Study on antibacterial activity against acne-involved bacteria and stability of naphthoquinone rich *Plumbago indica* root extract

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ชื่อวิทยานิพนธ์ การศึกษาฤทธิ์ต้านเชื้อแบคทีเรียที่ก่อให้เกิดสิวและความคงตัวของสารสกัดรากเจตมูลเพลิงแดงที่มีสารแนพโธควิโนนในปริมาณสูง
ผู้เขียน นายเสริมวุฒิ แก้วบำรุง
สาขาวิชา เภสัชศาสตร์
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บทคัดย่อ

สารประกอบแนพโธควิโนน 3 ชนิด ได้แก่ plumbagin, 3,3'-biplumbagin และ elliptinone ซึ่งแยกได้จากสารสกัดรากเจตมูลเพลิงแดงถูกแยกเพื่อนำมาใช้เป็นสารมาตรฐานในการวิเคราะห์ปริมาณสารแนพโธควิโนน และในการเตรียมสารสกัดรากเจตมูลเพลิงแดงที่มีสารแนพโธควิโนนในปริมาณสูง วิธีวิเคราะห์ปริมาณสารประกอบแนพโธควิโนนในสารสกัดรากเจตมูลเพลิงแดงด้วยเทคนิค reverse phase high-performance liquid chromatography ได้ถูกพัฒนาขึ้น โดยใช้คอลัมน์ชนิด Phenomenex® ODS column (150 × 4.6 mm, 5 μm particle size) กับโมบายเฟสซึ่งเป็นตัวทำละลายผสมระหว่าง 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% และกราฟมาตรฐานของสารประกอบแนพโธควิ-โนนทั้ง 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 อย่างไรก็ตามสารสกัดรากเจตมูลเพลิงแดงที่มีสารแนพโธควิ-โนนในปริมาณสูงมีฤทธิ์ใกล้เคียงกับสาร 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

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ABSTRACT

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 increase 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).

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CHAPTER 1

INTRODUCTION

1.1 Background

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 belong a common disease in adolescents and does not harmful for human life but it may cause of permanent scar and lead to significant emotional distress. (National Institute of Arthritis and Musculoskeletal and Skin Diseases, 2006)

Although mechanism of acnes has never been clearly proven, believe 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 *Staphylococcus epidermidis* (Baron, 1996). *P. acnes* has been described as the most important organism for development of inflammatory acne, which produce a variety of enzyme, include lipase and protease to hydrolysis 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 therapy to treat acne vulgaris includes topical comedolytic (retinoid and derivative, benzoyl peroxide, azaleic acid) and antibiotics (doxycycline, tetracycline, minocycline, clindamycin and erythromycin) for both oral and topical (Krautheim, 2004).

Focusing on topical therapy retinoid and derivatives including tretinoid, isotretinoin, adapalene, tazarotene, motretinide, retinoyl □-glucuronide and retinaldehyde present as effective therapy with their mechanism to suppress the development of new microcomedones, inhibit inflammatory reactions *via* inhibition of the release of prostaglandins, leukotrienes and proinflammatory cytokines such as interferon gamma and IL-1□. Benzoyl peroxide has strong antimicrobial, slight anti-inflammatory and anticomedogenic effects. Azaleic acid has effect on chemotaxis suppression, anti-inflammation, modifies epidermal keratinization and exhibits 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.

For many year, medicinal plants became an 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 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 (Kaewbumrung and Panichayupakaranant, 2008). *P. indica* was therefore selected for this study.

Plumbagin, the naphthoquinone from the root part of *P. indica*, was well known as the most active compound against several bacterial strains (Mallavadhani *et al.,* 2002). So, plumbagin and its derived derivatives were used as a standard marker to prepare the naphthoquinone-rich *P. indica* root extract. In addition, the quantitative analytical method was necessary for monitoring naphthoquinone contents in the extract. The simple, rapid and high degree of sensitivity, precision and accuracy than would be developed and used as a valuable informative tool for naphthoquinones determination. Moreover, the solubility and stability of the extract were then studied at various solvents and conditions which useful as the necessary information for anti acne preparations from *P. indica* root extract in the future.

1.2 Objectives

1) To isolate naphthoquinones from *Plumbago indica* roots and evaluate their antibacterial activity against *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis*

2) To develop an 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

1.3 Literature review

Medicinal plants had been studied for a long time to evaluate their pharmaceutical activities and finding of 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 is 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).

After that, 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 an 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 (ปริศนา นิยมคำ, 2549).

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).

1.3.1 *Plumbago indica* L.

Figure 1-1 *Plumbago indica* L.

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

Family name: Plumbaginaceae

Synonym: *Plumbago rosea* L.

Common name: ปิดปิวแดง (northern-east, northern), ไฟใต้ดิน (southern), คุ้ยวู่ (Khanchanaburi), Rose colored leadwort, Indian leadwort, Fire plant, Official leadwort, Kan-gyok-ni (Burmese)

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 are 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).

1.2.1.1 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).

1.2.1.2 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 1-2), plumbaginol (Figure 1-3), leucodelphinidin (Figure 1-4) and steroids (e.g. campesterol, sitosterol and stigmasterol) (Figure 1-5) (Schmelzer and Gurib-Fakim, 2008).

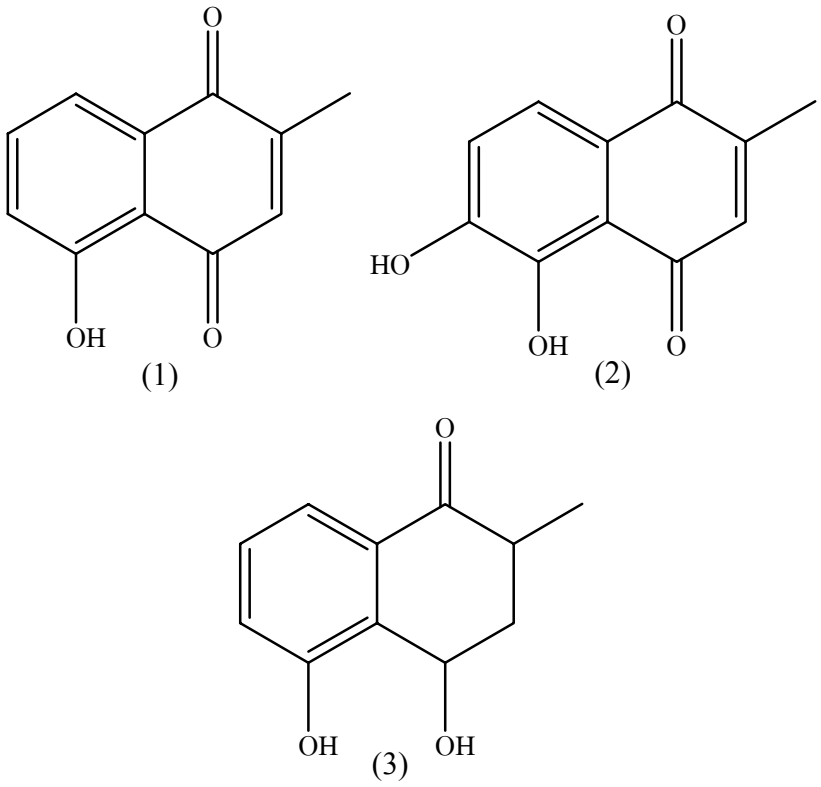

Figure 1-2 Chemical structures of naphthoquinone from *P. indica* include plumbagin (1), 6-hydroxy plumbagin (2) and isoshinanolone (3)

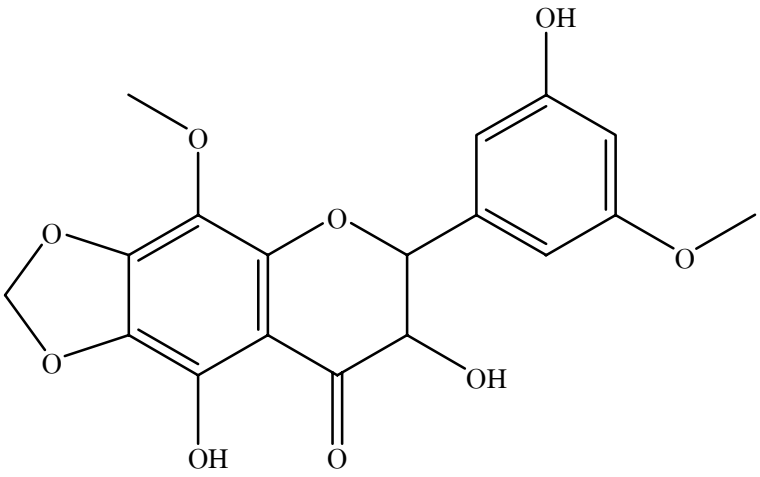

Figure 1-3 Chemical structures of plumbaginol

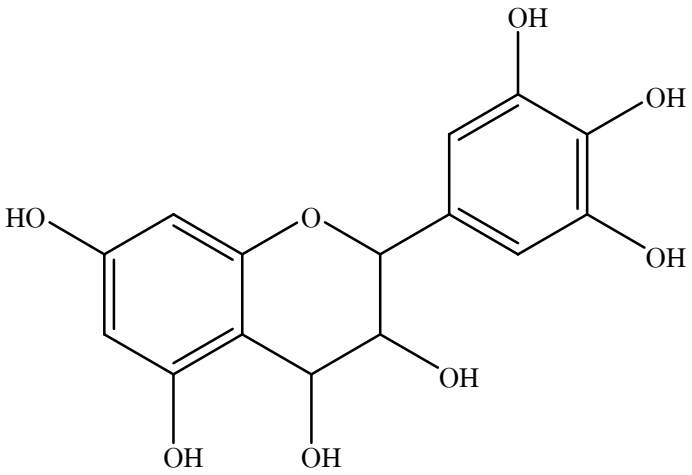

Figure 1-4 Chemical structures of leucodelphinidin

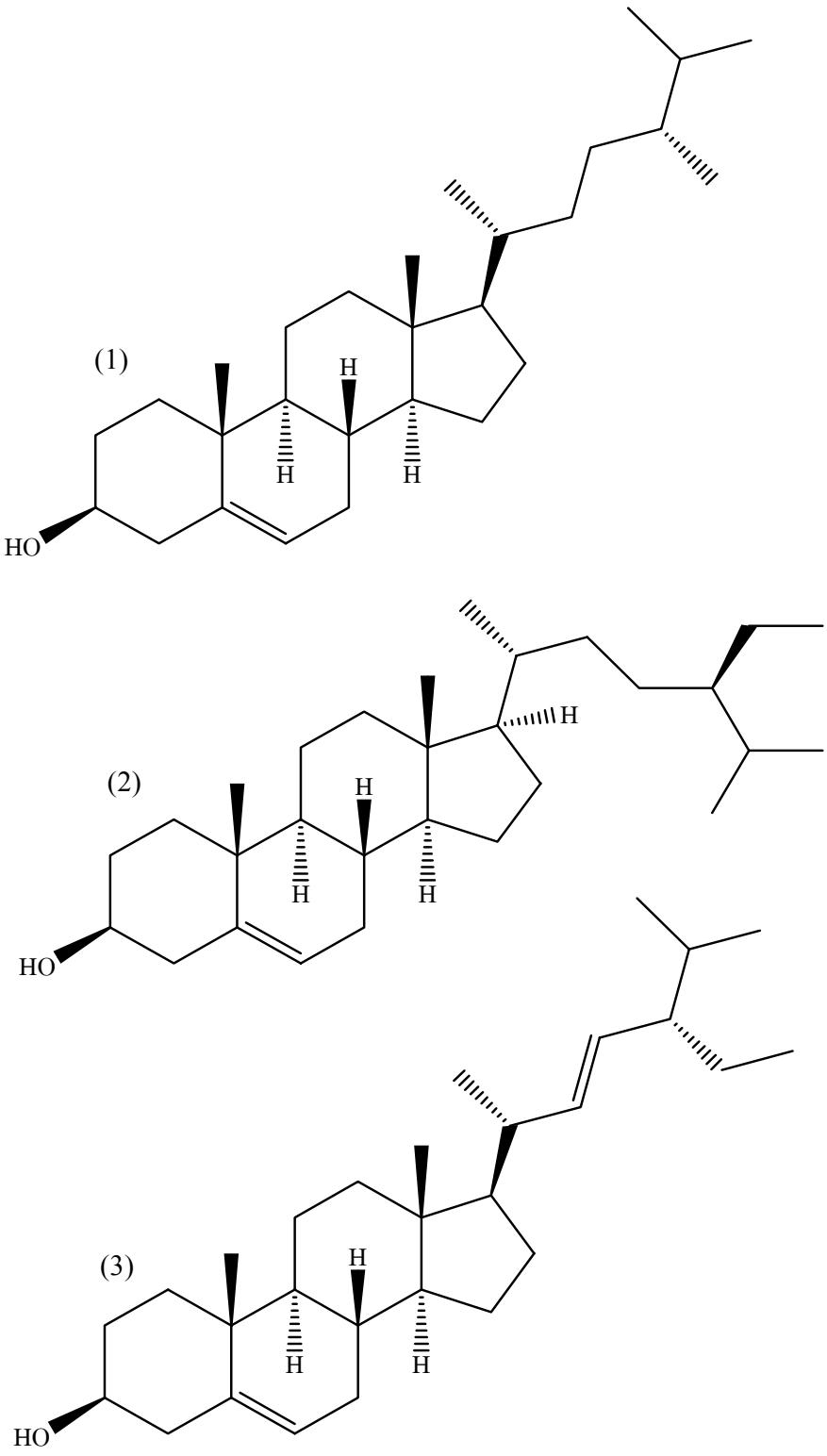

Figure 1-5 Chemical structures of steroids from *P. indica* include campesterol (1), sitosterol (2) and stigmasterol (3)

1.2.1.3 Pharmacological properties of *Plumbago indica*

Antifungal activity

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

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 value 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).

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).

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 antiestrogenic activity. Histological studies of the uteri further confirmed the estrogenic activity of the acetone extract (Sheeja *et al*., 2009).

