Mitragyna speciosa: Hairy Root Culture for Triterpenoid Production and High Yield of Mitragynine by Regenerated Plants

Siriwan Phongprueksapattana[a], Waraporn Putalun[b], Niwat Keawpradub[a], and Juraithip Wungsintaweekul[a,*]

[a] Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand. Fax: +66 74 42 82 20. E-mail: juraithip.w@psu.ac.th

[b] Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand

* Author for correspondence and reprint requests

Z. Naturforsch. **63 c**, 691–698 (2008); received March 25/May 2, 2008

Hairy root cultures of *Mitragyna speciosa* were established by infection of *Agrobacterium rhizogenes* ATCC 15834 and maintained in McCown woody plant medium (WPM) supplemented with 0.5 mg/l naphthaleneacetic acid. The hairy roots were identified for the rooting genes loci of *rol*A and *rol*B by polymerase chain reaction. For studying the secondary metabolite production, the *n*-hexane extract of the hairy roots was prepared and the compounds were isolated by silica gel column chromatography, affording triterpenoids (ursolic acid and oleanolic acid) and phytosterols (β-sitosterol and stigmasterol). The shoots from the hairy root cultures were regenerated and differentiated to the plantlets. For micropropagation, shoot multiplication was successfully induced from the axillary buds of the regenerated plantlets in WPM supplemented with 0.1 mg/l thidiazuron. The mitragynine contents of 5-month-old regenerated plants and *in vitro* plantlets (germinated from seeds) were determined using the TLC-densitometric method. The regenerated plants contained (14.25 ± 0.25) mg/g dry wt mitragynine, whereas the *in vitro* plantlets contained (4.45 ± 0.09) mg/g dry wt.

Key words: Mitragyna speciosa, Ursolic Acid, Mitragynine, Hairy Root Culture

Introduction

Mitragyna speciosa (Roxb.) Korth. (Rubiaceae) is an endemic plant found in tropical Southeast Asia. It is of particular medicinal importance and known as "Kratom" in Thailand. In folklore medicine, Kratom has been used as an opium-substitute for pain relief and treatment of diarrhea (Jansen and Prast, 1988). Many studies reported the wide variety of indole alkaloids from Kratom's leaves including mitragynine (**1**) (Shellard, 1974) (Fig. 1). In addition, it also contains flavone, flavonol, flavonoid (Harvala and Hinou, 1988), and polyphenolic compounds (Hinou and Harvala, 1988), and triterpenoids such as ursolic acid (**2**) and oleanolic acid (**3**) (Said *et al*., 1991). The pharmacological activities of mitragynine and its derivatives such as analgesic activity, antinociceptive activity (Watanabe *et al*., 1997), antistress activity, muscle-relaxant activity (Aji *et al*., 2001) and inhibition of gastric acid secretion (Tsuchiya *et al*., 2002) have been reported from studies in tested animals. The mechanism of action of mitragynine as analgesic is binding to the opioid receptors, similar to morphine (Thongpraditchote *et al*., 1998). Interestingly, mi-

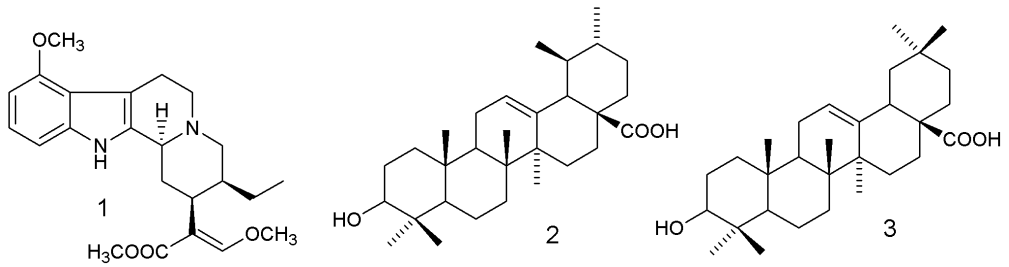

Fig. 1. Chemical structures of mitragynine (**1**), ursolic acid (**2**) and oleanolic acid (**3**).

tragynine has a characteristic of less addiction than morphine. Therefore, it highlights the relevance to be an alternative to opioid analgesic drugs. Contrary to the potential of *M. speciosa*, it is recognized as an illegal plant, and culturing of this plant is prohibited in Thailand. As such, the basic knowledge of mitragynine biosynthesis is very little. From the biosynthesis point of view, the steps of mitragynine formation are still unknown.

