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Optimal Detection of Aristolochic Acid I in *Krai-krue* by High Performance thin Layer Chromatography

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Abstract

Aristolochic acids (AA) are toxic substances found in *Krai-kruea* (*Aristolochia sp.*). They are nitrophenanthrene carboxylic acids which contain aristolochic acid I (AA-I) and aristolochic acid II (AA-II) as major components. The detection of AA is needed in order to avoid using AA-containing herbal raw materials. In this study, high performance thin layer chromatography (HPTLC) was optimized for AA-I screening in *Krai-kruea*. Three mobile phase systems were tried with HPTLC to obtain the best separation and reverse-phase thin layer chromatography (RP-TLC) was used to confirm the identification. For HPTLC, a mixture of chloroform, methanol, and acetic acid (65:20:1, v/v) was the most optimal mobile phase system with the lower limit of detection (LOD) of 8 ng, while a mixture of acetonitrile, methanol, and water (3:0.5:1, v/v) was the most suitable one for RP-TLC with LOD of 15 ng. According to the system, the Rf values of AA-I in Krai-kruea samples were 0.55 and 0.48 with HPTLC and RP-TLC, respectively. In summary, these HPTLC and RP-TLC systems can be used to detect AA-I in *Krai-kruea* single herb and HPTLC has greater sensitivity than RP-TLC.

Key words: **aristolochic acid, *Krai-kruea*, HPTLC, RP-TLC, herbal formulas**

Introduction

Aristolochic acids (AA) are nitrophenanthrene carboxylic acids found in plants of *Aristolochia sp.* in which aristolochic acid I (AA-I) and aristolochic acid II (AA-II) are major components. AA and their derivatives have been isolated and determined by several spectral and chemical methods.[1-3] In 2009, the U.S. Food and Drug Administration (USFDA) issued a strong warning to consumers to avoid botanical products that contain AA because the toxicities of AA had been reported.[4] In a short-term toxicity study, rats treated orally

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with 10 mg/kg AA, AA-I and AA-II every 3 days for 19 days had pathological changes, i.e. liver and kidney cell enlargement and lesions.[5] For an acute toxicity study, rats and mice treated orally or intravenously with high-dose AA died from acute renal failure within 15 days.[6] Due to these toxicities, many countries have eliminated any drugs or herbal products that contain AA. Therefore, several analytical methods including high performance liquid chromatography (HPLC),[7-10] capillary zone electrophoresis[11] and UHPLC[12] have been developed to detect these toxic compounds in products that are suspected of containing AA. However, such previous methods have several limitations such as the ionization of the carboxylic acid group when using alkaline mobile phase in reverse-phase HPLC analysis, the inconvenience to control pH and power supply in capillary zone electrophoresis, the use of expensive tools and solvents, the requirements of many steps in sample preparation when analyzing with HPLC, etc. In this study, we modified the method of AA detection using the TLC technique of Ohno T. et al as it was a more convenient, faster and cheaper procedure that could be an alternative method for detecting such compounds.[13]

In Thailand, *Krai-kruea* (KK), a dried root of either *Aristolochiapierrei* Lecomte or *Aristolochia tagala* Cham. (*Aristolochiaceae*), has been used as an ingredient of many traditional herbal medicines. It is used as an antipyretic, tonic, appetizer, anti-inflammatory, muscle relaxant, appetizing, and analeptic.[14] In 2011, the National Drug Committee removed KK from every herbal recipe on the List of Herbal Medicinal Products A.D. 2011. However, this crude drug can still be bought from local herbal dispensaries. So the detection of AA is needed for herbal medicine manufacturers in order to avoid using AA-containing herbal raw materials. Thin layer chromatography (TLC) is one of the chromatographic techniques for the separation and identification of mixture compounds based on the use of appropriate stationary and mobile phases. This technique is simple, cost-effective and reliable. In this study we, therefore, attempted to optimize a simple and rapid TLC method to detect AA-I in KK samples. Furthermore, we also tried to detect AA-I in *Yahom Nawakod* (or *Homnawakod*, HNK) Ayurved Siriraj, a herbal formula that contains KK, which is a representative sample of complex multiple herbal formulas. *Yahom Nawakod* is a famous remedy for the treatment of nausea, vomiting, and vertigo among elderly persons and has been included on the List of Herbal Medicinal Products since A.D. 2006.