1.3.2 Plumbagin

Plumbagin, the most active naphthoquinone derived from the species of *Plumbago, Drosera* and *Diospyros*, had 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:

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).

Anti-inflammatory activity

Plumbagin exhibited an 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 A induced IL-2, IL-4, IL-6 and IFN-γ cytokines (Checker *et al.,* 2009).

Antimalarial activity

It has been reported that plumbagin exhibited 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).

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 on an 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 and coworker (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*.

Mutagenic activity

Plumbagin was reported as an antimutagenic activity against *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).

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 antiovulatory action (Kini *et al.*, 1997).

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 diestrus 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).

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).

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).

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).

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*).*

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).

1.3.3 *Propionibacterium acnes*

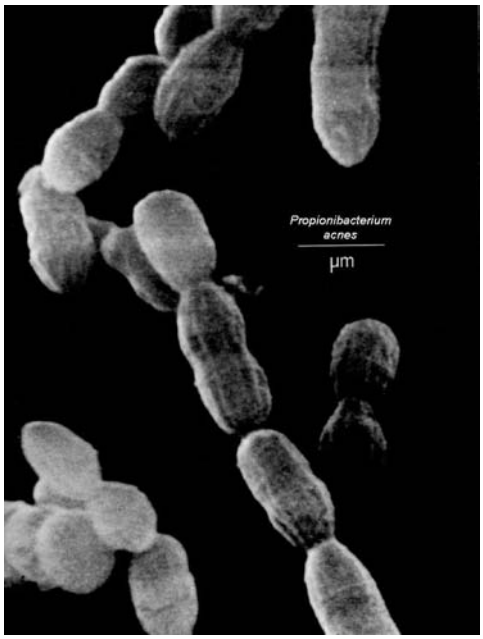

Figure 1-6 *Propionibacterium acnes*

Propionibacterium acnes (synonym: *Corynebacterium acnes* and *Bacillus acnes*) is a commensal, non-sporulating bacilliform (rod-shaped), gram-positive bacterium found in a variety of locations on the human body including the skin, mouth, urinary tract and areas of the large intestine. *P. acnes* is most commonly associated with its implicated role as the predominant cause of the common inflammatory skin condition acne vulgaris. It is primarily anaerobic and has an optimal growing temperature of 37 °C.

P. acnes' genome codes for a wide variety of metabolic products. Metabolic analysis has shown that *P. acnes* had the ability to live in anaerobic as well as "microaerobic" conditions. It has the key metabolic requirements to carry out oxidative phosphorylation, Krebs

cycle, Embden-Meyerhof pathway and the pentose phosphate pathway. Under *in vitro* anaerobic conditions, *P. acnes* can grow permissively on media such as glucose, glycerol, ribose, fructose, mannose and N-acetylglucosamine. *In vivo*, *P. acnes* produce various lipases to digest excess skin oil and sebum in the pilosebaceous units (regions that contains the hair follicle and sebaceous gland) of adolescent and adult human skin. For energy, *P. acnes* can employ a fermentative process yielding byproducts like short-chain fatty acids and propionic acid from which it gets its name. In addition to fermentation, *P. acnes* can utilize various other anaerobic pathways deriving energy with the help of enzymes such as nitrate reductase, dimethyl sulfoxide reductase and fumarate reductase (Rosenberg, 1969)

CHAPTER 2

METERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The dried roots of *P. indica* (Figure 2-1) were purchased from an herbal drug store in Hat-Yai, Thailand. 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. Thai traditional knowledge of herbal medicines was used to distinguish *P. indica* roots from *P. zeylanica* roots. The roots of *P. indica* are turning dark when dried and the roots cause skin irritation and make the skin redness when contact to the skin, while this phenomenon is not take place with *P. zeylanica* roots. The roots were then reduced to powder using a grinder and a sieve no. 45.

Figure 2-1 Dried roots of *P. indica*

2.1.2 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.

2.1.3 Chemicals

2.1.3.1 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.

2.1.3.2 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)

2.1.3.3 For HPLC analysis

- Milli-Q grade 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)

2.1.4 Instruments

The equipments used in this study were listed in Table 2-1.

Table 2-1 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

2.2 Methods

2.2.1 Preparation of ethyl acetate extract of *P. indica* root

The dried powder of *P. indica* roots (0.5 kg) was extracted with ethyl acetate (1 l ×3) under reflux conditions for 1 hour. The pooled extracts were evaporated to dryness under reduced pressure. The dark brown semisolid with acrid odor was obtained.

2.2.2 Isolation of naphthoquinones from *P. indica* root

The ethyl acetate extract of *P. indica* root (20 g) was subjected to silica gel vacuum chromatography (Figure 2-2) 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 *in vaccuo* 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.

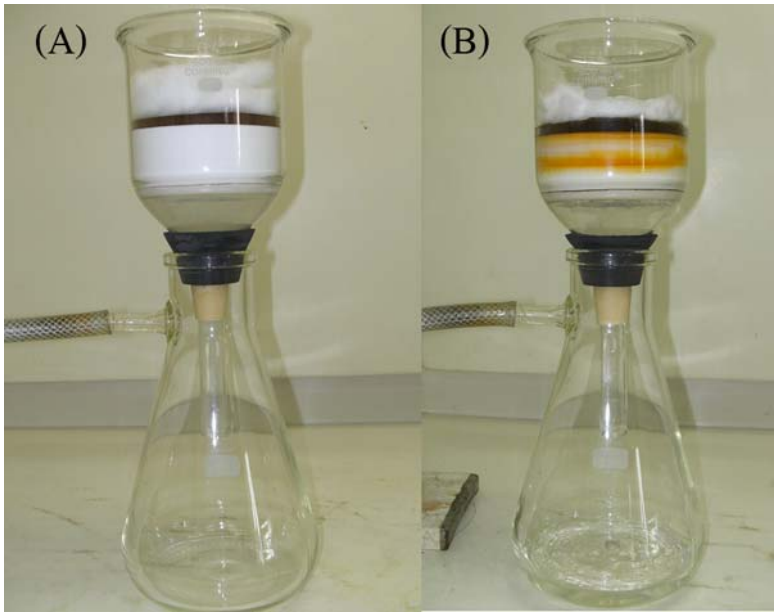

Figure 2-2 Vacuum silica gel chromatography (A) packed column before elution (B) after elution with a mixture of hexane and ethyl acetate

2.2.3 HPLC method

The HPLC analytical method was developed from the method used to quantitative analysis of 4 naphthoquinones, 1,4-naphthoquinone, lawsone, juglone and plumbagin in *Dionaea muscipula* Ell. crude extract (Babula *et al.,* 2006).

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 25ºC 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.

2.2.3.1 Standard solution

Separate stock solutions of the reference standards, 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.

2.2.3.2 Sample preparation

P. indica dried powder (500 mg) was extracted with ethanol (25 ml) under reflux conditions for an hour (×3). The extract was then filtered and the solvent was evaporated to dryness *in vacuo*. The sample was then accurately weighted and dissolved in methanol to prepared stock sample solution at concentration of 10 mg/ml. The samples were filtered through

0.45 □m membrane filter and analyzed immediately after extraction in order to avoid possible chemical degradation.

2.2.4 Validation of analytical method

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

2.2.4.1 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.

2.2.4.2 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 curve was analyzed using the linear least-squares regression equation derived from the peak area.

2.2.4.3 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 calculated actual recoveries. The amount of each analyte was determined in triplicate and percentage recoveries were then calculated.

2.2.4.4 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).

2.2.4.5 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.

2.2.5 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.

2.2.6 Determination of fractionation method

2.2.6.1 Preparation of *P. indica* root extract

P. indica dried powder (1 kg) was successively extracted with the suitable solvent from 2.2.5 (5 l ×3) under reflux conditions for 1 hour. The pooled extracts were dried *in vacuo* and subsequently divided to purify by different technique.

2.2.6.2 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 *in vacuo.*

2.2.6.3 Fractionation by silica gel vacuum chromatography

A sintered glass column (13 cm in diameter) was packed with silica gel approximately 5 cm high. The *P. indica* extract (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 *in vacuo.*

2.2.6.4 Partition by liquid-liquid extraction

Partition with purified water

The *P. indica* extract (20 g) was dissolved in ethyl acetate (150 ml) and then repeatedly partitioned with water (150 ml). The pooled ethyl acetate phases were then dried *in vacuo.*

Partition with 5% acetic acid in water

The *P. indica* extract (20 g) was dissolved in ethyl acetate (150 ml) and then repeatedly partitioned with 5% acetic acid in water (150 ml). The pooled ethyl acetate phases were then dried *in vacuo.*

2.2.7 Antibacterial activity against acne-involved bacteria

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.

2.2.7.1 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 inoculum was 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 inoculum was added to each well then incubated at properly conditions as described in 2.2.7.

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.

2.2.7.2 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.

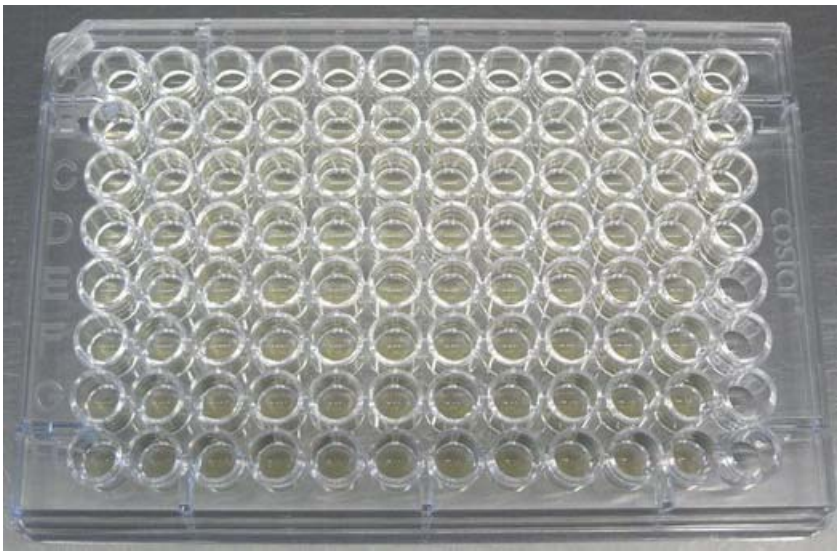

Figure 2-3 Sterilized 96-well plates for determination of MIC before added Alamar blue

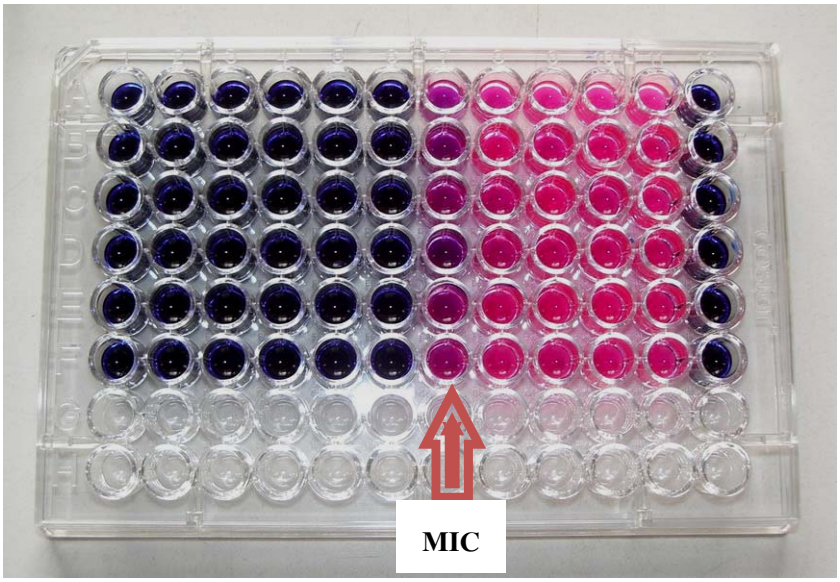

Figure 2-4 Determination of MIC using 96-well plates after added Alamar blue and incubate for 5 hours at 37°C

2.2.8 Determination of solubility (British Pharmacopoeia Commission, 2001)

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 follow (Table 2-2).

Table 2-2 The solubility term used to evaluated naphthoquinones rich *P. indica* root extract solubility

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

2.2.9 Stability evaluation of naphthoquinone- rich *P. indica* root extract

(Sakunpak, *et al.,* 2009)

2.2.9.1 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.

2.2.9.2 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.

2.2.9.3 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.

2.2.9.4 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.

2.2.10 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 $p<0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation of naphthoquinones from *P. indica* root

Dried powder of *P. indica* root was extracted with ethyl acetate under reflux conditions to yield ethyl acetate extract (Figure 3-1). 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 3-1 Crude 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 compound gave red to purple spot when sprayed with 10% KOH in methanol (Figure 3-2). These pure compounds were then subjected to determination of their chemical structures by NMR and MS.

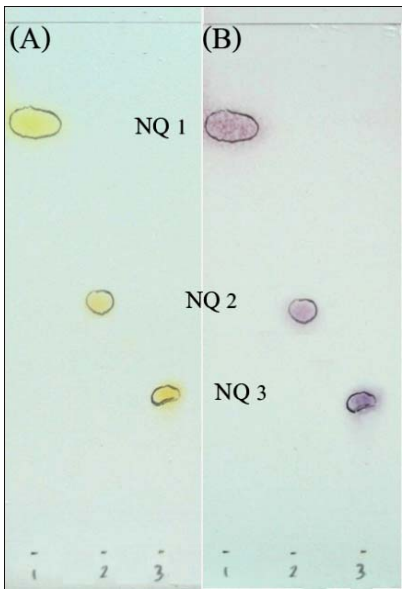

Figure 3-2 TLC chromatogram of (1) NQ 1, (2) NQ 2 and (3) NQ 3 developed in hexane and ethyl acetate (9.6:0.4 v/v), (A) before spray with 10% KOH and (B) after spray with 10% KOH

3.2 Structure identification

3.2.1 Identification of NQ 1

NQ 1 had the same retention time as the standard plumbagin when determined by the HPLC system (Figure 3-3). NQ 1 was therefore identified as plumbagin (Figure 3-4) when compared its spectral data with those previously reported by Philip *et al*. (1999).

