state of cell development characterized by a large central vacuole occupying up to ninety percent of the cell volume, the nucleus is to one side and the cell is specialized is function.

Embryo culture. *In vitro* development or maintenance of isolated mature or immature embryos (either zygotic or somatic).

Embryogenesis. The process of zygotic/somatic embryo initiation and development.

Explant. The starting plant material, taken from its original site and transferred to and artificial medium for growth or maintenance.

Friability. A term indicating the tendency for plant cells to separate from one another.

Induction. Initiation of a plant structure, organ, or a process which occurs under either *in vitro* other conditions.

***In vitro*.** Conditions of growth in a tissue culture vessel; an artificial or simulated conditions (literally means "in glass").

***In vitro* propagation.** Propagation of plants in a controlled artificial environment, using plastic or glass culture vessels, aseptic techniques, and a defined culture medium.

***In vivo*.** Conditions of growth, which occur under natural conditions in the field or greenhouse.

Meristem culture. *In vitro* of a generally shiny dome-like structure measuring less than 0.1 mm in length when excised, most often excised from the shoot apex.

Meristemming. A popular, not in scientific usage, referring to the *in vitro* clonal propagation of plants from various explant sources including shoot tips, leaf sections and calli; shoot apical meristems are seldom used.

Micropropagation. This terum is synonymous with *in vitro* propagation.

Morphogenesis.

a) The process of growth and development of differentiated structures.
b) The evolution of a structure from an undifferentiated to a differentiated state.

Organ culture. The maintenance and growth of organ primodia or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function.

Passage. The transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and, therefore, dilution of cells, whether deliberate or not, may occur. Passage is synonymous with the term "subculture".

Plant tissue culture. The growth or maintenance of plant cells, tissues, organs, or whole plants *in vitro*.

Protoplast. A plant cell from which the outer retaining cellulose cell wall has been removed either by mechanical or enzymic means.

Semi-solid. A gelled medium, usually achieved using agar or agarose. (An agar medium is not correctly described as solid).

Shoot apical meristem. Undifferentiated tissues, located within the shoot tip, generally appearing as a shiny dome-like structure distal to the youngest leaf primodium and measuring< 0.5 mm in length when excised.

Shoot tip (apex) culture. A structure consisting of the shoot apical meristem plus one to several primodial leaves, usually measuring from 0.1-1.0 mm in length; in instances where more mature leaves are included the structure can measure up to several centimeters high in length.

Somaclonal variation. A broad term used to describe the variability in phenotype observed in the regenerated cell lines or plants from cultured tissue. The basis of such variation is considered to be the result of either gene mutations or changes in gene expression or a combination of both which occurs at various stages of the tissue culture process particularly during callus development and tissue dedifferentiation.

Somatic embryo. A vegetative embryo derived by mitotic divisions of totipotent cells in the absence of fertilization.

Table 9 Compositions of plant tissue culture media

Constituent	**LS**	**MS**	**B5**	**N**	**SH**
Macronutrients					
(mg/l)					
$(NH_4)_2SO_4$	-	-	134	-	-
NH_4NO_3	1,650	1,650	-	720	-
KNO_3	1,900	1,900	2,500	950	2,500
$CaCl_2.2H_2O$	435	435	150	166	200
$MgSO_4.7H_2O$	370	370	250	185	400
KH_2PO_4	170	170	-	68	-
$NaH_2PO_4.H_2O$	-	-	150	-	-
Na_2EDTA	37.3	37.3	-	37.35	15
$FeSO_4.7\ H_2O$	27.8	27.8	-	27.85	20
Micronutrients					
(mg/l)					
$MnSO_4.4H_2O$	16.9	16.9	10	18.95	10
$ZnSO_4.7H_2O$	8.6	8.6	2	10	1
H_3BO_3	6.2	6.2	3	10	5
KI	0.75	0.75	0.75	-	1
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25	0.1
$CuSo_4.5H_2O$	0.025	0.025	0.025	0.025	0.2
$CoCl_2.6\ H_2O$	0.025	0.025	0.025	-	0.1
Vitamins (mg/l)					
Thiamine HCl	0.4	0.8	10	0.5	5
Nicotinic acid	-	-	1	0.5	5
Pyridoxin HCl	-	-	1	0.5	0.5
Folic acid	-	-	-	0.5	-
Biotin	-	-	-	0.05	-
Other (mg/l)					
(iso-) or myo-inositol	100	1,000	100	2.0	1,000
Glycine	-	-	-	5.0	-
Sucrose	30,000	30,000	20,000	20,000	30,000

Abbreviations: LS = Linsmaier and Skoog, 1965
MS = Murashige and Skoog, 1962
B5 = Gamborg and Eveleigh, 1970
N = Nitsch et al., 1968
SH = Schenk and Hildebran, 1972

Medium preparation

Various stock solutions of LS, MS, B5, N, SH and plant growth regulators were prepared at the concentrations show in Table 10.