In the present study, we aimed to induce the *in vitro* cultures of *M. speciosa* including the hairy root culture and to propagate plants containing high yield of mitragynine. The hairy root cultures were obtained from infection of *M. speciosa* with a wild-type *Agrobacterium rhizogenes* ATCC 15834. Secondary metabolites accumulated in the *M. speciosa* hairy roots were isolated and their structures elucidated by NMR spectroscopic methods. As we focused on the mitragynine biosynthesis in *M. speciosa*, the *in vitro* hairy root cultures and *in vitro* regenerated plantlets obtained from this study can serve as plant models for further biosynthetic studies.

Experimental

Bacterial strain and chemicals

Agrobacterium rhizogenes ATCC 15834 was obtained from the Microbiology Resource Center, Pathumtani, Thailand. Mitragynine was isolated from *M. speciosa* leaves (Keawpradub, 1990). Ursolic acid was purchased from Sigma-Aldrich Pte., Ltd. (Singapore). McCown woody plant medium (WPM) and plant agar were from Duchefa Biochemie (Haarlem, The Netherlands). *N*-Benzyladenine (BA), 1-naphthaleneacetic acid (NAA) (95% GC), and 6-furfurylamino-purine (kinetin) were purchased from Fluka Chemie (Buchs, Switzerland). Thidiazuron (TDZ) was purchased from Supelco (Bellefonte, PA, USA). Bacterial media were purchased from Himedia Laboratories (Mumbai, India). All other chemicals were standard commercial products of analytical grade.

Plant materials

M. speciosa seeds were collected from Hat Yai District, Songkhla, Thailand. They were surface-sterilized by rinsing with 70% (v/v) ethanol for 5 min, rinsing with 20% (v/v) Clorox® (NaClO; Selangor, Malaysia) for 5 min and finally rinsing with sterile distilled water thoroughly. Sterile seeds were germinated on WPM supplemented with 1.0 mg/l BA and incubated at 25 °C under 16 h daily light. Two-month-old plantlets were used for bacterial infection.

Induction of M. speciosa hairy root cultures

A. rhizogenes ATCC 15834 were prepared freshly on yeast extract (YE) solid medium [containing 5.0 g/l beef extract, 1.0 g/l peptone, 5.0 g/l sucrose, 50 ml/l of 10% (w/v) $MgSO_4$ solution and 15 g/l agar] at 28 °C for 16 h. A single bacterial colony was inoculated into 5 ml YE broth medium, placed on a rotary shaker (218 rpm) and incubated at 28 °C for 16 h. The bacterial suspension was harvested by centrifugation at 4,000 rpm for 5 min and the pellet was re-suspended in sterile WPM. The OD_{600} value was adjusted to 0.5–0.6. The explants (stems and leaves) were wounded by a needle and submerged in bacterial suspension for 30 min. The infected explants were thoroughly washed with sterile water and transferred to WPM containing 0.7% (w/v) plant agar. After 3 d of infection, explants were transferred to solid WPM containing 500 mg/l cefotaxime disodium (M&H, Bangkok, Thailand) and, for further culture, the cefotaxime disodium concentrations were decreased to 250, 100 and 50 mg/l each week. Cultures free of *A. rhizogenes* were transferred to hormone-free solid WPM. Hairy roots were initiated within 10 d after infection, at (25 ± 2) °C under darkness. Hairy roots were excised from explants and maintained in liquid WPM. For untransformed roots, the seedling roots were cut and cultured in liquid WPM. Both types of culture were kept at (25 ± 2) °C in the darkness and rotary-shaken at 80 rpm.

Identification of transformed hairy roots by PCR analysis

Genomic DNA was isolated from untransformed roots and hairy roots using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The transformed genes in hairy roots were identified by PCR analysis for the rooting locus genes *rol*A and *rol*B. For *rol*A, forward primer 5′-CAGAATGGAATTAGCCGGACTA-3′ and reverse primer 5′-CGTATTAATCCCGTAGGTTTGTTT-3′ were used for amplification of a 300-bp fragment. For *rol*B, forward primer 5′-ATGGATCCCAAATTGCTATTCCTTCCACGA-3′ and reverse primer 5′-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3′ were used for amplification of a 780-bp

fragment. The PCR reactions were performed in a total volume of 50 μl, containing 50 pg of genomic DNA, 0.5 μM of each primer, 200 μM dNTP, 2.5 U of Taq DNA polymerase (New England Biolab, MA, USA), and 1x ThermolPol buffer (New England Biolab). PCR conditions were 94 °C for 2 min, 24 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min for *rol*A and 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min for *rol*B and a final extension at 72 °C for 10 min. PCR fragments were analyzed by 1.2% (w/v) agarose gel electrophoresis and visualized using UV transluminator (312 nm) after ethidium bromide staining.

