Research Report

Screening of antibacterial activity of herbal extracts against *Propionibacterium acnes* and establishment of standardization method

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ABSTRACT

Ethyl acetate and methanol extracts of eighteen Thai medicinal plants were investigated for their antibacterial activity against *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Thirteen plant extracts were capable of inhibiting the growth of *P. acnes* and *S. epidermidis*, while fourteen plant extracts exhibited inhibitory effect on *S. aureus*. Based on the broth dilution method, the ethyl acetate extract of *Plumbago indica* L. roots showed the strongest antibacterial effect against *P. acnes*, *S. aureus* and *S. epidermidis*, with minimum inhibitory concentration (MIC) values of 4.9, 312.5 and 2.4 µg/ml, respectively, and minimum bactericidal concentration (MBC) values of 39.1, 312.5 and 78.1 µg/ml, respectively. Three naphthoquinones including plumbagin, 3,3'-biplumbagin and elliptinone were isolated from the roots of *Plumbago indica* L. and used as standard naphthoquinones for determination and preparation of the naphthoquinone-rich *P. indica* root extract. The reversed-phase high-performance liquid chromatographic method was developed for quantification of plumbagin, 3,3'-biplumbagin and elliptinone in the root extract. The method involved the use of a Phenomenex® ODS column (150 × 4.6 mm, 5 µm particle size) with the mixture of methanol and 5% v/v aqueous acetic acid (80:20, v/v) as the mobile phase, flow rate 0.85 ml/min, and peaks were detected at 260 nm. The parameters of linearity, precision, accuracy, and limit of detection and quantification of the method were evaluated. The recovery of the method was 98.5 – 100.6% and good linearity ($R^2 > 0.9990$) was obtained for all naphthoquinones. The low limit of detection and quantification, high degree of specificity as well as intraday and interday precision (% R.S.D. was less than 5%) were also achieved. Fractionation of ethanol extract using a vacuum silica gel chromatography eluted with a mixture of hexane and ethyl acetate (9.2:0.8, v/v) afforded a naphthoquinone-rich *P. indica* root extract. The total content of naphthoquinones was increased from 0.58 mg/g to 138.58 mg/g of the extract. For antibacterial activity against acne-involved bacteria, including *Propionibacterium acnes* (DMST 14916, 21823 and 21824), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 14990), plumbagin exhibited the strongest antibacterial activity with the minimum inhibitory concentration (MIC) values of 0.39,

12.50, 12.50, 3.12 and 0.024 μg/ml and the minimum bactericidal concentration (MBC) values of 25, 50, 50, 12.50 and 3.12 μg/ml, respectively. However, the naphthoquinone-rich *P. indica* root extract showed closely activity to plumbagin which MIC values of 1.56, 12.50, 12.50, 12.50 and 0.78 μg/ml and MBC values of 50, 50, 50, 25 and 6.25 μg/ml, respectively. In addition, the antimicrobial activity of the naphthoquinone-rich extract was also much more potent than the *P. indica* ethanol extract. Solubility evaluation of the naphthoquinone-rich extract in various solvents found that the extract was freely soluble in chloroform, ethyl acetate, and ethanol, slightly soluble in methanol and propylene glycol, very slightly soluble in hexane and practically insoluble in water. The naphthoquinone-rich extract exhibited good stability when kept in well-sealed closed containers protected from light and stored in cool place (4°C).

บทคัดย่อ

จากการทดสอบฤทธิ์ต้านเชื้อแบคทีเรีย *Propionibacterium acnes*, *Staphylococcus aureus*, และ *Staphylococcus epidermidis* ของสารสกัดสมุนไพรไทย18 ชนิด ด้วย ethyl acetate และ methanol พบว่ามีสารสกัด 13 ชนิดที่สามารถยับยั้งการเจริญของเชื้อ *P. acnes* และ *S. epidermidis* ได้ ในขณะที่มีสารสกัด 14 ชนิดสามารถยับยั้งการเจริญของเชื้อ *S. aureus* ได้ และจากการทดสอบด้วยเทคนิค broth dilution method พบว่าสารสกัดรากเจตมูลเพลิงแดง ด้วย ethyl acetate มีฤทธิ์ยับยั้งเชื้อ *P. acnes, S. aureus* และ *S. epidermidis* ได้ดีที่สุด ด้วยค่า minimum inhibitory concentration (MIC) เท่ากับ 4.9, 312.5 and 2.4 μg/ml และค่า minimum bactericidal concentration (MBC) เท่ากับ 39.1, 312.5 and 78.1 μg/ml ตามลำดับ สารประกอบแนพโธควิโนน 3 ชนิด ได้แก่ plumbagin, 3,3'-biplumbagin และ elliptinone ซึ่งแยกได้จากสารสกัดรากเจตมูลเพลิงแดง ถูกแยกเพื่อนำมาใช้เป็นสารมาตรฐานในการวิเคราะห์ปริมาณสารแนพโธควิโนน และในการเตรียมสารสกัดรากเจตมูลเพลิงแดงที่มีสารแนพโธควิโนนในปริมาณสูง วิธีวิเคราะห์ปริมาณสารประกอบแนพโธควิโนนในสารสกัดรากเจตมูลเพลิงแดงด้วยเทคนิค reverse phase high-performance liquid chromatography ได้ถูกพัฒนาขึ้น โดยใช้คอลัมน์ชนิด Phenomenex® ODS column (150 × 4.6 mm, 5 μm particle size) กับ โมบายเฟสซึ่งเป็นตัวทำละลายผสมระหว่าง methanol และ 5% aqueous acetic acid ในอัตราส่วน 80:20 v/v โดยกำหนดอัตราการไหลไว้ที่ 0.85 ml/min และตรวจวัดสัญญาณที่ความยาวคลื่น 260 nm การประเมินความถูกต้องของวิธีวิเคราะห์ (method validation) ในหัวข้อ linearity, precision, accuracy และ limit of detection and quantification (LOD และ LOQ) พบว่า % recovery ของการวิเคราะห์สารทั้ง 3 ชนิด อยู่ในช่วง 98.5 - 100.6% และกราฟมาตรฐานของสารประกอบแนพโธควิโนนทั้ง 3 ชนิด มี linearity ที่ดี โดยมีค่า R^2 มากกว่า 0.9990 นอกจากนี้ ระบบดังกล่าวยังมีค่า LOD และ LOQ ที่ค่อนข้างต่ำ และระบบดังกล่าวยังมีความจำเพาะเจาะจง รวมถึงความเที่ยงสูง (ค่า % R.S.D. ทั้ง intraday และ interday precision น้อยกว่า 5%) การแยกสารสกัดรากเจตมูลเพลิงแดงด้วย ethanol ให้บริสุทธิ์ขึ้นโดยใช้ vacuum silica gel chromatography ชะด้วยสารละลายผสมของ hexane และ ethyl acetate (9.2:0.8, v/v) ทำให้ได้สารสกัดรากเจตมูลเพลิงแดงที่มีสารแนพโธควิโนนในปริมาณสูง โดยสารสกัดที่ได้มีปริมาณสารแนพโธควิโนนรวมเพิ่มขึ้นจาก 5.80 mg/g เป็น 138.58 mg/g ในการทดสอบฤทธิ์ต้านเชื้อแบคทีเรียที่ก่อให้เกิดสิว 3 ชนิด คือ *Propionibacterium acnes* (DMST 14916, 21823 and 21824), *Staphylococcus aureus* (ATCC 25923) และ *Staphylococcus*

epidermidis (ATCC 14990) พบว่า สาร plumbagin มีฤทธิ์ดีที่สุดในการยับยั้งเชื้อทั้ง 3 ชนิดโดยมีค่า minimum inhibitory concentration (MIC) เท่ากับ 0.39, 12.50, 12.50, 3.12 และ 0.024 μg/ml และค่า minimum bactericidal concentration (MBC) เท่ากับ 25, 50, 50, 12.50 และ 3.12 μg/ml ตามลำดับ อย่างไรก็ตามสารสกัดรากเจตมูลเพลิงแดงที่มีสารแนพโธควิโนนในปริมาณสูงมีฤทธิ์ใกล้เคียงกับสาร plumbagin โดยมีค่า MIC เท่ากับ 1.56, 12.50, 12.50, 12.50 และ 0.78 μg/ml และ มีค่า MBC เท่ากับ 50, 50, 50, 25 และ 6.25 μg/ml ตามลำดับ และยังมีฤทธิ์ดีขึ้นกว่าสารสกัดหยาบด้วย ethanol ค่อนข้างมาก การประเมินค่าการละลายของสารสกัดในตัวทำละลายต่างๆ พบว่า สารสกัดสามารถละลายได้ดีใน chloroform, ethyl acetate และ ethanol ละลายได้น้อยใน methanol และ propylene glycol ละลายได้น้อยมากใน hexane แต่ไม่ละลายในน้ำ นอกจากนั้น การทดสอบความคงตัวของสารสกัด พบว่าสารสกัดมีความคงตัวดีตลอดระยะเวลา 4 เดือน เมื่อเก็บในภาชนะปิดสนิทป้องกันแสง และที่ 4 องศาเซลเซียส

INTRODUCTION

Acne vulgaris is a skin disorder of the pilosebaceous units, which most numerous on the face and upper back. It is characterized by open and closed comedones (blackheads and whiteheads) and inflammatory lesions including papules, pustules and nodules (Strauss *et al.*, 2007). Although, acnes is a common disease in adolescents and does not harmful for human life, it may cause permanent scar and lead to significant emotional distress. (National Institute of Arthritis and Musculoskeletal and Skin Diseases, 2006).

Although mechanism of acne has never been clearly proven, it is believed that adrenal androgens stimulate the lipid production of sebaceous glands and produce a more rapid turnover of follicular epithelium. Later, it becomes more cohesive. Thus the cells adhere to one another and form the follicular impaction known as the microcomedo, the precursor of all other acne lesions (Shalita, 2004). The follicles can then be colonized by some skin normal flora bacteria include *Propionibacterium acnes, Staphylococcus aureus* and *S. epidermidis* (Baron, 1996). *P. acnes* has been described as the most important organism for development of inflammatory acne, which produces a variety of enzyme, including lipase and protease to hydrolyze sebum and release inflammatory compounds to stimulate immunological response (e.g., mast cells degranulation and neutrophil chemotaxis) resulting in inflammatory acne (Serena *et al.*, 2006).

The current therapies of acne vulgaris are uses of topical comedolytic (retinoid and derivative, benzoyl peroxide, azaleic acid), and antibiotics (doxycycline, tetracycline, minocycline, clindamycin, and erythromycin). They are used both oral and topical administration (Krautheim, 2004). Focusing on the topical use, retinoid and its derivatives including tretinoid, isotretinoin, adapalene, tazarotene, motretinide, retinoyl β-glucuronide and retinaldehyde are an effective therapy. Their mechanism is suppressing the development of new microcomedones, inhibition of inflammatory reactions *via* inhibition of prostaglandins, leukotrienes and proinflammatory cytokines such as interferon gamma and IL-1α releasing. Benzoyl peroxide exhibits strong antimicrobial, slight anti-inflammatory and anticomedogenic effects. Azaleic acid possesses

chemotaxis suppression, anti-inflammation, modify epidermal keratinization, and antibacterial properties against both aerobic and anaerobic bacteria (Krautheim, 2004).

While the risk of antibiotic resistant was increased in prevalence within the dermatologic setting (Swanson, 2003), the topical comedolytic drugs also have several side effects such as skin irritation, abnormal skin pigmentation, skin burning, skin dryness, peeling and photo sensitivity (Russell, 2000). Thus, the new sources of anti acne drugs have been investigated.

Currently, medicinal plants become extensively sources for study and research on active compounds against several bacterial strains (Chomnawong *et al.*, 2005). We recently studied on antibacterial activity of 20 medicinal plants, including *Allium sativum* L. (Alliaceae), *Arcangelisia flava* (L.) Merr. (Menispermaceae), *Azadirachta indica* A. Juss. (Meliaceae), *Cassia fistula* L. (Fabaceae), *Cassia siamea* Lam. (Fabaceae), *Eugenia cumini* (L.) Druce. (Myrtaceae), *Eupatorium odoratum* L. (Asteraceae), *Gynura pseudochina* (L.) DC. (Compositae), *Impatiens balsamina* L. (Balsaminaceae), *Mimusops elengi* L. (Sapotaceae), *Morinda citrifolia* L. (Rubiaceae), *Muntingia calabura* L. (Muntingiaceae), *Nelumbo nucifera* Gaertn. (Nelumbonaceae), *Phyllanthus emblica* L. (Phyllanthaceae), *Plumbago indica* L. (Plumbaginaceae), *Psidium guajava* L. (Myrtaceae), *Punica granatum* L. (Lythraceae), *Quercus infectoria* Oliv. (Fagaceae), *Rhinacanthus nasutus* L. (Acanthaceae) and *Uncaria gambia* Roxb. (Rubiacea) against acne involved bacteria, *P. acnes, S. aureus* and *S. epidermidis*. The result showed that crude ethyl acetate extract of *P. indica* root exhibited the strongest antibacterial activity against *P. acnes*, *S. aureus* and *S. epidermidis*, with minimum inhibitory concentration (MIC) values of 4.9, 312.5 and 2.4 μg/ml, respectively, and minimum bactericidal concentration (MBC) values of 39.1, 312.5 and 78.1 μg/ml, respectively (Table 1 - Table 3) (Kaewbumrung and Panichayupakaranant, 2008). *P. indica* was therefore selected for this study.

Table 1 Inhibition zone of the plant extracts

Medicinal plants	Inhibition zone (mm)					
	P. acnes		*S. aureus*		*S. epidermidis*	
	EtOAc ext.	MeOH ext.	EtOAc ext.	MeOH ext.	EtOAc ext.	MeOH ext.
A. sativum	19.1 ± 0.24	n.i.	9.1 ± 0.57	n.i.	12.0 ± 0.24	n.i.
A. indica var. *indica*	n.i.	n.i.	n.i.	n.i.	n.i.	11.0 ± 0.20
C. fistula	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
D. membranacea	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
E. odoratum	21.8 ± 1.68	8.3 ± 2.10	10.8 ± 0.42	n.i.	8.7 ± 0.43	n.i.
G. pseudochina var. *hispida*	n.i.	7.5 ± 0.35	n.i.	n.i.	n.i.	n.i.
I. balsamina	n.i.	n.i.	n.i.	9.3 ± 0.36	n.i.	n.i.
M. elengi	n.i.	n.i.	n.i.	16.6 ± 0.18	n.i.	22.6 ± 0.11
M. alba	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
M. calabura	n.i.	n.i.	n.i.	13.0 ± 0.18	n.i.	13.6 ± 0.23
M. citrifolia	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
N. nucifera	n.i.	n.i.	n.i.	11.0 ± 0.14	n.i.	12.9 ± 0.15
P. emblica	17.4 ± 0.24	18.7 ± 3.17	12.4 ± 0.36	14.6 ± 0.08	13.4 ± 0.15	18.1 ± 0.32
P. granatum	n.i.	11.0 ± 0.71	n.i.	17.4 ± 0.13	n.i.	n.i.
P. guajava	n.i.	15.1 ± 1.27	8.0 ± 0.19	13.8 ± 0.10	n.i.	15.5 ± 0.22
P. indica	56.2 ± 1.93	13.4 ± 1.09	12.0 ± 0.14	n.i.	13.4 ± 0.15	n.i.
S. siamea	n.i.	8.4 ± 0.27	n.i.	n.i.	n.i.	n.i.
S. cumini	17.9 ± 1.47	16.9 ± 1.10	12.1 ± 0.36	16.3 ± 0.18	13.1 ± 0.32	18.7 ± 0.22
Tetracycline	60.2 ± 0.65		26.7 ± 0.45		30.6 ± 0.54	

n.i. = no inhibition zone

Table 2 Minimum inhibitory concentration of the herbal extracts

Medicinal plants	Minimum inhibitory concentration (μg/ml)					
	P. acnes		*S. aureus*		*S. epidermidis*	
	EtOAc ext.	MeOH ext.	EtOAc ext.	MeOH ext.	EtOAc ext.	MeOH ext.
A. sativum	312.0	-	78.0	-	19.5	-
A. indica var. *indica*	-	-	-	-	-	1.25×10^{3}
E. odoratum	312.0	5.0×10^{3}	625.0	-	625.0	-
G. pseudochina var. *hispida*	-	1.25×10^{3}	-	-	-	-
I. balsamina	-	-	-	2.5×10^{3}	-	-
M. elengi	-	-	-	625.0	-	312.0
M. calabura	-	-	-	2.5×10^{3}	-	2.5×10^{3}
N. nucifera	-	-	-	1.25×10^{3}	-	625.0
P. guajava	-	625.0	625.0	312.0	-	312.0
P. emblica	2.5×10^{3}	312.0	312.0	312.0	312.0	312.0
P. granatum	-	156.0	-	625.0	-	-
P. indica	4.9	625.00	312.5	-	2.4	-
S. siamea	-	1.0×10^{4}	-	-	-	-
S. cumini	312.0	1.25×10^{3}	312.0	625.0	156.0	312.0
Tetracycline	0.15		0.30		0.04	

- = not perform due to no inhibition zone

Table 3 Minimum bactericidal concentration of the herbal extracts

Medicinal plants	Minimum bactericidal concentration (µg/mL)					
	P. acnes		*S. aureus*		*S. epidermidis*	
	EtOAc ext.	MeOH ext.	EtOAc ext.	MeOH ext.	EtOAc ext.	MeOH ext.
A. sativum	625.0	-	312.0	-	78.0	-
A. indica var. *indica*	-	-	-	-	-	1.25×10^{3}
E. odoratum	625.0	5.0×10^{3}	1.25×10^{3}	-	1.25×10^{3}	-
G. pseudochina var. *hispida*	-	1.0×10^{4}	-	-	-	-
I. balsamina	-	-	-	5.0×10^{3}	-	-
M. elengi	-	-	-	625.0	-	625.0
M. calabura	-	-	-	2.5×10^{3}	-	2.5×10^{3}
N. nucifera	-	-	-	1.25×10^{3}	-	625.0
P. guajava	-	1.25×10^{3}	625.0	625.0	-	625.0
P. emblica	5.0×10^{3}	625.0	625.0	312.0	625.0	312.0
P. granatum	-	1.25×10^{3}	-	1.25×10^{3}	-	-
P. indica	39.1	1.2×10^{3}	312.5	-	78.1	-
S. siamea	-	$>10^{4}$	-	-	-	-
S. cumini	312.0	1.25×10^{3}	1.25×10^{3}	625.0	1.25×10^{3}	312.0
Tetracycline	4.9		9.7		4.9	

- = not perform due to no inhibition zone

Plumbagin, a well known naphthoquinone isolated from the roots of *P. indica*, is an active compound against several bacterial strains (Mallavadhani *et al.*, 2002). In this study, plumbagin and its derivatives are therefore used as the standard markers for establishment of HPLC quantitative analysis of naphthoquinones in *P. indica* extract and preparation of naphthoquinone-rich *P. indica* root extracts. Solubility and stability of the

naphthoquinone-rich *P. indica* root extracts were also determined. The objectives of this study are as follows:

1) To isolate naphthoquinones from *P. indica* roots and evaluate their antibacterial activity against *P. acnes, S. aureus* and *S. epidermidis*
2) To develop the HPLC analytical method for simultaneous determination of naphthoquinones in *P. indica* root extracts
3) To prepare naphthoquinone-rich *P. indica* root extracts and investigate their properties

LITERATURE REVIEW

Medicinal plants have been studied for a long time to evaluate and search for their pharmaceutical activities and active compounds. Especially in Thailand, which is rich in ethnobotanical knowledge and many plants have been used for traditional medicines. But, there are a few researches, which study on antibacterial activity against *P. acnes.*

There was a study on anti-*P. acnes* activity of 19 medicinal plants including, *Andrographis paniculata* Nees. (Acanthaceae), *Azadirachta indica* A. Juss. (Meliaceae), *Barleria lupulina* Lindl. (Acanthaceae), *Carthamus tinctorius* L. (Asteraceae), *Centella asiatica* (L.) Urban. (Mackinlayaceae), *Clinacanthus nutans* (Burm. f.) Lindau. (Acanthaceae), *Cymbopogon citratus* (DC.) Stapf. (Graminae), *Eupatorium odoratum* L. (Asteraceae), *Garcinia mangostana* L. (Clusiaceae), *Hibiscus sabdariffa* L. (Malvaceae), *Houttuynia cordata* Thunb. (Saururaceae), *Lawsonia inermis* L. (Lythraceae), *Lycopersicon esculentum* L. (Solanaceae), *Murdannia loriformis* Hassk. (Commelinaceae), *Psidium guajava* L. (Myrtaceae), *Senna alata* (L.) Roxb. (Fabaceae), *Senna occidentalis* L. (Fabaceae), *Senna siamea* (Lam.) Irwin&Barneby (Fabaceae) and *Tagetes erecta* L. (Compositae). It was found that *G. mangostana* fruit peel extract exhibited the strongest antibacterial activity against *P. acnes* with the same MIC and MBC values of 39 µg/ml and the active compound was mangostin (Chomnawang *et al.*, 2005).

