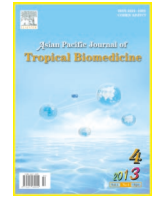




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The identities and anti-herpes simplex virus activity of *Clinacanthus nutans* and *Clinacanthus siamensis*

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Comments

This is a good study providing the scientific interesting information and development of the natural product for antiviral drug. The result obtained from this study indicated that combination of pharmacognostic and molecular data are suitable for identification and these Thai herbal medicines have a potential for development of antiviral agent deriving from the natural product.

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ABSTRACT

Objective: To distinguish the difference among the *Clinacanthus nutans* (Burm. f.) Lindau (*C. nutans*) and *Clinacanthus siamensis* Bremek (*C. siamensis*) by assessing pharmacognosy characteristics, molecular aspect and also to evaluate their anti-herpes simplex virus (HSV) type 1 and type 2 activities. **Methods:** Macroscopic and microscopic evaluation were performed according to WHO Geneva guideline. Stomatal number, stomatal index and palisade ratio of leaves were evaluated. Genomic DNA was extracted by modified CTAB method and ITS region was amplified using PCR and then sequenced. Dry leaves were subsequently extracted with *n*-hexane, dichloromethane and methanol and antiviral activity was performed using plaque reduction assay and the cytotoxicity of the extracts on Vero cells was determined by MTT assay. **Results:** Cross section of midrib and stem showed similar major components. Leaf measurement index of stomatal number, stomatal index and palisade ratio of *C. nutans* were 168.32±29.49, 13.83±0.86 and 6.84±0.66, respectively, while *C. siamensis* were 161.60±18.04, 11.93±0.81 and 3.37±0.31, respectively. The PCR amplification of ITS region generated the PCR product approximately 700 bp in size. There were 34 polymorphisms within the ITS region which consisted of 11 Indels and 23 nucleotide substitutions. The IC₅₀ values of *C. nutans* extracted with *n*-hexane, dichloromethane and methanol against HSV-1 were (32.05±3.63) µg/mL, (44.50±2.66) µg/mL, (64.93±7.00) µg/mL, respectively where as those of *C. siamensis* were (60.00±11.61) µg/mL, (55.69±4.41) µg/mL, (37.39±5.85) µg/mL, respectively. Anti HSV-2 activity of *n*-hexane, dichloromethane and methanol *C. nutans* leaves extracts were (72.62±12.60) µg/mL, (65.19±21.45) µg/mL, (65.13±2.22) µg/mL, respectively where as those of *C. siamensis* were (46.52±4.08) µg/mL, (49.63±2.59) µg/mL, (72.64±6.52) µg/mL, respectively. **Conclusions:** The combination of macroscopic, microscopic and biomolecular method are able to authenticate these closely related plants and both of them have a potency to be an anti-HSV agent.

KEYWORDS

Clinacanthus nutans, *Clinacanthus siamensis*, Microscopic analysis, Biomolecular analysis, Internal transcribed spacer, Herpes simplex virus, Plaque reduction assay

1. Introduction

Medicinal plants are the source of a large number of chemical compounds used as drug in primary health care of the world's population since prehistoric times. *Clinacanthus nutans* (Burm. f.) Lindau (*C. nutans*) and *Clinacanthus siamensis* Bremek. (*C. siamensis*) are medicinal plants in family Acanthaceae which can be found in Thailand^[1]. The fresh leaves have been used for relieving of insect bites

and skin rashes, and extracts from fresh leaves have been traditionally used for treatment of herpes simplex virus (HSV) infection in primary health care^[2,3]. Since a number of experiments have reported for their antiviral activities, *C. nutans* (Thai name: Phaya Yo) is suggested to be used as antiviral agent against HSV. *C. siamensis* is known in Thailand by the name of "Lin ngu hao". It is found throughout Thailand and used as traditional medicine to relieve painful swelling. Several phytochemical studies

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on *C. nutans* have been reported but none on *C. siamensis*. Acyclovir (ACV) is an effective drug used for HSV infection. The widespread use of ACV may lead to drug resistant[4,5]. Screening the HSV inhibitory agents from herbal medicine that are reputed to have this efficacy may be useful in order to obtain an alternative agent for inhibiting the HSV infection and the patients may not have to spend expensively for the antiviral drug.