Isolation of ursolic acid and phytosterols

Dried hairy roots of *M. speciosa* (37.4 g) were macerated with 300 ml methanol for 3 d and filtered. The marc was re-macerated and the methanol fractions were pooled and evaporated to dryness. The residue (8.52 g) was dissolved in 100 ml methanol, partitioned with 100 ml *n*-hexane for three times, and evaporated. The crude *n*-hexane extract (403 mg) was further purified by loading on the top of a silica gel column (3 × 18 cm; Scharlau, La Jota, Spain) and eluting with *n*-hexane/ethyl acetate (9:1, 8:2, 7:3, 5:5, v/v), ethyl acetate, ethyl acetate/methanol (8:2, 5:5, v/v) and methanol. From TLC analysis, 11 fractions were obtained. Fractions F6 and F10 were further purified. F6 was re-crystallized using chloroform/methanol (7:3, v/v). White needle crystals were obtained, affording MSF1 (6.1 mg). F10 was washed with *n*-hexane. A white amorphous solid was obtained, affording MSF2 (10.11 mg).

Concerning the structure of MSF2, the ^{1}H NMR spectra exhibited signals typical for the structure of triterpenoid compounds. The NMR data were obtained as follows.

^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$): 3.20 (t, 1H, J = 7.5 Hz, H-3), 5.24 (m, J = 3.5 Hz, 1H, H-12), 2.20 (d, J = 11.0 Hz, 1H, H-18), 2.02–1.15 (m, H-22), 0.98 (s, 3H, Me-23), 0.78 (s, 3H, Me-24), 0.93 (s, 3H, Me-25), 0.82 (s, 3H, Me-26), 1.10 (s, 3H, Me-27), 0.86 (d, J = 6.5 Hz, 3H, Me-29), 0.95 (d, J = 6.5 Hz, 3H, Me-30).

^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$): 38.4 (C-1), 26.5 (C-2), 78.5 (C-3), 39.2 (C-4), 55.0 (C-5), 18.0 (C-6), 32.8 (C-7), 39.2 (C-8), 47.3 (C-9), 36.7 (C-10), 23.9 (C-11), 125.2 (C-12), 137.9 (C-13), 41.8 (C-14), 23.9 (C-15), 23.0 (C-16), 47.6 (C-17), 52.6 (C-l8), 38.8 (C-19), 38.7 (C-20), 30.4 (C-21), 36.6 (C-22), 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 180.5 (C-28), 16.7 (C-29), 20.9 (C-30).

DEPT 90 experiment CH ($CDCl_3/CD_3OD$): 78.5 (C-3), 55.0 (C-5), 47.3 (C-9), 125.2 (C-12), 52.6 (C-l8), 38.8 (C-19), 38.7 (C-20).

DEPT 135 experiment ($CDCl_3/CD_3OD$) CH_2: 38.4 (C-1), 26.5 (C-2), 18.0 (C-6), 32.8 (C-7), 23.9 (C-11), 23.9 (C-15), 23.0 (C-16), 30.4 (C-21), 36.6 (C-22); CH_3: 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 16.7 (C-29), 20.9 (C-30).

Quantification of ursolic acid content

The ursolic acid content was determined using the HPLC method as described by Chen *et al.* (2003). Dried hairy root powder (100 mg) was refluxed thrice with 50 ml of *n*-hexane for 1 h at 50 °C and filtered. Pooled *n*-hexane fractions were evaporated to dryness. For HPLC analysis, the residue was dissolved in 5 ml of acetonitrile, filtered through a 0.45 μm membrane prior to HPLC injection. An HPLC system (Agilent 1100 Series LC System, Agilent Technologies, Wilmington, USA) was equipped with a C18 reverse phase column (3.9 × 300 mm, 10 μm, Bondapak, Waters, USA) and a UV detector (photodiode array) set at 206 nm. Ursolic acid was eluted isocratically with acetonitrile/0.1% (v/v) H_3PO_4 in water (70:30, v/v) with a flow rate of 1 ml/min at 15.5 min. A calibration curve of authentic ursolic acid (Sigma-Aldrich) was established. Linearity of the calibration curve was observed in the range 15–120 μg/ml with r^2 of 0.9999 (% RSD of 0.09–0.45%). Each calibration point was established in triplicate.