Niyomkam (2006) has reported on a screening of antibacterial activity against *P. acnes* of 18 Thai medicinal plants including, *Alpinia galangal* (L.) Willd. (Zingiberaceae), *Andrographis paniculata* Nees. (Acanthaceae), *Azadirachta indica* A. Juss. (Meliaceae), *Boesenbergia pandurata* (Roxb.) Holtt. (Zingiberaceae), *Centella asiatica* (L.) Urban. (Mackinlayaceae), *Cinnamomum verum J. Presl.* (Lauraceae), *Cymbopogon citratus* (DC.) Stapf. (Graminae), *Dioscorea membranacea* Pierre. (Dioscoreaceae), *Morus alba* L. (Moraceae), *Ocimum americanum* L. (Lamiaceae), *Ocimum sanctum* L. (Lamiaceae), *Piper betle* L. (Piperaceae), *Plumbago zeylanica* L. (Plumbaginaceae), *Punica granatum* L. (Lythraceae), *Rhinacanthus nasutus* L. (Acanthaceae), *Syzygium aromaticum* (L.) Merrill & Perry (Myrtaceae), *Senna alata* (L.) Roxb. (Fabaceae) and *Zingiber officinalis*

Roscoe. (Zingiberaceae). The result demonstrated that crude ethyl acetate extract of *A. galanga* rhizome exhibited the strongest activity against *P. acnes* with MIC and MBC values of 156 and 312 μg/ml, respectively with the active compound was 1'-acetoxychavicol acetate (Prisana Niyomkam, 2006).

Plumbago is a genus in Plumbaginaceae family. It's characterized by herbs perennial or rarely annual, rarely shrubs. Stems are usually branched and growing to 0.5-2 m tall. The leaves are spirally arranged, simple, entire, 0.5-12 cm long, with a tapered base and often with a hairy margin. The flowers are white, blue, purple, red, or pink, with a tubular corolla with five petal-like lobes; they are produced in racemes. The flower calyx has glandular hairs, which secrete sticky mucilage that is capable of trapping and killing insects. The ovary is ellipsoid, ovoid, or pyriform. There are about 25 species around the world, native to warm temperate to tropical regions, but 2 species including *Plumbago indica* L. and *Plumbago zeylanica* L. were found in Thailand (Schlauer, 1997; Schmelzer and Gurib-Fakim, 2008).

***Plumbago indica* L.**

Scientific name: *Plumbago indica* L. (Figure 1)

Family name: Plumbaginaceae

Synonym: *Plumbago rosea* L.

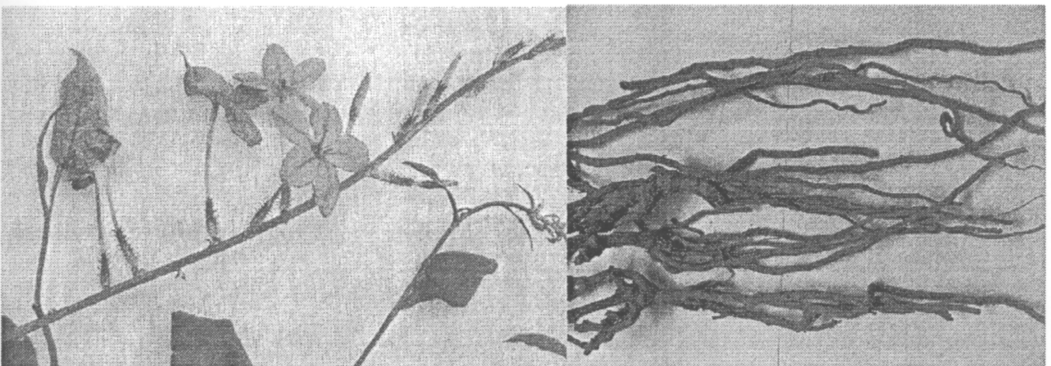

Figure 1 *Plumbago indica* L.

P. indica is a shrubby and evergreen plant, which frequently grows to the height of 0.5 – 2 m. Petiole base with-out auricles; leaf blade narrowly ovate to elliptic-ovate, papery, base rounded to obtuse, apex acute. Inflorescences 35–90 flowered; peduncle 1–3 cm, not glandular; rachis 10–40cm, not glandular; bracts ovate, 2–3 × 1.5–2 mm, apex acuminate; bractlets obovate-elliptic to ovate, 2–2.5 × 1.5–2 mm, apex acute. Flowers heterostylous. Calyx 7.5–9.5 mm, glandular almost throughout, tube is 2 mm in diameter at middle. Corolla red to dark red, tube is 2–2.5 cm, apex rounded and mucronate. Anthers blue, 1.5–2 mm. Ovary ellipsoid-ovoid, indistinctly angular. Style basally pilose; short-styled forms with style arms partly exserted, stigmatic glands without enlarged apex; long-styled form with style arms completely exserted from corolla throat, stigmatic glands capitates (คณะอนุกรรมการจัดทำตำราอ้างอิงยาสมุนไพรไทย, 2551; Schmelzer and Gurib-Fakim, 2008).

Medicinal properties of *Plumbago indica*

In Thai traditional medicine, *P. indica* roots were used for gastric stimulant, flatulence, hemorrhoid, appetizer and adaptation of uterus after delivered. In large doses, it is acro-narcotic poison. Locally, it is used for wound healing, tinea versicolor and ringworm (คณะอนุกรรมการจัดทำตำราอ้างอิงยาสมุนไพรไทย, 2551).

In eastern Africa and India *P. indica* was traditionally used for gastric stimulant, abortifacient and oral contraceptive. An infusion of roots is taken to treat dyspepsia, colic, cough and bronchitis. A liniment made from bruised root mixed with a little vegetable oil was used as a rubefacient to treat rheumatism and headache (Schmelzer and Gurib-Fakim, 2008).

Chemical constituents of *Plumbago indica*

The root of *P. indica* contains naphthoquinone plumbagin (2-methyl juglone). Other compounds isolated from the aerial parts include isoshinanolone, 6-hydroxy plumbagin (Figure 2), plumbaginol (Figure 3), leucodelphinidin (Figure 4), and steroids (e.g. campesterol, sitosterol and stigmasterol) (Figure 5) (Schmelzer and Gurib-Fakim, 2008).

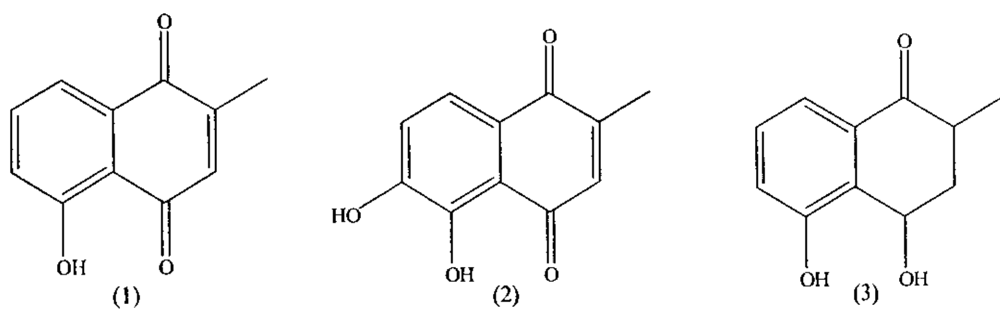

Figure 2 Chemical structures of naphthoquinone from *P. indica*: plumbagin (1), 6-hydroxy plumbagin (2) and isoshinanolone (3)

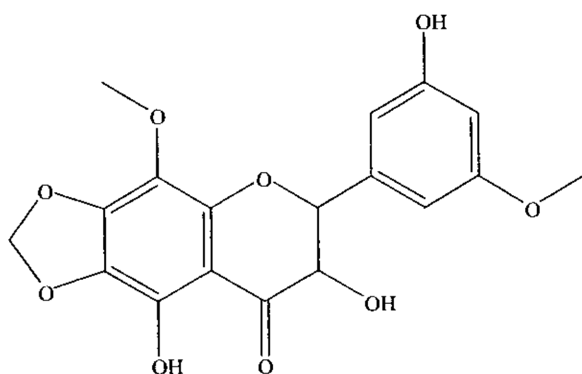

Figure 3 Chemical structures of plumbaginol

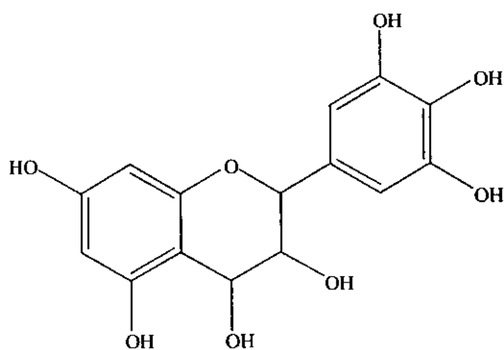

Figure 4 Chemical structures of leucodelphinidin

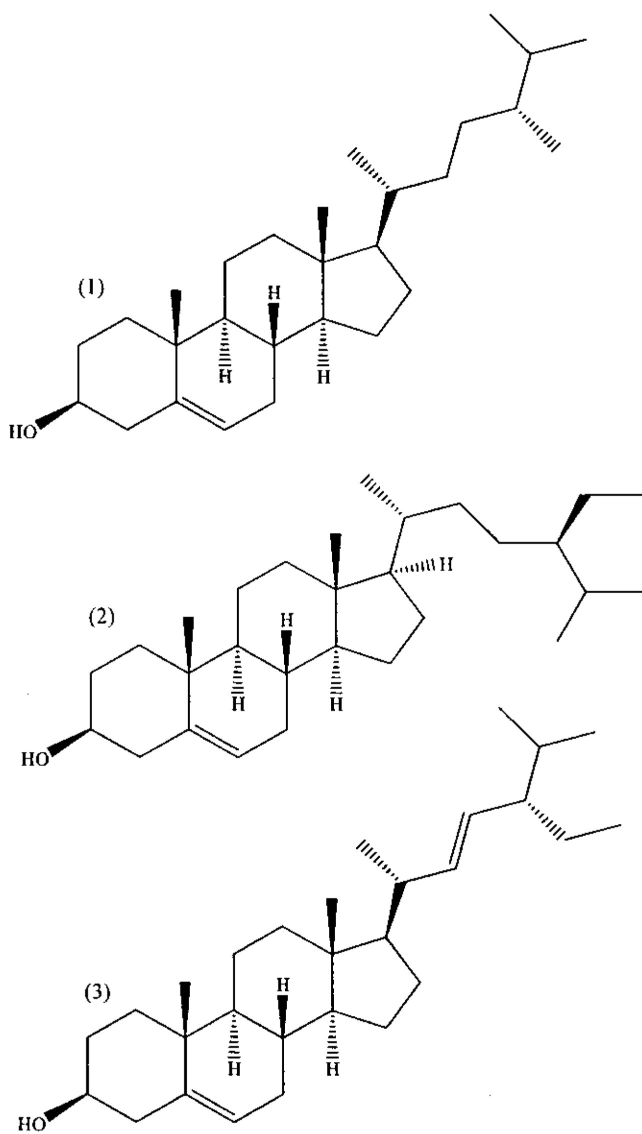

Figure 5 Chemical structures of steroids from *P. indica*: campesterol (1), sitosterol (2) and stigmasterol (3)

Pharmacological properties of *Plumbago indica*

1. Antifungal activity

Hydroalcoholic (80% ethanol) extract of *P. indica* roots possessed potent antifungal activity against *Aspergillus niger* and *Candida albicans* (Valsaraj *et al.*, 1997). In addition, plumbagin had been reported as the active compound against *C. albicans* with MIC and MFC (Minimum fungicidal concentration) values of 0.78 and 1.56 µg/ml, respectively (Figueiredo *et al.*, 2003).

2. Antibacterial activity

Hydroalcoholic (80% ethanol) extract of *P. indica* roots exhibited antibacterial activity against *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *S. aureus* with MIC values of 6.25 mg/ml for *B. subtilis* and 12.5 mg/ml for *P. aeruginosa, E. coli* and *S. aureus* (Valsaraj *et al.*, 1997). Moreover, plumbagin had been reported as the active compound against *S. aureus* with MIC and MBC values of 1.56 and 25.0 µg/ml, respectively (Figueiredo *et al.*, 2003).

3. Antiparasite activity

P. indica roots extract showed a macrofilaricidal property against *Setaria digitata*, a filarial parasite of cattle. Complete inhibition of motility was observed at concentrations range between 0.02 and 0.05 mg/ml. Fractionation of the crude extract resulted in the isolation of the active molecule plumbagin (Paiva *et al.*, 2003).

3. Antifertility activity

Acetone extract of *P. indica* stems exhibited activity in interrupting the normal estrous cycle of female Albino rats at two dose levels, 200 and 400 mg/kg. The rats exhibited prolonged diestrous stage of the estrous cycle with consequent temporary inhibition of ovulation. The anti-ovulatory activity was reversible on withdrawal of the extract. The effective acetone extract was further studied on estrogenic functionality in rats. The acetone extract showed significant estrogenic and anti-estrogenic activity. Histological studies of the uteri further confirmed the estrogenic activity of the acetone extract (Sheeja *et al.*, 2009).

Pharmacological properties of plumbagin

Plumbagin, the most active naphthoquinone derived from the species of *Plumbago, Drosera* and *Diospyros*, has been wildly studied on pharmacological activities. In small doses, it is a sudorific and stimulates the central nervous system, while in large doses may cause death from respiratory failure and paralysis. The pharmacological activities of plumbagin have been reported as follows:

1. Antitumor activity

Plumbagin exhibited anticancer activity against melanoma cells line (Bowes cell) and breast cancer cells line (MCF7) with IC_{50} values of 1.39 and 1.28 μM, respectively (Nguyen *et al.*, 2004).

For breast cancer cells, plumbagin exhibited cell proliferation inhibition by inducing cells to undergo G_2-M arrest and autophagic cell death. Blockade of the cell cycle was associated with increased p21/WAF1 expression and Chk2 activation, and reduced amounts of cyclin B1, cyclin A, Cdc2, and Cdc25C. Plumbagin also reduced Cdc2 function by increasing the association of p21/WAF1/Cdc2 complex and the levels of inactivated phospho-Cdc2 and phospho-Cdc25C by Chk2 activation (Kuo *et al.*, 2006).

Anticancer effect of plumbagin had been reported against human non-small cell lung cancer cells A549 with IC_{50} value of 11.69 μM. It exhibited effective cell growth inhibition by inducing cancer cells to undergo G_2-M phase arrest and apoptosis. Blockade of cell cycle was associated with increased levels of p21 and reduced amounts of cyclinB1, Cdc2, and Cdc25C. Plumbagin treatment also enhanced the levels of inactivated phosphorylated Cdc2 and Cdc25C. Blockade of p53 activity by dominant-negative p53 transfection partially decreased plumbagin-induced apoptosis and G_2-M arrest, suggesting it might be operated by p53- dependent and independent pathway. Plumbagin treatment triggered the mitochondrial apoptotic pathway indicated by a change in Bax/Bcl-2 ratios, resulting in mitochondrial membrane potential loss, cytochrome-c release, and caspase-9 activation (Hsu *et al.*, 2006).

2. Anti-inflammatory activity

Plumbagin exhibited the immunomodulatory effects by inhibition of T cell proliferation in response to polyclonal mitogen Concanavalin A (Con A) by blocking cell cycle progression (IC_{50} value of 50 nM). It also suppressed expression of early and late activation markers CD69 and CD25, respectively in activated T cells. The inhibition of T cell proliferation by plumbagin was accompanied by a decrease in the levels of Con An induced IL-2, IL-4, IL-6 and IFN-γ cytokines (Checker *et al.*, 2009).

3. Antimalarial activity

It has been reported that plumbagin possessed anti-*Plasmodium falciparum* activity by inhibition of isolated *P. falciparum* enzyme, succinate dehydrogenase (SDH), with IC_{50} value of 5 mM. It also inhibited *in vitro* growth of *P. falciparum* with IC_{50} value of 0.27 mM (Paiva *et al.*, 2003).

4. Antibacterial activity

Plumbagin has been reported as an Anti-*Helicobacter pylori* agent with MIC value of 4.0 µg/ml, which more potent than that of metronidazole (MIC value of 32 µg/ml) (Park *et al.*, 2006).

Farr and coworker (1985) reported the antibacterial activity of plumbagin against wild-type *E. coli* strain AB1157 with 99.9% killed by exposure to 1.0 mM plumbagin for 1 hour at 37°C. Antibacterial mechanism of plumbagin may be due to its toxicity by generated active oxygen species and may damage DNA besides a pathway *via* H_2O_2.

In contrast, Jamieson and coworkers (1994) conducted tests in wild-type strain *Saccharomyces cerevisiae* S150-2B and mutated strains using disruption mutations in the genes encoding of two superoxide dismutases, Cu/ZnSOD (*SOD*1) and mitochondrial MnSOD (*SOD*2), and showed that the *SOD*1 mutant was 100-fold more sensitive to plumbagin than its parent, while the sensitivity of the *SOD*2 strain to plumbagin was indistinguisable from that of the wild-type strain. Thus, Cu/ZnSOD was the prinicipal superoxide dismutating genes target.