Both *C. nutans* and *C. siamensis* are in the same genus *Clinacanthus*, and some of their morphologies are similar to each other. Traditionally authentication of medicinal plant relies upon morphological, physical and chemical inspection. Pharmacognosy evaluation using multidisciplinary characteristics such as macroscopic, microscopic and chemical fingerprinting can be used for plant authentication. However, many closely related species have similar morphology and chemical analysis may be limited by the amount of material, growth conditions as well as many other variables such as the storage condition and the harvest processing. Therefore, additional DNA based methods have been developed for medicinal plants authentication and DNA markers have now become a popular mean for identification and authentication of plant species due to the less effects caused by age, physiological condition and environment factors. Moreover, small amount of sample is sufficient for analysis and can be detected at any phase of plant development[6]. Sequence variation of the internal transcribe spacer (ITS) region in the nuclear DNA has been applied widely for phylogenetic evaluation at species level and can be developed to be a pattern for identification of herbal plants. Even more, this method can be further applied for investigation the adulteration of herbal drugs[7].

Due to their similar morphologies and the reports about identification and efficacies on anti-HSV-1 and HSV-2 activity of these two medicinal plants are quite limit. Hence, the aim of this present study has been conducted using pharmacognotic characteristic and sequence variation in the ITS region in order to distinguish between *C. nutans* and *C. siamensis* and assessing their *in vitro* antiviral activity against HSV-1 and HSV-2.

2. Materials and methods

2.1. Plants

C. nutans (Burm. f.) Lindau and *C. siamensis* Bremek. were collected from different sources of Thailand. The plants were authenticated by the expert-Associate Prof. Dr. Nijisiri Ruangrungsi and deposited at College of Public Health Sciences, Chulalongkorn University.

2.2. Macroscopic and microscopic evaluation

The mature branch including flower of *C. nutans* and *C. siamensis* was collected for macroscopic observation and illustrated on drawing paper with pencil and black

ink pen. Microscopic evaluation was carried out under the appropriate magnitude using a photomicroscope attached with digital camera (Canon Power Shot A640). The stem and midrib from mature leaf were crossed section into thin pieces, mounted onto a slide and then observed under microscope. The recorded images were illustrated in a size proportional to the original. Leaf measurement was followed the method described in Pulok with some modifications[8]. The mature leaves were clearly bleached by boiling in sodium hypochlorite solution (sodium hypochlorite: water, 1:1, v/v). Then, cut the small piece of leaf on the center next to the midrib and peeled the lower side of the leaf then, evaluated under microscope. The stomatal number was counted within a 1 mm² for 30 fields, and stomatal index was calculated. To determine the palisade ratio, after bleaching process, the fraction of leaf was placed uppermost on the slide, evaluated under microscope and counted the round light green palisade cells in four continuous boundary epidermal cells then, calculated for the proportion of palisade cells in one epidermal cell. The stomatal number, stomatal index and palisade ratio were presented as mean±SD.

2.3. Molecular evaluation

Genomic DNA of individual young leaves of plant samples were extracted by modified CTAB method described by Dellaporta and used as DNA template for PCR amplification[9]. The ITS region (including ITS1–5.8s –ITS2) was amplified using the universal ITS5 forward primer (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 reverse primer (5′-TCC TCC GCT TAT TGA TAT GC-3′)[10]. PCR amplification was carried out in 20 µL reaction mixture containing approximately 1–2 µL of genomic DNA, 1×PCR Buffer (100 mmol/L KCl, 20 mmol/L Tris-HCl, pH 8.0), 2.5 mmol/L MgCl₂, 100 µmol/L dNTPs, 0.2 µmol/L of each primers and 0.5 units/µL of *Taq* polymerase (Promega). The amplified was carried out under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C denaturation for 30 seconds, 55 °C annealing for 30 seconds, and 72 °C extension for 30 seconds, then followed by final extension at 72 °C for 5 min. Five microliter of PCR products were evaluated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transilluminator. The PCR products were then purified by QIAquick PCR purification kit (QIAGEN) prior sequencing. The ITS sequences from sense and antisense were analyzed using CLC DNA Workbench 6.0.

