Using 6-Gingerol Content and Gene Mapping for Identification of Two Types of Ginger Used in Thai Traditional Medicine

Pimolvan Tappayuthpijarn MSc*, Intouch Sakpakdeejaroen MSc*,
Torsak Seelanan PhD**, Arunporn Itharat PhD*

** Department of Applied Thai Traditional Medicine, Faculty of medicine, Thammasat University, Rungsit Campus, Klongluang, Pathumthani, Thailand*
*** Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand*

Background: *Two species of ginger were used in Thai traditional medicine as Zingiber officinale Roscoe and Zingiber ligulatum Roxb.*
Objective: *To investigate for identification two types of ginger by morphological and microscopic characters, DNA profiles, and determination of 6-gingerol content by HPLC.*
Material and Method: *Fresh rhizomes of two gingers, their ages more than one year were collected from 12 sources in 4 parts of Thailand. The fresh leaves were also collected for studying DNA profile by amplified fragment length polymorphism (AFLP) method.*
Results: *The morphological characters of two types of gingers were almost corresponded to AFLP patterns and they were identified as Z. officinale Rocs. and Z. ligulatum Roxb. Microscopic examinations of dried rhizomes from the both species showed the same pattern. By means of HPLC and TLC methods, 6-gingerol content was found only in Z. officanale in range of 2.58-17.04% but disappeared in Z. ligulatum.*
Conclusion: *Determination of 6-gingerol content by HPLC or TLC pattern can be used to identified two types of ginger used in Thai Traditional Medicine.*

Keywords: *Zingiber officinale, Zingiber ligulatum, DNA mapping, 6-gingerol*

J Med Assoc Thai 2012; 95 (Suppl. 1): S135-S141
Full text. e-Journal: *http://jmat.mat.or.th*

Two species of ginger are used in Thai traditional medicine. One of ginger is *Zingiber officinale* Roscoe, Thai name called Khing or Khing Haeng which is widely used as food, carminative, stimulant and mixing in polyherbs remedies for balancing conditions of patients[1,2]. Another ginger is *Zingiber ligulatum* Roxb., sometimes called in Thai as Khing Klang or Khing Haeng which is only used as carminative. Normally the identification of plants used their flowers but in case of *Zingiber* sp. flowers were found only once a year or rarely found. *Zingiber* sp. flowers attached on inflorescence only in one day, especially *Z. officinale* flowers still alive only two hours and its petal is too delicate to preserve as in original appearance[3]. Normally, only aerial parts were used to determine species of *Zingiber*. Thus, *Z. ligulatum* Roxb. had often misunderstood and misused as *Z. officinale* Roscoe.

The objectives of the present study are to identify type of gingers which used by Thai traditional healers, in accordance with morphological and microscopic characters, amplified fragment length polymorphism (AFLP) fingerprints and to determine of 6-gingerol content in two types of gingers by HPLC and TLC technique.

Correspondence to:
Itharat A,Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Rungsit Campus, Klongluang Pathumtani 12120, Thailand.
Phone & Fax: 0-2926-9749
E-mail: iarunporn@yahoo.com

Material and Method

Fresh rhizomes of gingers used by traditional healers were collected from 12 sources in 4 parts of Thailand and propagated for at least 1 year before experiments.

Identification of gingers

The morphological characteristic of leaves, stems, rhizomes and flowers were observed and used for identification according to plant taxonomic

references[3-8]. The specimens were compared to voucher specimen at the Thai Forest Herbarium (BKF), National Park, Wildlife and Plant Conservation Department, Bangkok, Thailand.

Microscopic examination of gingers

Dried rhizomes of each ginger were blended, put through sieve No. 60 and examined under microscope. Cells and tissues stained with iodine solution or phloroglucinol solution were pictured and drawn[9,10].

Identification of DNA profiles

The fresh leaves were studied for DNA profiles by amplified fragment length polymorphism (AFLP) method. Five young leaves of each ginger was collected, cleaned and extracted for DNA by using DNA kit; NucleoSpin® Plant II (Macherey-Nagel, Germany) according to the method of Vos P. et al 1995[11]. Amplified Fragment Length Polymorphisms (AFLPs) were performed by modified method of Vos P et al (1955) and DNA band were examined by silver staining method[11,12].