Kamal et al. (1995) conducted *in vivo* anti-*S. aureus* test in female mice and showed that plumbagin was noticed to increase in the activity up to 8 weeks with 25 µg/kg body weight, due to its ability to stimulate the response on oxygen radical

release by macrophages. While at high dose (50 μg/kg body weight), it has direct inhibitory activity against *S. aureus.*

5. Antimutagenic activity

Plumbagin showed antimutagenic activity on *Salmonella typhimurium* TA98 when induced by 2-nitrofluorene (2NF), 3-nitrofluoranthene (3-NFA) and 1-nitropyrene (1-NP) (Edenharder and Tang, 1997). Moreover, for *Escherichia coli* WP2s (*uvrA trpE*), plumbagin was not mutagenic when presence of plasmid pKM101 (Kato *et al.*, 1994).

6. Antifertility activity

Plumbagin containing albumin microspheres were implanted to 20 days pregnant albino rats and found that their ovaries showed clear inhibition of growth of graffian follicules and degeneration of the mature follicles, and corpus luteum were observed and result to failed to conceive, the antifertility action of plumbagin seemed to be related to its anti-ovulatory action (Kini *et al.*, 1997).

7. Abortifacient activity

Plumbagin administered by intubation to albino female rats at 10 mg/kg for 15 days significantly inhibited mating and prolonged duration of estrus cycle and diestrous phase. Plumbagin showed a dose-related abortifacient activity in rats administered 5-20 mg/kg orally from Day 5 to 11 of pregnancy. At doses 10-20 mg/kg from days 1 to 5 of pregnancy, plumbagin caused a significant anti-implantation effect. No gross teratogenic effects were noticed in pups born to female rats that had received 5 or 10 mg/kg plumbagin from days 1 to 5 of pregnancy (Premakumari *et al.*, 1977).

8. Reproductive toxicity

Plumbagin has demonstrated reproductive toxicity in male and female animals. Teratogenic effects were not seen in limited studies. Only one of 12 female Long-Evans rats intubated with plumbagin at 10 mg/kg for 10 days conceived, bearing a litter of five pups. All 12 control animals conceived, producing an average litter size of six pups. One animal in the plumbagin group died of hemorrhage that the authors suspected was caused by competitive inhibition of vitamin K activity, needed for the synthesis of clotting factors (Azad Chowdhury *et al.*, 1982).

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Plumbagin given orally at 10 mg/kg for 10 days to adult female rats of the Holtzman strain caused a highly significant decrease in the weight of ovaries as compared with the controls (Santhakumari and Suganthan, 1980).

Plumbagin administered intra-peritoneal at a dose of 10 mg/kg for 60 days caused selective testicular lesions in dogs. The wet weights of testes and epididymides were decreased. In addition, the seminiferous tubule and Leydig cell nuclei diameter were significantly decreased and cellular heights of epididymides were drastically curtailed (Bhargava, 1984).

Oral administration of plumbagin to male gerbils at 10 mg/day for 20 days caused a decrease in the wet weight of seminal vesicle and prostate glands. The cell height of the secretory epithelium was also decreased, and little secretion in the lumen of these glands was observed (Bhargava, 1984).

Plumbagin caused a decrease in the number of spermatids, resting and pachytene spermatocytes, and a significant reduction in seminiferous tubule and Leydig cell nuclei diameter when given orally to immature Wistar rats at 10 mg/kg for 32 days (Bhargava, 1986).

9. Cardiotonic action

Plumbagin produced a triphasic inotropic response in guinea-pig papillary muscle. Plumbagin did not cause any positive inotropy under anoxic conditions, and the positive inotropic effect was markedly inhibited by oxidative phosphorylation uncouplers (Itoigawa *et al.*, 1991).

10. Hypolipidemic and antiatherosclerotic effects

When administered to hyperlipidaemic rabbits, plumbagin reduced serum cholesterol and LDL cholesterol by 53 to 86 percent and 61 to 91 percent, respectively. Furthermore, plumbagin treatment prevented the accumulation of cholesterol and triglycerides in liver and aorta and regress atheromatous plaques of the thoracic and abdominal aortas (Sharma *et al.*, 1991).

11. Effects on microsomal enzymes

Plumbagin exhibited a potent, dose dependent inhibitory activity against aromatase cytochrome P450 in human placental microsomes. However, plumbagin showed relatively weak reducing effects in the presence of microsomal membranes, suggesting that the inhibitory effects on monooxygenase reactions were not due to the formation of superoxide radicals (Muto *et al.*, 1987).

MATERIALS AND METHODS

Plant material

The dried roots of *P. indica* (Figure 6) were purchased from an herbal drug store in Hat-Yai, Thailand, in 2008. The roots were identified by comparison with the herbarium specimen (specimen no. SKP 148 16 09 01) that deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The roots were then reduced to powder using a grinder and a sieve no. 45.

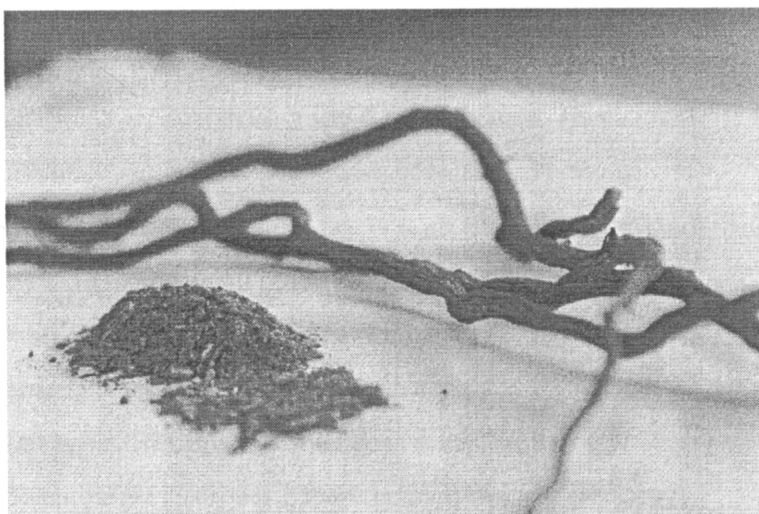

Figure 6 Dried roots and root powder of *P. indica*

Microorganism

Three strains of *Propionibacterium acnes* (DMST 14916, DMST 21823 and DMST 21824) were obtained from Department of Medical Science Center, Thailand. They were cultured on Brain Heart Infusion agar (Becton Dickinson, USA) and incubated in anaerobic conditions using Anaerocult® A (Merck, Germany Germany) and

anaerobic jar (Merck, Germany) at 37°C for 72 hours. *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 14990) were obtained from Department of Microbiology, Faculty of Medicine, Prince of Songkla University, Thailand, and Thailand Institute of Scientific and Technological research, respectively. They were cultured on Mueller-Hinton and incubated in aerobic conditions at 37°C for 24 hours. All tested bacteria were stored in glycerol broth at -20°C and subcultured twice in properly conditions for each bacterium before used.

Chemicals

Chemicals for extraction and purification

- Amberlite® IRA-67 (Sigma, USA)
- Chloroform, commercial grade (Lab scan Asia, Thailand)
- Dichloromethane, analytical grade (Lab scan Asia, Thailand)
- Diethylether, analytical grade (Lab scan Asia, Thailand)
- Ethanol, commercial grade (Lab scan Asia, Thailand)
- Ethyl acetate, commercial grade (Lab scan Asia, Thailand)
- Hexane, commercial grade (Lab scan Asia, Thailand)
- Isopropanol, analytical grade (Lab scan Asia, Thailand)
- Methanol, commercial grade (Lab scan Asia, Thailand)
- Methanol, HPLC grade (Lab scan Asia, Thailand)
- Silica gel 60 (SiO_2 60, 230-400 mesh) (Merck, Germany)

*All commercial grade organic solvents were distilled before use.

Chemicals for antibacterial activity test

- 0.85 % w/v Sodium chloride solution
- Bacto agar (Merck, Germany)
- Brain heart infusion broth (Becton Dickinson, USA)
- McFarland solution
- Mueller-Hinton broth (Merck, Germany)

- Resazurin sodium, Alama blue (Sigma, Switzerland)
- Standard tetracycline disc (6 mm 30 mg/disc) (Oxoid, UK)
- Tetracyclin HCl standard (Fluka, Switzerland)

Chemicals for HPLC analysis

- Water was purified in a Milli-Q system (Millipore, Bedford, USA)
- Methanol, HPLC grade (Lab scan Asia, Thailand)
- Acetic acid, glacial AR grade (Lab scan Asia, Thailand)

Instruments

The equipments used in this study were listed in Table 1.

Table 4 General information of equipments

Instrument	Model	Company
Autoclave	Huxley Incubator vertical type	Huxley Medical Instruments, Taiwan
Bio-safety cabinet	Holten Lamin Air	Thermo electron corporation, UK
Hot air oven	DIN 12880-KI	Memmert, Germany
HPLC	Agilent 1100 series	Palo Alto, USA
Incubator	General purpose incubator 189L	Shellab, USA
Mass spectrometer	MAT 95XL	Thermo Finnigan, USA
NMR spectrometer	UNITY INOVA	Varian, USA
Rotary evaporator	N-N Series	EYELA, Japan
Vortex	G-560E	Scientific Industries, USA
Water bath	WB-14	Memmert, Germany

Methods

1. Preparation of ethyl acetate extracts of *P. indica* roots

The dried powder of *P. indica* roots (0.5 kg) was extracted three times with ethyl acetate (1 L) under reflux conditions for 1 hour. The pooled extracts were evaporated to dryness under reduced pressure at 40°C. The dark brown semisolid with acrid odor was obtained. This extract was used for the isolation of the three naphthoquinone compounds. The crude ethyl acetate extract was stored in an airtight container, protected from light, at room temperature until required.

2, Isolation of naphthoquinones from *P. indica* roots

The crude ethyl acetate extract of *P. indica* root (20 g) was subjected to silica gel vacuum chromatography and eluted with hexane (500 ml/fraction) until the obtained fraction became colorless. After that, a mixture hexane and ethyl acetate (9.8:0.2 v/v; 500 ml/fraction) was used as the eluent to produce yellow fractions. The pooled yellow fractions were then dried under reduced pressure at 40°C, and crystallized in methanol with small amount of water to produce NQ 1 (200 mg).

After NQ 1 was eluted, the vacuum silica gel chromatography was then eluted with a mixture of hexane and ethyl acetate (8:2 v/v; 250 ml/fraction). The pooled fractions 1-3 (fraction A) and 4-7 (fraction B) were further purified by silica gel column chromatography. Elution of fraction A with a mixture of hexane and ethyl acetate (9.2:0.8 v/v; 15 ml/fraction) produced 40 fractions. The pooled fractions 14-35 were rechromatographed with the same chromatographic conditions. The pooled naphthoquinone fractions were then subjected to gel filtration chromatography (Sephadex® LH-20) eluted with methanol to produce a light yellowish crystal of NQ 2 (14 mg).

Elution of fraction B with a mixture of hexane and ethyl acetate (9.5:0.5 v/v; 15 ml/fraction) produced 45 fractions. The pooled fractions 26-38 were rechromatographed with the same chromatographic conditions to produce an orange-yellow crystal of NQ 3 (26 mg).

The structures of NQ 1, NQ 2 and NQ 3 were determined by NMR and MS. The pure compounds of NQ 1, NQ 2 and NQ 3 were then used as indicative markers for

development of HPLC analytical method and prepared the naphthoquinone-rich *P. indica* root extract.

3. HPLC analysis of naphthoquinones

The HPLC analytical method has been developed from the method used to quantitative analysis of four naphthoquinones, including 1,4-naphthoquinone, lawsone, juglone and plumbagin in *Dionaea muscipula* crude extract.

HPLC analysis was carried out using Agilent 1100 liquid chromatographic system (Palo Alto, USA) equipped with isocratic pump (G1312A), auto sampler (G1313A) and photodiode array (PDA) detector (model G1315B). Data analysis was performed using Agilent ChemStation for LC 3D software (Agilent, USA). The wavelength used for quantitative determination of the naphthoquinones was set as 260 nm. Separation was achieved isocratically at 25°C on a Phenomenex® ODS column (150 × 4.6 mm, 5 μm particle size). The mobile phase consisted of methanol and 5% acetic acid in Milli-Q grade water (80:20 v/v) and was pumped at a flow rate of 0.85 ml/min. The injection volume was 10 μl.

3.1 Standard solution

Separate stock solution of the reference standard, plumbagin, 3,3'-biplumbagin and elliptinone were made in methanol. A working solution of the standards was subsequently prepared in methanol and diluted to provide a series of the standard ranging from 0.03 to 10 μg/ml for 3,3'-biplumbagin and elliptinone and 0.01 to 30 μg/ml for plumbagin. The calibration curves were constructed for each of the target analyzes.

3.2 Sample preparation

P. indica dried powder (500 mg) was extracted three time with ethanol (25 ml) under reflux conditions for 1 hour. The pooled extracts were then evaporated to dryness under reduced pressure at 40°C. The residue was then accurately weighed, reconstituted and volume adjusted to 10 mL with methanol to produce a solution of sample at concentration of 10 mg/ml. The sample was filtered through a 0.45 μm membrane filter and analyzed immediately in order to avoid possible chemical degradation. All samples were analyzed in triplicate.

4. Method validation

The analytical method validation for naphthoquinones derived from *P. indica* was examined for specificity, linearity, accuracy, precision, LOD and LOQ.

4.1 Specificity

Peak identification was carried out using the standard and photodiode-array detector. The UV spectra were taken at various points of the peaks to check peak homogeneity.

4.2 Linearity

Calibration curves were constructed on three consecutive days by analysis of a mixture containing each of the standard compounds at six concentrations. Plumbagin was performed at 0.01, 0.1, 5, 10, 20 and 30 μg/ml. 3,3'-Biplumbagin and elliptinone were performed at 0.0313, 0.125, 0.5, 1, 5 and 10 μg/ml. The standard curves were analyzed using the linear least-squares regression equation derived from the peak area.

4.3 Accuracy

Plumbagin at concentrations of 0.1, 5 and 20 μg/ml, 3,3'-biplumbagin and elliptinone at concentrations of 0.125, 0.5 and 5 μg/ml were prepared and added into *P. indica* root extract at a ratio of 1:1 (v/v). Prior to analyze fortification, the background levels of plumbagin, 3,3'-biplumbagin and elliptinone in the extract of *P. indica* roots were determined so as to calculate actual recoveries. The amount of each analyte was determined in triplicate and percentage recoveries were then calculated.

4.4 Precision

Precision experiments were conducted for intraday and interday. The solution of one sample was used to achieve repeatability testing. The data of repeatability was the content of six injections separately in the same day. The data used to calculate % RSD of interday precision was the content of three samples analyzed in three days (three injections in succession each day).

4.5 Limit of detection (LOD) and quantification (LOQ)

Serial dilutions of sample solution standards were made with methanol and analyzed with the HPLC method. LOD and LOQ were determined by means of signal to noise ratio of 3:1 and 10:1, respectively.

5. Determination of solvent for extraction

P. indica dried powder (500 mg) was separately extracted with ethyl acetate, ethanol, isopropanol, dichloromethane and diethyl ether (25 ml) under reflux conditions for 1 hour (×3). After filtration, the pooled extracts of the same solvent were evaporated to dryness under reduced pressure, adjusted to 10 ml with methanol, and subjected to HPLC analysis. The experiments were performed in triplicate.

6. Determination of fractionation method

6.1 Preparation of *P. indica* root extract

P. indica dried powder (1 kg) was successively extracted three times with the ethanol (5 L) under reflux conditions for 1 hour. The pooled extracts were dried under reduced pressure at 40°C and subsequently subjected to fractionate by different methods.

6.2 Fractionation by anion exchange chromatography

The anion exchange resin (Amberlite® IRA-67, Sigma, USA) was treated with methanol and loaded into a column (10 × 126 cm). The column was washed twice with water and methanol, respectively. The *P. indica* extract (20 g) was dissolved in methanol and loaded into the column with a flow rate of 5 ml/min. The column was eluted with methanol until other impurity bands were completely washed out. The naphthoquinones were then eluted with 10% acetic acid in methanol. The pooled naphthoquinone fractions were dried under reduced pressure at 40°C.

6.3 Fractionation by silica gel vacuum chromatography

A sintered glass column (13 cm in diameter) was packed with silica gel approximately 5 cm high. The crude ethanol extract of *P. indica* roots (20 g), which pre-

adsorbed on the silica gel, was loaded as a thin layer on the surface of column. The column was eluted with a mixture of hexane and ethyl acetate (9.2:0.8 v/v; 500 ml) with the aid of a vacuum pump. The pooled fractions of naphthoquinones were then dried under reduced pressure at 40°C.

6.4 Partition by liquid-liquid extraction

6.4.1 Partition with purified water

The crude ethanol extract of *P. indica* roots (20 g) was suspended water (150 ml) and partitioned with ethyl acetate (150 ml) until the ethyl acetate phase was colorless. The pooled ethyl acetate fractions were then dried under reduced pressure at 40°C.

6.4.2 Partition with 5% acetic acid in water

The crude ethanol extract of *P. indica* roots (20 g) was suspended 5% acetic acid in water (150 ml) and partitioned with ethyl acetate (150 ml) until the ethyl acetate phase was colorless. The pooled ethyl acetate fractions were then dried under reduced pressure at 40°C.

7. Antibacterial activity assay

The standard naphthoquinones and naphthoquinone-rich extract were evaluated for antibacterial activity against acne-involved bacteria include *Propionibacterium acnes* (DMST 14916, DMST 21823 and DMST 21824), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 14990). Three strains of *P. acnes* were grown in Brain heart infusion agar (BHI) and incubate at 37°C for 72 hours in anaerobic conditions (Anaerocult® A and Anaerobic jar, Merck, Germany). *S. aureus* and *S. epidermidis* were grown in Mueller-Hinton agar (MH agar) and incubate at 37°C for 24 hours in aerobic conditions.

7.1 Determination of MIC

MIC value of the standard naphthoquinones and naphthoquinone-rich *P. indica* root extract were determined using broth dilution method (Wiegan *et al.*, 2008).

The test compounds were dissolved in DMSO at the concentration of 100 µg/ml (stock solution) and then it was serial diluted with BHI or MH broth to gave the final concentrations between 0.025 and 100 µg/ml. Tetracycline hydrochloride and DMSO were used as a positive and negative control, respectively. The test was performed in 96-well plates. Two-fold dilutions were prepared directly in 96-well plate (NUNC, Denmark), as follows: 100 µl of the working solution of compounds or extract was added to well 1 of the dilution series. To each remaining well, 50 µl of BHI or MH broth were added. With a sterile pipette tip, 50 µl of the mixture was transferred from well 1 to well 2. After thorough mixing, 50 µl of the mixture was transferred to well 3. This process was continued until the last final concentration was obtained. The last well received no antimicrobial agent and served as a growth control.

The inoculums were prepared in sterile physiological saline solution and adjusted turbidity to 0.5 McFarland standard (1.5×10^8 cfu/ml). It was then further diluted 1:100 in sterile broth to contain 1.5×10^6 cfu/ml and 50 µl of the adjusted inoculums were added to each well then incubated at properly conditions as described above.

After incubation period, all wells were added with 50 µl Alamar blue (10 µg/ml) and incubated for 5 hours before determined MIC value. The blue color wells mean bacteria were inhibited by test sample and cannot growth in these medium, while pink wells mean test samples cannot inhibit bacteria growth. The lowest concentration that did not show any growth of bacteria was taken as the MIC.

