Standardization of *Nelumbo nucifera* plumule and evaluation of neferine content

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ดีบัวในเมล็ดบัวหลวงเป็นเครื่องยาสมุนไพรไทย ที่ใช้ในการรักษาความผิดปกติของระบบประสาทอาการนอนไม่หลับ ภาวะมีไข้สูงกระสับกระส่าย และโรคหัวใจและหลอดเลือด เนเฟอรีน เป็นสารอัลคาลอยด์หลักที่พบในดีบัว และมีการรายงานว่ามีฤทธิ์ทางเภสัชวิทยาหลายอย่าง การกำหนดมาตรฐานพืชสมุนไพรมีความจำเป็นสำหรับการควบคุมคุณภาพ ประสิทธิศักย์ และความปลอดภัย การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อที่จะจัดทำข้อกำหนดทางเภสัชเวทของดีบัวและการประเมินปริมาณสารเนเฟอรีนด้วยวิธีทินเลเยอร์โครมาโทกราฟี-เดนซิโทเมทรี และการวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ การศึกษาภาคตัดขวางของเครื่องยาดีบัวพบลักษณะที่สำคัญได้แก่ เซลล์ผิว ช่องอากาศ พาเรนไคมา และท่อลำเลียง นอกจากนี้การศึกษาจุลกายวิภาคศาสตร์ของเนื้อเยื่อของผงยาดีบัวพบปากใบชนิด อะโนโมไซติก พาเรนไคมาที่มีเม็ดแป้ง และชั้นของใบเลี้ยง ในการศึกษาได้มีการจัดทำลายพิมพ์องค์ประกอบทางเคมีและค่าคงที่ทางเคมีและกายภาพ ผลการศึกษาพบว่ามีปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด น้ำหนักที่หายไปเมื่อทำแห้ง ปริมาณน้ำ ปริมาณสารสกัดด้วยเอทานอลและสารสกัดน้ำมีค่าเท่ากับร้อยละ 3.86, 0.54, 9.91, 10.02, 16.54 และ 28.27 กรัม ตามลำดับ นอกจากนี้ได้มีการตรวจสอบความถูกต้องของวิธีการหาปริมาณสารเนเฟอรีนในดีบัวด้วยวิธีทินเลเยอร์โครมาโทกราฟี เดนซิโทเมทรี และวิธีการวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ โดยผลการทดสอบของทั้งสองวิธีพบว่าค่าความเป็นเส้นตรงของความเข้มข้นอยู่ระหว่าง 0.25-3.0 ไมโครกรัมต่อจุด และ มีค่าความถูกต้อง ความเที่ยงตรง และความคงทน ที่อยู่ในเกณฑ์เหมาะสม การวิเคราะห์ปริมาณเนเฟอรีนด้วยวิธีทินเลเยอร์โครมาโทกราฟี เดนซิโทเมทรี มีค่าเท่ากับ 0.496 ± 0.186 กรัม ในดีบัว 100 กรัม และด้วยวิธี การวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ มีค่าเท่ากับ 0.495 ± 0.190 กรัม ในดีบัว 100 กรัม นอกจากนั้นจากการนำสารสกัดดีบัวทั้ง 3 ส่วน คือ สารสกัดด้วยเอทานอล สารสกัดแยกส่วนด้วยไดคลอโรมีเทน และสารสกัดแยกส่วนด้วยเอทานอลมาทดสอบการแสดงความเป็นพิษต่อเซลล์มะเร็งโดยใช้เซลล์มะเร็งทั้งหมด 5 ชนิด คือ มะเร็งเต้านม (BT-474) มะเร็งปอด (CHAGO-K1) มะเร็งลำไส้ (SW-620) มะเร็งกระเพาะอาหาร (KATO-3) มะเร็งตับ (HEP-G2) และเซลล์ปกติ 1 ชนิด คือ เซลล์ปอด (Wi-38) ผลการทดสอบพบว่าสารสกัดดีบัวทั้ง 3 ส่วน ไม่แสดงความเป็นพิษต่อเซลล์มะเร็งและเซลล์ปกติ โดยมีค่า IC_{50} มากกว่า 20 ไมโครกรัมต่อมิลลิลิตร ข้อมูลที่ได้จากการศึกษาในครั้งนี้ สามารถนำไปใช้ในการตรวจเอกลักษณ์และการกำหนดมาตรฐานของดีบัว และการพัฒนาข้อกำหนดในโมโนกราฟของพืชชนิดนี้ต่อไปในอนาคต

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KANOKWAN UACHAROEN: Standardization of *Nelumbo nucifera* plumule and evaluation of neferine content. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 116 pp.

Plumule of *Nelumbo nucifera* Gaertn., a traditional Thai medicine, is primarily used for nervous disorder, insomnia, high fevers with restlessness, and cardiovascular disease. Neferine is one of the main bisbenzylisoquinoline alkaloid components in *N. nucifera* plumule and has been reported to possess several pharmacological properties. Standardization of plant materials is necessary for an assurance of quality, efficacy and safety based on various parameters. This study aimed to investigate the pharmacognostic specification of *N. nucifera* plumule and determine the neferine content using TLC densitometry and TLC image analysis. Transverse section of this crude drug showed the anatomical characteristics of epidermis, air chamber, parenchyma and vascular bundle. Additionally, the histological study of powder showed anomocytic stomata, parenchyma with starch granule and layer of cotyledons. TLC fingerprint and physicochemical constants were also established. The average values of total ash, acid insoluble ash, loss on drying, moisture content, ethanol and water extractive contents were found to be 3.86, 0.54, 9.91, 10.02, 16.54 and 28.27 g% respectively. Moreover, TLC densitometry and TLC image analysis were validated for determination of neferine content in *N. nucifera* plumule. The calibration range was between 0.25-3.0 µg/spot and exhibited suitable accuracy, precision and robustness in both methods. Neferine content in *N. nucifera* plumule were 0.496 ± 0.186 g% by TLC densitometry and 0.495 ± 0.190 g% by TLC image analysis. Additionally, *N. nucifera* plumule ethanolic extract, dichloromethane fractionate and ethanolic fractionate were tested for *in vitro* cytotoxic activity against 5 human cancer cell lines including breast ductal carcinoma (BT-474), undifferentiated lung carcinoma (CHAGO -K1), colon adenocarcinoma (SW-620), gastric carcinoma (KATO-3), hepatocarcinoma (HEP-G2) and one human normal cell line (lung fibroblast, Wi-38). *N. nucifera* plumule ethanolic extract, dichloromethane fractionate and ethanolic fractionate exhibited no significant activity against the five cancer cell line as well as one normal cell line with an IC_{50} more than 20 µg/ml. Information obtained from this study can be used for the identification and standardization of *N. nucifera* plumule and also toward monograph development on this plant.

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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

°C	Degree Celsius
Cm	Centimeter
DMSO	Dimethyl sulfoxide
G	Gram
hR_f	Hundreds of retention factors
IC_{50}	Half maximal inhibitory concentration
ICH	International Conference on Harmonization
LOD	Limit of detection
LOQ	Limit of Quantitation
mg	Milligram
min	Minute
ml	Milliliter
nm	nanometer
R^2	Correlation coefficients
R_f	Retention factors
RSD	Relative standard deviation
SD	Standard deviation
TLC	Thin layer chromatography
UV	Ultraviolet

v/v	Volume in a volume
WHO	World Health Organizations
µg	Microgram
µl	Microliter
β	Beta
α	Alpha
ATCC	American type culture collection
BT-474	Ductal carcinoma breast
Chago-K1	Undifferentiated lung carcinoma
SW-620	Colon adenocarcinoma
KATO-III	Gastric carcinoma
Hep-G2	Liver hepatoblastoma
Wi-38	Lung fibroblast

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Medicinal plants have long been used as a major source for treatment of human diseases [1]. The World Health Organization (WHO) estimated that about 80% of the world population still depend on the use of medicinal plants in traditional health systems for their primary health care needs [2]. The use of medicinal plants for medicinal purposes are traditionally considered harmless and increasingly being consumed by people without prescription. However, some of medicinal plants can cause health problems, some are not effective and some may interact with other drugs [3]. Therefore, traditional use of medicinal plants needs to be systematically investigated and standardized from the perspective of quality, safety and efficacy.

Nelumbo nucifera Geartn. is an important medicinal plant and used in traditional Thai medicine. It is the one of only two species of aquatic plant in the family Nelumbonaceae [4]. It has many common names such as Indian lotus, Chinese water lily and sacred lotus, which is known as Bua-luang in Thailand. Different parts (leaves, flowers, rhizomes, fruits and seeds) of this plant have been reported to possess various medicinal values to treat a variety of conditions, including diarrhea, abnormal bleeding, poor digestion, fever, and insomnia [5].

The one important part of *N. nucifera* is seed which used in folk medicine for the treatment of tissue inflammation, cancer, skin diseases, leprosy, poison antidote and generally prescribed to children as diuretics [6]. The seed of *N. nucifera* comprised of three parts including integuments (3.74%), plumule (3.03%) and cotyledons (93.23%) [7]. Plumule is found only in seed of *N. nucifera* and reported to have antioxidant activities [5] and recognized as a cooling food with anti-inflammatory activities [8]. Many studies revealed the major active constituents in plumule of *N. nucifera* and their biological activities. Several major alkaloid compounds (e.g. liensinine, isoliensinine, neferine) were shown a significant cytoprotective effect against oxidative stress [9]. Two major compositions (neferine and isoliensinine) had extensive cardiovascular activity; antiarrhythmic, antithrombic and antihypertensive [10]. Four alkaloids (lotusine, liensinine, isoliensinine and neferine) were determined in *N. nucifera* plumule and reported as major bioactive alkaloids [11]. Additionally, some constituents isolated from *N. nucifera* have been reported on anticancer potential. Isolated compounds from leaves exhibited cytotoxicity against human tumor cells. Alkaloid compounds from buds and leaves inhibited activity of melanoma cells. Aporphine alkaloids from leaves inhibited the proliferation of melanoma, prostate and gastric cancer cells. Bioactive alkaloid from embryos induced hepatocellular carcinoma cell apoptosis [12-15].

Neferine is one of the major bioactive alkaloids in *N. nucifera* plumule. Neferine content determination has been reported by various methods such as TLC [16], NMR spectroscopy [10], LC–ESI-MS/MS [17] and high-speed counter-current chromatography [18]. TLC densitometry and TLC Image analysis have many advantages as low cost, rapid and easy technique [19].

In spite of the several medicinal health benefits of *N. nucifera* plumule, the basic information of the standardization parameters of this plant part is still limited. Therefore, the objective of this study is to investigate the standardization parameters of *N. nucifera* plumule, to determine the neferine content using TLC densitometry and Image analysis and to evaluate the anticancer potential of this crude drug.

1.2 Research gap

Standardization of *N. nucifera* plumule, its neferine content and *in vitro* anticancer efficacy have never been reported in Thailand.

1.3 Objectives of the study

1.3.1 To evaluate macroscopic and microscopic characteristics of *N. nucifera* plumule in Thailand.

1.3.2 To determine standardization parameters of *N. nucifera* plumule in Thailand.

1.3.3 To evaluate neferine content in *N. nucifera* plumule by TLC densitometry and TLC image analysis.

1.3.4 To evaluate cytotoxicity against various cancer cell lines of *N. nucifera* plumule by MTT assay.

1.4 Expected benefits and applications

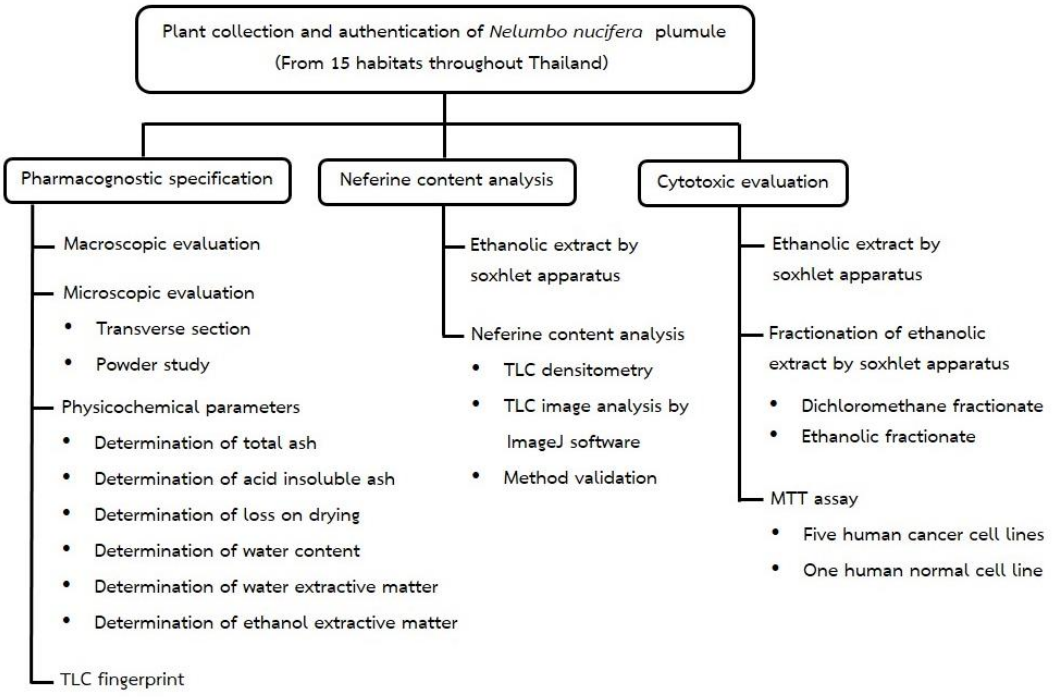
1.4.1 Able to provide scientific data of specification parameters of *N. nucifera* plumule in Thailand.

1.4.2 Able to provide scientific data of neferine content in *N. nucifera* plumule in Thailand.

1.4.3 Able to be a tool for the quality control of Thai medicinal crude drugs.

1.4.4 Able to provide scientific data in cytotoxicity of *N. nucifera* plumule against various cancer cell lines.

1.5 Conceptual framework



CHAPTER II

LITERATURE REVIEWS

2.1 *Nelumbo nucifera* Geartn.

2.1.1 Classification of *N. nucifera* [20, 21]

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Proteales

Family: Nelumbonaceae

Genus: *Nelumbo*

Species: *Nelumbo nucifera* Gaertn.

2.1.2 Synonyms: *Nelumbium speciosum* Willd., *Nelumbo speciose* Willd., *Nelumbium Nelumbo* (Linn.) Duce [4]

2.1.3 Vernacular names: Sanskit – Ambuja [6]; Hindi - Kamal, Kanwal; English - Sacred lotus; Bengali - Padma; Nepalese - Pales waa; Sinhalese - Nelum; Japanese - Hasu; Chinese - Lienoou; German - Indische lotusblume; French - Lotus sacred, Nelumbo; Arabian and Persian - Nilufer [22], Indian lotus, Chinese water lily [7]; Thailand - Bua-Luang [23].