2.4. Anti-herpes simplex virus type 1 and type 2 activities evaluation

Ten grams of dried powdered leaves of *C. nutans* and *C. siamensis* was subsequently extracted first with *n*-hexane (400 mL), then dichloromethane (400 mL) and finally with methanol (400 mL) until exhausted in soxhlet extraction apparatus. All the three fractions were evaporated at the temperature not exceeding 40 °C to give the corresponding *n*-hexane fraction, dichloromethane fraction and

methanol fraction. The crude extracts were dissolved in dimethylsulfoxide (DMSO) in the stock solution of 1000 µg/mL then two-fold serially diluted with minimum essential media (MEM) to give the desired concentration ranging from 100 µg/mL to 12.5 µg/mL. Acyclovir (ACV, Sigma) was used as the positive control.

Herpes simplex virus type 1 (KOS) and type 2 (Baylor 186) and Vero cells were obtained from Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Vero cells were cultured in MEM supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics.

The plaque reduction assay was performed by modified method described by Phrutivorapongkul *et al.*^[11]. Vero cells (10⁵ cells/mL) in 96-well tissue culture plate in triplicate were infected with 30 plaque forming unit (PFU)/25 µL per well of HSV-1 (KOS) or HSV-2 (Baylor 186). After 1 h inoculation at room temperature for virus adsorption, the overlay media containing various concentrations of the plant extracts were added. The infected cultures were further incubated at 37 °C for 2 d. The infected cells were fixed and stained with 5% (v/v) formalin and 0.03% (v/v) methylene blue solution. The numbers of plaques were counted and the percent inhibition was determined compared with the control. The 50% inhibitory concentration (IC₅₀) was determined from the curve relating the plaque number to the concentration of the extract compared to acyclovir.

The cytotoxicity assay of plant extracts was evaluated. The culture medium containing the extract at various concentrations ranging from 100–1600 µg/mL were added in Vero cells monolayer in 96-well tissue culture plate and cells were further grown at 37 °C for 2 d. After incubation, the viability of cells was evaluated by MTT assay^[12,13]. The percentage of cell viability was evaluated by comparing to the number of cell control. The concentration of the extract reducing cell viability by 50% (50% cytotoxicity concentration, CC₅₀) was determined. The selectivity index (SI) was also determined by the ratio between the CC₅₀ and IC₅₀ value. The higher of SI indicated the potential of safer therapy of the given extract.

3. Results

3.1. Macroscopic and microscopic evaluation

The morphological evaluation of *C. nutans* and *C. siamensis* was illustrated and described in Figure 1. *C. nutans* is a shrub with short hair branches. Leaves are pale green, simple, opposite, narrowly elliptic-oblong with acute apex size 2.5–13.0 cm long and 0.5–1.5 cm wide. There are 6–7 pairs of side veins and quite prominent below. The stem is straight green with white internodes and vertical strips throughout the entire stem. Flowers are in dense cymes at the top of the branches, often terminating drooping horizontal branches but themselves erect. Each flower has glandular-pubescent calyx, corolla glandular-pubescent, about 3.5 cm, dull red with green base and yellow streaks on

lower lip. There are 2 stamens appressed against the upper lip.

Microscopically, the stomata of both plants could be found only on the lower epidermis of the leaf with other components (glandular trichomes, lithocysts). The plant stomata was classified as diacytic type which the stomata was accompanied by two subsidiary cells at right angle to it and the epidermal cell components were illustrated in Figure 2. The average stomatal number, stomatal index in the area of 1 mm² and palisade ratio of *C. nutans* were 168.32±29.49, 13.83±0.86 and 6.84±0.66, respectively while *C. siamensis* were 161.60±18.04, 11.93±0.81 and 3.37±0.31, respectively. The stem cross section of *C. nutans* and *C. siamensis* revealed that there were lithocysts interposed in the cortical parenchyma which lies beneath epidermis. Groups of collenchyma lies above parenchyma of cortex and pinniform parenchyma which is located above the phloem tissues. The xylem fibers align gather in group and were interposed longitudinally with parenchyma ray. There was xylem vessels with a wide lumen interposed sparsely in the xylem fibers. The cells in the ground tissue were polygonal shape in various sizes (Figure 3).

