CHAPTER I
INTRODUCTION

In recent years, the natural product markets have grown rapidly (Da-cheng, Shi-lin et al. 2010). Due to the high market demands, adulteration and substitution of medicinal materials widely occur in many countries (Ernst 2002, Mitra and Kannan 2007). Therefore, authentication of raw materials plays an important role for efficacy and safety of consumers. After the discovery that consumption of *Aristolochia* containing herbal products causes severe nephropathy and associated malignancies, the intake of botanical products containing *Aristolochia* plants becomes one of global concerns (Arlt, Stiborova et al. 2002).

The genus *Aristolochia*, a member of the family Aristolochiaceae, consists of about 500 species and is widely distributed in tropical and subtropical area including Asia, Africa, Europe and America (Heywood 1993, González 1997). Several *Aristolochia* species have been medicinally used in many traditional drug formulas including Chinese traditional medicines, Indian folk medicines and European medicines. For example, in China, *Aristolochia contorta* Bunge was used as an antitussive and a purgative against rabies (James A. Dook and Ayensu 1985), *A. debilis* Siebold & Zucc as a sedative (James A. Dook and Ayensu 1985), *A. fangchi* Y.C. Wu ex L.D. Chow & S.M. Hwang as an antirheumatic and a diuretic (Heinrich, Chan et al. 2009), *A. manshuriensis* Kom for treating problems relating to the urine and the bladder problems (Heinrich, Chan et al. 2009). *A. gigantea* Mart. et Zucc. was used as abortifacients and in the treatment of wounds and skin diseases in Brazil (Holzbach and Lopes 2010), *A. tagala* Cham. and *A. indica* L. were used as emmenagogue, antirheumatism and anti-snake bite in India (S.K. Jain and DeFilpips 1991).

In Thailand, there are 22 species of *Aristolochia* (Smitinand 2014) as follows:

1) *A. arenicola* Hance
2) *A. baenzigeri* B.Hansen & Phuph.
3) *A. curtisii* King ex Gamble
4) *A. dinghoui* Favio González & O. Poncy
5) *A. grandis* Craib
6) *A. hansenii* Phuph.
7) *A. helix* Phuph.
8) *A. gigantea* Mart.
9) *A. kerrii* Craib
10) *A. kongkandae* Phuph.
11) *A. labiata* Willd.
12) *A. littoralis* Paradi.
13) *A. longeracemosa* B.Hansen & Phuph.
14) *A. macrophylla* Lam.
15) *A. perangustifolia* Phuph.
16) *A. pierrei* Lecomte
17) *A. poomae* Phuph.
18) *A. pothieri* Pierre ex Lecomte
19) *A. ringens* Vahl
20) *A. tagala* Cham.
21) *A. versicolor* S.M. Hwang
22) *A. yalaensis* Phuph.

However, three species were used in Thai traditional medicine in term of crude drug "Krai-Krue" (Athikomkulchai and Ruangrungri 2001, Sathornviriyapong, Picheansoonthon et al. 2007).

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 (ยาอำมฤควาที), 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 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 legally issued

to manufacturer to remove Krai-Krue from all formulas within one years after April 19th, 2013 (Control 2013, Health 2013). However, despite warnings, Krai-Krue still be bought from local dispensaries.

The genus *Aristolochia* contains aristolochic acids and its derivatives. These compounds are kidney targeting carcinogenic substances and are found in the whole plant through DNA adduct mechanism. The toxicity is known as "Aristolochic Acid Nephropathy (AAN)" (IARC 2012). Therefore identification of *Aristolochia* plants by accurate and efficient analysis method is very important for safety of customer.

Due to some limitations of classical plant authentication methods, for example, microscopic and macroscopic approaches need experts. Recently, many molecular biological technologies have been used as useful tools for DNA analysis of medicinal plants (Li, Cao et al. 2011, Techen, Parveen et al. 2014). For example, SCAR markers to detect adulterants of saffron (Marieschi, Torelli et al. 2012), RAPD for authentication of *Cuscuta reflexa* (Khan, Mirza et al. 2010), RAPD and AFLP for identification of *Withania* species (Mir, Koul et al. 2013), multiplex PCR for discrimination of *Artemisia* herbs (Lee, Doh et al. 2008). They can provide accurate identifications for plant samples that are not distinguishable by morphology or their names.

In recent years, DNA barcoding is become one of the methods for identification an organism at family, genus and species levels (Li, Cao et al. 2011). DNA barcoding is the latest technique involves using the analysis of short standardized regions of genome. It is not only an effective tool to establish centralized sequence database of organism but also is the gold standard for authentication and identification of organism (Group 2009, Hollingsworth 2011). A Consortium for the Barcode of Life (CBOL) Plant Working Group proposed four DNA regions for land plants. Nucleotide sequence of *mat*K region and *rbc*L region have been proposed to be core barcode to identify angiosperm at species levels and ITS (or ITS2) and *trn*H-*psb*A have been proposed as alternative DNA barcode (Hollingsworth 2011, Fazekas, Kuzmina et al. 2012). The United States Pharmacopoeia and Chinese Pharmacopoeia also include this technique as one of raw material identification (Song, Yao et al. 2009, Li, Cao et al. 2011).

However, the limited number of DNA sequences has restricted the development of rapid molecular identification techniques for these herbs.

Normally, the whole plants in the genus *Aristolochia* produce aristolochic acids (AAs). Aristolochic acids are a family of nitrophenanthrene carboxylic acids. Two major substances found are aristolochic acid I (AAI) and aristolochic acid II (AAII). AAI is commonly found at higher concentration than AAII (NTP 2011). Therefore, the presence of aristolochic acid I is an important chemical marker to identify herbs and herbal products derived from *Aristolochia* species (Blatter and Reich 2004, Li, Au et al. 2012, Phadungrakwittaya, Akarasereenont et al. 2012). High-performance thin layer chromatography (HPTLC) pattern is referred by European Pharmacopoeia and British Pharmacopoeia for screening of aristolochic acids in herbal products since 2012 with the presence of aristolochic acid I at levels equal to or greater than 2 ppm (Commission and Britain 2012, Pereira Sena, Ashton-Prolla et al. 2012).

In the present study, four DNA barcodes (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) of eleven *Aristolochia* species collected in Thailand were successfully generated. The nucleotide sequences of ITS2 was utilized for the discrimination of three *Aristolochia* species used in Thai traditional medicine under the name "Krai-Krue" using multiplex PCR. Application of ITS2 nucleotide polymorphisms, in combination with the presence of aristolochic acid I as chemical marker, was used to investigate seven crude drug samples from various local dispensaries. Combination of multiplex PCR and HPTLC analysis was successful for the identification of Krai-Krue herbs. Randomly purchased 23 Krai-Krue containing formulas were also analyzed by HPTLC.