CHAPTER VI

CHEMICAL PROFILES OF KRAI-KRUE AND THAI TRADITIONAL FORMULAS

6.1 Introduction

Chemical profiling is a basic approach for herbal material identification used as complement to other methods for better quality control. The major chemical constituents in the whole plant of *Aristolochia* are the human carcinogens, aristolochic acid I (AAI) and aristolochic acid II (AAII) (NTP 2011). Aristolochic acid I (AAI) has been used as a chemical marker for the quality control of herbs and herbal products containing *Aristolochia* species (Blatter and Reich 2004, Li, Au et al. 2012, Phadungrakwittaya, Akarasereenont et al. 2012). Since 2012, High-performance thin layer chromatography (HPTLC) pattern is recommended by European Pharmacopoeia and British Pharmacopoeia for screening of aristolochic acids at levels equal to or greater than 2 ppm (Commission and Britain 2012, Pereira Sena, Ashton-Prolla et al. 2012). In the present study, the HPTLC was performed to detect aristolochic acid I in seven Krai-Krue crude drugs and twenty-three Krai-Krue containing formulas available in market.

6.2 Materials and Methods

6.2.1 Crude drug "Krai-Krue" and Thai traditional formulas containing Krai-Krue

Krai-Krue samples and Krai-Krue containing Thai traditional formulas were purchased from various local dispensaries in Thailand as shown in Table 14.

Table 14 Details of Krai-Krue crude drugs and samples analyzed in this study.

Sample	Code	Date of manufacture	Purchased location (Thailand, Province)	Proportion of Krai-Krue in formula
Krai-Krue	C1	27/12/12	Bangkok	-
Krai-Krue	C2	27/12/12	Bangkok	-
Krai-Krue	C3	16/04/13	Nakhon Si Thammarat	-
Krai-Krue	C4	27/07/13	Phetchaburi	-
Krai-Krue	C5	17/09/13	Ayutthaya	-
Krai-Krue	C6	20/08/14	Bangkok	-
Krai-Krue	C7	20/08/14	Bangkok	-
Ya Ummaruekawatee	R151	23/12/11	Sakaeo	0.1000
	R153	23/02/13	Bangkok	0.1000
Ya Kheawhom	R122	09/08/13	Bangkok	0.0526
	R123	06/03/13	Bangkok	0.0526
Ya Tatbunjob	R072	26/07/13	Bangkok	0.0370
	R073	15/01/13	Bangkok	0.0370
	R075	10/04/12	Mahasarakham	0.0370
Ya Hom Nawakod	R052	17/08//13	Bangkok	0.0185
	R053	28/01/13	Bangkok	0.0185
	R054	12/07/12	Prachinburi	0.0185
Ya Wisumpayayai	R112	05/08/13	Bangkok	0.0185
	R113	15/10/12	Bangkok	0.0185
Ya Treehom	R043	22/04/13	Bangkok	0.0156
Ya Prasa Ganplu	R082	24/07/13	Bangkok	0.0154
	R083	01/12/12	Bangkok	0.0154
Ya Prasa Jettapungkee	R093	02/04/12	Bangkok	0.0152
Ya Munthatat	R103	09/04/13	Bangkok	0.0108
Ya Hom Inthajuk	R062	12/06/13	Bangkok	0.0102

	R063	02/07/13	Bangkok	0.0102
	R064	16/07/12	Prachinburi	0.0102
Ya Juntaleela	R012	25/05/13	Bangkok	0.1212
	R013	20/05/13	Bangkok	0.1212
Ya Hom Kaelomwingwean	R143	28/08/13	Bangkok	N/A

([a] Manufacturing date of the products, N/A = Not available)