Midrib cross section of *C. nutans* and *C. siamensis* revealed multicellular and glandular trichomes on the epidermis which was interposed sparsely with lithocysts. Collenchyma located next to the epidermis. Palisade mesophyll and chlorenchyma lied next to the collenchyma. Xylem vessels arranged longitudinally and were interposed by parenchyma ray in the central portion of the polygonal shape parenchyma. Phloem tissue lied semicircular next to the xylem vessels in the central portion of the parenchyma (Figure 4).

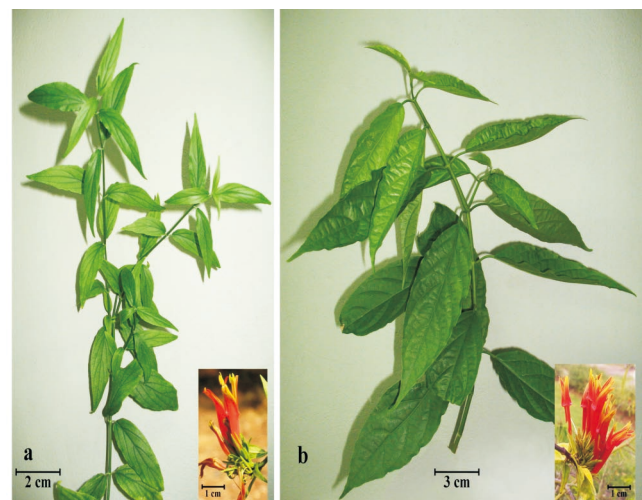


Figure 1. Branch and flower illustration of *C. nutans* and *C. siamensis*.
a: *C. nutans*; b: *C. siamensis*.

3.2. Molecular evaluation

Universal PCR primers designed from highly conserved regions flanking the ITS region was used for PCR amplification and generated the PCR product approximately 700 bp in size. Sequence analysis of *C. nutans* from 3

different locations (CN–MU, CN–CU, CN–NS) showed 98% homology and there were 7 and 11 single nucleotide polymorphism (SNP) within the ITS1 and ITS2 region, respectively. Where those of *C. siamensis* (CS–MU, CS–CU, CS–CS) showed 97% homology and there were 7 and 15 SNPs within the ITS1 and ITS2 region were observed. Sequence comparison of ITS region between *C. nutans* and *C. siamensis* showed 97% homology and 68% GC content. There were 34 polymorphisms within the ITS region which consisted of 11 Indels and 23 nucleotide substitutions.

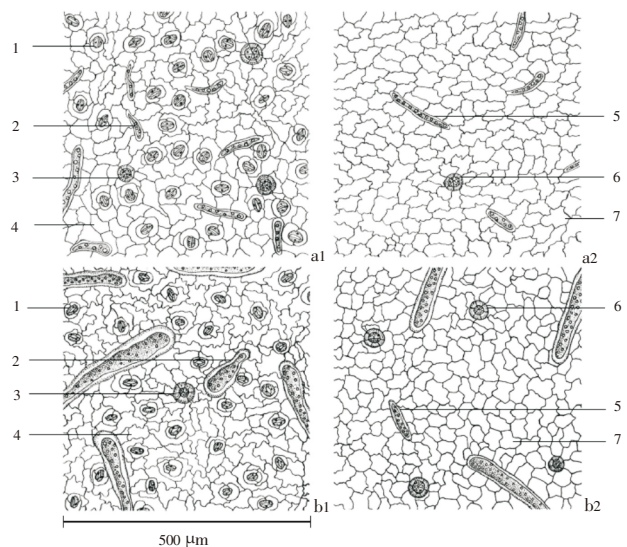


Figure 2. Epidermis of the leaf of *C. nutans* and *C. siamensis* with 20× magnification. a: *C. nutans*; b: *C. siamensis*; a1, b1: lower epidermis; 1: diacytic type stoma; 2: lithocyst; 3: glandular trichome; 4: epidermal cell; a2, b2: upper epidermis; 5: lithocyst; 6: glandular trichome; 7: epidermal cell.

