IDENTIFICATION AND SPECIFICATION OF WAN CHAK MOTLUK (*CURCUMA* SPP.) IN THAILAND

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Thesis
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IDENTIFICATION AND SPECIFICATION OF WAN CHAK MOTLUK (*CURCUMA* SPP.) IN THAILAND

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ABSTRACT

Wan chak motluk has been used as a remedy for gynecological diseases. Nowadays, the products derived from Wan chak motluk are popularly commercialized. In this study, Wan chak motluk which is cultivated and sold in Thailand was investigated for taxonomic status, chemical identification and biological activities. Thirteen samples were surveyed and collected from various cultivated parts of Thailand. The results showed that Wan chak motluk is classified into 2 groups of the genus *Curcuma*. They are *Curcuma comosa* Roxb and *Curcuma* sp. The inflorescences and florets are distinguishing characteristics. Generally, *C. comosa* possesses smaller and shorter lateral rhizomes than *Curcuma* sp. However, variation of rhizomes could be found and some rhizomes were not easily identified by consumers or traders. The general microscopic characteristics of these groups were quite similar and could not be used as a tool for differentiation. Thin-layer chromatographic method was appropriate as an identifying method using 2 solvent systems, DCM: MeOH (99:1) and DCM: EtOAc: MeOH (93:4:7), and 2 reference standards, 1,7-diphenyl-5-hydroxy-(1*E*)-l-heptene and 5-hydroxy-7-(4-hydroxyphenyl)-l-phenyl-(1*E*)-l-heptene.

For the screening of the biological activities of those alcoholic extracts, the extracts of *C. comosa* possessed significant uterotrophic activity, while *Curcuma* sp. showed effects not significantly different from the control (ovariectomized rat). *C. comosa* and *Curcuma* sp. showed moderate free radical scavenging activity (ED_{50} 8.93-25.86 and 151.43-273.03 µg/ml, respectively) and cytotoxicity by brine shrimp lethality assay (LC_{50} 0.84-39.03 and 151.43-273.03 µg/ml, respectively). Though the physicochemical properties of *C. comosa* raw materials were varied, all these alcoholic extracts possessed acceptable uterotrophic activity. This work provides the specifications together with thin-layer chromatographic fingerprints to be the fundamental standard for the research and development of Wan chak motluk raw materials and products on a commercialized scale. Additionally, the possible toxicity of Wan chak motluk should be cautioned.

KEY WORDS: WAN CHAK MOTLUK / *CURCUMA COMOSA* / SPECIFICATION/ BRINE SHRIMP LETHALITY TEST/ FREE RADICAL SCAVINGING ACTIVITY

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การพิสูจน์เอกลักษณ์และการจัดทำข้อกำหนดของว่านชักมดลูกในประเทศไทย
(IDENTIFICATION AND SPECIFICATION OF WAN CHAK MOTLUK (*CURCUMA* SPP.) IN THAILAND)

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บทคัดย่อ

ว่านชักมดลูกเป็นสมุนไพรที่ใช้สำหรับรักษาโรคสตรีมาเป็นเวลานาน และได้มีการพัฒนาเป็นผลิตภัณฑ์อย่างแพร่หลายในปัจจุบัน งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาลักษณะทางอนุกรมวิธานของว่านชักมดลูกควบคู่ไปกับการทดสอบองค์ประกอบทางเคมีโดยวิธีรงขเลขผิวบาง (thin-layer chromatography) และฤทธิ์ทางชีวภาพ โดยการรวบรวมเหง้าว่านชักมดลูกจากแหล่งปลูกต่างๆ ในประเทศไทย 13 ตัวอย่าง ผลการศึกษาพบความแตกต่างของลักษณะช่อดอกและดอกย่อยเป็น 2 กลุ่ม ระบุได้เป็น *Curcuma comosa* Roxb. และ *Curcuma* sp. โดยทั่วไปลักษณะเหง้าของ *C. comosa* มีแขนงเล็กและสั้นกว่า *Curcuma* sp. ทั้งนี้ลักษณะภายนอกของเหง้าว่านชักมดลูกทั้ง 2 ชนิดมีความหลากหลายและอาจจำแนกได้ยากโดยเฉพาะสำหรับผู้บริโภคและผู้ค้าขายสมุนไพร ในการตรวจสอบทางเอกลักษณ์ จุลทรรศน์ลักษณะของว่านชักมดลูกทั้ง 2 ชนิดมีความคล้ายกันซึ่งไม่อาจใช้แยกความแตกต่างได้ เมื่อศึกษาองค์ประกอบทางเคมีโดยวิธีรงขเลขผิวบาง สามารถใช้แยกพืช 2 ชนิดอย่างชัดเจน โดยใช้ระบบ นำพา DCM : MeOH (99:1) และ DCM : EtOAc : MeOH (93:4:7) และใช้สารเทียบ 1,7-diphenyl-5-hydroxy-(1E)-l-heptene และ 5-hydroxy-7-(4-hydroxyphenyl)-l-phenyl-(1E)-l-heptene

นอกจากนี้การศึกษาฤทธิ์ทางชีวภาพจากสารสกัดอัลกอฮอล์ของว่านชักมดลูกทั้ง 2 ชนิด พบว่า *C. comosa* แสดงฤทธิ์คล้ายฮอร์โมนเอสโตรเจน โดยทำให้มดลูกของหนูทดลองหนาตัวขึ้น ในขณะที่ *Curcuma* sp. แสดงฤทธิ์ต่อมดลูก ไม่แตกต่างจากกลุ่มควบคุม (ovariectomized rat) อีกทั้ง *C. comosa* และ *Curcuma* sp. แสดงฤทธิ์ต้านอนุมูลอิสระจาก DPPH ได้ดี (ED_{50} 8.93-25.86 และ 13.48-27.78 μg/ml ตามลำดับ) และแสดงความเป็นพิษต่อเซลล์ (cytotoxicity) โดยวิธี brine shrimp lethality assay (LC_{50} 0.84-39.03 และ 151.43-273.03 μg/ml ตามลำดับ)

แม้ว่าคุณสมบัติทางกายภาพและเคมีของตัวอย่าง *C. comosa* ที่ศึกษาจะมีค่าแตกต่างกัน แต่สารสกัดอัลกอฮอล์แสดงฤทธิ์คล้ายฮอร์โมนเอสโตรเจนได้เช่นกัน ข้อกำหนดของ *C. comosa* และ *Curcuma* sp. ที่จัดทำขึ้น เป็นข้อมูลพื้นฐานในการวิจัย การพัฒนาวัตถุดิบและการผลิตว่านชักมดลูกในระดับอุตสาหกรรม อย่างไรก็ตามควรศึกษาความเป็นพิษเพิ่มเติม เพื่อความปลอดภัยของผู้บริโภค

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ABBREVIATIONS

AR grade	analytical reagent grade
AS/S	anisaldehyde/sulfuric acid
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BW	bodyweight
CI	confidence intervals
cm	centimeter(s)
DCM	dichloromethane
diam.	diameter
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
ED_{50}	effective dose at 50%
EtOAc	ethyl acetate
EtOH	ethanol
g	gram (s)
GC-MS	gas chromatography-mass spectrometry
h	hour(s)
Hex	hexane
hR_f	mobility relative to front x 100
kg	kilogram(s)
KOH	potassium hydroxide
LC_{50}	lethality concentration at 50%
MeOH	methanol
mg	milligram
min	minute(s)
ml	milliliter(s)
nm	nanometer(s)
No.	number

ABBREVIATIONS (Continued)

OVX	ovariectomized
THP	Thai Herbal Pharmacopoeia
TLC	thin layer liquid chromatography
USP	The United State Pharmacopoeia
UV	ultraviolet
wt	weight
°C	degree Celsius
μg	microgram(s)
μl	microliter(s)
λ	wavelength

CHAPTER I
INTRODUCTION

In this decade, alternative and complementary medicines are increasing popularity worldwide for health promotion and adjuvant therapy. Scientists from many countries are working on a common pharmacopoeia of qualitative standards for herbal drugs and nutraceuticals. Wan chak motluk is one of the popular Thai medicinal plants which has been used for remedy of gynecological diseases. It was recommended for women suffering from uterine inflammation and pain, used for treatment of postpartum uterine bleeding. It also used as emmenagogue, and abortifacient. Besides, it can be used to treat hemorrhoid, used as cholagogue and carminative. Moreover, its rhizome ground with alcohol is externally used for relieving scrotum inflammation in man (1). Currently, this herb is popularly commercialized in several dosage forms such as capsules and hydro-alcoholic extracts. Wan chak motluk was previously defined as *Curcuma comosa* Roxb., Zingiberaceae family (2). The extracts of plant showed various biological activities supporting its traditional uses such as estrogenic (3, 4), choloretic (5), uterine relaxing (6), anti-inflammatory activities (7, 8) and showed effect on male organ (9, 10). Besides, diarylheptanoids and a phloracetophenone glucoside were isolated from *C. comosa* (11, 12). Wan chak motluk is cultivated in the central, north and northeastern parts of Thailand and its rhizome is used as the raw material for traditional herbal medicines industries. It is usually cultivated in the same area with other species of *Curcuma* such as *C. aromatica* Salisb. (Wan nang kham) which is quite similar in the external appearance. The adulteration or mistaken identification of the raw materials is a serious problem for both manufacturer and researcher. According to the World Health Organization guidelines for medicinal plants, the quality of both raw materials and herbal products are emphasized to ensure the efficacy and safety (13). Several international pharmacopoeias have provided monographs of the medicinal plants which including quality parameters and standards such as WHO monographs on selected medicinal plants (13, 14). Fingerprint analysis by TLC which has been accepted by WHO as a methodology for the assessment of herbal medicines could be used for qualitative control of plant materials (15). In Thailand, two volumes of Thai herbal Pharmacopoeia (THP) have been published in 1995 and 2000 (16, 17) but the monograph of Wan chak motluk has not yet been established.

Plants are the primary sources of antioxidants and many antioxidants have been reported in Zingiberaceous plants (18-21) Antioxidants play an important role to protect the body from the formation of free radicals which is one of the major causes of the damage of the cells. Besides, brine shrimp lethality assay was very useful tool and a convenient monitor for screening cytotoxicity in plants which correlated to antitumor activity and toxicity (22, 23).

In the present study, the identification of Wan chak motluk from different locations in Thailand was conducted. Its specification according to WHO guidelines for medicinal plants and THP was established. Finally, its free radical scavenging activity by DPPH (24) and cytotoxicity by brine shrimp lethality assay, together with uterotrophic activity was also evaluated.

CHAPTER II
LITERATURE REVIEW

‘Wan chak motluk’ is Thai common name which has been used as traditional medicine. It is not only indigenously used by traditional healers but also used as the raw material in traditional medicine manufacture. It is commonly used in the forms of single herbal- and polyherbal–capsules or hydro-alcoholic extracts. It is recommended for women suffering from uterine inflammation and pain (1) and for inflammation in postpartum uterine bleeding (1, 25, 26). It is also used as emmenagogue (25, 26) and abortifacient (26). Besides, it has been applied for treatment of hemorrhoid, used as cholagogue (25, 26) and carminative (26). Moreover, its rhizome ground with alcohol is externally applied for relieving scrotum inflammation in man (1).

Wan chak motluk has previously been defined in “Thai Plant Names” into two species which are *Curcuma comosa* Roxb. and *Curcuma xanthorrhiza* Roxb (2). Only *C. comosa* is found in wild native and cultivated throughout many parts of Thailand. *C. xanthorrhiza* is originated from Java, Indonesia and spread to several area in the Indo-Malaysia territory (27-29). It is rarely found in cultivation in Thailand.

Curcuma is one of the economically important genus of the family Zingiberaceae and consists of approximately 80 species (30). More than 40 species of *Curcuma* are found in Thailand (31). Only 13 species of *Curcuma* are mentioned in “Thai Plant Names” such as *C. aromatica* Salisb., *C. comosa* Roxb., *C. longa* L., *C. xanthorrhiza* Roxb. and *C. zedoaria* (Berg) Rosc. (2).

The genus *Curcuma* is predominantly found in the Asian tropical. The geographic distribution of this genus ranges from India to Thailand, Indochina, Malaysia, Indonesia and Northern Australia. The similarities of their growth habit, leaf-shapes, and the flowers among *Curcuma* species are so great that it is generally difficult to distinguish the species at both vegetative and reproductive stages (32).

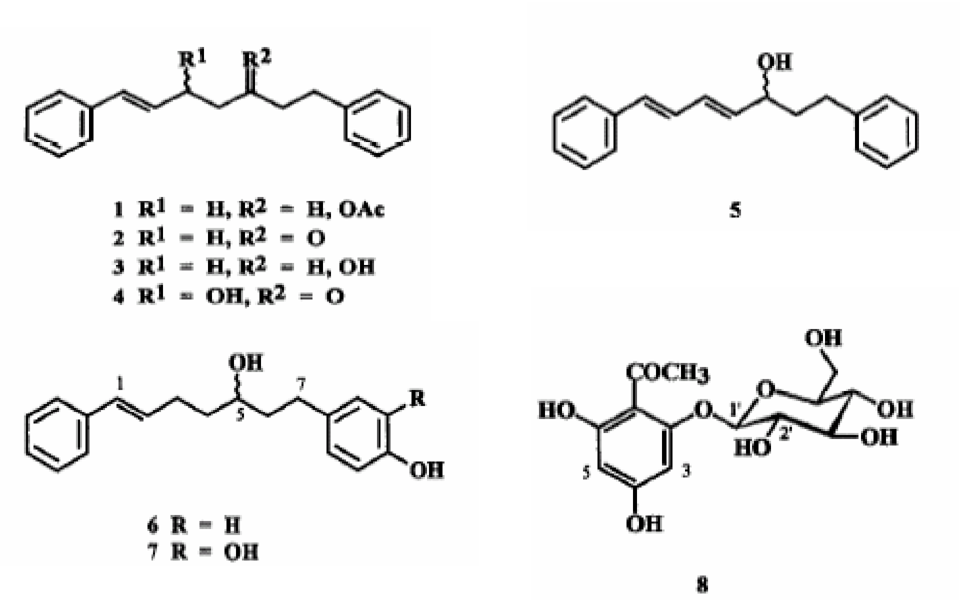
1. *Curcuma comosa* Roxb.

1.1 Botanical character

C. comosa is a perennial herb with large oval rhizome with pale yellow inside. Leaves are broad-lanceolate to oblong, green with purple midrib above, glabrous both sides, with long petiole. Inflorescences blossom in May. Spike-like is clavate, with pinkish white fertile bracts and copious, bright pink comas. Flowers are pale yellow, lobes of corolla pinkish white (27, 28). This plant can grow in the low plains to those 650-900 meters above sea level (33). *C. comosa* is also found in Myanmar, India and neighboring tropical countries (27, 28).

1.2 Chemical studies

In 1994, diarylheptanoids [1-5] (figure 1) were firstly isolated from methanolic extract of *C. comosa* (11). However, diarylheptanoids had previously been found in *C. xanthorrhiza, Alpinia officinarum* Hance (34) and *A. katsumadai* (Zingiberaceae) (35). In 1997, Suksamrarn et al., obtained a new phloracetophenone glucoside [8] from the ethyl acetate and butanol extract of *C. comosa* rhizome. Moreover, three known diarylheptanoids [3, 6, 7] were isolated from the ethyl acetate extract as well (12).



[1] 1,7-diphenyl-3-acetoxy-6(*E*)-heptene

[2] 1,7-diphenyl-6(*E*)-hepten-3-one

[3] 1,7-diphenyl-5-hydroxy-(1*E*)-l-heptene

[4] 1,7-diphenyl-4(*E*),6(*E*)-heptadien-3-ol

[5] 1,7-diphenyl-6(*E*)-hepten-3-one-5-ol

[6] 5-hydroxy-7-(4-hydroxyphenyl)-l-phenyl-(1*E)*-l-heptene

[7] 7-(3,4-dihydroxyphenyl)-5-hydroxy-l-phenyl-(1*E*)-l-heptene,

[8] 4,6-dihydroxy-2-O-(β-D-glucopyranosyl)acetophenone

Figure 1 Structure of chemical compositions in *Curcuma comosa* Roxb. rhizome.

1.3 Biological activities

Choleretic and hypolipidemic activity

Choleretic properties of four extracts of *C. comosa* (hexane, butanol, ethyl acetate and water) were investigated in male Wistar rats. Intraduodenal administration of the butanol and ethyl acetate extracts (100-1,000 mg/kg BW) exhibited the strong effective in producing a choleretic effect whereas the hexane and aqueous extracts had less effect. Moreover, the ethyl acetate extract exhibited a dose-dependent stimulation of bile acid secretion (5). An isolated compound from ethyl acetate and butanol extracts of *C. comosa*, phloracetophenone glucoside, was considered to possess this choleretic activity (12).

Effects on plasma cholesterol level of an ethyl acetate extract of *C. comosa* in normal male Swiss albino mice and hypercholesterolaemic female hamsters were investigated. The extract at a dose of 500 mg/kg BW effectively lowered both plasma cholesterol and triglyceride (36, 37).

Estrogenic activity

Uterotrophic activity of four extracts of *C. comosa* (hexane, butanol, ethyl acetate and water) was investigated in female Wistar rats. Intraperitoneal administration of the hexane extract (480 mg/kg BW) exhibited the most effective in increasing uterine weight and glycogen content. The butanol and ethyl acetate extracts produced weak effect whereas the aqueous extract had no effect. The hexane extract induced cornification of vaginal epithelium and increased growth and keratinization of vaginal mucosa. Moreover, the extract effectively induced an increase in specific estradiol binding site in the uterine nuclei (3, 4).

As estrogen has a variety of influences on male organs, effect of hexane and ethyl acetate extract (500 mg/kg BW) on reproductive organs were investigated in male Wistar rats. Intragastric administration of the hexane extract effectively decreased on weights of testes, epididymis, ventral prostate, seminal vesicles and levator ani muscle whereas the ethyl acetate did not affect the organ in immature male rats weights. In mature rat, although sperm concentration and motility in the cauda epididymides were also significantly suppressed. Fertility was not significantly affected (9, 10).

Uterine relaxant activity

The effect of 95 % ethanolic extract on contraction of intact and isolated rat uterus was markedly shown to reduce uterine contraction previously induced by oxytocin and other agents (6).

Anti-inflammatory activity

The hexane extract (1 ng/ml – 10 µg/ml) of *C. comosa* significantly suppressed the levels of nitric oxide released from LPS (lipopolysaccharide)-activated microglia that demonstrated to possess anti-inflammatory activity (7).

Pretreatment with hexane or ethanol or two diarylheptanoids, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene) of *C. comosa* significantly decreased the release of pro-inflammatory cytokines, tumor necrosis factor α (TNF- α) and interleukin-1β, from phorbol-12-myristate-13-acetate (PMA)-stimulated PBMC and U937 cells (8).

Antimicrobial activity

Antimicrobial activity of acetone and ethyl acetate extract of *C. comosa* rhizome (8-128 µg/ml) were investigated by agar diffusion method. The two extracts exhibited no effect on all tested bacteria, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* (38).

Nematocidal activity

Jurgens et al. reported that three fractions (hexane, dichloromethane and water) from methanolic extract of *C. comosa* were evaluated against *Caenorhabditis elegans*. Both the hexane and dichloromethane fractions possessed antinematode activity while the aqueous fraction exhibited negative result (11).

1.4 Toxicity

Toxicity of ethanolic extract (containing 2.05 % phloracetophenone) at the dose of 100, 200, 400 and 800 mg/kg BW was investigated in Wistar rats for 90 consecutive days. Intragastric administration of the extract did not affect growth and food consumption. Decreases of hematocrit and hemoglobin in rat receiving the highest dose were still within normal range. Histopathological examination of visceral organs revealed no abnormality related to the extract except hyperplasia and hyperkeratosis of the gastric epithelium (39).

Acute toxicity of choleretic compound, phloracetophenone (2,4,6-trihydroxyacetophenone; THA) (0.1-6 g/kg) was investigated in three different animal species (mice, rat and hamster). The mice and hamsters were more sensitive to THA than rats (LC_{50} values were 338, 365 and 498 mg/kg BW, respectively). Intraperitoneal administration showed more toxic than the intragastric. This investigation has been concluded that THA was acutely toxic to all tested animals. Moreover, subacute toxicity of THA (37-300 mg/kg BW) in mice for 30 consecutive days was investigated by intragastric administration. High doses of THA induced periportal hepatocyte degeneration. In the ethinylestradiol (EE)-induced cholestasis, THA enhanced the hepatic clearance of sulfobromophthalein and decreased the elevasted plasma alkaline phosphatase in EE-cholestatic rats to control levels. However, all increases were slightly remained within the normal ranges. Therefore the THA at biologically active choleretic dose had low toxicity (40).

2. Quality control of herbal medicine

Analysis of herbal drugs is necessarily performed to safeguard the public because of three main reasons, biochemical variation of the drug-furnishing organisms, deterioration from treatment and storage, and substitution or adulteration as a result of ignorance, carelessness, or fraud. World Health Organization (WHO) has emphasized the need to ensure quality control of medicinal plant products by using modern techniques and by applying suitable standards (13).

Pharmacognostic characters of medicinal plants were required for quality control of the raw material used in manufacturer products. Many reports of investigation in their microscopy have been published such as *Mitracarpus vilosus* (S.W.) D.C. (41) and *Gisekia pharnacioides* L. (42). Anatomical and morphological characters of *Psidumi guajava* L. leaf was also performed to help the authentication (43). Besides, rhizome and root anatomy of black cohosh (*Actaea racemosa* L.) (44) and three *Phyllanthus* species (45) were comparative investigated to protect the adulterants from other related species.

3. Chemical analysis by thin-layer chromatography

In any traditional formularies or even single medicinal plant, many unknown active components can be detected. The chromatographic fingerprints represent pharmacologically active and chemically characteristic components.

Fingerprint analysis has been accepted by WHO as a methodology for the assessment of herbal medicines (13). Various pharmacopoeias such as British Herbal Pharmacopoeia (BHP), European Pharmacopoeia (EP) and THP, etc. are employing thin-layer chromatography as a means for assessing quality and purity (46). Chromatography is a method of analysis in which a mobile phase passes over a stationary phase in such a way that a mixture of substances is separated into its components. This technique is based on different partition and retention of analyses between the stationary phase and mobile phase. It suffices to mention that the R_f value (rate of flow, distance moved by solute divided by distance moved by solvent front) of compound, determined under specific condition, is characteristic and can be used as an aid to identity (47).

Thin-layer chromatography (TLC) is a mode of liquid chromatography in which the sample is applied as a small spot or steak to the origin of a thin absorbent layer supported on a glass, plastic, or metal plate. Compounds resolved on the TLC plate are visualized using either general or specific method. Quenching zones in short wavelength (254 nm) and fluorescence zones in long wavelength (366 nm) ultraviolet light are indicated all compounds containing chromophores (48). A visual comparison of the size and pattern of the spots may serve for qualitative or semi quantitative estimation (49).

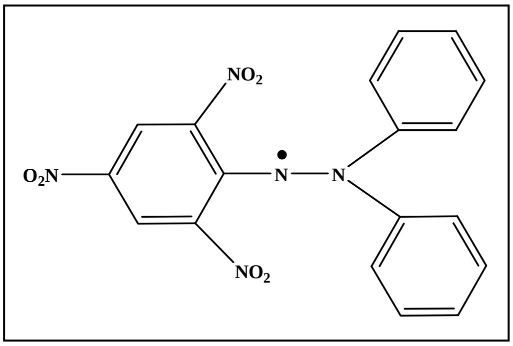
4. Free radical scavenging activity

Antioxidant

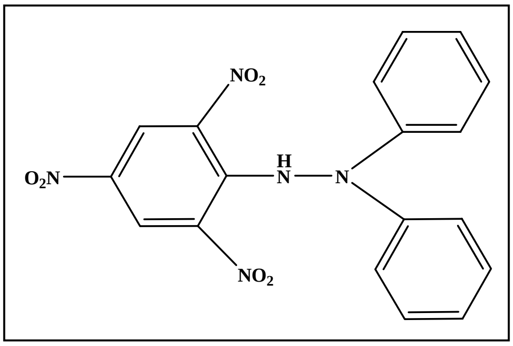
Antioxidant compounds play an important role as a health-protecting factor. They help to protect the body from the formation of free radicals which cause the damage of the cells, impairing the immune system and leading to infections and various degenerative diseases such as heart disease and cancer. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced antioxidants like vitamin C, vitamin E, phenolic acids, carotenes and phytoestrogens have been recognized as having the potential to reduce disease risk. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are presented in biological systems from a wide variety of sources (50). Many antioxidants can be obtained from Zingiberaceous plants such as curcumin and its derivatives from *C. longa* L. and *C. zedoaria* (Berg.) Rosc., diarylheptanoids and scopoletin from *C. mangga* Valeton & van Zijp, xanthorrhizol from *C. xanthorrhiza* Roxb., dehydrozingerone from *Zingiber officinale* L. and panduratin A from *Boesenbergia pandurata* Schult (18-21, 51, 52).