6.2.2 High performance thin layer chromatography (HPTLC)

Detection of aristolochic acid I was determined using a CAMAG Linomat 5 automatic sample spotter (Muttenz, Switzerland) under a flow of nitrogen gas for Krai-Krue crude drugs and manually spotted for Krai-Krue containing formulas. The test solution was prepared by extracting 2.25 g of the powdered herbal drug with 10 ml of anhydrous formic acid, water, methanol (1:9:40 V/V/V), then sonicated at room temperature for 10 min and centrifuged at 14,000 rpm for 5 min. The clear solution was used as the test solution for 1 µL (for C1-C5), 20 µL (for C6-C7 and all formulas). The solution samples were spotted in the bands of width 8 mm with a CAMAG microlitre syringe on a HPTLC Silica gel 60 F_{254} glass plate (20x10 cm). The plate was developed in a CAMAG glass twin-through chamber (20x20 cm) which was presaturated with 25 ml mobile phase of an upper layer of the mixture of anhydrous formic acid, water, ethyl acetate, toluene (1:1:10:20 V/V/V/V) for 30 min at room temperature. Solvent fronts of the mobile phases were allowed to ascend 8 cm above the line of sample application. Subsequently, developed HPTLC plates were air dried and sprayed with a 100 g/L solution of stannous chloride in dilute hydrochloric acid until the plate is slightly wet, and then heated at 100°C for 1 min. The chromatograms were observed under long ultraviolet wavelengths (365 nm). HPTLC plates were air dried and scanned with a CAMAG TLC scanner 3 and analyzed by winCATS software version 1.4.4.

Aristolochic acid I (Sigma-Aldrich, USA) was used as standard at concentration 2 and 5 ppm for 20 µL. All materials and reagents were of analytical grade.

6.3 Results

HPTLC chromatogram of standard aristolochic acid of AAI was developed using the mobile phase and the detection method described above. The result showed that Rf value of AAI was 0.46. The chromatogram of AAI showed greenish-blue zone. The lighted yellow zone, a characteristic fluorescence of AAII, was observed at high concentration of AAI (Figure 11). The linear calibration curves of AAI were within the concentration range of 0.5-20 ppm. A linear calibration equation, y= 396.28x+823.09 was obtained with a correlation coefficient of 0.9835 (Figure 12).

HPTLC profiles of extracts of Krai-Krue samples and Thai traditional formulas were examined. Standard AAI at concentration 2 and 5 ppm were used as reference markers. Seven Krai-Krue crude drugs were tested. The result showed that only C1-C5 have the same Rf value as standard AAI. (Figure 13). The AAI contents of crude drug extracts of C1-C5 were 0.135, 0.225, 0.144, 0.141 and 0.159 %w/w, respectively.

The solutions of extracts prepared from twenty-three formulas were used as test samples. The chromatogram showed that thirteen formulas (R151, R123, R072, R073, R053, R054, R093, R112, R113, R043, R062, R063, R064) have a band with a greenish-blue color and an Rf value of AAI. While the other ten formulas (R153, R122, R075, R052, R082, R083, R103, R012, R013, R143) did not show (Figure 14 and Table 15).

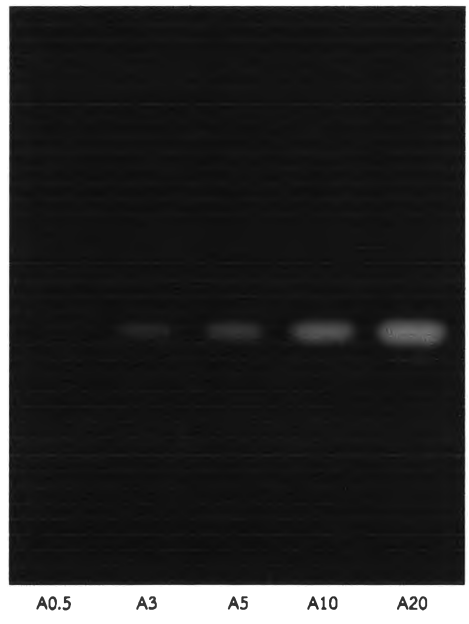

Figure 11 HPTLC profiles of standard aristolochic acid I at concentration 0.5, 3, 5, 10 and 20 ppm respectively (lane 1-5).

