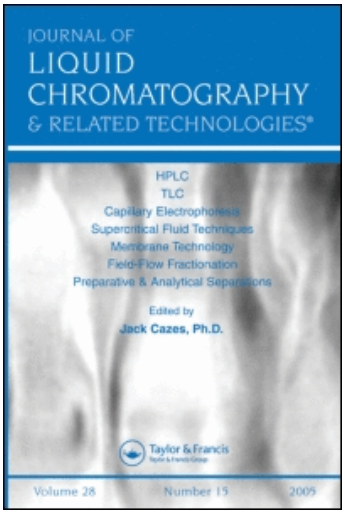


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# APPLICATION OF TWO DIMENSIONAL THIN LAYER CHROMATOGRAPHY PATTERN COMPARISON FOR FINGERPRINTING THE ACTIVE COMPOUNDS IN THE LEAVES OF *VITEX TRIFOLIA* LINN POSSESSING ANTI-TRACHEOSPASMOLYTIC ACTIVITY

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# APPLICATION OF TWO DIMENSIONAL THIN LAYER CHROMATOGRAPHY PATTERN COMPARISON FOR FINGERPRINTING THE ACTIVE COMPOUNDS IN THE LEAVES OF *VITEX TRIFOLIA* LINN POSSESSING ANTI-TRACHEOSPASMOLYTIC ACTIVITY

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□ *We have developed one approach to fingerprint and estimate the active compounds in the leaves of* Vitex trifolia *Linn possessing anti-tracheospasmolytic assay using two-dimensional TLC pattern comparison. Based on the two-dimensional TLC pattern and the activity of the centrifugal partition chromatography fractions, we concluded that the semi polar compounds were responsible for anti-tracheospasmolytic activity. The best non-polar/semi polar mobile phases for the two-dimensional TLC using silica gel as the stationary phase were chloroform/methanol (9/1) as the first mobile phase and ethyl acetate/chloroform/methanol (28/28/44) as the second mobile phase.*

**Keywords** asthma, centrifugal partition chromatography, fingerprinting, thin layer chromatography, tracheospasmolytic assay, *Vitex trifolia* Linn

## INTRODUCTION

*Vitex trifolia* Linn is used as one ingredient of *jamu*, an Indonesian traditional functional food. *Jamu* is consumed to enhance the body

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condition against several diseases such as dry cough, fever, and headaches. In this preparation both water soluble and insoluble parts are used.[1]

Different groups of constituents have been reported present in this plant. Several researchers reported the presence of phenolic acids *p*-hydroxybenzoic acid, vanillic acid, coumaric acid, protocatechuic acid, caffeic acid, and ferulic acid; glucosides agnusid and aucubin; flavonoids luteolin-7-glycoside and homoorientin; and alkaloid Vitricin.[2,3] The leaf extract was also reported to contain myristic, palmitic, stearic, palmitoleic, oleic linoleic acid; glucoside casticin; flavonoids artemetin, tetramethylquercetagetin, isoorientin, and Lu-glucoronide; and diterpenoids vitetrifolin, difuran, dihydrosolidagenone, and abietatriene.[4–6]

Despite the extensive researches on the health promoting activity of this plant; e.g., anti-inflammation, anti-microbial activity, and anti-cancer;[7–9] very little research is focused on the treatment of asthma. Asthma is currently increasing worldwide in both frequency and severity. It affects about 5% of the population of industrialized nations and approximately 20.5 million Americans including 6.2 million children had asthma in 2004 in the U.S.[10]



This plant has been reported to have anti-tracheospasmolytic activity, which is related to the suppression of trachea contraction in asthma.[11–13] Vitoesin A and vitexicarpin are the only compounds, which had been isolated from *Vitex trifolia* Linn and possessing anti-tracheospasmolytic activity.[11] There are other active compounds present in this plant which still not yet be isolated and identified. However, it is time consuming to isolate and identify all possible active compounds from this plant. Therefore, the fingerprinting of the active constituents will be more useful in order to warranty the quality of the extract.

In this research we have developed two dimensional (2D) thin layer chromatography (TLC) for a detection tool. TLC has been known as the fast tool for the detection of compounds. Another advantage of TLC is the capability to detect more compounds than HPLC, although the resolution is poorer. In this regard, the compounds which can not be eluted still can be detected. Moreover, the compounds having no UV absorption, e.g., sugar, still can be detected by reagent spraying.

The 2D TLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extracts. Thus, it can be used as a tool in the quality control in order to warranty that the active compounds are extracted. The active compounds are not independent on each other as they could have a synergism/antagonism effect. Therefore, the use of the mixture is preferred in a traditional healer rather than a single pure compounds.