7.2 Determination of MBC

The incubation mixtures that showed positive result of inhibitory effect were streaked on BHI or MH agar. The MBC was detected as the lowest concentration which no colonies of test bacteria formed on the cultivation medium.

8. Determination of solubility

To evaluate the solubility of naphthoquinone-rich *P. indica* root extract, the solvents include ethyl acetate, ethanol, chloroform, methanol, hexane, propylene glycol and distilled water were used. The naphthoquinone-rich *P. indica* root extract was accurately weighed to 10 mg and placed in a vessel of at least 100 ml capacity. Various

solvents were examined by adding increments of 10 μl, shaking frequently and vigorously for 10 minutes. The volume of solvent was recorded when a clear solution was obtained. The sample would be added continuously until 10 ml if the solution was become cloudy or non-dissolve. After 10 ml of solvents were added, the sample which remained non-dissolve would be repeated in a 100 ml volumetric flask, shake vigorously and observe for 24 hours. The samples which remained non-dissolve within 24 hours observed would be reported as practically insoluble.

Descriptive term of solubility and approximate volume of solvents required to completely dissolve a solute (in milliliters per gram of solute) were drawn as shown in Table 2 (British Pharmacopoeia Commission, 2001).

Table 5 Solubility terms in British Pharmacopoeia Commission 2001

Solubility term	Volume of solvent (ml) to dissolve 1 g of sample
Very soluble	less than 1
Freely soluble	from 1 to 10
Soluble	From 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	from 100 to 1000
Very slightly soluble	from 1000 to 10,000
Practically insoluble	more than 10,000

9. Stability evaluation of naphthoquinone- rich *P. indica* root extracts
(Sakunpak, et al., 2009)

9.1 Effect of light on stability of the extract

The naphthoquinone-rich *P. indica* root extracts were accurately weighed to 2 mg and kept in sealed microtubes (Axygen Scientific, USA). The extracts were then stored at 25 ± 2°C either protected from light or exposed to 36 Watts fluorescent light (40 cm distance) for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 16 weeks and subjected to quantitative analysis of naphthoquinone content using HPLC. The experiments were performed in triplicate.

9.2 Effect of temperature on stability of the extract

The naphthoquinone-rich *P. indica* root extracts were accurately weighed to 2 mg and kept in sealed microtubes and protected from light. The extracts were then stored at 4 ± 2°C and room temperature (30 ± 2°C) for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12 and 16 weeks and subjected to quantitative analysis of naphthoquinone content using HPLC. The experiments were performed in triplicate.

9.3 Effect of pH on stability of the extract

The naphthoquinone-rich *P. indica* root extracts were accurately weighed to 4 mg and dissolved in phosphate buffer solution to achieve pH values of 5.5, 7.0, and 8.0. The sample solutions were then kept in well-closed containers, protected from light and stored at 25 ± 2°C for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 16 weeks and subjected to quantitative analysis of naphthoquinone content using HPLC. The experiments were performed in triplicate.

9.4 Effect of accelerated condition on stability of the extract

The naphthoquinone-rich *P. indica* root extracts were accurately weighed to 2 mg and kept in sealed microtubes and protected from light. The extracts were then stored at 45 ± 2°C and 75% humidity for 4 months. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 16 weeks and subjected to quantitative analysis of naphthoquinone content using HPLC. The experiments were performed in triplicate.

10. Statistical analysis

Values are expressed as mean ± standard deviation (SD). Data were analyzed by Student's *t*-test. The level of statistical significance was taken at $p<0.05$.

RESULTS AND DISCUSSION

Isolation of naphthoquinones from *P. indica* roots

Dried powder of *P. indica* root was extracted with ethyl acetate under reflux conditions to yield ethyl acetate extract (Figure 7). The extract (20 g) was then fractionated by silica gel vacuum chromatography, silica gel column chromatography and gel filtration chromatography to produce three naphthoquinones, NQ 1 (200 mg), NQ 2 (14 mg) and NQ 3 (26 mg).

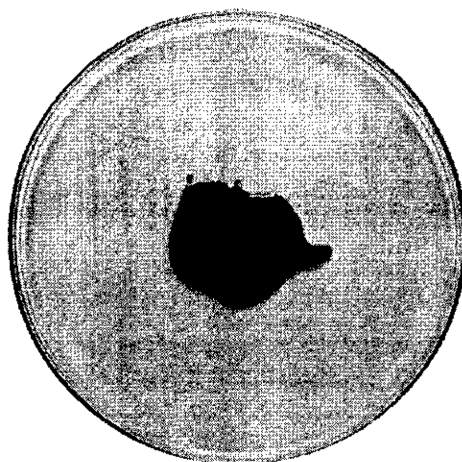

Figure 7 Crude ethanol extract of *P. indica*

Thin layer chromatography (TLC) chromatogram of NQ 1, NQ 2 and NQ 3 developed in a mixture of hexane and ethyl acetate (9.5:0.5) gave Rf values of 0.8, 0.46 and 0.35, respectively, and all compounds gave red to purple spot when sprayed with 10% KOH in methanol. These pure compounds were then subjected to determination of their chemical structures by NMR and MS.

Structure determination

1. Identification of NQ 1

NQ 1 had the same retention time as the standard plumbagin when determined by the HPLC system (Figure 8). NQ 1 was therefore identified as plumbagin (Figure 9) when compared its spectral data with those previously reported by Philip *et al.* (1999).

The ^{1}H NMR spectrum of NQ 1 (Table 3, Figure 10) exhibited a methyl group at δ 2.17 (3H, d, J = 1.7, 11-CH_3) coupling with olefinic protons at δ 6.78 (1H, m, J = 1.7, 3-H) and a hydroxyl group at δ 11.95 (1H, s, 5-OH) bearing on aromatic ring at δ 7.23 (1H, dd, J = 8.0, 1.5, 6-H), δ 7.58 (1H, dd, J = 8.0, 7.6, 7-H) and δ 7.60 (1H, dd, J = 7.6, 1.5, 8-H).

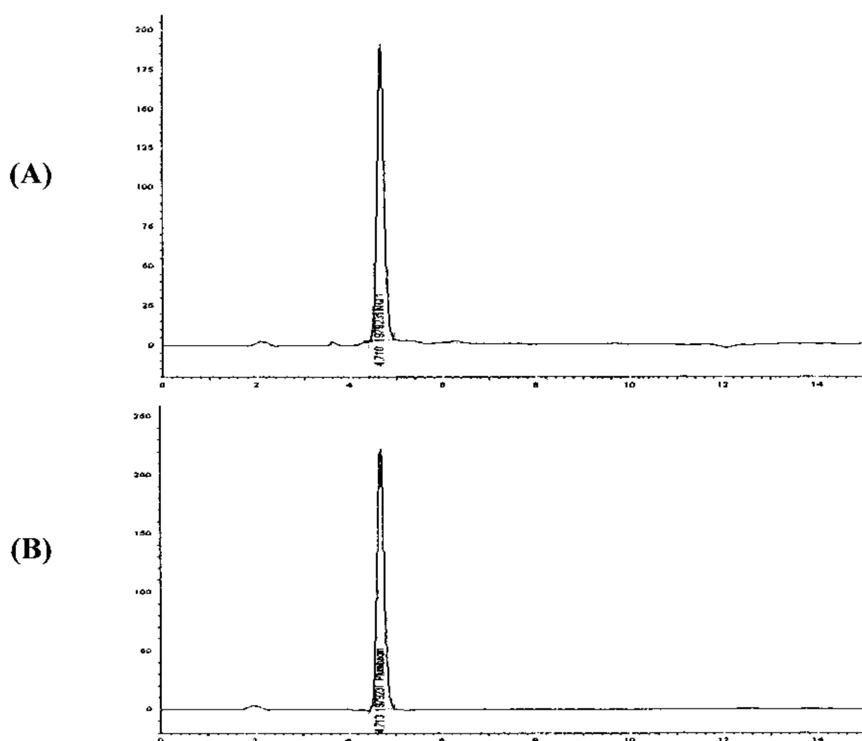

Figure 8 HPLC chromatograms of (A) NQ 1 and (B) plumbagin determined by the developed method; mobile phase consist of methanol and 5% aqueous acetic acid (80:20 v/v), flow rate 0.85 ml/min and detection wavelength set at 260 nm

Table 6 ^{1}H NMR ($CDCl_3$; 500 MHz) spectral data of NQ 1 and plumbagin

Positions	NQ 1	Plumbagin (Philip *et al.*, 1999)
	^{1}H (mult.; *J* in Hz)	^{1}H (mult.)
1	-	-
2	-	-
3	6.78 (d; 1.7)	6.81 (d)
4	-	-
5	11.95 (s)	11.95 (s)
6	7.23 (dd; 8.0, 1.5)	7.25 (m)
7	7.58 (dd; 8.0, 7.6)	7.62 (m)
8	7.60 (dd; 7.6, 1.5)	7.62(m)
9	-	-
10	-	-
11	2.17 (d; 1.7)	2.20 (s)

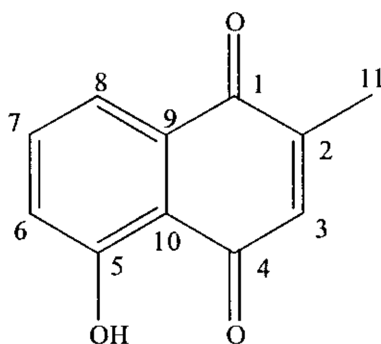

Figure 9 Chemical structure of plumbagin

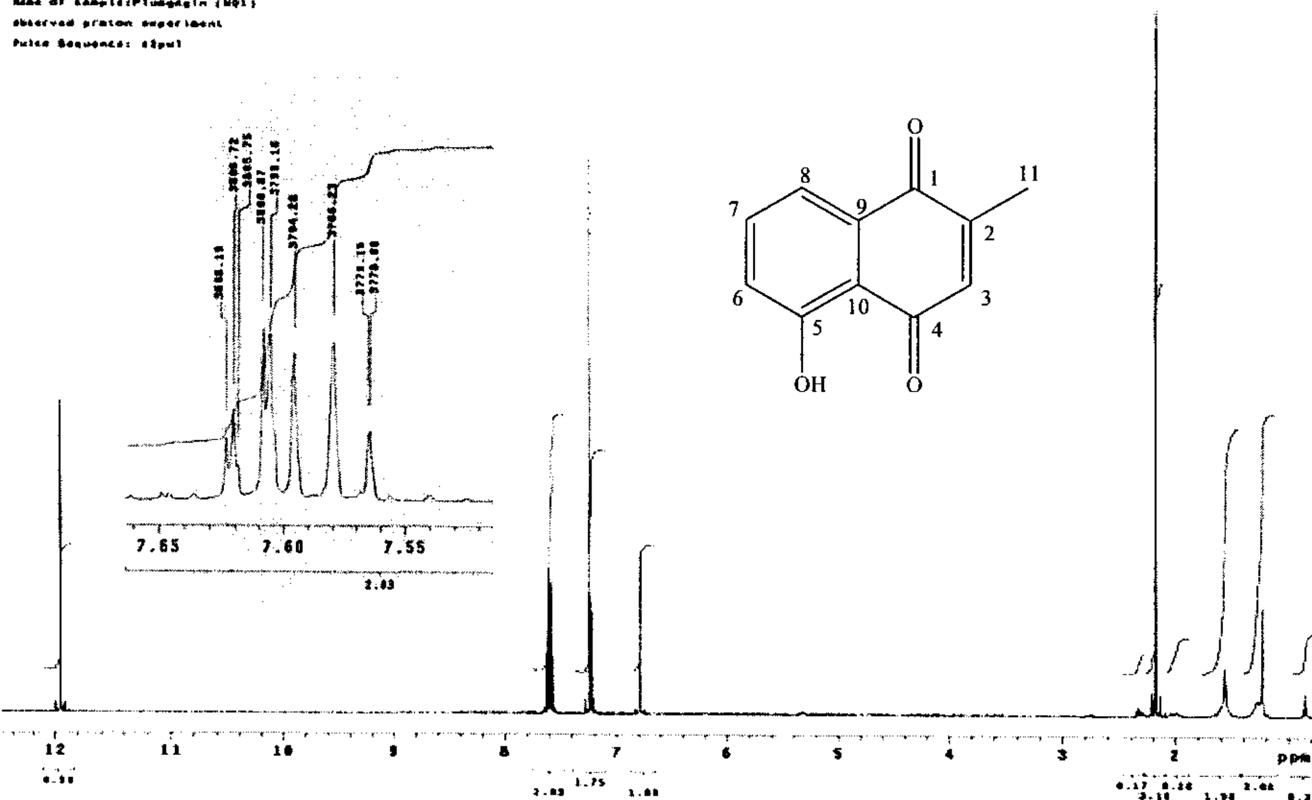

Figure 10 ^{1}H-NMR ($CDCl_3$; 500 MHz) of NQ 1

2. Identification of NQ 2

The ^{1}H NMR spectrum of NQ 2 (Table 4, Figure 11) exhibited two methyl groups at δ 2.00 (6H, s, 11-CH_3, 11'-CH3), aromatic protons at δ 7.28 (2H, d, J = 7.8, 6-H, 6'-H), δ 7.65 (2H, dd, J = 8.3, 7.8, 7-H, 7'-H), δ 7.71 (2H, d, J = 8.3, 8-H, 8'-H) and hydroxyl groups at δ 11.89 (2H, s, 5-OH, 5'-OH). The ^{13}C NMR spectrum of NQ 2 (Table 4, Figure 12) revealed 22 carbons; two methyl, six methines, fourteen quarternary with four carbonyl and ten methines. NQ 2 has a molecular formula of $C_{22}H_{14}O_6$ as established by mean of the EI mass spectrum (Figure 13), m/z (rel. int. %): 374 $[M]^{+}$ (100), 359 $[M^{+}\text{-}CH_3^{\bullet}]$ (80), 345 $[M^{+}\text{-}CH^{\bullet}]$ (6), 330 $[M^{+}\text{-}CH_3^{\bullet}]$ (16), 317 $[M^{+}\text{-}CH^{\bullet}]$ (6), 303 $[M^{+}\text{-}CH^{\bullet}]$ (10), 120 $[M^{+}\text{-}C_{10}HO_4^{\bullet}]$ (10), 92 $[M^{+}\text{-}CO^{\bullet}]$ (13), 67 $[M^{+}\text{-}C_2H^{\bullet}]$ (5) and the data of high resolution mass spectroscopy indicated the exact mass of NQ 2 was 374.0790 g/mol. Thus, NQ 2 was identified as 3,3'-biplumbagin (Figure 14).

Table 7 ^{1}H NMR ($CDCl_3$; 500 MHz) and ^{13}C NMR ($CDCl_3$; 125 MHz) spectral data of NQ 2

Positions	^{1}H NMR (mult.; *J* in Hz)	^{13}C NMR
1, 1'	-	183 (-C=O)
2, 2'	-	147 (C)
3,3'	-	139 (C)
4, 4'	-	187 (-C=O)
5, 5'	11.89 (s)	161 (-C-OH)
6, 6'	7.28 (d; 7.8)	124 (CH)
7, 7'	7.65 (dd; 8.3, 7.8)	136 (CH)
8, 8'	7.71 (d; 8.3)	119 (CH)
9, 9'	-	132 (C)
10, 10'	-	116 (C)
11, 11'	2.00 (s)	14.5 (CH_3)