The culture media were then prepared by mixing the stock solutions and added sucrose into the distilled water, as described in Table 11. The pH of each medium was adjusted to its desired value with 0.1 N potassium hydroxide or 0.1 N hydrochloric acid. The media were solidified with 0.8% (w/v) agar (Difco, Detroit Michigan, USA) for solid media. The media were sterilized by autoclaving at 121°C (15 lb/in^2) for 15-20 minutes. Liquid media were also prepared similarly but agar was omitted.

Table 10 Preparation of stock solution of LS MS B5 N and SH

	LS	MS	B5	N	SH
Stock1 (Macronutrients) g/1000 ml	Stock1 (Macronutrients) g/1000 ml	Stock1 (Macronutrients) g/1000 ml	Stock1 (Macronutrients) g/1000 ml	Stock1 (Macronutrients) mg/250 ml	Stock1 (Macronutrients) mg/250 ml
NH_4NO_3	33	33	-	-	-
KNO_3	38	38	50	9500	50
$MgSO_4.7H_2O$	7.4	7.4	5	1850	8
KH_2PO_4	3.4	3.4	-	-	-
$NaH_2PO_4.H_2O$	-	-	3	-	-
$(NH_4)_2SO_4$	-	-	2.68	-	-
KH_2PO_4	-	-	-	680	-
NH_4NO_3	-	-	-	7200	-
$NH_4H_2PO_4$	-	-	-		6
Stock2 (Micronutrients) mg/100 ml	Stock2 (Micronutrients) mg/100 ml	Stock2 (Micronutrients) mg/100 ml	Stock2 (Micronutrients) mg/100 ml	Stock2 (Micronutrients) mg/100 ml	Stock2 (Micronutrients) mg/100 ml
H_3BO_3	620	620	300	100	500
$MnSO_4.4H_2O$	-	-	-	250	
$MnSO_4.H_2O$	1,690	1,690	1,000	-	1,000
$ZnSO_4.7H_2O$	860	860	200	100	100
$Na_2MoO_4.2H_2O$	25	25	25	2.5	10
$CuSO_4.5H_2O$	2.5	2.5	2.5	0.25	20
$CoCl_2.6H_2O$	2.5	2.5	2.5	-	10
Stock3 (Ca stock) g/100 ml	Stock3 (Ca stock) g/100 ml	Stock3 (Ca stock) g/100 ml	Stock3 (Ca stock) g/100 ml	Stock3 (Ca stock) mg/250 ml	Stock3 (Ca stock) mg/250 ml
$CaCl_2.2H_2O$	8.7	8.7	3	1660	4
Stock4 (KI stock) mg/100 ml	Stock4 (KI stock) mg/100 ml	Stock4 (KI stock) mg/100 ml	Stock4 (KI stock) mg/100 ml	-	Stock4 (KI stock) mg/100 ml
KI	75	75	75		100
Stock5 (Vitamins) mg/100 ml	Stock5 (Vitamins) mg/100 ml	Stock5 (Vitamins) mg/100 ml	Stock5 (Vitamins) mg/100 ml	Stock5 (Vitamins) mg/50 ml	Stock5 (Vitamins) mg/100 ml
Thiamine HCl	4	8	1,000	10	50
i-Inositol	1,000	10,000	10,000	40	10,000
Nicotinic acid	-	-	100	10	50
Pyridoxine HCl	-	-	100	10	5
Glycine	-	-	-	100	-
Folic acid	-	-	-	10	-
Biotin	-	-	-	1	-
Stock6 (Fe-EDTA stock) g/500 ml	Stock5 (Vitamins) mg/100 ml	Stock5 (Vitamins) mg/100 ml	Stock5 (Vitamins) mg/100 ml	Stock6 (Fe-EDTA stock) mg/100 ml	Stock6 (Fe-EDTA stock) mg/100 ml
Na_2EDTA	3.73	3.73	3.73	373.5	150
$Fe_2SO_4.7H_2O$	2.78	2.78	2.78	278.5	200

Table 11 Preparation of LS MS B5 N and SH

	LS	MS	B5	N	SH
Distilled water	1,000 ml	1,000 ml	1,000 ml	1,000 ml	1,000 ml
Stock 1	50 ml	50 ml	50 ml	25	50
Stock 2	1 ml	1 ml	1 ml	10 ml	1 ml
Stock 3	5 ml	5 ml	5 ml	25 ml	5 ml
Stock 4	1 ml	1 ml	1 ml		1 ml
Stock 5	10 ml	10 ml	1 ml	2.5 ml	10 ml
Stock 6	5 ml	5 ml	5 ml	10 ml	10 ml
Sucrose	30 g	30 g	20 g	20 g	30 g
Final pH	5.6	5.8	5.5	5.6	5.6

Auxin (100 mg/l) as needed
Cytokinin (100 mg/l) as needed
Agar (solid medium) 8 g

VITA

Miss Orasa Choosakul was born on December 18, 1975 in Bangkok, Thailand. She received her Bachelor of Science in Agriculture (second class honors) in 1996 from Kasetsart University, Bangkok, Thailand.