2.1.4 Plant description [7, 24-26]

Nelumbo nucifera, Figure 1, is a member of the family Nelumbonaceae. Herbs perennial, aquatic. Petiol 1-2 m, terete, fistulous, glabrous or papillae hard and scattered; leaf blade abaxially blue-green, orbicular, 25-90 cm in diam., papery, glaucous, glacous, water-repellent, margin entire. Flowers 10-23 cm in diam.; peduncles longer than petioles, grabrous or sparsely spinulate. Tepals caduceus, pink or white, oblong elliptic to obovate, 5-10 x 3-5 cm. Stamens slightly longer than receptacle; filament slender; anther linear, 1-2 mm; connective appendage clavate, to 7 mm, incurved. Receptacle accrescent, turbinate, 5-10 cm in diam. Fruit oblong to ovoid, 1.0-2.0 x 7-15 cm, glabrous; pericarp thick, hardened.

2.1.5 Distribution of *N. nucifera*

N. nucifera is distributed in East, South and Southeast Asia and North Australia. This plant has been cultivated as a crop in Asia for more than 3000 years [27]. The species also shows important religious and culture significance [21]. It has earned the title of purity in Buddhist and Hindu traditions. Throughout Asia, many parts of the plant are used for nutrition and medicine [28].

2.1.6 Traditional uses of *N. nucifera*

N. nucifera has long been used in traditional medicine for its tremendous health benefit throughout the world. It is used to treat many diseases like diarrhea, high fever, hemorrhoids, sunstroke, vomiting of blood, uterine bleeding disorders, improving the skin condition, hypertension, hemoptysis, liver disorders and leprosy [29, 30].

2.1.7 Pharmacological activities of *N. nucifera*

Various pharmacological activities of many parts of *N.nucifera* have been screened scientifically such as anti-inflammatory activity, hepatoprotective activity, antioxidant activity etc. as shown in Table1.

Table 1 *Pharmacological activities of N. nucifera*

Pharmacological activities	**Part of plant**	**Pharmacological activities**	**Part of plant**
Anti-ischaemic activity [31]	seeds	Psychopharmacological activity [32]	rhizomes
Antioxidant activity [33-37]	seeds, rhizomes, flowers, leaves	Diuretic activity [38]	rhizomes
Hepatoprotective activity [33, 39]	seeds, leaves	Antipyretic activity [40, 41]	rhizomes, flowers
Anti-inflammatory activity [42-44]	seeds, rhizomes	Aldose reductase inhibitory activity [45]	flowers
Anti-fertility activity [46, 47]	seeds	Antibacterial activity [48]	flowers
Anti-arrhythmic activity [49-53]	seeds	Aphrodisiac activity [54]	flowers
Anti-fibrosis activity [55]	seeds	Antiplatelet activity [56]	flowers
Antiviral activity [57, 58]	seeds, leaves	Heart disease [59]	Leaves, seeds

Table 1 Pharmacological activities of *N. nucifera* (cont.)

Pharmacological activities	**Part of plant**	**Pharmacological activities**	**Part of plant**
Antiproliferative activity [60-62]	seeds	Anti-obesity activity [63]	leaves
Immunomodulatory [64]	seeds, rhizomes	Lipolytic activity [65]	Leaves, seeds
Antidiarrhoeal activity [66]	rhizomes	Hypocholesterolaemic activity [67, 68]	leaves
Hypoglycaemic activity [69-71]	rhizomes, flowers	Anticancer activity [72]	Leaves, seeds

2.1.8 Phytochemicals of *N. nucifera*

The various type of phytochemicals are isolated from many parts of *N. nucifera* as shown in Table 2. [7, 30, 43, 73, 74].

Table 2 Phytochemicals of N. nucifera

Part of plant studied	Phytochemicals of *Nelumbo nucifera*
Fruits and seeds	dauricine, l. iensinine, isoliensinine, neferine, lotusine, nuciferine, *n*-nornuciferine, *o*-nornuciferine, roemerine, pronuciferine, armepavine, D(–)-3 - bromo-*O*-methyl-armepavine, D–1,2,3,4-tetrahydro-6-methoxy-1-(p-methoxbenzly) -2-methyl-7-isoquino- linol, saponins, gallic acid
Leaves	procyanidin, anonaine, D(–)-3'-bromo-*o*-methyl-armepavine, coclaurine, norcoclaurine, D-1,2,3,4-tetrahydro-6-methoxy-1-(*p*-methoxybenzly)-2-methyl-7-isoquinolinol, liriodenine, dehydroemerine, dehydronuciferine, dehydroanonaine, nelumboside, remerine, quercetin-3-*O*-β-D-glucuronide, asimilobine, lirinidine, *N*-methyl-coclaurine, *N*-methylisococlaurine, quercetin, rutin, hyperoside, leucocyanidin, leucodelphinidin, isoquercitrin, (+)-catechin, astragalin

Table 2 Phytochemicals of *N. nucifera* (cont.)

Part of plant studied	**Phytochemicals of *Nelumbo nucifera***
Flowers	kaempferol, kaempferol 3-*O*-β-D-galactopyranoside, kaempferol 3-*O*-β-D-glucopyranoside, Kaempferol 7-*O*-β-D-glucopyranoside, kaempferol 3-*O*-β-L-rhamnopyranosyl-(1,6)-β-D-glucopyranoside, kaempferol 3-*O*-β-L-rhamnopyranosyl-(1⟶2)-β-D-glucopyranoside, kaempferol 3-*O*-β-L-rhamnopyranosyl-(1⟶2)-β-D-glucuronopyranoside, kaempferol 3-*O*-α-D-glucuronopyranoside, kaempferol 3-*O*-α-D-glucuronopyranosyl, methylester, arbutin, myricetin3', 5'-dimethylether 3-*O*-β-D glucopyranoside, nelumboroside A, nelumboroside B, quercetin 3-*O*-β-D-glucopyranoside, isorhamnetin 3-*O*-β-D-glucopyranoside, isorhamnetin 3-*O*-β-L-rhamnopyranosyl- (1⟶6)-β-D-glucopyranoside, β-sitosterol glucopyranoside
Rhizomes	betulinic acid

2.2 *Nelumbo nucifera* plumule

2.2.1 Description of *N. nucifera* plumule

N. nucifera plumule is the green germ of the mature lotus seed [75]. The plumule is cushioned and protected by two large enveloping cotyledons one long and one short, rolls into arrow shaped structure [27], somewhat club-shaped, 1-1.4cm long, and 0.2cm in diameter. It is brittle, easily broken, and with several holes on cross section. It has slight odor and bitter taste [76].

2.2.2 Traditional uses of *N. nucifera* plumule

N. nucifera plumule has long been used in traditional medicine for treatment of many diseases such as nerves disorders, insomnia, high fevers with restlessness, cardiovascular diseases such as hypertension and arrhythmia [4], tranquilizer and antihypertensive agent [77].

2.2.3 Pharmacological activities of *N. nucifera* plumule

Several pharmacologically active constituents that isolated from *N. nucifera* plumule are responsible for the medicinal values such as nerves disorder, insomnia, high fevers with restlessness, cardiovascular diseases (hypertension), arrhythmia, anti-HIV activity, anti-inflammatory activity [78], treatment of cholera [79]. Some constituents isolated from *N. nucifera* plumule have been reported on cytotoxicity activity such as bioactive alkaloid induces hepatocellular carcinoma cell apoptosis [12-15].

2.2.4 Phytochemicals of *N. nucifera* plumule

N. nucifera plumule contains various compounds including alkaloids (demethylcolaurine, isoliensinine, liensinine, lotusine, methylocrypalline, neferine, nuciferine, pronuciferine, *N*-nornuciferine, *O*-nornuciferine, liriodinine, *d*-methylcoclaurine, tetrandrine, remerine and *dl*-armepavine), flavonoids (galuteolin, hyperine, rutin), polysaccharides [78, 80-82], some microelements (Zn, Fe, Ca, and Mg) [75, 79] and glutathione [4].

2.3 Neferine

2.3.1 Description

Neferine is the major bisbenzylisoquinoline alkaloid isolated from the plumule of a traditional medicinal plant *N. nucifera* [83, 84].

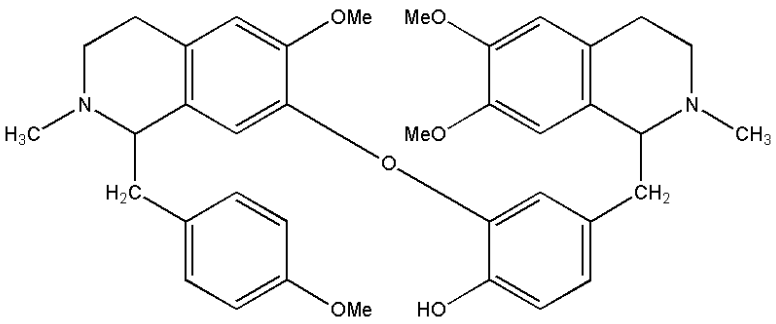


Figure 1 Chemical structure of neferine

IUPAC Name: 4-[[(1R)-6, 7-dimethoxy-2-methyl-3, 4-dihydro-1H-isoquinolin-1-yl] methyl]-2-[[(1R)-6-methoxy-1-[(4-methoxyphenyl) methyl]-2-methyl-3, 4-dihydro-1H-isoquinolin-7-yl] oxy] phenol

Chemical formula: $C_{38}H_{44}N_2O_6$ [10]

Molecular weight: 624.77 g/mol

Classification: Alkaloid

2.3.2 Pharmacological activities of neferine

Neferine which is a bisbenzylisoquinoline alkaloid isolated from the *N. nucifera* plumule displays multiple pharmacological activities [84]. The pharmacological activities of neferine are as follows:

2.3.2.1 Antidiabetic effects

Neferine isolated from the *N. Nucifera* plumule was comparable with rosiglitazone in enhancing insulin sensitivity and improving fasting blood glucose, triglycerides, and inflammatory cytokines in insulin-resistant rats. The mechanism of action might involve reducing release of tumor necrosis factor-alpha by activating the gamma peroxisome proliferator-activated receptor (PPAR) as well as decreasing insulin compensatory release from pancreatic islet cells [85]. Moreover, the previous study in neferine treated type 2 diabetic rats showed that body weight, blood pressure,

fasting blood glucose, insulin, total cholesterol and triglyceride were decreased and high density lipoprotein was increased [86].

2.3.2.2 Antidepressant activity

In vitro and animal data studies showed that neferine isolated from *N. nucifera* plumule might have antidepressant activity as indicated by its anti-immobility effects in mice in a forced swimming test. Neferine is a direct 5-hydroxytryptamine (5-HT) 1A receptor agonist and may inhibit 5-HT reuptake or activation of 5-HT metabolism [87].

2.3.2.3 Antiarrhythmic effects

Neferine, a major alkaloid component in the *N. nucifera* plumule has antagonized arrhythmias induced by aconitine in rats, calcium chloride in mice, and coronary occlusion-reperfusion in dogs. Neferine's anti-arrhythmic effect may involve by blocking human-ether-a-go-go-related gene potassium channels associated with repolarization of the cardiac potential. The effects of neferine on the electrical andmechanical activity were investigated in guinea pig papillary muscles and atria [81, 88, 89].

2.3.2.4 Anticoagulants and antiplatelets

In animal study, neferine isolated from *N. nucifera* has been noted to have inhibitory activity on platelet aggregation. Neferine effects on platelet

aggregation and TXA2/PGI2 and cAMP/cGMP balance were studied with methods of turbidimetry and RIA. Neferine was shown to significantly inhibit rabbit platelet aggregation induced by adenosine diphosphate (ADP), collagen, arachidonic acid (AA) and platelet-activating factor (PAF) with IC_{50} of 16, 22, 193 and 103 $mmol.L^{-1}$. Neferine was found to increase vascular 6-keto-PGF1 alpha and platelet cAMP levels in dose-dependent manner. The results suggest that the mechanism of neferine on platelet aggregation is related to regulation of TXA2/PGI2 and cAMP/cGMP balance [62].

2.3.2.5 Antihypertensives

Preliminary research on antihypertensives revealed that neferine and isoliensinine have been noted as two major constituents of the *N. nucifera* plumule that may have antihypertensive effects [10].

2.3.2.6 Antitumor

Neferine isolated from the *N. Nucifera* plumule has an effect on human osteosarcoma cells [90]. Moreover, neferine induces mitochondrial mediated ROS generation and leads to caspase-dependent apoptosis in HepG2 cells [91].

2.3.2.7 Anticancer

Neferine, an alkaloid from the *N. Nucifera* plumule, has enhancing anti-cancer activities in Hep3B cell, molecular mechanisms of cell cycle arrest, ER stress induced apoptosis and anti-angiogenic response [92]. It also induces reactive oxygen

species mediated intrinsic pathway of apoptosis in HepG2 cells [91] as well as inhibits human lung cancer cell growth by MAPK activation and cell cycle arrest [93].

2.3.2.8 Antioxidant

The study showed that neferine isolated from *N. Nucifera* plumule had anti-amnesic activity with antioxidant and anti-inflammatory capacities, as well as inhibition of ChEs and BACE1 [94].

2.4 Quality control methods for herbal material [3, 95-99]

Standardization is the gathering of complete data on medicinal plants including the qualitative and quantitative parts of analysis. Qualitative analysis covers the identification of the compound, whereas the quantitative analysis is performed by measuring the level of a chemical in a crude drug extract. The standardization will help in authentication of the plants and ensures reproducible quality of herbal medicines which will lead to safety and efficacy. The standardization parameters covers the following methods.

2.4.1 Macroscopic and microscopic evaluation [100-103]

The macroscopic characteristics are useful for determining the identity and purity of the herbal drug including shape, size, color, texture, fracture aspects and characteristics of the cut surface. This techniques may be used to discriminate

between the desired plant species or plant part and morphologically similar, yet distinguishable species that could occur as potential adulterants.

The microscopic characteristics helps to identify the herbal drug and may be crucial in the identification of adulterants. This analysis is necessary for powdered or fragmented drugs. Using microscope detecting various cellular tissues, trichomes, stomata, starch granules, calcium oxalate crystals are some of important parameters which play important role in identification of certain crude drug. Crude drug can also be identified microscopically by cutting the thin transverse section or longitudinal section. Some of the chemicals which are used in obtaining clear sections are phloroglucinol, chloral hydrate, etc. The microscopic characters are usually described by words or coupled with pictures drawn by hands or photos of both transverse sections and powder characteristics. The microscopic analysis should be supplemented with data from chemical and physico-chemical analysis.

2.4.2 Determination of total ash [97, 98, 104, 105]

The total ash is the amount of inorganic elements in plant. It is the residue remaining after the water and organic matters have been removed by incineration. Total ash consists of the elements and their salts for example carbonates, phosphates, oxides and silicates.