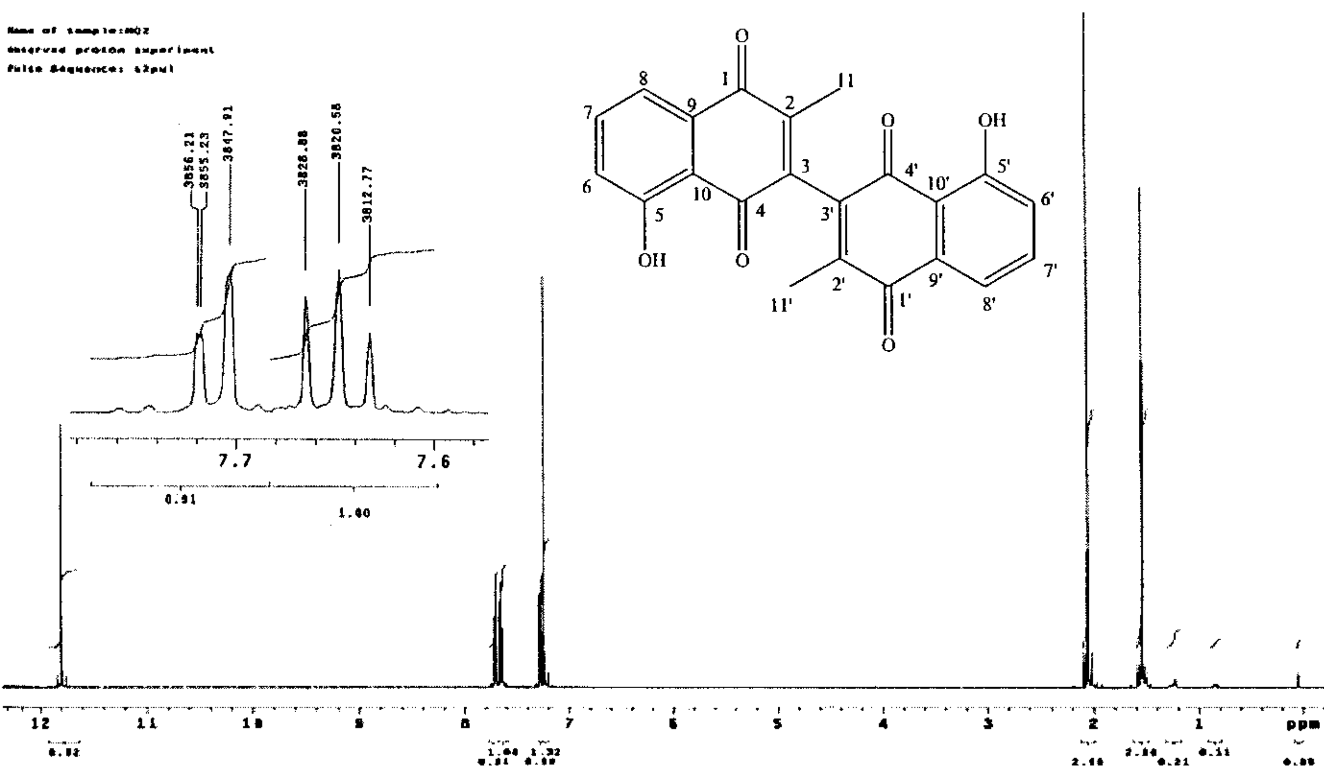

Figure 11 ^{1}H-NMR ($CDCl_3$; 500 MHz) of NQ 2

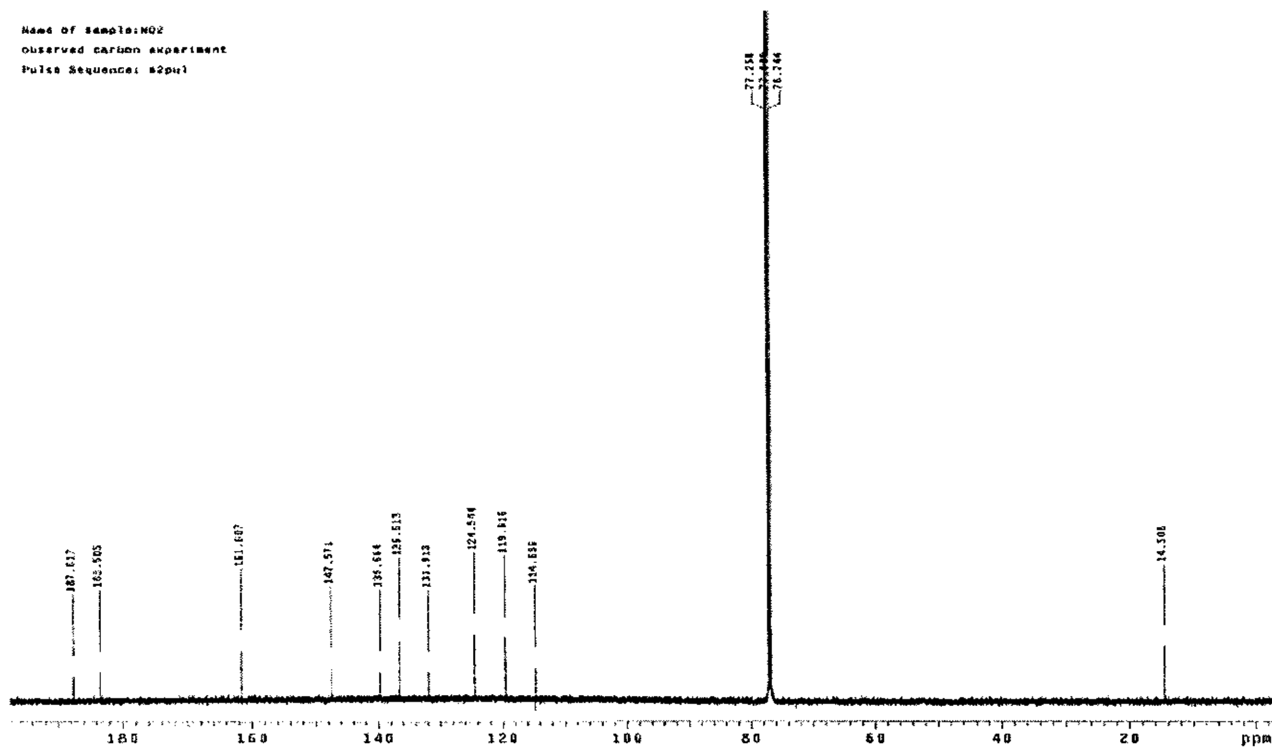

Figure 12 ^{13}C-NMR ($CDCl_3$; 125 MHz) of NQ 2

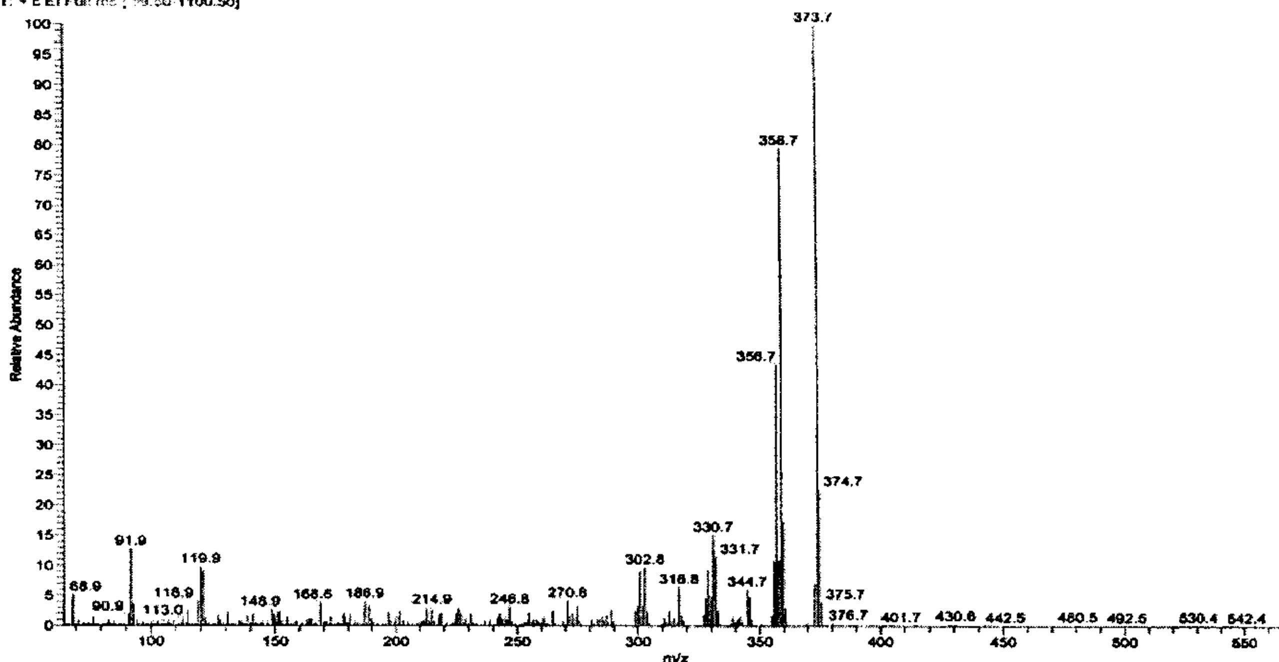

Figure 13 Mass spectroscopy of NQ 2

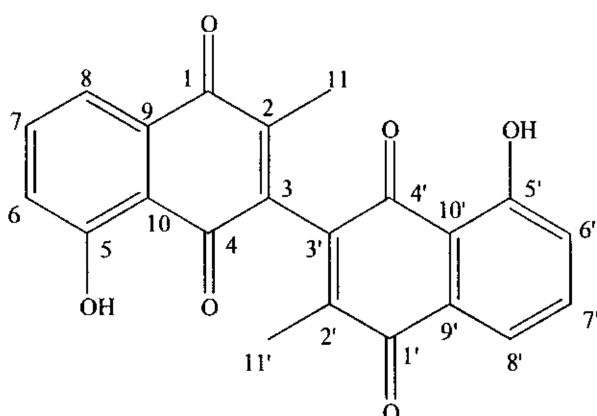

Figure 14 Chemical structure of 3,3'-biplumbagin

3. Identification of NQ 3

The ^{1}H NMR spectrum of NQ 3 (Table 5, Figure 15) exhibited two methyl groups at δ 1.99 (6H, d, J = 1.5, 11-CH_3, 11'-CH3) coupling with olefinic protons at δ 6.79 (2H, m, J = 1.5, 3-H, 3'-H), two hydroxyl groups at δ 12.56 (2H, s, 5-OH, 5'-OH) attach on two aromatic rings, which contain aromatic protons δ 7.18 (2H, d, J = 8.8, 8-H, 8'-H) and δ 7.27 (2H, d, J = 8.8, 7-H, 7'-H). The ^{13}C NMR spectrum of NQ 3 (Table 5, Figure 16) revealed 22 carbons; two methyl groups, six methines, fourteen quarternary carbons with four carbonyls and ten methines. NQ 3 has a molecular formula of $C_{22}H_{14}O_6$ as established by mean of the EI mass spectrum (Figure 17), *m/z* (rel. int. %): 374 $[M]^+$ (100), 346 $[M^+\text{-}CO^\bullet]$ (7), 331 $[M^+\text{-}CH_3^\bullet]$ (30), 303 $[M^+\text{-}CO^\bullet]$ (60), 278 $[M^+\text{-}C_2H^\bullet]$ (33), 250 $[M^+\text{-}CO^\bullet]$ (40), 221 $[M^+\text{-}C_2H_3^\bullet]$ (5), 165 $[M^+\text{-}C_3OH^\bullet]$ (11), 140 $[M^+\text{-}C_2H^\bullet]$ (15) and the data of high resolution mass spectroscopy indicated the exact mass of NQ 3 was 374.0790 g/mol. Therefore, NQ 3 was identified as elliptinone (Figure 18)

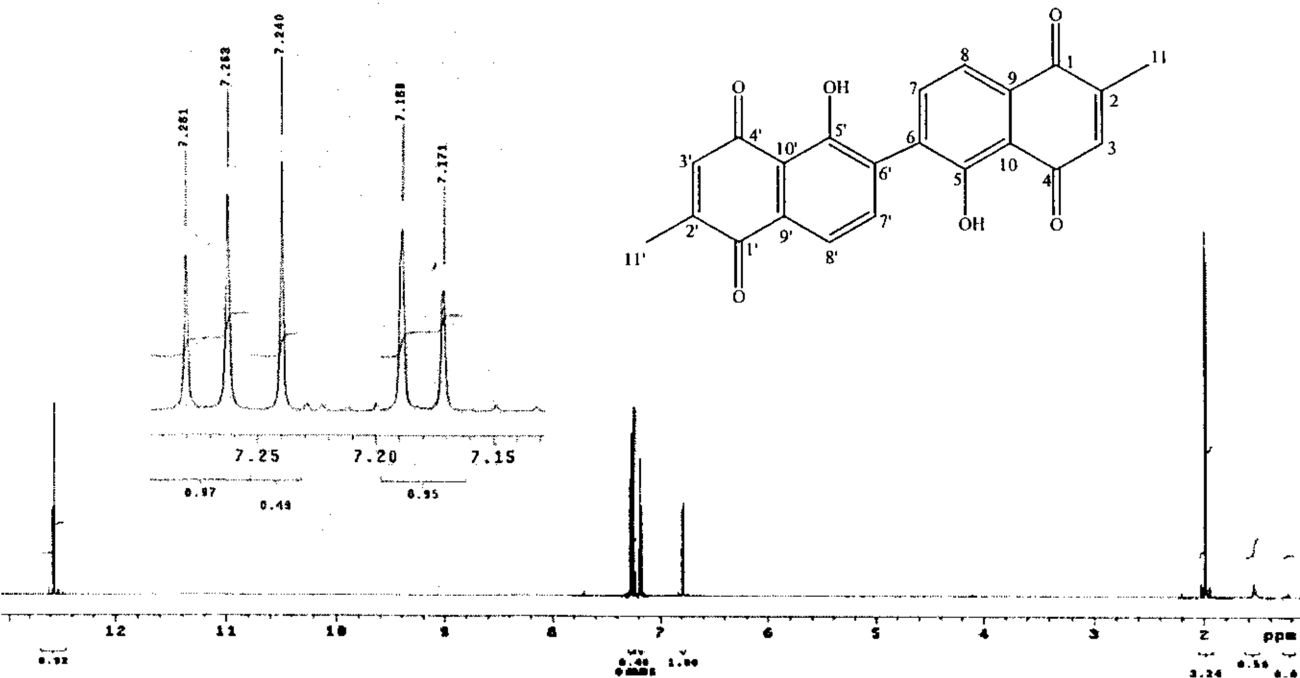

Figure 15 ^{1}H-NMR ($CDCl_3$; 500 MHz) of NQ 3

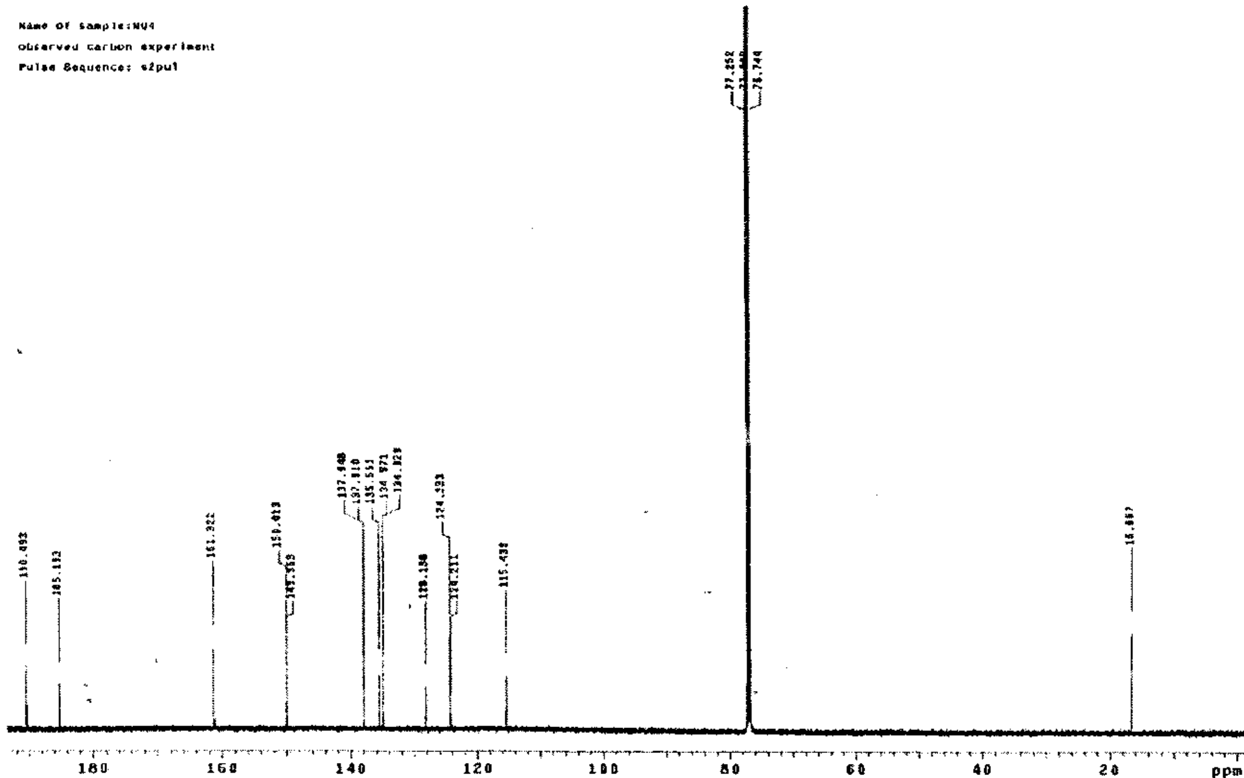

Figure 16 ^{13}C-NMR ($CDCl_3$; 125 MHz) of NQ 3

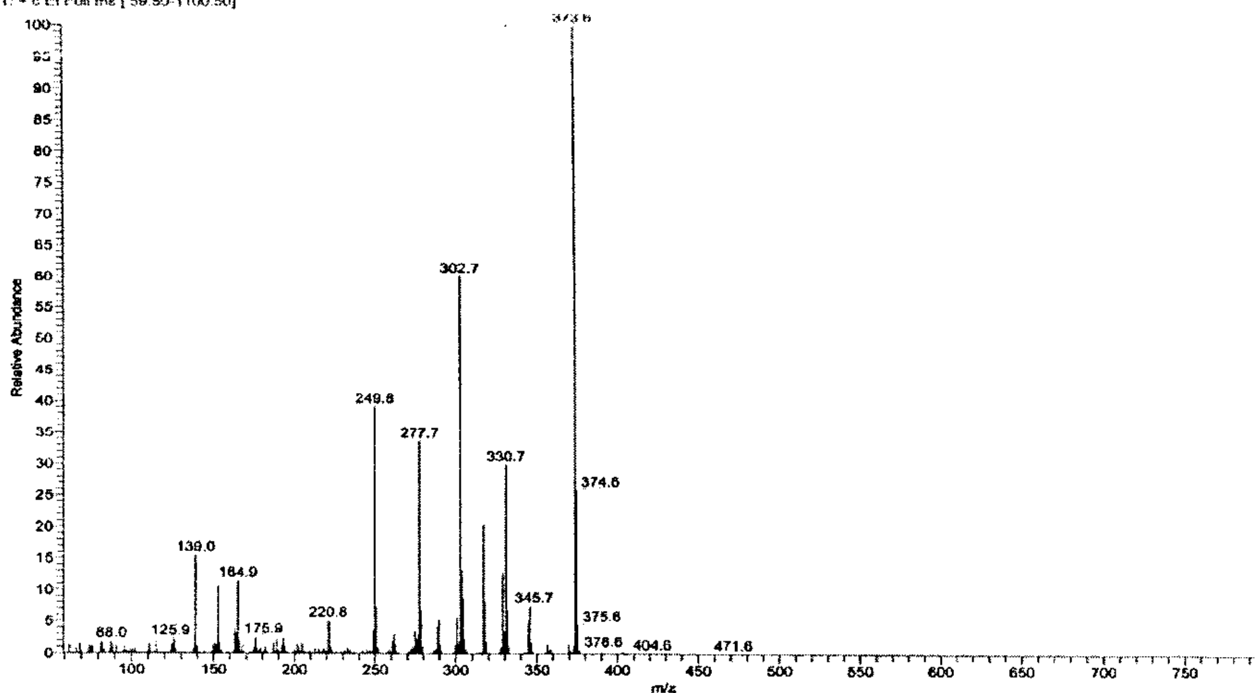

Figure 17 Mass spectroscopy of NQ 3

Table 8 ^{1}H NMR ($CDCl_3$; 500 MHz) and ^{13}C NMR ($CDCl_3$; 125 MHz) spectral data of NQ 3

Positions	^{1}H NMR (mult.; *J* in Hz)	^{13}C NMR
1, 1'	-	185.2 (-C=O)
2, 2'	-	150 (C)
3,3'	6.79 (m; 1.5)	134.9 (CH)
4, 4'	-	190.5 (-C=O)
5, 5'	12.56 (s)	161.3 (-C-OH)
6, 6'	-	128.1 (C)
7, 7'	7.27 (d; 8.8)	137.9 (CH)
8, 8'	7.18 (d; 8.8)	124.3 (CH)
9, 9'	-	135.6 (C)
10, 10'	-	115.4 (C)
11, 11'	1.99 (d; 1.5)	16.7 (CH_3)

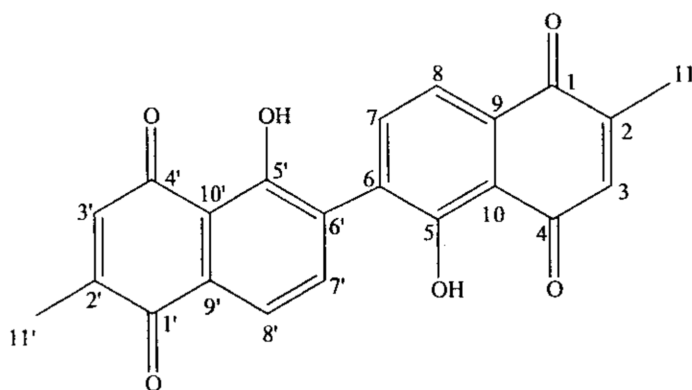

Figure 18 Chemical structure of elliptinone

HPLC quantitative analysis of naphthoquinones in *P. indica* root extracts

The optimal conditions for simultaneous quantitative determination of naphthoquinones in *P. indica* root extracts were performed using reverse phase HPLC system on Agilent HPLC 1100 series with Agilent ChemStation for LC 3D software. The three naphthoquinones were used as the indicative markers for quantitative analysis of *P. indica* root extracts. As these compounds have maximum absorption at 260 nm, this wavelength was then used for quantification. Samples were injected at 10 µl through a Phenomenex® ODS column (150 × 4.6 mm 5 µm particle size) and isocratically eluted with a mixtures of methanol and 5% aqueous acetic acid in the ratio of 80 : 20. The flow rate was used at 0.85 ml/min. The retention times of all naphthoquinones were within 15 minutes (Figure 1) and separated with satisfactory resolution. On the basis of the reverse phase HPLC analysis, plumbagin, the most polar compound would be firstly eluted at the retention time about 4 min followed by elliptinone and 3,3'-biplumbagin with the retention times about 6 and 10 min, respectively.