Determination of 6-gingerol content by High Pressure Liquid Chromatography (HPLC)

The dried rhizomes were determined for 6-gingerol content by using HPLC model Constametric® 4100 Bio with UV-visible detector (Spectromonitor® 4100) and automatic injector (Spectra System AS3500). HPLC column was Phenomenex® Luna 5µ C18 (2) 100A, size 150 x 4.60 mm 5 micron with guard catridges (C18) size 4 x 3.0 mm. Mobile phase (HPLC grade) was water-acetonitrile with gradient elution. 10 mg of dried ethanolic extract from each of ginger rhizome was dissolved in 1 ml of acetonitrile and analyzed for 6-gingerol[13,14]. HPLC data were analyzed by TSP PC 1000 software.

Identification of 6-gingerol by Thin Layer Chromatography (TLC)

Ten milligram of dried ethanolic extract from each ginger rhizome was dissolved in 1 ml of methanol and 1 µl was applied on precoated silica gel G F_{254} TLC plate. Chloroform: methanol 99:1 was used as mobile phase and detected by UV 254 and anisaldehyde-sulphuric acid reagent[13,15]. The 6-gingerol was used as authentic sample to be compared with ginger extract.

Results

Fresh rhizomes of gingers used by traditional healers were collected from 12 provinces which showed in Table 1.

Taxonomic classification of gingers

According to Flora of India[8], ginger No. 6, which had very short inflorescence raised from rhizome, could be classified to be in section *Cryptanthium,* Horan. This ginger, flowered in August (Fig. 1), was determined to be *Z. ligulatum*, Roxb with descriptions as followed: "Leafy stem up to 60 cm. Leaves 15-20 by 4-6 cm, oblong-lanceolate, glabrous beneath. Spike dense, subglobose; peduncle 5-7 cm; bract about 2 cm, flower bracts pink, outer ovate inner lanceolate, corolla segment pink, subequal, 2 cm, lip as long as the corolla-segments, obovate-cuneate yellow-white unspotted. Stamen yellow, shorter than the lip". It was notified that gingers number 5, 6 and 7 had almost the same vegetative characters such as petiole 1-2.5 cm long, ligule1-2 cm long, leaf with no pungent smell, young shoot red basally. Thus, it was identified by taxonomy characteristic as *Z. ligulatum.* All of these samples were called Khing Haeng by traditional healers.

Gingers in another section, *Lampuzium*, Horan had long inflorescence, covered with membranous bract, raised from rhizome. *Zingiber officinale* Rosc is classified to be in this section, and unfortunately this ginger very rarely flowers. The gingers No. 1, 2, 3, 4, 8 and 12 had almost the same characters as *Zingiber officinale* Rosc with descriptions as followed rhizomes 2-3 cm thick, palmately lobed, grayish-yellow within, pungent smell. Leafy shoot up to 1m tall. Leaves linear, glabrous except for short hair near base of each leaf-base, petiole 2 mm long; ligule 2-5 mm long, membranous, slightly bilobed; lamina 15-25 x 1.5-2.5 cm, narrowly lanceolate, acuminate, base attenuate. Ginger No.12 collected from Thung-sa-laeng-loung forest Petchaboon (Fig. 2) flowered once a year in September. Its flower had oblong spike, light-green bract with slightly incurved margin, pale yellow corolla, but no evidence whether the flower had dull purple labellum mottled with yellow. Thus, by the vegetative characters, sample No. 12, including No. 1, 2, 3, 4 and 8 could be identified as *Zingiber officinale* Rosc. However, these specimens were also compared with voucher specimen BKF number 48735.