The ^{1}H NMR spectrum of NQ 1 (Table 3-1, Figure 3-5) 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).

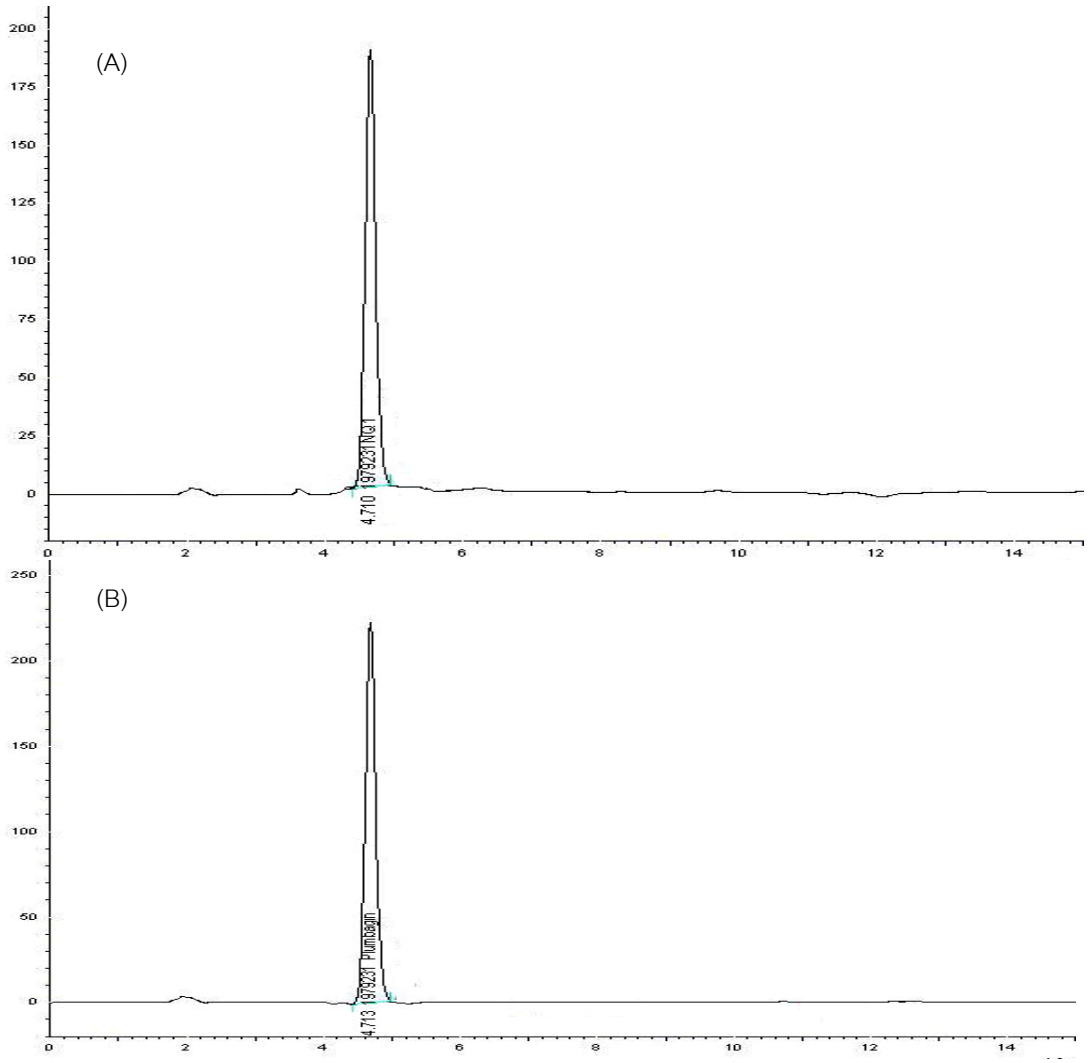

Figure 3-3 HPLC chromatogram of NQ 1 (A) compare to plumbagin (B) determined by the developed method; mobile phase consist of methanol and 5% aqueous acetic acid (80:20 v/v), flow rate 0.85 ml/min and detection wavelength set at 260 nm

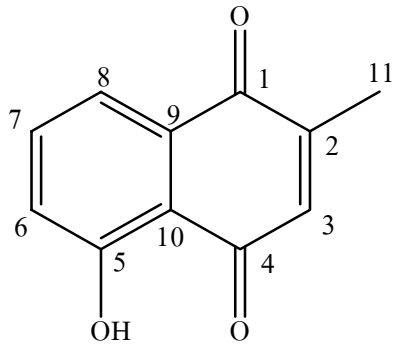

Figure 3-4 Chemical structure of plumbagin

Table 3-1 ^{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)

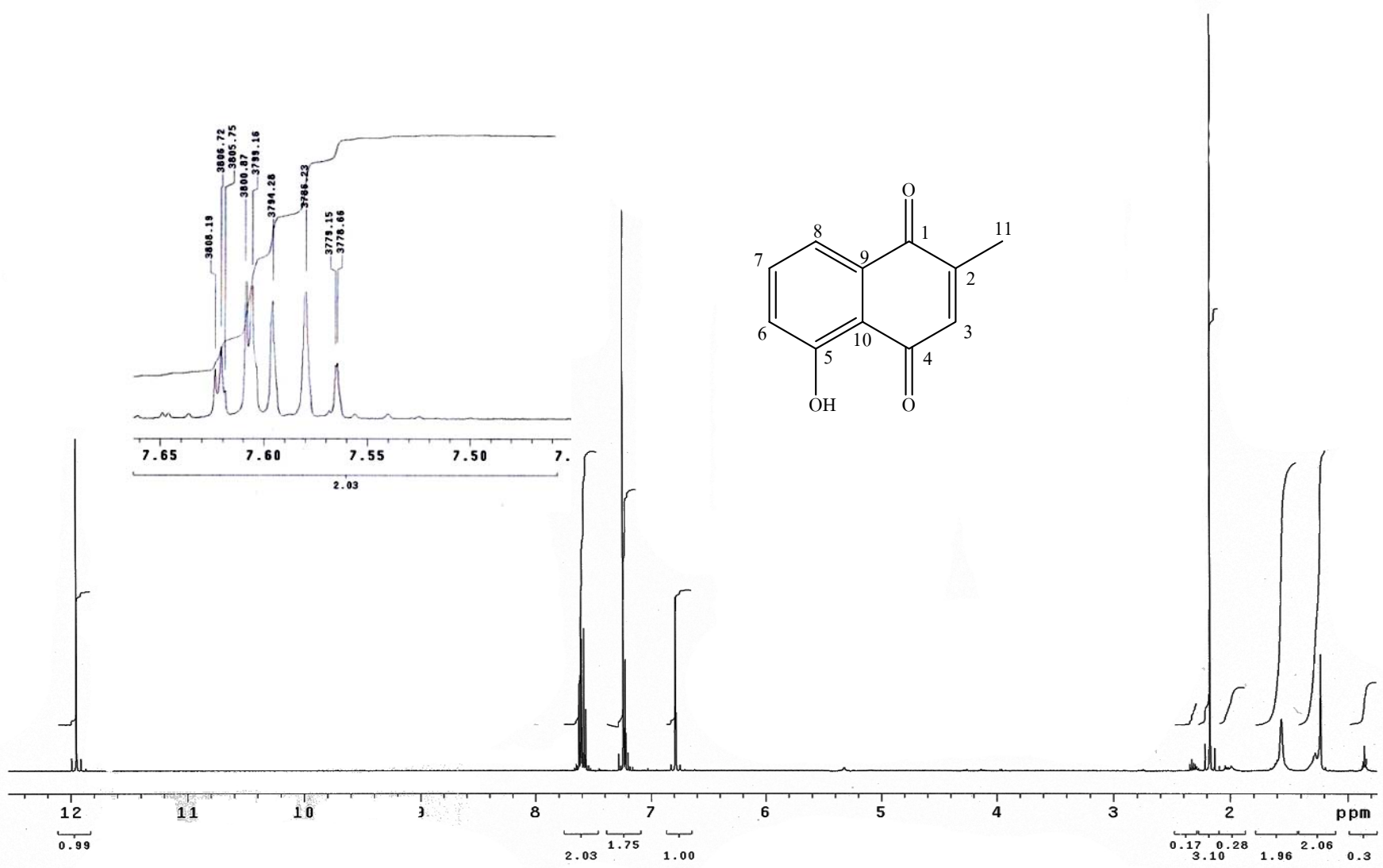

Figure 3-5 ^{1}H-NMR ($CDCl_3$; 500 MHz) of NQ 1

3.2.2 Identification of NQ 2

The ^{1}H NMR spectrum of NQ 2 (Table 3-2, Figure 3-6) 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 3-2, Figure 3-7) 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 3-8), *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). HRFAB MS m/z: 374.0787 $[M+H]^+$ (calculated for $C_{22}H_{14}0_6$, 374.0790). Thus, NQ 2 was identified as 3,3'-biplumbagin (Figure 3-9)

Table 3-2 ^{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)