3.3. Anti-herpes simplex virus type 1 and type 2 activities

Three extracts were isolated successively from the leaves of *C. nutans* and *C. siamensis* ranging from the least polar to the most polar (*n*-hexane, dichloromethane and methanol, respectively). The crude extracts yield of *C. nutans* and *C. siamensis* obtained from *n*-hexane were 0.36 g (3.58% w/w) and 0.16 g (1.59% w/w), respectively, dichloromethane crude extract of *C. nutans* and *C. siamensis* were 0.16 g (1.59% w/w) and 0.24 g (2.39 % w/w) respectively and methanol crude extract of *C. nutans* and *C. siamensis* were 1.72 g (17.15% w/w) and 1.70 g (16.95 % w/w) of dry weight of plant material, respectively.

Table 1

IC₅₀, SI and CC₅₀ of *C. nutans* and *C. siamensis* extracts against HSV-1 and HSV-2.

Extracts	<i>C. nutans</i>					<i>C. siamensis</i>				
	IC ₅₀ (µg/mL, mean±SD)		SI		CC ₅₀ (µg/mL)	IC ₅₀ (µg/mL, mean±SD)		SI		CC ₅₀ (µg/mL)
	HSV-1	HSV-2	HSV-1	HSV-2		HSV-1	HSV-2	HSV-1	HSV-2	
<i>n</i> -hexane	32.05±3.63	72.62±12.60	>50.36	>22.50	>1600	60.00±11.61	46.52±4.08	>27.31	>34.53	>1600
Dichloromethane	44.50±2.66	65.19±21.45	19.57	14.09	869	55.69±4.41	49.63±2.59	3.50	3.92	194
Methanol	64.93±7.00	65.13±2.22	>24.84	>24.59	>1600	37.39±5.85	72.64±6.52	>43.52	>22.14	>1600

To determine the anti-HSV-1 and HSV-2 activities, Vero cells were infected with 30 PFU/25 µL of HSV-1 (KOS) or HSV-2 (Baylor186). After incubation, the infected cells were fixed and stained and then the numbers of plaques were counted. Percent inhibition of plaque formation and therapeutic index were determined as shown in Table 1.

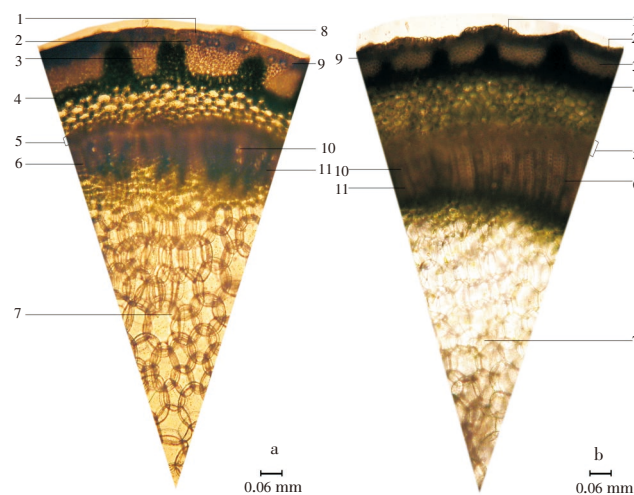


Figure 3. Stem cross section of *C. nutans* and *C. siamensis*. a: *C. nutans*; b: *C. siamensis*; 1: epidermis; 2: lithocyst; 3: group of collenchyma; 4: parenchyma of cortex; 5: phloem tissues; 6: parenchyma ray; 7: ground tissue; 8: multicellular trichome; 9: cortical parenchyma; 10: xylem vessel; 11: xylem fiber.