Quantification of mitragynine content

The TLC-densitometric method was developed by Keawpradub (1990) for determination of the mitragynine content. The regenerated plants were harvested, dried at 50 °C for 12 h, ground and used as material for preparation of the crude alkaloid extract. The dried powder (200 mg) was refluxed with 50 ml methanol three times at 60 °C for 1 h and filtered. The filtrates were pooled and evaporated to dryness. The dried residue was re-dissolved in 30 ml of 7% (v/v) acetic acid/H_2O and filtered. The acidic filtrate was washed with petroleum ether, and then the solution was basified with 25% (v/v) ammonia solution to pH 9. The filtrate was partitioned with 50 ml chloroform three times.

The chloroform fractions were pooled and evaporated to dryness. The crude alkaloid extract was dissolved in 5 ml chloroform and the solution (5 μl) was subjected to a TLC plate (Silica gel F_{254}, Merck). The mobile phase was chloroform/methanol (9:1). The R_F-value of mitragynine was 0.7. After removing from the tank, the TLC plate was dried and placed in a CAMAG TLC scanner (Muttenz, Switzerland) equipped with Cats version 4.01 software. The UV detector was set at 254 nm, and peak areas were integrated and converted to concentrations using a calibration curve. The linearity of the calibration curve of authentic mitragynine was in the range of 0.3–5.0 mg/ml with R^2 of 0.9984. The peak identity was performed by scanning the UV absorption at 200–600 nm.

Spectroscopy

^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured with a Unity Inova NMR spectrophotometer (Varian, Darmstadt, Germany). A mixture of $CDCl_3$ and CD_3OD (1:1) was used as solvent and tetramethylsilane (TMS) was used as the internal standard.

Results

Hairy root cultures of M. speciosa

The *M. speciosa* hairy root culture was established by infection of 2-month-old plantlets with *A. rhizogenes* ATCC 15834. Optimization of the site of infection, leaf veins and stems was performed for the explants. The result suggested that an appropriate explant was from the leaf vein with a percentage of hairy root induction of 85%, while a percentage of hairy root induction of 67% was found when using the stems as the explants. The hairy roots obtained from leaf veins appeared as thin roots and contained small pubescent. In contrast, the hairy roots obtained from the stems contained nodules (undeveloped hairy roots) and thick roots. Genotypes of the hairy roots were identified for the rooting locus genes *rol*A and *rol*B by polymerase chain reaction. The presence of *rol*A and *rol*B genes of *A. rhizogenes* ATCC 15834 in the transformed hairy roots was accounted by 4/6 clones (Fig. 2).

Due to the slow growth rate of the hairy roots in WPM, other types of media were manipulated.

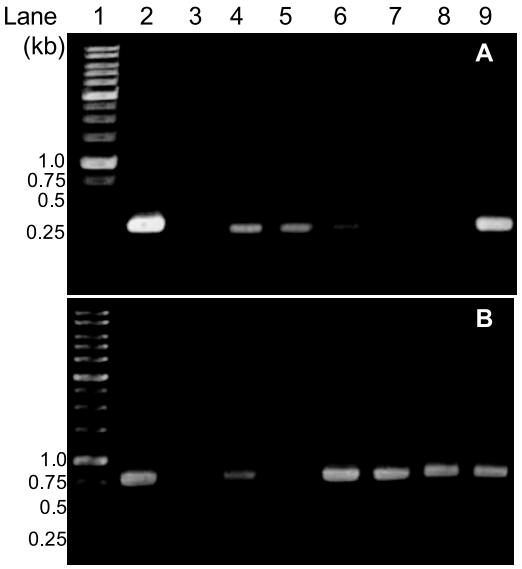

Fig. 2. PCR analyses of the hairy roots. PCR was performed with primers for the rooting locus genes (A) *rol*A (300 bp) and (B) *rol*B (780 bp). Lane 1, marker (10 kb DNA ladder); lane 2, *A. rhizogenes* ATCC 15834; lane 3, untransformed roots; lanes 4–9, transformed hairy roots (lines 1–6).