DPPH scavenging test

A simple method that has been developed to determine the antioxidant activity utilizes the stable free radical 2,2-diphenyl-1-picrylhydrazyl. The odd electron in the DPPH free radical gives a strong absorption maximum at 520 nm and is violet in color. When solution of DPPH is mixed with a substance that can donate a hydrogen atom, then this gives rise to the reduced form with turning from violet to yellow color (presented from a picryl group) shown in figure 2. The resulting decolorization is stoichiometric with respect to number of electrons captured (53).



Diphenylpicrylhydrazyl
(free radical : violet color)



Diphenylpicrylhydrazine
(nonradical : pale yellow color)

Figure 2 Structure of DPPH in free radical and reduced form.

5. Cytotoxicity by brine shrimp lethality assay

The brine shrimp (*Artemia salina* Leach) is well known animal as a food source for fish raised in home aquariums, aqua culture systems, and in laboratories. In salt water, embryogenesis begins and is completed between 16 to 36 h. The embryo emerges from the shell still covered in a hatching membrane. However, it soon develops antennae and mandibles, breaks free of the hatching membrane, and becomes an active, free swimming nauplius. The larva is reddish in color due to the presence of yolk and persists for 3 days (54). This organism likes many other primitive aquatic plants which are attracted to light, rising to the surface in the daytime, and sinking at night (55).

Brine shrimp toxicity has been used as a monitoring tool for pharmacologic activities in plant extracts which may be manifested as cytotoxicity. A number of papers have been published on the toxic effects of various chemicals and toxicants on brine shrimp. The first report of the use of the brine shrimp as a test organism appeared in 1956 by Michael et al. (56), and latter developed by many researchers (22, 23, 57). Mclaughlin et al. found a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity. It has been observed that ED_{50} values for cytotoxicities are generally about one-tenth the LC_{50} values found in the brine shrimp test. Furthermore, it is possible to detect and then monitor the fractionation of cytotoxic, as well as 3PS (P388) (*in vivo* murine leukemia) active extracts using the brine shrimp lethality bioassay rather than more complicated and expensive *in vitro* and *in vivo* antitumor assays (58). Brine shrimp bioassay has been used for the detection of fungal toxins (59), plant extract toxicity (22), heavy metal (60, 61), pesticides (62, 63), cytotoxicity testing of dental materials (55), mycotoxin (64-66)and anesthetics (67).

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Plant materials

Thirteen samples of rhizomes of Wan chak motluk were obtained within 2004-2006. Five samples were purchased from wholesalers who had procured those samples by contacting with farmer and 8 samples from the cultivated areas. Among all collected samples, 6 samples were obtained from the Central part, 5 samples from the Northeast part and 2 samples from the North part of Thailand. Coding and locations were shown in table 1.

Table 1 Source of Wan chak motluk collected from different locations.

No.	Code	Province	District	Local names	Region	Obtained date
1	KC01	Kanchanaburi	Thong Pha Phum	Wan chak motluk	C	25-03-2005
2	KC02	Kanchanaburi	Sai Yok	Wan chak motluk	C	07-07-2005
3	NT01	Nakhon Pathom	Kamphaeng Saen	Wan chak motluk	C	2004
4	NT02	Nakhon Pathom	Kamphaeng Saen	Wan chak motluk	C	25-03-2005
5	PB01	Phetchabun	Khao Kho	Wan chak motluk	C	07-07-2005
6	PB02	Phetchabun	Khao Kho	Wan chak motluk	C	04-12-2005
7	CM01	Chiang Mai	Wiang Hae	Wan chak motluk	N	07-07-2005
8	TK01	Tak	Phop Phra	Wan chak motluk	N	03-12-2005
9	KS01	Kalasin	Khao Wong	Wan thorahoed, Wan chak motluk tuamia	NE	16-12-2005
10	KS02	Kalasin	Khao Wong	Wan chak motluk tuaphu	NE	20-12-2005
11	SK01	Sakhon Nakhon	Sawang Dan Din	Wan chak motluk tuaphu	NE	29-06-2005
12	SK02	Sakhon Nakhon	Sawang Dan Din	Wan chak motluk tuaphu	NE	19-12-2005
13	SK03	Sakhon Nakhon	Sawang Dan Din	Wan thorahoed, Wan chak motluk tuamia	NE	26-12-2005

C = Central region, N = Northern region, NE = North-eastern region

1.2 Chemicals

1.2.1 Solvents

Methanol, AR grade	Merck,Germany
Ethanol, AR grade	Merck, Germany
Dichloromethane, AR grade	Merck, Germany
Hexane, AR grade	Merck, Germany
Ethyl acetate, AR grade	Merck, Germany
Dimethylsulfoxide (DMSO)	Merck, Germany
95% Ethanol, commercial grade	
Water, distilled	

1.2.2 Spraying reagent

Anisaldehyde sulfuric acied spray reagent	Merck, Germany

1.2.3 Reference standards

Chemical markers

1,7-diphenyl-5-hydroxy-(1*E*)-l-heptene (M1)

5-hydroxy-7-(4-hydroxyphenyl)-l-phenyl-(1*E)*-l-heptene (M2)

(Obtained from Prof. Dr. Apichart Suksamrarn, Department of Chemistry, Faculty of Science, Ramkhamhaeng University)

Possitive controls

Vitamin C	Sigma, USA
BHA	Sigma, USA
BHT	Sigma, USA
Berberine	Sigma, USA

1.2.4 Others

Hydrochloric acid	J.T. Baker, USA
Chloral hydrate	Unilab, Australia
Iodine	Univar, Australia
Aniline sulfate	
Diphenylpicrylhydrazyl radical (DPPH)	Sigma, USA
Sea salt	Sigma, USA
Glycerine water	Sigma, USA

1.3 Equipments

Soxhlet apparatus	Duran, Germany
Erlenmeyer flask	Pyrex, Germany
Separatory funnel	Pyrex, Germany
Filter paper No.1	Whatman, England
Ashless filter-paper No.42	Whatman, England
Silica gel 60 GF_{254}, precoated on TLC	Merck, Germany
Aluminium sheets	
Crucible	Din, Germany
Round bottom flask	Pyrex, USA
Sieve, nominal mesh aperture	Endecotts, England
Rotary evaporator	Buchi, Germany
Analytical balance	Mettler Toledo,
Muffle furnace	Vecstar, England
Microscope and camera lucida	Olympus, Japan
Desiccator	Duran, Germany
Spectrophotometer	Miltonroy®
UV detector	Camag, Switzerland
Ultrasonic bath	Selecta®
Micropipette	Eppendorf, Germany
Digital camera	Olympus
Microscope	Olympus
Free-hand microtome	
Hot plate	
Water bath	
Porous porcelain	
Slides	
Test tube	
Appendorf tube	
Microwell	

1.4 Solvent systems

DCM : MeOH = 99:1

DCM : EtOAc : MeOH = 93:4:7

1.5 Miscellaneous

Brine shrimp (*Artemia salina* Leach)

2. Methods

2.1 Plant identification

Observations of the rhizomes were done immediately after getting the specimens. All samples were planted in potteries in the nursery of the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University for further investigation of leaf and inflorescence. The morphological characteristics of rhizomes, leaves, inflorescences and florets were recorded. The herbarium specimens of available investigated samples were deposited at the herbarium of Department of Pharmaceutical Botany, Faculty of Pharmacy (PBM), and Department of Plant Science, Faculty of Science, Mahidol University. The Flora Indica (27), Flora of British India (28) and Flora of Java (29) were used as the references to identify Wan chak motluk samples. The collector numbers of herbarium specimens were shown in table 11.

2.2 Pharmacognostic studies

2.2.1 Anatomical and histological examination (13, 68)

Fresh rhizomes were sliced into thin sections by free-hand microtome. It was cleared by chloral hydrate solution, stained lignified cell wall with aniline sulfate solution, and then mounted with glycerine water. The sections were stained by iodine for determination of starch. They were examined under the microscope. Microphotography was also done with Olympus digital camera.

2.2.2 Powdered drug examination (13, 68)

Preparation of the powdered drugs

Fresh rhizomes were cleaned and cut into small pieces, then further dried in an oven at 60°C for 48 h. The dried materials were ground and passed through the sieve no. 40.

Powdered drug examination

The powdered rhizomes were examined by the same procedure as described in anatomical and histological examination (2.2.1).

2.2.3 Physicochemical properties

2.2.3.1 Loss on drying (13, 69, 70)

Loss in weight represents to content of water and small amounts of other volatile materials in sample. Three grams of powdered samples were accurately weighed in the crucible which was previously dried and tared. It was heated at 105°C in a hot air oven for 5 h. The crucible was allowed to cool in the desiccator before weighing. It was reheated until obtaining constant weight. Each sample was done in triplicate. Percentage of loss on drying was calculated with reference to dried material as follow.

$$\%\ \text{Loss on drying} = \left(\frac{\text{Loss weight (g)}}{\text{Weight of sample used (g)}}\right) \times 100$$

2.2.3.2 Total ash (69, 70)

Three grams of dried powdered samples were accurately weighed in the crucible which was previously dried and tared. It was ignited in muffle furnace by gradually increasing the heat to 450°C until it was white. The ash was allowed to cool in the desiccator. It was reheated at 105°C until consecutive weighing do not differ by more than 5 mg. Each sample was done in triplicate. Percentage of total ash was calculated with reference to dried material.

$$\% \text{ Total ash} = \left(\frac{\text{Total ash weight (g)}}{\text{Weight of sample used (g)}} \right) \times 100$$

2.2.3.3 Acid-insoluble ash (69, 70)

The total ash in crucible obtained from 2.2.3.2 was added with 3N hydrochloric acid solutions, and then boiled gently for 5 minutes. The insoluble matter was filtered on an ashless filter-paper then washed with hot water. It was transferred into the original crucible, dried on hot-plate and ignited at about 500°C. The residue was allowed to cool in the desiccator, and then weighed. It was reheated at 105°C until obtaining constant weight. Each sample was done in triplicate. Percent of acid-insoluble ash was calculated with reference to dried material.

$$\% \text{ Acid insoluble ash} = \left(\frac{\text{Acid insoluble ash weight (g)}}{\text{Weight of sample used (g)}} \right) \times 100$$

2.2.3.4 Extractive values (69, 70)

Ethanol-soluble extractive

Five grams of powdered samples were added with 100 ml of ethanol in closed flask for 24 h. The solution was shaken for 6 h then allowed to stand for 18 h. The filtrate (25 ml) was collected to porcelain dish. It was evaporated then heated at 105°C to dry. It was cooled in desiccator before weighing. It was reheated at 105°C until obtaining constant weight. Each sample was done in triplicate. The percentage of extract was calculated with reference to dried material.

Water-soluble extractive

The method was followed as described in ethanol-soluble extractive but water was used instead of ethanol.

Hexane-soluble extractive

Two grams of powdered samples were continuously extracted by hexane (250 ml) in an soxhlet apparatus for 20 h. The hexane solution was transferred to a tarred porcelain dish and allowed to evaporate. It was heated at 105°C then put in desiccator before weighted. It was reheated at 105°C until obtaining constant weight. Each sample was done in triplicate. The percentage of extract was calculated with reference to dried material.

Dichloromethane-soluble extractive

The method was followed as described in hexane-soluble extractive but dichloromethane was used instead of hexane.

2.2.3.5 Volatile oil content (69)

Twenty five grams of *Curcuma* sp. or 40.0 g of *C. comosa* dried powdered samples were added with 600 ml of distilled water in a 1,000 ml round-bottom flask. A few pieces of porous porcelain were filled. It was distilled for 5 h. The volatile oil content was recorded from graduated tube scale. Each sample was done in triplicate. The percentage of volatile oil was calculated with reference to dried powders.

2.3 Chemical analysis

2.3.1 Preparation of the extracts

Fifty grams of each dried powdered samples were extracted with 150 ml of 80% alcohol on a shaker at 25°C for 48 h and then filtered through Whatman no. 1 filter paper. The extraction was repeated three times then the extracts were combined. The filtrates were concentrated under reduced pressured using a rotary vacuum evaporator. Crude extracts were then evaporated on a water bath until dried.

2.3.2 Thin layer chromatographic fingerprint

Each tested solution was prepared by dissolving 10.0 mg of its extract in 1 ml of methanol to give a 10 mg/ml concentration. For reference standards, 1,7-diphenyl-5-hydroxy-(1*E*)-l-heptene (M1) and 5-hydroxy-7-(4-hydroxyphenyl)-l-phenyl-(1*E)*-l-heptene (M2) were used at concentration in 1 mg/ml. A 5 µl of tested solution was applied as a band of 8 mm in length on the stationary phase, precoated silica gel aluminium plate $60_{F254.}$ Two solvent systems were used as mobile phase to differentiate the compounds, they were DCM : MeOH (99:1) and DCM : EtOAc : MeOH (93:4:7). The plate was examined under short (254 nm) and long (366 nm) wavelength ultraviolet light and also detected with anisaldehyde/sulfuric acid spray reagent (AS/S).

2.4 Biological screening

2.4.1 Free radical scavenging activity (24)

Preparation of 0.06 mM DPPH

The DPPH solution was freshly prepared. DPPH (2.4 mg) was dissolved with 100 ml absolute ethanol in a volumetric flask. The solution was then sonicated for 2 min.

Preparation of test samples

Two milligrams of the test sample were dissolved in 5 ml of ethanol and diluted with ethanol to make a dilution series of 3.125 to 200 µg/ml. For each well, 500 µl of DPPH solution was added to the 500 ul test solution to finish the total volume of 1 ml. The experiment was carried out in triplicate. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and vitamin C were used as the positive controls.

DPPH scavenging assay

The reaction mixture (1 ml) was measured into three wells (A, B and C), each well contains the following reagents.

Solution A (Control)	500 µl of ethanol + 500 µl of DPPH solution
Solution B (Blank)	500 µl of ethanol + 500 µl of test sample
Solution C (Test sample)	500 µl of test sample in ethanol + 500 µl of DPPH solution

After each well was mixed and incubated in the dark at room temperature for 30 min, the absorbance of each well was measured at 520 nm with UV-Vis spectrophotometer.

Calculation of the percent inhibition of DPPH radical and ED_{50}

ED_{50} value was evaluated from the curve between % inhibition and concentration. Each sample was done in duplicate and the average of ED_{50} value was then calculated.

2.4.2 Cytoxicity by brine shrimp lethality assay (22, 23)

Alcoholic extracts of Wan chak motluk were determined for cytotoxicity using brine shrimp lethality assay.

Preparation of test samples

The alcoholic extracts of Wan chak motluk (2.0 mg) were thoroughly dissolved in 30 µl dimethyl sulfoxide (DMSO) using ultrasonic bath. Solution was diluted serially (1.25, 12.5, 125, 1,250 µg/ml) in sea water. Berberine was used as positive control. Control brine shrimp containing a mixture of DMSO and seawater were included in each experiment.

Brine shrimp (*Artemia salina*) hatching assay

Brine shrimp encysted eggs were hatched in a shallow rectangular dish (10x15 cm) filled with artificial sea water which was prepared from commercial sea salt. A plastic divider including two unequal compartments was used. The eggs were sprinkled into the compartment which was darkened, while the smaller compartment was illuminated. The incubation was at room temperature for 36 h to reach the metanauplii stage. The nauplii were attracted to the lighted side of divider and then collected with a Pasteur pipette.

Brine shrimp 24 h survival trials

For each well, 50 µl of sea water containing about 5-10 larvae were added to the 500 µl tested solution (obtain dilution series of 0.1, 1, 10, 100 µg/ml). The experiment was carried out in 6 replications. Tested brine shrimp was incubated at room temperature for 24 h. After The numbers of survivors and dead (non-motile) lavae in each well were counted and recorded under a stereomicroscope.

Data analysis

The lethal concentration 50% (LC_{50}) and the 95%confidence intervals were calculated by Probit analysis described by Finney (71). Extracts giving LC_{50} values greater than 1,000 µg/ml were considered to be non-cytotoxicity (22).

2.4.3 Uterotrophic activity (performed by Prof. Dr. Pawinee Piyachaturawat, Department of Physiology, Faculty of Science, Mahidol University).

The estrogenic property of Wan chak motluk was investigated by uterotrophic effect in immature ovariectomized female Wistar rats. Immature female Wistar rats were ovariectomized under ether anesthesia 5 days before used. They were treated with the dried alcoholic extracts at the dose 500 mg/kg BW by intraperitoneal injection for 2 consecutive days while estradiol 2.5 mg/kg BW was used as the positive control. The normal female rats (intact control) and ovariectomized rats (OVX) were included in this experiment for positive and negative controls. All animals were sacrificed and the uterine horns were rapidly dissected and recorded the weight.

CHAPTER IV
RESULTS

1. Plant identification

The common morphological characteristics of those thirteen samples of Wan chak motluk were observed. They were perennial herbs with underground stem as rhizome, producing short pseudostem and bearing inflorescence before the leaves appeared. Rhizomes were externally pale brown to dark brown and pale yellow to light brown inside. Leaves were simple, alternate, with long petiole, blade shape was broad lanceolate, base attenuate, apex apiculate, margin entire, upper surface was occasionally green with reddish purple band along midrib. Inflorescences were cylindrical shape and distinct from the leaves. The inflorescence consisted of sterile bracts which had another color than the fertile ones. This upper part of the inflorescence was crowned by a rosette of pink colored sterile bracts. Bracts were connate to each other at base and increasing in length to the apex. The flowers were tubular and white to pinkish white. Calyxes were cylindrical and minutely 3-toothed at apex. Corolla tubes were funnel-shaped, glabrous outside, with densely brown hairs inside about the middle part. Petals were 3 and minute hood at the apex. The upper corollas were lobe and the staminodes overlap, forming a hooded structure. The labellums were upcurved margins with yellow band thickening along the middle field. The stamens were short, white and situated under the hood. The anthers provided with two curved basal spurs and short crest at apex. The morphological characteristics were shown in figure 3 and data were recorded as followed.

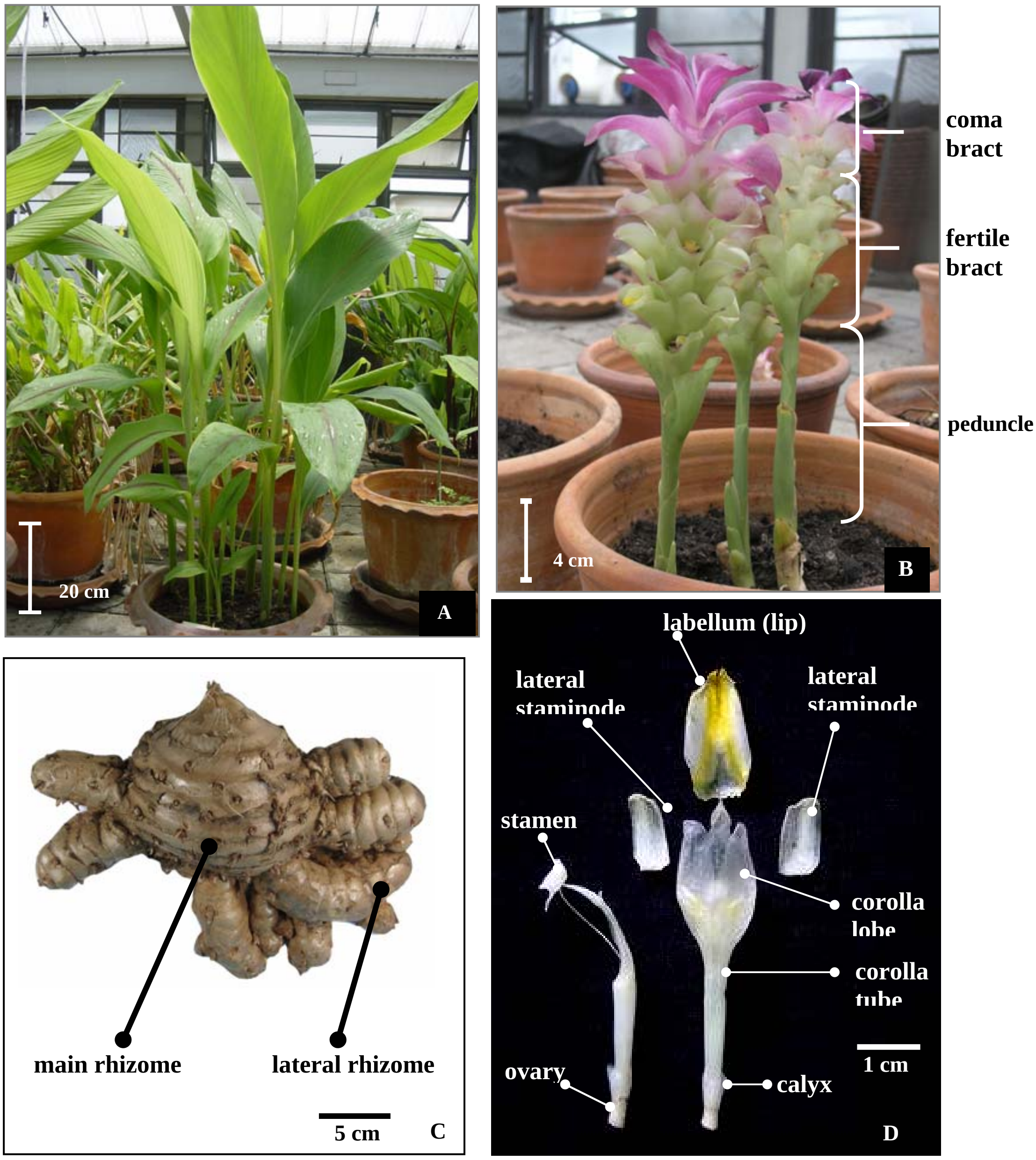


Figure 3 Morphological characteristics of Wan chak motluk: (A) pseudostem, (B) inflorescence, (C) rhizome, (D) floret.

1. Rhizome

A main rhizome ovoid, subglobose, 6-9 cm wide 7-15 long, lateral rhizome 1-2 cm long, < 2 cm diam. (figure 4A) ……………………………………NT01, NT02, KC01, KC02, CM01, SK03, KS01

B main rhizome ovoid, transversely globose 8-12 cm wide 8-15 long, lateral rhizome 3-10 cm or more long, > 2 cm diam. (figure 4B) ……………………….. PB01, PB02, SK01, SK02, TK01, KS02

A

B

Figure 4 Rhizomes of 2 groups of Wan chak motluk: (A) main rhizome ovoid, subglobose with short lateral rhizome, (B) main rhizome ovoid, transversely globlose with long lateral rhizome.

2 Pseudostem height

A ≤ 1 m ……………………………………..……KC01, KC02, CM01, SK03

B 1.1-2 m …………………………………..…. NT02, KS01, SK01, TK01

C > 2 m …………………………....….…..…….. PB01, PB02, SK02, KS02

3 Petiole/leaf

3.1 Petiole length

A <10 cm …………………………...……NT01, KC01, CM01, SK03, KS01

B 10-20 cm …………………………...... NT02, KC02, SK01, KS02, TK01

C > 20 cm ………………………………………...……. PB01, PB02, SK02

3.2 Leaf blade

3.2.1 Leaf blade width

A <10 cm …………………………..……………………….. NT01, NT02

B 10-15 cm ……………………………KC01, KC02, CM01, SK03, KS01

C >15 cm ……………..…..... PB01, PB02, SK01, SK02, TK01, KS02

3.2.2 Leaf blade length

A 30-40 cm …..……. NT01, NT02, KC01, KC02, CM01, SK03, KS01

B >40 cm ………………..……. PB01, PB02, SK01, SK02, TK01, KS02

3.2.3 Blade lower surface

A glabrous ………. NT01, NT02, KC01, KC02, CM01, SK03, KS01,
PB01, PB02, SK01, SK02, TK01, KS02

B pubescent ……………………..……………………...PB02, SK02

4 Inflorescence

4.1 Peduncle length

A <4 cm ……………………………...………NT01, NT02, CM01, SK03

B 5-7 cm ………………………..…………………..… KC01, KC02, PB02

C >8 cm ………………...……………… PB01, PB02, SK01, SK02, TK01

4.2 Fertile bract color

A white with pink at apex (figure 5A, 5B) …………..…. NT01, NT02, CM01, SK03, KC01, KC02

B green with pink at apex (figure 5C) ……….…. PB01, PB02, SK01, SK02, TK01

4.3 Coma bract color

A white with broad pink band at center (figure 5A) ……..….. NT02, SK03, KC01, KC02

B pink with white at base (figure 5B) …......….. NT01, NT02, CM01

C pink with green and white at base (figure 5C) ……….. PB01, PB02, SK01, SK02, TK01

A

B

C

Figure 5 Inflorescences of 3 groups of Wan chak motluk: (A) fertile bract white with pink at apex and coma bract white with board pink band at center, (B) fertile bract white with pink at apex and coma bract pink, (C) fertile bract green with pink at apex and coma bract pink.