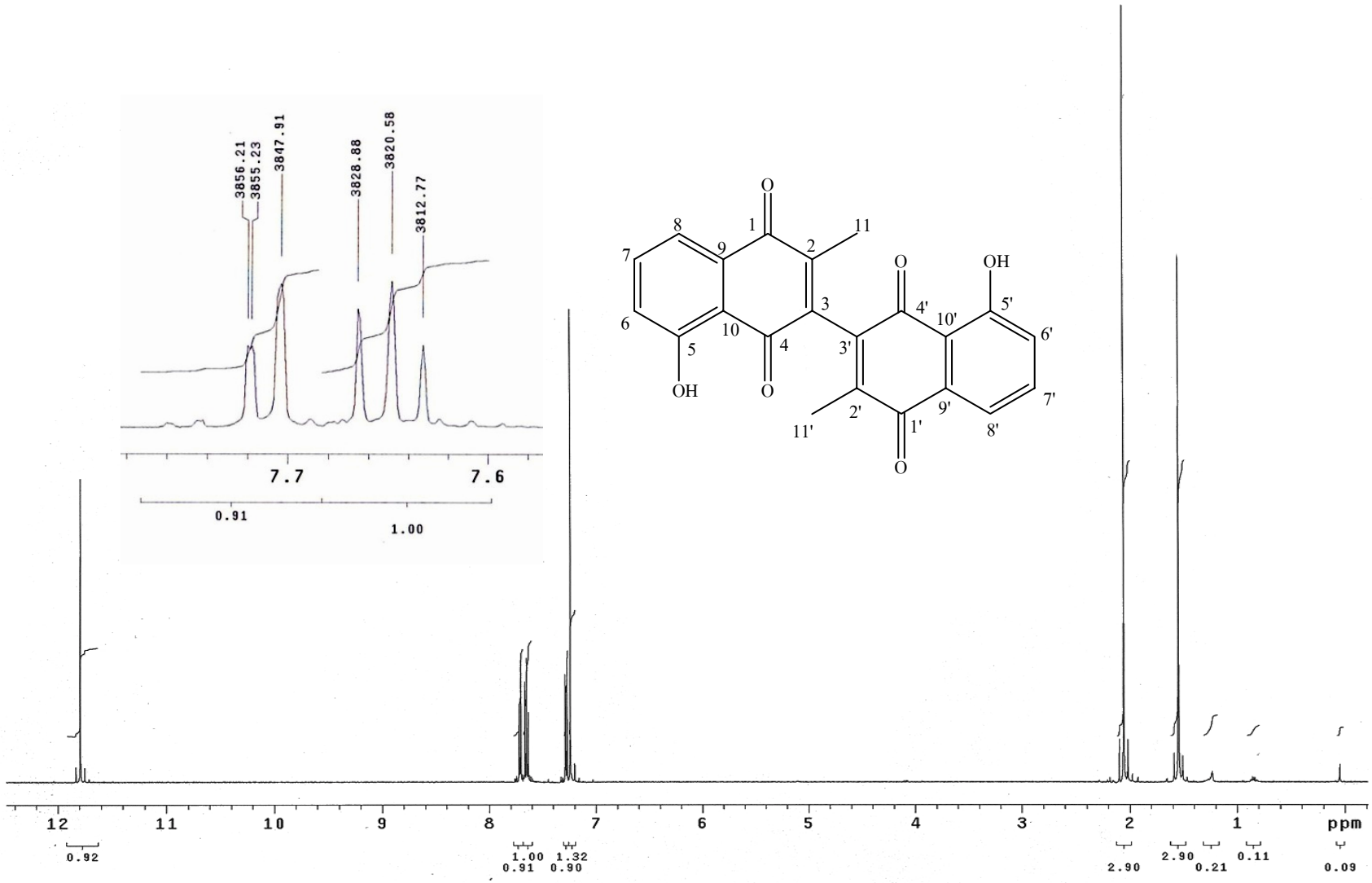

Figure 3-6 ^{1}H-NMR ($CDCl_3$; 500 MHz) of NQ 2

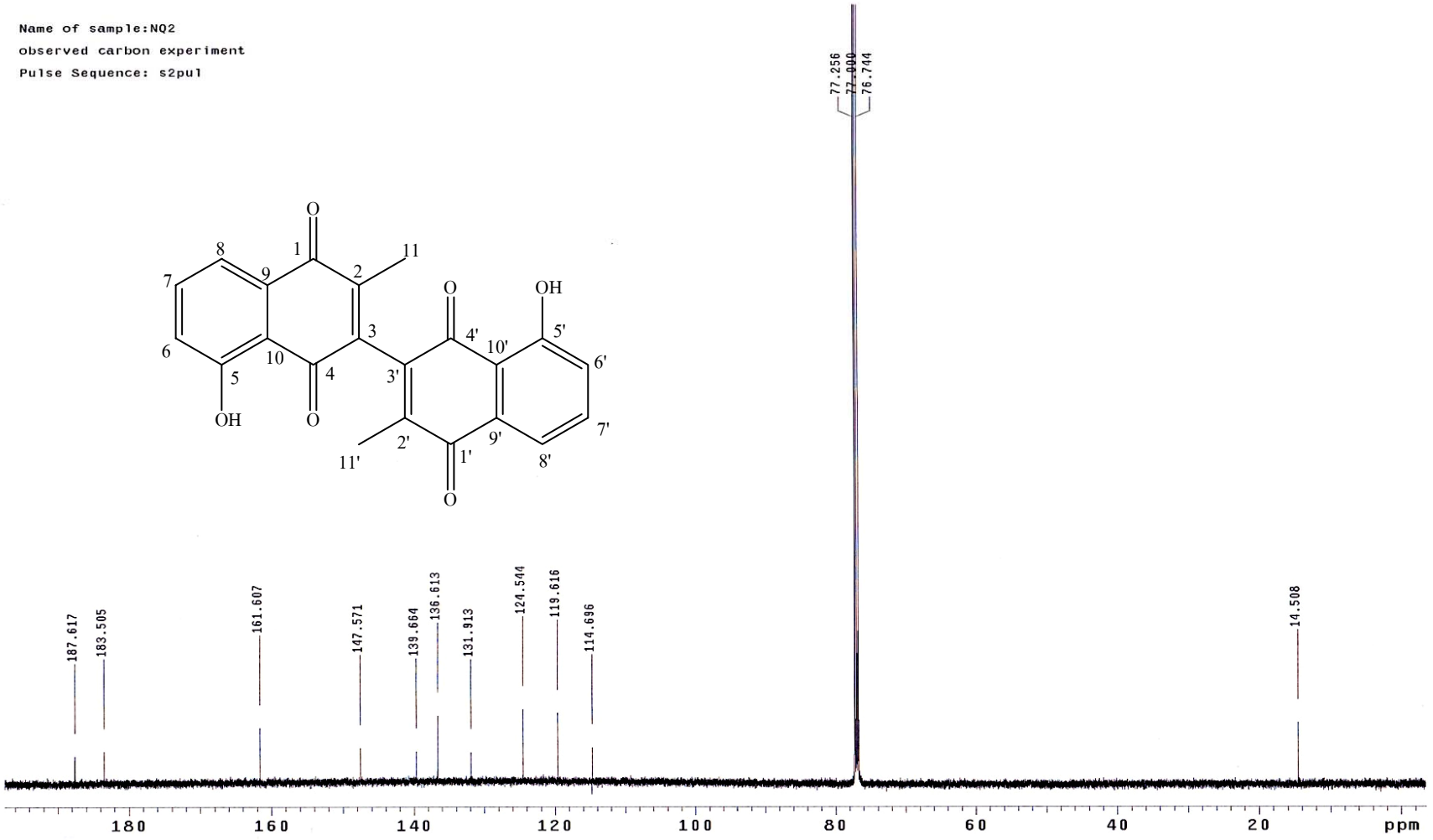

Figure 3-7 ^{13}C-NMR ($CDCl_3$; 125 MHz) of NQ 2

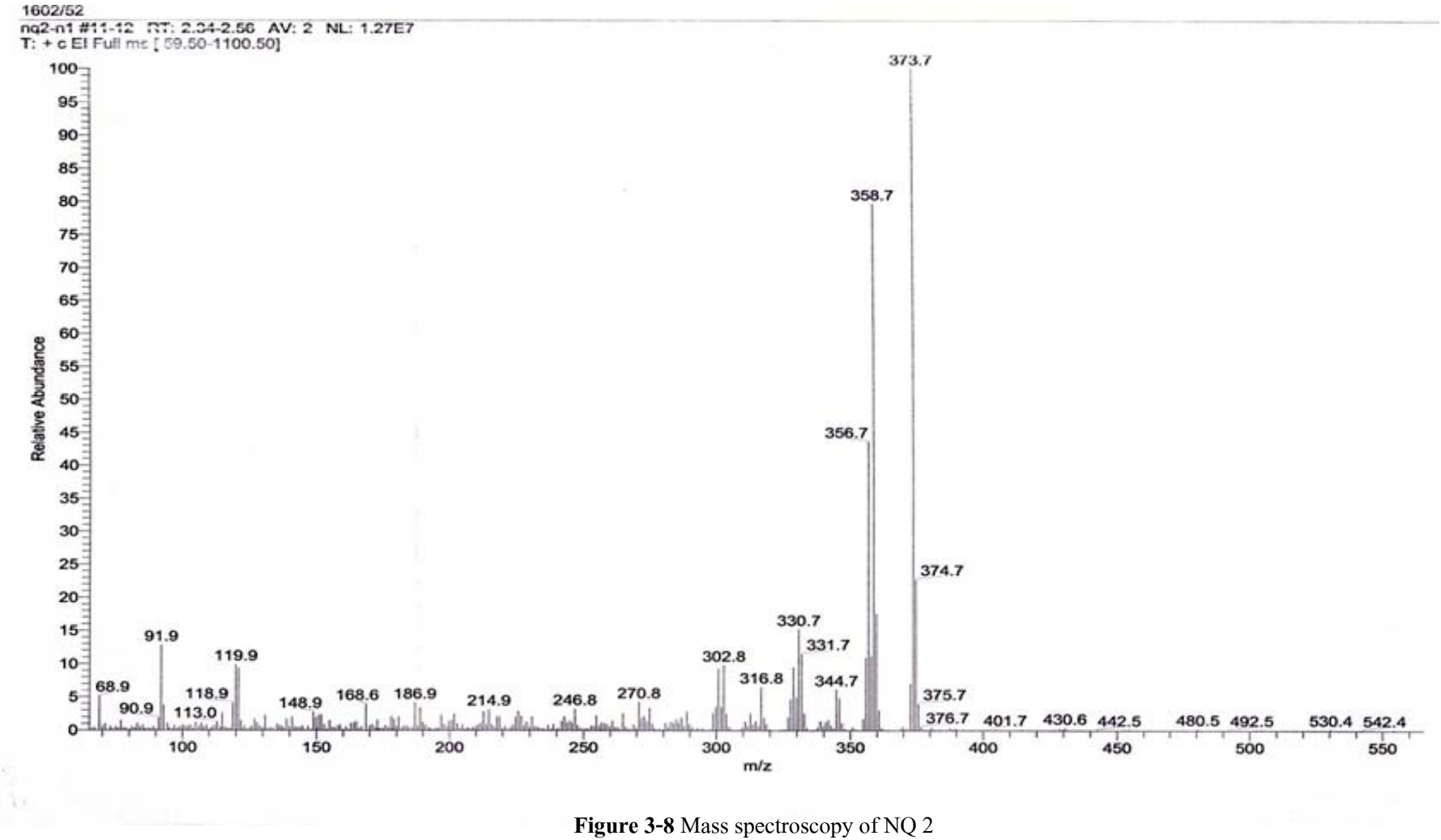

Figure 3-8 Mass spectroscopy of NQ 2

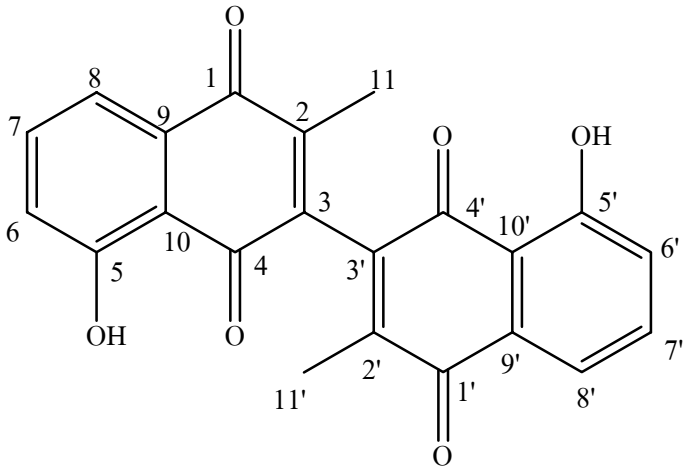

Figure 3-9 Chemical structure of 3,3'-biplumbagin

3.2.3 Identification of NQ 3

The ^{1}H NMR spectrum of NQ 3 (Table 3-3, Figure 3-10) 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 3-3, Figure 3-11) 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 3-12), *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). HRFAB MS m/z: 374.0797 $[M+H]^+$ (calculated for $C_{22}H_{14}0_6$, 374.0790). Therefore, NQ 3 was identified as elliptinone (Figure 3-13)

