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Facile Isolation and Purification of Thailandine, a Biologically Active Oxoaporphine Alkaloid, from *Stephania venosa* Leaves using Ion-pair Liquid-liquid Extraction

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ABSTRACT

Thailandine is a quaternary oxoaporphine alkaloid isolated from *Stephania venosa*. This compound has shown highly potent and broad range of biological effects, including cytotoxic, antimicrobial, antimalarial (*Plasmodium falciparum*, K1 strain) and antitubercular (*Mycobacterium tuberculosis* $H_{37}R_a$) activities. In this study, ion-pair extraction was applied to isolate and purify thailandine. The proposed method involved the extraction of dried *S. venosa* leaves with methanol. After solvent removal, the crude extract was dissolved in water and partitioned thrice with dichloromethane (1:1 v/v) to remove non and semi-polar substances. Dichloromethane was discarded and a volatile ion-pair reagent, trifluoroacetic acid, was added to the aqueous layer to form an ion-pair substance with thailandine. The ion-pair product was then transferred to the dichloromethane layer. After dichloromethane removal, thailandine was obtained as a red precipitate. Purity and identification were determined using NMR and ESI-HRMS. The results indicated that the proposed method could provide thailandine with high purity. The conditional optimizations showed that using 24 h maceration with methanol at room temperature for extraction and 1% v/v trifluoroacetic acid for ion-pair forming provided thailandine with the optimum yield of 0.18% based on dried weight. This study demonstrated the successful utilization of the ion-pair liquid-liquid extraction for thailandine isolation and purification.

Key words: Ion-pair liquid-liquid extraction, thailandine, *Stephania venosa*, oxoaporphine

INTRODUCTION

Stephania venosa, a member of the family Menispermaceae, is native to eastern and southern Asia, including Australia. This species is an herbaceous perennial vine with a large tuber on the ground. Its tubers have long been used in Thai traditional medicine for nerve tonics, aphrodisiac and appetizers, as well as for treatment of asthma, microbial infection and cancer. *S. venosa* leaves have been used to treat cancer, ringworm, tinea versicolor, scabies, chronic and fresh wound, melasma and acne (Boonyaprapat and Chokchaijarunporn, 1996). *S. venosa* has been reported to have a broad range of biological effects. The ethanolic extract of the tubers inhibit mushroom

tyrosinase, anti-inflammatory activity and also active against various microbial enteropathogens and dermatopathogens (Potduang *et al.*, 2005). The extract revealed antiproliferative activity at an IC_{50} of 40 μg mL^{-1}. Montririttigri *et al.* (2008) reported the inhibition of the extract to cell proliferation and apoptosis against SKOV3 human ovarian cancer cell line. Significant cytotoxic effects of the ethanolic extract were displayed against the breast adenocarcinoma cell line (MCF-7) (Keawpradub *et al.*, 2001) and the human small cell lung cancer (NCI-H187) (Leewanich *et al.*, 2011).

Previous studies reported the isolation of several isoquinoline alkaloids from the *S. venosa* tubers (Guinaudeau *et al.*, 1981, 1982; Pharadai *et al.*, 1985; Charles *et al.*, 1987; Banerji *et al.*, 1994; Likhitwitayawuid *et al.*, 1999; Ingkaninan *et al.*, 2006; Nantapap *et al.*, 2010; Makarasen *et al.*, 2011). Several of these alkaloids showed potent biological activities, including cytotoxic (Keawpradub *et al.*, 2001; Makarasen *et al.*, 2011), anti-proliferative (Nantapap *et al.*, 2010), antimalarial (Likhitwitayawuid *et al.*, 1999; Makarasen *et al.*, 2011), antitubercular (Makarasen *et al.*, 2011), antimicrobial (Makarasen *et al.*, 2011) and antiacetylcholinesterase (Ingkaninan *et al.*, 2006) activities.