All extractions of the two species at concentration of 100 µg/mL presented the anti HSV-1 and HSV-2 activities with more than 50% inhibition of plaque formation (30 PFU/25 µL). The lowest IC₅₀ values of *C. nutans* was (32.05±3.63) µg/mL of *n*-hexane extract that inhibited HSV-1 with selectivity index>50.36 and (65.13±2.22) µg/mL of methanol extract that inhibited HSV-2 with selectivity index>24.59 while those of *C. siamensis* was (37.39±5.85) µg/mL of methanol extract that inhibited HSV-1 with selectivity index>43.52 and (46.52 ±4.08) µg/mL of *n*-hexane extract that inhibited HSV-2 with selectivity index>34.53. Compared to acyclovir which was used as a positive control in plaque reduction assay expressed the IC₅₀ value at (0.09±0.02) µg/mL against HSV-1 and (0.43±0.04) µg/mL against HSV-2 (data not shown). Fifty percent cytotoxicity concentration (CC₅₀) of the extracts to Vero cells was also investigated. Among all extracts, dichloromethane extract of the both plants exhibited the highest value (869 and 194 µg/mL for *C. nutans* and *C. siamensis*, respectively). However, the highest IC₅₀ value that could inhibit the viral activity possessed the cytotoxicity in Vero cells.

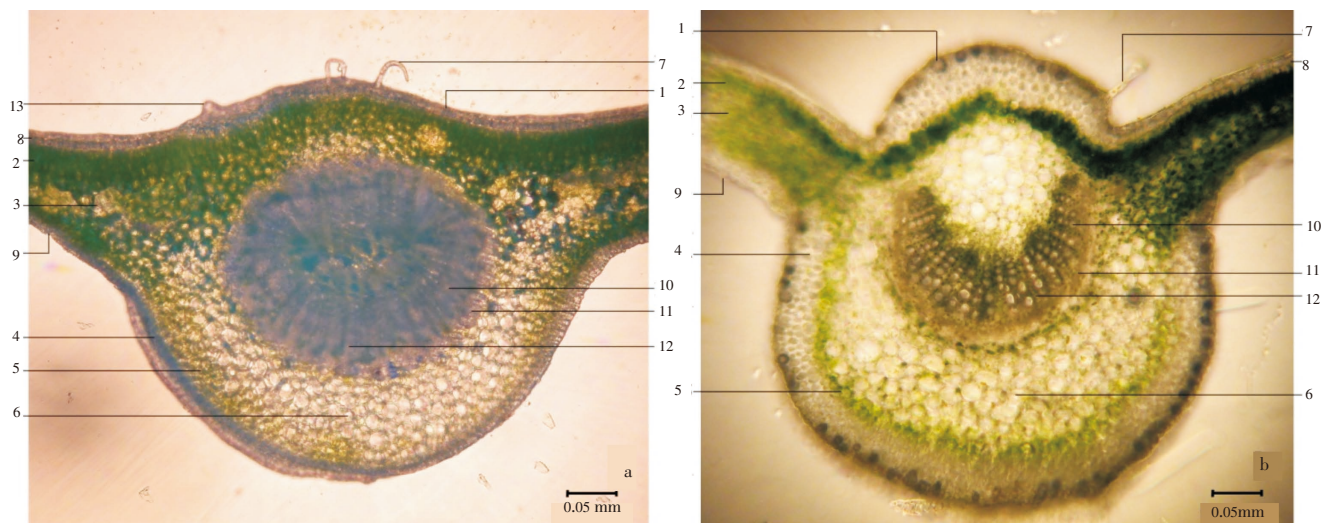


Figure 4. Midrib cross section of *C. nutans* and *C. siamensis*.

a: *C. nutans*; b: *C. siamensis*; 1: lithocyst; 2: palisade mesophyll; 3: spongy mesophyll; 4: collenchyma; 5: chlorenchyma; 6: parenchyma; 7: multicellular trichome; 8: epidermis; 9: stoma; 10: xylem vessel; 11: phloem tissue; 12: parenchyma ray; 13: glandular trichome.