The pattern comparison between plant fractions requires a reproducible fractionation method. Centrifugal partition chromatography (CPC) is the liquid-liquid chromatography, which can fractionate the plant extract

in a short time (less than 3 hours) and is reproducible because all parameter, e.g., centrifugal speed, pressure, and elution flow rate, can be controlled. Another advantage of CPC is all compounds will be eluted as it is a liquid-liquid chromatography. All compounds from both solvent phases can be recovered.[14]

The aim of this research is to develop a 2D TLC solvent system for the ethanol extract of the leaves of *Vitex trifolia* Linn. The optimum solvent system can be used to fingerprint and estimate the active compounds in the extract possessing anti-tracheospasmolytic activity by 2D TLC pattern comparison between the fractions. CPC will be used to fractionate the plant extract.

## EXPERIMENTAL

### Plant Material

Leaves of *Vitex trifolia* Linn were obtained from Gadjah Mada University, Indonesia. A voucher specimen is stored at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia.



### Chemicals

All organic solvents (p.a. grade) were purchased from J.T. Baker (Deventer, the Netherlands). Anisaldehyde was obtained from Acros Organic (Geel, Belgium).

### Instrumentation

A modular Sanki (Kyoto, Japan) Centrifugal Partition Chromatograph type LLN with six cartridges (total internal volume 125 mL) was used. It consisted of a power supply (model SPL), a triple-head constant-flow pump (model LBP-V) and a centrifuge (Model NMF). A Panasonic Pen recorder (Model VP 677222 A) was connected to a UVIS 200 detector (Linear Instruments, Reno, NV, USA). Fractions were collected by means of a superrac fraction collector (LKB 2211).

### Separation Methods

Based on the tracheospasmolytic assay, the fractionation was developed as shown in Figure 1. This method consists of the fractionation of ethanol extract by means of CPC. The ethanol extract was fractionated by CPC with the solvent system *n*-heptane/ethyl acetate/methanol/water (6:1:6:1).

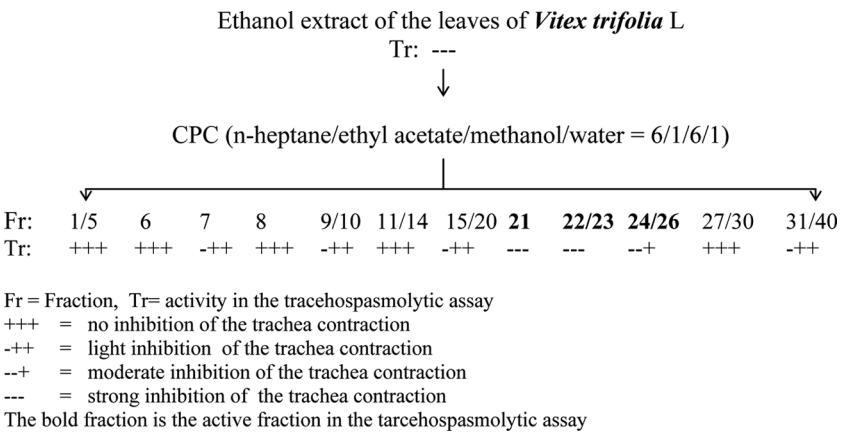


**FIGURE 1** The flowchart of the fractionation guided by the tracheospasmolytic assay.

Ascending mode elution was performed to collect 160 mL of eluate including the void volume. The inlet was changed from upper to lower layer and mode of elution was changed to descending to collect another 160 mL. The run was repeated for 3 times to inject 10 g of extract in total. The flow rate was set to 1 mL/ min, the fraction size was 4 mL, and the pressure of the pump was limited to 60 Bar.

The fractions were pooled based on TLC patterns. The TLC system that was used was chloroform/methanol (9:1). All of the fractions were evaporated and tested in the tracheospasmolytic assay. This fractionation procedure is repeated three times.



## The Searching of TLC Mobile Phase

### *Non-Polar TLC Mobile Phase*

The ethanol extract was spotted to the TLC plate ($4 \times 9$ cm). It was developed in a TLC chamber using several solvent systems such as toluene, *n*-pentane, chloroform, acetone, ethanol, diethyl ether, methanol, and ethyl acetate. It was then developed using several compositions of choloroform/methanol, chloroform/acetone, chloroform/ethanol, petroleum ether/methanol, petroleum ether/ethanol, petroleum ether/acetone, ethyl acetate/methanol, ethyl acetate/ethanol, and ethyl acetate/acetone. Each of those compositions were mixed using the ratio of 9:1, 8:2, 7:3, and 6:4. The extract was also developed using the mobile phase of ethyl acetate/chloroform/methanol (2/2/1) and ethyl acetate/chloroform/methanol (28/28/44). After developing, the TLC pates were observed under UV with $\lambda = 254$ and $\lambda = 366$ and then sprayed with anisaldehyde solution in sulfuric acid.