Materials and Methods

1. Chemicals and plant materials

All reagents were of analytical grade, including AA-I and AA-I sodium salt (AA-I salt) purchased from Sigma; acetic acid, 25 % ammonia solution and acetonitrile from Merck; chloroform, methanol and ethyl acetate from BDH, Scharlauand Riedel-deHaen,

respectively; KK samples and other plant materials from *Tai-hua-jan* drugstore; and a batch of HNK sample was prepared by the Herbal Medicine and Products Manufacturing Unit Ayurved Siriraj, Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2. Instruments

The HPTLC system (CamagMuttenz, Switzerland) consists of a semi-automatic sample spotter (Linomat 5), an automatic TLC plate developing equipment (ADC2) with a twin trough glass chamber and a densitometer (TLC Scanner 3). DigiStore 2 Documentation system was also used for the documentation. WINCATS software was used to control the system and analyze the results.

3. Methods

3.1 Preparation of standard solutions

The stock solutions of AA-I and AA-I sodium salt were prepared by dissolving 1 mg of AA-I in 1 ml of 1 M ammonium hydroxide (NH4OH) and 1 mg of AA-I sodium salt in 1 ml of distilled water. To determine the lower limit of detection (LOD), each stock solution of marker was diluted until the concentration of the analyte exhibited the signal height approximately three times the signal heights of blank samples.

3.2 Preparation of sample solutions

Amounts KK and HNK powder were accurately weighed for 5.49 mg and 300 mg, respectively.

The extracts of each sample were prepared using two kinds of solvents, 1 M NH_4OH in methanol and distilled water. The samples were extracted by vortex mixing for 30 minutes and then centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatant was filtered through a 0.2 μm polyvinylidene fluoride (PVDF) membrane filters for the analysis.

3.3 TLC analysis

By modifying the method of Ohno T et al, 2006, the mobile phases of chloroform/methanol (7:3, v/v), chloroform/methanol/acetic acid (65:20:1, v/v), and chloroform/methanol/ammonium hydroxide (10:10:1, v/v) were used for HPTLC analysis and acetonitrile/methanol/water (3:0.5:1, v/v) was used for RP-TLC analysis. The extracted solutions of KK, HNK, AA-I and AA-I sodium salt were subjected to HPTLC silica gel 60 F_{254} and TLC silica gel 60 RP-18F_{254s} plates which were pre-saturated in mobile phase for 5 minutes. The saturation time was 20 minutes and the developing distance for HPTLC and RP-TLC plate was 6.0 cm. After development, the plates were dried by cold air and detected under the ultraviolet light of 254, 323 and 366 nm. The existence of AA-I and AA-I salt in each sample was identified by comparing the Rf value and absorption spectrum of the sample with that of the AA-I and AA-II markers. To determine LOD, each known concentration of marker was diluted and run on the TLC plate until the lowest concentration exhibited approximately 3 times of noise response level.

Results

Identification of AA-I and AA-I salt

The identification of aristolochic acids in the samples is based on the similarity of Rf values and the absorption profiles to those of the reference markers (AA-I and AA-I sodium salt). Chromatograms of AA-I and AA-I salt were developed using three mobile phase systems for HPTLC plate and one mobile phase system for RP-TLC plate. The absorption spectrum of AA-I and AA-I salt developed from each mobile phase condition was scanned at the wavelength of 200-450 nm. Each marker compound had strong absorption at 251, 323 and 398 nm. Representative TLC finger prints of AA-I and AA-I salt developed from the solvent system of chloroform/methanol/acetic acid (65:20:1) were demonstrated in Figure 1, while Rf values and absorption spectrum of AA-I and AA-I salt standards of all mobile phases are shown in Table 1 and Figure 2, respectively.

Identification of aristolochic acids in KK using HPTLC analysis

All mobile phase systems could separate AA-I contained in KK. The peaks have the same UV absorption profile and Rf values as that of AA-I marker. The solvent system of chloroform/methanol/acetic acid (65:20:1) yielded the best separation and had a very

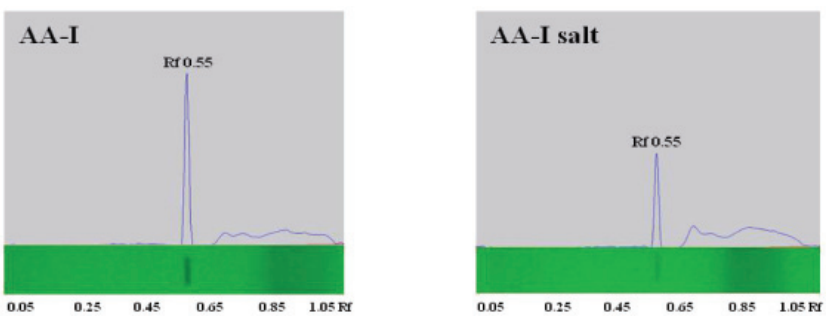

Fig. 1 Representative chromatogram and absorption spectrum of AA-I and AA-I salt developed in chloroform : methanol : acetic acid (65:20:1)