4.4.1 Corolla tube

A Upper part ≤1.2 cm diam., ratio of upper part to lower part length about 1.5:2 (figure 6A) ..…NT01, NT02, KC01, KC02, CM01, SK03

B Upper part >1.2 cm diam., ratio of upper part to lower part length about 2:1.5 (figure 6B)…. PB01, PB02, SK01, SK02, TK01

4.4.2 Labellum

A Glabrous without purple line (figure 6A) ....…NT01, NT02, KC01, KC02, CM01, SK03

B Hairy beside purple line on each side of thickening yellow mid-band (figure 6B).... PB01, PB02, SK01, SK02, TK01

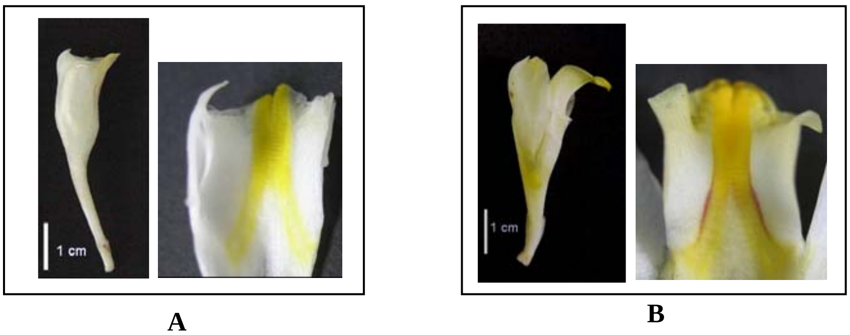


Figure 6 Florets of 2 groups of Wan chak motluk: (A) upper part of corolla tube shorter than lower part, without purple line on each side of thickening yellow band, (B) upper part of corolla tube longer than lower part, with purple line on each side of thickening yellow band.

All samples of Wan chak motluk were identified to be the member of Zingiberaceae family and genus *Curcuma*. They were differentiated into two groups. Wan chak motluk coded NT01, NT02, KC01, KC02, CM01 and SK03 were taxonomically identified as *Curcuma comosa* Roxb. but samples coded PB01, PB02, SK01, SK02 and TK01 were identified as *Curcuma* sp. All samples of *Curcuma* sp. possessed the same morphological characteristics within group and still under observation to obtain scientific name. However, the samples coded KS01 and KS02 did not bear the inflorescence after planting in the pots and could not be differentiated by the morphological characteristics only.

The differentiate characteristics were peduncle, color of fertile and coma bracts, and florets. For *C. comosa*, its peduncle was short, fertile bracts were white with pink at apex and coma bracts were pink or white with broad pink band at center. The other species possessed long peduncle, green in almost fertile bract and pink coma bract. The labellum of *C. comosa* was glabrous without purple lines, while the *Curcuma* sp. was hairy beside purple lines on each side of thickening yellow mid-band; pseudostem of *C. comosa* was generally not higher than those of *Curcuma* sp. Moreover, main rhizome of *C. comosa* were ovoid to subglobose with short lateral rhizome while *Curcuma* sp. were transversely globose with large and long lateral rhizome.

2. Pharmacognostic studies of the rhizomes

2.1 Macroscopic characteristics

The general macroscopic characteristics of Wan chak motluk which was identified to the *C. comosa* and *Curcuma* sp. were indistinctly different as followed.

C. comosa: Fresh rhizome composed of main and lateral rhizome. The main rhizomes were varied from ovoid to subglobose (7-15 x 6-9 cm) with small (not more than 2 cm diam.), short lateral rhizome (1-2 cm long) (figure 4A).

Curcuma sp.: Fresh rhizome composed of main and lateral rhizome. The main rhizomes were varied from ovoid to transversely globose (8-15 x 8-12 cm) with large (2-3.5 cm diam.), long lateral rhizome (2-10 cm long) (figure 4B).

Nevertheless, the external surface of both species was quite similar. It was wrinkled, showing annulations of scale leaf, pale brown to dark brown in color. Internal part was pale yellow to light brown showing a narrowed cortex (1-1.5 cm) which separated from broad central cylinder by a distinct endodermoid (figure 7). After exposed to the air, the color became darker with scattered, reddish brown fibrovascular bundles. The odor was slightly aromatic and slightly pungent taste.

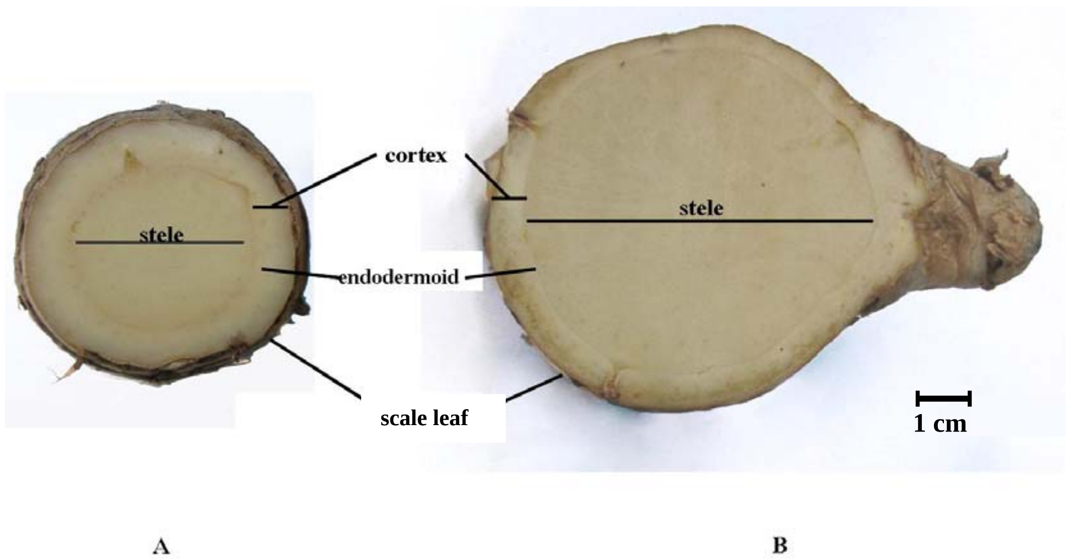


Figure 7 Internal appearance of rhizomes of Wan chak motluk : (A) *C. comosa*, (B) *Curcuma* sp.

2.2 Microscopic characters

The general microscopic characteristics of *C. comosa* and *Curcuma* sp. were quite similar as followed.

Anatomical and histological characteristics

Transverse sections of the rhizome showed of the following tissues passing from the periphery toward the center: (figure 8-10).

1. the scale leaf, composed of a layer of epidermis with scattered covering trichomes (about 1 mm long) and brownish parenchymatous cells of the mesophyll.

2. the periderm, composed of few layers of rectangular, thin-walled cork cells.

3. the cortex, composed of several layers of parenchymatous cells containing abundant starch granules and scattering orange oleoresin and a layer of the endodermoid at the innermost area.

4. the stele, a broad area of reserved parenchymatous cells; the fibrovascular bundles were scattered throughout the cortex and stele and occurred more numerously just beneath the endodermoid; slightly elongated lignified parenchymatous cells were often detected near the endodermoid.

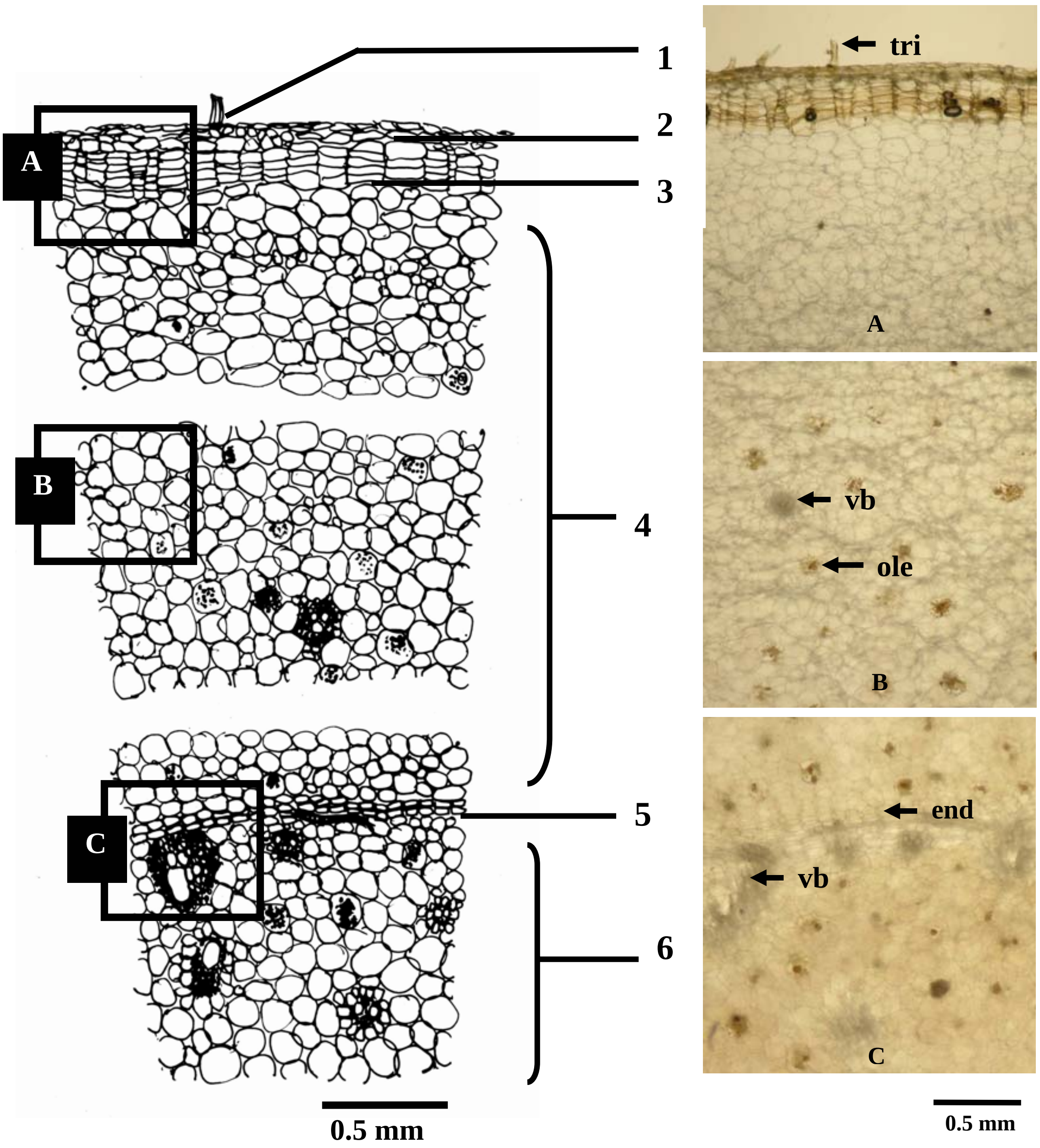


Figure 8 Transverse section of *Curcuma comosa* Roxb.

(A) sectional view of outer part of rhizome showing scale leaf with covering trichomes(tri), periderm and parenchymatous cells of cortex, vascular bundles (vb), (B) parenchymatous cells of cortex containing yellowish brown oil droplets and orange oleoresin (ole), (C) parenchymatous cells of stele showing endodermoid (end) and scattered vascular bundles.

1. covering trichomes, 2. scale leaf, 3. periderm, 4. cortex, 5. endodermoid, 6. stele.

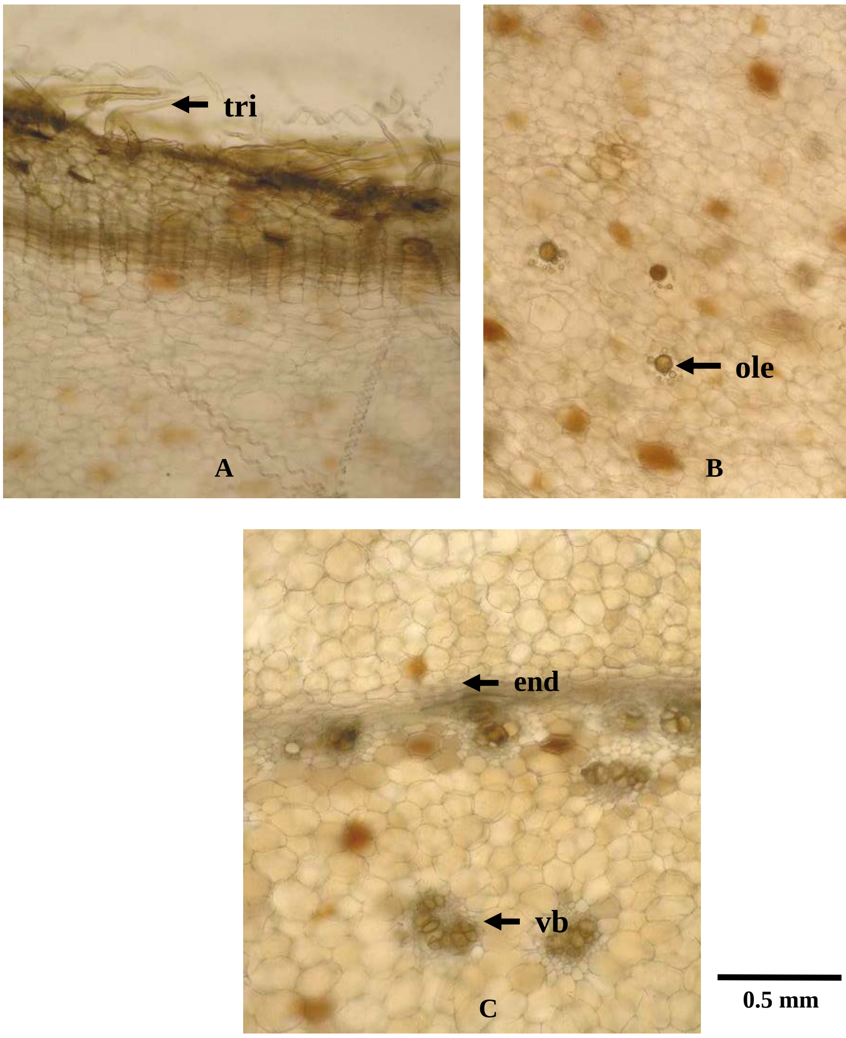


Figure 9 Anatomical feature of *Curcuma* sp.: (A) sectional view of outer part of rhizome showing scale leaf with covering trichomes (tri), periderm and parenchymatous cells of cortex, vascular bundles (vb), (B) parenchymatous cells of cortex containing yellowish brown oil droplets and orange oleoresin (ole), (C) parenchymatous cells of stele showing endodermoid (end) and scattered vascular bundles.

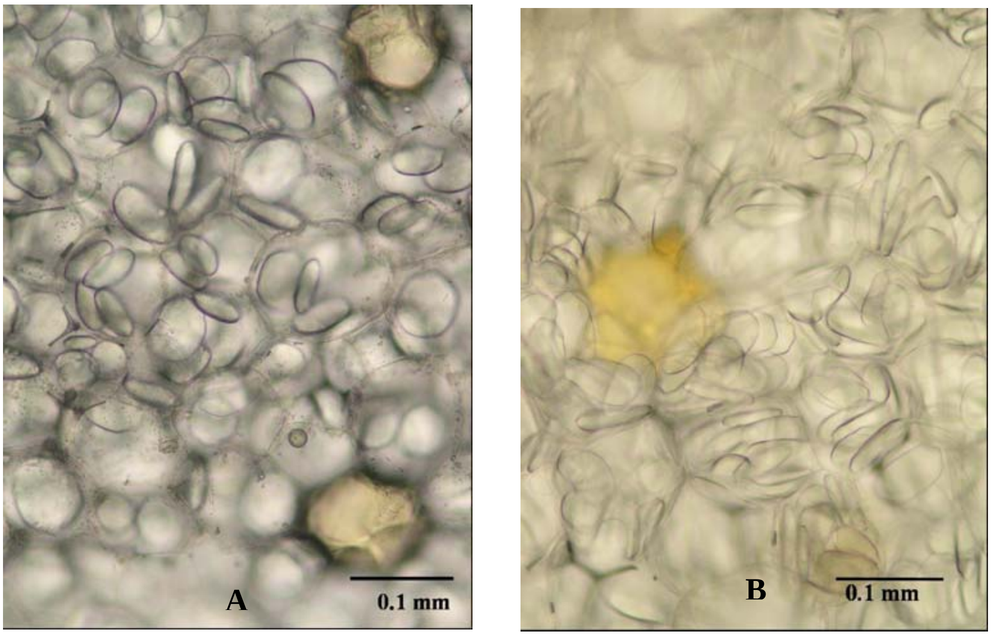


Figure 10 Starch granules of *Curcuma comosa* Roxb. and *Curcuma* sp.: (A) *Curcuma comosa*, (B) *Curcuma* sp.

Powdered drugs

The dried powdered drugs were pale brown to reddish-brown in color (figure 11). The odor of *C. comosa* group appeared sweet aromatic while *Curcuma* sp. was unpleasant aromatic. The taste of *Curcuma* sp. was more pungent and bitter.

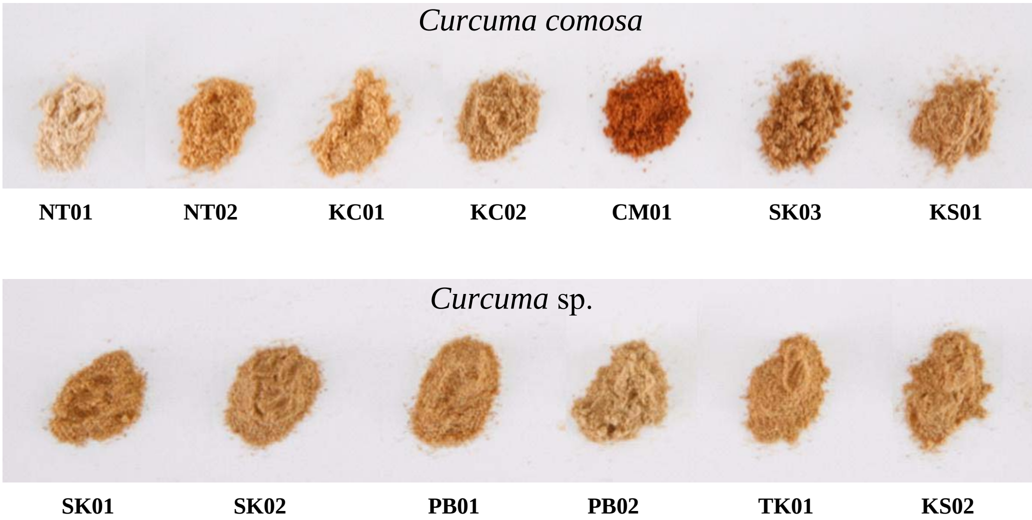


Figure 11 Powdered drugs of Wan chak motluk, *Curcuma comosa* Roxb. and *Curcuma* sp. from different locations.

The diagnostic characteristics of *C. comosa* (figure 12) and *Curcuma* sp. (figure 13) were abundant fragments of round to polygonal parenchymatous cells containing numerous starch granules, some yellowish brown oil droplets and orange oleoresin; fragments of annular, spiral, reticulate or scalariform vessels, commonly associated with rectangular, non-lignified parenchyma; fragments of cork cells in both surface and sectional views; abundant starch granules; some fragments from the scale leaf in surface view composed of epidermis with cicatrix and trichomes; occasional lignified parenchymatous cells. The abundant starch granules, found single, ranging from ovoid to subglobose with small hilum situated at the narrower end, 30-40 μ wide and 40-70 μ long.

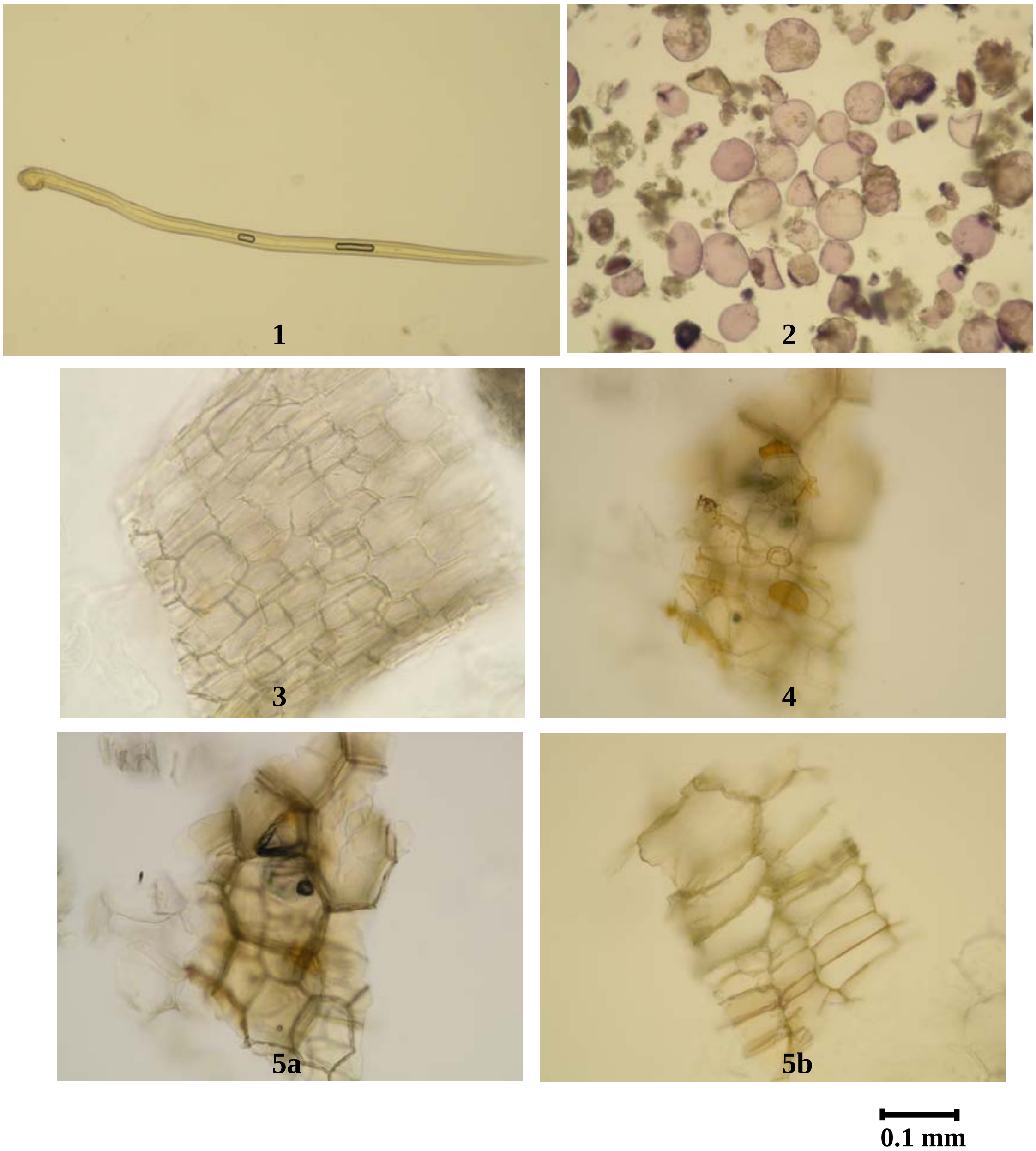


Figure 12 Microscopic characteristics of powdered drugs of *Curcuma comosa* Roxb.: 1. covering trichomes, 2. starch granules, 3. fragments of scale leaf in surface view, 4. epidermis of scale leaf in surface view with cicatrix, 5. cork (a) in surface view, (b) in sectional view.