Thailandine is a quaternary C-7 oxoaporphine alkaloid (Fig. 1) responsible for the red color in *S. venosa* latex. This compound was first isolated from the tuber in 1981 (Guinaudeau *et al.*, 1981). Surprisingly, although *S. venosa* has long been extensively studied, thailandine has never been reisolated until recently. Makarasen *et al.* (2011) isolated this compound from the aerial part of *S. venosa*. The isolate showed broad and very potent biological activities, including cytotoxicity against various cell lines with an IC_{50} less than 3.7 μg mL^{-1}. The compound was found to be non-toxic against vero cell. Thailandine also displayed antimalarial activity (*Plasmodium falciparum*, K1 strain) with an IC_{50} of 0.02 μg mL^{-1}, antitubercular activity (*Mycobacterium tuberculosis* $H_{37}R_a$) with an IC_{50} of 6.25 μg mL^{-1} and antimicrobial activity against various Gram-positive bacteria. To identify additional biological activities, detailed molecular mechanism studies and *in vivo* investigations, large amounts of thailandine are required. To our knowledge, there is no published report on the chemical synthesis of this compound. Therefore, isolation from the plant is the only source of thailandine. The typical isolation procedure for thailandine requires traditional silica gel column chromatography with polar organic solvent, sometimes an acid modifier, as the eluent (Makarasen *et al.*, 2011). Irreversible adsorption of thailandine on silica gel and decomposition induced by the silanol groups are inevitable problems. Moreover, isolation of this compound is labor-intensive and generates high amounts of toxic wastes. Therefore, we developed a simple, rapid and efficient procedure for the isolation and purification of thailandine from *S. venosa* leaves in this study.

Ion-pair liquid chromatography has long been used to increase the retention and improve the peak shape of charged analytes. The formation of ion-pair could raise the hydrophobicity of ionizable compounds, thus allowing for longer retention on non-polar liquid chromatography column (Dai and Carr, 2005, 2009; Dai *et al.*, 2005; Wang and Carr, 2007). Analogous to ion-pair

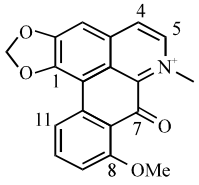

Fig. 1: Structure of thailandine

liquid chromatography, the formation of ion-pair between thailandine (quaternary ammonium salt) and an ion-pair reagent could increase the hydrophobicity of the ion-pair product. This procedure allows thailandine to be partitioned into the organic phase of common aqueous/organic solvent systems. In the current study, a volatile ion-pair reagent, Trifluoroacetic Acid (TFA), was selected primarily because of its high volatility and easy removal during the purification step.

In the proposed method, thailandine was extracted from the *S. venosa* leaves by maceration in methanol. Dichloromethane was used to remove pigments, non and semi-polar substances. Trifluoroacetic acid was then added to form the ion-pair. In the last step, thailandine in the form of the ion-pair product was transferred from the aqueous to dichloromethane layer and subsequently evaporated to obtain solid thailandine.

MATERIALS AND METHODS

Chemicals and reagents: All organic solvents used in sample preparation were of analytical grade (Lab Scan, Thailand). Water was produced by Milli-Q water (18.2 Mohm) (Millipore, Bedford, mA, USA). Methanol-d_4 used for NMR measurements was from Merck, Germany. Trifluoroacetic acid was from Fluka (Switzerland).

Materials: *S. venosa* leaves were collected from Bangkok, Thailand. Leaves were kept, air-dried and ground using a food blender before extraction. The voucher specimen (No. SN144956) was deposited at the Forest Herbarium (BKF) of the Royal Forest Department, Department of Forestry, Bangkok, Thailand.

Optimization: To determine the most effective extraction method, four techniques: macerations for 1 h and for 24 h; 60°C hot solvent extraction for 1 h; and sonication for 30 min were compared. Dried powdered leaves (1.0 g) were extracted with methanol (2×10 mL). The combined methanol extract was then evaporated under reduced pressure to dryness. To purify the extract, the crude methanolic extract was re-dissolved in water (10 mL) and partitioned thrice with dichloromethane (10 mL). The ratio of water:dichloromethane is 1:1 v/v. The combined dichloromethane layer containing non and semi-polar substances was discarded. UV absorbance at 480 nm of the aqueous layer containing thailandine was then measured using the microplate reader. Absorbance values, which indicate the relative amounts of thailandine, of the extracts from each method were compared and the most suitable extraction method was chosen.