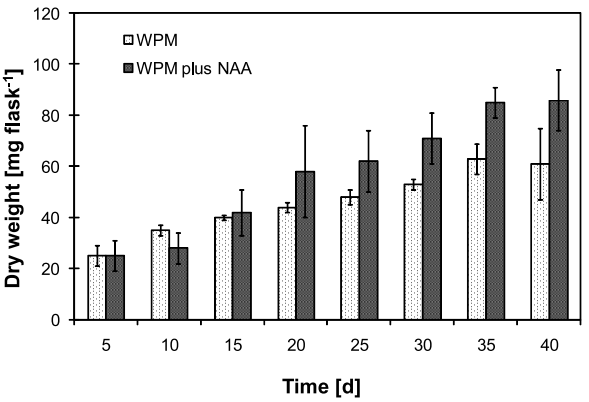

Fig. 3. Effect of NAA (0.5 mg/l) in WPM on the growth of the *M. speciosa* hairy root culture. Values are means of triplicate determinations. Error bars present standard deviation.

Hairy roots in half-strength WPM, B5 and MS media grew badly (data not shown). Comparison of the growth curves of the hairy roots in WPM and WPM plus NAA revealed that the addition of NAA (0.5 mg/l) to WPM stimulated the growth of hairy roots. As shown in Fig. 3, doubling time of the hairy roots was decreased from about 2

months of culture to about 30 d. The hairy roots were, therefore, maintained in WPM supplemented with NAA (0.5 mg/l) and used as materials for evaluation of secondary metabolites production.

Accumulation of triterpenoids and phytosterols in M. speciosa hairy roots

Preliminary screening by TLC after detection with Dragendorff's reagent showed that *M. speciosa* hairy roots were unable to produce alkaloids. To identify the secondary metabolites present in the hairy roots, an *n*-hexane extract was prepared and isolated by silica gel column chromatography. NMR data of MSF1 and MSF2 were accomplished by ^{1}H, 1D and 2D ^{13}C NMR analysis. Concerning the structure of MSF1, analysis of ^{1}H NMR data was in agreement with NMR data of published by Subhadhirasakul and Pechpongs (2005). From ^{13}C NMR spectra, peaks at 138.32 (C-22) and 129.21 (C-23) were observed. Both signals corresponded to the double bond in the structure of stigmasterol. Integration of the allylic protons H-22 and H-23 indicated that MSF1 was a mixture of β-sitosterol and stigmasterol, present in the ratio of 1:1.

The peaks at of δ 78.5 (C-3; COH), 125.2 (C-12), 137.9 (C-13), 180.5 (C-28; C=C) suggested that MSF2 contained ursolic acid (**2**). 2D-NMR: COSY, HMQC and HMBC indicated the correlation of ^{13}C-^{13}C, ^{1}H-^{13}C and long-length coupling ^{1}H-^{13}C, respectively. Analysis of NMR data of MSF2 was in agreement with a previous report on triterpenoids (Güvenalp *et al.*, 2006). The presence of the signal at δ 5.28 in the ^{1}H NMR spectrum indicated that MSF2 contained the isomer of ursolic acid, oleanolic acid. Integration of the H-12 signal in ^{1}H NMR spectrum indicated that MSF2 contained a mixture of ursolic acid and oleanolic acid in the ratio of 5:1.

To construct the growth curve and production curve, samples were taken every 5 d over a period of 40 d of culture. The ursolic acid content was determined from the hairy roots in comparison with the untransformed roots. The HPLC chromatograms of authentic ursolic acid, and the extracts from the untransformed roots and the hairy roots are shown in Fig. 4. The ursolic acid contents are summarized in Table I. The results indicated that both types of cultures were able to produce ursolic acid. It can be noted that the hairy roots, at 30 d, accumulated ursolic acid with the yield of (3.47 ± 0.03) mg/g dry weight (DW), whereas the untransformed roots produced (2.41 ± 0.01) mg/g DW.

Table I. Ursolic acid contents in transformed hairy roots and untransformed roots (n = 4).

Time [d]	Ursolic acid content [mg/g DW ± SD]	
	Transformed hairy roots	Untransformed roots
5	1.52 ± 0.00	1.58 ± 0.01
10	1.90 ± 0.02	1.74 ± 0.01
15	2.49 ± 0.02	1.89 ± 0.01
20	1.65 ± 0.01	1.69 ± 0.01
25	2.00 ± 0.04	2.09 ± 0.01
30	3.47 ± 0.03	2.41 ± 0.01
35	1.98 ± 0.03	1.74 ± 0.02
40	2.40 ± 0.01	2.01 ± 0.00