4. Discussion

Human beings use plants as medicine for the treatment of various diseases for thousands of year. It is essential to use the right herbal material for the correct pharmacological effect. Correct identification and quality control of the starting material is essential for herbal preparation. There are many methods for medicinal plant authentication. Morphological assessment is an effective tool for determining the identity of plant material as it is fast and inexpensive. However, it is subjective and depends on experience of the examiner when closely related species are involved. Since most herbal medicines are used as processed material and crude extracts, various methods have been developed for separation and identification. The use of chemical techniques to standardize and quality control has some limitation because of their variable sources and chemical complexity. Therefore, additional of DNA technologies have been applied for medicinal plant authentication and combinations of various methods have been employed for herbal drug technology development. The present study deals with the discrimination between the two closely related plants in genus *Clinacanthus* by using macroscopic, microscopic and molecular methods. According to the results, macroscopic and microscopic analysis of these two closely related plants revealed the similar morphology and cell components. However, the leaf measurement index (stomatal number, stomata index and palisade ratio) which are the important property for species identification showed different constant numbers. Similar to the previously pharmacognostic study indicated that the value of leaf measurement of *C. siamensis* leaf differ from *C. nutans* especially, the value of palisade ratio^[14]. There have been previous reports using leaf measurement index for identification of medicinal plants^[15,16]. In addition, molecular markers have been utilized in medicinal plants identification due to their powerful in elucidating the identity of the substitutes and adulterants of medicinal materials that often introduced intentionally or accidentally. Identifying of specific regions in the DNA can be used for different level of authentication as the genetic composition

is unique for each species and is not affected by physical and environmental factors^[17,18]. The ITS region is widely used in plant taxonomy and molecular phylogeny because it has a high degree of variation even between closely related species^[19]. According to the results, the ITS regions between *C. nutans* and *C. siamensis* are similar to those previously published, with sequence lengths of approximately 700 bp. Typically, published ITS1 sequences were approximately 300 bp, while ITS2 were 400–600 bp with the conservative 5.8S rDNA sequences. Due to the limitation in published ITS sequence of *C. nutans* and *C. siamensis*, comparable with that of traditional classification is still doubtful and need to be further investigated in number. However, ITS sequences are capable of providing many variable information, which plays a more important role in the authentications of medicinal herbs. The ITS sequences of *C. siamensis* showed considerable variations due to the high level of intra-individual variation. The sequence of the ITS region in this study were almost identical between *C. nutans* and *C. siamensis* (97–99% similarity) indicating closely related species and ITS region may not be suitable molecular marker for differentiation between *C. nutans* and *C. siamensis*. Not only the sequence variation of the ITS region was suitable for identification of various medicinal plants^[17–19], but other coding and spacer regions of nuclear, chloroplast and mitochondrial genes such as *rbcL*, *matK* and *trnK* gene have been also explored and used as molecular markers for authentication^[20–22]. It is generally accepted that multi-locus combinations will be required for species discrimination^[23]. DNA based techniques have been extensively applied in traditional Chinese medicine as well as Asian traditional medicine such as Ginseng^[17,24], especially in case of the identification of substituted or adulterated with other species that morphologically and phytochemically indistinguishable. In Thai medicinal plants, molecular markers were also extensively used for the identification of an important medicinal plants remedy^[25].

HSV infection is commonly found worldwide. ACV and other nucleoside derivatives have been used for treatment of HSV infections. A large number of anti-HSV agents from medicinal plants have been previously reported in several