Table 1 Rf-value of AA-I and AA-I salt standards

Plates	Solvent system	Rf-value	
		AA-I	AA-I salt
HPTLC	chloroform : methanol (7:3, v/v)	0.36	0.38
	chloroform : methanol : acetic acid (65:20:1, v/v)	0.55	0.55
	chloroform : methanol : ammonium hydroxide (10:10:1, v/v)	0.60	0.61
RP-TLC	acetonitrile : methanol : water (3:0.5:1, v/v)	0.48	0.46

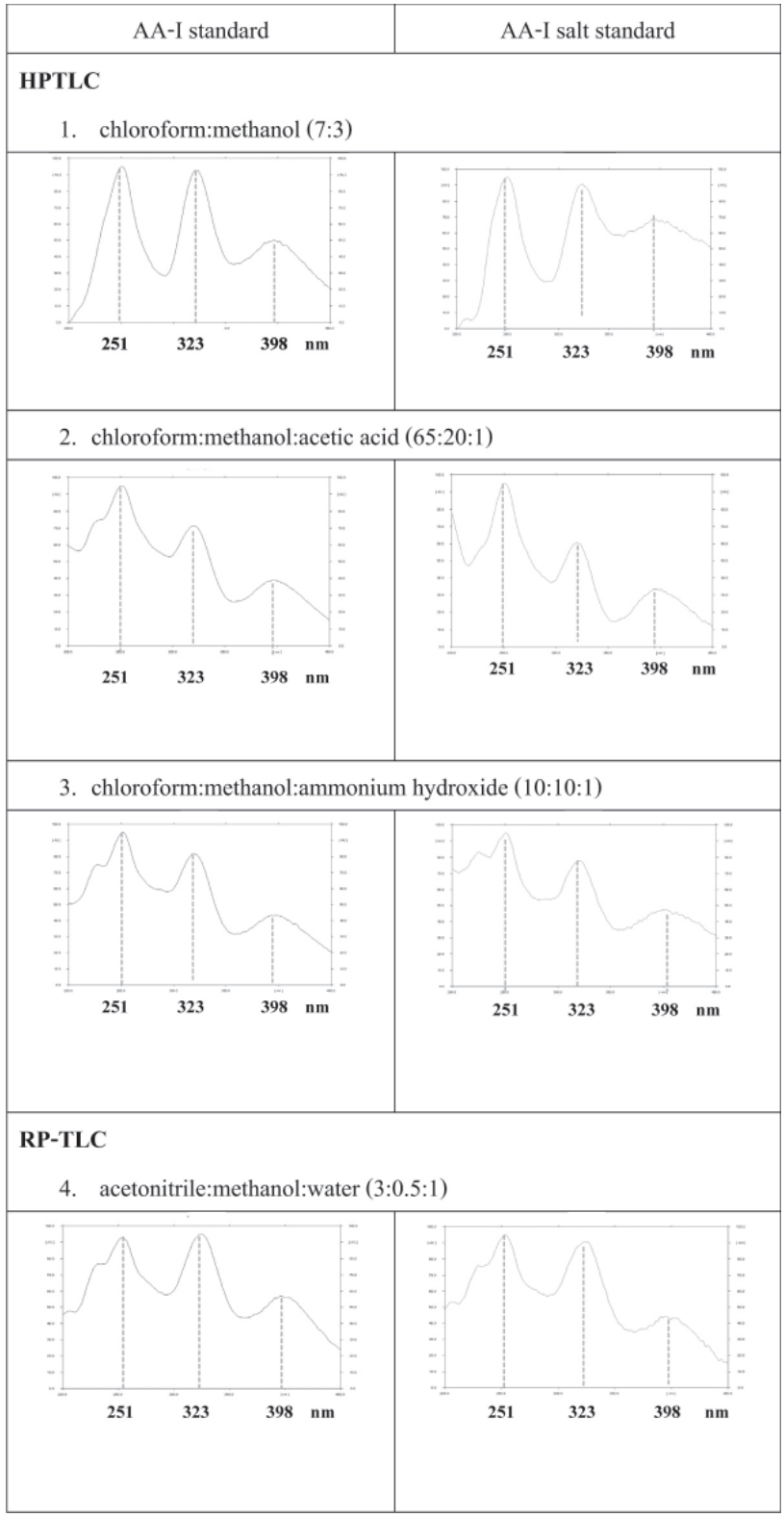

Fig. 2 UV absorption spectra of AA-I and AA-I sodium salt standards (scanned from 200 - 450 nm)

