CHAPTER V

APPLICATION OF MULTIPLEX PCR FOR IDENTIFICATION OF KRAI-KRUE

5.1 Introduction

Krai-Krue is a crude drug used as an ingredient in Thai folk medicinal formulas for tonic, muscle relaxant, diuretic, antipyretic, analgesic, anti-rheumatism, immunostimulant, emmenagogue, abortive agent and liver enhancer (Vuthithammavech 1997). It is also one of ingredients in 10 herbal recipes on the Thailand list of Herbal Medicinal Products A.D. 2006, for example, Ya hom Nawakod (ยาหอมนวโกฐ), Ya hom Inthajuk (ยาหอมอินทจักร์), Ya Ummaruekawatee (ยาอัมฤควาที), Ya Tatbunjob (ยาธาตุบรรจบ), Ya Wisumpayayai (ยาวิสัมพยาใหญ่), Ya Munthatat (ยามัณฑธาตุ), Ya Kheawhom (ยาเขียวหอม), Ya Treehom (ยาตรีหอม), Ya Prasaganplu (ยาประสะกานพลู), Ya Prasajettapungkee (ยาประสะเจตพังคี) (Health 2006). According to microscopic, morphological and chemical profiling approaches, Krai-Krue can be derived from dried roots of the three *Aristolochia* species, *A. pothieri* Pierre ex Lecomte (Vuthithammavech 1997, Athikomkulchai and Ruangrungri 2001), *A. pierrei* Lecomte and *A. tagala* Cham. (Sathornviriyapong, Picheansoonthon et al. 2007). In 2013, the National Drug Committee have issued an order that demands the removal of Krai-Krue from all formulas within one years after April 19th, 2013 (Control 2013, Health 2013). However, despite the warnings, Krai-Krue still be bought from local dispensaries.

In this study, a novel multiplex PCR technique was developed based on nucleotide sequence of ITS2 region for the discrimination of *Aristolochia* Krai-Krue herbs and was used to authenticate crude Krai-Krue drugs purchased from various local dispensaries.

5.2 Materials and Methods

5.2.1 Crude drugs named Krai-Krue and Thai traditional formulas containing Krai-Krue

Seven commercial crude drug samples claimed to be Krai-Krue (i.e. C1–C7) and twenty-three formulas claimed to contain Krai-Krue were randomly purchased from various traditional drug stores. The list of samples used in this study was shown in Table 12.

5.2.2 Genomic DNA extraction

Total genomic DNA was extracted from *20-50* mg of dried crude drug samples (as shown in Figure 9) using genomic DNA extraction kit as mentioned in Material and Methods of Chapters III.

5.2.3 Multiplex PCR

The multiplex PCR protocol was performed as mentioned in Material and Methods of Chapter IV. The amplification reaction was performed in 25 µL of reaction mixture GoTaq® Flexi DNA Polymerase, consisting of 5X PCR buffer, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 10 mM of each primer, 5U *Taq* polymerase (Promega, USA), and 3.5 µL of total genomic DNA as a template.

Table 12 Details of Krai-Krue crude drugs and formulas analyzed in this study.

Sample	Code	Date of manufacture	Purchased location (Thailand, Province)	Proportion of Krai-Krue in formulas
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)

5.3 Results

The multiplex PCR was performed under the conditions described above. The experimental DNA admixtures containing the genomic DNA of three *Aristolochia* species were prepared and subjected to multiplex PCR analysis to test the accuracy of this technique. After gel electrophoresis, the combined electrophoresis patterns were resolved, and PCR products observed on agarose gel represented different species in the mixtures (Figure 10 and Table 13). Each fragment exhibited the unique characteristic of the species. The DNA admixture containing *A. pothieri, A. pierrei and A. tagala* presented a combined pattern of four fragments: a 123-bp fragment from *A. pothieri*, a 191-bp fragment from *A. pierrei*, a 265-bp fragment from *A. tagala* and a 400-bp fragment from internal amplification control. The PCR products from C1-C5 were observed by two different sizes at 191-bp and 400-bp. The results indicated that C1-C5 were derived from *A. pierrei*, while the fragments of C6 and C7 at 400-bp in length indicated that they were not of *A. pothieri*, *A. pierrei* or *A. tagala*. They were probably derived from other species of Krai-Krue herbs.