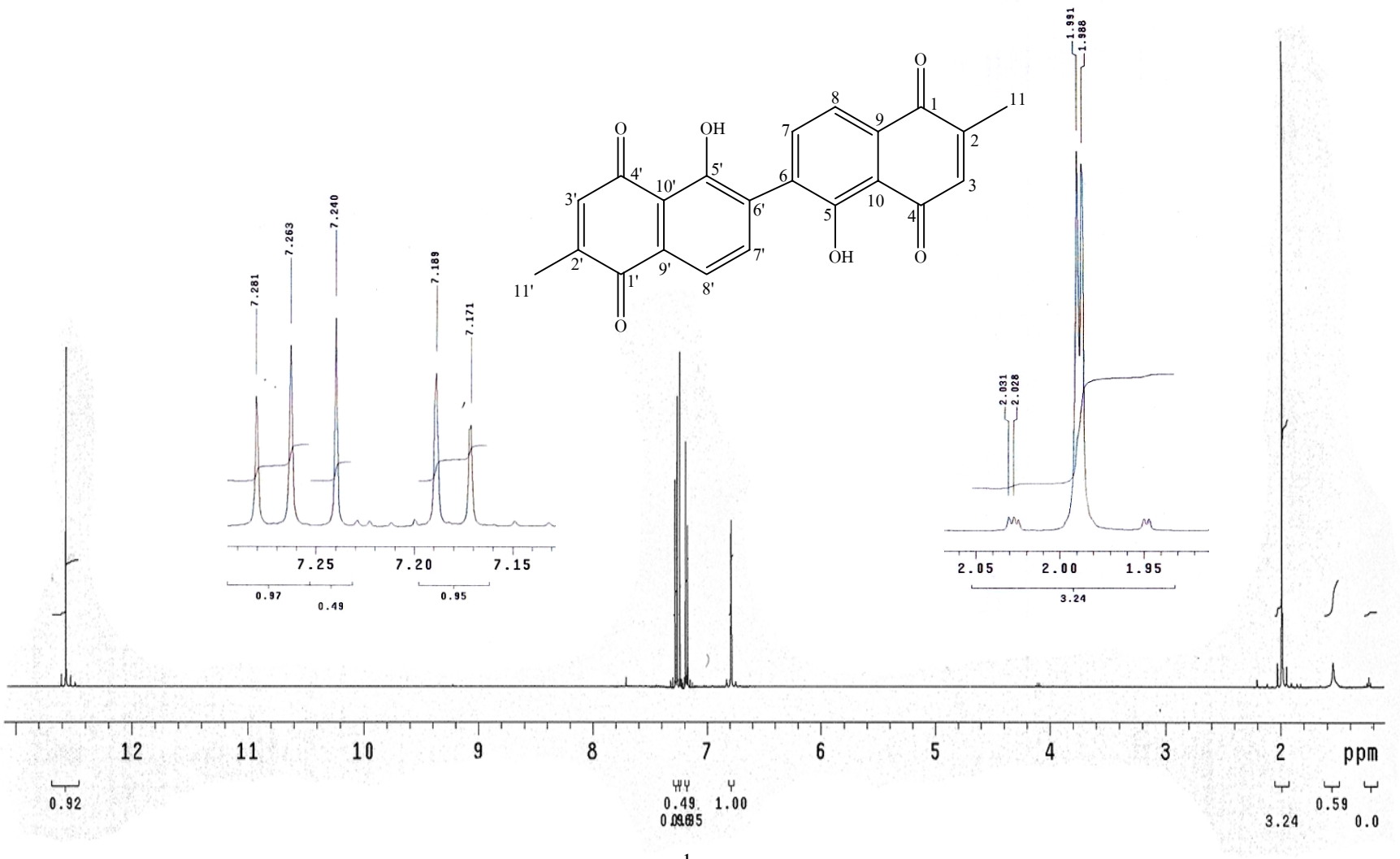

Figure 3-10 ^{1}H-NMR ($CDCl_3$; 500 MHz) of NQ 3

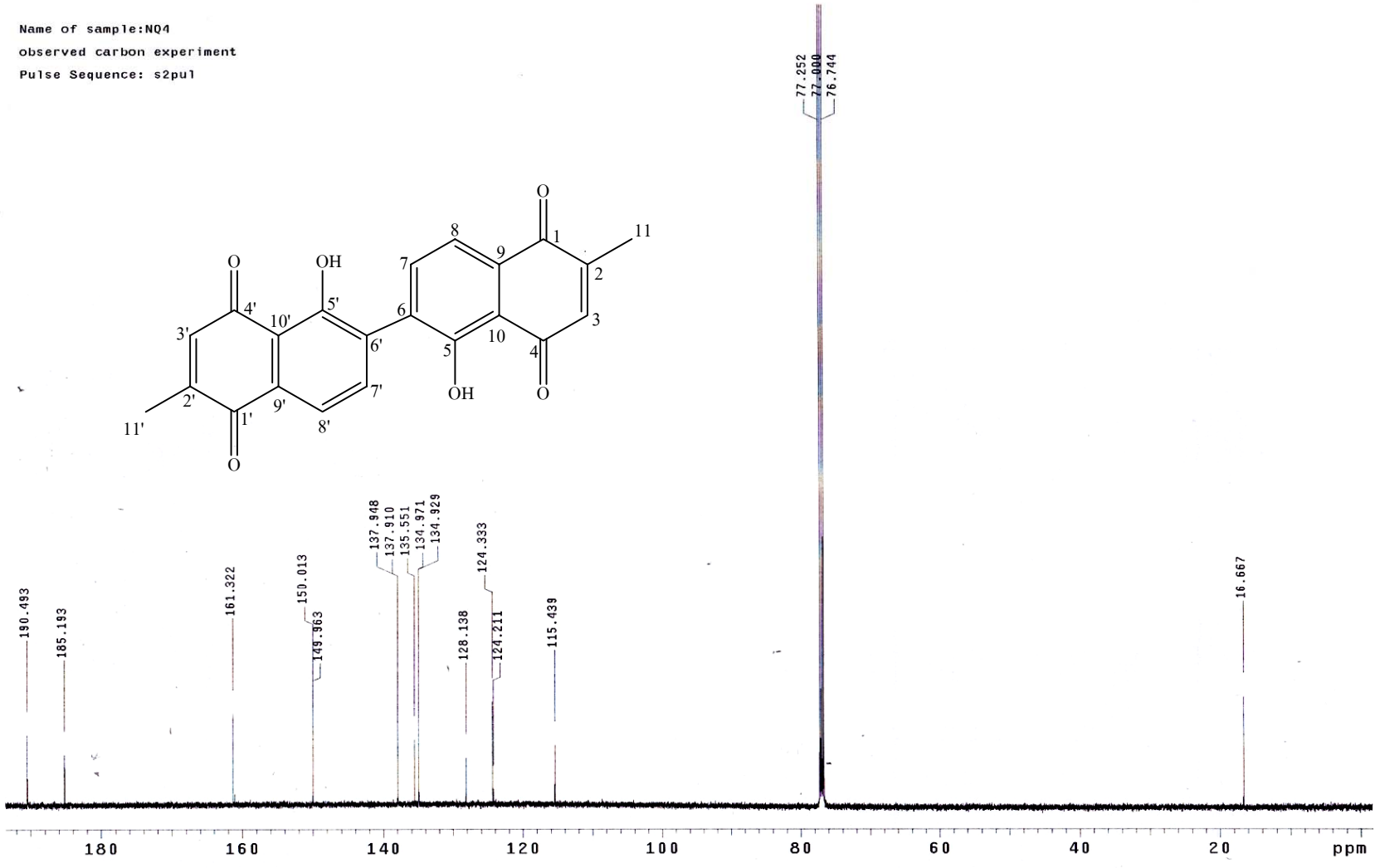

Figure 3-11 ^{13}C-NMR ($CDCl_3$; 125 MHz) of NQ 3

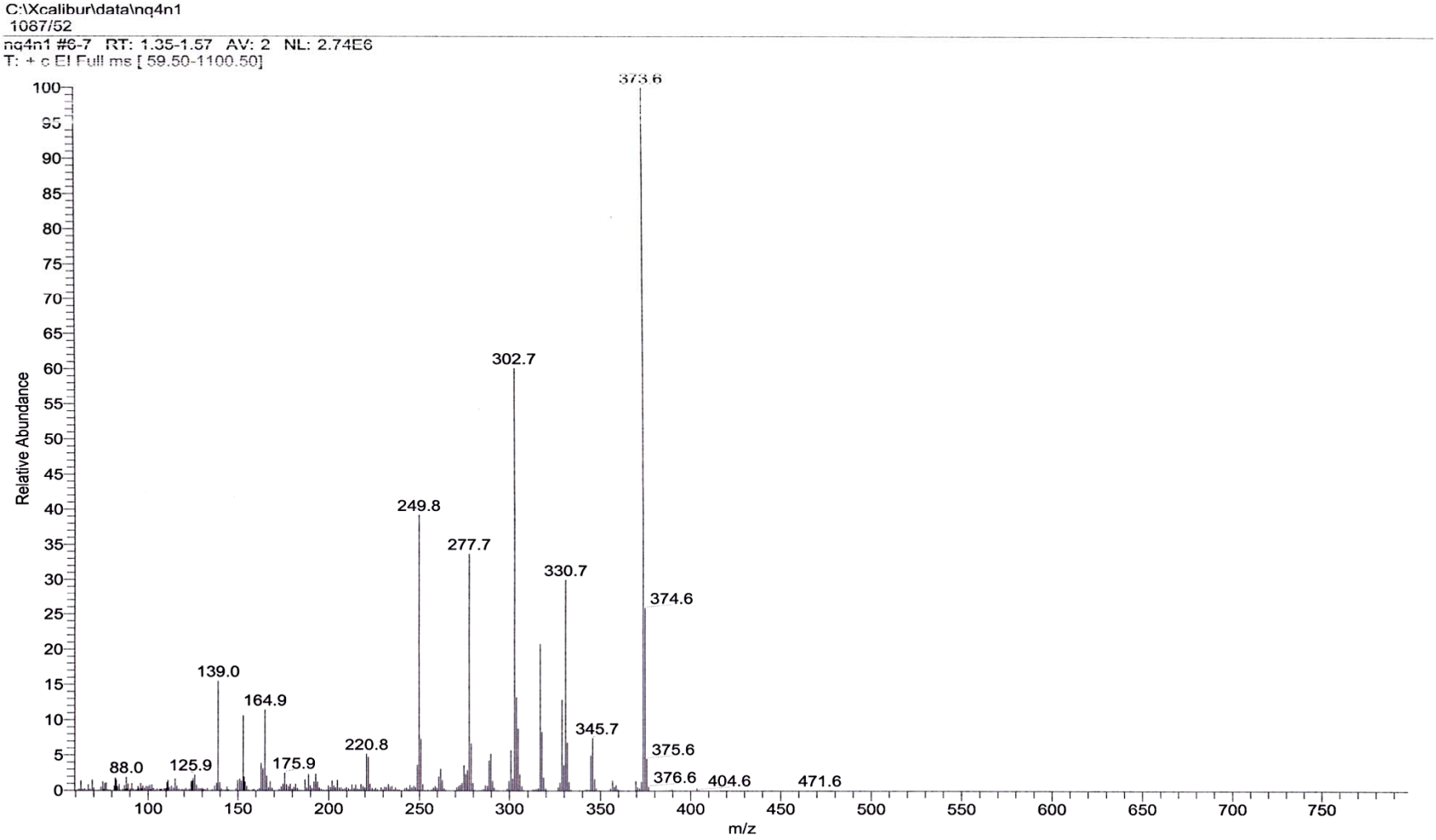

Figure 3-12 Mass spectroscopy of NQ 3

Table 3-3 ^{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)

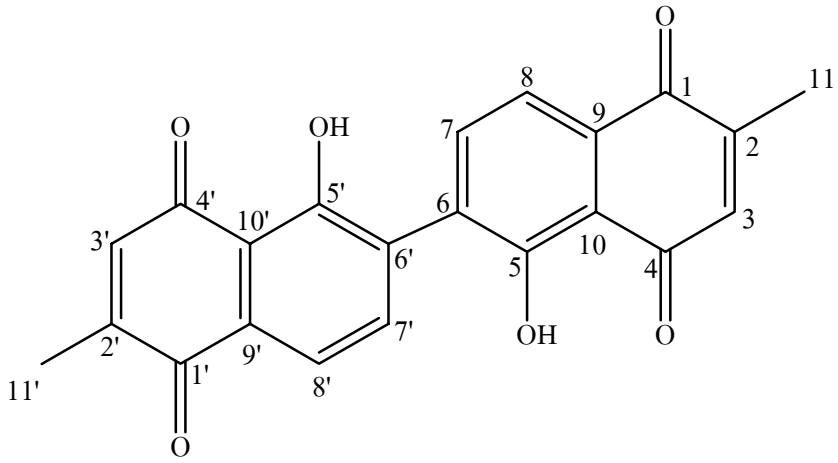

Figure 3-13 Chemical structure of elliptinone

3.3 HPLC quantitative determination 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 3-14) 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.

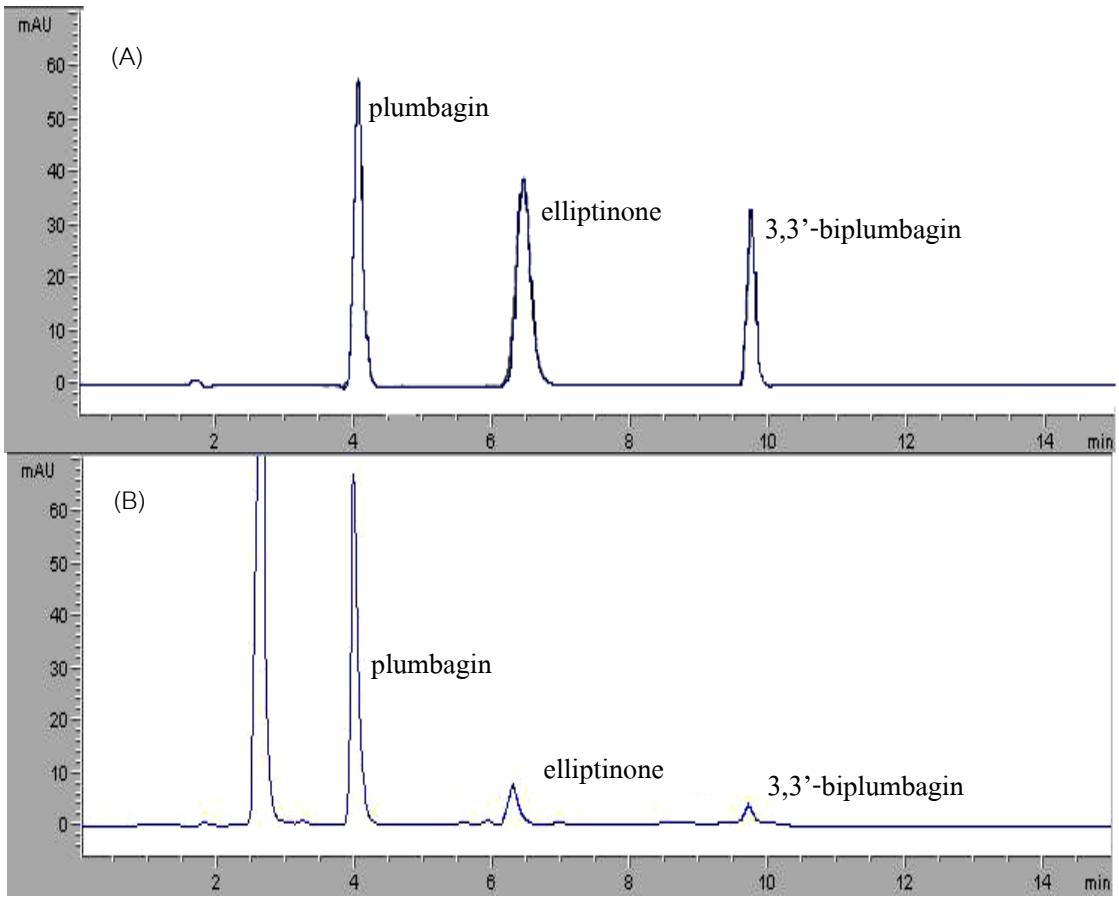

Figure 3-14 HPLC-chromatograms of standard naphthoquinones (A) and naphthoquinone-rich *P. indica* root extract (B)

3.4 Validation of analytical method

3.4.1 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 3-15 to 3-17). These results suggested that the HPLC method possessed good specificity.