Moreover, the Garden Bulletin Singapore, 1996[5] classified *Zingiber officinale* into 2 varieties as followed: one was a variety as *officinale*:- rhizome yellow externally, leafy shoots green basally, labellum dark purple mottled; another was a variety as *rubrum*. Theilade:- rhizome reddish externally, leafy shoots red

Table 1. Classification of types of ginger by relative of identification by taxonomy , DNA fingerprint by AFLP pattern, 6-gingerol content by HPLC and TLC

No.	Place of Collection	Identify by taxonomy	AFLP pattern	6-Gingerol content in HPLC	TLC pattern	Conclusion
1	Surin	*Zingiber officinale* (green shoot)	Group I	-	-	Gr1
2	Songkhla	*Zingiber officinale var:rubrum* (red shoot)	Group I	9.78 ± 0.01	Found	Gr1
3	Rayong	*Zingiber officinale var:rubrum* (red shoot)	Group I	2.58 ± 0.01	Found	Gr1
4	Roi-et	*Zingiber officinale var:rubrum* (red shoot)	Group III closely group I	11.54 ± 0.03	Found	Gr1
5	Sra-kaew	*Zingiber ligulatum*	Group II	-	-	Gr2
6	Trat	*Zingiber ligulatum*	Group II	-	-	Gr2
7	Chieng-Mai	*Zingiber ligulatum*	Group II	-	-	Gr2
8	Chantaburi	*Zingiber officinale* (green shoot)	Group IV	12.21 ± 0.02	Found	Gr3
9	Chantaburi	Can not identify as *Zingiber*	Group V	17.04 ± 0.04	Found	Gr4
10	Chantaburi	Can not identify as *Zingiber*	Group V	-	-	Gr5
11	Chantaburi	Can not identify as *Zingiber*	Group VI	-	-	Gr5
12	Petchaboon	*Zingiber officinale var. officinale* (red shoot and pungent)	Group I	5.40 ± 0.01	Found	Gr1

Fig. 1 Ginger No. 6 flower was determined as *Zingiber ligulatum* Roxb

Fig. 2 Ginger No. 12 flower was conferred as *Zingiber officinale* Rosc

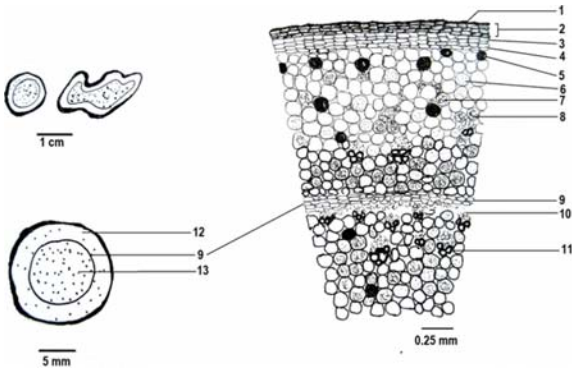
basally, labellum scarlet red mottled with cream. *Z. officinale* var *rubrum* differs vegetative from var. *officinale* by the smaller, red colored rhizomes which

have a stronger, more pungent smell, the red coloring of the basal parts of the leafy stems and petioles and leaves are larger than variety *officinale*. Ginger samples No. 2, 3 and 4 had red basally shoots especially in young shoot, more pungent smell than other samples while the number 1 and 8 had white green young shoots. These evidences supported ginger samples No. 2, 3 and 4 to be identified as *Z. officinale* var *rubrum* and No. 12 as *Zingiber officinale* var. *officinale.*

Sample No. 9, 10 and 11, were also called Khing by Thai traditional healers, had large stem and large leaves, lamina 20-25 x 2.5-4 cm, ligule 0.5-1 cm long, rhizomes with reddish externally and smell of ginger in combination with other volatile odor. In addition, the ginger No. 9 had petiole of 2.5 cm long while the other two gingers had sessile leaves. However, they should be identified by another technique. The taxonomic name were concluded in Table 1.