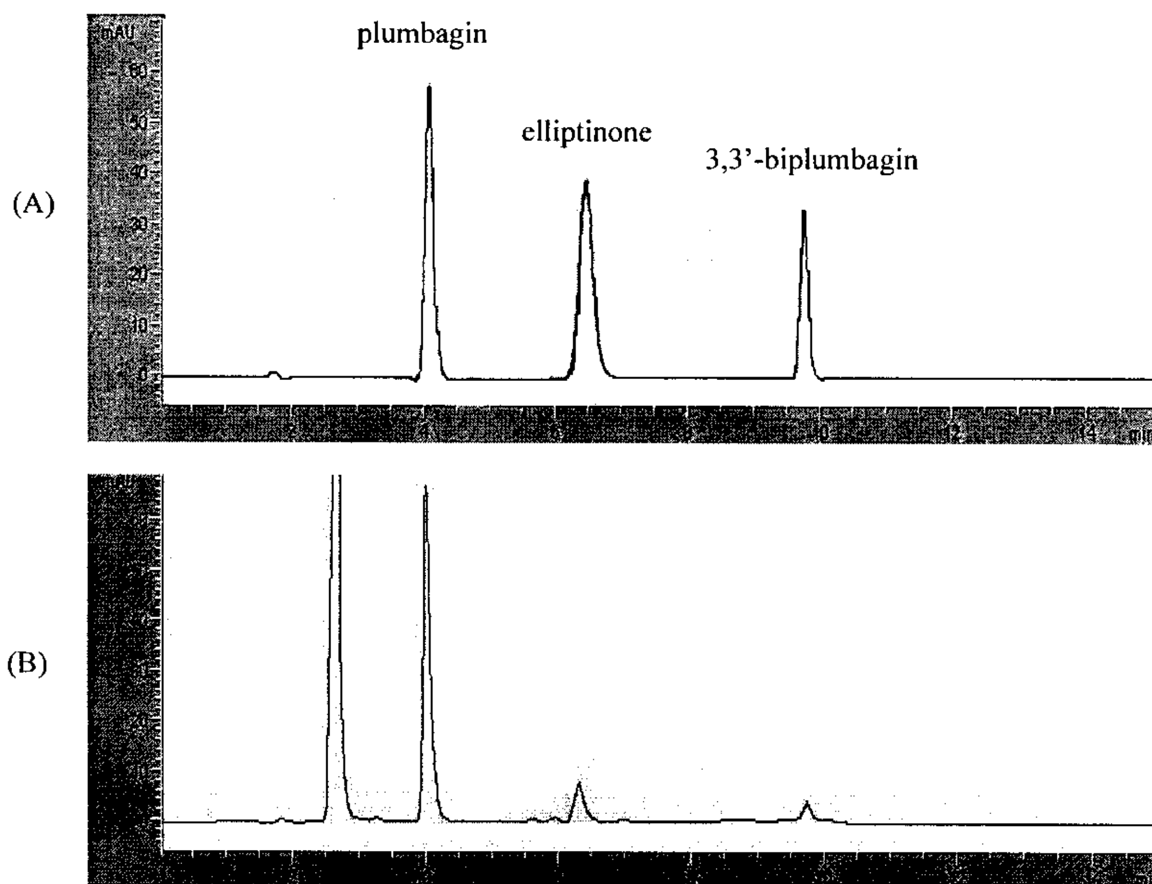

Figure 19 HPLC-chromatograms of (A) standard naphthoquinones and (B) naphthoquinone-rich *P. indica* root extracts

Validation of analytical method

1. Specificity

Utilizing the PDA makes it possible to obtain the UV spectra of the analyzed compounds. Specificity of the method was evaluated using UV-absorption spectra that were taken at three various points of the peaks to check peak homogeneity. The results indicated that the homogeneity of the three spectra at three different points of the peaks was observed (Figure 20 to 22). These results suggested that the HPLC method possessed good specificity.

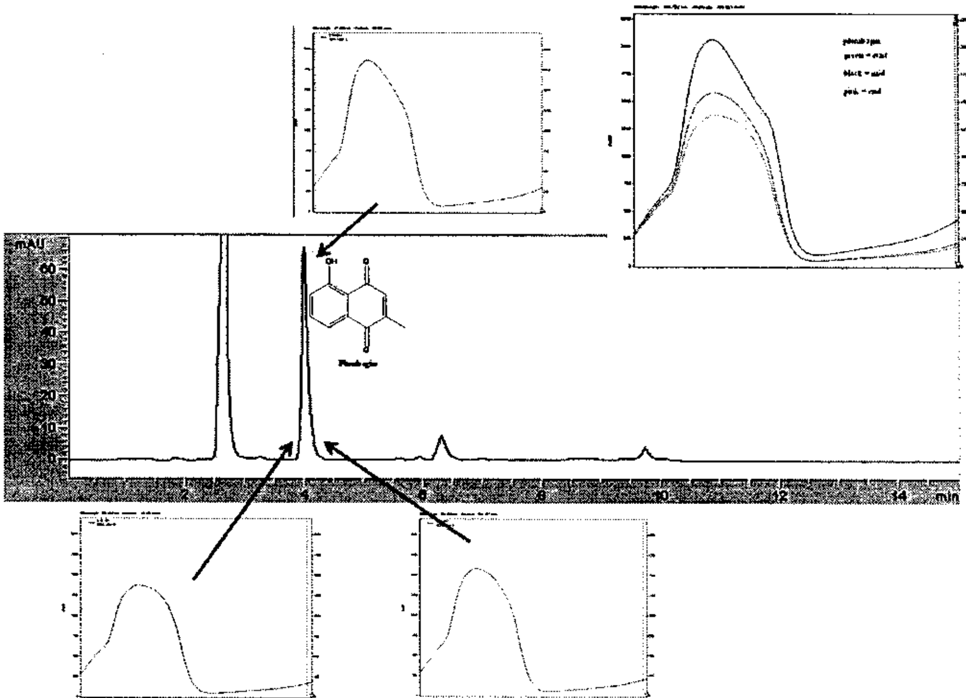

Figure 20 UV absorption spectra of plumbagin in *P. indica* root extracts

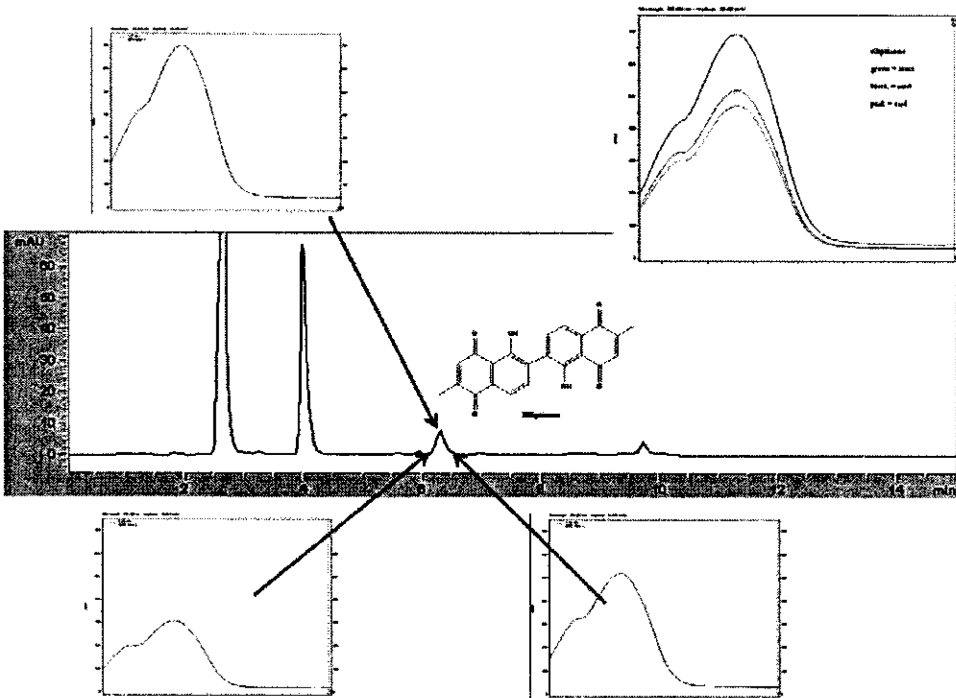

Figure 21 UV absorption spectra of elliptinone in *P. indica* root extracts

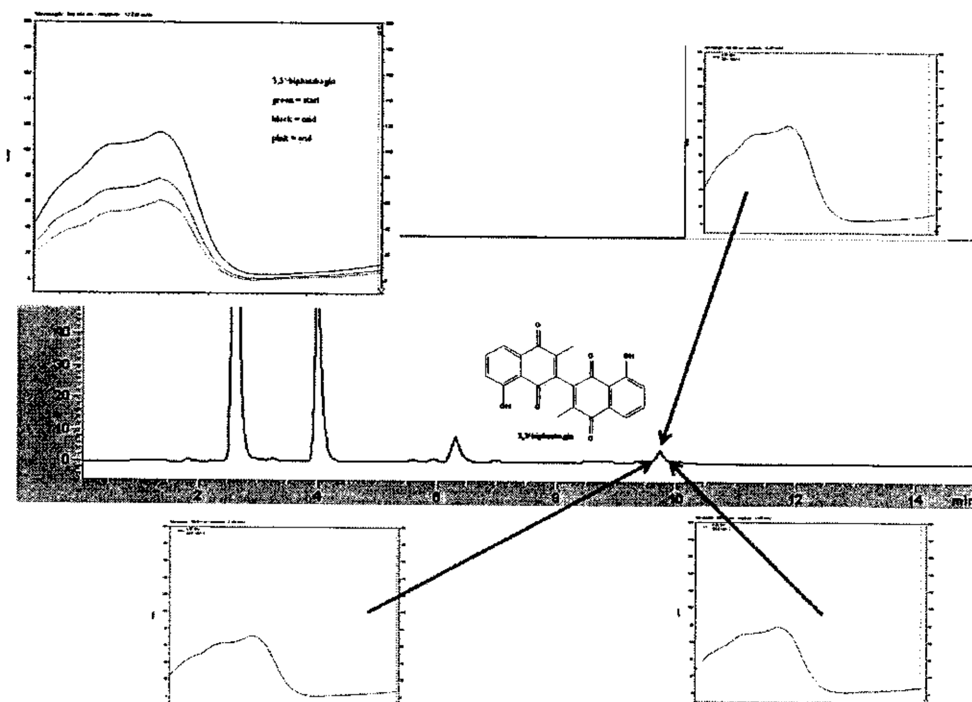

Figure 22 UV absorption spectra of 3,3'-biplumbagin in *P. indica* root extracts

2. Linearity

Linearity was evaluated using the standard naphthoquinone solutions over six concentrations. The concentrations of plumbagin were 0.01 - 30 µg/ml, and those of 3,3'-biplumbagin and elliptinone were 0.03 - 10 µg/ml. Three measurements for each concentration were performed. Excellent linearity was obtained for these three compounds, with the correlation coefficients of 0.9997, 0.9997, and 0.9999 for plumbagin, 3,3'-biplumbagin and elliptinone, respectively (Table 6).

Table 9 Retention time, linear ranges, and correlation coefficients (R^2) of calibration curves

Compounds	Retention time (min)	Linear range (µg/ml)	Y = aX + b linear model*	Linearity (R^2)
Plumbagin	4.0	0.01 – 30.0	Y = 41.27X + 11.807	0.9997
Elliptinone	6.3	0.03 – 10.0	Y = 58.305X + 8.203	0.9999
3,3'-Biplumbagin	9.8	0.03 – 10.0	Y = 41.821X + 7.120	0.9997

* Y = peak area; X = concentration (µg/ml)

3. Accuracy

The accuracy of the analytical method was studied by spiking technique. Plumbagin (concentrations of 0.1, 5 and 20 µg/ml), 3,3'-biplumbagin and elliptinone (concentrations of 0.1, 0.5 and 5 µg/ml) were spiked into *P. indica* sample solutions to evaluate recoveries of the standard compounds. The recoveries were closed to 100% (Table 7), which indicated for a good accuracy of the HPLC method.

Table 10 Recoveries of naphthoquinones from *P. indica* root extracts

Compounds	Spiked concentration (µg/ml)	Recovery (%)[a] (n = 3)
Plumbagin	0.1	99.9 ± 1.10
	5	99.7 ± 0.64
	20	100.3 ± 0.22
3,3'-Biplumbagin	0.1	98.6 ± 1.10
	0.5	98.8 ± 0.99
	5	100.4 ± 0.45
Elliptinone	0.1	98.6 ± 1.57
	0.5	98.9 ± 0.86
	5	100.6 ± 0.42

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

4. Precision

The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of the sample solutions. The percentage relative standard deviation (%R.S.D.) was determined. The % RSD values of intraday and interday analysis of all compounds were less than 5% (Table 8), which indicated for the high precision of the HPLC method.

Table 11 Intraday and interday precision data of *P. indica* root extracts

Compounds	Interday (n = 3 per day)				Intraday (n = 6)	
	Content (mg/g of extract)			% RSD	Content (mg/g)	% RSD
	Day 1	Day 2	Day 3			
Plumbagin	127.46	130.36	129.53	1.16	127.46	0.11
3,3'-biplumbagin	5.01	5.26	5.12	2.50	5.01	0.58
elliptinone	16.54	16.88	16.70	1.00	16.54	0.30

5. LOD and LOQ

The limit of detection represents the lowest concentration that can be detected by the analytical method, whereas the limit of quantification represents the lowest concentration that can be determined with acceptable precision and accuracy. Data of LOD and LOQ of the HPLC method for plumbagin, 3,3'-biplumbagin and elliptinone were shown in Table 9. The results indicated that the established HPLC method is sensitive for determination of these three compounds.

Table 12 LOD and LOQ of *P. indica* root extracts

Compounds	LOD [a] (μg/ml)	LOQ [b] (μg/ml)
Plumbagin	0.001	0.010
3,3'-biplumbagin	1.00	5.00
elliptinone	0.10	1.00

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

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

Determination of solvent for extraction

A few different extraction solvents include ethyl acetate, ethanol, isopropanol, dichloromethane and diethyl ether were examined to produce the highest content of naphthoquinones in *P. indica* root extracts. The result showed that although isopropanol produced the highest yield of the root extract, ethanol produced the highest content of total naphthoquinones (Table 10). Thus, ethanol was appropriately used for the extraction.

Table 13 Yield and naphthoquinones content in *P. indica* root extracts

Solvents	Yield (% w/w)	Content[a] (mg/g of extract; Mean ± S.D.)			
		Plumbagin	3,3'-biplumbagin	Elliptinone	Total naphthoquinones
EtOH	11.5	4.76 ± 0.067	0.34 ± 0.044	0.69 ± 0.035	5.80
EtOAc	10.9	4.63 ± 0.266	0.25 ± 0.021*	0.51 ± 0.070*	5.39
C_3H_7OH	11.8	3.50 ± 0.606*	0.23 ± 0.051*	0.49 ± 0.108*	4.22
CH_2Cl_2	9.1	3.85 ± 0.910	0.19 ± 0.030*	0.34 ± 0.054*	4.38
Diethylether	8.8	4.26 ± 0.576	0.16 ± 0.075*	0.47 ± 0.088*	4.89

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

* Significant difference ($P<0.05$) when compared with the ethanol extract

EtOH = ethanol; EtOAc = ethyl acetate; C_3H_7OH = isopropanol;

CH_2Cl_2 = dichloromethane

Determination of suitable fractionation method

The crude ethanol extract of *P. indica* roots was used for further determination of an appropriate fractionation method to produce naphthoquinone-rich *P. indica* root extract. The result exhibited that although anion exchange chromatographic method (Amberlite® IRA-67) produced the highest content of 3,3'-biplumbagin, it was difficult to eluted plumbagin and elliptinone from the anion exchange column. Thus, this method produced a low content of the total naphthoquinones (Table 11). Although the liquid-liquid extraction methods produced the highest yield of the extracts, the total

naphthoquinone content also lower than the silica gel vacuum chromatographic method. In addition, acidic water used for liquid-liquid extraction could not improve the content of naphthoquinones in the extract. The silica gel vacuum chromatographic method was therefore suitable for preparation of naphthoquinone-rich *P. indica* root extracts. This method was capable of increase the total naphthoquinone content up to 138.98 mg/g dry weight of the extract (Table 11).

Table 14 Naphthoquinone content in purified *P. indica* root extract from various methods

Purification methods	Yield[b] (% w/w)	Content[a] (mg/g of extract; Mean ± S.D.)			
		Plumbagin	3,3'-biplumbagin	Elliptinone	Total naphthoquinones
Crude	-	4.76 ± 0.067	0.34 ± 0.044	0.69 ± 0.035	5.80
VSC	2.46	124.42 ± 0.281	2.42 ± 0.014	12.13 ± 0.048	138.98
IRA-67	0.59	3.26 ± 0.066*	3.03 ± 0.056*	0.18 ± 0.052*	6.48
Water partition	11.15	38.52 ± 0.104*	1.70 ± 0.007*	4.94 ± 0.063*	45.16
Acid partition	14.78	28.37 ± 0.477*	1.39 ± 0.028*	4.18 ± 0.043*	33.93

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

[b] Yield (%) were calculated from weight of *P. indica* crude ethanol extract

* Significant difference (*P<0.05*) when compared with vacuum silica gel chromatography

Crude = *P. indica* crude ethanol extract

VSC = Vacuum silica gel chromatography

IRA-67 = Anion exchange resin (Amberlite® IRA-67)

Water partition = Partition between ethyl acetate and purified water

Acid partition = Partition between ethyl acetate and 5% acetic acid

The naphthoquinone-rich *P. indica* root extracts prepared by the silica gel vacuum chromatographic method was yellowish semisolid. Preparation of the extracts several batches revealed that the average yield of the extracts was 2.44 %w/w, compared to weight of the dried powdered roots. The extracts contained average total naphthoquinone content of 13.20% w/w (Table 12).

Table 15 The naphthoquinone content in naphthoquinone-rich *P. indica* root extracts

Lot.	Yield (% w/w)	Naphthoquinone content (% w/w ± S.D.)			
		Plumbagin	3,3'-biplumbagin	Elliptinone	Total naphthoquinones
1	2.38	11.69 ± 0.124	0.31 ± 0.066	1.26 ± 0.265	13.26
2	2.45	11.16 ± 1.271	0.46 ± 0.176	1.43 ± 0.081	13.05
3	2.50	11.31 ± 0.567	0.51 ± 0.131	1.48 ± 0.155	13.30
Average	2.44	11.39 ± 2.715	0.43 ± 1.056	1.39 ± 1.107	13.20 ± 0.13

Antibacterial activity against acne-involved bacteria

Antibacterial activity of naphthoquinone-rich *P. indica* root extracts, plumbagin, 3,3'-biplumbagin and elliptinone were evaluated against acne-involved bacteria including, *P. acnes, S. aureus* and *S. epidermidis.* The result revealed that plumbagin, 3,3'-biplumbagin and elliptinone possessed inhibitory effect against all tested bacteria, with MIC values of 0.024 - 12.50 µg/ml, 0.024 - 100 µg/ml and 0.78 - 100 µg/ml, respectively. The naphthoquinone-rich *P. indica* root extracts exhibited antibacterial activity against *P. acnes* and *S. aureus* with MICs was almost the same as those of plumbagin, but more potent than both 3,3'-biplumbagin and elliptinone. In addition, the antibacterial activities of the naphthoquinone-rich *P. indica* root extracts were much better than the crude ethanol extract of *P. indica* (Table 13).