2.4.3 Determination of acid insoluble ash [97, 98, 104, 105]

Total ash content is not sufficient to reflect the quality of herbal material. The acid insoluble ash content is another index to demonstrate the quality of herbal material. Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and re-incinerating that acid insoluble matters. Some elements including silica are not solubilized by hydrochloric acid then still remain.

2.4.4 Determination of loss on drying [98, 106]

The loss on drying is a wildly used method to determine and control the moisture content of herbal drugs by heating the sample. It also refers to the loss of any volatile matters. This method compares the weight of an herbal drug before and after it is dried, expressed as percentage weight by weight resulting from water and volatile matters.

2.4.5 Determination of water content [100, 106]

The water content determination is commonly measured properties of plant materials. The presence of excessive amounts of water in plant drugs is responsible for the growth of bacteria and fungi as well as the hydrolysis of constituents. The pharmacopoeial monographs limit the water content, especially in drugs that have the facility to absorb it, or in which the excessive amounts of water cause deterioration. The azeotropic method may be applied, which consists of distilling the crude drug with toluene or xylene. This method requires special

equipment. The water and the solvent are distilled together and separated in the receiving tube on cooling.

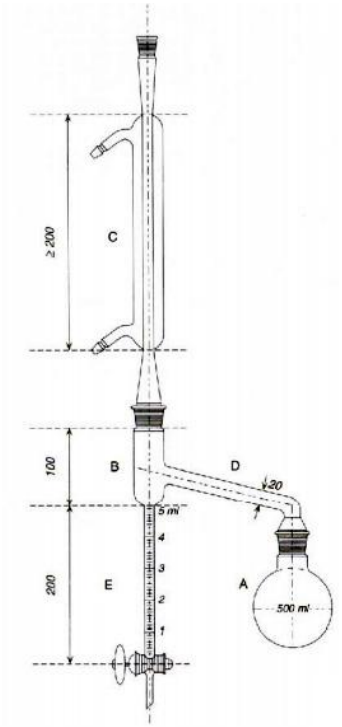


Figure 2 Azeotropic apparatus for determination of water content (dimentions in mm), (A) a glass flask, (B) a cylindrical tube, (C) a reflux condenser, (D) a receiving tube, (E) a graduated receiving tube

2.4.6 Determination of volatile oil [95]

The determination of volatile oils are characterized by their odor, appearance and capability to volatilize at room temperature. They are composed of a mixture of chemical compounds especially aromatic compounds such as the

oxygenated derivatives, sesquiterpenes, monoterpenes. Volatile oils are considered to be the fragrance of the medicinal herbs. This method will be applied by hydrodistillation using Clevenger equipment.

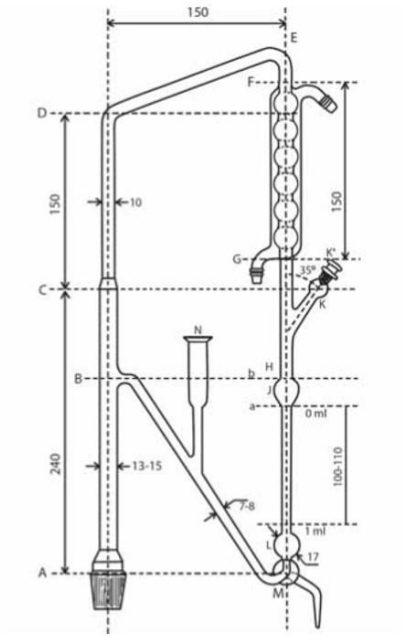


Figure 3 Clevenger apparatus for determination of volatile oil content

2.4.7 Determination of solvent extractive matters [97, 98, 104]

The determination of extractable matters refers to the amount of active constituents in a plant material when extracted with a specific solvent. This value provides an indication of the extent of non-polar, medium polar and polar components present in the plant material. It is employed for those plant materials for which no suitable or biological assay method exist. Substances extracted with water, with ethanol in various dilutions and, more rarely, with ether are usually

determined. The method is based on the solubility of active substances in a given solvent, and when these are not known, on the pharmacological activity of the extract obtained as a solvent.

2.4.8 Thin layer chromatographic identification (TLC fingerprint) [106-108]

Chromatographic technique is valuable additional information to establish the identity of plant material. This method is effective and convenient to perform, and the equipment required is inexpensive.

TLC fingerprint is the important key for herbal medicines made up of the complex mixture of constituents such as essential oils, resin, that no longer have any organic structure. It is a powerful and relatively rapid solution to distinguish between chemical classes. The basic parameter used to describe migration in TLC is the retention factor (Rf). The Rf is defined as the distance traveled by the compound divided by the distance traveled by the solvent.

$$hRf = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent front}} \times 100$$

The Rf for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant such as solvent system, adsorbent, thickness of the adsorbent, amount of material spotted and temperature. It is frequently used for evaluating medicinal plant materials and their preparations

and should be determined on the basis of published pharmacopoeial monograph or established experimentally for each individual plant material.

2.5 Quantitative analysis of neferine in *N. nucifera* plumule [11, 109-111]

Various chromatographic methods are used for neferine content analysis for example HPLC (High-Performance Liquid Chromatographic), CZE (Capillary Zone Electrophoresis). This study used TLC densitometry and TLC image analysis by ImageJ software.

2.5.1 TLC densitometry [112-115]

Densitometry measures the amount of a substance which is on the TLC plate based on the intensity of the different light absorption or fluorescence and then converts the signal into densitogram and measured for peak area. Densitometry can be used to scan wavelength ranging 190-800 nm. This method is most widely used, highly sensitive, versatile, convenient and reliable quantitative TLC method.

2.5.2 TLC Image analysis by ImageJ software [116-118]

This method uses ImageJ software to analyze TLC image due to its free download software application, java image processing and analytic program inspired by National Institutes of Health (NIH), USA. A digital image of a TLC chromatogram is obtained with digital camera and densities of the TLC spots are analyzed using ImageJ software. Combining digital photography with regular TLC, it is to improve the

quantitation of compound as well as provide the accuracy of the quantitative TLC analysis with lower cost than other commercial equipment. The pixel density of spot image can be used as a detector signal. The intensity is proportional to the amount of substance and can be in accordance with the peak area when quantified with reference densitometry.

2.6 Method validation

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories [119].

2.6.1 Accuracy

The accuracy expresses the closeness of the results obtained by the analytical procedure to agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

2.6.2 Specificity

The investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure.

2.6.3 Precision

Precision expresses the closeness of agreement between a series of measurements. Repeatability (intraday) and intermediate precision (interday) of the method is determined by analysis of three replicates under the same operating conditions. It is conducted and presented as relative standard deviation (RSD).

2.6.4 Limit of detection (LOD)

The LOD of an analytical procedure is the lowest amount of analyte in sample which can be detected but not necessarily quantitated as an exact value.

2.6.5 Limit of quantitation (LOQ)

The LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

2.6.6 Robustness

Robustness of an analytical procedure is the reliability of an analysis with respect to deliberate variations in method parameters. The evaluation of robustness should be considered during the development phase.

2.6.7 Linearity

The linearity of an analytical procedure is the ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

2.6.8 Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

2.7 Cytotoxicity evaluation

Cytotoxicity assays are widely used for screening of cytotoxicity activity of interested compounds. There are many methods to evaluate the cytotoxic effects such as MTT assay, lactate dehydrogenase (LDH or LD) assay, sulforhodamine B (SRB) assay and clonogenic assay (Table3). In this study, MTT assay is used.

Table 3 Cytotoxic methods

Method	Principle
MTT assay [120]	This assay measures the reducing potential of the living cell to transform the MTT tetrazolium salt into MTT formazan.
Lactate dehydrogenase (LDH or LD) Assay [121]	Lactate dehydrogenase (LD or LDH) catalyzes the reduction of pyruvate by NADH to form Lactate and NAD. The catalytic concentration is determined from the rate of decrease of NADH measured at 340 nm.
Sulforhodamine B (SRB) assay [122]	Using for cell density determination, based on the measurement of cellular protein of cultured cells content.
Clonogenic assay [123]	Cell biological technique for studying the effectiveness of specific agents on the survival and proliferation of cells. Treated and untreated cells are allowed to form colonies for 14 days and then stained with 0.3% crystal violet solution.

2.7.1 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay, originally described by Mosmann (1983) [120] for measuring cell survival and proliferation [124], has been used successfully to quantitate macrophage-mediated cytotoxicity. Colorimetric assay is used based on the capacity of succinate dehydrogenase in mitochondria of viable cells to transform the MTT tetrazolium salt into MTT formazan (5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan) [125]. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The advantages of this method is savings in costs, reagents and equipment, reducing labor through the elimination of sample processing steps for liquid scintilation and gamma counting as well as avoiding use of radioisotopes [126]. The results will be expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value). According to the American NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with IC_{50} value ≤ 20 μg/ml, while this value was deemed at ≤ 4 μg/ml for a pure compound [127].

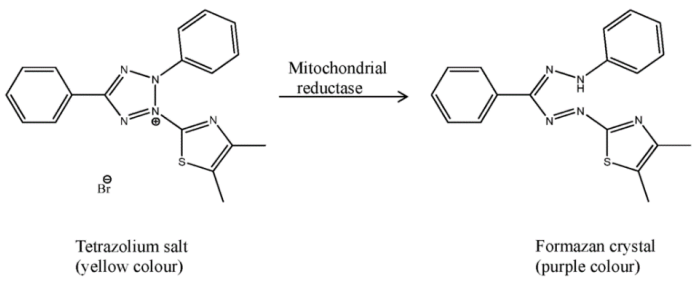


Figure 4 The formation of formazan crystals from the tetrazolium salt [128].

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Filter paper grade No.4	Whatman, England
Filter paper grade No. 40, Ashless	Whatman, England
TLC silica gel 60 GF_{254} (0.2 mm thickness, 20 x 10 cm)	Merck, Germany
Tissue culture flask 25 cm^2	Constar, USA
Tissue culture plate 96 well	Constar, USA

3.2 Chemicals and reagents

Acetic acid	BDH Chemicals Ltd., England
Bismuth nitrate	Sigma-Aldrich., Germany
Diethylamine (Analytical grade)	RCI Labscan, Thailand
Ethanol (Analytical grade)	Thailand
Ethyl acetate (Analytical grade)	RCI Labscan, Thailand
Neferine	Sigma-Aldrich., St. Louis, USA
Potassium iodide	BDH Chemicals Ltd., England
Toluene (Analytical grade)	RCI Labscan, Thailand

Dichloromethane (Analytical grade)	RCI Labscan, Thailand
Ultrapure water	Thailand
RPMI 1640	Biochrom Gmbh, Germany
Fetal calf serum	Biochrom Gmbh, Germany
Doxorubicin	Sigma-Aldrich., Germany
Normal saline	General Hospital Products Public Co., Ltd., Thailand
Trypsin	Sigma-Aldrich., Germany
MTT	Invitrogen, USA
EDTA	Sigma-Aldrich., Germany
Dimethylsulfoxide (DMSO)	RCI Labscan, Thailand

All of chemicals and reagents were analytical grade.

3.3 Equipments and instruments

Ashing Furnance	Carbolite, England
CAMAG Visualizer	CAMAG, Switzerland
Digital camera (Canon Power Shot A650 IS)	Canon Marketing Co. Ltd., Thailand
Hot air oven	WTC Binder, Germany
ImageJ software (version: 1.46r)	The National Institute of Mental Health, USA

Microscope	Zeiss, Germany
Rotary evaporation instrument	Buchi Glas Uster, Switzerland
Ultraviolet fluorescence analysis cabinet	Spectronics Corporation, USA
winCAT software (version: 1.4.6.2002)	CAMAG, Switzerland
CO_2 Incubator Forma Series II	Thermo Electron Corporation, USA
Plate mixer SH30	Bio-Active Co., Ltd.
Microplate reader Multiskan FC 540 nm	Thermo Scientific, USA
Hemocytometer deep 1/10 mm	BOECO, Germany
Invert microscope	Nikon, Japan

3.4 Cell Line

Five human cancer cell lines

BT-474 (Ductal carcinoma breast)	ATCC, USA
CHAGO-K1 (Undifferentiated lung carcinoma)	ATCC, USA
SW-620 (Colon adenocarcinoma)	ATCC, USA
KATO-3 (Gastric carcinoma)	ATCC, USA
Hep-G2 (Liver hepatoblastoma)	ATCC, USA

One human normal cell line

Wi-38 (Lung fibroblast)	ATCC, USA

3.5 Methods

3.5.1 Plant samples

3.5.1.1 Plant sample collection

Dried *N. nucifera* plumule samples was collected from 15 different locations in 5 parts (North, Central, North-East, East, South) of Thailand.

3.5.1.2 Plant sample authentication

N. nucifera plumule samples, was authenticated by specialist, Associate Professor Dr.Nijsiri Ruangrungsi, College of Public Health Sciences, Chulalongkorn University, Thailand.

3.5.1.3 Plant sample preservation

N. nucifera plumule samples was produced as herbarium specimens and deposited at College of Public Health Sciences, Chulalongkorn University.

3.5.2 Pharmacognostic specifications

Pharmacognostic parameters such as morphological characters, microscopical characters, physicochemical constants including ethanol soluble extractive value, water soluble extractive value, water content, loss on drying, total ash, acid insoluble ash and TLC fingerprint was performed following World Health Organization (WHO) guidline [95] with some modifications [96].

3.5.2.1 Macroscopic evaluation

Whole plant of *N. nucifera* Geartn. was demonstrated botanically by hand drawing. *N. nucifera* plumule samples was identified with size, shape, color and other by visual inspection.

3.5.2.2 Microscopic evaluation

Transverse section

N. nucifera plumule was transversely sectioned and investigated with the aid of microscope.

Powder study

The dried *N. nucifera* plumule was powdered and studied under microscope.

3.5.3 Physicochemical parameters

All the tests of physicochemical parameters was done in triplicate.

3.5.3.1 Determination of loss on drying

Three grams of dried powder of *N. nucifera* plumule samples was placed in a crucible. The drying was carried out in an oven at 105ºC until constant weight. The loss of weight was calculated in percentage of dried sample [95, 96].

3.5.3.2 Determination of total ash

Three grams of dried powder of *N. nucifera* plumule samples was placed in crucible and incinerated to ash at 500ºC until it is become white or gray color indicating the absence of carbon, cooled in a desiccator and weighed. The content of total ash was calculated in percentage of dried sample [95, 96].

3.5.3.3 Determination of acid insoluble ash

Twenty-five milliliters of hydrochloric acid (70g/l) was added to the crucible containing the total ash and boiled gently for 5 minutes. The insoluble matters was collected on an ashless filter-paper. The filter-paper containing the insoluble matters was transferred to the original crucible, dried on a hot-plate and incinerated again. After cooling down in a desiccator, the content of acid insoluble ash was weighted and calculated in percentage of dried sample [95, 96].