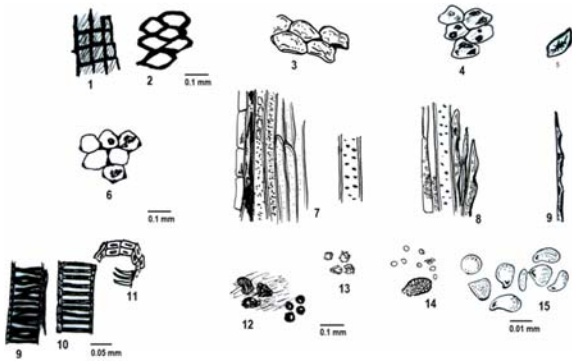
Microscopic examination of gingers

Cross-section of *Zingiber ligulatum* Roxb rhizome in Fig. 3 showed light-yellow oil droplets dispersed over cortex and stele. Parenchyma cells bearing with starch, crystal plates and orange oleoresin were scattered. Powder drug of both *Zingiber ligulatum* Roxb. (Fig. 4) and *Z. officinale* Rosc. (Fig. 5) had the same characters such as epidermal cork, parenchyma showing wrinkled wall, parenchyma with oil droplet, resin mass and crystal, collenchyma, vascular bundle showing septate fiber. The typical characters of *Zingiber* such as mainly starch grain showing beak and lining surface, dentate fiber in vascular bundle, reticulate and scalariform vessel were found in both species. Two type of ginger cannot be identified by microscopic method.



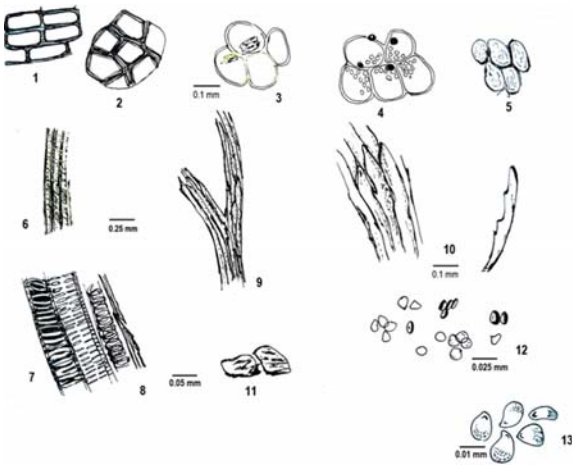
1. epidermal cell, 2. epidermal layer, 3. cork cambium, 4. phelloderm, 5. oil droplet, 6. parenchyma with starch, 7. parenchyma with crystal, 8. parenchyma with oleoresin, 9. endodermis, 10. phloem, 11. xylem, 12. cortex, 13. vascular bundle

Fig. 3 Cross-section of *Zingiber ligulatum* Roxb. Rhizome



1. top view of epidermal cork, 2. cork cell, 3. parenchyma showing wrinkled wall, 4. parenchyma with oil droplet, resin mass and crystal, 5. stone cell, 6. collenchyma with crystal and resin mass, 7. vascular bundle showing parenchyma and septate fiber, 8. fiber, 9. vascular bundle showing dentate fiber with crystal, 10. dentate fiber, 11. reticulate and scalariform vessel, 12. yellow-orange resinous mass, 13. orange-red oil droplet, 14. plate crystal, 15. starch, 16. starch showing beak and lining surface

Fig. 4 Powder drug of *Z. ligulatum* Roxb. Rhizome



1. cork cell, 2. top view of epidermal cork, 3. parenchyma resin mass, 4. parenchyma with oil droplet and starch, 5. parenchyma showing wrinkled wall, 6. vessel, 7. reticulate and scalariform vessel, 8. spiral vessel, 9. vascular bundle showing septate fiber, 10. dentate wall of fiber, 11. yellow-orange resinous mass, 12. starch, 13. starch showing beak and lining surface

Fig. 5 Powder drug of *Z. officinale* Rosc. Rhizome

Identification of DNA profiles

Based on AFLP patterns (Fig. 6, 7), six groups of gingers could be categorized. Group I was ginger No. 12 included No. 1, 2, 3. Group II was comprised of No. 5, 6 and 7. The other 4 groups showed distinctly different AFLP fingerprints such as group III (No. 4), group IV (No. 8), group V (No. 9 and 10) and group VI (No. 11). Nevertheless, AFLP patterns suggested that gingers in group I and no.4 were genetically closely related.

6-gingerol content by High Pressure Liquid Chromatography (HPLC)

The standard curve of 6-Gingerol analyzed by HPLC was shown in Fig. 8. The HPLC chromatogram of 12 samples of ginger extracts could be categorized into 2 groups (Fig. 9). Group A were ginger No. 2, 3, 4, 8, 9, 12 which showed 6-gingerol peak at retention time of 9.070 minutes and minor peaks as shown. Group B were ginger No. 1, 5, 6, 7, 10, 11 with unknown minor peak at 10.135 minutes. It could be concluded that 6-gingerol was disappeared in group B. The percent of 6-gingerol (w/w of ethanolic extract) found in the group A were calculated as 9.78 ± 0.01, 2.58 ± 0.01, 11.54 ± 0.03, 12.21 ± 0.02, 17.04 ± 0.04 and 5.40 ± 0.01 respectively (means ± SD).