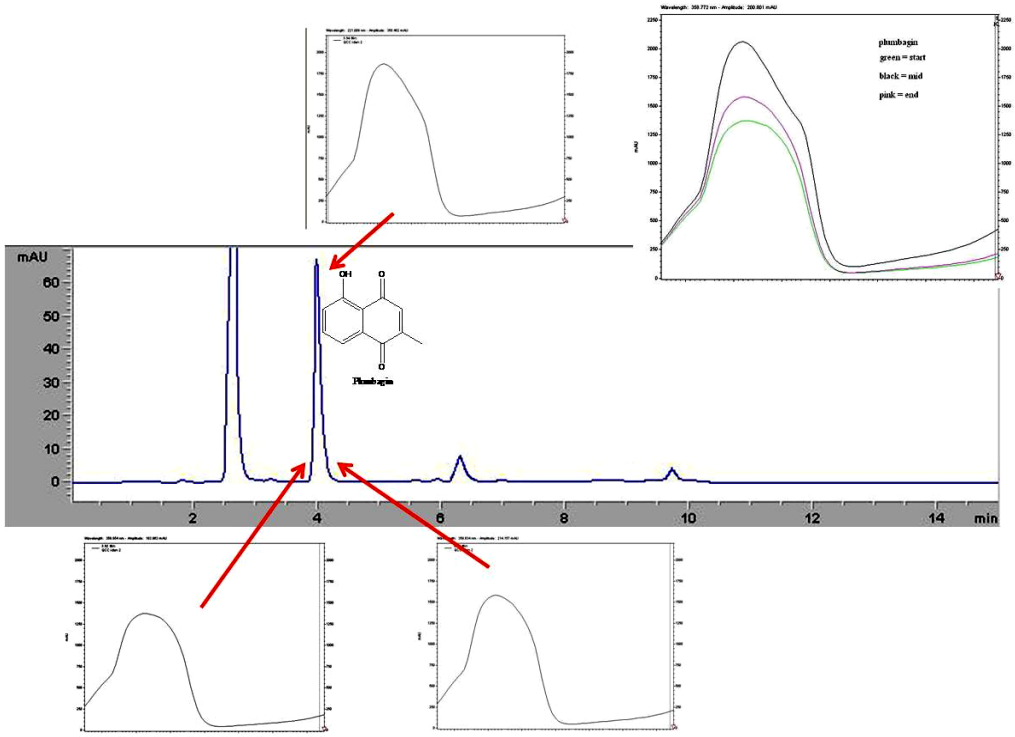

Figure 3-15 UV absorption spectra of plumbagin in *P. indica* root extract

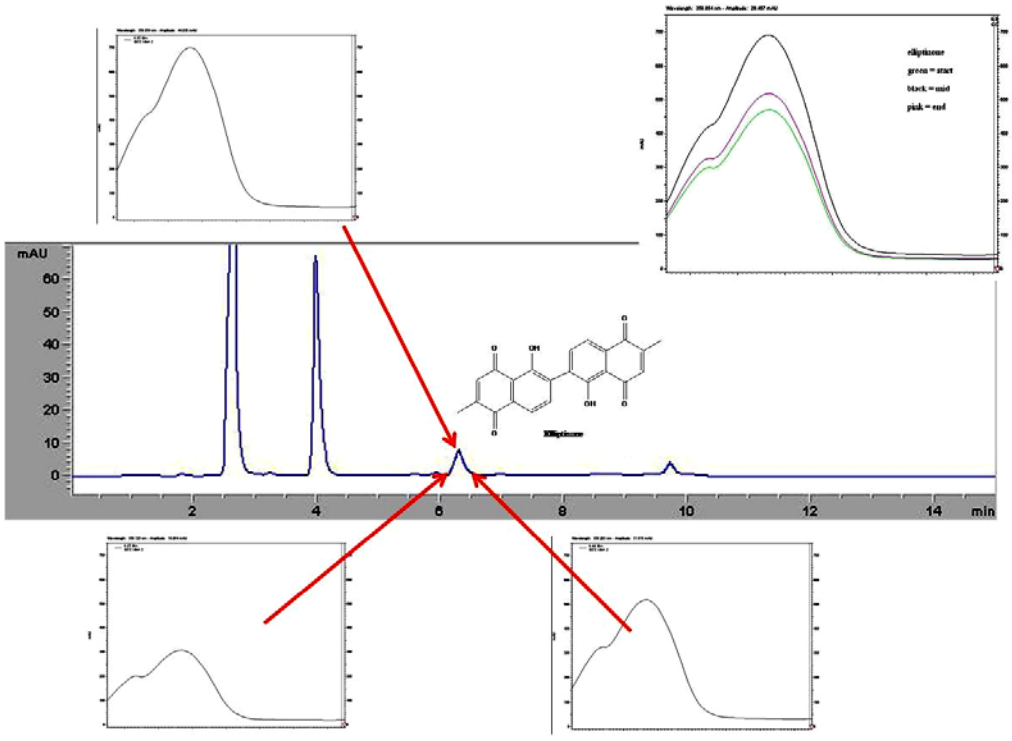

Figure 3-16 UV absorption spectra of elliptinone in *P. indica* root extract

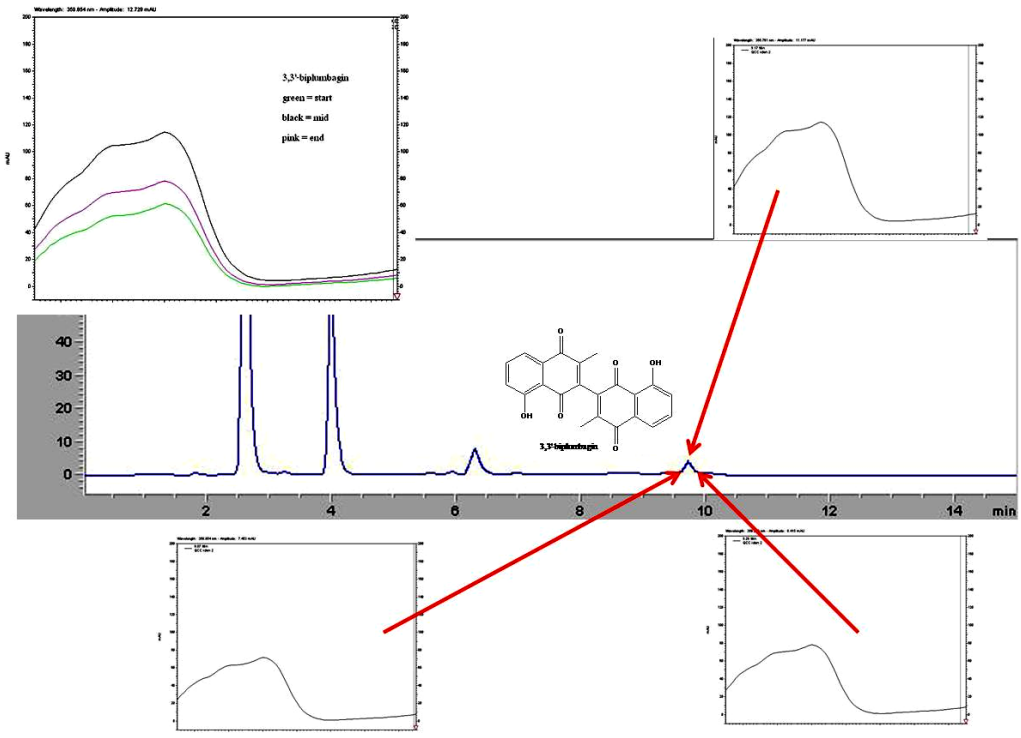

Figure 3-17 UV absorption spectra of 3,3'-biplumbagin in *P. indica* root extract

3.4.2 Linearity

Linearity was evaluated using the standard naphthoquinone isolated solutions over six calibration points (0.01 – 30 µg/ml for plumbagin and 0.03 – 10 µg/ml for 3,3'-biplumbagin and elliptinone) with three measurements for each calibration points. Excellent linearity was observed for all compounds over the evaluated ranges with the correlation coefficients of 0.9997, 0.9997 and 0.9999 for plumbagin, 3,3'-biplumbagin and elliptinone, respectively (Table 3-4).

Table 3-4 Retention time, linear ranges and correlation coefficients (R^2) of calibration curves

	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)

3.4.3 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 3-5), which indicated for a good accuracy of the HPLC method.

Table 3-5 Recoveries of naphthoquinones from *P. indica* root extract

Compounds	Spiked concentration (μg/ml)	Recovery (%)[a] (n = 3)
Plumbagin	0.1	99.88 ± 1.101
	5	99.66 ± 0.645
	20	100.32 ± 0.219
3,3'-biplumbagin	0.1	98.61 ± 1.100
	0.5	98.78 ± 0.991
	5	100.44 ± 0.453
Elliptinone	0.1	98.57 ± 1.572
	0.5	98.93 ± 0.856
	5	100.60 ± 0.420

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

3.4.4 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 3-6), which indicated for the high precision of the HPLC method.

Table 3-6 Intraday and interday precision data of *P. indica* root extract

	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

3.4.5 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. The LOD and LOQ for plumbagin, 3,3'-biplumbagin and elliptinone were exhibited (Table 3-7), indicate that the established HPLC method is sensitive for determination of these compounds

Table 3-7 LOD and LOQ of *P. indica* root extract

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

3.5 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 3-8). Thus, ethanol was appropriately used for the extraction.

Table 3-8 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
$C_2H_5O\ C_2H_5$	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; $C_2H_5O\ C_2H_5$ = diethyl ether

3.6 Simple purification method to improved naphthoquinone content

The ethanol extract was used for further study on 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 column. Thus, this method produced a low content of the total naphthoquinones (Table 3-9). Moreover, the liquid-liquid extraction methods produced the highest yielding of extracts but the total naphthoquinone content also lower than silica gel vacuum chromatography. In addition, acetic acid presence in the extraction not able to improved the content of naphthoquinones in the extract. Thus, silica gel vacuum chromatography was used to prepare naphthoquinone-rich *P. indica* root extract, which increase the total naphthoquinones content from 5.80 mg/g, in the crude ethanol extract, to 138.98 mg/g of extract (Table 3-9).

Table 3-9 The 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 aqueous solution

Table 3-10 The naphthoquinone content in naphthoquinone-rich *P. indica* root extract

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

3.7 Antibacterial activity against acne-involved bacteria

Antibacterial activity of naphthoquinone-rich *P. indica* root extract, plumbagin, 3,3'-biplumbagin and elliptinone were evaluated against acne-involved bacteria including *P. acnes, S. aureus* and *S. epidermidis*. The result demonstrated that, all tested bacteria were inhibited by plumbagin, 3,3'-biplumbagin and elliptinone with MIC value between 0.024 - 12.50 µg/ml, 0.024 - 100 µg/ml and 0.78 - 100 µg/ml, respectively. Naphthoquinone-rich *P. indica* root extract exhibited antibacterial activity against *P. acnes* and *S. aureus* close to that of plumbagin and more potent than both 3,3'-biplumbagin and elliptinone. Moreover, naphthoquinone-rich *P. indica* root extract gave much more potent antibacterial activity than *P. indica* crude ethanol (Table 3-11).

The MBC values of the naphthoquinone-rich *P. indica* root extract against all tested bacterial were found between 6.25 – 50 µg/ml (Table 3-12 and Figure 3-18). Although all tested bacteria were killed by plumbagin, 3,3'-biplumbagin and elliptinone with MBC value between 3.12 – 100 µg/ml, only *S. aureus* that survived within the concentration of 3,3'-biplumbagin and elliptinone below 100 µg/ml.

These results indicated that naphthoquinone-rich *P. indica* root extract possessed antibacterial activity against acne-involved bacteria more potent than 3,3'-biplumbagin and elliptinone, and its antibacterial activity was close to plumbagin. In addition, the total naphthoquinone content of naphthoquinone-rich *P. indica* root extract was 13.20 ± 0.13 % w/w. Thus, the naphthoquinone-rich *P. indica* root extract should contain total naphthoquinone not less than 13% w/w to give the acceptable antibacterial activity against acne-involved bacteria.

Table 3-11 The minimum inhibitory concentration against acne-involved bacteria

	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
NPE	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

NPE = naphthoquinone-rich *P. indica* root extract

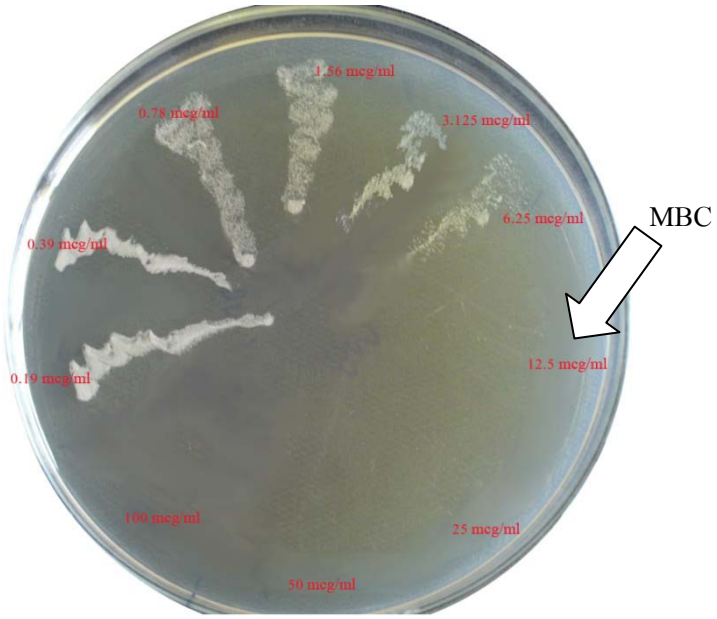

Figure 3-18 Determination of MBC after steak on agar and incubated at properly conditions. The MBC value indicated as the lowest concentration of sample which bacteria were killed.

Table 3-12 The minimum bactericidal concentration against acne-involved bacteria

	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
NPE	50	50	50	25	6.25
Ethanol extract	50	50	50	>100	100
Tetracycline	25	50	50	12.5	3.12

NPE = naphthoquinone-rich *P. indica* root extract

3.8 Solubility of naphthoquinone-rich *P. indica* root extract

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 extract is used to estimate the dissolution of the extract in various solvents. The result showed that the naphthoquinone-rich *P. indica* root extract is freely soluble in chloroform, ethyl acetate and ethanol. It is slightly soluble in methanol and propylene glycol, very slightly soluble in hexane and practically insoluble in water (Table 3-13). The naphthoquinone-rich *P. indica* root extract contains most likely moderate non-polar compounds therefore the suitable solvents for the naphthoquinone-rich *P. indica* root extract should be a moderate non-polar solvents such as ethyl acetate, ethanol and chloroform.

Table 3-13 Solubility of naphthoquinone-rich *P. indica* root extract 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 (distilled)	> 10,000	Practically insoluble

3.9 Stability of naphthoquinone-rich *P. indica* root extract

3.9.1 Effect of light on stability of the extract

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 4 months. The result demonstrated that under light condition, the color of naphthoquinone-rich *P. indica* root extract gradually faded. In contrast, the physical appearance of the extract kept in a container protected from light did not change through the period of 4 months. In addition, the significant decrease of 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 condition (Table 3-14 and Figure 3-19, 3-20). This finding suggests that the naphthoquinone-rich *P. indica* root extract should be kept in a well-sealed closed container, protected from light, in order to stabilize the physical appearance.

Table 3-14 Naphthoquinone content of naphthoquinone-rich *P. indica* root extract 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±0.723	101.45±0.723	2.11±0.176	2.11±0.176	11.50±0.586	11.50±0.586
1	86.30±1.223	86.47±1.280	2.09±0.116	2.09±0.012	11.79±0.336	11.50±0.292
2	81.74±2.727	76.69±9.410	2.15±0.208	2.08±0.068	11.78±0.335	11.33±0.316
3	66.51±14.970	63.48±3.549	2.08±0.056	2.07±0.262	11.37±0.098	11.21±0.156
4	70.37±8.539	83.94±1.659	2.14±0.047	2.06±0.054	11.82±0.370	11.43±0.466
6	60.22±7.048	77.12±1.339	2.03±0.122	2.07±0.090	11.57±0.470	11.60±0.236
8	59.18±17.499	75.29±3.239	2.12±0.176	2.06±0.022	11.31±0.440	11.64±0.059
12	55.37±10.122	74.99±2.798	2.07±0.193	2.04±0.013	11.41±0.367	11.27±0.526
16	54.53±2.720	30.50±0.438	2.08±0.231	2.08±0.040	11.54±0.170	11.21±0.220