Table 13 Details of commercially available crude drugs analyzed.

Claimed crude drugs	Code	Purchased location (Thailand, Province)	Purchase date	Detected species
Krai-Krue	C1	Bangkok	2012/08/27	*A. pierrei*
Krai-Krue	C2	Bangkok	2012/08/27	*A. pierrei*
Krai-Krue	C3	Nakhon Si Thammarat	2013/04/16	*A. pierrei*
Krai-Krue	C4	Phetchaburi	2013/07/22	*A. pierrei*
Krai-Krue	C5	Ayutthaya	2013/09/17	*A. pierrei*
Krai-Krue	C6	Bangkok	2014/12/20	N/D
Krai-Krue	C7	Bangkok	2014/12/20	N/D

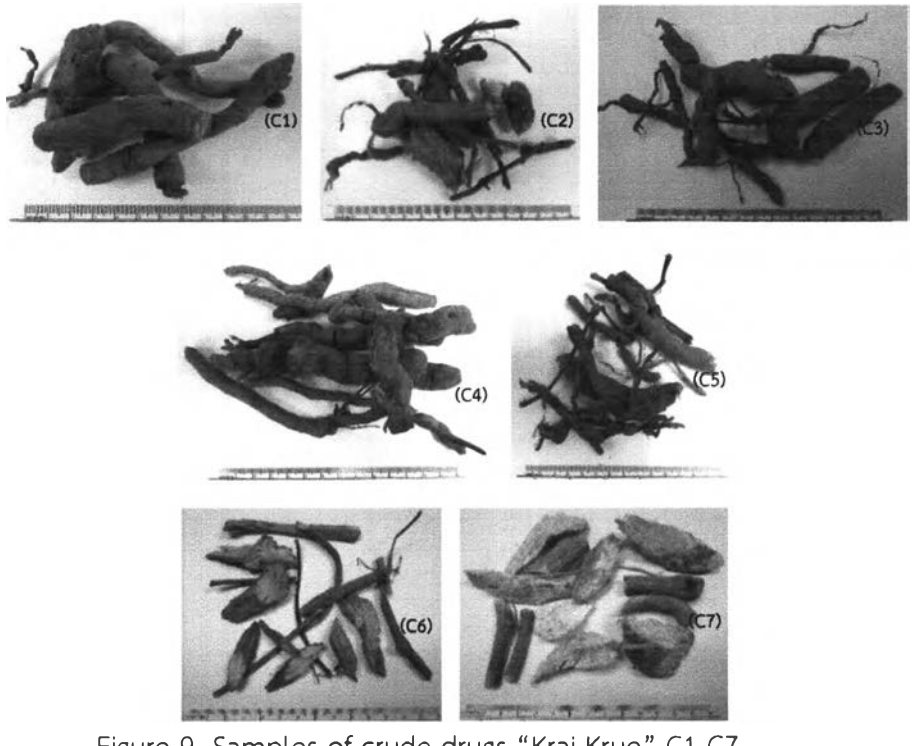

Figure 9 Samples of crude drugs "Krai-Krue" C1-C7

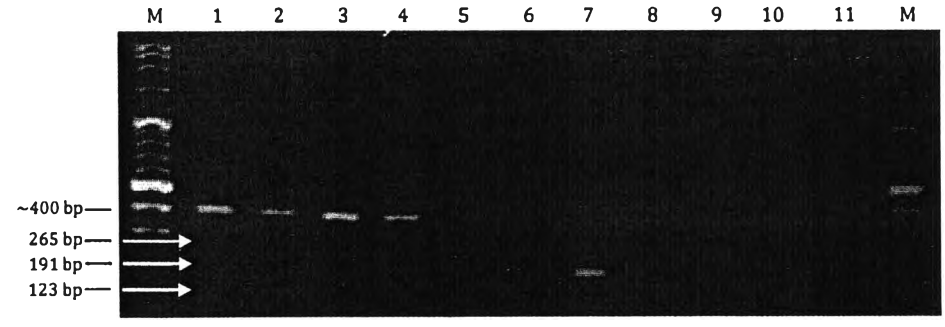

Figure 10 1.7% agarose gel electrophoresis image of species-specific PCR primers on ITS2 region of Krai-Krue herb. Seven commercially available crude drugs (C1 – C7) and DNA markers (M) in bp are indicated. Lane 1: mixed Krai-Krue, 2: *Aristolochia pothieri*, 3: *A. pierrei*, 4: *A. tagala*, 5-10: C1-C7 respectively.

5.4 Discussion

The nephrotoxic herb, many *Aristolochia* species, share a similar crude drug name in Thai as "Krai-Krue". The National Drug Committee has legally issued the announcement to manufacturer to remove Krai-Krue from registered formulas within one year after 19th April 2013. The identification of Krai-Krue herbs is challenging when they are in form of powders or small pieces. Unfortunately, using DNA barcoding of ITS2 sequence for identification of commercial Krai-Krue crude drugs failed (data not shown). The BLAST of C1-C7 sequencing results indicated that they were naturally contaminated by fungal and endophyte (data not shown). It might be affected by storaging for a long time or DNA is highly degraded or contaminated by microorganism, making the poor quality amplification of DNA for sequencing or fingerprinting (Singh, Srivastava et al. 2008, Gautam and Bhadauria 2009, Gautam, Sharma et al. 2011, Li, Zhang et al. 2011). Indeed, DNA barcoding can be used to identify individual species. However, there are the alternative molecular methods as well as multiplex PCR which provides reliable and rapid protocols, and can also be applied for species identification of closely related herb. Moreover, multiplex PCR reactions that can routinely amplify barcode markers will significantly reduce laboratory costs (Heubl 2013). For example,