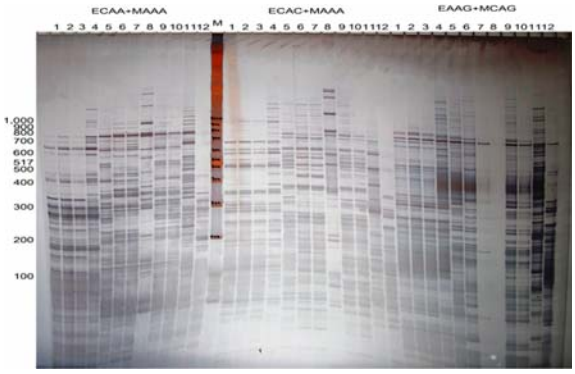


Fig. 6 AFLPs of 12 gingers DNA fingerprints generated by 3 pairs of selective primer, namely ECAA + MAAA ECAC + MAAA and EAAG + MCAG. Lane No. referred to sample No. and M is 1-kb DNA ladder

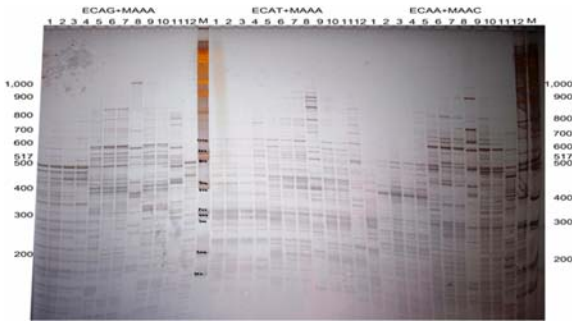


Fig. 7 AFLPs of 12 gingers DNA fingerprints generated by 3 pairs of selective primer, namely ECAG + MAAA, ECAT + MAAA and ECAA + MAAC. Lane No. referred to sample No. and M is 1-kb DNA ladder

Determination of 6-gingerol content by Thin Layer Chromatography (TLC)

TLC fingerprints (Fig. 10, 11) revealed that 6-gingerol (Rf value as 0.76) was found strikingly in sample No. 2, 3, 4, 8, 9 and 12. The gingerol was visible as red-purple spot when spaying with anisaldehyde reagent (Fig. 10) and shined as blue spot in UV 365 .

Discussion

According to the present study, 12 samples of Khing used by Thai traditional healers could be

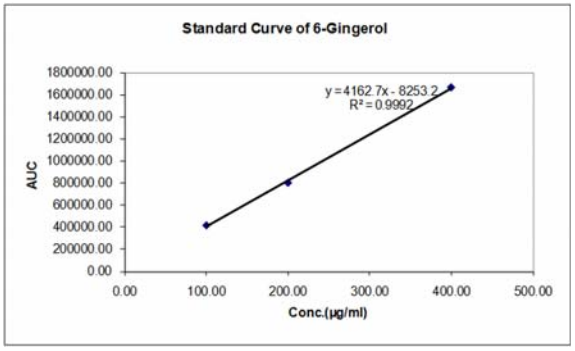


Fig. 8 Standard Curve of 6-Gingerol analyzed by HPLC

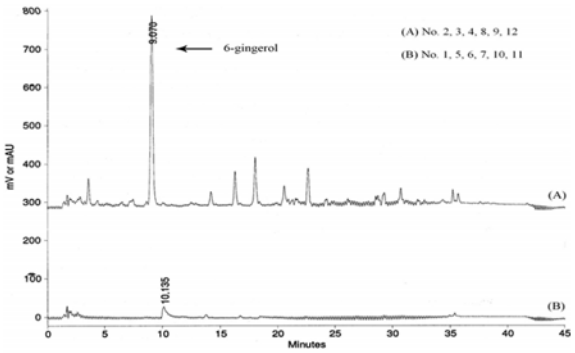


Fig. 9 HPLC chromatogram of 12 samples of ginger extracts. Mobile phase: water-acetronitrile with gradient elution as follows: 0 min min (50:50), 17 min (35:65), 32 min (0:100), 38 min (0:100), 43 min (55:45), 48 min (55:45); Flow rate 1.0 ml/min; UV detector at (55:45), 8 256 nm