M. speciosa plant regeneration containing high yields of mitragynine

During the establishment of *M. speciosa* hairy root culture, shoot regeneration was observed when the hairy roots contained part of the stem (cut from the original explant). After 2 months, the regenerated shoots were removed and placed in the hormone-free solid WPM. The roots were then initiated after culture for 10 d to complete the plantlets. The regenerated plantlets were then used as materials for micropropagation. Axillary buds were excised from the stems and used as explants for shoot multiplication. Since the hairy roots have been obtained from infection with *A. rhizogenes* ATCC 15834, therefore, the regenerated plantlets were determined for the presence of the *rol*A and the *rol*B genes. However, the data indicated that the regenerated plantlets did not carry the genes from *A. rhizogenes.*

To increase the number of shoots in the *M. speciosa* plantlet, the axillary buds were excised and manipulated in solid WPM supplemented with three kinds of cytokinin. In this study, BA (1 mg/l) and TDZ (0.1 and 0.5 mg/l) were used for the shoot inductions. After 4 weeks, the shoot numbers were counted and calculated as the shoots number per explant. The results indicated that the shoot numbers of (2.8 ± 1.5), (6.3 ± 1.6) and (6.3 ± 1.3) per explants were obtained from WPM supplemented with 1 mg/l of BA, 0.1 mg/l of TDZ and 0.5 mg/l of TDZ, respectively. These results showed that TDZ was an appropriate plant growth

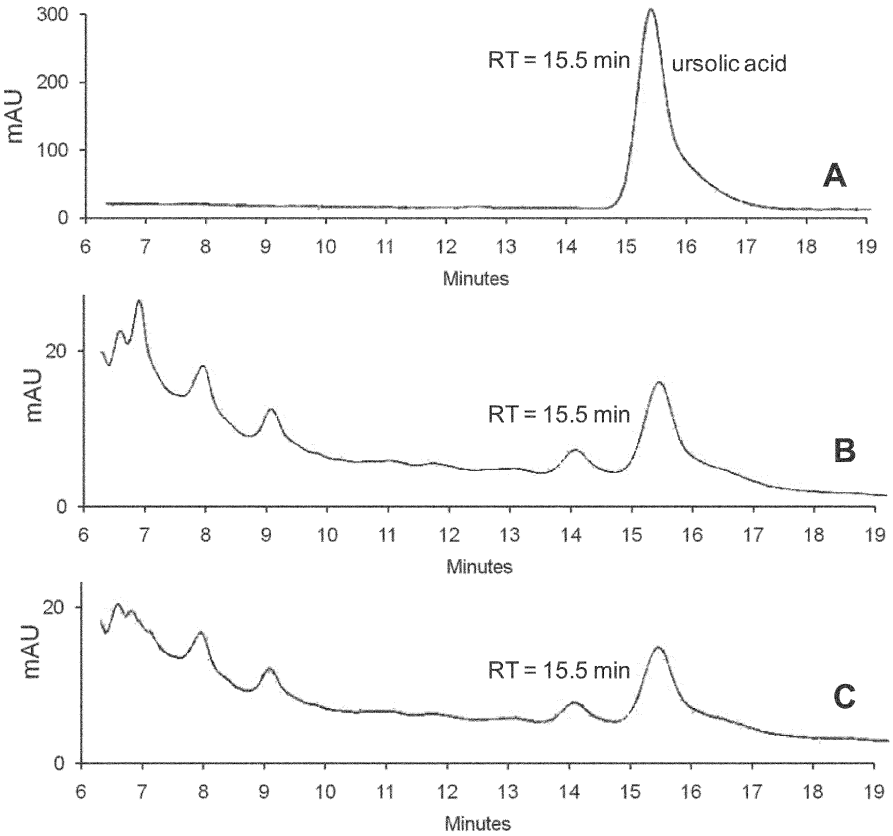

Fig. 4. HPLC chromatograms of (A) authentic ursolic acid, (B) the *n*-hexane extracts of the transformed hairy roots and (C) the untransformed hairy roots.

regulator for shoot multiplication. Root initiation of the regenerated plantlet of *M. speciosa* was simply performed. The shoots (containing 2–4 leaves) were cut from the explants and placed on hormone-free WPM. Roots were initiated after 1 week of culture.

To evaluate the mitragynine production in the regenerated plantlets, the *in vitro* plants, obtained from seeds germination in WPM supplemented with 1 mg/l BA and grown under controlled conditions, were used as control. Mitragynine contents of *in vitro* plantlets and regenerated plantlets were determined. A simple and rapid method of mitragynine quantification was established using the TLC-densitometric method. The extract from the whole plant of the *in vitro* and the regenerated plantlets (5 months old) were prepared for alkaloid extracts and determined for mitragynine contents. As shown in Table II, the mitragynine content in the regenerated plantlet was about 3.2 times higher than that in the *in vitro* plantlet. From this experiment it can be concluded that mitragy-

Table II. Mitragynine content determined from 5-month-old plants.