MBC values of the naphthoquinone-rich *P. indica* root extracts against all tested bacterial were in the ranges of 6.25 - 50 µg/ml, which almost equal to those of plumbagin (Table 14). These results suggested the extracts possessed bactericidal activity against *P. acnes* and *S. aureus*, due to their MBCs were not higher than MICs more than two times.

Based on HPLC analysis, the total naphthoquinone content of naphthoquinone-rich *P. indica* root extracts was 13.20 ± 0.13 % w/w. Thus, the naphthoquinone-rich *P. indica* root extracts should contain total naphthoquinone not less than 13% w/w in order to give the good antibacterial activity against acne-involved bacteria.

Table 16 Minimum inhibitory concentration against acne-involved bacteria

Compounds or Extracts	Minimum inhibitory concentration (μg/ml)				
	P. acnes			*S. aureus*	*S. epidermidis*
	DMST 14916	DMST 21823	DMST 21824	ATCC 25923	ATCC 14990
Plumbagin	0.39	12.50	12.50	3.12	0.024
Elliptinone	6.25	50	50	100	0.78
3,3'-biplumbagin	1.56	25	25	100	0.024
NPEs	1.56	12.50	12.50	12.50	0.78
Ethanol extract	6.25	50	50	>100	3.12
Tetracycline	0.19	0.39	0.39	0.39	0.049

NPEs = naphthoquinone-rich *P. indica* root extracts

Table 17 Minimum bactericidal concentration against acne-involved bacteria

Compounds or Extracts	Minimum bactericidal concentration (μg/ml)				
	P. acnes			*S. aureus*	*S. epidermidis*
	DMST 14916	DMST 21823	DMST 21824	ATCC 25923	ATCC 14990
Plumbagin	25	50	50	12.50	3.12
Elliptinone	50	50	50	>100	100
3,3'-biplumbagin	50	50	50	>100	50
NPEs	50	50	50	25	6.25
Ethanol extract	50	50	50	>100	100
Tetracycline	25	50	50	12.5	3.12

NPEs = naphthoquinone-rich *P. indica* root extracts

Solubility of naphthoquinone-rich *P. indica* root extracts

Solubility is commonly expressed as a maximum equilibrium amount of a solute that can normally dissolve per amount of solvent or a maximum concentration of a saturated solution. These maximum concentrations are often expressed as grams of

solute per 100 ml of solvent. The solubility test of the naphthoquinone-rich *P. indica* root extracts is used to estimate the dissolution of the extract in various solvents. The result showed that the naphthoquinone-rich *P. indica* root extracts were freely soluble in chloroform, ethyl acetate, and ethanol. They were slightly soluble in methanol and propylene glycol, very slightly soluble in hexane and practically insoluble in water (Table 15). This implies that the naphthoquinone-rich *P. indica* root extracts contained most likely moderate non-polar compounds. The suitable solvents for the naphthoquinone-rich *P. indica* root extract should be therefore moderate non-polar solvents such as ethyl acetate, ethanol, and chloroform.

Table 18 Solubility of naphthoquinone-rich *P. indica* root extracts in various solvents

Solvent	Volume of solvent (ml) to dissolve 1 g of sample	Solubility term
Hexane	8,000	Very slightly soluble
Chloroform	2	Freely soluble
Ethyl acetate	2	Freely soluble
Methanol	700	Slightly soluble
Ethanol	2	Freely soluble
Propylene glycol	500	Slightly soluble
Water	> 10,000	Practically insoluble

Stability of naphthoquinone-rich *P. indica* root extracts

1. Effect of light on stability of the extracts

The extracts were kept in the well-closed containers and stored either under fluorescent light or protected from light, at room 30 ± 2°C for a period of four months. The result exhibited that under light conditions, color of the naphthoquinone-rich *P. indica* root extracts gradually faded. In contrast, the physical appearance of the extracts did not change through the period of four months when they were kept in the container protected from light. In addition, significant decrease of the plumbagin content was observed in the first week of both light and light protected conditions, while the content of 3,3'-biplumbagin and elliptinone were not significantly changed in the period of four

months for both conditions (Table 16, Figure 23 and 24). This finding suggests that the naphthoquinone-rich *P. indica* root extracts should be kept in a well-sealed closed container, protected from light, in order to stabilize the physical appearance.

Table 19 Naphthoquinone content of naphthoquinone-rich *P. indica* root extracts stored under light and protected from light conditions

Weeks	Naphthoquinone content (µg/ml; Mean ± S.D.)					
	Plumbagin		3,3'-biplumbagin		Elliptinone	
	Protected from light	Light	Protected from light	Light	Protected from light	Light
0	101.45±0.72	101.45±0.72	2.11±0.18	2.11±0.18	11.50±0.59	11.50±0.59
1	86.30±1.22	86.47±1.28	2.09±0.12	2.09±0.01	11.79±0.34	11.50±0.29
2	81.74±2.73	76.69±9.41	2.15±0.21	2.08±0.07	11.78±0.34	11.33±0.32
3	66.51±14.97	63.48±3.55	2.08±0.06	2.07±0.26	11.37±0.10	11.21±0.16
4	70.37±8.54	83.94±1.66	2.14±0.05	2.06±0.05	11.82±0.37	11.43±0.47
6	60.22±7.05	77.12±1.34	2.03±0.12	2.07±0.09	11.57±0.47	11.60±0.24
8	59.18±17.50	75.29±3.24	2.12±0.18	2.06±0.02	11.31±0.44	11.64±0.06
12	55.37±10.12	74.99±2.80	2.07±0.19	2.04±0.01	11.41±0.37	11.27±0.53
16	54.53±2.72	30.50±0.44	2.08±0.23	2.08±0.04	11.54±0.17	11.21±0.22

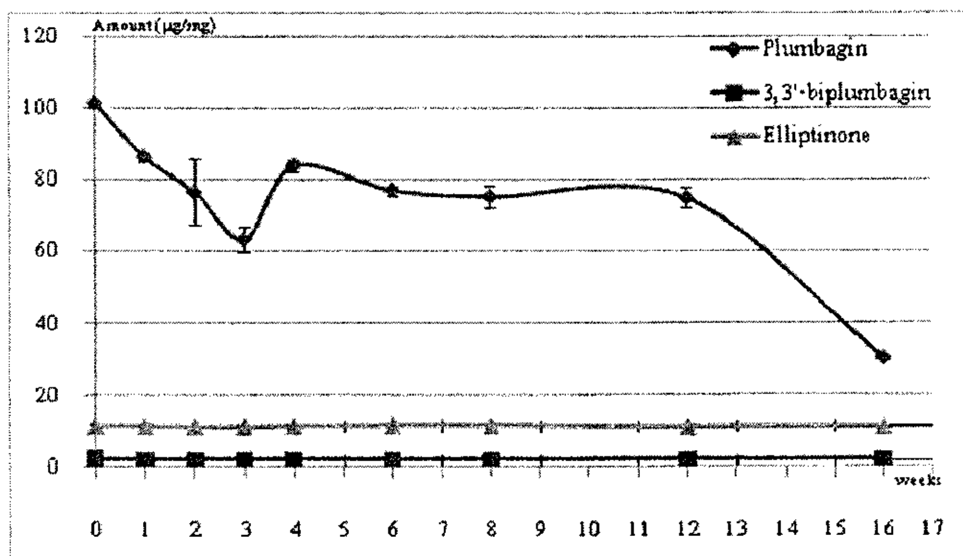

Figure 23 Stability profile of naphthoquinone content of naphthoquinone-rich *P. indica* root extracts stored under light conditions

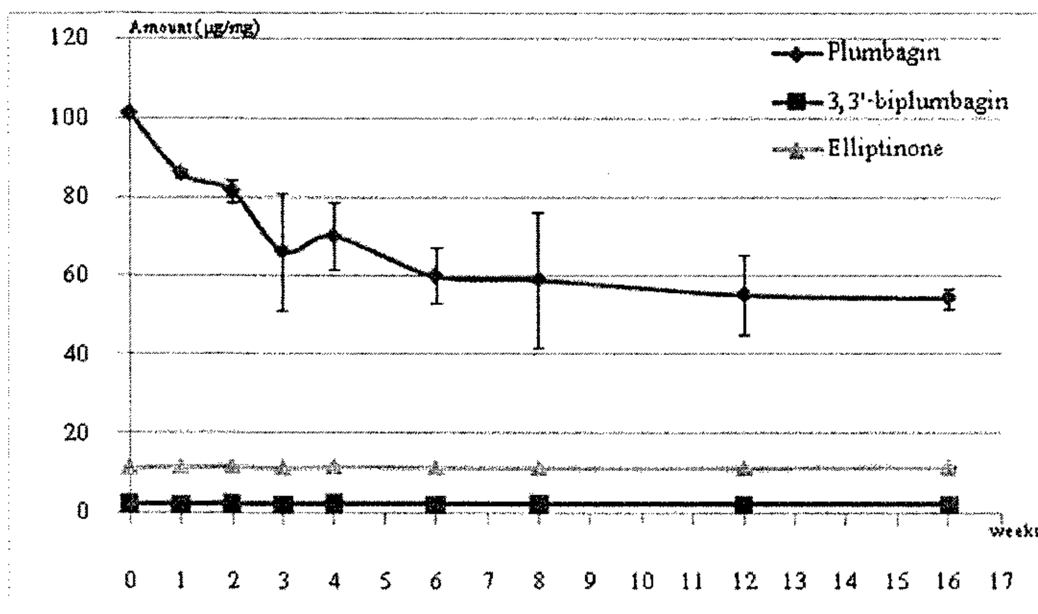

Figure 24 Stability profile of naphthoquinone content of naphthoquinone-rich *P. indica* root extracts stored under dark conditions

Effect of temperature on stability of the extracts

The effect of temperature on the stability of naphthoquinone-rich *P. indica* root extracts was examined under two temperatures, 4 ± 2°C and 30 ± 2°C, protected from light. The result revealed that significant decrease of plumbagin content was observed in the first week when the extracts were stored at 30 ± 2°C. In contrast, the content of plumbagin was not significantly change when the extracts were stored at 4 ± 2°C. However, the content of 3,3'-biplumbagin and elliptinone were not significantly change through the period of four months under both temperatures (Table 17 and Figure 25 and 26). This finding suggests that the naphthoquinone-rich *P. indica* root extracts should be kept at 4 ± 2°C in order to stabilize the naphthoquinone content.

Table 20 Naphthoquinone content of naphthoquinone-rich *P. indica* root extracts stored at 4°C and 30°C

Weeks	Naphthoquinone content (µg/ml; Mean±S.D.)					
	Plumbagin		3,3'-biplumbagin		Elliptinone	
	4°C	30°C	4°C	30°C	4°C	30°C
0	101.45±0.72	101.45±0.72	2.11±0.18	2.11±0.18	11.50±0.59	11.50±0.59
1	107.76±5.95	96.79±1.64	2.18±0.10	2.07±0.10	11.75±0.07	11.94±0.28
2	105.48±2.76	73.14±14.68	2.13±0.11	2.03±0.05	11.62±0.30	11.83±0.50
3	104.11±7.78	69.02±14.02	2.20±0.15	2.12±0.02	11.66±1.19	11.39±0.78
4	104.27±9.17	86.93±10.54	2.12±0.12	2.12±0.14	11.70±0.27	11.66±0.32
6	109.03±0.12	55.96±13.30	2.09±0.19	2.10±0.03	11.69±0.48	11.45±0.63
8	105.18±11.17	53.10±5.59	2.17±0.15	2.11±0.04	11.48±0.67	11.73±1.40
12	105.62±11.12	53.10±5.59	2.13±0.08	2.12±0.01	11.63±0.72	11.68±1.45
16	107.14±5.14	49.11±6.89	2.26±0.25	2.11±0.09	11.49±0.49	11.39±0.17

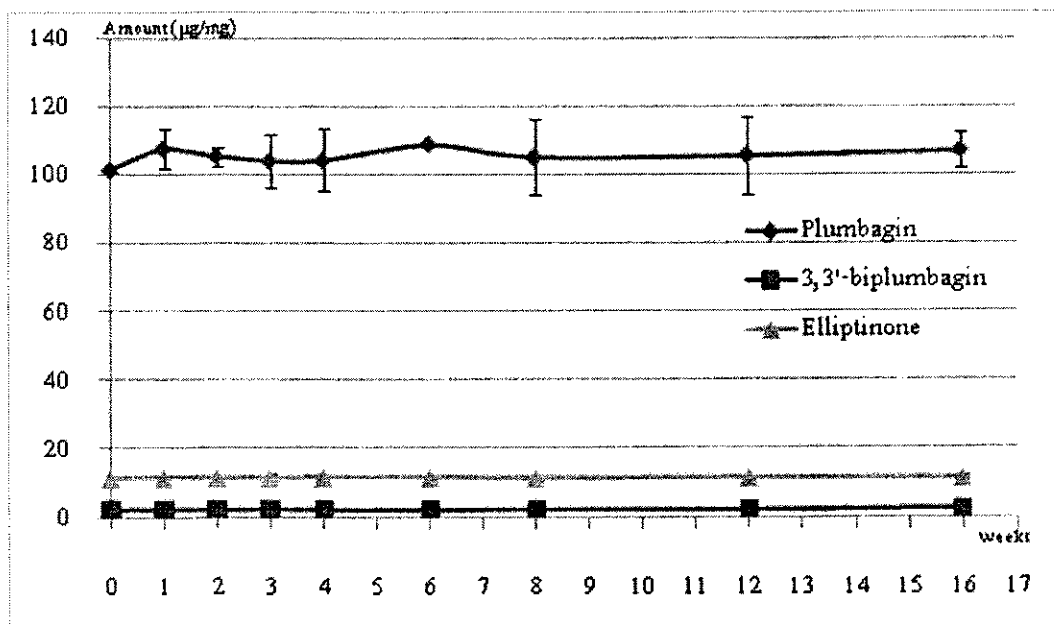

Figure 25 Stability profile of naphthoquinone content of naphthoquinone-rich *P. indica* root extracts stored at 4 ± 2°C

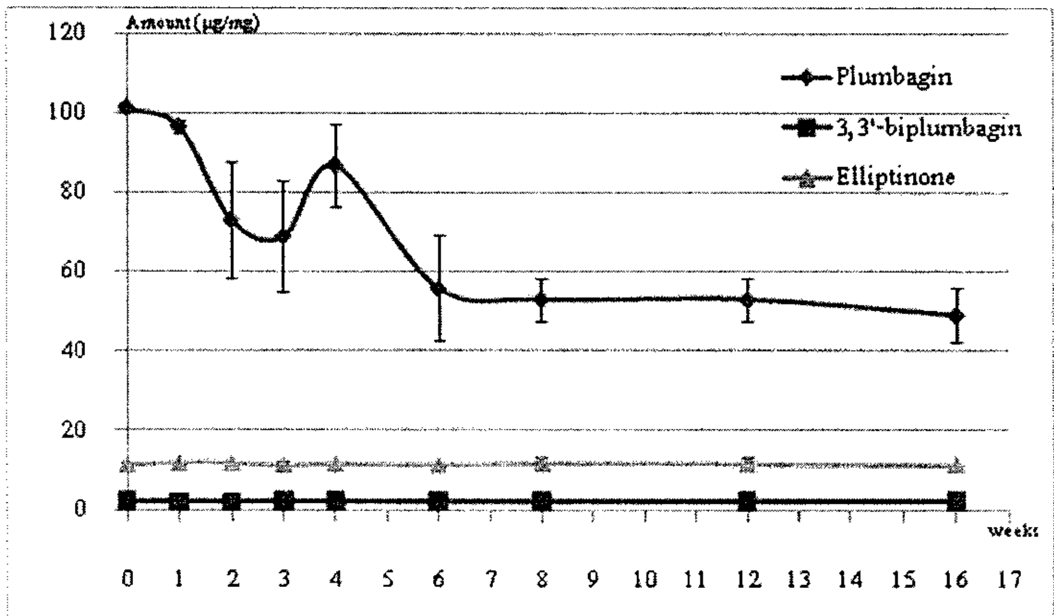

Figure 26 Stability profile of naphthoquinone content of naphthoquinone-rich *P. indica* root extracts stored at 30 ± 2°C

Effect of pH on stability of the extracts

Evaluation of acid-base stability of naphthoquinone-rich *P. indica* root extracts in solutions with three different pH values; 5.5, 7.0, and 8.0 was examined. The results showed that the solution with pH 5.5 was not change in physical appearance. In contrast, the color of the solutions with pH 7.0 and 8.0 was changed from yellow to orange (Figure 27 and 28). In addition, the naphthoquinone content was decreased in all tested pH (Table 18 - 20, and Figure 29 - 31). However, the naphthoquinones were more stable in the solution with pH 5.5 than in the solution with 7.0 and 8.0. It implies that all these three naphthoquinones are not stable when they are in solution. Thus, preparation of naphthoquinone-rich *P. indica* root extracts in an aqueous solution should be performed carefully.