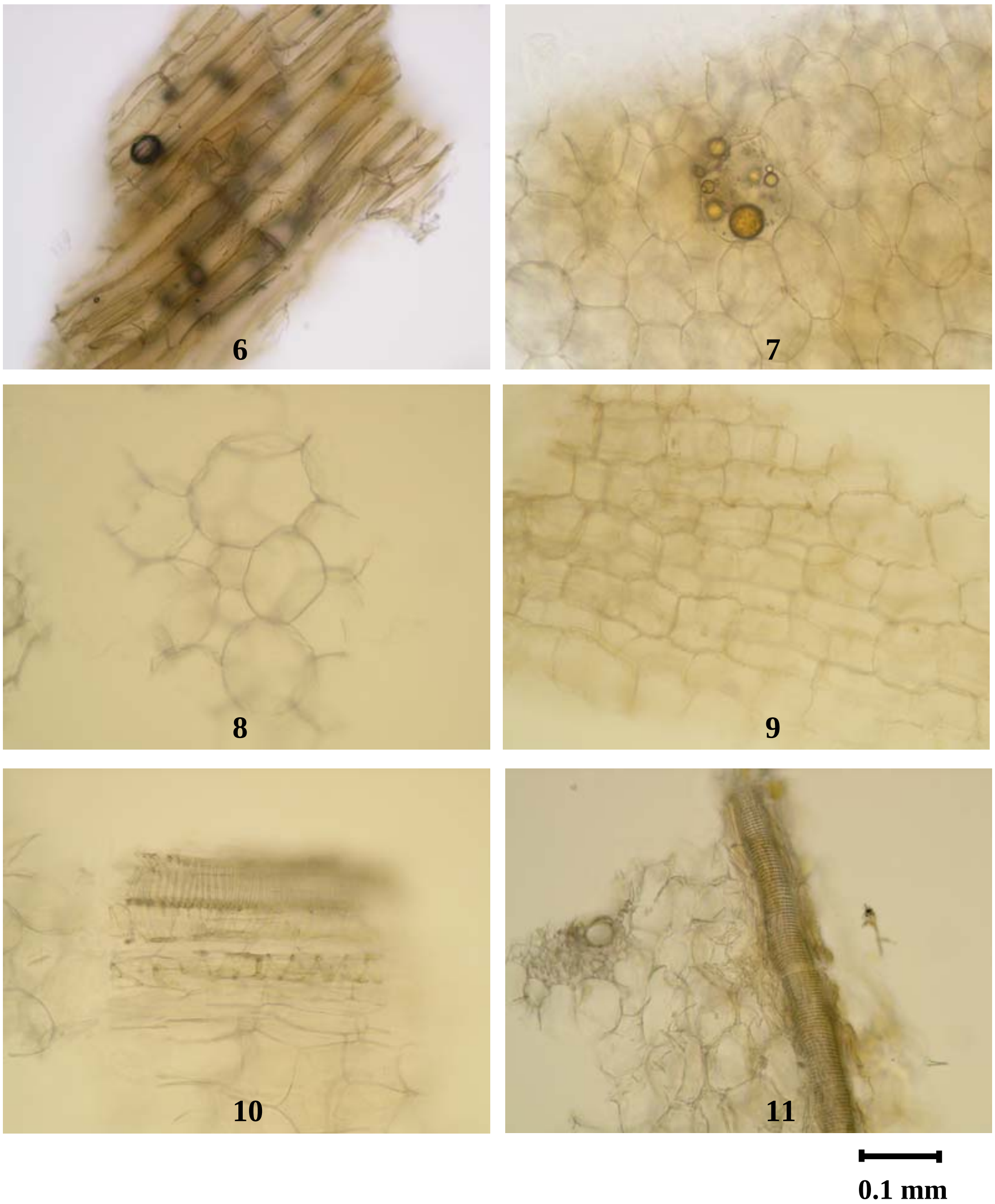
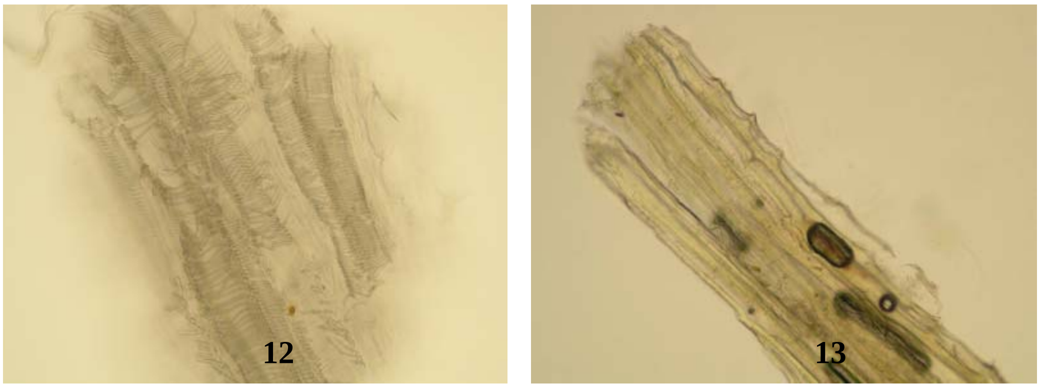


Figure 12 Microscopic characteristics of powdered drugs of *Curcuma comosa* Roxb.: 6. longitudinal parenchymatous cell from scale leaf in surface view,
7. parenchymatous cell containing brownish orange oils, 8. non-lignified parenchymatous cell, 9. rectangular parenchymatous cells, 10. group of vessels associated with parenchymatous cells, 11. fragment of reticulate vessel associated with parenchymatous cells and sectional view of vascular bundle. (continued)



0.1 mm

Figure 12 Microscopic characteristics of powdered drugs of *Curcuma comosa* Roxb.: 12. group of vessel, 13. fragment of lignified-parenchyma from outermost area of the stele in longitudinal view. (continued)

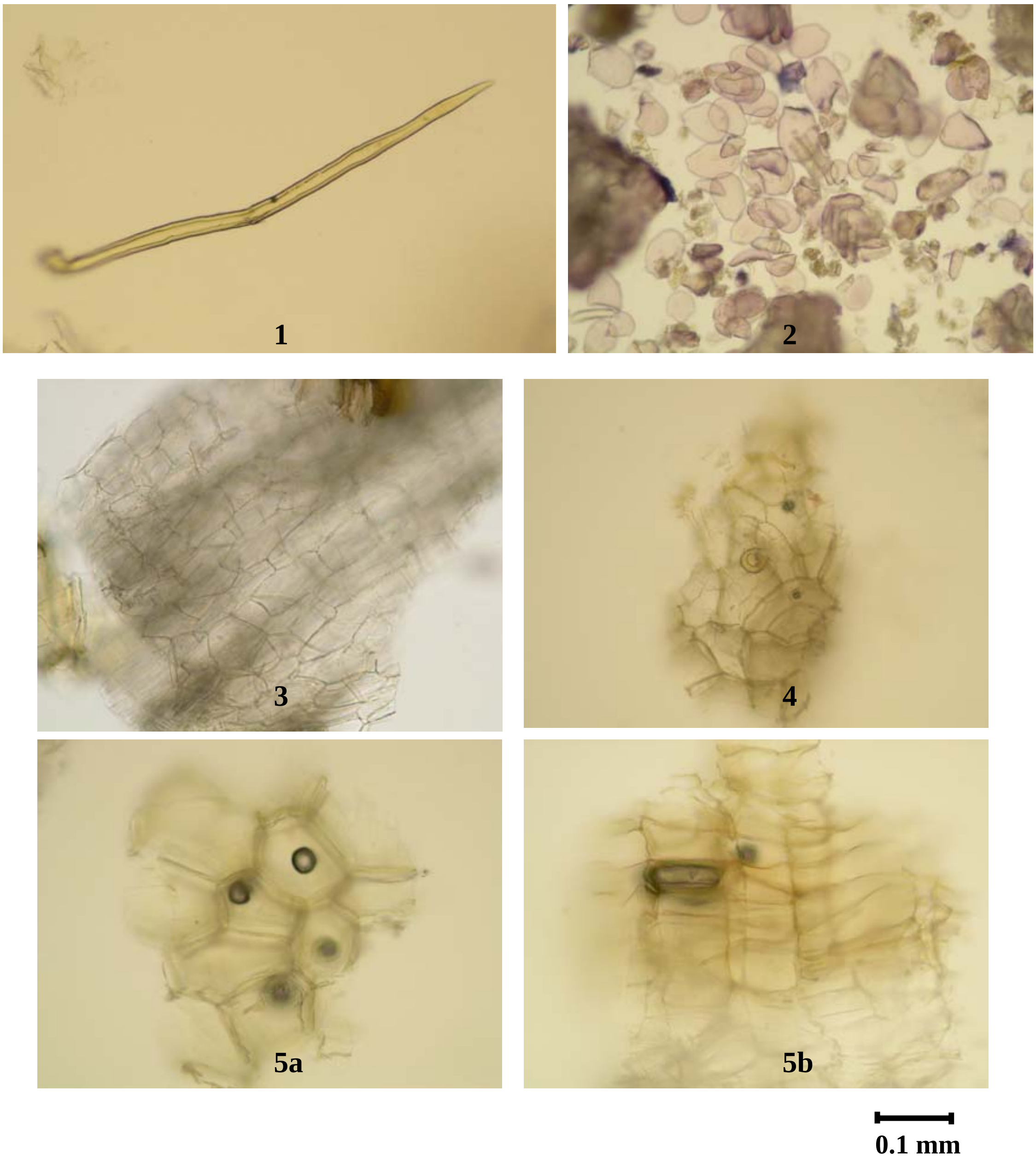


Figure 13 Microscopic characteristics of powdered drugs of *Curcuma* sp. :
1. covering trichomes, 2. starch granules, 3. fragments of scale leaf in surface view, 4. epidermis of scale leaf in surface view with cicatrix, 5. cork (a) in surface view, (b) in sectional view.

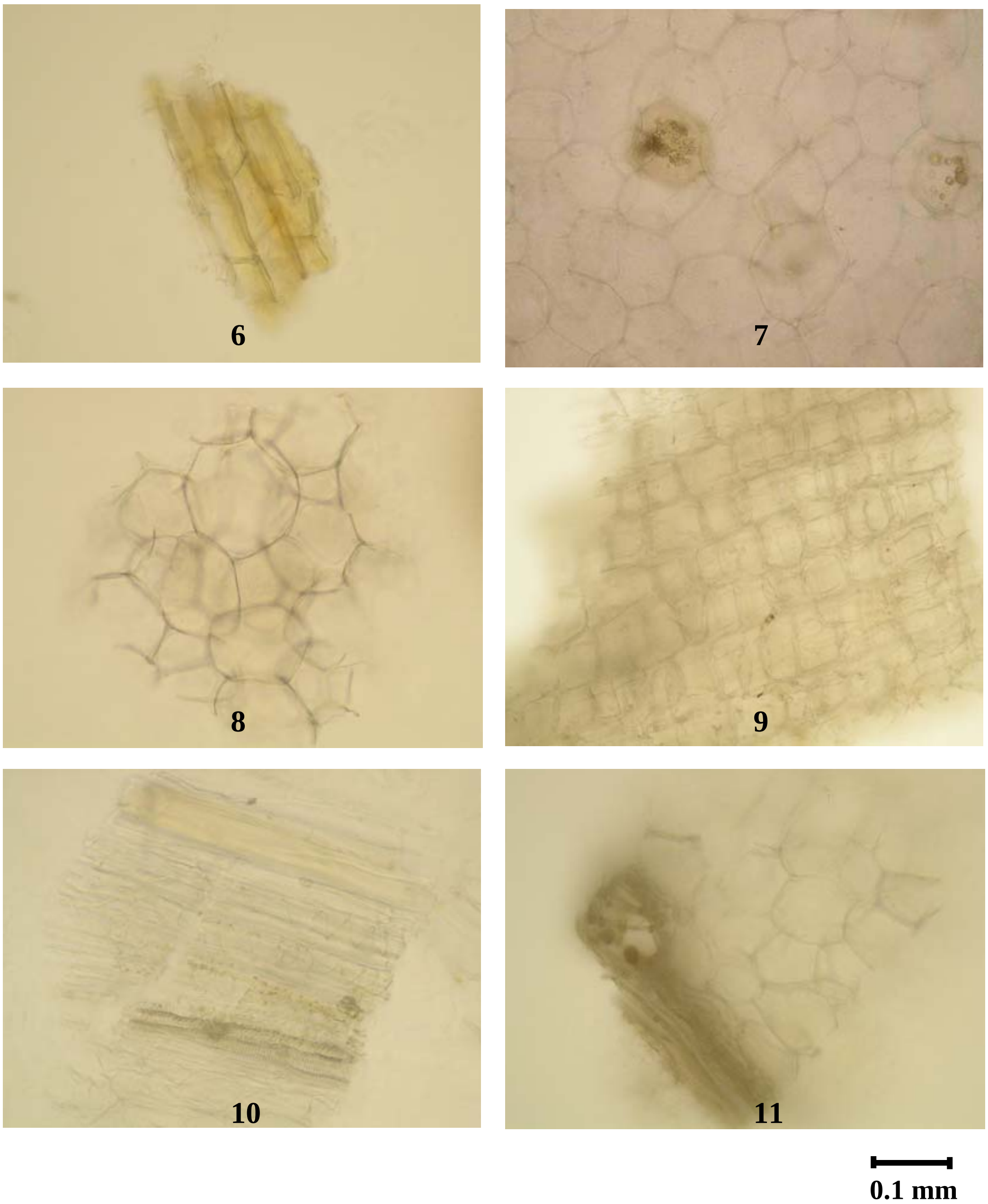


Figure 13 Microscopic characteristics of powdered drugs of *Curcuma* sp.:
6. longitudinal parenchymatous cell from scale leaf in surface view,
7. parenchymatous cell containing brownish orange oils, 8. non-lignified parenchymatous cell, 9. rectangular parenchymatous cells, 10. group of vessels associated with parenchymatous cells, 11. fragment of reticulate vessel associated with parenchymatous cells and sectional view of vascular bundle. (continued)

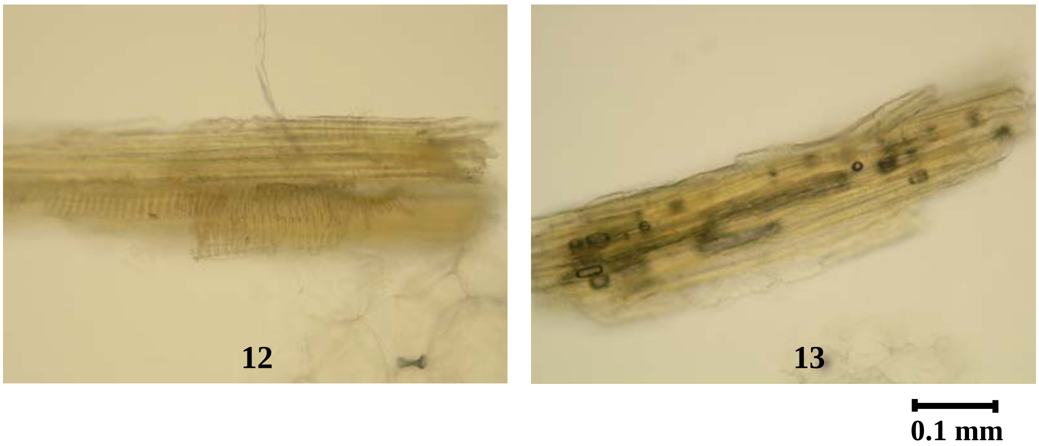


Figure 13 Microscopic characteristics of powdered drugs of *Curcuma* sp.:
12. group of vessel, 13. fragment of lignified-parenchyma from outermost area of the stele in longitudinal view. (continued)

2.3 Physicochemical properties

The physicochemical properties of Wan chak motluk were shown in table 2. The extractive values of the powdered drugs were evaluated by water, ethanol, dichloromethane and hexane. Water extractive values of *C. comosa* and *Curcuma* sp. ranged from 12.89-19.06 and 10.74-15.00 % (v/w), ethanol extractive values ranged from 6.16-22.12 and 2.71-15.03 % (v/w), dichloromethane extractive values ranged from 7.61-15.33 and 3.38-11.94 % (v/w) and hexane extractive values ranged from 5.69-14.84 and 1.51-7.58 % (v/w) respectively. The sample of *C. comosa* from Chiangmai (CM01) showed the highest values in all extractive.

They were also determined for physical properties as loss on drying, total ash and acid insoluble ash. *C. comosa* and *Curcuma* sp. showed ranging 4.62-10.62 and 6.13-10.00 % (w/w) for loss on drying, 5.68-10.86 and 3.70-8.50 % (w/w) for total ash, 0.73-2.91 and 0.61-1.82 % (w/w) for acid insoluble ash.

Pulverized powdered drug of rhizome of *C. comosa* coded number NT01, NT02 and KS01 contained 0.25 % (v/w) volatile oil while SK03 contained 0.46 % (v/w) volatile oil. However, volatile oil content of samples coded KC01, KC02 and CM01 could not be detected. The volatile oil contents of *Curcuma* sp. were higher, ranging from 1.4 to 2.0 % (v/w).

Table 2 Physicochemical properties of Wan chak motluk powder collected from different locations.

Plant species	Location	Chemical extractive value (%)				Loss on drying (%)	Total ash (%)	Acid insoluble ash (%)	Volatile oils (%)
		Water	EtOH	DCM	Hex				
C. comosa	NT01	17.95 ± 0.06	10.11 ± 0.48	7.61 ± 0.09	5.69 ± 0.07	10.27 ± 0.27	5.92 ± 0.11	1.86 ± 0.05	0.25
	NT02	15.28 ± 0.19	6.16 ± 0.16	9.57 ± 0.10	7.21 ± 0.18	7.01 ± 0.26	6.19 ± 0.05	1.64 ± 0.03	0.25
	KC01	15.58 ± 0.17	9.30 ± 0.13	13.79 ± 0.05	9.29 ± 0.34	8.12 ±0 .50	5.94 ± 0.01	1.68 ± 0.09	nd
	KC02	16.22 ± 0.35	9.89 ± 0.17	13.67 ± 0.03	9.43 ± 0.42	7.38 ± 0.24	7.98 ± 0.01	2.45 ± 0.24	nd
	CM01	19.06 ± 1.36	22.12 ± 0.37	15.33 ± 0.06	14.84 ± 0.27	10.62 ± 0.19	10.86 ± 0.92	2.91 ± 0.09	nd
	SK03	12.89 ± 0.41	7.89 ± 0.22	11.55 ± 0.17	8.37 ± 0.06	7.08 ± 0.16	5.68 ±0.10	0.73 ± 0.03	0.46
	KS01	14.13 ± 0.58	6.90 ± 0.35	9.61 ± 0.39	7.11 ± 0.13	4.62 ± 0.10	7.26 ± 0.17	1.82 ± 0.11	0.25
Curcuma sp.	PB01	15.00 ± 1.01	5.87 ± 0.20	6.88 ± 0.08	3.71 ± 0.24	9.46 ± 1.42	4.75 ± 0.01	1.27 ± 0.05	2.00
	PB02	11.36 ± 0.32	2.91 ± 0.03	4.19 ± 0.15	1.85 ± 0.07	7.48 ± 0.38	5.03 ± 0.21	1.17 ± 0.11	1.40
	SK01	12.04 ± 0.18	15.03 ± 0.17	11.94 ± 0.22	7.58 ± 0.64	8.80 ± 0.15	8.50 ± 0.01	1.54 ± 0.01	1.80
	SK02	10.74 ± 0.06	2.71 ± 0.01	3.38 ± 0.09	1.51 ± 0.01	6.13 ± 1.09	5.37 ± 0.01	1.27 ± 0.04	1.67
	TK01	14.57 ± 1.47	3.62 ± 0.07	6.00 ± 0.11	2.28 ± 0.10	8.95 ± 0.50	4.68 ± 0.05	0.77 ± 0.06	1.70
	KS01	13.35 ± 0.24	5.09 ± 0.10	3.53 ± 0.16	3.93 ± 0.23	10.00 ± 0.17	3.70 ± 0.01	0.61 ± 0.01	1.80

nd = not detectable

3. Chemical analysis

Pulverized rhizomes of Wan chak motluk were extracted with 80% alcohol on shaker for 48 h and repeated 3 times. The percentage of alcoholic extracts was shown in table 3.

Table 3 Percent ethanolic extract of Wan chak motluk rhizomes from different locations.

Plant species	Location	Alcoholic extract (%)	Range (%)
C. comosa	NT01	9.32	9.32 - 25.70
	NT02	11.38	
	KC01	14.49	
	KC02	14.12	
	CM01	25.70	
	KS01	13.53	
	SK03	15.83	
Curcuma sp.	PB01	11.20	8.13 – 28.21
	PB02	8.54	
	SK01	28.21	
	SK02	8.13	
	TK01	11.15	
	KS02	15.20	

Thin-layer chromatographic fingerprint

TLC chromatograms of 13 samples of alcoholic extract of Wan chak motluk were compared in 2 solvent systems (DCM : MeOH = 99:1 and DCM : EtOAc : MeOH = 93:4:7) with 3 detectors (254 nm, 366 nm UV light and AS/S) (figure 14-17). TLC patterns of Wan chak motluk were classified into 2 groups which were 7 samples of *C. comosa* group (NT01, NT02, KC01, KC02, CM01, SK03, KS01) and 6 samples of *Curcuma* sp. group (PB01, PB02, SK01, SK02, TK01, KS02). TLC chromatograms of *C. comosa* group in the solvent system of DCM : MeOH (99:1) contained 17 bands that revealed 8 quenching bands at 254 nm (band 1, 2, 3, 7, 10, 11, 14, 15, 17), 7 fluorescence bands at 366 nm (band 2, 4, 6, 9, 13, 14, 16) and also detected by AS/S spraying reagent as shown in table 4. They gave a major band (band 11) at the same hR_f value (50.00) with M1 under 254 nm and showed a deep green color while M1 gave slightly green color with AS/S spraying reagent. TLC patterns of *Curcuma* sp. group contained 11 bands that revealed 5 quenching bands at 254 nm (band 3, 4, 6, 8, 9), 4 fluorescence bands at 366 nm (band 1, 2, 6, 10) and also detected by AS/S spraying reagent (table 5). Three major bands were given under 254 nm (band 6, 8, 9). They also gave a band (band 8) as the same hR_f value (52.83) with M1 under 254, 366 nm but showed brown color with AS/S spraying reagent.

For M2, the solvent system DCM : EtOAc : MeOH = 93:4:7 was used. TLC chromatograms of *C. comosa* contained 13 bands that revealed 6 quenching bands at 254 nm (band 2, 4, 5, 6, 11, 12), 9 fluorescence bands at 366 nm (band 1, 2, 3, 5, 7, 8, 10, 12, 13) and also detected by AS/S spraying reagent (table 6). They gave intense band (band 6) at the same hR_f value (45.28) with M2 under 254 nm and showed deep violet color while M2 gave brown color with AS/S spraying reagent. The major band was exhibited in band 12. TLC patterns of *Curcuma* sp. contained 12 bands that revealed 6 quenching bands at 254 nm (band 2, 4, 5, 6, 11, 12), 5 fluorescence bands at 366 nm (band 2, 5, 6, 7, 11) and also detected by AS/S spraying reagent (table 7). They gave a band (band 6) the same hR_f value (45.28) with M2 under 254 and 366 nm. Two major bands were given in band 11 and 12. Besides, the TLC patterns of samples of *C. comosa* and *Curcuma* sp. showed consistency in each group.

Table 4 TLC chromatogram of *Curcuma comosa* Roxb. group in the solvent system of DCM : MeOH (99:1).