Sample	Mitragynine content[a] [mg/g DW ± SD]
In vitro plantlet[b]	4.45 ± 0.09
Regenerated plant[c]	14.25 ± 0.25
Leaves from regenerated plant	12.5 ± 0.25

[a] Samples were determined in triplicate.
[b] From plantlets that germinated from seeds and grew in WPM supplemented with BA (1 mg/l).
[c] From regenerated plants that were maintained in the hormone-free WPM.

nine was produced and accumulated mostly in leaves.

Discussion

The main objective of this investigation was to establish model plants for mitragynine biosynthesis in *M. speciosa*, a monoterpenoid indole alkaloid. Hairy root cultures were performed by infection with the wild-type *A. rhizogenes*. Since the susceptibility of plant cells to *A. rhizogenes* is dependent upon age and differentiation status of plant tissue (Sevón and Oksman-Caldentey, 2002), the hairy root culture used in this study was induced from the meristematic tissues such as stems and leaves due to ease of differentiation. Theoretically, the hairy root culture should grow relatively fast and in plant growth regulator-free medium (Guillon *et al.*, 2006). However, from this study, the hairy roots could grow only slowly and required a low concentration of NAA (0.5 mg/l) for promoting their growth. This evidence is unusual for a normal hairy root culture (Sevón and Oksman-Caldentey, 2002). Concerning case of other Rubiaceous plants, an *Ophiorrhiza pumila* hairy root culture in B5 medium [containing 2% (w/v) sucrose] could grow fast and produce camptothecin (Saito *et al.*, 2007). It can be suggested that the hormonal balance in *M. speciosa* hairy roots was not appropriate for root proliferation (Guillon *et al.*, 2006).

The hairy roots of *M. speciosa* could accumulate triterpenoids (ursolic acid and oleanolic acid) and phytosterols (β-sitosterol and stigmasterol) but none of the alkaloids were found in this culture. Nevertheless, many studies have reported that alkaloids such as mitraphylline and rhynchophylline have been found in the root bark of *M. speciosa* (Houghton and Shellard, 1974; Shellard *et al.*, 1978). It can be suggested that the intermediates of alkaloids were probably unstable and degraded during culture. However, the hairy roots accumulated ursolic acid, which is pharmacologically important for antibacterial, anti-inflammatory, antiviral activities, etc. (Liu, 1995). It is produced in the late linear phase at a yield of (3.47 ± 0.03) mg/g DW. It can be noted that *M. speciosa* hairy roots could accumulate high yields of ursolic acid when compared to a *Uncaria tomentosa* cell suspension culture [(1.68 ± 0.04) mg/g DW] (Feria-Romero *et al.*, 2005). From this evidence, it can be concluded that the organ culture such as hairy roots preferred to produce a higher amount of triterpenoids.

From the biosynthetic point of view, *M. speciosa* hairy roots could not produce any alkaloid, especially mitragynine. It may be caused by a lack of precursors and enzymes that are involved in the synthesis of monoterpenoid indole alkaloids, since primary metabolites need special cell compartments for storage and degradation (Luckner, 1990). The presence of ursolic acid, coexisting with oleanolic acid, indicated that at least isoprene units have been produced in this culture. These isoprene units are precursors for both triterpenoids and phytosterols (Luckner, 1990), which means that their biosynthesis was active in this culture. Therefore, the *M. speciosa* hairy root culture is not only useful for ursolic acid production but may also have the potential to be a model plant culture for triterpenoid biosynthesis. Unexpectedly, plantlet regeneration of *M. speciosa* was obtained. In this study, TDZ was shown to be an efficient cytokinin for shoot multiplication. TDZ acts as a substitute for both auxin and cytokinin, requirements to induce organogenesis and somatic embryogenesis in many plant species (He *et al.*, 2007). The regenerated plantlets produced and accumulated a considerable amount of mitragynine, when compared to the *in vitro* plantlets. It was postulated that this may be caused by the effect of TDZ. Nevertheless, it is still unknown.

Acknowledgement

This work was supported by grants from the Prince of Songkla University (PSU) and the strengthening program of the Faculty of Pharmaceutical Sciences, PSU (FY 2006). Partial financial support from The Thailand Research Fund (TRF, Grant no. RMU4980023) is also acknowledged.

Aji A. M., Effraim K. D., and Onyeyili A. P. (2001), Anti-stress activity of *Mitragyna africanus* (Wild) stem bark extract. Sciences **1**, 105–107.