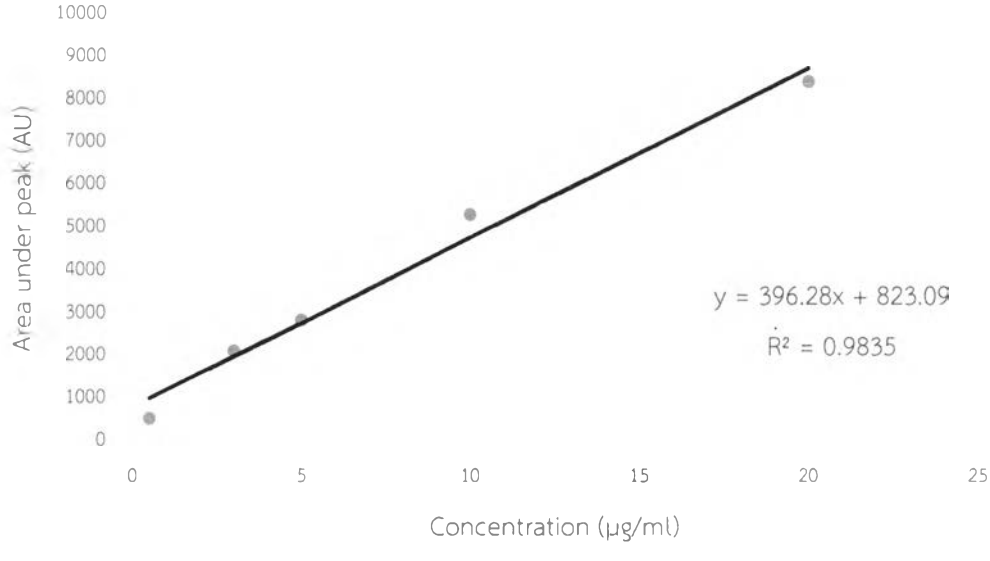

Figure 12 Calibration curve of AAI by TLC-densitometric method.