To optimize the amount of trifluoroacetic acid used for ion-pair formation, dried powdered leaves (1.0 g) were extracted twice by maceration in methanol (10 mL) for 24 h. After removal of methanol and purification with dichloromethane as described previously, various amounts of trifluoroacetic acid i.e., 50, 70, 100 and 150 μL (0.5, 0.7, 1.0 and 1.5% v/v, respectively) were added to the aqueous layer. The solution was then partitioned with dichloromethane (3×10 mL). The combined dichloromethane phase was evaporated to dryness under reduced pressure and re-dissolved in methanol (2 mL). The methanolic solution was then diluted four times with methanol before UV measurement at 480 nm on a microplate reader. Absorbance values, which indicate the relative amounts of thailandine, were compared.

Plant extraction, purification and ion-pair formation: The flow chart for extraction of thailandine from *S. venosa* was shown in Fig. 2. Dried powdered leaves (10 g) of *S. venosa* were extracted twice with methanol (100 mL) by maceration for 24 h. The combined methanolic extract

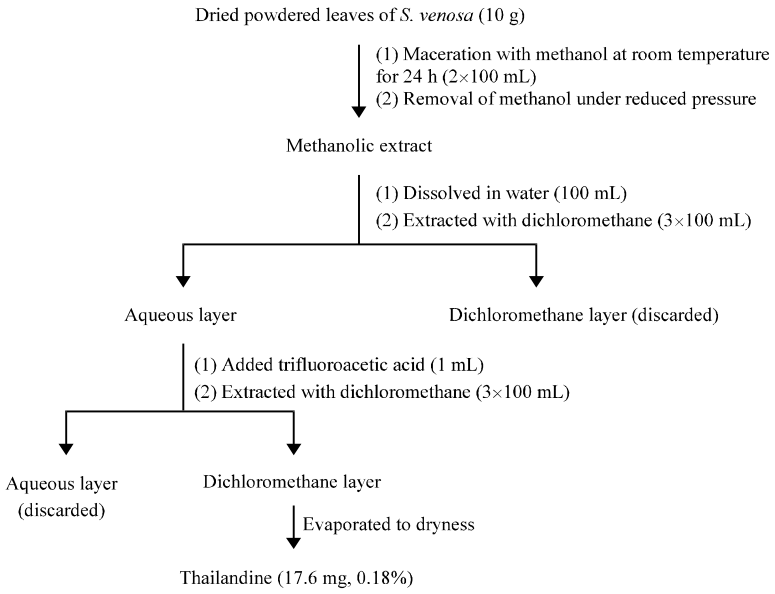

Fig. 2: Ion-pair liquid-liquid extraction of thailandine from *S. venosa*

was evaporated to dryness under reduced pressure to give the crude extract (0.92 g, 9.2%). The crude methanolic extract was re-dissolved in water (100 mL) and the solution was partitioned thrice with dichloromethane (100 mL). The dichloromethane layer was discarded. Trifluoroacetic acid (1 mL) was added to the dark-red aqueous solution containing thailandine and the solution was partitioned thrice with dichloromethane (100 mL). The combined dichloromethane layer, containing thailandine in ion-pair form, was dried over anhydrous sodium sulfate and filtered. After the evaporation of dichloromethane under reduced pressure, thailandine was obtained as a red amorphous solid (17.6 mg, 0.18%).

Instrumental: ^{1}H and ^{13}C NMR spectra were recorded on Bruker NMR 400 MHZ (Bruker Biospin, Switzerland) using methanol-d_4 as solvent and internal reference. ESI-HRTOFMS was performed on Bruker MicroTOF (Bruker Daltonics, Germany) at a resolution of 10000. UV absorbance was recorded on a microplate reader (Spectra max plus, USA) at 480 nm.

RESULTS AND DISCUSSION

Thailandine is the red component of the latex of *S. venosa* tubers and aerial parts. Since the tubers are very expensive and almost extinct from the forest, the *S. venosa* leaves were used as material source. This practice provided not only a sustainable source but also a value-added advantage.

The components in the methanolic extract were initially screened by using ESI-TOFMS (see supporting information; Fig. 1). Thailandine (m/z = 320) was found to be the major component along with a small amount of an alkaloid at m/z 306 ($C_{18}H_{12}NO_4$), which should be oxostephanine as reported earlier (Makarasen *et al.*, 2011). Thailandine is a quaternary aporphine alkaloid with a permanent positive charge, whereas oxostephanine is an isoquinoline alkaloid. Therefore, thailandine could be isolated from other alkaloids using an ion-pair liquid-liquid extraction (IP-LLE).