No.	hR_f value	UV (nm)		Anisaldehyde/sulfuric acid
		254	366	
1	3.70	-	-	+ (pink)
2	8.33	++	++ (blue)	+ (violet)
3	12.96	+	-	+ (violet)
4	16.67	-	+ (blue)	-
5	18.52	-	-	+ (orange)
6	22.22	-	+ (orange)	-
7	24.07	+	-	+ (pink)
8	31.48	-	-	+ (pink)
9	37.04	-	++ (blue)	-
10	40.74	+	-	-
11	**50.00**	**+++**	**-**	**++ (deep green)**
12	60.19	-	-	+ (pink)
13	62.96	-	+ (green)	-
14	68.52	+	++ (dark)	+ (brown)
15	74.07	++	-	+ (green)
16	77.78	-	+++ (blue)	-
17	79.63	++	-	+ (violet)

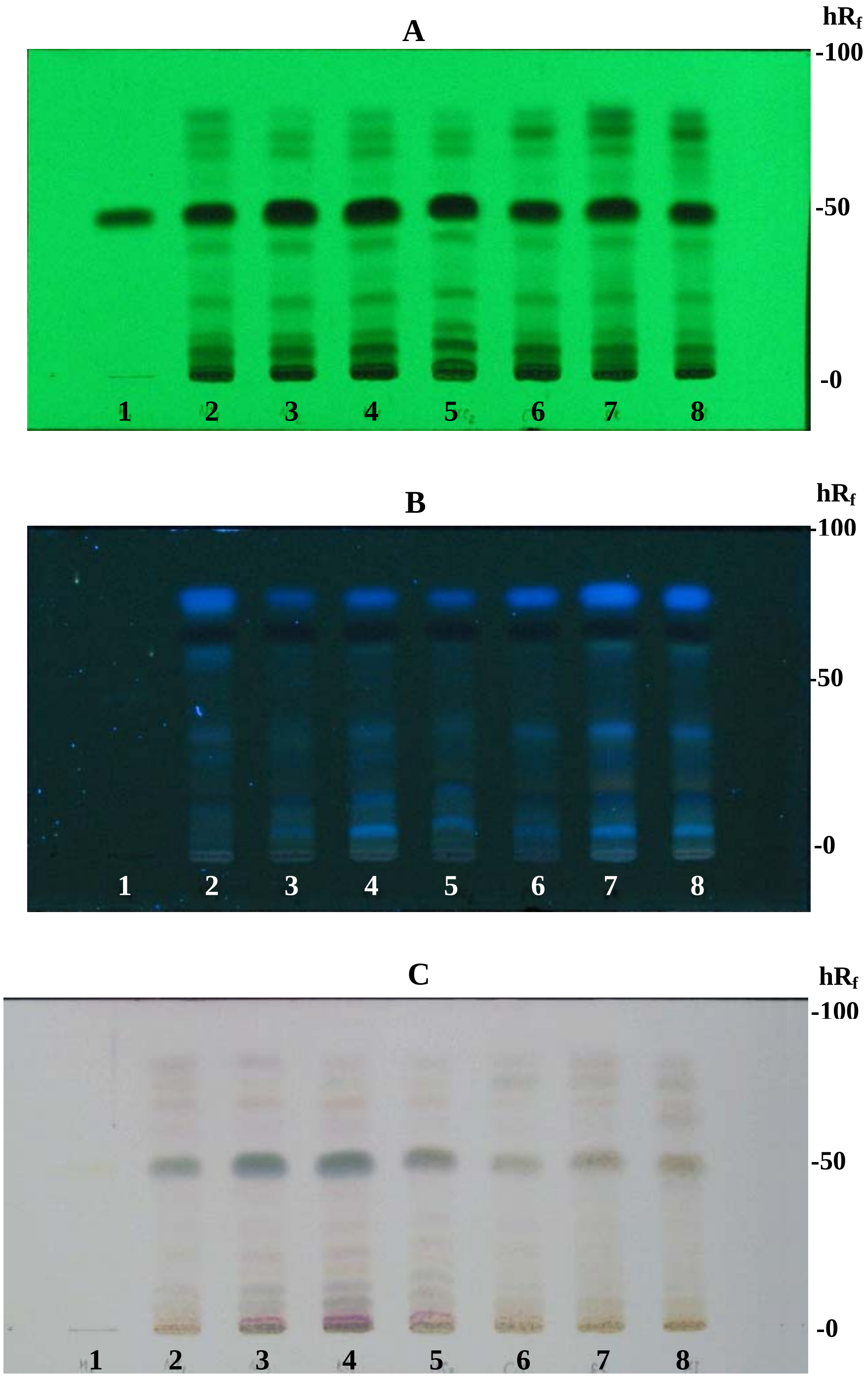


Figure 14 Thin-layer fingerprints of alcoholic extract of *Curcuma comosa* Roxb. in the solvent system of DCM : MeOH (99:1).

Absorbent : silica gel 60 GF_{254}

(A) 254 nm **(B)** 366 nm **(C)** Anisaldehyde/sulfuric acid

1 = M_1 2 = NT01 3 = NT02 4 = KC01

5 = KC02 6 = CM01 7 = SK03 8 = KS01

Table 5 TLC chromatogram of *Curcuma* sp. group in the solvent system of DCM : MeOH (99:1).

No.	hR_f value	UV (nm)		Anisaldehyde/sulfuric acid
		254 nm	366 nm	
1	4.72	-	++ (blue)	-
2	11.32	-	+++ (green)	-
3	16.04	+	-	++ (orange)
4	21.70	+	-	+ (pink)
5	33.96	-	-	+ (pink)
6	40.57	+	+ (green)	++ (orange)
7	47.17	-	-	++ (violet)
8	**52.83**	**++**	**-**	**+++ (brown)**
9	66.04	++	-	+++ (violet)
10	67.92	-	+ (blue)	-
11	77.36	-	-	+++ (orange)

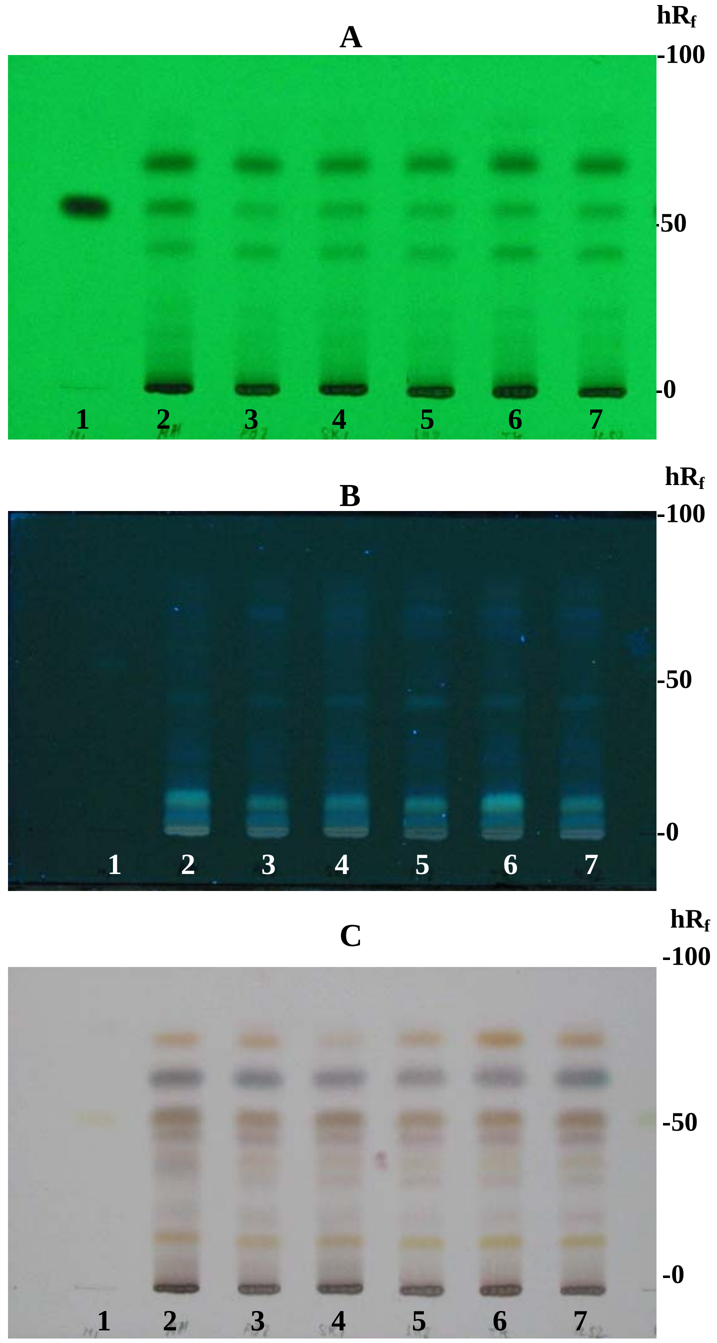


Figure 15 Thin-layer fingerprints of alcoholic extract of *Curcuma* sp. in the solvent system of DCM : MeOH (99:1).

Absorbent : silica gel 60 GF_{254}

(A) 254 nm **(B)** 366 nm **(C)** Anisaldehyde/sulfuric acid

1 = M_1 2 = PB01 3 = PB02 4 = SK01

5 = SK02 6 = TK01 7 = KS02

Table 6 TLC chromatogram of *Curcuma comosa* Roxb. group in the solvent system of DCM : EtOAc : MeOH (93:4:7).

No.	hR_f value	UV (nm)		Anisaldehyde/sulfuric acid
		254 nm	366 nm	
1	3.77	-	+ (green)	-
2	11.32	-	+ (green)	+ (orange)
3	22.64	++	+++ (green)	+++ (pink)
4	27.36	-	-	++ (brown)
5	39.62	-	+++ (blue)	-
6	**45.28**	**+++**	**-**	**++ (brown)**
7	50.94	-	+++ (blue)	-
8	56.60	+	+ (green)	+ (violet)
9	61.32	-	-	+ (pink)
10	66.98	+	-	+ (orange)
11	71.70	-	++ (blue)	-
12	77.36	+++	+ (orange)	+++ (deep green)
13	84.91	++	+++ (blue, dark)	++ (brown)

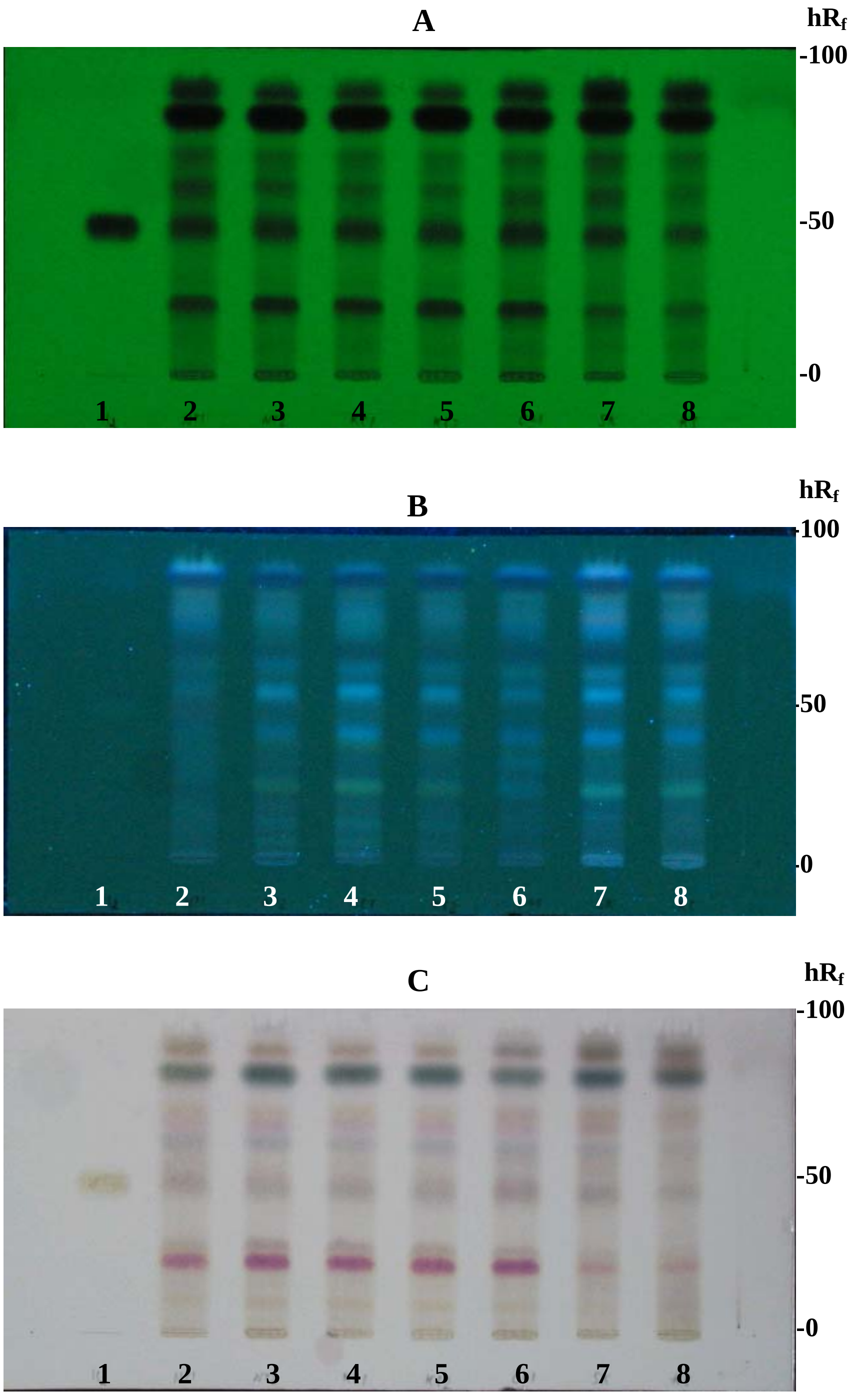


Figure 16 Thin-layer fingerprints of alcoholic extract of *Curcuma comosa* Roxb. in the solvent system of DCM : EtOAc : MeOH (93:4:7).

Absorbent : silica gel 60 GF_{254}

(A) 254 nm **(B)** 366 nm **(C)** Anisaldehyde/sulfuric acid

1 = M_2	2 = NT01	3 = NT02	4 = KC01
5 = KC02	6 = CM01	7 = SK03	8 = KS01

Table 7 TLC chromatogram of *Curcuma* sp. group in the solvent system of DCM : EtOAc : MeOH (93:4:7).

No.	hR_f value	UV (nm)		Anisaldehyde/sulfuric acid
		254 nm	366 nm	
1	7.55	-	-	+ (pink)
2	11.32	+	++ (green)	-
3	14.15	-	-	+ (violet)
4	16.98	+	-	+ (pink)
5	30.19	+	+ (blue)	-
6	**45.28**	**+**	**+++ (green)**	**-**
7	49.06	-	+++ (blue)	-
8	54.72	-	-	++ (orange)
9	66.04	-	-	++ (pink)
10	75.47	-	-	+++ (brown)
11	79.25	++	++ (blue)	++ (violet)
12	83.02	++	-	-

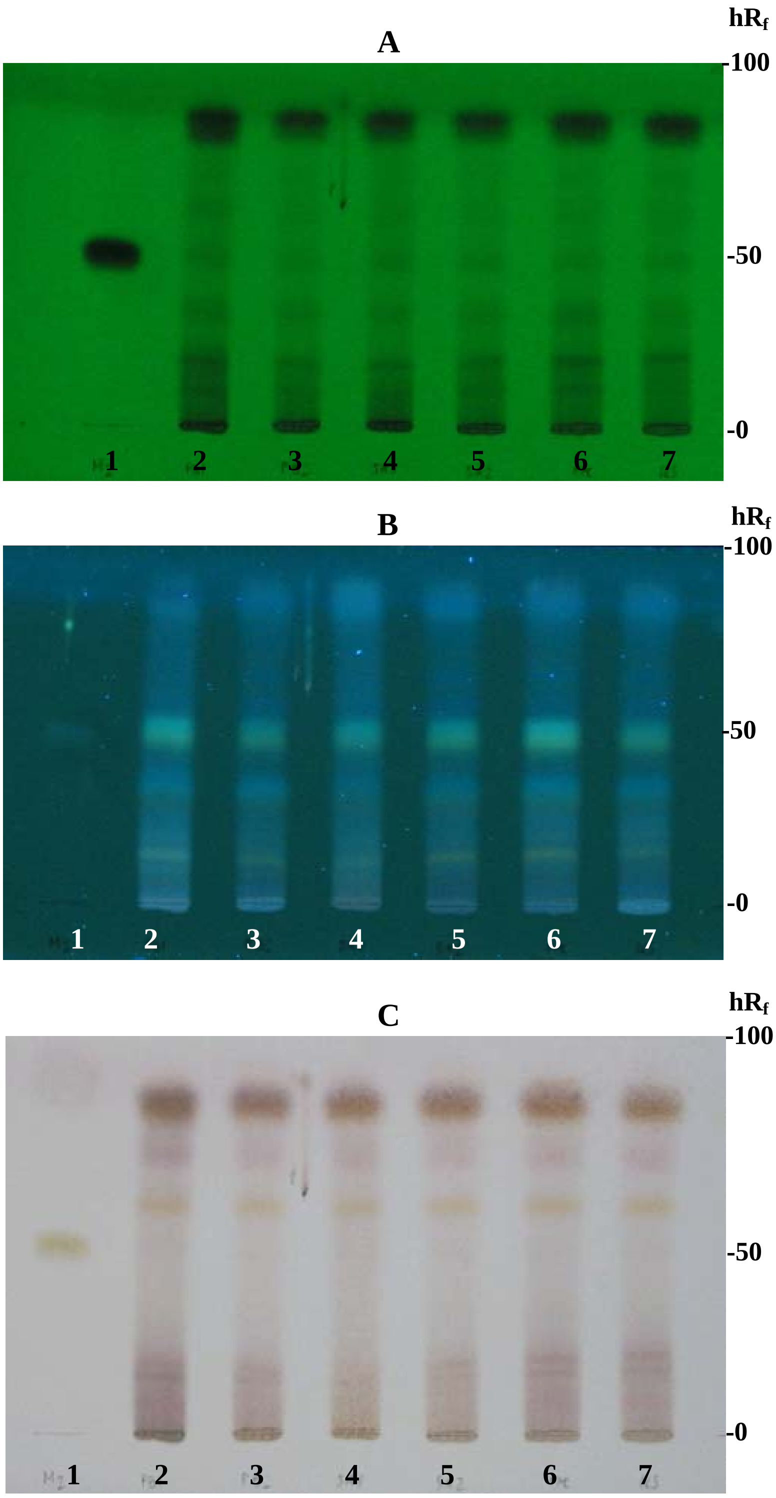


Figure 17 Thin-layer fingerprints of alcoholic extract of *Curcuma* sp. in the solvent system of DCM : EtOAc : MeOH (93:4:7).

Absorbent : silica gel 60 GF_{254}

(A) 254 nm **(B)** 366 nm **(C)** Anisaldehyde/sulfuric acid

1 = M_2	2 = PB01	3 = PB02	4 = SK01
5 = SK02	6 = TK01	7 = KS02	

4. Free radical scavenging activity

The result of free radical scavenging activity by DPPH assay of *C. comosa* and *Curcuma* sp. was shown in table 8 and figure 18. The alcoholic extracts of both *C. comosa* and *Curcuma* sp. showed moderately antioxidant activity compared with three reference standards. The ED_{50} of alcoholic extracts of *C. comosa* ranged from 8.93-25.86 µg/ml and those of *Curcuma* sp. ranged from 13.48-27.78 µg/ml more than vitamin C, BHA and BHT which showed ED_{50} 3.53, 4.64 and 11.75 µg/ml**,** respectively.

Table 8 Free radical scavenging activity of alcoholic extract of Wan chak motluk.

Plant species	Location	ED_{50} (µg/ml) ± S.E.	Range (µg/ml)
C. comosa	NT01	19.35 ± 0.14	8.93 – 25.86
	NT02	15.70 ± 1.21	
	KC01	20.07 ± 0.24	
	KC02	17.72 ± 1.17	
	CM01	8.93 ± 0.36	
	KS01	14.56 ± 1.35	
	SK03	25.86 ± 4.91	
Curcuma sp.	PB01	27.78 ± 0.38	13.48 – 27.78
	PB02	22.08 ± 0.56	
	SK01	18.63 ± 2.16	
	SK02	13.48 ± 1.34	
	TK01	17.90 ± 1.09	
	KS02	15.10 ± 0.05	
BHA	**4.64 µg/ml**		
BHT	**11.75 µg/ml**		
Vitamin C	**3.53 µg/ml**		

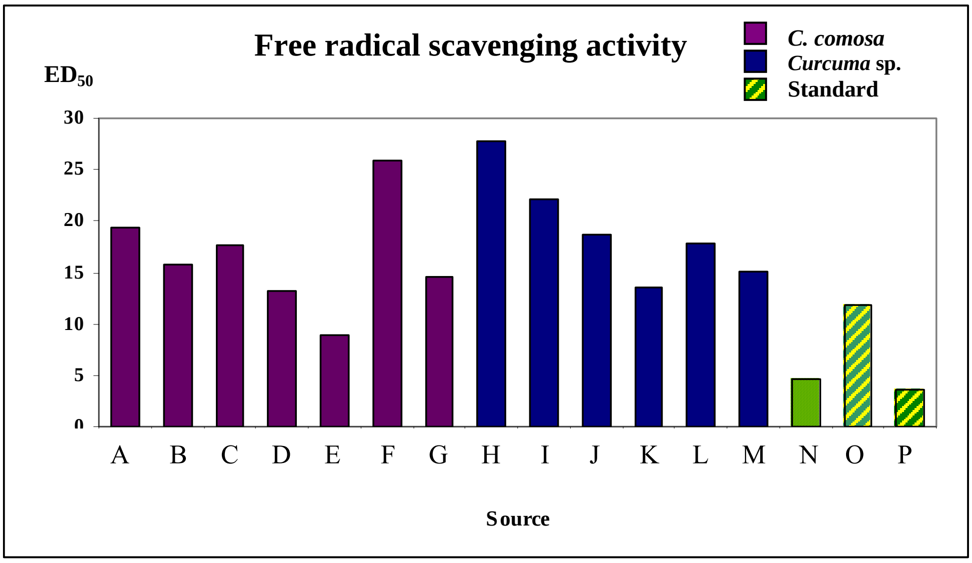


Figure 18 Free radical scavenging activity of *C. comosa* (A-G) and *Curcuma* sp. (H-M).

A = *C. comosa* from Kamphaeng Saen, Nakhon Pathom(NT01)
B = *C. comosa* from Kamphaeng Saen, Nakhon Pathom (NT02)
C = *C. comosa* from Thong Pha Phum, Kanchanaburi (KC01)
D = *C. comosa* from Sai Yok, Kanchanaburi (KC02)
E = *C. comosa* from Wiang Hae, Chiang Mai (CM01)
F = *C. comosa* from Sa wang Dan Din, Sakhon Nakhon (SK03)
G = *C. comosa* from Khao Wong, Kalasin (KS01)
H = *Curcuma* sp. from Khao Kho, Phetchabun (PB01)
I = *Curcuma* sp. from Khao Kho, Phetchabun (PB02)
J = *Curcuma* sp.from Sawang Dan Din, Sakhon Nakhon (SK01)
K = *Curcuma* sp. from Sawang Dan Din, Sakhon Nakhon (SK02)
L = *Curcuma* sp. from Phop Phra, Tak (TK01)
M = *Curcuma* sp. from Khao Wong, Kalasin (KS02)
N = Reference standard, BHA
O = Reference standard, BHT
P = Reference standard, vitamin C

5 Cytotoxicity

All alcoholic extracts of Wan chak motluk exhibited cytotoxicity to brine shrimp (22). LC_{50} values for Wan chak motluk are reported in table 9.

Among all Wan chak motluk samples, *C. comosa* group possessed cytotoxicity higher than those of *Curcuma* sp. group by LC_{50} values (0.84-39.03 and 151.43-273.03 µg/ml, respectively). Samples of *C. comosa* coded NT02, KC01 and KC02 exhibited LC_{50} values lower than 10 µg/ml, thus they possessed high toxicity.

Table 9 The cytotoxicity of alcoholic extract of Wan chak motluk on brine shrimps.