3.5.3.4 Determination of water extractive matter

Five grams of dried powder of *N. nucifera* plumule samples was accurately weighed. The sample was macerated with 70 ml of water in closed flask for 24 hours, shaking during the first 6 hours and allowing to stand for 18 hours. The marc was weighed with small portion of water and the filtrate 100 ml. Twenty milliliters of the filtrate was transferred to a pre weighed beaker, evaporated to dryness on a water bath, dried in on oven at 105ºC for 6 hours and cooled in a desiccator. The content of water soluble extractable matter was calculated in percentage [95, 96].

3.5.3.5 Determination of ethanol extractive matter

Five grams of dried powder of *N. nucifera* plumule samples was accurately weighed. The sample was macerated with 70 ml of ethanol in closed flask for 24 hours, shaking during the first 6 hours and allowing to stand for 18 hours. The marc was weighed with small portion of ethanol and the filtrate 100 ml. Twenty milliliters of the filtrate was transferred to a pre weighed beaker, evaporated to dryness on a water bath, dried in on oven at 105ºC for 6 hours and cooled in a desiccator. The content of ethanol soluble extractable matter was calculated in percentage [95, 96].

3.5.3.6 Determination of water content

Five grams of dried powder of *N. nucifera* plumule samples was added with 200 ml of water-saturated toluene in flask. The flask was heated until the water was distilled

over. The condenser tube was allowed to cool and the toluene and water in receiving tube was separated completely, the volume of water distilled over can be read [95, 96].

3.5.3.7 Determination of volatile oil

Ground samples was weighed 100 g and added 600 ml with distilled water in round bottomed flask. Then continue distillation about 4-6 hours, stop the heater, wait at least 10 minutes and read volume of oil that was separated in the receiving tube.

3.5.3.8 Thin layer chromatographic fingerprint

The extract of *N. nucifera* plumule was obtained by maceration of one gram of crude drug is 20 ml of ethanol for 6 hours under shaking followed by 18 hours standing at room temperature. After filtration and evaporating, the residue was dissolved in 1 ml of ethanol and 3-5 µl was applied to the TLC plate coat with silica gel G60 F_{254}. The TLC plate was developed in the chamber with the suitable solvent system of toluene: ethyl acetate: diethylamine (7:2:1 v/v). After development, the TLC plate was removed and observed the produced spots, under ultraviolet light at 254 and 365 nm as well as detection by spraying with Dragendorff's reagent and heating at 110°c for 5-10 minutes.

3.5.4 Quantitative analysis of neferine

3.5.4.1 Preparation of the ethanolic extract of *N. nucifera* plumule

The acculate 5 g of dried powder of *N. nucifera* plumule was exhaustively extracted with 250 ml of 95% ethanol in a Soxhlet apparatus. The prepared extract was filtered, evaporated *in vacuole* till dryness and kept at 4ºC. Forty millgrams of the extracts samples was dissolved in 1 ml of 95% ethanol and stored at 4ºC for further TLC analysis.

3.5.4.2 Preparation of neferine standard solution

One milligram of neferine standard was dissolved in 1 ml of 95 % ethanol. This stock solution was diluted serially for calibration curves.

3.5.4.3 TLC densitometry

Three microliters of the extract solution and neferine standard solutions was applied to silica gel G60 F_{254} TLC plates 10 × 20 cm. The plate was placed in a chromatography tank previously saturated for 45 min with a mobile phase of toluene: ethyl acetate: diethylamine (7:2:1 v/v). The plate was developed in a normal vertical chromatography tank at ambient temperature to a distance 8 cm. The plates was allowed to dry at room temperature and then the spots will be scanned by CAMAG TLC scanner at 285 nm. A calibration curve was obtained by plotting the area under the peak against neferine standard concentration and regression equations

was computed by winCATS standard program. The quantitative analysis was performed in triplicate.

3.5.4.4 TLC image analysis

Developed TLC plate was further observed under ultraviolet light 254 nm in Spectroline® UV viewing cabinet. The photograph was taken using digital camera and save as Tiff file format. The intensity of spot on TLC plate was transformed to chromatographic peak by ImageJ software. The amounts of neferine was determined based on the calibration curve of the neferine standard performed in the same TLC plate. The tests was done in triplicate.

3.5.5 Method validation

3.5.5.1 Calibration curve

Calibration curve was constructed by plotting peak area *versus* five different levels of standard neferine. Linear regression and correlation coefficients was analyzed by winCATS standard program.

3.5.5.2 Accuracy

The accuracy of an analytical procedure was analyzed by adding three different concentrations of standard neferine (low, medium and high levels) into sample. All tests was done in triplicate. The accuracy was calculated as percentage recovery of neferine by the formula:

$$\% \text{ Recovery} = [A / (B+C)] \times 100$$

A = the amount of neferine that found after spiking standard solution

B = the amount of neferine that found before spiking standard solution

C = the amount of neferine standard actually added

3.5.5.3 Precision

The precision of the TLC method for neferine determination was evaluated by repeatability (intra-day) and intermediate precision (inter-day). The sample with three different concentrations of neferine was analyzed on the same day and three different days respectively. All sample tests was done in triplicate. The precision was evaluated as the relative standard deviation (RSD) according to the following equation:

$$\% \text{ RSD} = \text{Standard deviation } (\sigma) \times 100 / \text{Mean}$$

3.5.5.4 Limit of detection (LOD)

The limit of detection was estimated based on the residual standard deviation of regression line and the slope of the calibration curve as:

$$LOD = 3.3\ \sigma/S$$

Where, **σ** = the residual standard deviation of regression line

S = the slope of the calibration curve

3.5.5.5 Limit of quantitative (LOQ)

The limit of quantitative was estimated based on the residual standard deviation of regression line and the slope of the calibration curve as:

$$LOD = 10\ \sigma/S$$

Where, **σ** = the residual standard deviation of regression line

S = the slope of the calibration curve

3.5.5.6 Robustness

Robustness in this study was performed by making small deliberate changes in the ratio of mobile phase. Mobile phase ratio was varied with toluene: ethyl acetate: diethylamine (7:2:1 v/v), (7.1:1.9:1 v/v), (6.9:2.1:1 v/v). The tests was done in triplicate. The robustness was illustrated by %RSD of peak area.

3.5.5.7 Specificity

The specificity was performed by comparison of absorbance spectra of the standard neferine and each sample (peak identity) along with comparison of matching absorbance spectra at apex, up slope and down slope of the peak (peak purity) by using CAMAG TLC scanner 4.

3.5.5.8 Data analysis

The quality parameters was conducted as grand mean ± pooled standard deviation. The neferine content between TLC densitometry and TLC image analysis was statistically compared using paired *t*-test.

3.5.6 Cytotoxicity determination

The cytotoxic activity was done at the institute of Biotechnology and Genetic Engineering, Chulalongkorn University. Five human cancer cell lines and 1 human normal cell line including BT-474 (breast ductal carcinoma), CHAGO -K1 (undifferentiated lung carcinoma), SW-620 (colon adenocarcinoma), KATO-3 (gastric carcinoma), HEP-G2 (hepatocarcinoma) and WI-38 (lung fibroblast) respectively, was purchased from American Type Culture Condition (ATTC). Cell survival was measured using the MTT assay.

3.5.6.1 Fractionation of *N. nucifera* plumule crude ethanolic extract

Crude ethanolic extract of a sample of *N. nucifera* plumule was fractionated by dissolving with dichloromethane. The crude ethanolic extract, dichloromethane fractionate and ethanolic fractionate was dissolved in DMSO to obtain the concentrations of 0.01, 0.1, 1, 10, 100 and 200 µg/ml.

3.5.6.2 Preparation of doxorubicin (positive control drug)

Five milligrams of doxorubicin was dissolved in normal saline to obtain the concentrations of 0.001, 0.01, 0.1, 1 and 10 µg/ml.

3.5.6.3 Preparation of MTT solution

MTT was dissolved in normal saline at 5 mg/ml, stored in the dark at 4°C and used within 1 month.

3.5.6.4 Preparation of cell line

The cell lines was cultured in tissue culture flask in RPMI-1640 supplemented with 5% (v/v) fetal calf serum and incubated at 37°C in 5 % CO_2 for 3 days. Two hundreds microliters of cells was seeded in a 96-well culture plates at a density of $1x10^4$ cells/well and cultured in a 5 % CO_2 incubator at 37°C and 100% relative humidity for 24 h.

3.5.6.5 MTT Method

The sample solution (2 µl) was dispensed into the appropriate wells. This analysis was performed in 4 replicates (control cells group, n=4; each sample treatment group, n=4; Doxorubicin/DMSO n=4). Culture plates was incubated for 72 h prior to the addition of MTT solution (10 µl). Plates were incubated for 4 h at 37°C, 5 % CO_2 incubator. After incubation, the formazan produced in the cells is captured as dark crystals in the bottom of the wells. All of the culture medium supernatant was removed from wells. DMSO (150 µl) was added to dissolve the resulting formazan and mixed for 2-3 minutes on an orbital shaker. Following formazan solubilization, the absorbance was measured using a microplate reader at 540 nm. The cell survival was calculated in percentage by the following formula:

$$\text{Percentage of cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

The cytotoxic activity was expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value).

CHAPTER IV
RESULTS

4.1 *N. nucifera plumules* examination

4.1.1 Macroscopic evaluation

Nelumbo nucifera plumules (Figure 5) were the green germ of the mature lotus seed. The plumules were cushioned and protected by two large enveloping cotyledons one long and one short, rolls into arrow shaped structure, somewhat club-shaped, 1-1.4cm long, and 0.2cm in diameter. It was brittle, easily broken, and with several holes on cross section. It had slight odor and bitter taste. The whole plant drawing of *N. nucifera* Gaertn. was illustrated in Figure 6

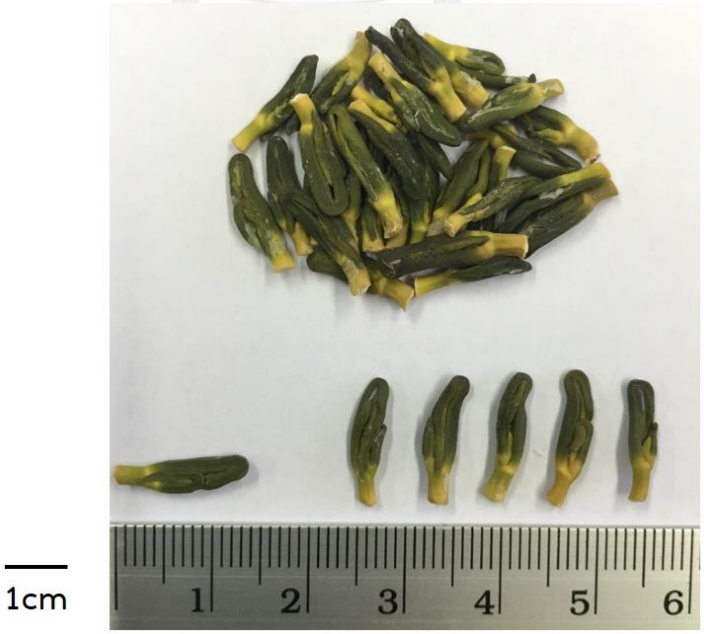


Figure 5 *Nelumbo nucifera* plumule crude drug

Figure 6 *Nelumbo nucifera* Gaertn.

4.1.2 Microscopic evaluation

4.1.2.1 Powder microscopy

The powder microscopy of *N. nucifera* plumule showed the present of anomocytic type stomata, parenchyma surface with starch granule, starch granule (round shape), starch granule (hilum shape) and layer of cotyledons in surface view as shown in Figure 7.

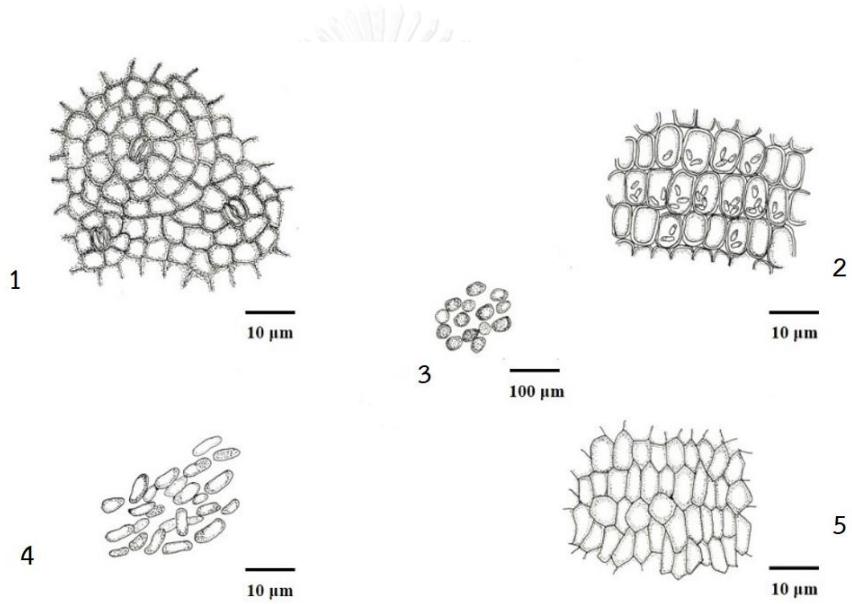


Figure 7 Histological character of *N. nucifera* plumule powders

1. anomocytic type stomata
2. Parenchyma surface with starch granule
3. Starch granule (round shape)
4. Starch granule (hilum shape)
5. Layer of cotyledons in surface view

4.1.2.2 Transverse section

Transverse section of *N. nucifera* plumule showed the arrangement of different type of cells including epidermis, air chamber, parenchyma and vascular bundle as illustrated in Figure 8.

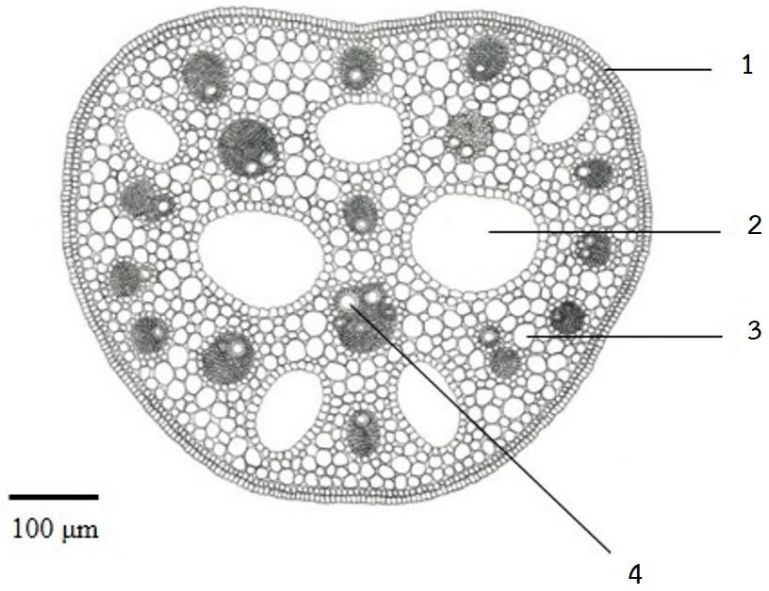


Figure 8 Transverse section of *N. nucifera* plumule

1. Epidermis
2. Air Chamber
3. Parenchyma
4. Vascular bundle

4.1.3 Physicochemical evaluation

Physicochemical properties of *N. nucifera* plumules from 15 different sources throughout Thailand including total ash, acid insoluble ash, loss on drying, extractive matter contents, moisture and volatile oil were evaluated for the pharmacognostic specifications as presented in Table 4.