First, thailandine was extracted with methanol from dried powdered leaves using four extraction methods: macerations for 1 h and for 24 h, 60°C hot extraction for 1 h and sonication for 30 min. After removal of methanol, the extract residue was purified by partitioning with water and dichloromethane (1:1). Non and semi-polar substances, including pigments, waxes and non-permanently charged alkaloids, were partitioned to dichloromethane, while thailandine remained in the aqueous phase. Repeated purification using ethyl acetate was found to be unnecessary (data not shown) and sometimes caused an emulsion problem. After purification, the UV absorbance at 480 nm of the aqueous phase from each extraction method was measured and extraction efficiency was compared (Fig. 3). Maceration for 1 h gave the lowest extraction efficiency followed by sonication for 30 min. Maceration for 24 h and 60°C hot extraction for 1 h yielded comparable efficiencies. However, in terms of large scale preparation, maceration for 24 h at room temperature was the better choice.

Another crucial factor for this IP-LLE method is the ion-pair forming reagent. Ion-pair forming has long been utilized mostly in chromatography and liquid-liquid extraction. After ion-pair forming, the ion-pair product is more hydrophobic than charged analyte which is very beneficial. In chromatography, the peak shape is improved and the retention time is extended on typical revered-phase liquid chromatography. Ion-pair formation was proved to be very useful for extraction, fractionation and isolation, particularly for quaternary alkaloid compounds. Quaternary alkaloids possess permanent positive charge on nitrogen atom which thus has high polarity in nature. Solubility in aqueous phase of these compounds causes the difficulty to extract out with common organic solvents. Using ion-pair forming method, the ion-pair product is more hydrophobic which then possible to extract into non-polar immiscible phase of liquid extraction systems. Recently, using small hydrophilic anionic additives, such as perfluorinated acids (e.g., trifluoroacetic acid, pentafluoropropionic acid, heptafluorobutyric acid), perchlorate, hexafluorophosphate, tetrafluoroborate, as ion-pair reagents has been paid attention instead of usual surfactants (Dai *et al*., 2005; Kazakevich *et al*., 2005; Flieger, 2006, 2010; Cecchi and Passamonti, 2009; Dai and Carr, 2009; Flieger and Czajkowska-Zelazko, 2011). These small anions could disrupt the solvation shell of positively charge analytes, thus increasing the apparent analyte hydrophobicity (Kazakevich *et al*., 2005). These small anionic counter ions were recently applied to IP-LLE for quaternary ammonium alkaloids from various sources (Kato *et al*., 1996;

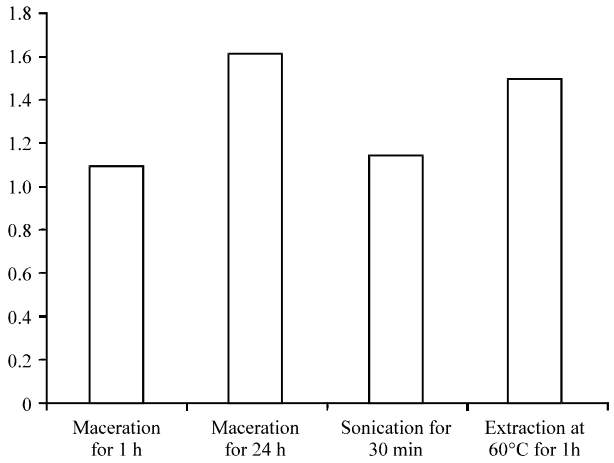

Fig. 3: UV absorbance at 480 nm of the extract from each extraction method

Tanahashi *et al*., 2000; Bourdat-Deschamps *et al*., 2004; Storme *et al*., 2008; Jerz *et al*., 2008, 2010; Wybraniec *et al*., 2009, 2010). With these successful evidences in using small anionic counter ions in IP-LLE, trifluoroacetic acid-quaternary alkaloid ion-pair complex was applied for the extraction of bioactive thailandine compound from *S. venosa* leaves.