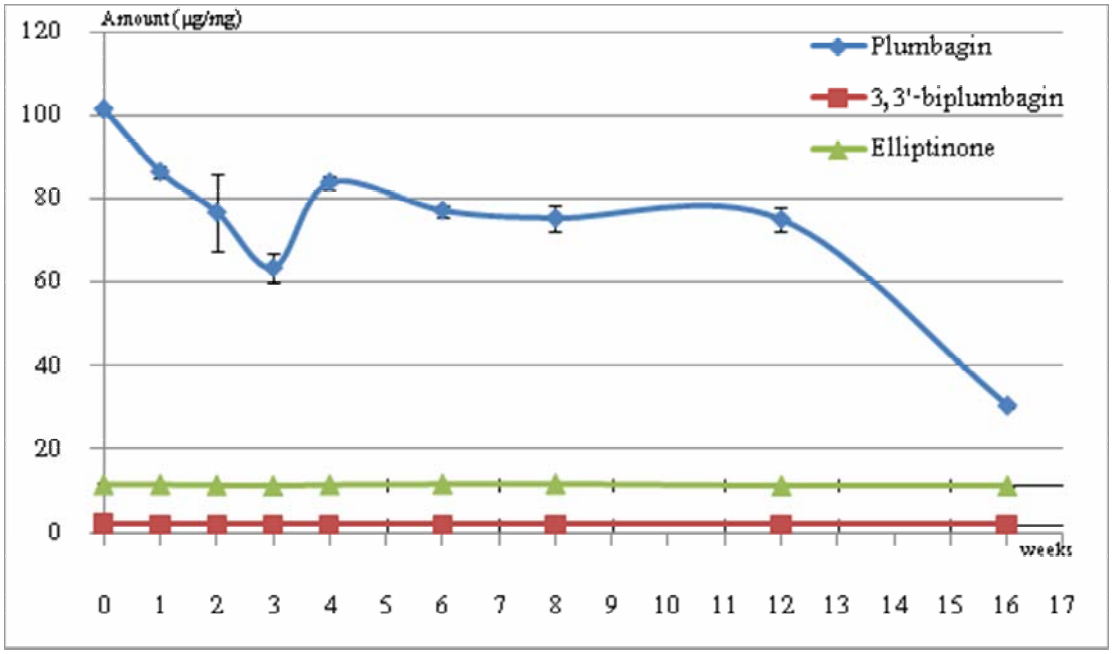

Figure 3-19 Stability profile of naphthoquinone content at light condition

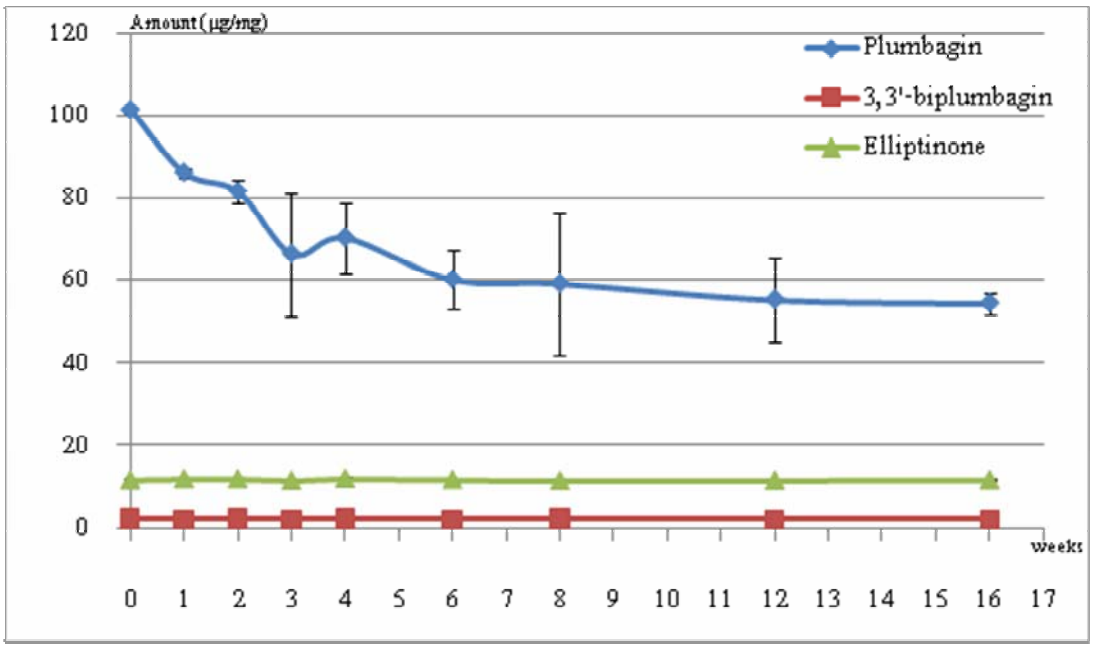

Figure 3-20 Stability profile of naphthoquinone content at dark condition

3.9.2 Effect of temperature on stability of the extract

The effect of temperature on the stability of naphthoquinone-rich *P. indica* root extract was examined under two temperatures, 4 ± 2°C and 30 ± 2°C, protected from light. The result found that the significant decrease of plumbagin content was observed in the first week for 30±2°C storage but it was not significantly changed for 4 ± 2°C condition, while the content of 3,3'-biplumbagin and elliptinone were not significantly changed in the period of four months for both conditions through the period of 4 months (Table 3-15 and Figure 3-21, 3-22). This finding suggests that the naphthoquinone-rich *P. indica* root extract should be kept at 4 ± 2°C in order to stabilize the naphthoquinone content.

Table 3-15 Naphthoquinone content of naphthoquinone-rich *P. indica* root extract stored under 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±0.723	101.45±0.723	2.11±0.176	2.11±0.176	11.50±0.586	11.50±0.586
1	107.76±5.947	96.79±1.639	2.18±0.097	2.07±0.102	11.75±0.074	11.94±0.284
2	105.48±2.757	73.14±14.683	2.13±0.110	2.03±0.054	11.62±0.304	11.83±0.497
3	104.11±7.782	69.02±14.016	2.20±0.150	2.12±0.022	11.66±1.191	11.39±0.777
4	104.27±9.168	86.93±10.545	2.12±0.124	2.12±0.144	11.70±0.272	11.66±0.316
6	109.03±0.121	55.96±13.303	2.09±0.191	2.10±0.030	11.69±0.477	11.45±0.633
8	105.18±11.168	53.10±5.586	2.17±0.148	2.11±0.040	11.48±0.668	11.73±1.405
12	105.62±11.119	53.10±5.586	2.13±0.076	2.12±0.012	11.63±0.724	11.68±1.452
16	107.14±5.139	49.11±6.893	2.26±0.247	2.11±0.089	11.49±0.488	11.39±0.169

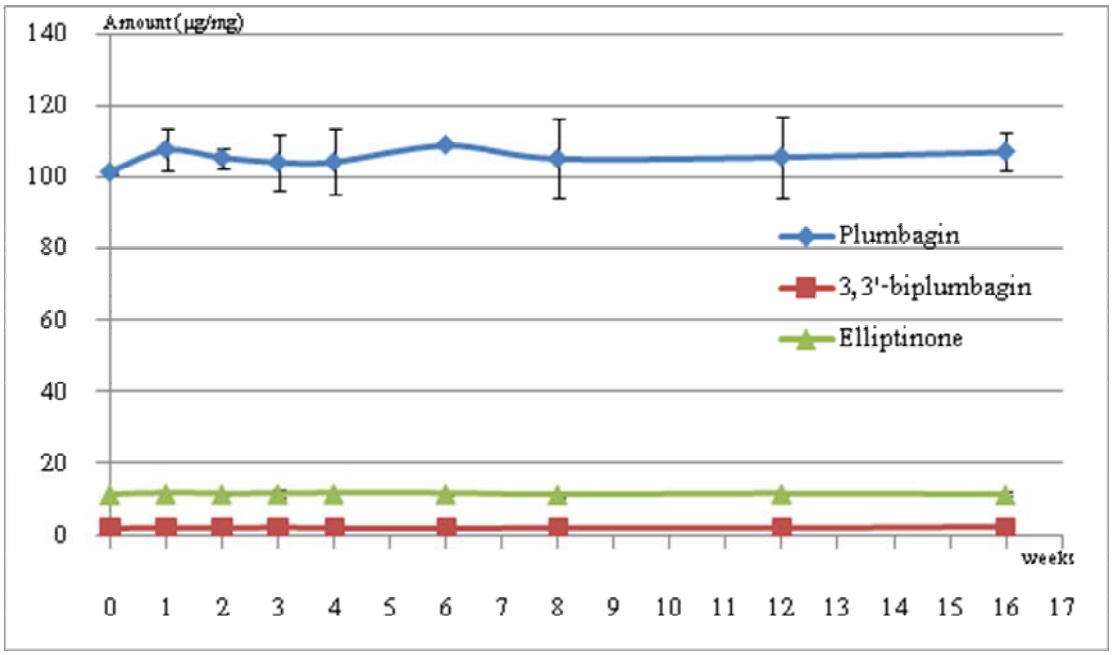

Figure 3-21 Stability profile of naphthoquinone content at 4 ± 2°C condition

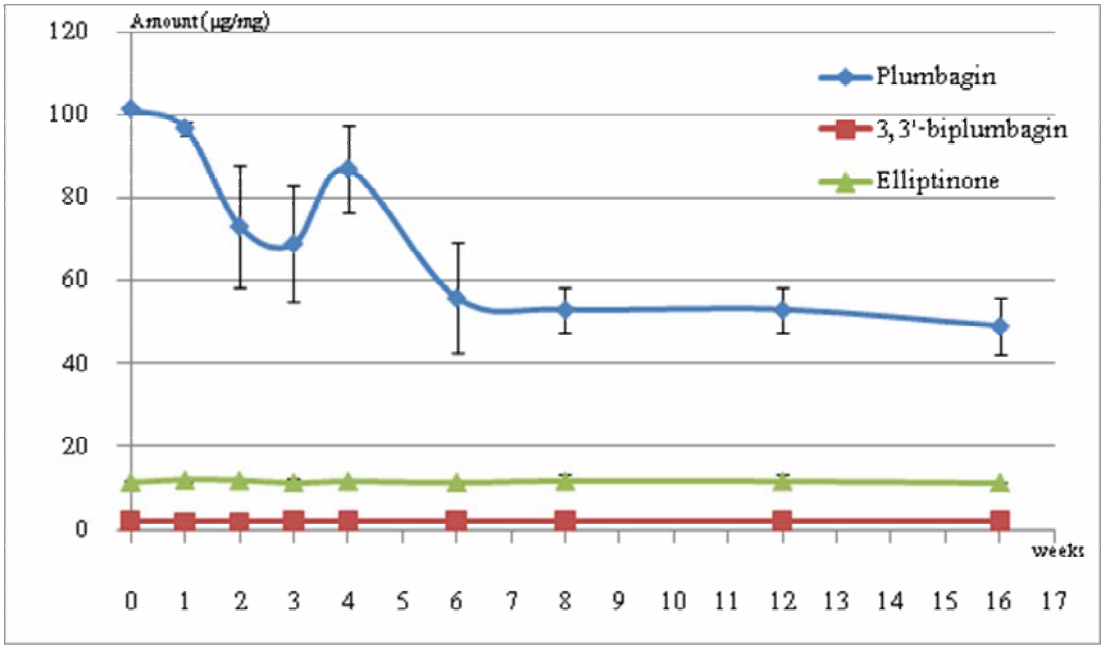

Figure 3-22 Stability profile of naphthoquinone content at 30 ± 2°C condition

3.9.3 Effect of pH on stability of the extract

The acid-base stability evaluation of naphthoquinone-rich *P. indica* root extract in solution was determined at three different pH values; 5.5, 7.0 and 8.0. It was found that at pH 5.5 was not change in physical appearance but pH 7.0 and 8.0 the color of sample changed from yellow to orange (Figure 3-23, 3-24). In addition, the naphthoquinone content was decrease under all tested pH. At pH 5.5, the content of elliptinone was significantly decreases after 4 weeks and at pH 7.0 and 8.0, it significantly decreases from the first week (Table 16 to 18; Figure 3-25 to 3-27). It implied that all naphthoquinones are not stable when they are in solution. However, naphthoquinones were more stable in the solution at pH 5.5 than 7.0 and 8.0. Thus, the preparation of naphthoquinone-rich *P. indica* root extract in an aqueous solution should be performed carefully.

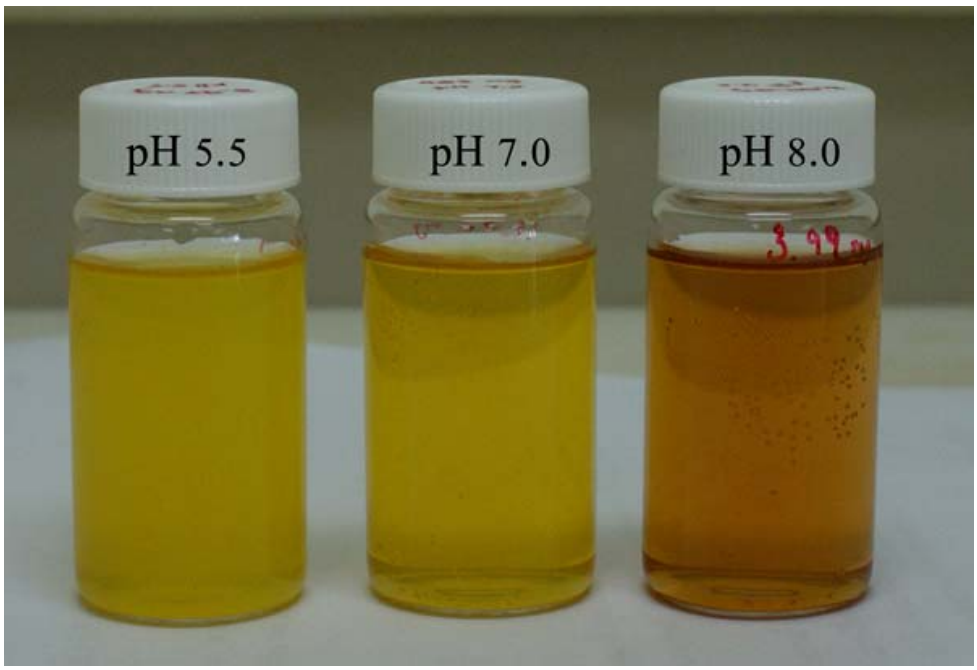

Figure 3-23 Extracts for stability under pH variation test at week 0

Figure 3-24 Extracts for stability under pH variation test after 16 weeks storage

Table 3-16 Plumbagin content of naphthoquinone-rich *P. indica* root extract in solution at 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 ± 0.696	121.06 ± 0.167	104.20 ± 2.832
1	121.74 ± 2.482	96.40 ± 10.653	11.79 ± 0.704
2	119.63 ± 0.231	37.10 ± 1.769	5.35 ± 1.325
3	112.82 ± 4.209	23.38 ± 1.156	2.66 ± 0.343
4	110.80 ± 4.070	15.35 ± 1.016	0.00
6	105.37 ± 6.083	7.19 ± 0.879	0.00
8	98.78 ± 4.139	2.01 ± 0.974	0.00
12	98.78 ± 4.139	0.75 ± 0.752	0.00
16	87.50 ± 0.385	1.17 ± 0.178	0.00