Plant species	Location	LC_{50} value in µg/ml	95% CI
	NT01	12.97	6.89-21.09
	NT02	<1.0	nd
	KC01	3.71	0.18-8.30
C. comosa	KC02	**0.84**	0.00-6.31
	CM01	30.94	20.34-46.36
	KS01	**39.03**	24.84-61.14
	SK03	11.56	6.06-17.94
	PB01	151.43	102.61-235.38
	PB02	**273.03**	185.64-418.37
Curcuma sp.	SK01	270.65	190.22-392.66
	SK02	189.94	129.83-297.39
	TK01	270.89	185.87-399.75
	KS02	172.01	108.73-274.47

The results are reported as LC_{50} value in µg/ml with the corresponding 95% confidence intervals (95%CI)

nd = not detectable

6. Uterotrophic activity

After immature ovariectomized female rats treated with alcoholic extracts of Wan chak motluk at the dose 500 mg/kg BW for 2 consecutive days, those uterine weight were evaluated. Figure 20 shows the effect of alcoholic extracts of *C. comosa* and *Curcuma* sp. on uterine wet weight. *C. comosa* was found to be effective in increasing uterine weight, while *Curcuma* sp. showed uterotrophic effect no significant differences when compared with ovariectomized rat control.

Among *C. comosa* from various sources, samples from Nakhon Pathom showed the most effectiveness.

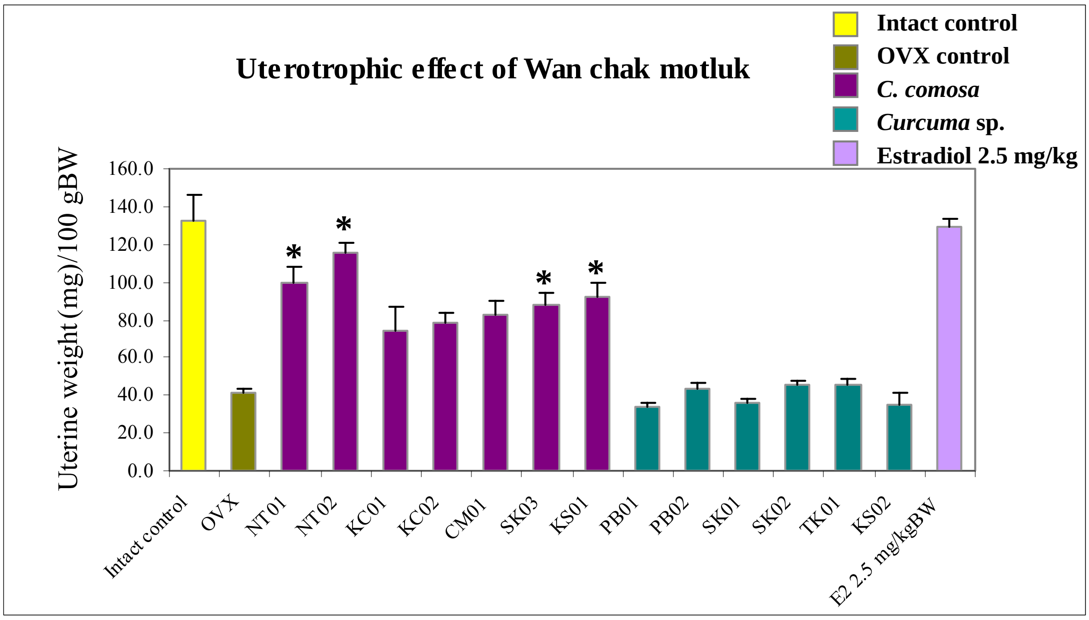


Figure 19 Uterotrophic activity of *C. comosa* and *Curcuma* sp. in immature ovariectomized rats (500 mg/kg BW day for 2 consecutive days)

CHAPTER V
DISCUSSION

Generally, *Curcuma* exhibits large morphological variations, especially in the group with flowers bloomed before leaves. They show similar patterns of morphology, particularly the rhizome which led to confusion and adulteration. The classification of *Curcuma* or even other, *Zingiber*, has traditionally been achieved based on morphological data. (30, 32). The present study demonstrated that the raw materials of Wan chak motluk in Thailand were taxonomically and chemically identified into 2 species, *C. comosa* and *Curcuma* sp. Thus *Curcuma* sp. rhizome could be adulterated to *C. comosa*.

The raw materials Wan chak motluk that were sold in the market might be whole rhizome without leaf, thin dried pieces or powder. The differentiation by the manufacturers was quite difficult. Besides, this study showed the similarity of the microscopic characteristics between *C. comosa* and *Curcuma* sp. which were also found among other *Zingiber* such as *Z. officinale* Rosc., *Z. montanum* (Koenig) Link ex Dietr. and *C. longa* L. (16, 68). Their anatomical and powdered drugs characteristics were quite similar including the shape and size of starch granules (72). Thus, it was more hardly differentiated when material was in form of powder.

TLC has been developed and found to be convenient and effective method for identification of many plants such as *Panax* spp. (73), *Cirsium* spp. (74), *Rhodiola* spp. (75) and *Pueraria* spp. (76). From this study, the conventional taxonomic techniques together with chemical identification by TLC may resolve the taxonomic confusion prevailing between these 2 species, *C. comosa* and *Curcuma* sp. Thus, this method was recommended to be rapidly preliminary tool to identify and distinguish different plant species under the trade name Wan chak motluk in the market. Furthermore, quantitative measurements for their quantity of compounds in samples possible by means of densitometry, and high performance liquid chromatography (HPLC) were suggested for further investigation (77).

Volatile oil contents of *C. comosa* and *Curcuma* sp. showed about 0.25 and 2 %(v/w), respectively. While *C. longa* and *Z. montanum* were previously reported about 6.0 and 13.0 % (v/w), respectively (16). However, the quality of *C. comosa* and *Curcuma* sp. oils should be investigated using TLC or gas chromatography-mass spectrometry (GC-MS) (78).

For their present biological activities, both *C. comosa* and *Curcuma* sp. presented moderate free radical scavenging activity of by DPPH compared to three reference standards, BHA, BHT and vitamin C. A low EC_{50} value indicates strong free radical scavenging activity in a sample. Thus, they both have potential antioxidant activity as well as found in other species of *Curcuma* (18-21, 51, 52). For cytotoxic investigation, they both showed moderate active to brine shrimp according to Meyer et al. (22). Thus, if *C. comosa* or *Curcuma* sp. will be later used as medicine, they could be investigated for acute and chronic toxicity. Besides, they might display capability to be antitumor agent (54).

In this investigation, ethanolic extract of *C. comosa* group also exhibited the uterotrophic activity which had been showed in previously investigations. Piyachaturawat et al. reported that the most effective uterotrophic activity were achieved in the first step of continuous extraction which is hexane extract (3, 4) and decreasing in butanol and ethyl acetate extract whereas the last step, the aqueous extract had no effect. Although the physicochemical properties of *C. comosa* were varied, all those alcoholic extract possessed acceptable uterotrophic activity.

According to all present, along with their previously investigations such as choleretic (5), lowering cholesterol activity (36, 37) and anti-inflammatory activity (7, 8), would be basic information for all further applications. These important findings would lead to the development of Wan chak motluk into different products for the health of the people, especially women. From all activities especially uterotrophic activity, *C. comosa* was approved and recommended for manufacturing and using in household remedy. However, *Curcuma* sp. showed no significant differences when compared with OVX control, it still exhibited free radical scavenging activity. More investigations should be explored to support commercial uses.

CHAPTER VI
CONCLUSION

1. Identification of Wan chak motluk, *C. comosa* and *Curcuma* sp.

Morphological characteristics

Thirteen raw material samples of Wan chak motluk which were cultivated and sold in Thailand were taxonomically identified into two species of genus *Curcuma*. They were *C. comosa* Roxb. and *Curcuma* sp.

Key to species

A Rhizome ovoid to subglobose; lateral rhizome short ≤2 cm; fertile bract white with pink at apex; coma bract pink or white with board pink band at center; ratio of upper part to lower part of corolla tube about 1.5:2; labellum not hairy beside purple lines on each side of thickening yellow band …..………….. *Curcuma comosa*

AA Rhizome ovoid to transversely globose; lateral rhizome long >2 cm; fertile bract green with pink at apex; coma bract pink with green at base; ratio of upper part to lower part corolla tube about 2:1.5; labellum hairy beside purple lines on each side of thickening yellow band .... *Curcuma* sp.

The microscopic characteristics of *C. comosa* and *Curcuma* sp. rhizomes were quite similar. Their anatomical characteristics composed of thin scale leaf with scattered covering trichomes attached to the epidermis, periderm, narrow cortex which separated from the broad zone of the stele by a layer of endodermoid. These areas composed of parenchymatous cells containing starch granules, oils and oleoresin cells with scattered fibrovascular bundles. The fibrovascular bundles and lignified, elongated parenchyma were clouded beneath the endodermoid.

Chemical characteristics

The samples of Wan chak motluk can be differentiated into *C. comosa* and *Curcuma* sp. by TLC chromatograms. Seven samples of *C. comosa* (NT01, NT02, KC01, KC02, CM01, SK03 and including KS01) showed different TLC patterns from other 6 samples of *Curcuma* sp. (PB01, PB02, SK01, SK02, TK01 and including KS02) under detection with 254, 366 nm and AS/S spraying reagent, using the suitable solvent systems, DCM : MeOH (99:1) and DCM : EtOAc : MeOH (93:4:7).

2. The specification of *C. comosa* and *Curcuma* sp.

Specification of *Curcuma comosa* Roxb.

Common name Wan chak motluk (Thailand)

Other name Wan thorahoed, Wan chak motluk tuamia (North east)

Family Zingiberaceae

Distribution Widely distributed and cultivated in the Central and North-eastern part of Thailand, and also found in India.

Description

Root nodulose. **Rhizome** ovoid to subglobose, 7-15 x 6-9 cm, pale yellow inside; lateral rhizome 1-2 cm long, not more than 1 cm diam. **Pseudostem** 1.0-1.2 m high. **Leaves** simple, alternate, with long petiole, 10-20 cm long, blade broad lanceolate, 30-40 x 10-12 cm, base attenuate, apex apiculate, margin entire, upper surface green with reddish purple band along midrib when young and disappear when mature, lower surface green, glabrous both sides. **Inflorescences** produced before the leaf, spike-like, cylindric, 20 x 8 cm, peduncle short, 2-5 cm long, radical, usually gradually enlarged to the apex; bract numerous, connate to each other at base, gradually increasing in length to the apex of spike; fertile bract obovate to ovate-elliptic, white with pink at apex, each one supported a cyme of several flowers; coma bract oblong-lanceolate, bright pink to reddish pink. Flowers 3.5-5.5 cm long, calyx cylindric, 6-8 mm long, apex minutely 3-toothed, white; corolla tube funnel-shaped, white, glabrous outside, with densely brown hairs inside about the middle part. corolla lobes 3, dorsal petal 1-1.5 x 1 cm, apex with minutely hood, 1 mm long, white with pink at apex, lateral petals ovate, 1.3 x 0.75 cm, apex obtuse, white; lateral staminodes 2, petaloid, ovate-elliptic to oblong, 1.1-1.3 x 0.6 cm, thickening along the middle, white with yellow apex; labellum broad ovate, 1.5-2.0 x 1.4-1.5 cm, apex bilobed and deflexed, white with bright yellow V shape at base; filament hairy below apex, anther oblong, 4.5 x 2.5 mm, with a pair of spur at base, 4 mm long, white, open by longitudinal slits; ovary short cylindric, 3.5 x 3 mm, base rounded, with light brown hairs on the upper half, 3 locules, ovules numerous; style filiform; stigma protrudes above the anther, obtriangular shape, lips ciliate; stylodial glands 2, linear, apex obtuse or acute, yellow.

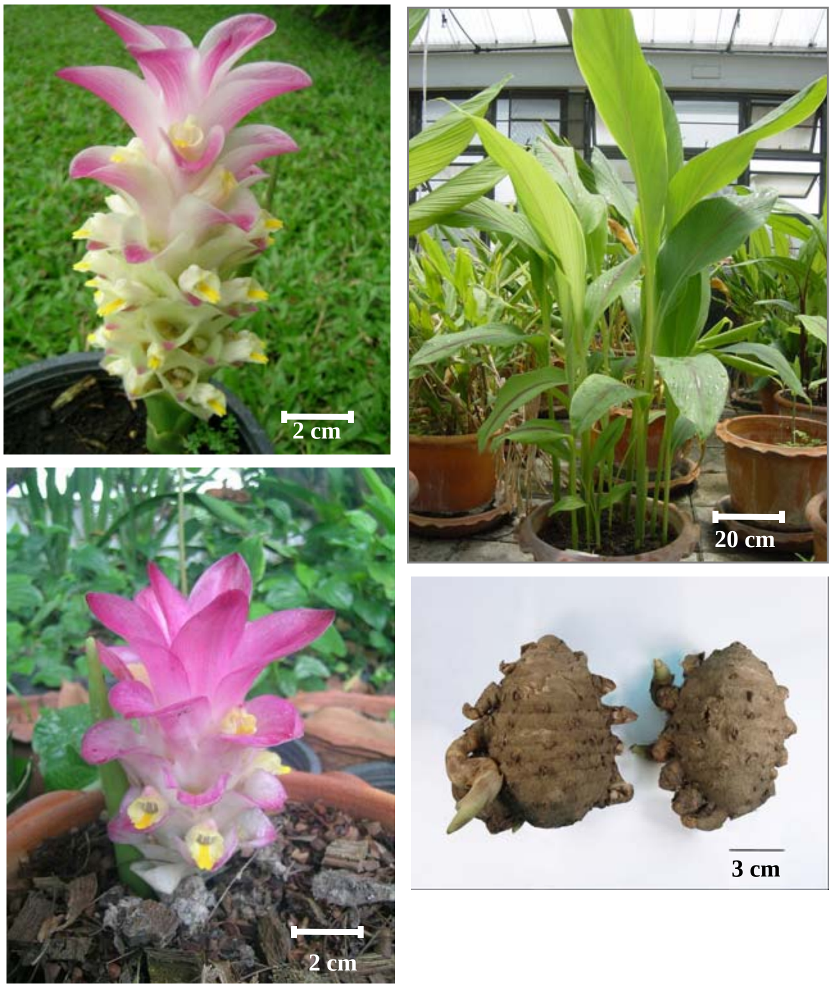


Figure 20 Morphological characteristics of *Curcuma comosa* Roxb.

Organoleptic properties

Color, light brown to dark brown; odor, sweet aromatic; taste, slightly bitter and pungent sensation.

Macroscopic characteristics

The fresh rhizome composed of main and lateral rhizome. The main rhizomes were varied from ovoid to subglobose (7-15 x 6-9 cm) with small (not more than 2 cm diam.), short lateral rhizome (1-1.5 cm long). The external surface was wrinkled, showing annulations of the scale leaf, pale brown to dark brown in color. Internal part was pale yellow to light brown showing a narrowed cortex (1-1.5 cm) which separated from broad central cylinder by a distinct endodermoid. After exposed to the air, the internal color became darker with scattered spots of reddish brown fibrovascular bundles.

Microscopic characteristics

Anatomical and histological

Transverse sections of the rhizome showed of the following tissues passing from the periphery toward the center:

1. The scale leaf, composed of a layer of epidermis with scattered covering trichomes (about 1 mm long) and brownish parenchymatous cells of the mesophyll.
2. The periderm, composed of few layers of rectangular, thin-walled cork cells.
3. The cortex, composed of several layers of parenchymatous cells containing abundant of starch granules and scattering orange oleoresin. The innermost area was a layer of tangentially elongated parenchyma of the endodermoid. Slightly elongated lignified parenchymatous cells were often detected near the endodermoid.
4. The stele, a broad area of reserved parenchymatous cells. The fibrovascular bundles were scattered throughout the cortex and stele and occurred more numerously just beneath the endodermoid.

Powdered drugs

A pale brown to reddish-brown in color with a pleasant aromatic odor, slightly bitter and pungent taste.

The diagnostic characteristics of *C. comosa* are :-

(a) The abundant *starch granules*, which are found scattered; they are simple, fairly large, flattened, oblong to oval in outline with small hilum situated at the narrower end; they occur clumped together in groups, 30-40 μ wide and 40-70 μ long.

(b) The abundant *parenchyma* composed of large, ovoid to elongated cells, thin-walled cells; the cells are filled with numerous starch granules, some contain globules of yellowish brown oil droplets and orange oleoresin.

(c) The fragments of *cork* occur in both surface and sectional view, the cells are thin-walled; polygonal in surface view and rectangular in sectional view.

(d) Few fragments of *vessels*, which are found singly or in small groups; the walls are slightly lignified and show annular or spiral thickening, they are generally found associated with ground, non-lignified parenchymatous cells.

(e) The very occasional fragments of yellowish-brown *scale*, which are usually found in surface view; they composed of epidermal layer with cicatrixs and underlying with elongated parenchymatous cells with parallel bands of vascular bundles.

Loss on drying : 4.62-10.62 % (w/w)

Total ash : 5.68-10.86 % (w/w)

Acid-insoluble ash : 0.73-2.91 % (w/w)

Water-soluble extractive : 12.89-19.06 % (v/w)

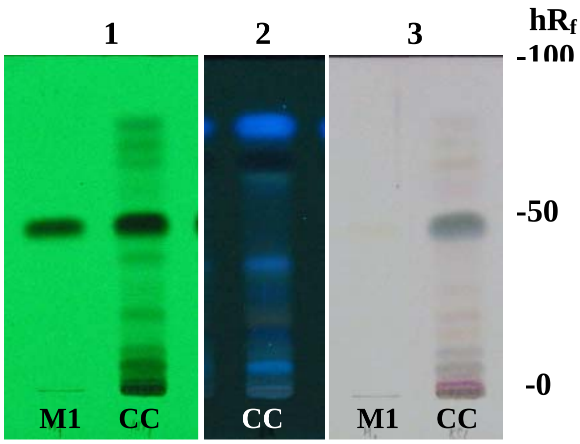
Alcohol-soluble extractive : 6.16-22.12 % (v/w)

Dichloromethane extractive : 7.61-15.33 % (v/w)

Hexane extractive : 5.69-14.84 % (v/w)

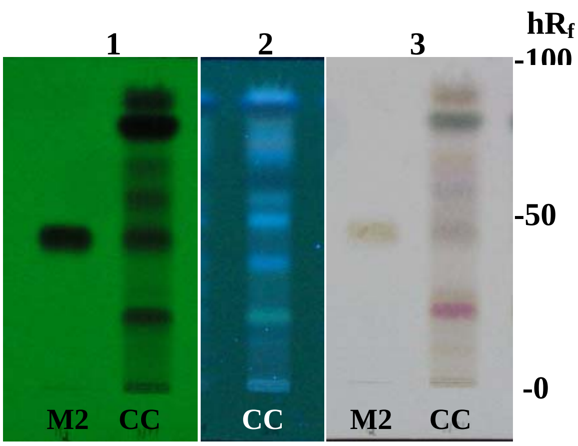
Volatile oil content: 0.25-0.46 % (v/w)

Chemical analysis by TLC method



Solvent system:

DCM : MeOH = 99:1



Solvent system:

DCM : EtOAc : MeOH = 93:4:7

Figure 21 Thin-layer fingerprints of alcoholic extract of *Curcuma comosa* Roxb. (CC)

Detectors : (1) 254 nm, (2) 366 nm, (3) Anisaldehyde/sulfuric acid

Absorbent : silica gel 60 GF_{254}

M1 : Reference standard, 1,7-diphenyl-5-hydroxy-(1*E*)-l-heptene

M2 : Reference standard, 5-hydroxy-7-(4-hydroxyphenyl)-l-phenyl-(1*E)*-l-heptene

Biological activity

Free radical scavenging activity : The alcoholic extract showed moderate antioxidant activity, $ED_{50} =$ 8.93-25.86 µg/ml compared with ED_{50} of the positive controls, BHA = 4.64 µg/ml, BHT=11.75 µg/ml and vitamin C = 3.53 µg/ml.

Cytotoxicity by brine shrimp lethality test : The alcoholic extract exhibited moderate to high toxic (LC_{50} = 0.84-39.03 μg/ml) to brine shrimp The berberine was used as a positive control.

Uterotrophic activity : The alcoholic extract showed effective result in increasing uterine weight in rat.

Specification of *Curcuma* sp.

Common name Wan chak motluk (Thailand)

Other name Wan chak motluk tuaphu (North east)

Family Zingiberaceae

Distribution Central, North-eastern and Northern part of Thailand, and India, Burma.

Description

Root nodulose. **Rhizome** ovoid to subglobose, 8-15 x 8-12 cm, pale yellow inside; lateral rhizome, 3-10 cm long, 2.5-4 cm diam. **Pseudostem** 1.2-2 m high. **Leaves** simple, alternate, with long petiole, 20-30 cm long, blade broad lanceolate, 40-50 x 15-20 cm, base attenuate, apex apiculate, margin entire, upper surface green, rarely appearing reddish purple band along midrib, lower surface green, glabrous both sides. **Inflorescences** produced before the leaf, spike-like, cylindric, peduncle long, 5-9 cm, usually gradually enlarged to the apex; bract numerous, connate to each other at base, gradually increasing in length to the apex of spike; fertile bract obovate to ovate-elliptic, green with pink at apex, each one supported a cyme of several flowers; coma bract oblong-lanceolate, purplish pink to reddish pink. Flowers 4-5 cm long, calyx cylindric, 7 mm long, apex minutely 3-toothed, white; corolla tube funnel-shaped, 3.5-4 cm long, white, glabrous outside, with densely brown hairs inside about the middle part; corolla lobes 3, dorsal petal ovate, 1.5 x 1.2 cm, apex with minutely hood, 1 mm long, white with pink at apex, lateral petals ovate, 1.3-1.5 x 1 cm, apex obtuse, white; lateral staminodes 2, petaloid, ovate-elliptic to oblong, 1.1-1.5 x 0.5-1 cm, thickening along the middle, white with yellow apex; labellum broad ovate, 1.5-2.5 x 1.3-2 cm, apex bilobed and deflexed, white with bright yellow V shape at base, purple line with hair beside yellow mid-band; filament hairy below apex, anther oblong, 4-4.5 x 2.3-2.7 mm, with a pair of spur at base, 4 mm long, white, open by longitudinal slits; ovary short cylindric, 4.5 x 3 mm, base rounded, with light brown hairs on the upper half, 3 locules, ovules numerous; style filiform; stigma protrudes above the anther, obtriangular shape, lips ciliate; stylodial glands 2, linear, apex obtuse or acute, yellow.

Figure 22 Morphological characteristics of *Curcuma* sp.

Organoleptic properties

Color, light brown to dark brown; odor, unpresant aromatic; taste, pungent and bitter sensation.

Macroscopic characteristics

The fresh rhizome composed of main and lateral rhizome. The main rhizomes were varied from ovoid to transversely globose (8-15 x 8-12 cm) with large and long lateral rhizome (2-3.5 cm diam. and 2-10 cm long). The external surface was wrinkled, showing annulations of the scale leaf, pale brown to dark brown in color. Internal part was pale yellow to light brown showing a narrowed cortex (1-1.5 cm) which separated from broad central cylinder by a distinct endodermoid. After exposed to the air, the internal color became darker with scattered, reddish brown fibrovascular bundles.

Microscopic characteristics

Anatomical and histological

Transverse sections of the rhizome showed of the following tissues passing from the periphery towards the center:

1. The scale leaf, composed of a layer of epidermis with scattered covering trichomes (about 1 mm long) and brownish parenchymatous cells of the mesophyll.
2. The periderm, composed of few layers of rectangular, thin-walled cork cells.
3. The cortex, composed of several layers of parenchymatous cells containing abundant of starch granules and scattering orange oleoresin. The innermost area was a layer of tangentially elongated parenchyma of the endodermoid. Slightly elongated lignified parenchymatous cells were often detected near the endodermoid.
4. The stele, a broad area of reserved parenchymatous cells. The fibrovascular bundles were scattered throughout the cortex and stele and occurred more numerously just beneath the endodermoid.

Powdered drugs

A pale brown to dark brown in color with unpleasant aromatic odor, bitter and pungent taste.

The diagnostic characteristics of *Curcuma* sp. are :-

(a) The abundant *starch granules*, which are found scattered; they are simple, fairly large, flattened, oblong to oval in outline with small hilum situated at the narrower end; they occur clumped together in groups, 30-40 μ wide and 40-70 μ long.

(b) The abundant *parenchyma* composed of large, ovoid to elongated cells, thin-walled cells; the cells are filled with numerous starch granules, some contain globules of yellowish brown oil droplets and orange oleoresin.

(c) The fragments of *cork* occur in both surface and sectional view, the cells are thin-walled; polygonal in surface view and rectangular in sectional view.