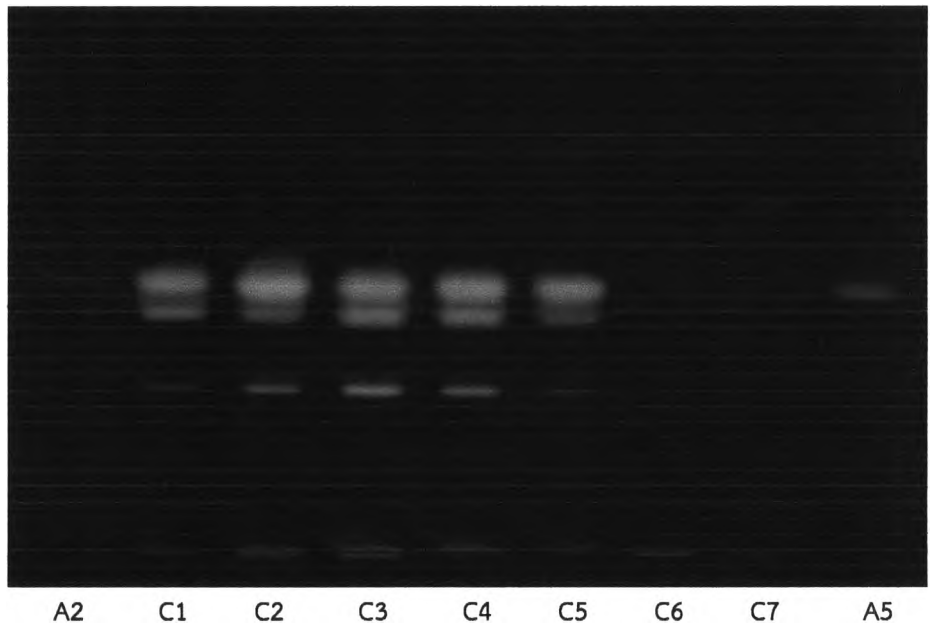

Figure 13 HPTLC profile of Krai-Krue herbs; lane 1 (A2) is standard AAI 2 ppm (20 µl), lane 2-8 are Krai-Krue from traditional herb stores (1 µl for C1-C5 and 20 µl for C6-C7) and lane 9 (A5) is standard AAI 5 ppm (20 µl).

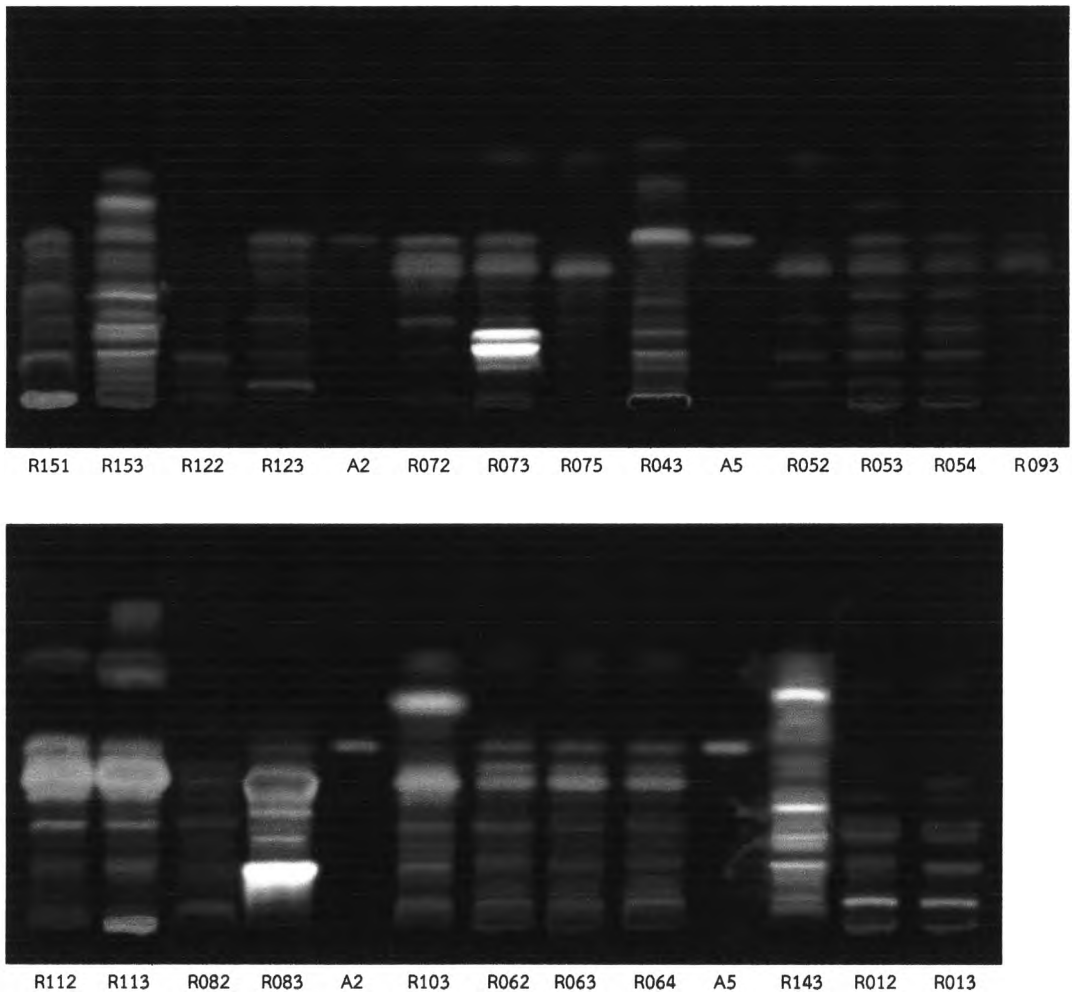

Figure 14 HPTLC profiles of 23 available formulas containing Krai-Krue.