discrimination of Korean *Artemisia iwayomogi* from other *Artemisia* herbs (Lee, Doh et al. 2008), identification of Mediterranean olive (Consolandi, Palmieri et al. 2008), authentication of *Dendrobium* species used in China (Chiang, Yu et al. 2012), authentication of *Anemarrhena asphodeloides* (Jigden, Wang et al. 2010) and discrimination of Phyllanthus taxa used in China (Lee, Li et al. 2006).

The multiplex PCR technique could be successfully applied to distinguish the three *Aristolochia* plants used as Krai-Krue herbs. The results disclosed that there are still crude drugs derived from *A. pierrei* in local dispensaries in Thailand. Two samples are not derived from *A. pothieri*, *A. pierrei* or *A. tagala*, and could not be identified at species level. Likewise for the formulas purchased from traditional drug stores, multiplex PCR could not be used for detection of Krai-Krue herbs. The possible causes might be the DNA degradation after formulation processes including heat, grinding, mixing and too low amounts of Krai-Krue in the formulas. However, the failure of this process probably causes from the limitation of multiplex PCR such as competition of primers and limited of resources in reaction (Edwards and Gibbs 1994). Nested PCR has been used in cases in which direct PCR proved impossible. However, combination with other identification tools such as chemical fingerprint is needed to confirm that whether they are derived from *Aristolochia* species.

5.5 Conclusion

This multiplex PCR method could serve as a rapid and reliable identification tool of raw material Krai-Krue herbs with specific for *A. pothieri*, *A. pierrei* and *A. tagala*. Although, various identification methods are available, each method has its own pros and cons; and no single method can be sufficient to authenticate herbal materials. To assure the identification results, another identification tool is need such as chemical assessment by HPTLC with standard chemical marker.