Table 4 Physicochemical specifications of *N. nucifera* plumules

Parameter (% by weight)	**Mean ± SD***
Total ash	3.86 ± 0.17
Acid insoluble ash	0.54 ± 0.04
Loss on drying	9.91 ± 0.09
Water soluble extractives	28.27 ± 2.01
Ethanol soluble extractives	16.54 ± 1.34
Moisture	10.02 ± 0.72
volatile oil	0

*Results were expressed as grand mean ± pooled SD from 15 samples in triplicate

4.1.4 Thin layer chromatographic fingerprint

The TLC fingerprint profile of *N. nucifera* plumule using toluene: ethyl acetate: diethylamine (7:2:1) as mobile phase was shown in Figure 9.

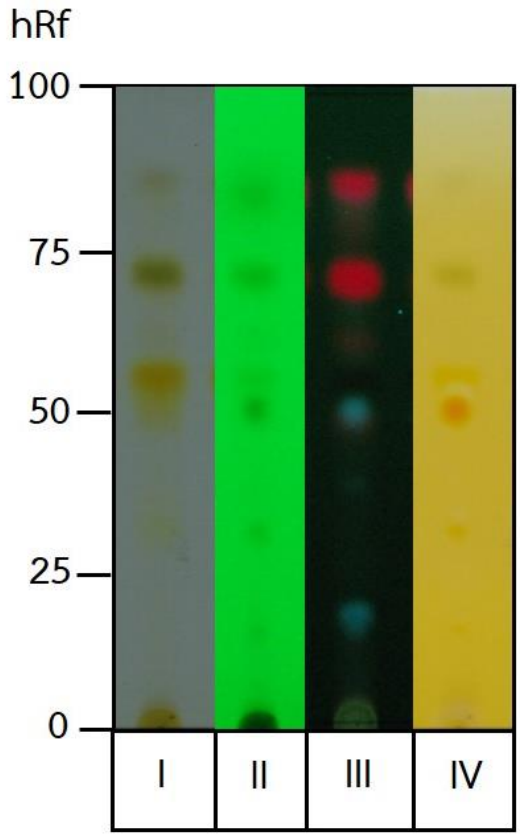


Figure 9 TLC fingerprint of ethanol extract of *N. nucifera* plumule

Solvent system Toluene: Ethyl acetate: Diethylamine (7: 2: 1)

Detection I = detection under daylight

II = detection under UV light 254 nm

III = detection under UV light 365 nm

IV = detection with Dragendorff's reagent

4.2 Ethanolic extract of *N. nucifera* plumule

The dried powders of *N. nucifera* plumule from 15 sources were extracted with 95 % ethanol by Soxhlet apparatus. The percent yield of crude extracts were demonstrated in Table 5. The average percent yield of *N. nucifera* plumule ethanolic extract was 39.94 ± 2.52 g/100g.

Table 5 The percent yields of ethanolic extract of *N. nucifera* plumule crude drug from 15 sources in Thailand

Source	**weight of sample** (g)	**weight of extractive value** (g)	**% yield** (g/100g of crude drug)
1	5.00	2.21	44.12
2	5.00	1.92	38.30
3	5.00	1.97	39.47
4	5.00	2.05	40.97
5	5.00	2.08	41.55
6	5.00	2.01	40.26
7	5.00	1.95	39.08
8	5.00	1.81	36.14
9	5.00	1.93	38.53
10	5.00	2.09	41.88
11	5.00	1.93	38.56
12	5.00	1.87	37.49
13	5.00	1.83	36.61
14	5.00	2.24	44.71
15	5.00	2.07	41.39
		Average	39.94 ± 2.52

4.3 The amount of neferine in *N. nucifera* plumule by TLC densitometry

The neferine contents in *N. nucifera* plumules from 15 different locations were determined in triplicate by TLC densitometry. The neferine contents in crude drugs were calculated and the average content was 0.496 ± 0.186% (Table 6).

Table 6 The amount of neferine in *N. nucifera* plumule by TLC densitometry (% by dried weight)

Source	Neferine in the ethanol extract (g/g of crude extract)				Yield of the ethanol extract (g/100 g of dried crude drug)	Neferine in *N.nucifera* plumule (g/100 g of dried crude drug)
	1	2	3	Mean		
1	0.016	0.015	0.016	0.016	44.116	0.690
2	0.016	0.016	0.017	0.016	38.304	0.617
3	0.012	0.014	0.014	0.013	39.472	0.522
4	0.021	0.020	0.021	0.021	40.974	0.843
5	0.017	0.016	0.018	0.017	41.552	0.709
6	0.014	0.013	0.015	0.014	40.256	0.565
7	0.007	0.006	0.006	0.006	39.082	0.253
8	0.007	0.007	0.006	0.007	36.144	0.247
9	0.013	0.013	0.013	0.013	38.534	0.505
10	0.015	0.014	0.016	0.015	41.880	0.636
11	0.010	0.010	0.010	0.010	38.556	0.384
12	0.011	0.010	0.010	0.011	37.492	0.399
13	0.008	0.008	0.009	0.008	36.606	0.301
14	0.012	0.010	0.012	0.011	44.712	0.512
15	0.006	0.006	0.006	0.006	41.388	0.253
					Mean	0.496
					SD	0.186

4.3.1 Method validation of TLC densitometry

4.3.1.1 Calibration curve

The calibration curve of neferine (0.25-3.0 µg/spot) by TLC densitometry was polynomial. The regression equation was $y = -1231.4x^2 + 8186.6x + 817.55$ and coefficient of determination (R^2) was 0.9994 (Figure 10).

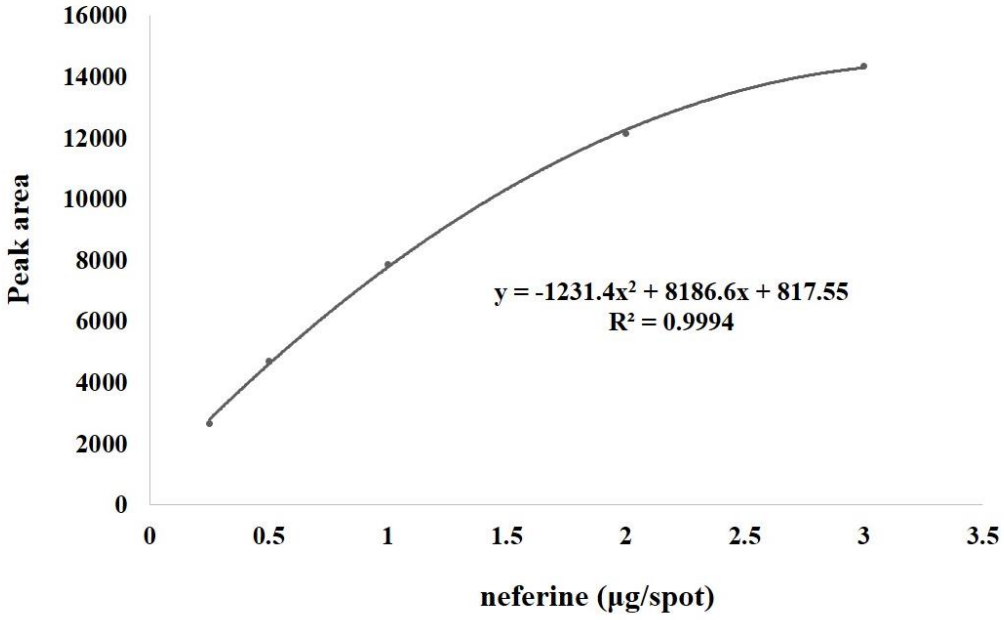


Figure 10 The calibration curve of neferine by TLC densitometry

4.3.1.2 Accuracy

The accuracy of neferine quantitation by TLC densitometry was evaluated in percentage of recovery. The recovery of neferine was performed on sample spiked with three different concentrations of neferine (0.50, 1.00, 2.00 µg/spot). The recovery method was done in triplicate. The results were between 94.07- 104.72% (Table 7).

Table 7 Recovery of neferine by TLC densitometry (n=3)

Neferine added (µg/spot)	**Neferine found** (µg/spot)	**% Recovery**
0.00	0.36	-
0.50	0.90	104.72
1.00	1.28	94.07
2.00	2.36	100.03

4.3.1.3 Precision

The precision of neferine quantitation by TLC densitometry was evaluated in triplicate of each concentration group. The result was presented as the percentage of relative standard deviation which represented the error of the method. The repeatability was performed by three different concentrations on the same day. The intermediate precision was determined by three different concentrations on the different days. The repeatability and intermediate precision were between 4.094 - 11.236% and 5.870 - 11.881% respectively (Table 8).

Table 8 Repeatability and intermediate precision of neferine by TLC densitometry (n=3)

Neferine (µg/spot)	**Repeatability** (%RSD)	**Intermediate precision** (%RSD)
0.90	4.09	9.75
1.28	10.83	5.87
2.36	11.24	11.88

4.3.1.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

In this study, limit of detection and limit of quantitation in TLC densitometry were measured based on the residual standard deviation. The value of slope of regression line and residual standard deviation were 8252.067 and 1091.496. The LOD and LOQ were found to be 0.067 and 0.202 µg/spot respectively.

4.3.1.5 Robustness

Robustness of the TLC densitometry was performed by introducing small changes in the mobile phase complements (toluene: ethyl acetate: diethylamine). Each variation was determined in triplicate. The robustness value was 5.52%RSD. The peak area of neferine were between 11053.52 and 12341.04 (Table 9).

Table 9 Robustness of the TLC densitometry (n=3)

Mobile phase (v/v)	**Peak area of neferine** (Pixel)
7.0: 2.0: 1.0	11053.52
7.1: 1.9: 1.0	11635.18
6.9: 2.1: 1.0	12341.04
Mean ± SD	11676.58 ± 644.76
%RSD	5.52

4.3.1.6 Specificity

The specificity was executed by peak identity and peak purity checking. The identity in absorbance spectra determined at the peak apex among neferine standards and a spot in the samples at the same Rf value was illustrated in Figure 11 and the purity in absorbance spectra determined at up-slope, apex and down-slope of the sample peak was shown in Figure 12. The absorption spectra of neferine in all samples and standard were identical with the maximum absorption at 285 nm which represented the method specificity.

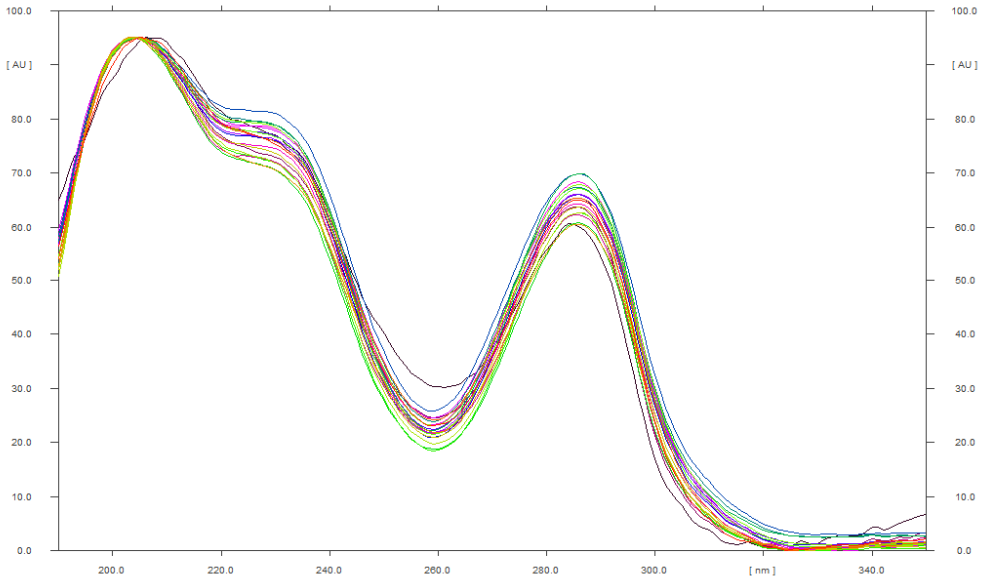


Figure 11 UV absorbance spectra of 5 neferine standards and 15 ethanolic extracts of *N.nucifera* plumule

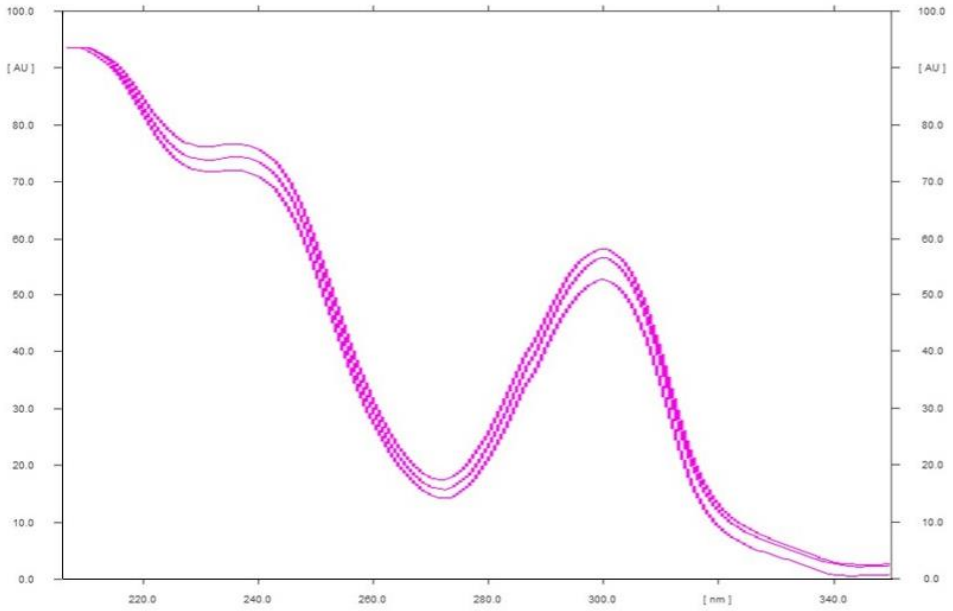


Figure 12 UV absorbance spectra of neferine in ethanolic extract of *N.nucifera* plumule using up-slope, apex and down-slope of the peak

4.4 The amount of neferine in *N. nucifera* plumule by TLC image analysis

The neferine contents in *N. nucifera* plumules from 15 different locations were determined in triplicate by TLC image analysis. The neferine contents in crude drugs were calculated and the average content was 0.495 ± 0.190 % (Table 10).