In this study, trifluoroacetic acid was selected as ion-pair forming reagent because of its high volatility, thus enabling easy removal. Various amounts of trifluoroacetic acid at 0.5, 0.7, 1.0 and 1.5% v/v were added to the aqueous phase and the solution was partitioned with dichloromethane. The formation of an ion-pair between thailandine and trifluoroacetic acid significantly decreased the polarity of thailandine and allowed it to be partitioned into dichloromethane layer while other polar substances remained in the aqueous phase; this is the key step to a successful IP-LLE. After removal of dichloromethane and trifluoroacetic acid, the red amorphous solid of thailandine was dissolved in methanol and UV absorbance at 480 nm was measured. The UV absorbance values at various percentages of added trifluoroacetic acid were compared (Fig. 4). The more absorbance intensity indicated the more amount of extracted thailandine, therefore 1.0% v/v of trifluoroacetic acid was found to be the optimal amount for ion-pair formation.

Using the optimized extraction method and trifluoroacetic acid amount, the purity of the obtained thailandine was assessed using NMR and ESI-TOFMS spectroscopy (see supporting information). Results indicated that the proposed method could provide thailandine with high purity and 0.18% yield.

Structural identification was accomplished by HR-MS and ^{1}H-and ^{13}C-NMR. All NMR data were matched with those reported for thailandine in the literature (Makarasen *et al*., 2011).

Thailandine: ^{1}H-NMR (400 MHZ, CD_3OD) δ ppm: 8.72 (d, J = 6.5 Hz, 1H, H-5), 8.36 (d, J = 6.5 Hz, 1H, H-4), 8.16 (d, J = 8.0 Hz, 1H, H-11), 7.71 (dd, J = 8.3, 8.3 Hz, 1H, H-10), 7.6 (s, 1H, H-3), 7.22 (d, J = 8.4 Hz, 1H, H-9), 6.65 (s, 2H, H-1'), 4.87 (s, 3H, Me), 3.99 (s, 3H, OMe); ^{13}C NMR (100 MHZ, CD_3OD) δ ppm: 178 (C-7), 160 (C-8), 157.5 (C-1), 151.6 (C-2), 141.1 (C-6a), 140.6 (C-5), 136.2 (C-10), 133 (C-11a), 125.3 (C-4), 122.9 (C-6b), 118.8 (C-7a and C-11), 112.9 (C-9), 108 (C-11b), 105.3 (C-1'), 102.8 (C-3), 55.4 (OMe), 48.6 (Me); ESI-HRMS m/z $[M+H]^+$ calcd for $C_{19}H_{14}NO_4$: 320.0917; found 320.0922 (see supporting information; Fig. 2-4).

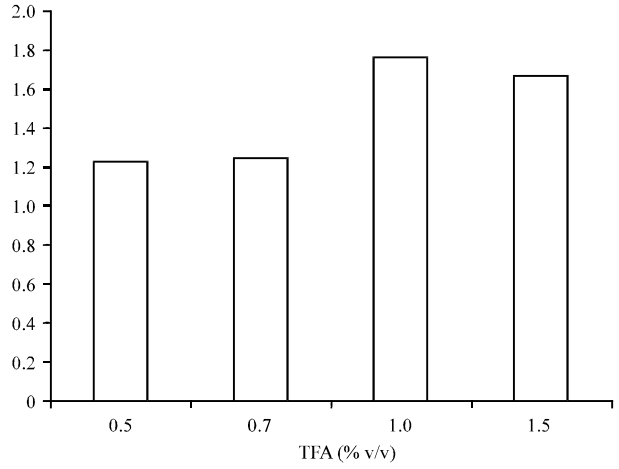

Fig. 4: UV absorbance at various percentages of trifluoroacetic acid added

CONCLUSION

This study presented the successful method of thailandine extraction from dried *S. venosa* leaves by ion-pair liquid-liquid extraction. Methanol was used as the extraction solvent. In the purification step, dichloromethane was used to remove non and semi-polar substances, including non-permanently charged alkaloids. Trifluoroacetic acid was used to form ion-pair with thailandine, thus allowing thailandine to be partitioned into the dichloromethane layer. Thailandine was obtained with high purity. Findings of this study demonstrate that the IP-LLE method is a rapid and efficient method for thailandine extraction that generates a low amount of toxic wastes and is not labor intensive.

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