Chen J. H., Xia Z. H., and Tan R. X. (2003), High-performance liquid chromatographic analysis of bioactive triterpenes in *Perilla frutescens*. J. Pharm. Biomed. Anal. **32**, 1175–1179.

Feria-Romero I., Lazo E., Ponce-Noyola T., Cerda-García-Rojas C. M., and Ramos-Valdivia A. C. (2005), Induced accumulation of oleanolic acid and ursolic acid in cell suspension cultures of *Uncaria tomentosa*. Biotechnol. Lett. **27**, 839–843.

Güvenalp Z., Kilic N., Kazaz C., Kaya Y., and Demirezer O. (2006), Chemical constituents of *Galium tortumense*. Turk. J. Chem. **30**, 515–523.

Guillon S., Tremouillaux G. J., Pati P. K., Rideau M., and Gantet P. (2006), Hairy root research: recent scenario and exciting prospects. Plant Biol. **9**, 341–346.

Harvala C. and Hinou J. (1988), Flavonol derivatives from the leaves of *Mitragyna speciosa*. Pharmazie **43**, 372.

He S. S., Liu C. Z., and Saxena P. K. (2007), Plant regeneration of an endangered medicinal plant *Hydrastis canadensis* L. Sci. Hort. **113**, 82–86.

Hinou J. and Harvala C. (1988), Polyphenolic compounds from the leaves of *Mitragyna speciosa*. Fitoterapia **59**, 156.

Houghton P. J. and Shellard E. J. (1974), The alkaloidal pattern in *Mitragyna rotundifolia* from Burma. Planta Med. **26**, 104–112.

Jansen K. L. R. and Prast C. J. (1988), Ethnopharmacology of kratom and the *Mitragyna* alkaloids. J. Ethnopharmacol. **23**, 115–119.

Keawpradub N. (1990), Alkaloids from the fresh leaves of *Mitragyna speciosa*. Master's Thesis, Graduate School, Chulalongkorn University, Bangkok, Thailand.

Liu J. (1995), Pharmacology of oleanolic acid and ursolic acid. J. Ethnopharmacol. **49**, 57–68.

Luckner M. (1990), Secondary Metabolism in Microorganisms, Plants and Animals, 2nd ed. Interdruck Graphischer Großbetrieb, Leipzig, Germany.

Said I. M., Chun N. C., and Houghton P. (1991), Ursolic acid from *Mitragyna speciosa*. Planta Med. **57**, 398.

Saito K., Sudo H., Yamazaki M., Koseki-Nakamura M., Kitajima M., Takayama H., and Aimi N. (2007), Feasible production of camptothecin by hairy root culture of *Ophiorrhiza pumila*. Plant Cell Rep. **20**, 267–271.

Sevón N. and Oksman-Caldentey K. M. (2002), *Agrobacterium rhizogenes*-mediated transformation: Root cultures as a source of alkaloids. Planta Med. **68**, 859–868.

Shellard E. (1974), Alkaloids of *Mitragyna* with special reference to those of *Mitragyna speciosa*. Bull. Narcotics **26**, 41–55.

Shellard E. J., Houghton P. J., and Resha M. (1978), The *Mitragyna* species of Asia, part XXXII. The distribution of alkaloids in young plants of *Mitragyna speciosa* Korth. grown from seed obtained from Thailand. Planta Med. **34**, 253–263.

Subhadhirasakul S. and Pechpongs P. (2005), Terpenoid and two steroids from *Mammea siamensis*. Songklanakarin J. Sci. Technol. **27**, 555–561.

Thongpraditchote S., Matsumoto K., Tohda M., Takayama H., Aimi N., Sakai S., and Watanabe H. (1998), Identification of opioid receptor subtypes in antinociceptive actions of supraspinally-administered mitragynine in mice. Life Sci. **62**, 1371–1378.

Tsuchiya S., Miyashita S., Yamamoto M., Horie S., Sakai S. I., Aimi N., Takayama H., and Watanabe K. (2002), Effect of mitragynine, derived from Thai folk medicine, on gastric acid secretion through opioid receptor in anesthetized rats. Eur. J. Pharmacol. **443**, 185–188.

Watanabe K., Yano S., Horie S., and Yamamoto L. T. (1997), Inhibitory effect of mitragynine, an alkaloid with analgesic effect from Thai medicinal plant *Mitragyna speciosa*, on electrically stimulated contraction of isolated guinea-pig ileum through the opioid receptor. Life Sci. **60**, 933–942.