Table 10 The amount of neferine in *N. nucifera* plumule by TLC image analysis (% by dried weight)

Source	Neferine in the ethanol extract (g/g of crude extract)				Yield of the ethanol extract (g/100 g of dried crude drug)	Neferine in *N.nucifera* plumule (g/100 g of dried crude drug)
	1	2	3	Mean		
1	0.016	0.018	0.018	0.017	44.116	0.758
2	0.014	0.018	0.015	0.016	38.304	0.606
3	0.014	0.011	0.014	0.013	39.472	0.512
4	0.022	0.019	0.021	0.021	40.974	0.849
5	0.013	0.017	0.018	0.016	41.552	0.661
6	0.014	0.013	0.011	0.013	40.256	0.507
7	0.008	0.009	0.007	0.008	39.082	0.295
8	0.008	0.008	0.008	0.008	36.144	0.286
9	0.013	0.018	0.012	0.014	38.534	0.546
10	0.016	0.018	0.015	0.016	41.880	0.687
11	0.010	0.009	0.009	0.009	38.556	0.365
12	0.010	0.010	0.010	0.010	37.492	0.370
13	0.006	0.010	0.007	0.007	36.606	0.267
14	0.009	0.012	0.010	0.010	44.712	0.467
15	0.006	0.006	0.006	0.006	41.388	0.246
					Mean	0.495
					SD	0.190

4.4.1 Method validation of TLC image analysis

4.4.1.1 Calibration curve

The calibration curve of neferine (0.25-3.0 µg/spot) by TLC image analysis method was polynomial. The regression equation was $y = -710.14x^2 + 14664x - 373.75$ and coefficient of determination (R^2) was 0.9989 (Figure 13).

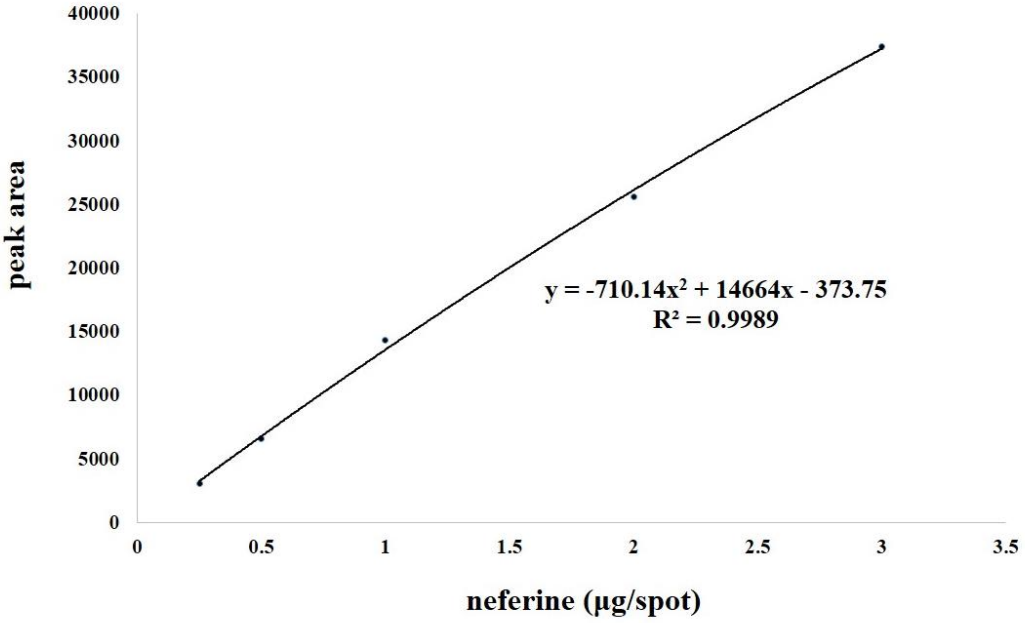


Figure 13 The calibration curve of neferine by TLC image analysis

4.4.1.2 Accuracy

The accuracy of neferine quantitation by TLC image analysis was evaluated in percentage of recovery. The recovery of neferine was performed on sample spiked with three different concentrations of neferine (0.50, 1.00, 2.00 µg/spot). The recovery method was done in triplicate. The results were between 96.442 - 110.809% (Table 11).

Table 11 Recovery of neferine by TLC image analysis (n=3)

Neferine added (µg/spot)	**Neferine found** (µg/spot)	**% Recovery**
0.00	0.33	-
0.50	0.91	110.81
1.00	1.38	103.96
2.00	2.24	96.44

4.4.1.3 Precision

The precision of neferine quantitation by TLC image analysis was evaluated in triplicate of each concentration group. The result was presented as the percentage of relative standard deviation which represented the error of the method. The repeatability was performed by three different concentrations on the same day. The intermediate precision was determined by three different concentrations on the different days. The repeatability and intermediate precision were between 1.447 - 3.588% and 1.495 - 3.914% respectively (Table 12).

Table 12 Repeatability and intermediate precision of neferine by TLC image analysis (n=3)

Neferine (µg/spot)	**Repeatability** (%RSD)	**Intermediate precision** (%RSD)
0.91	3.59	3.91
1.38	2.68	3.28
2.24	1.45	1.49

4.4.1.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

In this study, limit of detection and limit of quantitation in TLC image analysis were measured based on the residual standard deviation. The value of slope of regression line and residual standard deviation were 17237.013 and 1213.019. The LOD and LOQ were found to be 0.073 and 0.220 µg/spot respectively.

4.4.1.5 Robustness

Robustness of the TLC image analysis was performed by introducing small changes in the mobile phase complements (toluene: ethyl acetate: diethylamine). Each variation was determined in triplicate. The robustness value was 7.44%RSD. The peak area of neferine were between 23843.19 and 27357.36 (Table 13).

Table 13 Robustness of the TLC image analysis (n=3)

Mobile phase (v/v)	**Peak area of neferine** (Pixel)
7.0: 2.0: 1.0	27357.36
7.1: 1.9: 1.0	24462.15
6.9: 2.1: 1.0	23843.19
Mean ± SD	25220.90 ± 1875.93
%RSD	7.44

4.5 The comparison of neferine contents between TLC densitometry and TLC image analysis

The neferine contents between TLC densitometry and TLC image analysis by imageJ were compared by paired t-test statistical analysis. The comparison was found that the neferine by two methods were not significantly different ($P > 0.05$).

Table 14 Comparison of neferine contents between TLC densitometry and TLC image analysis

Source	**Neferine in *N. nucifera* plumule** (g/100 g of dried crude drug)	
	TLC densitometry	**TLC image analysis**
1	0.690	0.760
2	0.620	0.610
3	0.520	0.510
4	0.840	0.850
5	0.710	0.660
6	0.570	0.510
7	0.250	0.290
8	0.250	0.290
9	0.500	0.550
10	0.640	0.690
11	0.380	0.370
12	0.400	0.370
13	0.300	0.270
14	0.510	0.470
15	0.250	0.250
Average	**0.496±0.186**	**0.495±0.190**

4.6 MTT assay

The percent yields of dichloromethane fractionate and ethanolic fractionate of *N. nucifera* plumule crude ethanolic extract were found to be 20.13% w/w. and 69.73 % w/w respectively. *N. nucifera* plumule crude ethanolic extracted, dichloromethane fractionated and ethanolic fractionated part were tested for *in vitro* cytotoxic activity against 5 human cancer cell lines and 1 human normal cell line including breast ductal carcinoma (BT-474), undifferentiated lung carcinoma (CHAGO - K1), colon adenocarcinoma (SW-620), gastric carcinoma (KATO-3), hepatocarcinoma (HEP-G2) and lung fibroblast (Wi-38) respectively. The cytotoxic effects of six concentrations (0.01, 0.1, 1, 10, 100, 200 µg/mL) of each sample were evaluated by micro-culture tetrazolium assay (MTT) and IC_{50} values were calculated. The results were shown in Table 15. The crude ethanolic extract, dichloromethane fractionate and ethanolic fractionate of *N. nucifera* plumule exhibited no significant activity against five cancer cell lines with IC_{50} more than 20 µg/ml. The criteria of cytotoxicity for the crude extract, as established by the U.S. National Cancer Institute (NCI), is an IC_{50}<20 µg/mL in the preliminary assay. The U.S. National Cancer Institute (NCI) defined plants which give the extracts with the IC_{50} values of < 20 µg/ml and < 4 µg/ml for pure compounds as the plants with cytotoxic activity [127, 129]. However the crude ethanolic extract showed more potential to against colon adenocarcinoma (SW-620) (IC_{50} = 38.63 µg/ml). All samples were not cytotoxic to tested normal cell.

Table 15 IC_{50} of *N. nucifera* plumule fractionate extract on 5 human cancer cell lines and 1 human normal cell line

Sample/Cell	**IC_{50}** (µg/ml)					
	BT474	CHAGO -K1	Hep-G2	KATO-3	SW620	Wi-38
Crude ethanolic extract	108.96	115.58	101.70	185.12	38.63	161.42
Dichloromethane fractionate	170.37	135.91	130.84	>200	127.61	>200
Ethanolic fractionate	>200	>200	>200	>200	>200	>200
Doxorubicin	0.40	0.50	0.07	8.81	0.23	0.17

IC_{50}: The concentration at which cell viability was reduced by 50%

CHAPTER V

DISCUSSION AND CONCLUTION

Standardization of plant materials by evaluation of pharmacognostic parameters including macroscopic characteristics, microscopic characteristics, physicochemical parameters and TLC fingerprint is important for identification, authentication and detection of adulteration and also assurance of quality control of crude drugs.

The plumule of *N. nucifera* is useful in traditional medicine for the treatment of various ailments, so it is important to standardize this plant part for quality, efficacy and safety use as a medicinal plant material. This present study evaluated macroscopic characteristics, microscopic characteristics and physicochemical constants of *N. nucifera* plumule. Neferine content was also determined by two methods of TLC densitometer and TLC image analysis.

In this study, microscopic evaluation including transverse section of *N. nucifera* plumule and powder study revealed the presence of various important characteristics of this plant. Trichome was not found in the powder of *N. nucifera* plumule but present in leaf powder of this plant [130]. Therefore, trichome can be used to differentiate between plumules and leaves of *N. nucifera* samples in the powder form.

The result showed that 5 important cells including anomocytic type stomata, parenchyma surface with starch granule, starch granule (round shape), starch granule (hilum shape) and layer of cotyledons in surface view were observed in the powder of *N. nucifera* plumule. The powder characters of *N. nucifera* plumule were also mentioned in Chinese Pharmacopoeia which consisted of epidermal cells sub rectangular, radially prolonged, thin-walled; mesophyll cells sub rounded, thin-walled, containing numerous starch granules and green pigments; radicle cells rectangular, arranged tidily, walls extremely thin, and some cells containing fatty oil droplets [131] which all 3 cells (epidermal cells, mesophyll cells, radicle cells) not found in this study. On the basis of microscopic aspects, the presence of histological characters can serve as useful parameters for the identification of *N. nucifera* plumule. Microscopic study of medicinal plants materials is necessary for the identification of the broken and powdered materials [132].

Physicochemical parameters could be useful for the quality control and quality assurance of plant materials. The total ash is particularly important in the evaluation of purity of plant sample. Alcohol and water soluble extractive values were determined to find out the amount of water and alcohol soluble components. In present study, the moisture content of *N. nucifera* plumule is not too high (10.02 ± 0.72%w/w) and has similar value to *N. nucifera* seed (10.50 %w/w) [6], but higher value than *N. nucifera* leaves, thus it could discourage bacterial, fungi or yeast growth,

as the general requirement for moisture content in crude is not more than 14%w/w [133]. Total ash and acid insoluble ash values of *N. nucifera* plumule were lower value than *N. nucifera* leaves while water and alcohol soluble extractive values were higher value than of *N. nucifera* leaves (Table 16). It indicated that *N. nucifera* plumule contained lower amount of inorganic matters such as carbonates, phosphates, silicates and silica than leaves of this plant but contained higher amount of chemical constituents soluble in water and alcohol than leaf part of this plant. The moisture content and total ash of *N. nucifera* plumule should not more than 12.0% and 5.0% were mentioned in Chinese Pharmacopoeia [131].

Table 16 Physicochemical specifications of *N. nucifera* leaf and plumule

Parameter	N. nucifera leaf			N. nucifera plumule
Total ash	4.26±0.15	9.62±0.07	9.03±0.02	3.86 ± 0.17
Acid insoluble ash	2.31±0.09	2.61±0.24	1.28±0.23	0.54 ± 0.04
Water soluble ash	ND	ND	ND	ND
Alcohol soluble extractive	7.84±0.27	6.24±0.22	2.12±0.04	16.54 **±** 1.34
Water soluble extractive	8.71±0.18	9.51±0.24	16.45±0.54	28.27 **±** 2.01
Moisture content	4.62±0.33	7.06±0.11	ND	10.02 ± 0.72
Loss on drying	ND	7.69±0.07	ND	9.91 ± 0.09
Reference	[130]	[134]	[104]	

*ND (Not Determined)

The TLC fingerprint profile of *N. nucifera* plumule used toluene: ethyl acetate: diethylamine (7:2:1) as mobile phase. The TLC fingerprint profile of compounds

present in plant materials is useful in standard setting up for evaluating the quality and purity as well as screening analysis of plant materials. The advantages of TLC are its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation [135]. Thus, TLC is a convenient method of determination the quality and possible adulteration of plant materials [136]. In this study, TLC was used to construct the fingerprint of *N. nucifera* plumule which exhibited clear pattern of compounds in all investigations; under visible light, UV 254 nm, UV 365 nm and after spraying with Dragendorff's reagent.

In this study, Soxhlet apparatus with 95% ethanol was used to extract constituents from *N. nucifera* plumule powder for the determination of neferine content. The percentage of extract yields was 39.94±2.52 g% of dried crude drug. The advantages of this extraction include continuous process, basic, simple and cheap [137]. The disadvantages include time consuming, uses large amount of solvent, the Soxhlet apparatus cannot provide agitation to accelerate the process, not suitable for thermolabile compounds as long time heating may lead to degradation of compounds and exposure to hazardous and flammable liquid organic solvents with potential toxic emissions during extraction [138].

At present, several studies have been reported on the determination of neferine in both quantitative and qualitative aspects such as TLC, HPLC, and GC-MS.

Until now, the TLC densitometry method and TLC image analysis method have not been reported on the method validation for determination of neferine.