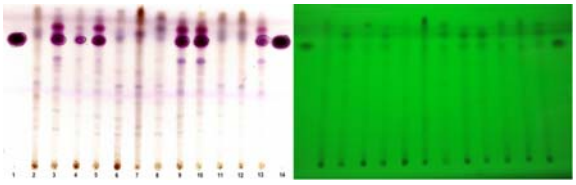


Fig. 10, 11 TLC fingerprints showed 6-gingerol in lane 1 and 14 and 12 samples of ginger extract in lane 2-13. Adsorbent: Silica gel G F_{254}. Mobile phase: Chloroform: methanol = 9:1. Detected by spraying with anisaldehyde-sulphuric acid reagent then heat 105°C 5 min (Fig. 10) and under UV 254 (Fig. 11)

categorized in 5 groups. The morphological characteristics of these groups were found to be almost related to AFLP of gene and 6-gingerol content. Group I were samples No. 1, 2, 3, 4, 8 and 12 which conferred as *Zingiber officinale* Rosc. These gingers had pungent smell of Khing and had the vegetative characteristics representing this species. The AFLP of samples No. 1, 2, 3, 4 and 12 were almost the same but differed in No. 8. It may be concluded that it is new variety of *Zingiber officinale* Rosc, because DNA fingerprint showed significant different with 1, 2, 3, 4, 12. Group II were No. 6 and 7 which flowered in August and were determined as *Z. ligulatum* Roxb. The DNA fingerprints of sample No. 6, 7 were almost the same as No. 5. Ginger No. 5 leaves had longer petiole than No. 6 and 7. However, morphology, DNA fingerprint, disappearrance of 6-gingerol in these ginger, the No. 5, 6, 7 should be concluded as *Z. ligulatum* Roxb. Each of other 3 species as No. 9, 10, 11 which was totally different in DNA fingerprints, also had difference morphology characters with *Z.officinale* Rosc and *Z. ligulatum*. They should be other species because they cannot be identified as *Zingiber*. Ginger No. 9 had ginger smell, long petiolate leaves but had same pattern in DNA finger print as No. 10. This plant showed the highest content of 6-gingerol but gingers No. 10 and 11 with leafy shoot up to 1 meter tall and sessile leaves had no pungent smell which related to 6-gingerol content. Thus No. 9, 10 and 11 should be further investigated on morphology and also identified for botanical names.

The 6-gingerol, one of main active ingredients, could be found in samples No. 2, 4, 8 and 12 which were conferred as *Zingiber officinale* Rosc and in another unknown species, sample No. 9. Due to limited time, the sample No. 1, aged only 8 month, had no 6-gingerol content. This plant showed green shoot although it were identify as *Z offinale*. The peak of 6-gingerol in the HPLC patterns were also corresponded to the spot in TLC fingerprints. The 6-gingerol was doubtless disappeared in *Z. ligulatum* Roxb. and in the unknown sample No. 10,11.

Conclusion

These results revealed that more than 2 species of *Zingiber* had been used by Thai traditional healers. The main active principle, 6-gingerol could be detected only in samples which were conferred as *Zingiber officinale* Rosc. The morphological characteristics of these samples were almost corresponded to AFLP patterns of gene. Another Khing Haeng was determined as *Zingiber ligulatum* Roxb. had no pungent smell and also had no 6-gingerol content. There were three unknown species of *Zingiber* that were used by Thai traditional healers and only one unknown ginger contained 6-gingerol.

Acknowledgement

This work was supported by the National Research University Project of Thailand Office of Higher Education Commission and Thammasat University.

Potential conflicts of interest

None.