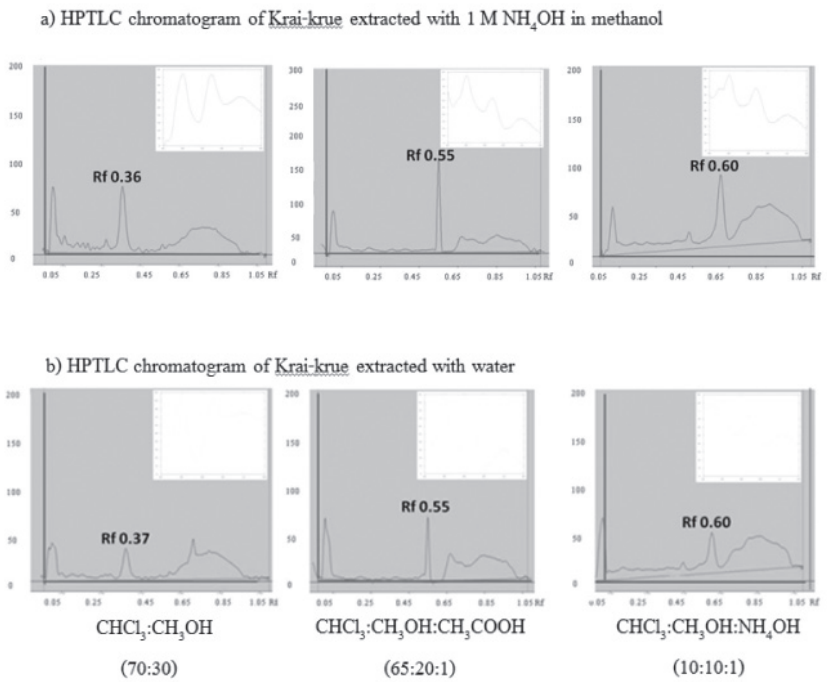

Fig. 3 HPTLC chromatogram of *Krai-krue* in different solvent systems detected under UV 323 nm. Absorption spectrum scanned from wavelength 200-450 nm was demonstrated in the right corner of each chromatogram.

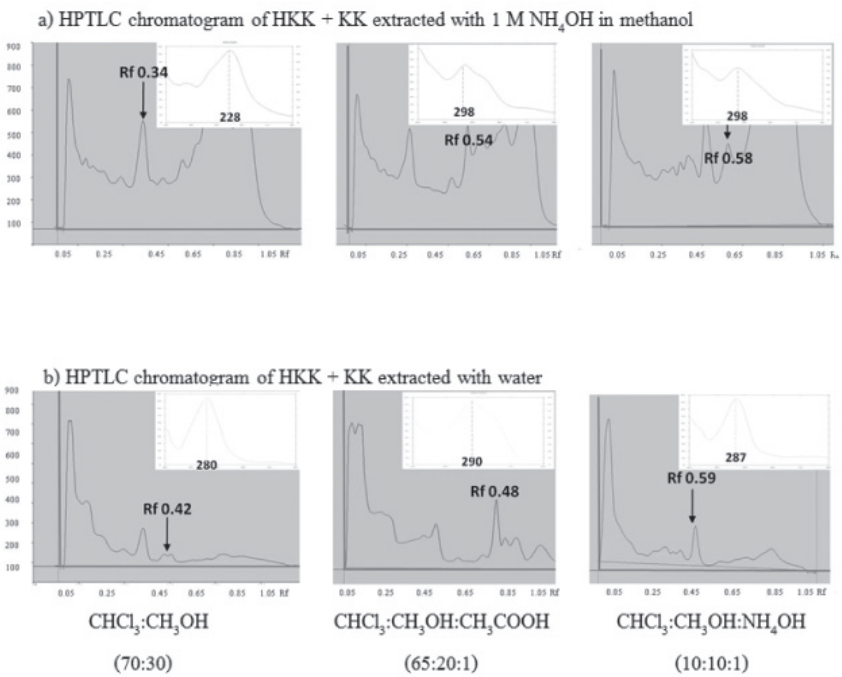

Fig. 4 HPTLC chromatogram of HNK detected under UV323 nm. Absorption spectrum scanned from wavelength 200-450 nm was demonstrated in the right corner of each chromatogram.