The λmax of neferine was found at 285 nm. Method validity with respect to specificity, accuracy, precision, LOD, LOQ, calibration range and robustness according to the ICH guideline were investigated. In this study, TLC densitometry and TLC image analysis were developed and validated for the identification and quantification of neferine content in *N. nucifera* plumule samples. The overall results of the accuracy of TLC densitometry and image analysis showed best recoveries (99.61 ± 5.34 and 103.74 ± 7.19 %), indicated that the methods were accurate. The results of repeatability (9.48 ± 3.61 and 2.99 ± 1.22 %RSD), intermediate precision (8.52 ± 2.81 and 2.63 ± 1.15 %RSD) and robustness for mobile phase variation (5.52 and 7.44 %RSD) indicated that the method were precise and robust. These results can indicated that the proposed methods are precise, accurate and robust for determination of neferine content in *N. nucifera* plumule. Moreover the methods are simple, rapid and economic which can be used for analysis in quality control of *N. nucifera* plumule.

Neferine content in *N. nucifera* plumule in this study was found to be 0.496 g% of dried crude drug (range from 0.25-0.84 g% of dried crude drug) by TLC densitometry and 0.495 g% of dried crude drug (range from 0.25-0.85 g% of dried crude drug) by TLC image analysis which similar to neferine content by CE method (0.146-0.854 g% of dried crude drug) [139]. In previous studies, neferine content from *N.*

nucifera plumule were reported on different extraction method including maceration (0.676 g% of dried crude drug), refluxing (0.873 g% of dried crude drug), microwave-assisted extraction (0.791 g% of dried crude drug) and ultrasound-assisted extraction (0.866 g% of dried crude drug) [140]. Additionally, influence of different solvents on the neferine extraction was also reported including 95% aqueous ethanol (0.839 g% of dried crude drug), 70% aqueous ethanol (0.882 g% of dried crude drug), 50% aqueous ethanol (0.667 g% of dried crude drug), 0.25% hydrochloric acid (0.881 g% of dried crude drug), 0.25% sulphuric acid (0.883 g% of dried crude drug), 90% aqueous methanol (0.780 g% of dried crude drug) and chloroform (0.798 g% of dried crude drug) [140].

In order to evaluate of the cytotoxic effect of *N. nucifera* plumule crude ethanolic extract, dichloromethane fractionate and ethanolic fractionate on human cancer cells by using MTT assays. Six cells lines were selected to be investigated in this study; five human cancer cell lines included breast ductal carcinoma (BT-474), undifferentiated lung carcinoma (CHAGO -K1), colon adenocarcinoma (SW-620), gastric carcinoma (KATO-3), hepatocarcinoma (HEP-G2) and one human normal cell line was lung fibroblast (Wi-38). The tested samples were crude ethanolic extract, dichloromethane fractionate and ethanolic fractionate. The results showed that all tested samples were exhibited no significant cytotoxic activity against the five cancer cells line with an IC_{50} more than 20 µg/ml (38.63-185.12 µg/ml) and the normal lung

fibroblast (WI-38) cells at 161.42 µg/ml. The crude ethanolic extract showed more potency on all tested cancer cell death than dichloromethane fractionate and ethanolic fractionate respectively. *N. nucifera* plumule crude ethanolic extract and its fractionates were non-toxic to human normal cell. US NCI guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation of less than 20 µg/ml after the exposure time of 72 hours [127, 129]. In previous studies, isolated compounds from *N. nucifera* have been reported on cytotoxic activity such as luteolin, alphitolic acid, maslinic acid and *N*-methylasimilobine from leaves which exhibited cytotoxic against human tumor cells with IC_{50} less than 20 µM; alkaloid compounds (*N*-methylated aporphine-type alkaloids nuciferine, *N*-methyllasimilobine, (-)-Lirinidine and 2-hydroxy-1-methoxy-6a,7-dehydroaporphine) from flower buds and leaves which inhibited B16 melanoma cells with IC_{50} less than 20 µM; 7-hydroxydehydronuciferine aporphine alkaloids from leaves which inhibited the proliferation of melanoma, prostate and gastric cancer cells at IC_{50} 62.9±0.1 µM and 80.8±0.2 µM; alkaloid compound (liensinine, isoliensinine and neferine) from plumules induces hepatocellular carcinoma cell apoptosis with wide range of IC_{50} between 4.2-72.9 µg/ml [12-15]. Although some constituents isolated from *N. nucifera plumule* have cytotoxic activity but there are some compounds from this plant exhibited no cytotoxic effect such as liriodenine aporphine alkaloid from leaves which exhibited no cytotoxic activity against AGS and DU-145 cells (prostate and gastric cancer cells) [14]; methanol

and acetone leaf extracts showed less activity against human breast cancer cell (MCF7) [72].

CONCLUSION

The present study was undertaken with an aim of pharmacognostic standardization of *N. nucifera* plumule and method validation of TLC densitometry and TLC image analysis for determination of neferine content in this plant. The finding generated from this study would be useful in quality assurance and also for preparation of monograph on the *N. nucifera* plumule.

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APPENDICES

APPENDIX A

Physicochemical parameters of *Nelumbo nucifera* plumule

Table 17 Physicochemical parameters of *N. nucifera* plumule

Source	No.	Moisture (g%)	Loss on drying (g%)	Ash value (g%)		Extractive value (g%)	
				Total ash	Acid insoluble ash	Water	Ethanol
1	1	12.000	10.939	3.780	0.440	18.309	16.280
	2	12.000	10.976	3.756	0.447	18.839	17.029
	3	10.800	10.982	3.776	0.383	21.768	16.589
2	1	10.667	9.642	3.854	0.786	32.268	15.497
	2	9.333	9.870	3.770	0.802	31.089	15.339
	3	11.000	9.970	3.900	0.806	28.499	14.808
3	1	10.667	10.095	3.976	0.818	29.389	16.380
	2	10.000	10.381	3.857	0.765	30.697	16.128
	3	11.333	10.330	3.847	0.787	29.846	16.318
4	1	10.000	9.459	3.831	0.509	31.597	16.320
	2	10.000	9.616	3.889	0.599	32.745	16.088
	3	9.000	9.472	3.941	0.524	28.838	16.029
5	1	9.000	9.285	3.851	0.536	30.977	13.768
	2	9.600	9.096	3.973	0.599	31.078	14.178
	3	9.400	9.011	3.165	0.576	30.739	14.148
6	1	11.800	11.068	3.774	0.724	29.549	17.500
	2	10.800	11.131	3.644	0.637	27.019	17.597
	3	12.000	10.994	3.957	0.687	27.178	17.358
7	1	9.333	10.753	3.960	0.703	31.439	18.699
	2	11.000	10.662	3.851	0.681	30.837	19.220
	3	10.000	10.827	3.940	0.636	29.558	18.937
8	1	9.000	9.682	4.120	0.457	29.366	17.838
	2	10.200	9.696	4.163	0.533	28.327	17.420
	3	9.600	9.736	4.080	0.480	26.968	17.839
9	1	11.000	10.091	3.763	0.527	30.158	18.299
	2	10.600	10.092	3.717	0.548	30.439	18.207
	3	10.600	10.286	3.714	0.631	30.287	18.100

Table 17 Physicochemical parameters of *N.nucifera* plumule (cont.)

Source	No.	Moisture (g%)	Loss on drying (g%)	Ash value (g%)		Extractive value (g%)	
				Total ash	Acid insoluble ash	Water	Ethanol
10	1	10.200	9.473	3.667	0.523	19.680	19.180
	2	8.400	9.404	3.811	0.514	30.585	19.287
	3	9.600	9.431	3.901	0.529	27.737	19.078
11	1	10.333	9.823	3.800	0.437	29.177	16.399
	2	8.667	9.863	3.793	0.330	28.949	16.519
	3	11.333	9.752	3.779	0.380	28.928	16.309
12	1	10.200	9.088	3.876	0.447	24.148	17.209
	2	9.300	9.119	3.900	0.380	24.770	17.199
	3	9.000	9.038	3.866	0.463	22.130	17.588
13	1	10.000	10.016	3.797	0.330	29.025	12.469
	2	8.667	9.936	3.717	0.383	29.035	17.529
	3	9.667	9.909	4.523	0.513	26.508	17.618
14	1	10.800	10.118	3.696	0.363	30.519	16.028
	2	10.600	10.089	3.680	0.357	27.847	16.019
	3	9.800	10.099	3.676	0.367	29.508	16.248
15	1	8.333	9.042	4.053	0.487	25.699	15.398
	2	7.333	8.933	4.063	0.427	30.175	17.408
	3	8.000	8.875	4.029	0.443	29.958	14.979
Min-Max		7.889-11.600	8.875-11.131	3.165-4.523	0.330-0.818	19.639-31.060	14.031-19.182
Grand mean± pooled SD		10.021± 0.718	9.914± 0.090	3.855± 0.172	0.540± 0.043	28.271± 2.005	16.764± 0.855

Grand mean formula: $\bar{\bar{x}} = \frac{n_1\bar{x}_1 + n_2\bar{x}_2 + \ldots + n_k\bar{x}_k}{n_1 + n_2 + \ldots + n_k}$

Pooled SD formula: $s_p = \sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2 + \cdots + (n_k-1)s_k^2}{n_1 + n_2 + \cdots + n_k - k}}$

APPENDIX B

Data of quantitative analysis of *Nelumbo nucifera* plumule

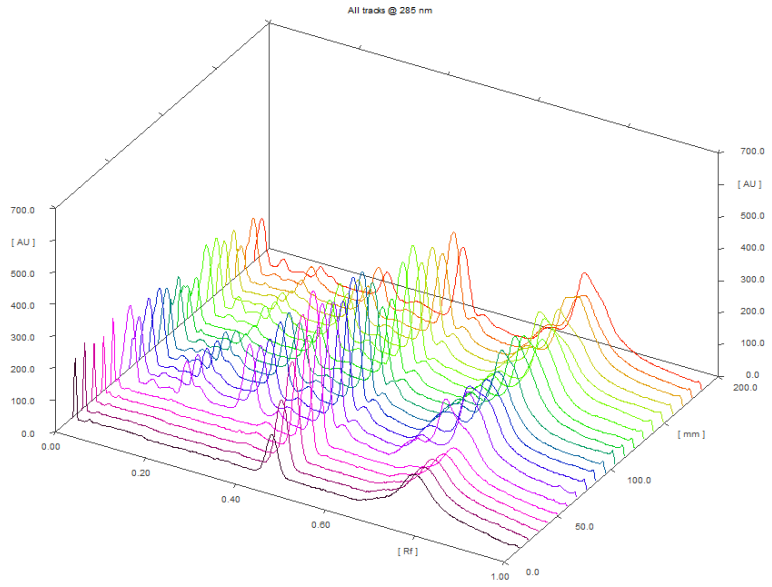

Figure 14 3D TLC densitogram (standard neferine No. 1-5, sample No. 6-20)

Table 18 Accuracy by TLC densitometry

Neferine added (μg/spot)	1	2	3	Average	%Recovery			
0.00	0.35	0.32	0.41	0.36	1	2	3	Average
0.50	0.87	0.89	0.94	0.90	101.88	108.63	103.64	104.72
1.00	1.43	1.17	1.23	1.28	106.22	88.36	87.62	94.07
2.00	2.58	2.43	2.06	2.36	109.74	104.56	85.79	100.03
							Average	99.61
							SD	5.34

Table 19 Repeatability by TLC densitometry

Neferine added	Repeatability					
(µg/spot)	1	2	3	Mean	SD	%RSD
0.00	0.35	0.32	0.41	0.36	0.04	11.77
0.50	0.87	0.89	0.94	0.90	0.04	4.09
1.00	1.43	1.17	1.23	1.28	0.14	10.83
2.00	2.58	2.43	2.06	2.36	0.27	11.24
					Average	9.48
					SD	3.61

Table 20 Intermediate precision by TLC densitometry

Neferine added	Intermediate precision					
(µg/spot)	1	2	3	Mean	SD	%RSD
0.00	0.36	0.32	0.32	0.34	0.02	6.56
0.50	0.90	0.87	0.75	0.84	0.08	9.75
1.00	1.28	1.23	1.14	1.21	0.07	5.87
2.00	2.36	1.87	2.05	2.09	0.25	11.88
					Average	8.52
					SD	2.81

Table 21 Robustness by TLC densitometry

Mobile phase (v/v)	Robustness					Peak area of neferine (Pixel)
	0.25	**0.5**	**1**	**2**	**3**	
7.0: 2.0: 1.0	2644.54	4831.57	7348.31	11184.23	14065.12	11053.52
7.1: 1.9: 1.0	3149.44	4941.52	7774.11	10669.89	14167.96	11635.18
6.9: 2.1: 1.0	2796.63	5162.95	7804.19	11432.83	15076.75	12341.04
mean	2863.54	4978.68	7642.20	11095.65	14436.61	11676.58
sd	259.01	168.79	254.96	389.11	556.76	644.76
%RSD	9.05	3.39	3.34	3.51	3.86	5.52

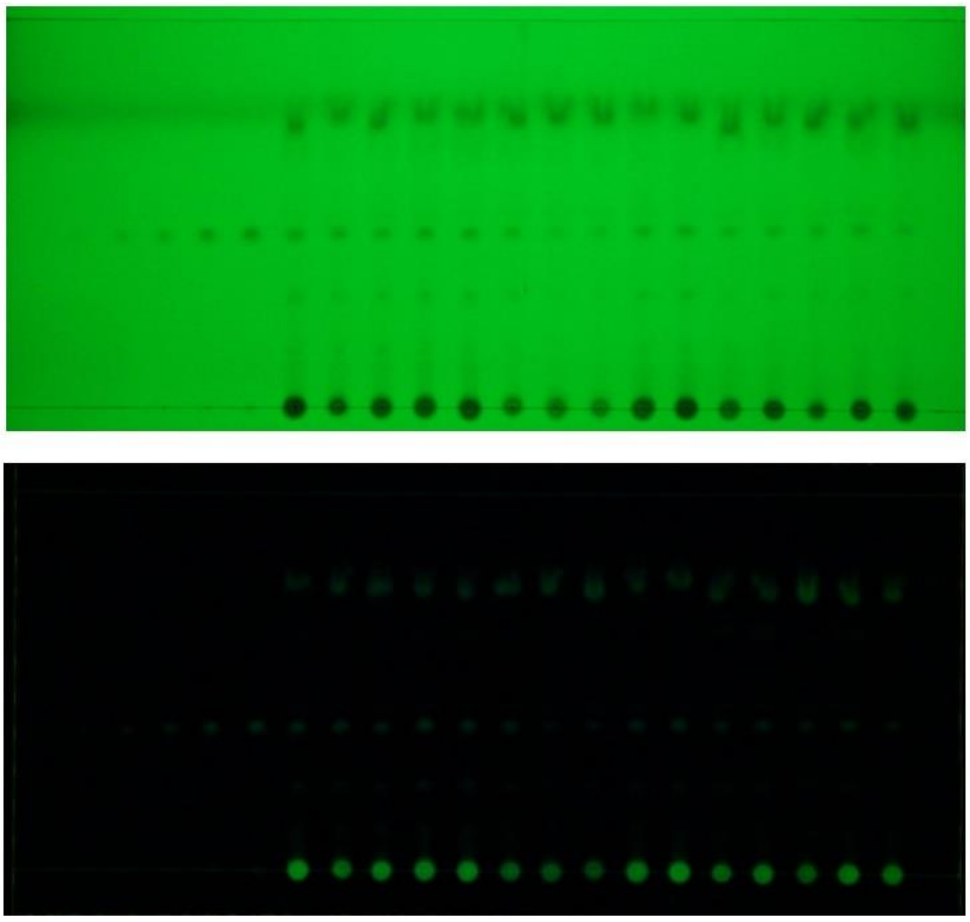

Figure 15 The TLC plate (standard neferine No. 1-5, sample No. 6-20) visual under UV 254 nm (A), with subtract background by imageJ software (B)