studies^[26–28], it was found that natural products from medicinal plant extracts are good and potential sources for promising anti-HSV drug including *C. nutans* and *C. siamensis*. Twenty medicinal plant extracts from Thailand have been evaluated for their anti-HSV activity, 11 out of 20 inhibited more than 50% plaque formation of HSV-1^[29]. Methanolic extracted of *C. siamensis* was shown to have more than 90% antiviral activity against HSV-1 and HSV-2 at the concentration 50 µg/mL^[30]. Moreover a large number of bioactive constituents of the extracts from *C. nutans* and *C. siamensis* leave, like polyphenolic, glycoside, terpenes, were found to be promising anti-HSV agents^[30–32]. Three chlorophyll derivatives isolated from the chloroform extract of *C. nutans* leaves were individually tested for anti-HSV activity and the results indicated that these compounds have anti herpes simplex activity in pre-viral entry step^[33]. There were various assays to study on antiviral activities of medicinal plants, Plaque reduction assay has always been used for *in vitro* antiviral activities. In this study, the result indicates that *n*-hexane, dichloromethane and methanol extracts of *C. nutans* and *C. siamensis* dry leaves can inhibit the plaque forming of both HSV-1 and HSV-2. The obtained results revealed that methanolic extract of *C. nutans* exhibit only a slight anti-HSV-1 activity ($IC_{50}=64.93$ µg/mL) compare to the *n*-hexane ($IC_{50}=32.05$ µg/mL) and selectivity index, determined by the CC_{50} to IC_{50} against HSV-1 was >50.36 . By contrast, methanolic extract of *C. siamensis* exhibited the strongest activity against HSV-1 ($IC_{50}=37.39$ µg/mL) with selectivity index value >43.52 while the *n*-hexane exhibit only a slight anti-HSV-1 activity ($IC_{50}=60.00$ µg/mL). Comparison to the anti-HSV-2 activity, *n*-hexane, dichloromethane and methanol extracts of *C. nutans* exhibited slight activity (72.62, 65.19, 65.13 µg/mL, respectively) whereas *n*-hexane and dichloromethane exhibited better activity (46.52 and 49.63 µg/mL) than methanolic extract (72.64 µg/mL) of *C. siamensis*. Many variables can affect the IC_{50} value obtained in the test, these include host cell line, viral inoculums titer, strain of virus, incubation time. Control HSV strains with known IC_{50} value must be include in each test. By comparing the ability of *C. nutans* and ACV to inactivate HSV-2, the results showed that the ethanolic extracts of *C. nutans* was able to inhibit plaque formation by HSV-2 in baby hamster kidney cell line and it was further tested for clinical trial in patients with genital herpes, in which the result exhibited that using the extract, the lesion could completely heal within 7 days which is much greater than the placebo group^[34]. In contrast to the study done by YooSook, organic solvent extracts of *C. nutans* did not show anti HSV-2 activity^[28]. The results in study of HSV-1 resistant to either ACV or foscarnet from *C. siamensis* ethanolic extract suggested that *C. siamensis* ethanolic extract had effect to the herpes simplex virus ACV-resistant AR3 with IC_{50} at 62.5 µg/mL.

In conclusion, macroscopic, microscopic and biomolecular method are able to authenticate the closely related plants; *C. nutans* and *C. siamensis*. Accordingly, these medicinal plants can be a source for isolation of anti-HSV compounds and the extraction of these medicinal plants exhibit antiviral activity against both HSV-1 and HSV-2, suggesting that

these Thai medicinal plants have a potential to be an antiviral agent.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Both *C. nutans* and *C. siamensis* are closely related species and have similar morphology. Assessing their identities by using pharmacognostic, molecular aspects and their antiviral activity are interesting.

Research frontiers

Studies are being performed in order to identify the characteristics of two closely related medicinal plants; *C. nutans* and *C. siamensis* by using many aspects in pharmacognostic, molecular and biological activity for identification.

Related reports

According to the previous investigation, there are some information about these two medicinal plants. However, comparison of their characteristics by using several aspects still limited.

Innovations and breakthroughs

This study aims to distinguish the similar characteristics of two closely related medicinal plants using difference characteristics such as macroscopic, microscopic, DNA sequence and biological activity in order to differentiate these two plants.

Applications

This study provides the information regarding to the comparison of two closely related plants species and their potential to be an anti-viral agent from Thai medicinal plant.

Peer review

This is a good study providing the scientific interesting information and development of the natural product for antiviral drug. The result obtained from this study indicated

that combination of pharmacognostic and molecular data are suitable for identification and these Thai herbal medicines have a potential for development of antiviral agent deriving from the natural product.

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