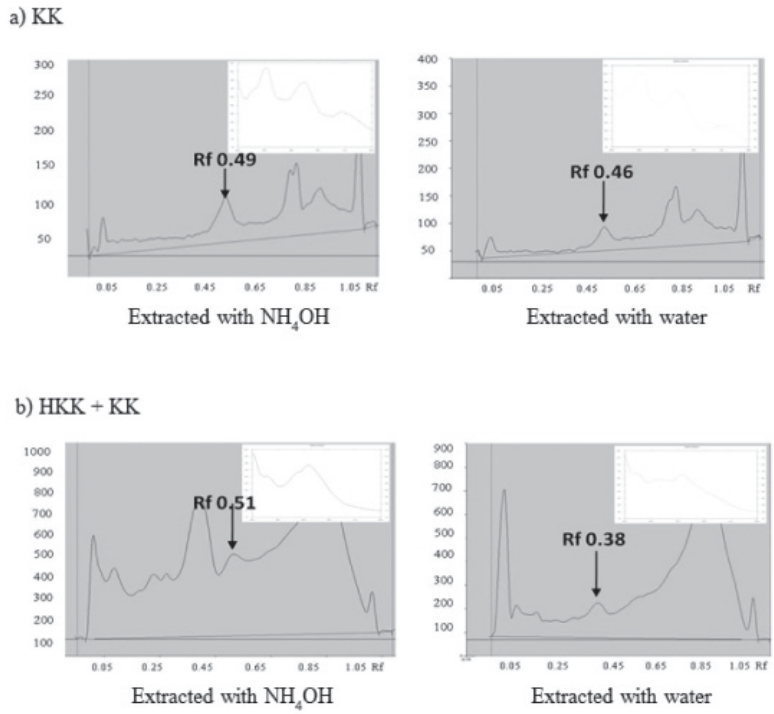

Fig. 5 Chromatogram of KK and HNK in RP-TLC analysis detected under UV 323 nm. Absorption spectrum scanned from wavelength 200-450 nm was demonstrated in the right corner of each chromatogram.

sharp peak (Figure 3).

Identification of aristolochic acids in HNK using HPTLC analysis

Although the chromatogram of HNK in all mobile phase systems showed a suspected peak that had the Rf value near to the AA-I marker; however, their absorption spectra were not similar to those of the markers (Figure 4).

Identification of aristolochic acid compound in KK and HNK by RP-TLC analysis

Using RP-TLC with solvent system of acetonitrile/methanol/water (3:0.5:1) give the results corresponding to HPTLC analysis. Chromatogram of KK showed better separation than that of HNK. Although the peak with the Rf value similar to that of the markers was exhibited in both KK and HNK, the absorption spectrum of the peak of the recipe was not similar to those of the markers (Figure 5).

Lower limit of detection (LOD)

By diluting the concentration of AA-I and AA-I salt until the lowest concentration yielded the average response approximately 3 times as high as the baseline noise level, HPTLC analysis had the lower limit of detection at 8 ng for AA-I and 5 ng for AA-I salt. For RP-TLC analysis, the lower limits detected for both AA-I and AA-I salt were 15 ng.

Discussion and Conclusion

In this study, the samples were prepared based on the traditional usage of Thai traditional herbal remedies in form of decoction or herbal tea. However, due to difficulty in dissolving AA-I in water, ammonia was used in the sample preparation. Specimens of KK and a Thai herbal recipe (HNK) were, therefore, extracted with tow kinds of solvents, ammonia and water. The results showed that the Rf value and absorption spectrum of AA-I were similar for the samples extracted with either ammonia or water. The intensity of AA-I peak of ammonia extract was higher than that of water extract.