Table 22 Accuracy by TLC image analysis

Neferine added (μg/spot)	1	2	3	Average	%Recovery			
0.00	0.34	0.31	0.32	0.33	**1.00**	**2.00**	**3.00**	**Average**
0.50	0.88	0.92	0.95	0.91	104.71	112.82	114.90	110.81
1.00	1.42	1.36	1.35	1.38	105.97	103.60	102.31	103.96
2.00	2.26	2.26	2.21	2.24	96.72	97.67	94.94	96.44
							Average	103.74
							SD	7.19

Table 23 Repeatability by TLC image analysis

Neferine added	Repeatability					
(µg/spot)	1	2	3	Mean	SD	%RSD
0.00	0.34	0.31	0.32	0.33	0.01	4.27
0.50	0.88	0.92	0.95	0.91	0.03	3.59
1.00	1.42	1.36	1.35	1.38	0.04	2.68
2.00	2.26	2.26	2.21	2.24	0.03	1.45
					Average	2.99
					SD	1.22

Table 24 Intermediate precision by TLC image analysis

Neferine added	**Intermediate precision**					
(µg/spot)	**1**	**2**	**3**	**Mean**	**SD**	**%RSD**
0.00	0.33	0.34	0.33	0.33	0.01	1.85
0.50	0.91	0.96	0.99	0.95	0.04	3.91
1.00	1.38	1.30	1.37	1.35	0.04	3.28
2.00	2.24	2.18	2.21	2.21	0.03	1.50
					Average	2.63
					SD	1.15

Table 25 Robustness by TLC image analysis

Mobile phase (v/v)	Robustness					Peak area of neferine (Pixel)
	0.25	**0.5**	**1**	**2**	**3**	
7.0: 2.0: 1.0	3031.16	5963.07	11543.37	23044.65	32605.09	27357.36
7.1: 1.9: 1.0	3187.63	5521.30	11242.43	21635.99	29597.06	24462.15
6.9: 2.1: 1.0	3091.92	5926.64	11884.52	22544.45	31261.71	23843.19
mean	3103.57	5803.67	11556.77	22408.36	31154.62	25220.90
sd	78.88	245.22	321.25	714.12	1506.87	1875.93
%RSD	2.54	4.23	2.78	3.19	4.84	7.44

APPENDIX C

Data of cytotoxicity determination of *Nelumbo nucifera* plumule

Table 26 Cytotoxicity effect of *N. nucifera* plumule crude ethanolic extract on 5 human cancer cell lines and 1 human normal cell line

	BT474		CHAGO-K1		Hep-G2	
Crude ethanolic extract (µg/ml)	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**
0.01	0.825	109	0.692	91	1.182	84
0.1	0.633	84	0.720	95	1.272	90
1	0.598	79	0.744	98	1.397	99
10	0.660	87	0.576	76	1.430	101
100	0.411	54	0.385	51	0.715	51
200	0.164	22	0.213	28	0.157	11
DMSO	0.755	100	0.757	100	1.414	100

	KATO-3		SW620		Wi-38	
Crude ethanolic extract (µg/ml)	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**
0.01	1.297	84	1.192	80	0.522	97
0.1	1.296	84	1.262	85	0.526	97
1	1.394	90	1.283	86	0.505	94
10	1.492	97	1.318	88	0.453	84
100	1.267	82	0.285	19	0.429	79
200	0.715	46	0.135	9	0.199	37
DMSO	1.544	100	1.490	100	0.540	100

*PS (Percentage of cell survival)

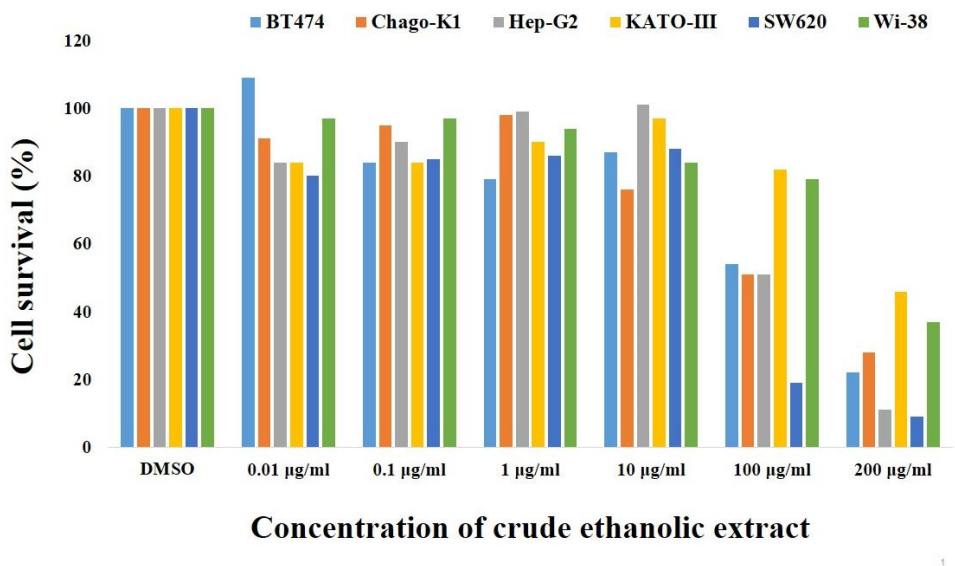

Figure 16 Cytotoxicity effect of *N. nucifera* plumule crude ethanolic extract on 5 human cancer cell lines and 1 human normal cell line

Table 27 Cytotoxicity effect of *N. nucifera* plumule dichloromethane fractionate on 5 human cancer cell lines and 1 human normal cell line

Dichloromethane fractionate (μg/ml)	BT474		CHAGO-K1		Hep-G2	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
0.01	0.884	117	0.808	107	1.392	98
0.1	0.689	91	0.811	107	1.377	97
1	0.712	94	0.852	113	1.420	100
10	0.777	103	0.864	114	1.432	101
100	0.703	93	0.539	71	1.080	76
200	0.283	37	0.230	30	0.124	9
DMSO	0.755	100	0.757	100	1.414	100

Dichloromethane fractionate (μg/ml)	KATO-3		SW620		Wi-38	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
0.01	1.468	95	1.350	91	0.538	100
0.1	1.427	92	1.372	92	0.537	99
1	1.431	93	1.348	90	0.458	85
10	1.545	100	1.363	91	0.508	94
100	1.366	88	1.045	70	0.426	79
200	0.872	56	0.194	13	0.298	55
DMSO	1.544	100	1.490	100	0.540	100

*PS (Percentage of cell survival)

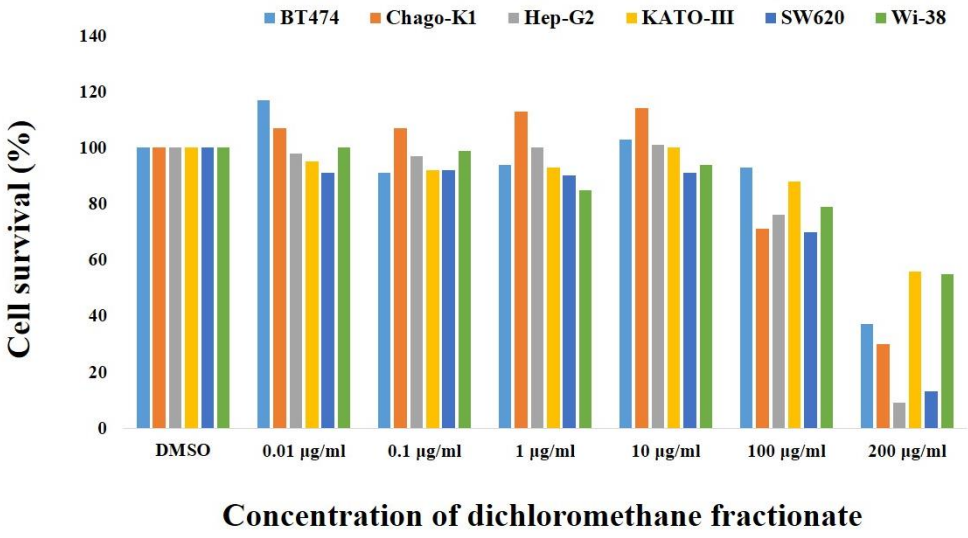

Figure 17 Cytotoxicity effect of *N. nucifera* plumule dichloromethane fractionate on 5 human cancer cell lines and 1 human normal cell line

Table 28 Cytotoxicity effect of *N. nucifera* plumule ethanolic fractionate on 5 human cancer cell lines and 1 human normal cell line

Ethanolic fractionate (μg/ml)	BT474		CHAGO-K1		Hep-G2	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
0.01	0.704	113	0.586	101	0.846	96
0.1	0.656	105	0.531	91	0.860	97
1	0.797	128	0.527	91	1.028	116
10	0.657	106	0.583	100	0.909	103
100	0.512	82	0.509	88	0.746	84
200	0.413	66	0.564	97	0.617	70
DMSO	0.622	100	0.581	100	0.885	100

Ethanolic fractionate (μg/ml)	KATO-3		SW620		Wi-38	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
0.01	0.695	98	1.205	100	0.642	107
0.1	0.736	103	1.169	97	0.610	101
1	0.752	106	1.249	103	0.625	104
10	0.902	127	1.469	121	0.564	94
100	0.856	120	1.513	125	0.528	88
200	0.676	95	1.069	88	0.436	72
DMSO	0.712	100	1.209	100	0.602	100

*PS (Percentage of cell survival)

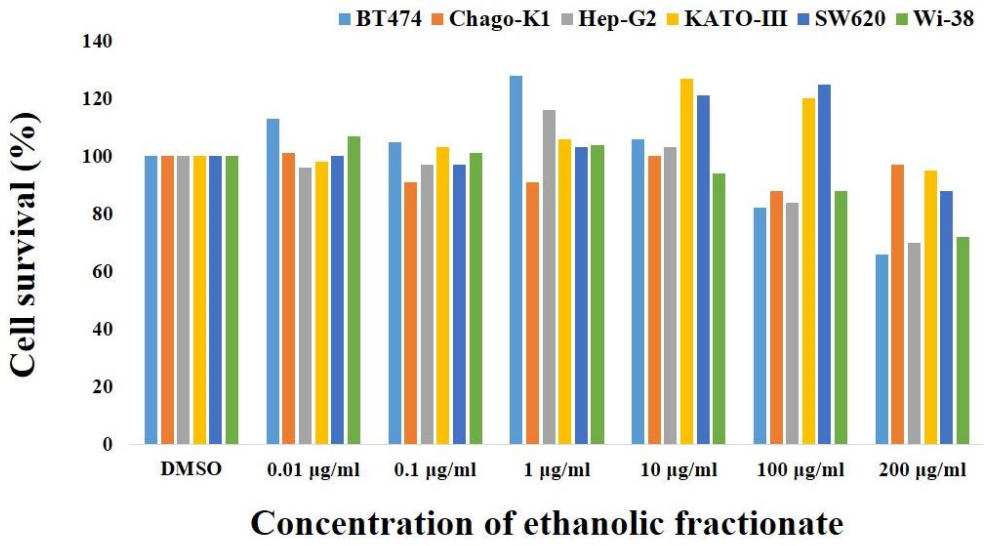

Figure 18 Cytotoxicity effect of *N. nucifera* plumule ethanolic fractionate on 5 human cancer cell lines and 1 human normal cell line

Table 29 Cytotoxicity effect of doxorubicin on 5 human cancer cell lines and 1 human normal cell line

	BT474		CHAGO-K1		Hep-G2	
Doxorubicin (μg/ml)	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**
0.001	0.949	104	1.233	102	1.989	103
0.01	0.868	95	1.203	100	1.740	90
0.1	0.760	83	1.093	91	0.867	45
1	0.234	26	0.333	28	0.111	6
10	0.146	16	0.092	8	0.415	21
Control	0.912	100	1.204	100	1.939	100

	KATO-3		SW620		Wi-38	
Doxorubicin (μg/ml)	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**	**Absorbance** (Mean)	**PS**
0.001	1.687	102	1.469	95	0.967	110
0.01	1.637	99	1.523	98	0.951	108
0.1	1.334	80	0.760	49	0.504	57
1	1.085	65	0.521	34	0.172	19
10	0.766	46	0.213	14	0.237	27
Control	1.660	100	1.551	100	0.882	100

*PS (Percentage of cell survival)

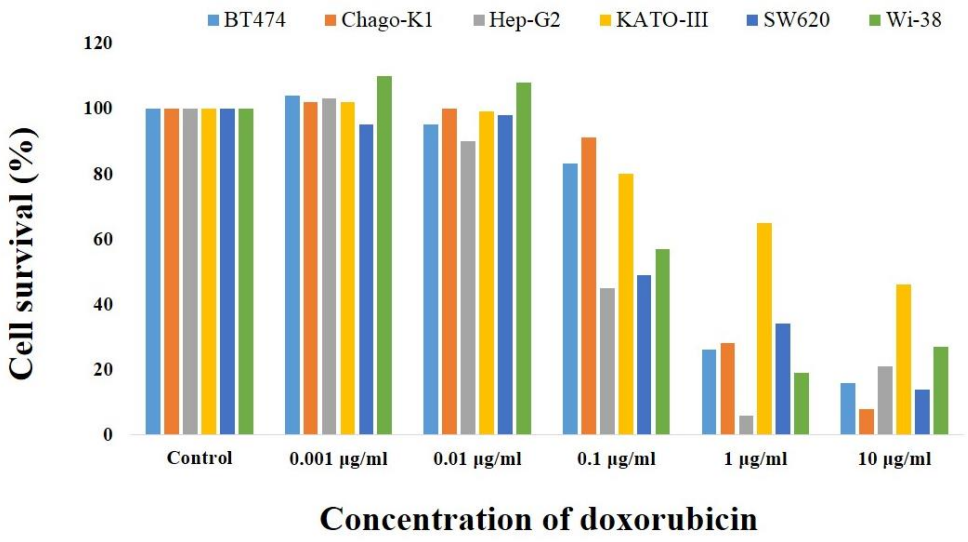

Figure 19 Cytotoxicity effect of doxorubicin on 5 human cancer cell lines and 1 human normal cell line

VITA

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Publication

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Poster presentation

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