Table 3-17 3,3'-biplumbagin content of naphthoquinone-rich *P. indica* root extract in solution at 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 ± 0.012	2.67 ± 0.090	1.09 ± 0.044
1	2.44 ± 0.034	0.40 ± 0.216	0.03 ± 0.097
2	2.30 ± 0.057	0.09 ± 0013	0.09 ± 0.007
3	1.76 ± 0.150	0.00	0.00
4	1.38 ± 0.035	0.00	0.00
6	1.08 ± 0.025	0.00	0.00
8	0.94 ± 0.158	0.00	0.00
12	0.93 ± 0.157	0.00	0.00
16	0.77 ± 0.315	0.00	0.00

Table 3-18 Elliptinone content of naphthoquinone-rich *P. indica* root extract in solution at 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 ± 0.459	13.00 ± 0.222	11.30 ± 0.376
1	13.58 ± 0.229	4.70 ± 0.382	0.71 ± 0.202
2	13.58 ± 0.229	3.52 ± 0.202	0.71 ± 0.202
3	11.73 ± 0.569	1.80 ± 0.123	0.00
4	11.36 ± 0.346	1.04 ± 0.103	0.00
6	10.84 ± 1.113	0.70 ± 0.042	0.00
8	9.47 ± 0.541	0.00	0.00
12	9.85 ± 0.269	0.00	0.00
16	9.69 ± 0.161	0.00	0.00

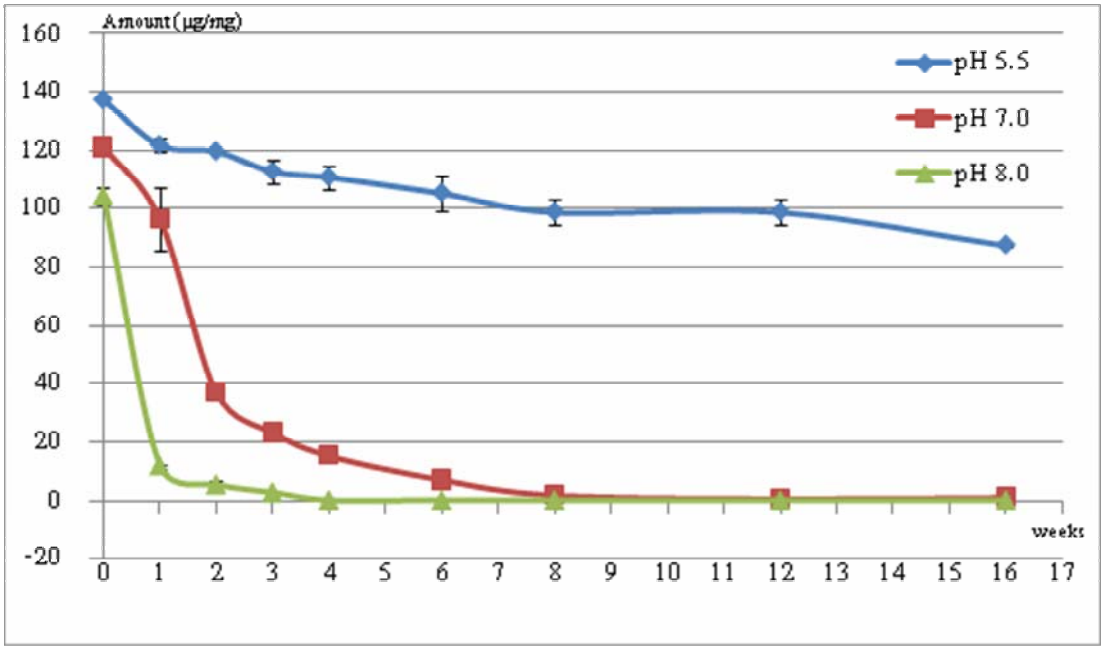

Figure 3-25 Stability profile of plumbagin content at various pH conditions

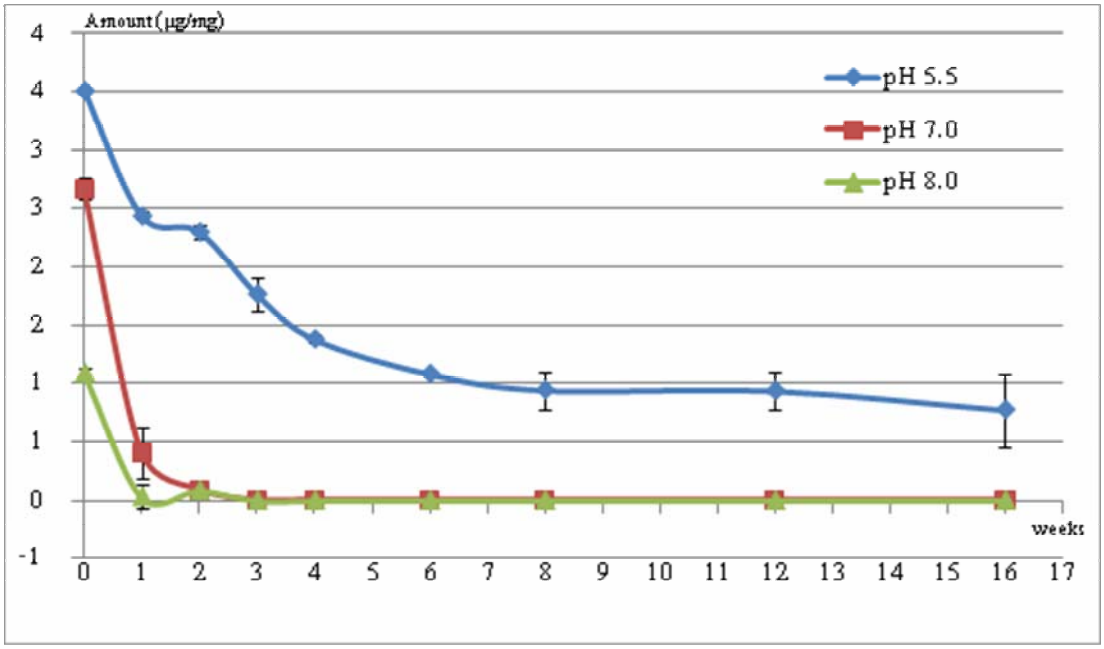

Figure 3-26 Stability profile of 3,3'-biplumbagin content at various pH conditions

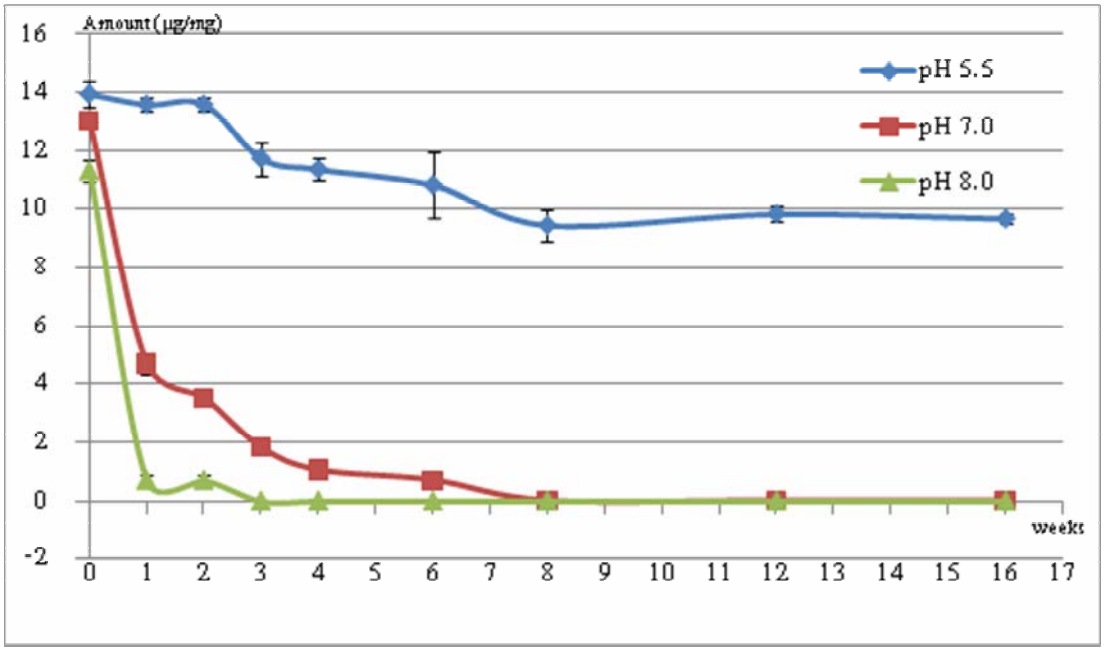

Figure 3-27 Stability profile of elliptinone content at various pH conditions

3.9.4 Effect of accelerated condition on stability of the extract

The accelerate stability test of the naphthoquinone-rich *P. indica* root extract was carried out using a stability chamber at 45 ± 2°C and 75% humidity. The result demonstrated that the physical appearance was not change through the period of 4 month. In addition, the significant decrease of plumbagin content was observed in the first week, while the content of 3,3'-biplumbagin and elliptinone were not significantly changed in the period of 4 months (Table 3-19; Figure 3-28). This result implies that the naphthoquinone-rich *P. indica* root extract was unstable when kept in well-closed containers, protected from light and stored at room temperature for at least two years.

Table 3-19 Naphthoquinone content of naphthoquinone-rich *P. indica* root extract store under accelerate condition

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

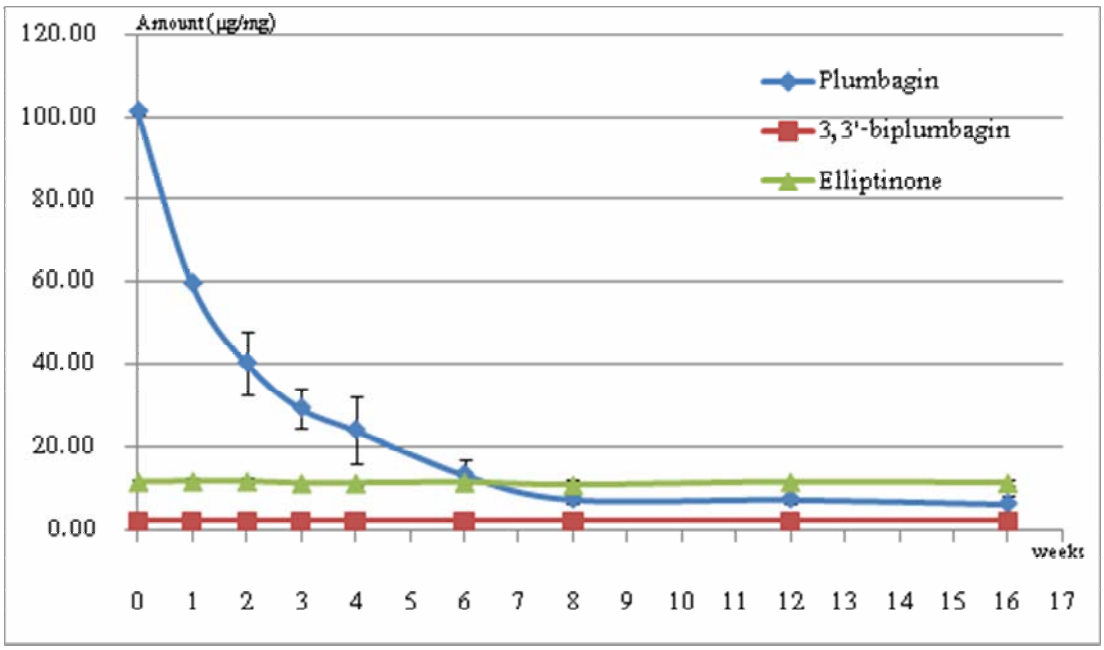

Figure 3-28 Stability profile of naphthoquinone content under accelerated condition (45 ± 2°C and 75% humidity)

CHAPTER 4

CONCLUSION

1. 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. This is the first of isolation of 3,3'-biplumbagin and elliptinone from *P. indica* roots.

2. A simple, specific, precise, accurate, rapid and reproducible HPLC method has been developed and validated for simultaneous quantitative determination of naphthoquinones, plumbagin, 3,3'-biplumbagin and elliptinone in *P. indica* root extracts.

3. Ethanol is a suitable solvent for extraction of naphthoquinones from *P. indica* roots.

4. Fractionation of the ethanol extract of *P. indica* roots using silica gel vacuum chromatography eluted with a mixture of hexane and ethyl acetate (9.2:0.8 v/v; 500 ml) afforded the naphthoquinone-rich *P. indica* root extract, which contained the total naphthoquinone content up to 138.58 mg/g of extract.

5. The naphthoquinone-rich *P. indica* root extract possessed antibacterial activity against all tested bacteria with the MIC values between 0.78 and 12.50 µg/ml. The extract showed satisfactory antibacterial activity against *P. acnes* and *S. aureus* with MIC values closed to those of plumbagin.

6. The standard specification of naphthoquinone content in naphthoquinone-rich *P. indica* root extract was established as the extract should contain total naphthoquinone content not less than 13% w/w, and the major active compound plumbagin should be not less than 11% w/w.

7. The naphthoquinones-rich *P. indica* root extract contains most likely moderate non-polar naphthoquinones therefore the suitable solvents to dissolve the extract should be moderate non-polar solvents such as ethyl acetate, ethanol and chloroform.

8. Stability evaluations of the naphthoquinones-rich *P. indica* root extracts in several conditions in the period of 4 months found that the extract possessed a satisfactory stability at 4°C condition. Thus, the extract should be stored in well-sealed closed containers, protected from light and cooled place (4°C) to stabilized total naphthoquinone content. The aqueous solutions of the extract are not stable either in acid or base conditions.

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

Muelle- 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

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