HPTLC and RP-TLC analyses could detect AA-I in a single herb but not in a multiple herbal recipe. This may be due to the complexity of a lot of ingredients in the recipe. Although several mobile phase systems were tried in this study to obtain the best separation for the identification of AA-I in KK specimen and one Thai herbal recipe containing KK, the peak of AA-I was isolated and detected only in the KK specimen but not in the recipe. The best solvent system for HPTLC analysis was chloroform/methanol/acetic acid (65:20:1, v/v) while the best solvent system for RP-TLC analysis was acetonitrile/methanol/water (3:0.5:1, v/v). The lower limits of detection values of AA-I were found in nanogram, which were 8 ng and 15 ng for HPTLC and RP-TLC, respectively. The HPTLC analysis revealed better sensitivity than that using the RP-TLC method. The addition of a small amount of acetic acid in the mobile phase may help to separate acidic compounds in the samples.15 The chromatogram of HNK revealed that the complexity of recipes could lead to an unclear separation. This undesirable result might be attributed to the multiple components of many herb species blended together in the recipe. Therefore, to improve the AA-I detection in multiple herbal recipe, the sample should be cleaned before the analysis to remove the interfering compounds.

The method developed in this study is a primary tool for rapid identification of AA-I in herbal materials to ensure their safety on the basis of the absence of AA. However, to complete the qualitative and quantitative analyses of AA in the recipe containing KK, further development should be performed in the step of sample purification to obtain a better resolution of AA chromatogram.

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บทคัดย่อ

การตรวจหาที่มีประสิทธิภาพสูงสุดของสาร aristolochic acid I ในสมุนไพรไคร้เครือด้วยเทคนิคโครมาโทกราฟีชั้นบางสมรรถนะสูง

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Aristolochic acids (AA) เป็นสารมีพิษที่พบในพืชสกุล *Aristolochiaceae* จัดเป็นสารกลุ่ม nitrophenanthrene carboxylic acid ซึ่งมีสาร aristolochic acid I (AA-I) และ aristolochic acid II (AA-II) เป็นองค์ประกอบหลัก วิธีตรวจสอบที่ง่ายรวดเร็ว และมีความไวในการตรวจหาสารดังกล่าวในวัตถุดิบสมุนไพรเพื่อหลีกเลี่ยงการใช้ จึงมีความจำเป็น เทคนิคโครมาโทกราฟีแบบชั้นบางสมรรถนะสูง (เอชพีทีแอลซี) ได้ ถูกนำมาปรับเพื่อหาสภาวะที่เหมาะสมและมีประสิทธิภาพสูงสุดในการตรวจหาสาร AA-I ในสมุนไพรไคร้เครือ ในการทดลองได้หาวัฏภาคเคลื่อนที่ที่เหมาะสมเพื่อให้เกิดการแยกของสารที่ดี 3 ระบบสำหรับวัฏภาคนิ่งชนิด เอชพีทีแอลซี และยืนยันผลการตรวจพบโดยใช้วัฏภาคนิ่งชนิด อาร์พีทีแอลซี ผลการทดลองพบว่าวัฏภาคเคลื่อนที่ คลอโรฟอร์ม : เมทานอล: กรดอะซิติก (65 : 20 : 1) เหมาะสมสำหรับวัฏภาคนิ่งชนิด เอชพีทีแอลซีที่สุด โดยมีค่าต่ำสุดที่วิธีสามารถตรวจพบได้เท่ากับ 8 นาโนกรัมและส่วนผสมของอะซิโตไนไตรล์:เมทานอล : น้ำ (3 : 0.5 : 1) เป็นวัฏภาคเคลื่อนที่ที่เหมาะสมสำหรับการ วิเคราะห์ด้วยวัฏภาคนิ่งชนิด อาร์พีทีแอลซี โดยมีค่าต่ำสุดที่วิธีสามารถตรวจพบได้เท่ากับ 15 นาโนกรัม จากทั้ง 2 สภาวะดังกล่าวข้างต้น พบว่า ค่า Rf ของสาร AA-I ในสมุนไพรไคร้เครือวิเคราะห์ด้วยเทคนิค เอชพีทีแอลซี และอาร์พีทีแอลซีมีค่าเท่ากับ 0.55 และ 0.48 ตามลำดับ จากผลการศึกษาสรุปได้ว่า วัฏภาคนิ่งชนิด เอชพีทีแอลซี และ อาร์พีทีแอลซี สามารถตรวจหา AA-I ในสมุนไพรไคร้เครือได้แต่วัฏภาคนิ่งชนิด เอชพีทีแอลซีมีความไวในการตรวจหาสาร AA-I มากกว่าชนิด อาร์พีทีแอลซี

คำสำคัญ: **Aristolochic acid, ไคร้เครือ, เอชพีทีแอลซี, อาร์พีทีแอลซี, ยาจากสมุนไพร**