References

1. Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z. Medicinal plants in therapy. Bull World Health Organ 1985; 63: 965-81.
2. Itharat A, Singchangchai P, Ratanasuwan P Study on wisdom of folk doctors in Southern of Thailand. Songkla, Thailand: Faculty of Pharmaceutical Science, Prince of Songkla University; 2542: 126-7.
3. Sabu M. Zingiberaceae and Costaceae of South India. Indian Association for Angiosperm Taxonomy; 2006: 228-45.
4. Larsen K. A preliminary checklist of the Zingiberaceae of Thailand. Thai Forest Bulletin (Botany) 1996; 24: 35-49.
5. Theilade I. Revision of the genus *Zingiber* in peninsular Malaysia. Gard Bull Singapore 1998; 48: 207-36.
6. Larsen K. Annotated key to the genera of Zingiberaceae of Thailand. Nat Hist Bull Siam Soc 1980; 28: 151-69.
7. Backer CA, Bakhuizen van den Brink RC. Flora of Java. New York: Springer; 1980.
8. Hooker JD. Flora of British India. Vol. 4. South

Carolina: Nabu Press; 1984: 243-9
9. Evans WC. Trease and evans pharmacognosy. 15th ed. Edinburgh: W.B. Saunders; 2002.
10. Thai Herbal Pharmacopoeia. Nonthaburi, Thailand: Department of Medical Sciences, Ministry of Public Health; 2000: 2.
11. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, et al. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 1995; 23: 4407-14.
12. Yildirim F, Akkaya MS. DNA fingerprinting and genetic characterization of Anatolian Triticum sp. using AFLP markers. Genet Resour Crop Evol 2006; 53: 1033-42
13. Master document of Zingiber Officinale. Bangalore, India: Natural Remedies. Research Center; 2006.
14. He X, Bernart MW, Lian L, Lin L. High-performance liquid chromatography–electrospray mass spectrometric analysis of pungent constituents of ginger. J Chromatogr A 1998; 796: 327-34.
15. Wagner H, Bladt S. Plant drug analysis: a thin layer chromatography atlas Berlin, Germany: Springer-Verlag; 1995: 293-301.

การใช้ปริมาณ 6-gingerol และ gene mapping สำหรับการพิสูจน์เอกลักษณ์ของขิง สองพันธุ์ที่ใช้ในยาไทย

พิมลวรรณ ทัพยุทธพิจารณ์, อินทัช ศักดิ์ภักดีเจริญ, ต่อศักดิ์ สีลานันท์, อรุณพร อิฐรัตน์

***ภูมิหลัง**: ขิง 2 ชนิดที่ใช้ในแพทย์แผนไทย คือพันธุ์ Zingiber officinale Roscoe และ Zingiber ligulatum Roxb.*
***วัตถุประสงค์**: เพื่อศึกษาวิธีการพิสูจน์ เอกลักษณ์ของขิงทั้งสองชนิด ด้วย ลักษณะสัณฐานวิทยาและผงยา โดยดูด้วยกล้องจุลทรรศน์ ลักษณะของ DNA profiles และ การวัดปริมาณของปริมาณ 6-gingerol ด้วยเทคนิค HPLC.*
***วัสดุและวิธีการ**: เหง้าขิงแห้ง ทั้งสองชนิด ที่มีอายุมากกว่า 1 ปี เก็บจาก 12 แหล่ง จาก 4 ภาคของประเทศไทย ใบสดเก็บมา เพื่อใช้ศึกษาลักษณะดีเอ็นเอ ด้วยเทคนิค เอ เอฟ แอล พี*
***ผลการศึกษา**: ลักษณะรูปร่างของขิงสองชนิดมีความสัมพันธ์กับลักษณะของดีเอ็นเอ ซึ่งสามารถพิสูจน์เอกลักษณ์เป็นขิง ชนิด Z. officinale Rocs. and Z. ligulatum Roxb. การตรวจสอบด้วยกล้องจุลทรรศน์ของเหง้าแห้ง ทั้งสองพันธุ์ มีลักษณะเหมือนกัน การใช้ปริมาณ 6-gingerol ที่วิเคราะห์ด้วยเทคนิค HPLC และ TLC พบว่า 6-gingerol จะพบเฉพาะ Z. officanale มีปริมาณ 2.58-17.04% แต่ไม่พบในชนิด Z. ligulatum*
***สรุป**: การวัดปริมาณ 6-gingerol ด้วยเทคนิค HPLC หรือ TLC สามารถใช้ ระบุชนิดของขิงทั้งสองชนิดที่ใช้ในยาไทยได้*