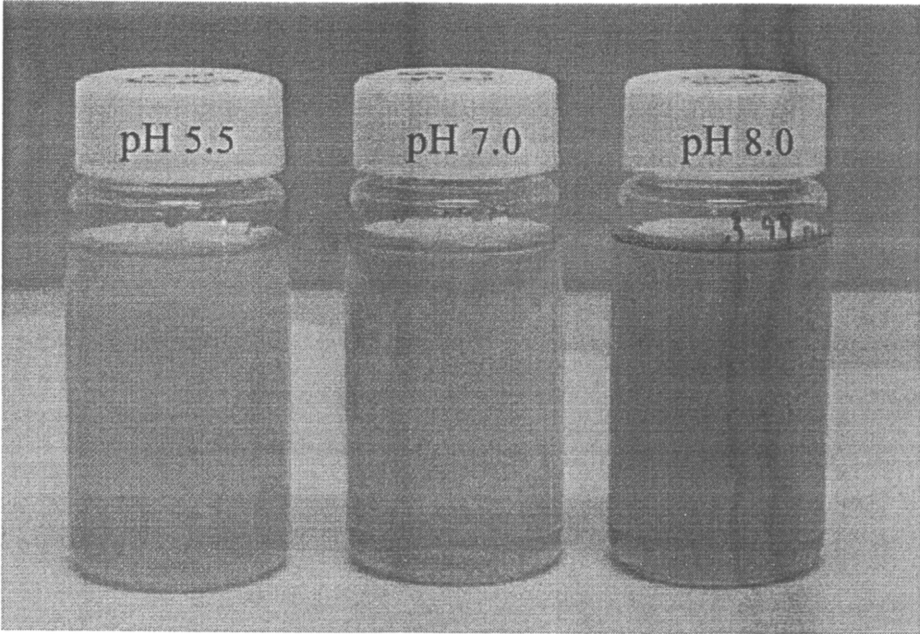

Figure 27 Solution of naphthoquinone-rich *P. indica* root extracts in various pH at week 0

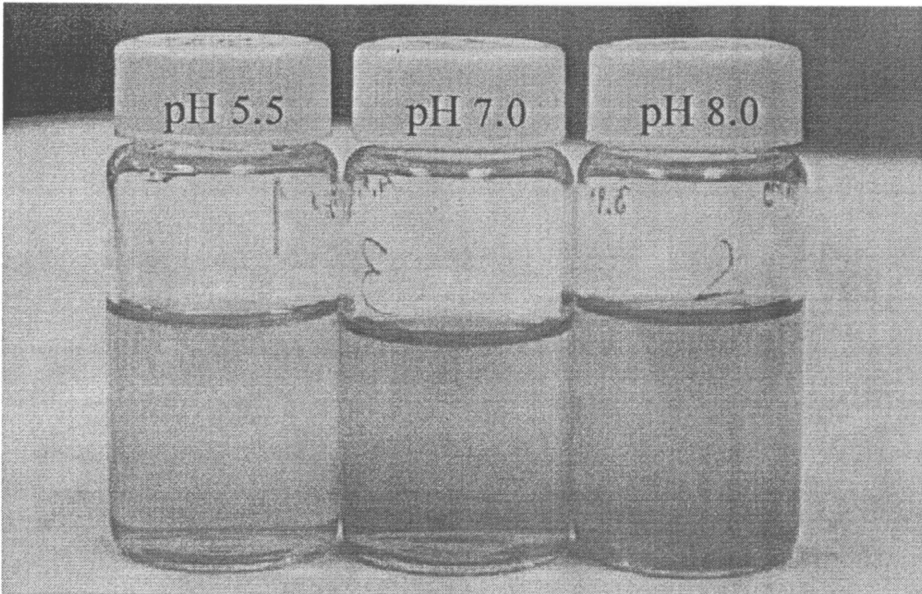

Figure 28 Solution of naphthoquinone-rich *P. indica* root extracts in various pH after 16-week storage

Table 21 Plumbagin content of naphthoquinone-rich *P. indica* root extracts in solution with pH 5.5, 7.0 and 8.0

Weeks	Plumbagin content (µg/ml; Mean±S.D.)		
	pH 5.5	pH 7.0	pH 8.0
0	137.46 ± 0.70	121.06 ± 0.17	104.20 ± 2.83
1	121.74 ± 2.48	96.40 ± 10.65	11.79 ± 0.70
2	119.63 ± 0.23	37.10 ± 1.77	5.35 ± 1.32
3	112.82 ± 4.21	23.38 ± 1.16	2.66 ± 0.34
4	110.80 ± 4.07	15.35 ± 1.02	0.00
6	105.37 ± 6.08	7.19 ± 0.88	0.00
8	98.78 ± 4.14	2.01 ± 0.97	0.00
12	98.78 ± 4.14	0.75 ± 0.75	0.00
16	87.50 ± 0.38	1.17 ± 0.18	0.00

Table 22 3,3'-biplumbagin content of naphthoquinone-rich *P. indica* root extracts in solution with pH 5.5, 7.0 and 8.0

Weeks	3,3'-biplumbagin content (µg/ml; Mean ± S.D.)		
	pH 5.5	pH 7.0	pH 8.0
0	3.51 ± 0.01	2.67 ± 0.09	1.09 ± 0.04
1	2.44 ± 0.03	0.40 ± 0.22	0.03 ± 0.10
2	2.30 ± 0.06	0.09 ± 0.01	0.09 ± 0.01
3	1.76 ± 0.15	0.00	0.00
4	1.38 ± 0.04	0.00	0.00
6	1.08 ± 0.02	0.00	0.00
8	0.94 ± 0.16	0.00	0.00
12	0.93 ± 0.16	0.00	0.00
16	0.77 ± 0.32	0.00	0.00

Table 23 Elliptinone content of naphthoquinone-rich *P. indica* root extracts in solution with pH 5.5, 7.0 and 8.0

Weeks	Elliptinone content (μg/ml; Mean ± S.D.)		
	pH 5.5	pH 7.0	pH 8.0
0	13.93 ± 0.46	13.00 ± 0.22	11.30 ± 0.38
1	13.58 ± 0.23	4.70 ± 0.38	0.71 ± 0.20
2	13.58 ± 0.23	3.52 ± 0.20	0.71 ± 0.20
3	11.73 ± 0.57	1.80 ± 0.12	0.00
4	11.36 ± 0.35	1.04 ± 0.10	0.00
6	10.84 ± 1.11	0.70 ± 0.04	0.00
8	9.47 ± 0.54	0.00	0.00
12	9.85 ± 0.27	0.00	0.00
16	9.69 ± 0.16	0.00	0.00

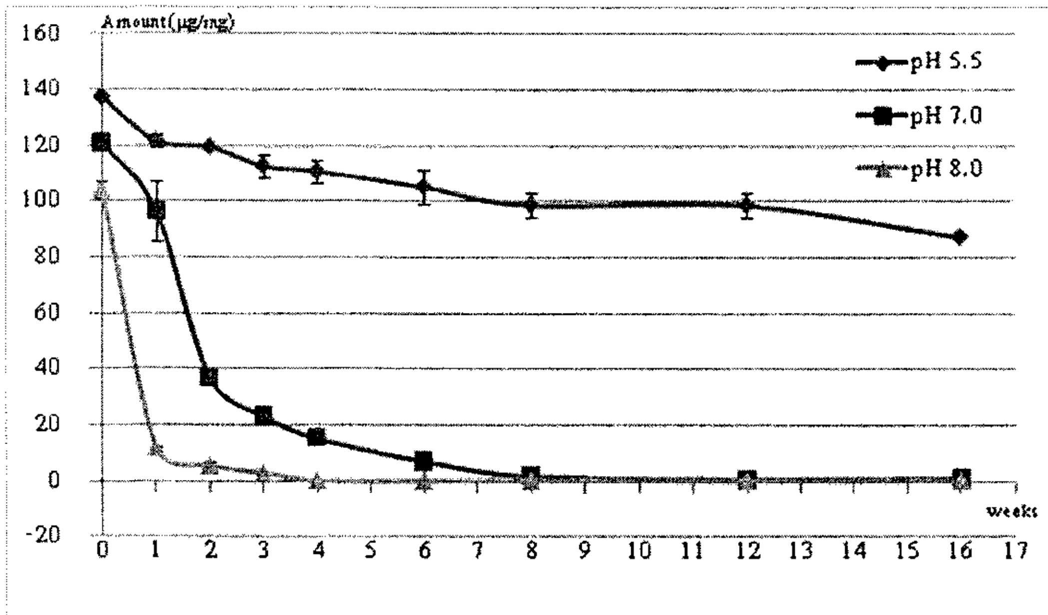

Figure 29 Stability profile of plumbagin content in the solution of naphthoquinone-rich *P. indica* root extracts in various pH

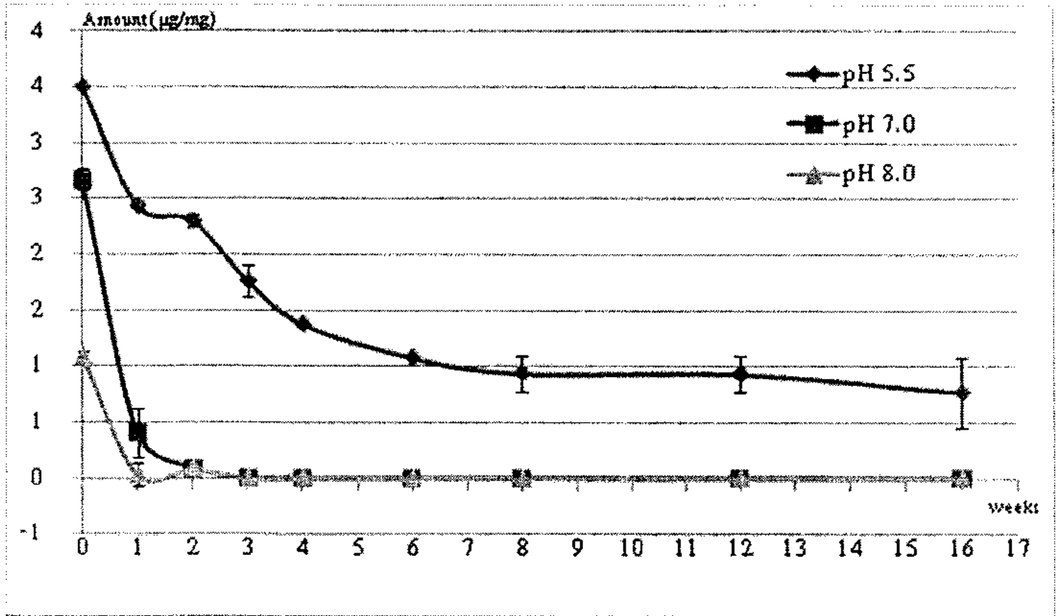

Figure 30 Stability profile of 3,3'-biplumbagin content in the solution of naphthoquinone-rich *P. indica* root extracts in various pH

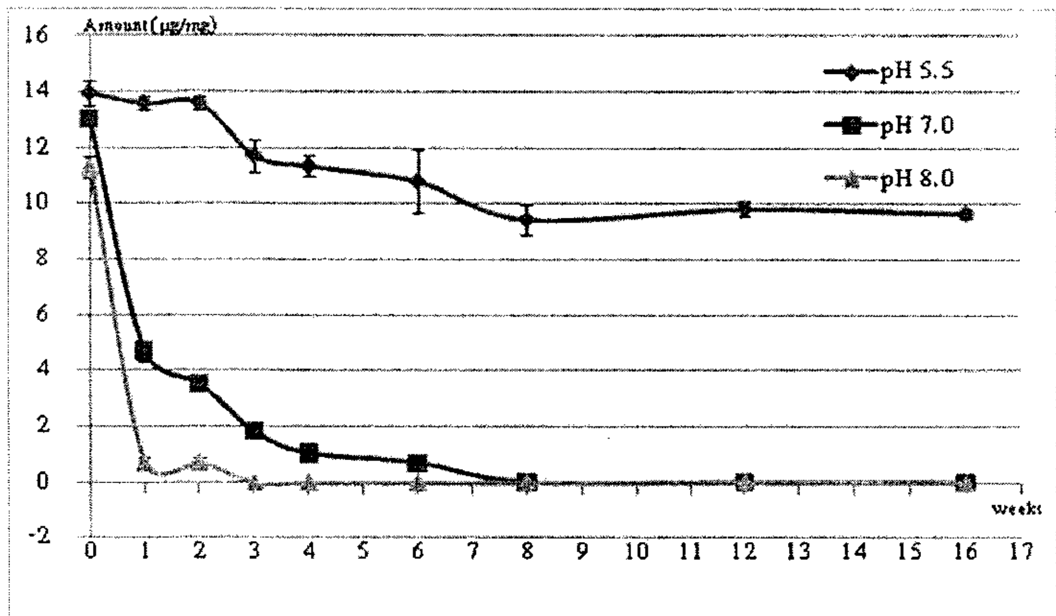

Figure 31 Stability profile of elliptinone content in the solution of naphthoquinone-rich *P. indica* root extracts in various pH

Effect of accelerated conditions on stability of the extract

The accelerated stability test of the naphthoquinone-rich *P. indica* root extracts was carried out using a stability chamber at 45 ± 2°C with 75% humidity. The result demonstrated that the physical appearance of the extracts was not change through the period of 4 months. Although the content of 3,3'-biplumbagin and elliptinone were not significantly changed in the period of 4 months, significant decrease of plumbagin content was observed in the first week (Table 21; Figure 32). This result indicated that the naphthoquinone-rich *P. indica* root extracts were not stable when kept in the high temperature and humidity conditions.

Table 24 Naphthoquinone content of naphthoquinone-rich *P. indica* root extracts stored under accelerate conditions

Weeks	Naphthoquinone content (μg/ml; Mean ± S.D.)		
	Plumbagin	3,3'-biplumbagin	Elliptinone
0	101.45 ± 0.723	2.11 ± 0.176	11.50 ± 0.568
1	59.64 ± 0.433	2.06 ± 0.282	11.60 ± 0.739
2	40.24 ± 7.599	2.08 ± 0.197	11.58 ± 0.755
3	29.30 ± 4.818	2.06 ± 0.025	11.09 ± 0.377
4	23.96 ± 7.989	2.08 ± 0.090	11.13 ± 0.387
6	13.25 ± 3.418	2.09 ± 0.112	11.34 ± 0.368
8	7.29 ± 0.933	2.05 ± 0.330	10.66 ± 1.577
12	7.29 ± 0.933	2.03 ± 0.322	11.41 ± 0.368
16	6.17 ± 2.064	2.05 ± 0.057	11.19 ± 0.608

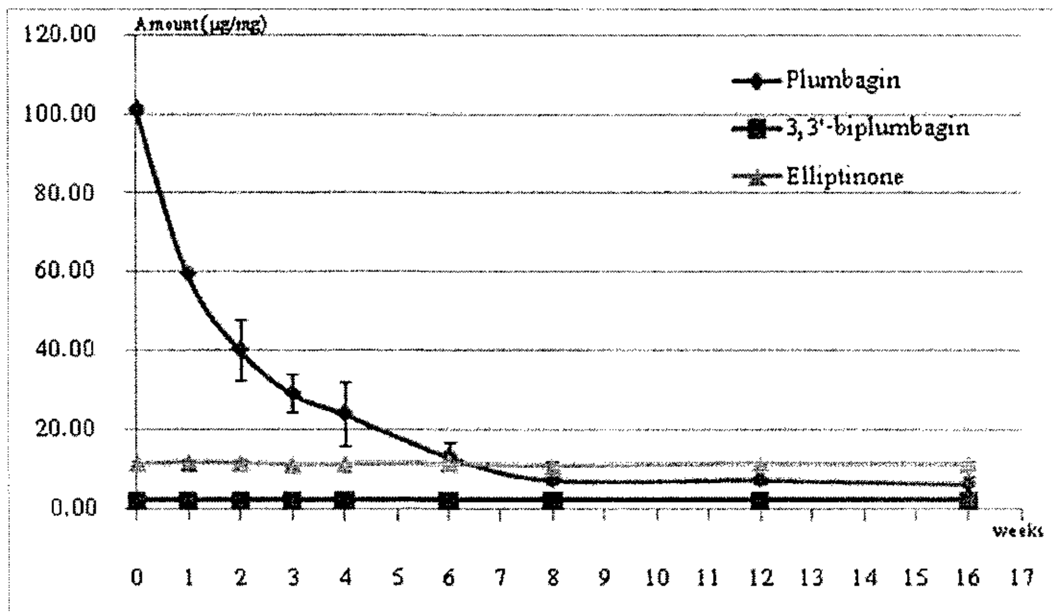

Figure 32 Stability profile of naphthoquinone content under accelerated conditions (45 ± 2°C and 75% humidity)

CONCLUSIONS

1. The ethyl acetate of *P. indica* exhibited the highest antibacterial activity against acne involved bacteria including *P. acnes*, *S. aureus*, and *S. epidermidis*.
2. Plumbagin, 3,3'-biplumbagin and elliptinone were isolated from *P. indica* roots and used as the indicative markers for quality control of *P. indica* root extracts. For 3,3'-biplumbagin and elliptinone, this is the first report of discovery in *P. indica* roots.
3. A simple, specific, precise, accurate, and reproducible HPLC method has been developed to quantify the naphthoquinones in *P. indica* root extracts. The simultaneous quantitative determination of plumbagin, 3,3'-biplumbagin and elliptinone provides useful marker information for the quality control of *P. indica* root extracts.
4. Ethanol is a suitable solvent for the extraction of naphthoquinones from *P. indica* roots.
5. Vacuum silica gel chromatographic method is a suitable fractionation method for preparation of naphthoquinone-rich *P. indica* root extracts.
6. The naphthoquinone-rich *P. indica* root extracts exhibited satisfactory antibacterial activity against all tested bacteria, with the MIC values almost the same as those of plumbagin.
7. The naphthoquinone content in naphthoquinone-rich *P. indica* root extracts was set as to contain total naphthoquinone content not less than 13% w/w.
8. The naphthoquinones-rich *P. indica* root extracts contain most likely moderate non-polar naphthoquinones. The suitable solvents of the extracts should be moderate non-polar solvents, such as ethyl acetate, ethanol, and chloroform.
9. Stability evaluations of the naphthoquinones-rich *P. indica* root extracts in several conditions through the period of 4 months revealed that the extract should be kept in well-sealed closed containers, protected from light, and stored in cooled place (4°C). The aqueous solutions of the extract are not stable either in acid or base conditions.

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APPENDIX

Preparation of Brain Heart Infusion agar (BHI agar)

Brain Heart Infusion	37 g
Bacto agar	15 g
Distill water	1000 ml

Preparation of Brain Heart Infusion broth (BHI broth)

Brain Heart Infusion	37 g
Distill water	1000 ml

Preparation of Mueller-Hinton agar

Mueller Hinton Agar	38 g
Distill water	1000 ml

Preparation of Mueller-Hinton broth

Mueller Hinton Broth	21 g
Distill water	1000 ml

Preparation of Glycerol broth

Tryptose	10 g
NaCl	5 g
Beef extract (Protose BE)	3 g
Yeast extract	5 g
Cysteine HCl	0.4 g
Glucose	1 g
Na_2HPO_4	4 g
Glycerol	150 ml
Distill water	850 ml

Preparation of 0.5 McFarland standard

1 % v/v H_2SO_4	99.5 ml
1.175 % w/v $BaCl_2$	0.5 ml

PPP001

IN VITRO ANTIBACTERIAL ACTIVITY OF SELECTED THAI MEDICINAL PLANTS USED TO TREAT ACNE VULGARIS

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Objective: To evaluate antibacterial activity of Thai medicinal plants against acnes-involved bacteria, including *Propionibacterium acnes* a major cause of acne vulgaris and skin normal flora bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Methods: Ethyl acetate and methanol extracts of 20 selected Thai medicinal plants were tested for antibacterial activity against *P. acnes*, *S. aureus* and *S. epidermidis* using disc diffusion method. The antibacterial active extracts were subsequently subjected to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using broth dilution method.

Results: The disc diffusion method demonstrated that 19 plant extracts were capable of inhibiting the growth of *P. acnes*, while 22 and 20 plant extracts inhibited the growth of *S. aureus* and *S. epidermidis*, respectively. The ethyl acetate extract of *Plumbago indica* roots exhibited the strongest antibacterial activity against *P. acnes* and *S. epidermidis* with MIC values of 4.88 and 2.44 μg/mL and MBC values of 39.10 and 78.10 μg/mL, respectively. Although the ethyl acetate extracts of *Rhinacanthus nasutus*, *Allium sativum*, *Arcangelisia flava* and the methanol extract of *Quercus infectoria* exhibited the strongest inhibitory activity against *S. aureus* with the same MIC value of 78.10 μg/mL, only the ethyl acetate extract of *R.*

nasutus showed the strongest bactericidal activity with the MBC equal to the MIC value.

Conclusions: Ethyl acetate extract of *P. indica* roots exhibited the strongest antibacterial activity against *P. acnes* and *S. epidermidis*. It would be an interesting for further study on an alternative treatment of acne vulgaris.