(d) Few fragments of *vessels*, which are found singly or in small groups; the walls are slightly lignified and show annular or spiral thickening, they are generally found associated with ground, non-lignified parenchymatous cells.

(e) The very occasional fragments of yellowish-brown *scale*, which are usually found in surface view; they composed of epidermal layer with cicatrixs and underlying with elongated parenchymatous cells with parallel bands of vascular bundles.

Loss on drying : 6.13-10.00 % (w/w)

Total ash : 3.70- 8.50 % (w/w)

Acid-insoluble ash : 0.61- 1.54 % (w/w)

Water-soluble extractive : 10.74-15.00 % (v/w)

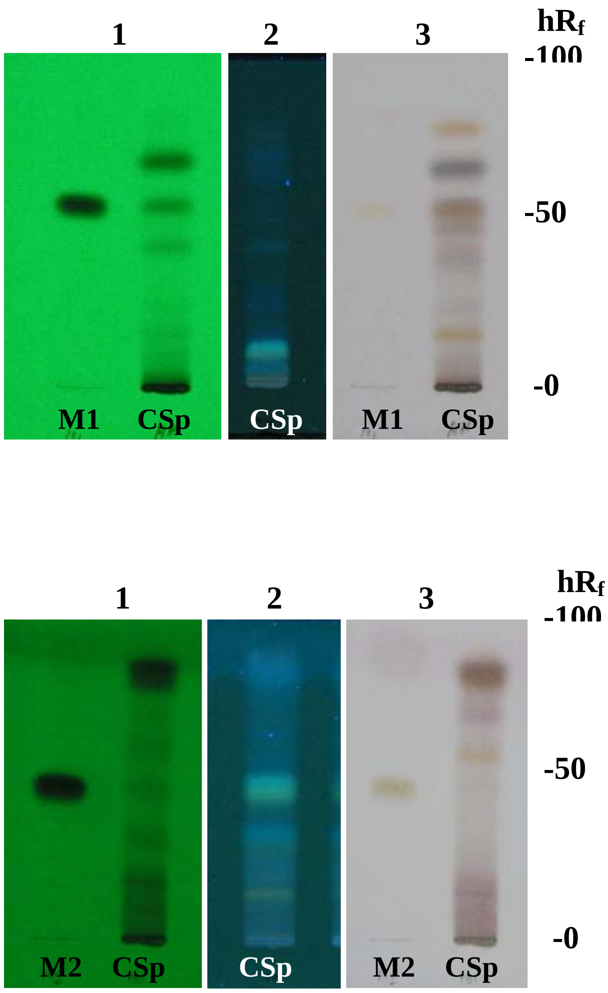
Alcohol-soluble extractive : 2.71-15.03 % (v/w)

Dichloromethane extractive : 3.38-11.94 % (v/w)

Hexane extractive : 1.51-7.58 % (v/w)

Volatile oil content: 1.4-2.0 % (v/w)

Chemical analysis by TLC method



Solvent system:

DCM : MeOH = 99:1

Solvent system:

DCM : EtOAc : MeOH

= 93:4:7

Figure 23 Thin-layer fingerprints of alcoholic extract of *Curcuma* sp. (CSp)

Detectors : (1) 254 nm, (2) 366 nm, (3) Anisaldehyde/sulfuric acid

Absorbent : silica gel 60 GF_{254}

M1 : Reference standard, 1,7-diphenyl-5-hydroxy-(1*E*)-l-heptene

M2 : Reference standard, 5-hydroxy-7-(4-hydroxyphenyl)-l-phenyl-(1*E*)-l-heptene

Biological activity

Free radical scavenging activity : The alcoholic extract showed moderately antioxidant activity, ED_{50} = 13.48-27.78 µg/ml compared with ED_{50} of the positive controls, BHA = 4.64 µg/ml, BHT=11.75 µg/ml and vitamin C = 3.53 µg/ml.

Cytotoxicity by brine shrimp lethality test : The alcoholic extract exhibited moderate to high toxic (LC_{50} = 151.43-273.03 µg/ml) to brine shrimp. The berberine was used as a positive control.

Uterotrophic activity : The alcoholic extract showed uterotrophic effect not significantly different from ovariectomized control.

3. Biological activities of *C. comosa* and *Curcuma* sp.

The alcoholic extracts of *C. comosa* and *Curcuma* sp. showed moderate antioxidant activity. *C. comosa* showed free radical scavenging activity by DPPH higher than those of *Curcuma* sp., ED_{50} values were 8.93-25.86 and 13.48-27.78 µg/ml, respectively. The extracts exhibited cytotoxicity to brine shrimp, *C. comosa* showed moderate to high toxic and higher than those of *Curcuma* sp. (LC_{50} = 0.84-39.03 and 151.43-273.03 µg/ml, respectively), The alcoholic extract of *C. comosa* was found to be effective in increasing uterine weight, while *Curcuma* sp. showed effect not significantly different from ovariectomized control.

REFERENCES

1. สมาคมโรงเรียนแพทย์แผนโบราณ สำนักวัดพระเชตุพนวิมลมังคลาราม. ประมวลสรรพคุณยาไทย (ภาคสาม) ว่าด้วยพฤกษชาติวัตถุธาตุและสัตว์วัตถุนานาชนิด. กรุงเทพมหานคร: ไพศิลป์การพิมพ์; 2520.
2. ส่วนพฤกษศาตร์ป่าไม้ กรมป่าไม้. ชื่อพรรณไม้แห่งประเทศไทย (เต็ม สมิตินันทน์ ฉบับแก้ไขเพิ่มเติม พ.ศ. 2544). พิมพ์ครั้งที่ 2. กรุงเทพมหานคร: บริษัท ประชาชน; 2544.
3. Piyachaturawat P, Ercharuporn S, Suksamrarn A. Uterotrophic effect of *Curcuma comosa* in rats. International Journal of Pharmacognosy 1995;33(4):334-8.
4. Piyachaturawat P, Ercharuporn S, Suksamrarn A. Estrogenic activity of *Curcuma comosa* extract in rats. Asia Pacific Journal of Pharmacology 1995;10:121-6.
5. Piyachaturawat P, Gansar R, Suksamrarn A. Choleretic effect of *Curcuma comosa* rhizome extracts in rats. International Journal of Pharmacognosy 1996;34(3):174-8.
6. อนุกูล สวัสดิ์พาณิชย์. ผลของสารสกัดด้วยเอธานอลจากว่านชักมดลูกต่อการหดตัวของกล้ามเนื้อมดลูกหนูขาวทั้งภายในและที่แยกออกจากร่างกาย [วิทยานิพนธ์ปริญญาวิทยาศาสตร์มหาบัณฑิต สหสาขาวิชาเภสัชวิทยา]. กรุงเทพมหานคร: บัณฑิตวิทยาลัยจุฬาลงกรณ์มหาวิทยาลัย; 2537.
7. Jantaratnotai N, Utaisincharoen P, Piyachaturawat P, Chongthammakun S, Sanvarinda Y. Inhibitory effect of *Curcuma comosa* on NO production and cytokine expression in LPS-activated microglia. Life Science 2006;78(6):571-7.

8. Sodsai A, Piyachaturawat P, Sophasan S, Suksamrarn A, Vongsakul M. Suppression by *Curcuma comosa* Roxb. of pro-inflammatory cytokine secretion in phorbol-12-myristate-13-acetate stimulated human mononuclear cells. International Immunopharmacology 2007;7(4):524-31.
9. Piyachaturawat P, Timinkul A, Chuncharunee A, Suksamrarn A. growth suppressing effect of *Curcuma comosa* extract on male reproductive organs in immature rats. Pharmaceutical Biology 1998;36(1):44-9.
10. Piyachaturawat P, Timinkul A, Chuncharunee A, Suksamrarn A. Effect of *Curcuma comosa* extract on male fertility in rats. Pharmaceutical Biology 1999;37(1):22-7.
11. Jurgens TM, Frazier EG, Schaeffer JM, Jones TE, Zink DL, Borris RP, et al. Novel nematocidal agents from *Curcuma comosa*. Journal of natural products 1994;57(2):230-5.
12. Suksamrarn A, Eiamong S, Piyachaturawat P, Byrne LT. A phloracetophenone glucoside with choleretic activity from *Curcuma comosa*. Phytochemistry 1997;45(1):103-5.
13. World Health Organization. WHO monographs on selected medicinal plants. Geneva: WHO; 1999.
14. Busse W. The significance of quality for efficacy and safety of herbal medicinal products. Drug Information Journal 2000;34:15-23.
15. Pascual ME, Carretero ME, Slowing KV, Villar A. Simplified screening by TLC of plant drugs. Pharmaceutical Biology 2002;40(2):139-43.
16. Thai Pharmacopoeia Committee. Thai herbal pharmacopoeia Vol.1. Bangkok: Prachachon; 1995.
17. Thai Pharmacopoeia Committee. Thai herbal pharmacopoeia Vol.2. Bangkok: Prachachon; 2000.
18. Kalpana C, Sudheer AR, Rajasekharan KN, Menon VP. Comparative effects of curcumin and its synthetic analogue on tissue lipid peroxidation and antioxidant status during nicotine-induced toxicity. Singapore medical journal 2007;48(2):124-30.

19. Sohn JH, Han KL, Lee SH, Hwang JK. Protective effects of panduratin A against oxidative damage of tert-butylhydroperoxide in human HepG2 cells. Biological & pharmaceutical bulletin 2005;28(6):1083-6.

20. Kuo P-C, Damu AG, Cherng C-Y, Jeng J-F, Teng C-M, Lee E-J, et al. Isolation of a natural antioxidant, dehydrozingerone from *Zingiber officinale* and synthesis of its analogues for recognition of effective antioxidant and antityrosinase agents. Archives of Pharmacal Research 2005;28(5):518-8.

21. Lim CS, Jin D, Mok H, Oh SJ, Lee JU, Hwang JK, et al. Antioxidant and antiinflammatory activities of xanthorrhizol in hippocampal neurons and primary cultured microglia. Journal of neuroscience research 2005;82(6):831-8.

22. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Medica 1982;45(1):31-4.

23. Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). Planta Medica 1993;59(3):250-2.

24. Yamasaki K, Hashimoto A, Kokusenya Y, Miyamoto T, Sato T. Electrochemical method for estimating the antioxidative effects of methanol extracts of crude drugs. Chemical & pharmaceutical bulletin 1994;42(8):1663-5.

25. วุฒิ วุฒิธรรมเวช. คัมภีร์เภสัชรัตนโกสินทร์. กรุงเทพมหานคร: ศิลป์สยามบรรจุภัณฑ์และการพิมพ์; 2547.

26. สุนทรี สิงหบุตรา. สรรพคุณสมุนไพร 200 ชนิด. กรุงเทพมหานคร: โอ.เอส.พริ้นติ้งเฮ้าส์; 2535.

27. Roxburgh W. Flora indica: or, Descriptions of Indian plants. New Delhi: Today & Tomorrow's Printers & Publishers; 1971.

28. Backer JG. Scitaminee. In : Hooker JD, ed. Flora of British India. Ashford,: L. Reeve; 1894.

29. Backer CA, Brink RCBVD. Flora of Java: spermatophytes only. Netherlands: N.V.P Noordhoff; 1968.
30. Sasikumar B. Genetic resources of *Curcuma*: diversity, characterization and utilization. Plant Genetic. Resources: C&U 2005;3:230–51.
31. Larsen K, Larsen SS. Gingers of Thailand. Chiangmai: Queen Sirikit Botanic Garden; 2006.
32. Apavatjrut P, Anuntalabhochai S, Sirirugsa P, Alisid C. Molecular markers in the identification of some early flowering *Curcuma* L. (Zingiberaceae) species. Annals of Botany 1999;84(4):529-34.
31. สมพร (ภูติยานันต์) หิรัญรามเดช, เจมส์ แฟรงคลิน แมกซ์เวล. เอกสารวิจัยการสำรวจและเก็บตัวอย่างพันธุ์ไม้สมุนไพรบริเวณดอยสุเทพ-ปุย. เชียงใหม่: มหาวิทยาลัยเชียงใหม่; 2534.
34. Uehara S, Yasuda I, Akiyama K, Morita H, Takeya K, Itokawa H. Diarylheptanoids from the rhizomes of *Curcuma xanthorrhiza* and *Alpinia officinarum*. Chemical & pharmaceutical bulletin 1987;35(8):3298-304.
35. Kuroyanagi M, Noro T, Fudushima S, Aiyama R, Ikuta A, Itokawa H, et al. Study on the constituents of *Alpinia katsumadai* Hayata. Chemical & pharmaceutical bulletin 1983;31:1544-50.
36. Piyachaturawat P, Teeratagolpisal N, Toskulkao C, Suksamrarn A. Hypolipidemic effect of *Curcuma comosa* in mice. Artery 1995;22(5):233-41.
37. Piyachaturawat P, Charoenpiboonsin J, Toskulkao C, Suksamrarn A. Reduction of plasma cholesterol by *Curcuma comosa* extract in hypercholesterolaemic hamsters. Journal of Ethnopharmacology 1999;66(2):199-204.
38. อนิรุตร์ สงวนสุข. ศุภเดช กิตติมั่นคง และเอกนันท์ กีรติศรัณย์. ฤทธิ์ต้านจุลชีพของสารสกัดจากสมุนไพรว่านชักมดลูกและผลมะคำดีควาย [ปริญญานิพนธ์เภสัชศาสตร์บัณฑิต สาขาเทคโนโลยีการผลิตยา]. กรุงเทพมหานคร: คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย; 2547.

39. Piyachaturawat P, Tubtim C, Chuncharunee A, Komaratat P, Suksamrarn A. Evaluation of the acute and subacute toxicity of a choleretic phloracetophenone in experimental animals. Toxicol Letters 2002;129(1-2):123-32.
40. Chivapat S, Hirunsaree A, Junsuwanitch N, Padungpat S, Rangsripipat A, Niumsakul S, et al. Subchronic toxicity of Wan chak motluk (*Curcuma comosa* Roxb.) extract. Proceedings of the 3rd symposium of the family Zingiberaceae; Khon Kaen: Thailand; 2003.
41. Jegede IA, O.F. K, Ibrahim JA, Ugbabe G, J.I. O. Pharmacognostic investigation of leaves of *Mitracarpus vilosus* (S.W.) D.C. African journal of biotechnology 2005;4(9):957-9.
42. Musa KY, Katsayal AU, Ahmed A, Mohammed Z, Danmalan UH. Pharmacognostic investigation of the leaves of *Gisekia pharnadioides*. African journal of biotechnology 2005;5(10):956-57.
43. Rivera-Arce E, Gattuso M, Lozoya X. Anatomical identity parameters of the crude drug *Psidii guajavae folium*. Pharmaceutical Biology 2003;41(7):516-21.
44. Applequist WL. Rhizome and root anatomy of potential contaminants of *Actaea racemosa* L. (black cohosh). Flora 2003;198:358-65.
45. Khatoon S, Rai V, Rawat AKS, Mehrotra S. Comparative pharmacognostic studies of three *Phyllanthus* species. Journal of Ethnopharmacology 2006;104:79-86.
46. Chaudhri RD. Herbal drugs industry: a practical approach to industrial pharmacognosy. New Delhi: Eastern; 1996.
47. Evans WC, editor. Trease and Evans pharmacognosy. Edinburgh: W.B. Saunders; 2002.
48. Fried B. Thin-layer chromatography. New York: Marcel Dekker; 1999.
49. Liang Y-Z, Xie P, Chan K. Quality control of herbal medicines. Journal of Chromatography B Biomedically Relevant Plant Components: Active Principles and Toxicants 2004;812(1-2):53-70.
50. Prakash A. Antioxidant activity. Medallion Laboratories 2001;19(2):1-4.

51. Mau JL, Lai EYC, Wang NP, Chen CC, Chang CH, Chyau CC. Composition and antioxidant activity of the essential oil from *Curcuma zedoaria.* Food Chemistry 2003;82:583-91.
52. Abas F, Lajis N, Shaari K, Israf D, Stanslas J, Yusuf U, et al. A labdane diterpene glucoside from the rhizomes of *Curcuma mangga*. Journal of natural products 2005;68(7):1090-3.
53. Molyneux P. The use of the stable free radical diphenylpicryhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin Journal of Science and Technology 2004;26(2):211-9.
54. Sam TW. Bioactive natrual products : detection, isolation, and structural determination. In: Colegate SM, Molyneux RJ, editors. Boca Raton: CRC Press; 1993.
55. Pelka M, Danzl C, Distler W, Petschelt A. A new screening test for toxicity testing of dental materials. Journal of Dentistry 2000;28:341-5.
56. Michael AS, Thompson CG, Abramovitz M. *Artemia salina* as a test organism for a bioassay. Science 1956;123(3194):464.
57. Vanhaecke P, Persoone G, Claus C, Sorgeloos P. Proposal for a short-term toxicity test with *Artemia nauplii*. Ecotoxicologyl and Environmental Safety 1981;5:382-7.
58. Mclaughlin jL, Rogers LL, Anderson JE. The use of biological assays to evaluate botanicals. Drug Information Journal 1998;32:513-24.
59. Harwing J, Scott P. Brine shrimp (*Artemia nauplii* L.) larvae as a screening system for fungal toxins. Applied Microbiology 1971;21:1011-6.
60. Martinez M, Del Ramo J, Torreblanca A, Diaz-Mayans J. Effect of cadmium exposure on zinc levels in the brine shrimp *Artemia parthenogenetica*. Aquaculture 1999;172(3-4):315-25.
61. Wisely B, Blick R. Mortality of marine invertebrate larvae in mercury, copper, and zinc solutions. Australian Journal of Marine and Freshwater Research 1967;18(1):63-72.
62. Lieberman M. A brine shrimp bioassay for measuring toxicity and remediation of chemicals. Journal of Chemical Education 1999;76(12):1689.

63. Barahona MV, Sanchez-Fortun S. Toxicity of carbamates to the brine shrimp *Artemia salina* and the effect of atropine, BW284c51, iso-OMPA and 2-PAM on carbaryl toxicity. Environmental Pollution 1999;104(3):469-76.
64. Hartl M, Humpf H-U. Toxicity assessment of fumonisins using the brine shrimp (*Artemia salina*) bioassay. Food and Chemical Toxicology 2000;38(12):1097-102.
65. Curtis RF, Coxon DT, Levett G. Toxicity of fatty acids in assays for mycotoxins using the brine shrimp (*Artemia salina*). Food and Cosmetics Toxicology 1974;12(2):233-5.
66. Prior MG. Evaluation of Brine Shrimp (*Artemia salina*) larvae as a bioassay for mycotoxins in animal feedstuffs. Canadian Journal of Comparative Medicine 1979;43(4):352-5.
67. Simon SA, Parmentier JL, Bennett PB. Anesthetic antagonism of the effects of high hydrostatic pressure on locomotory activity of the brine shrimp *Artemia.* Comparative Biochemistry and Physiology Part A: Physiology 1983;75(2):193-9.
68. Specification of Thai medicinal plants. Bangkok: Faculty of Pharmacy, Mahidol University, 1986.
69. Department of Medical Sciences, Ministry of Public Health. Thai pharmacopoeia. Bangkok: Department of Medical Sciences;1987.
70. The Stationery Office. British pharmacopoeia. London: The Stationary Office; 2005.
71. Finney DJ. Probit Analysis. Cambridge: Cambridge University Press.; 1971.
72. Wallis T. Textbook of pharmacognosy. London: Churchill; 1967.
73. Xie P, Chen S, Liang YZ, Wang X, Tian R, Upton R. Chromatographic fingerprint analysis—a rational approach for quality assessment of traditional Chinese herbal medicine. Journal of Chromatography A 2006;1112:117-80.
74. Ganzera M, Pocher A, Stuppner H. Differentiation of *Cirsium japonicum* and *C. setosum* by TLC and HPLC-MS. Phytochemical analysis 2005;16(3):205-9.

75. Yousef GG, Grace MH, Cheng DM, Belolipov IV, Raskin I, Lila MA. Comparative phytochemical characterization of three Rhodiola species. Phytochemistry 2006;67(21):2380-2391.

76. Chen SB, Liu HP, Tian RT, Yang DJ, Chen SL, Xu HX, et al. High-performance thin-layer chromatographic fingerprints of isoflavonoids for distinguishing between Radix Puerariae Lobate and Radix Puerariae Thomsonii. Journal of Chromatography A 2006;1121:114-9.

77. Suntornsuk L. High performance liquid chromatography of phytochemical substances. Thai Journal of Phytopharmacy 2000;7(1):1-12.

78. Singh G, Singh OP, Maurya S. Chemical and biocidal investigations on essential oils of some Indian Curcuma species. Progress in Crystal Growth and Characterization of Materials 2002;45(1):75-81.

APPENDIX

APPENDIX

Reagents for microscopical analysis

1. Chloral (solution of the hydrate) (Europ. Ph. I)

A 80.0 g chloral hydrate is dissolved in 20 ml water with heating.

Bleaching agent: The samples are treated with a few drops of the solution and are boiled briefly over a pilot flame.

2. Iodine solution

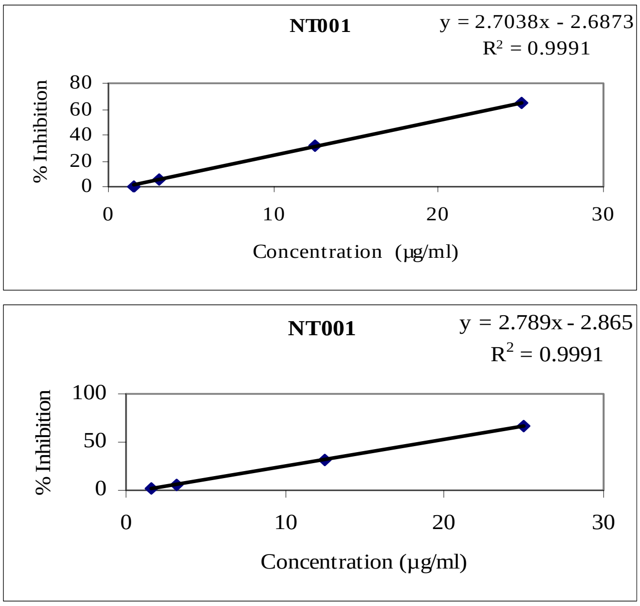
For the identification of starch, the usual 0.1N iodine solution. The aqueous sample is treated with a small amount of iodine solution from the side, and the discoloration of the cell elements is observed. Starch particles become dark-blue to blue-violet (iodine-inclusion compound); reversible on heating. To prevent drying, 1-2 drops glycerol are added after staining.

Reagent for chromatography and other chemical analysis

1. Anisaldehyde-sulfuric acid

For various natural materials, especially terpene derivatives. 0.5 ml anisaldehyde is mixed with 10 ml glacial acetic acid, 85 ml methanol, and 5 ml conc. sulfuric acid in the indicated sequence. After 10 ml of this mixture is sprayed on the plate, the plate is heated to 100-110°C for 10 min. Detection takes place in daylight.