Table 15 The detection of AAI in Krai-Krue crude drugs and Krai-Krue containing formulas collected from the herb and traditional medicine markets analyzed in this study.

Sample	Code	Date of manufac-ture	Purchased location (Thailand, Province)	Proportion of Krai-Krue in formula	Detection of AAI
Krai-Krue	C1	27/12/12	Bangkok	-	+
Krai-Krue	C2	27/12/12	Bangkok	-	+
Krai-Krue	C3	16/04/13	Nakhon Si Thammarat	-	+
Krai-Krue	C4	27/07/13	Phetchaburi	-	+
Krai-Krue	C5	17/09/13	Ayutthaya	-	+
Krai-Krue	C6	20/08/14	Bangkok	-	-
Krai-Krue	C7	20/08/14	Bangkok	-	-
Ya Ummaruekawatee	R151	23/12/11	Sakaeo	0.1000	+
	R153	23/02/13	Bangkok	0.1000	-
Ya Kheawhom	R122	09/08/13	Bangkok	0.0526	-
	R123	06/03/13	Bangkok	0.0526	+
Ya Tatbunjob	R072	26/07/13	Bangkok	0.0370	+
	R073	15/01/13	Bangkok	0.0370	+
	R075	10/04/12	Mahasara-kham	0.0370	-
Ya Hom Nawakod	R052	17/08//13	Bangkok	0.0185	-
	R053	28/01/13	Bangkok	0.0185	+
	R054	12/07/12	Prachinburi	0.0185	+
Ya Wisumpayayai	R112	05/08/13	Bangkok	0.0185	+
	R113	15/10/12	Bangkok	0.0185	+
Ya Treehom	R043	22/04/13	Bangkok	0.0156	+
Ya Prasa Ganplu	R082	24/07/13	Bangkok	0.0154	-
	R083	01/12/12	Bangkok	0.0154	-
Ya Prasa Jettapungkee	R093	02/04/12	Bangkok	0.0152	+
Ya Munthatat	R103	09/04/13	Bangkok	0.0108	-
Ya Hom Inthajuk	R062	12/06/13	Bangkok	0.0102	+
	R063	02/07/13	Bangkok	0.0102	+

	R064	16/07/12	Prachinburi	0.0102	+
Ya Juntaleela	R012	25/05/13	Bangkok	0.1212	-
	R013	20/05/13	Bangkok	0.1212	-
Ya Hom Kaelomwingwean	R143	28/08/13	Bangkok	Not available	-

6.4 Discussion

In Thailand, dried root of the three *Aristolochia* species, *A. pothieri* Pierre ex Lecomte (Athikomkulchai and Ruangrungri 2001), *A. pierrei* Lecomte and *A. tagala* Cham. (Sathornviriyapong, Picheansoonthon et al. 2007), have been reported as sources of medicinal crude drugs called "Krai-Krue".

In order to ensure consumer safety, chemical profile is a basic approach for herbal material identification. As far as chemical substances are concerned, chemical profiling using aristolochic acid I as standard reference were also used to analyze raw material of Krai-Krue by thin-layer chromatography (TLC) (Sathornviriyapong, Picheansoonthon et al. 2007), high-performance thin layer chromatography (HPTLC) (Phadungrakwittaya, Akarasereenont et al. 2012), high-performance liquid chromatography (HPLC) and liquid chromatography/mass spectroscopy (LC/MS) (Tripatara, Onlamul et al. 2012), and ultra-high performance liquid chromatography (UHPLC) (Wattanarangsan 2012).

From these techniques, the optimum HPTLC system for detection of AAI in Krai-Krue and Ya Homnawakod was conducted with chloroform, methanol and acetic acid (65:20:1 v/v) as mobile phase with Rf value 0.55. The lower limit of detection (LOD) at 8 ng for AA-I and 5 ng for AA-I salt. In addition, For RP-TLC analysis, acetonitrile, methanol and water (3:0.5:1 v/v) were used as mobile phase. AAI appeared at Rf value 0.48 with the lower limits detected for both AA-I and AA-I salt were 15 ng (Phadungrakwittaya, Akarasereenont et al. 2012).

The amount of AA-I determined by UHPLC in *A. tagala* was 0.237 %w/w. Limit of detection (LOD) and limit of quantification (LOQ) were 0.8 and 2.0 µg/mL

(Wattanarangsan 2012). Whereas other study found that the amount of AA-I in *A. tagala* was 0.24 %w/w and *A. tagala* and Ya Homnawakod showed the same profiles of HPLC and LC/MS (Tripatara, Onlamul et al. 2012). However, HPTLC is a method of choice for screening of AAI due to a more convenient, rapid and cheaper procedure.

In this study, HPTLC screening method of aristolochic acid recommended by British Pharmacopoeia and European Pharmacopoeia was performed. The results showed that 1 µl of C1-C5 extracts possibly contain aristolochic acid I and the source of these crude drugs are *Aristolochia* plants. In contrast to 20 µl of C6 and C7, there are no band chromatogram at the same Rf value as aristolochic acid I. This method is suitable for screening of *Aristolochia* containing herbal products and it requires more test to quantify AAI in products.

As the results, the amount of AAI in C1-C5 were 0.135, 0.225, 0.144, 0.141 and 0.159 %w/w, respectively. the average amount of AAI in C1-C5 were 0.1608 %w/w. According to proportion of Krai-Krue in formulas ranged from 0.0102-0.1212, the amount of AAI in formulas ranged from 0.00164-0.01949 %w/w. The results agreed with previous study that chromatogram of AAI was shown at the same Rf value but the UV absorbance of that was not detected. It might be caused by the amount of AAI is too low for HPTLC analysis or there are some substances from other plants in recipes that interfered the UV absorbance of AAI (Phadungrakwittaya, Akarasereenont et al. *2012)*. Therefore more sensitivity methods such as liquid chromatography (LC) or liquid chromatography coupled with mass spectrometry are recommended for confirmation of AAI (Commission and Britain 2012).

All formulas used as samples in this study were randomly purchased for both of before and during the regulation of removing Krai-Krue from herbal formula was announced. The ratios of Krai-Krue to all ingredients of 22 formulas were calculated as shown in Table 14except for one formula (R143), the detail of ingredients could not be found. The chromatograms of 20 µl showed that AAI were detected in

only 13 formula including R151, R123, R072, R073, R053, R054, R093, R112, R113, R043, R062, R063 and R064 which were manufactured before and during the regulation. The other ten formulas (R153, R122, R075, R052, R082, R083, R103, R012, R013 and R143), AAI did not contain AAI Figure 14 HPTLC profiles of 23 available formulas containing Krai-Krue. (Figure 14 and Table 14) which may due to the source of Krai-Krue (not *Aristolochia* sp.), small amounts of Krai-Krue in the formulas and also the production processes which affected the stability of the finished products. Because of the complexity of different ingredients in the recipes and the unassignable target peak, spectra could not be detected by CAMAG TLC Scanner 3. In addition, the different chemical patterns were found for the same formula indicating that the standardization and quality control of registered herbal products in Thailand are needed.

6.5 Conclusion

The HPTLC method was found to be a simple and rapid method for AAI of suspected *Aristolochia* containing crude drugs and formulas. These data should be useful for further regulation and legislation. Moreover, this technique should be included as a primary step for the standardization and quality control of plant raw materials and herbal formulations available in the market.