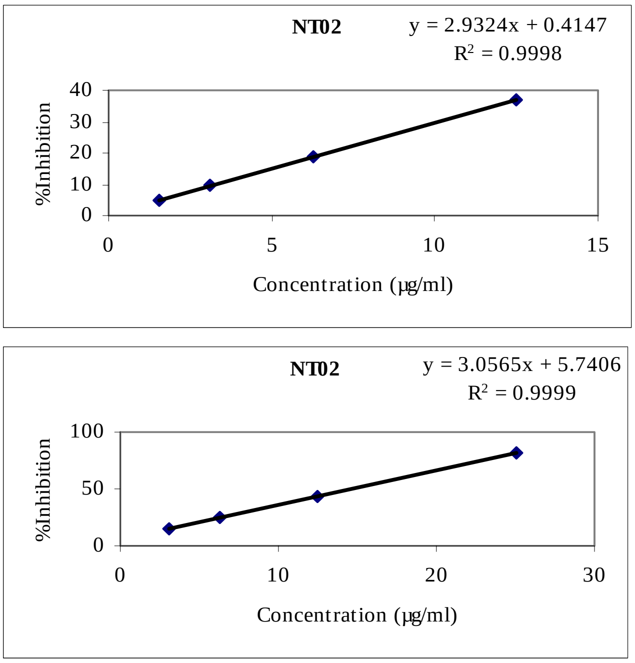
Calibration curve of antioxidant activity using DPPH of Wan chak motluk



ED_{50}= 19.49 μg/ml

ED_{50}= 18.95 μg/ml

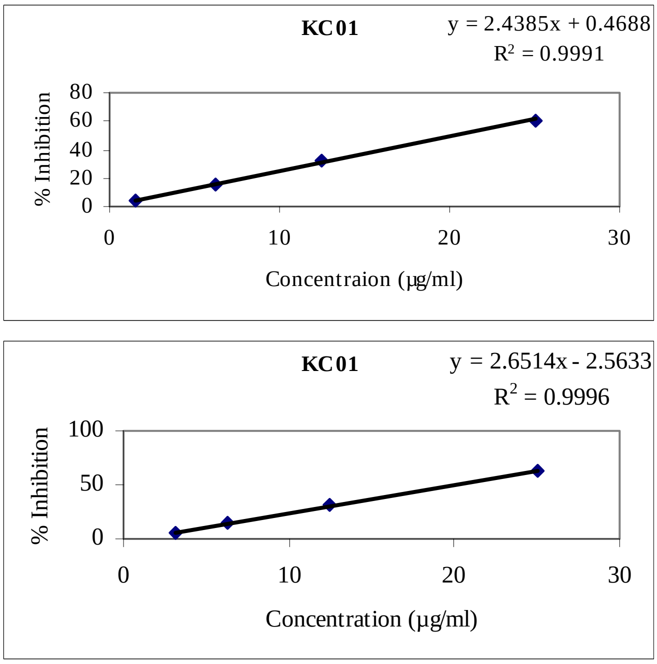
Figure 24 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma comosa* Roxb. (NT01)



ED_{50}= 16.91 μg/ml

ED_{50}= 14.48 μg/ml

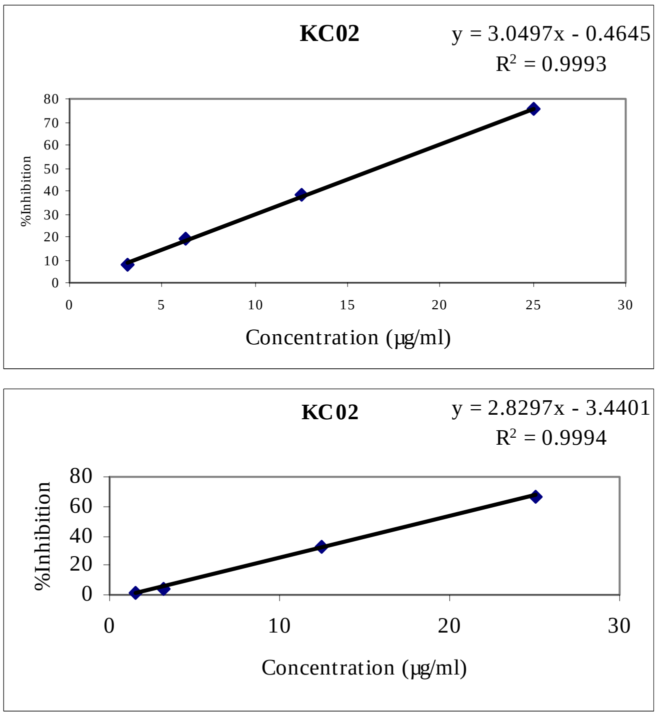
Figure 25 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma comosa* Roxb. (NT02)



ED_{50}= 20.31

ED_{50}= 19.82

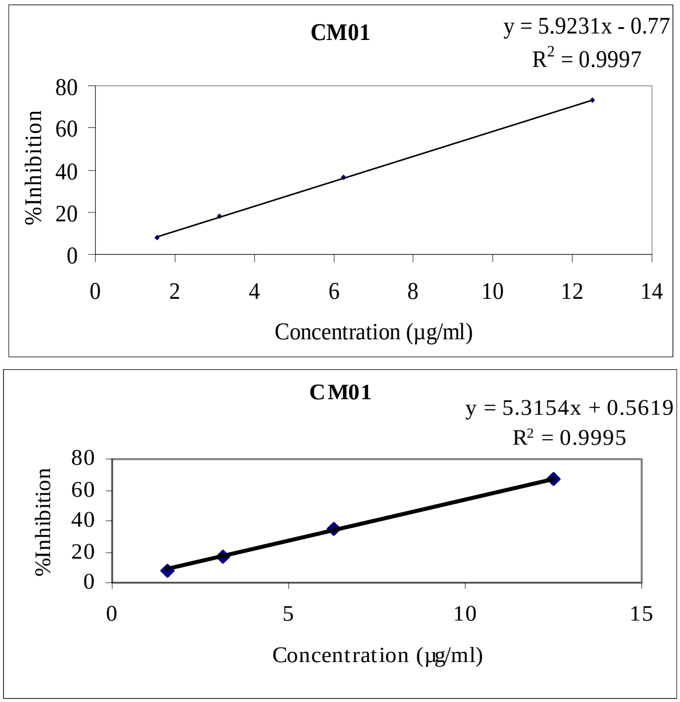
Figure 26 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma comosa* Roxb. (KC01)



ED_{50}= 16.55

ED_{50}= 18.89

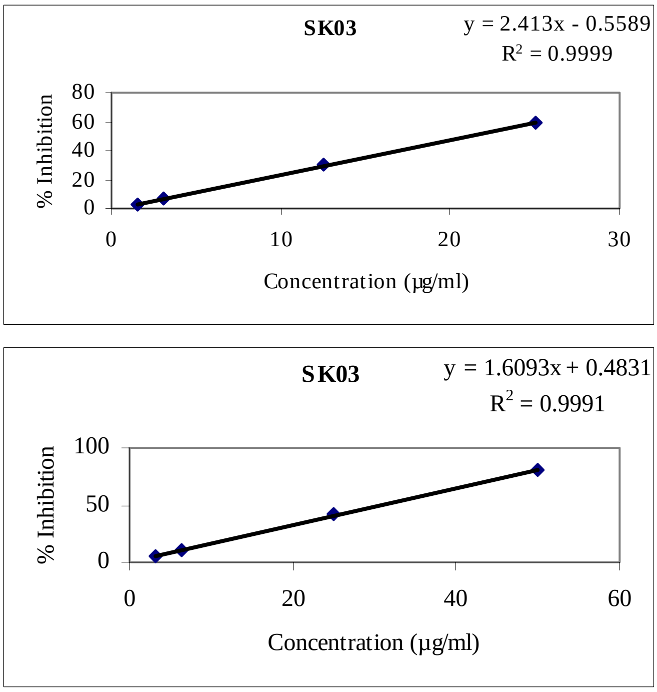
Figure 27 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma comosa* Roxb. (KC02)



ED$_{50}$= 8.57

ED$_{50}$= 9.30

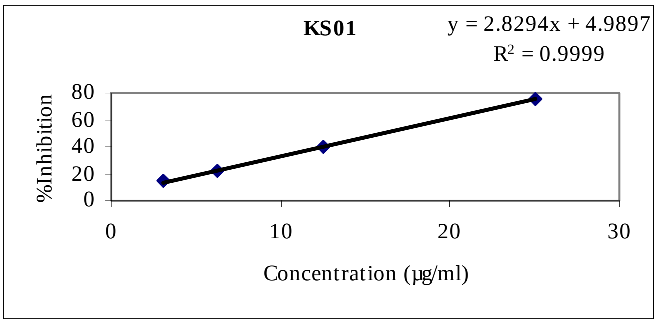
Figure 28 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma comosa* Roxb. (CM01)



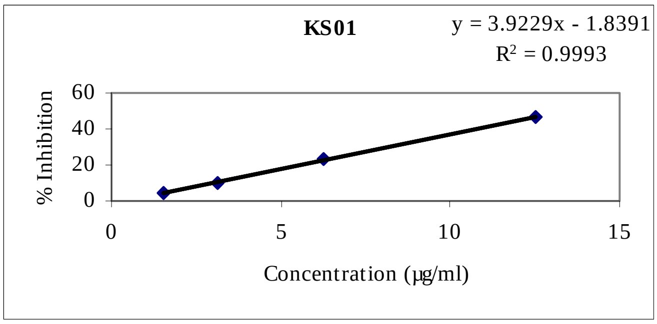
ED$_{50}$= 20.95

ED$_{50}$= 30.77

Figure 29 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma comosa* Roxb. (SK03)

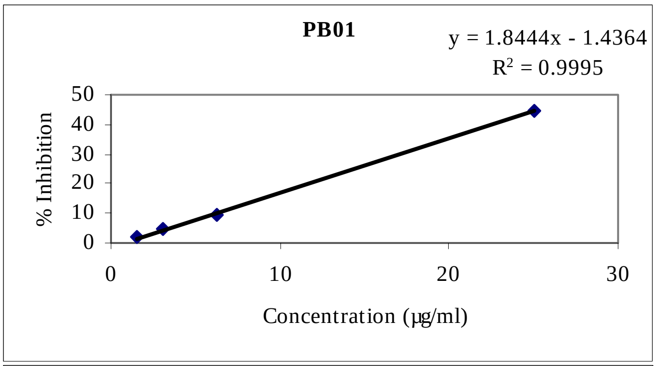


ED_{50}= 13.21

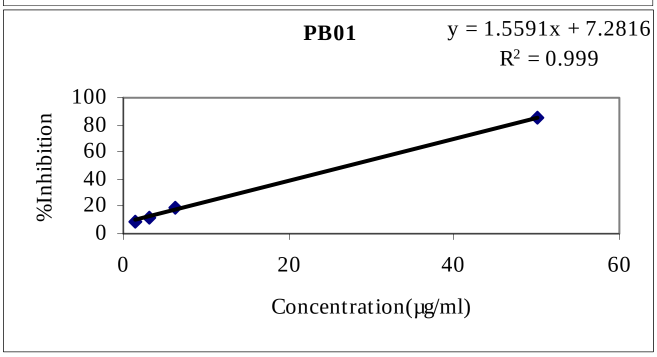


ED_{50}= 15.91

Figure 30 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma comosa* Roxb. (KS01)

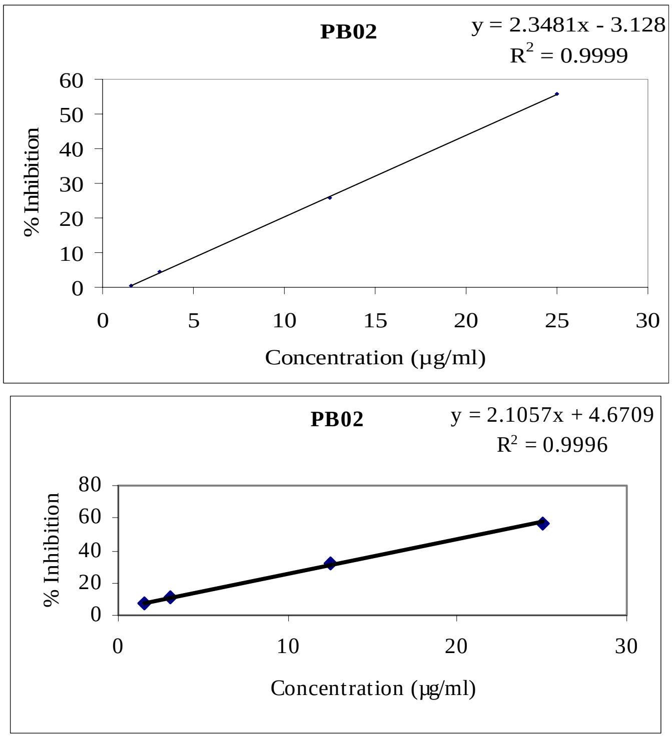


ED_{50}= 27.89



ED_{50}= 27.40

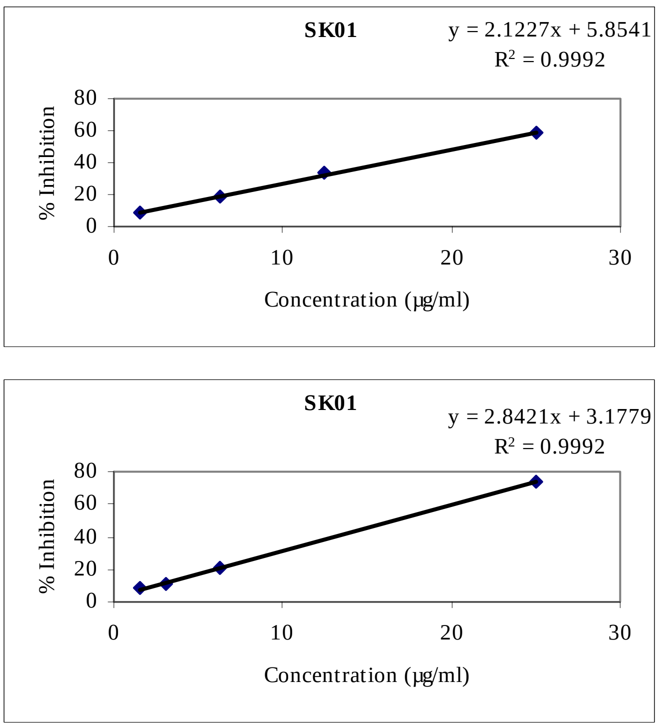
Figure 31 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma* sp. (PB01)



ED_{50}= 22.63

ED_{50}= 21.53

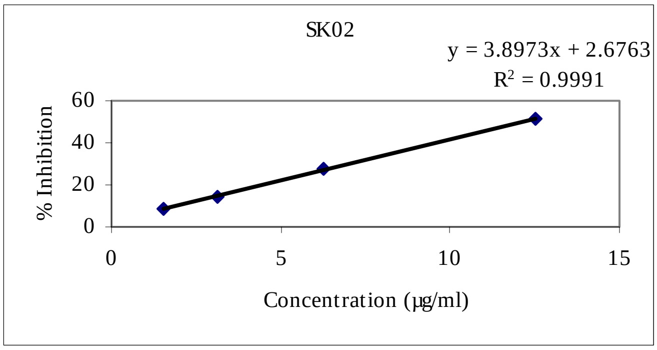
Figure 32 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma* sp. (PB02)



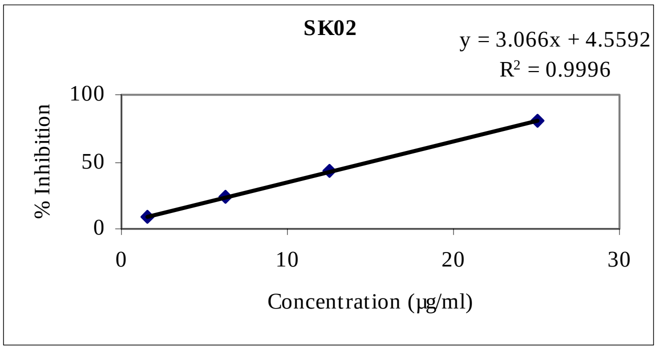
ED_{50}= 20.80

ED_{50}= 16.47

Figure 33 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma* sp. (SK01)

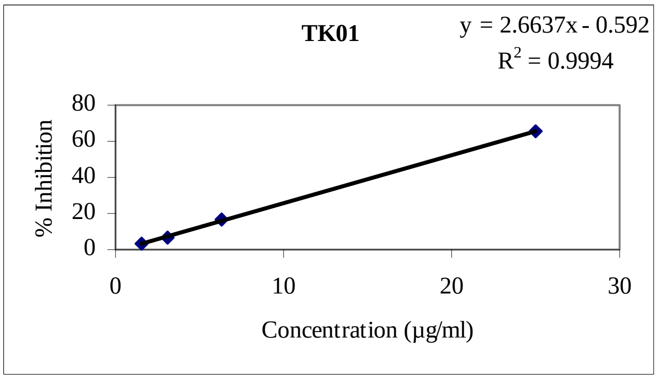


ED_{50}= 12.14

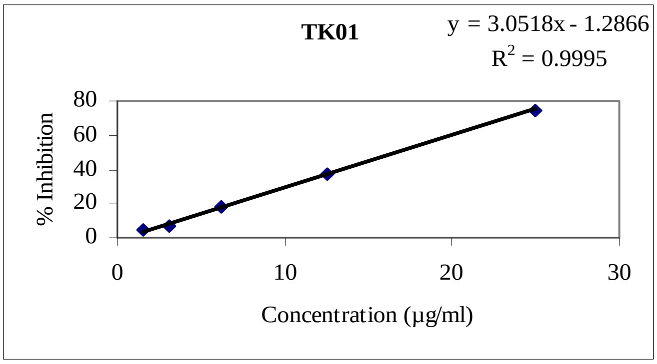


ED_{50}= 14.82

Figure 34 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma* sp. (SK02)

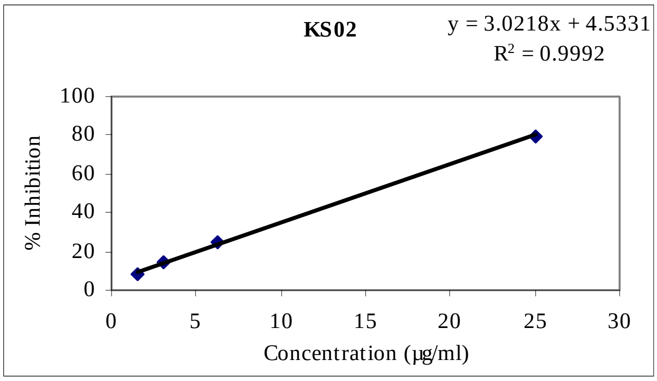


ED_{50}= 18.99

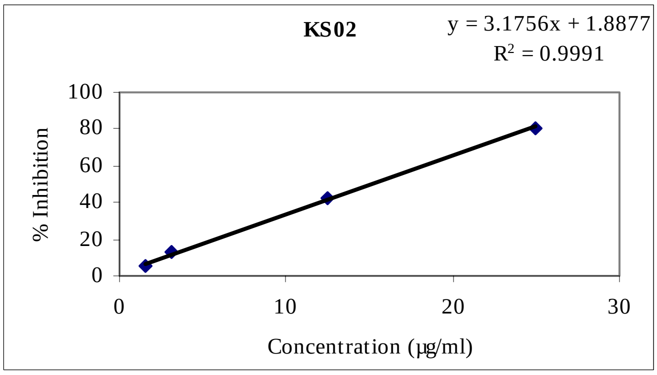


ED_{50}= 16.80

Figure 35 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma* sp. (TK01)



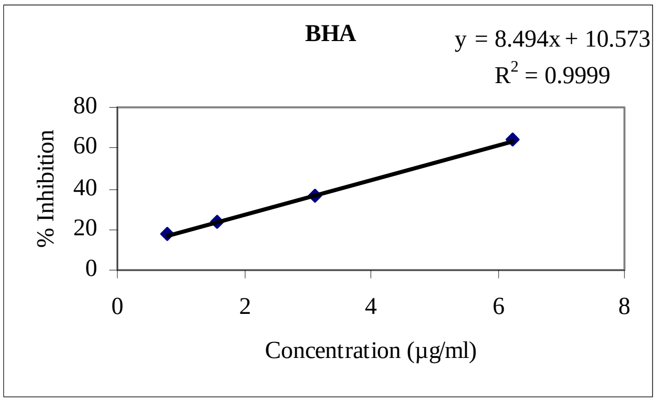
ED_{50}= 15.05



ED_{50}= 15.15

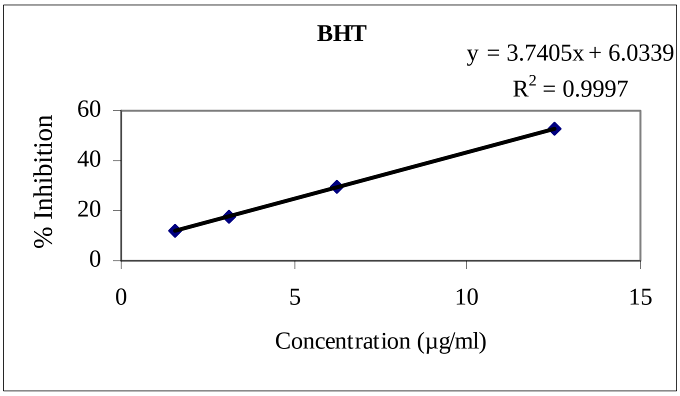
Figure 36 A calibration curve of DPPH scavenging activity of alcoholic extract of *Curcuma* sp. (KS02)

Calibration curve of antioxidant activity using DPPH of reference standard



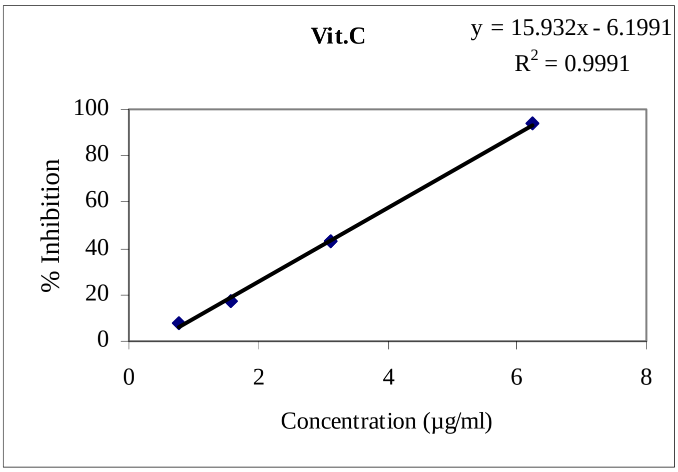
ED_{50}= 4.64

Figure 37 A calibration curve of DPPH scavenging activity of BHA



ED_{50}= 11.75

Figure 38 A calibration curve of DPPH scavenging activity of BHT



ED_{50}= 3.53

Figure 39 A calibration curve of DPPH scavenging activity of vitamin C

Data from uterotrophic activity of Wan chak motluk from various locations

Table 10 Uterotrophic activity of Wan chak motluk from various sources in immature ovariectomized rats.

Treatment	Body weight (g)	Uterine weight (mg)	Uterine wt. (mg/100 gBW)	n
	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	
Intact Control	98.8 ±2.5	130.9 ±13.3	132.7 ±13.5	17
OVX	101.4 ±3.5	42.0 ±2.1	41.8 ±2.1	15
OVX + NT01	111.7 ±3.2	112.1 11.3	99.8 ±8.0	6
OVX + NT02	98.2 ±5.5	112.4 ±2.6	115.3 ±5.4	6
OVX + KC01	95.1 ±2.7	71.3 ±12.4	74.4 ±12.8	6
OVX + KC02	97.8 ±3.1	76.5 ±5.5	78.3 ±5.2	5
OVX + CM01	98.5 ±4.1	81.5 ±7.6	83.0 ±7.3	6
OVX + SK03	109.4 ±2.5	96.8 ±7.4	88.4 ±6.1	5
OVX + KS01	99.6 ±1.6	92.4 ±6.9	92.7 ±6.6	5
OVX + PB01	109.7 ±1.4	37.4 ±2.5	34.1 ±2.4	6
OVX + PB02	109.6 ±0.7	47.2 ±3.5	43.1 ±3.2	6
OVX + SK01	110.2 ±2.5	39.7 ±2.7	36.0 ±2.3	6
OVX + SK02	101.6 ±2.5	46.2 ±2.5	45.5 ±2.5	6
OVX + TK01	107.8 ±3.0	49.0 ±4.4	45.2 ±3.2	6
OVX + KS02	107.8 ±4.5	37.1 ±7.2	35.0 ±6.8	6
OVX + E_2 2.5 mg/kgBW	98.0 ±2.1	126.5 ±3.9	129.6 ±4.0	19

Table 11 Collector numbers of herbarium specimens of Wan chak motluk.

No.	Code	Province	Living specimen No.	Collector No.
1	KC01	Kanchanaburi	KC01	SCMU 308, 309
2	KC02	Kanchanaburi	KC02	-
3	NT01	Nakhon Pathom	NT01	SCMU 300, 301
4	NT02	Nakhon Pathom	NT02	WICH 04
5	PB01	Phetchabun	PB01	SCMU 307, 310
6	PB02	Phetchabun	PB02	WICH 05
7	CM01	Chiang Mai	CM01	SCMU 304
8	TK01	Tak	TK01	-
9	KS01	Kalasin	KS01	-
10	KS02	Kalasin	KS02	-
11	SK01	Sakhon Nakhon	SK01	WICH 01
12	SK02	Sakhon Nakhon	SK02	WICH 03, 06, SCMU 305
13	SK03	Sakhon Nakhon	SK03	WICH 02, 07, SCMU 303

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