***Polar TLC Mobile Phase***

The ethanol extract was developed in a TLC chamber using several mobile phases: ethanol/methanol (3/7), ethanol/methanol (1/9), ethanol/methanol (4/6), ethyl acetate/formic acid/acetic acid/water (100/11/11/27), acetone/formic acid/acetic acid/water (100/11/11/27), chloroform/methanol/formic acid/acetic acid/water (90:10:10:15:5), chloroform/methanol/formic acid/acetic acid/water (80/20/10/15/5), ethanol/formic acid/acetic acid/water (100:10:15:5), water/ethanol/butanol (1/1/9), water/ethanol/butanol (3/3/9), water/ethanol/butanol (5/5/9), water/methanol/butanol (1/1/9), chloroform/methanol/water (90:10:1). After developing, the TLC pates were observed under UV with $\lambda = 254$ and $\lambda = 366$ and then sprayed with anisaldehyde solution in sulfuric acid.

## *In vitro* Inhibition of Antigen-Induced Contraction of Guinea-Pig Trachea, the Tracheospasmolytic Assay[12]

*Sensitization.* Male Hartley guinea pigs (650–750 g) were given, via injection, 3 mg ovalbumin in 300 μL saline (0.9% NaCl). The procedure was performed at least 2 weeks before the experiment.

*Antigen-induced contraction.* The animals were sacrificed by a sharp blow to the head and tracheae were isolated, carefully trimmed of excess fatty and connective tissue, and cut into strips of equal width (one full cartilage ring, ~2 mm). The preparation was performed under 50 mM $Na^+/K^+$ phosphate buffer. Each preparation was placed in a 20-mL water-jacketed organ bath containing Krebs buffer, which was maintained at 37°C and constantly aerated with 95% $O_2$/5% $CO_2$. The Krebs buffer contained, besides the usual composites, 3 μM indomethacin. The tissues were placed under 0.5 g of passive tension and equilibrated for 60 min, during which they were washed every 15 min with fresh indomethacin containing Krebs solution. Before the challenge with ovalbumin, the tissues were incubated with or without the testing plant extract for 30 min. Tissues were contracted with a series of four concentrations of ovalbumin (5, 50, 500, and 5000 ng/mL) for 15 min each. After one hour, carbachol (10 μM) was added to the preparations, and the antigen induced response, which was recorded continuously, was expressed as a percentage of this reference carbachol contraction.



## RESULTS AND DISCUSSION

### Search for TLC Mobile Phase

To be able to detect the active compound, the using of two dimensional TLC was more promising compared to the one dimensional TLC, as the

resulting separation will be better. However, the success of the detection using TLC is really depending on the right choice of mobile phase. The mobile phase should be able to separate the sample, thus the compound will be able to be detected. In this experiment, both non-polar/semi polar and polar mobile phases were tried to be standardized to separate the ethanol crude extract of the leaves of *Vitex trifolia* L. The stationary phase that was used was silica gel.

## Search for Non Polar/Semi Polar Mobile Phase

For the first step of the search for non-polar/semi polar mobile phase, several compositions of solvents ranging from a very non-polar to the polar solvent was applied for the mobile phase in TLC. As shown in Table 1, chloroform/methanol (9/1) and chloroform/methanol (8/2) have 13 spots in TLC. This number is the biggest compared to the other solvents. Therefore, this mixture can be used as the first mobile phase in the two



**TABLE 1** The number of spots detected in TLC after developed using several non-polar/semi polar mobile phases. The spots were observed under UV with $\lambda = 254$ and 366 and after spraying with anisaldehyde solution in sulfuric acid

| Mobile Phases | Number of Spots | Mobile Phases | Number of Spots |
|---|---|---|---|
| Chloroform/MeOH | | Petroleum ether/acetone | |
| (9/1) | 13 | (9/1) | 3 |
| (8/2) | 13 | (8/2) | 5 |
| (7/3) | 12 | (7/3) | 5 |
| (6/4) | 12 | (6/4) | 4 |
| Chloroform/acetone | | Ethyl acetate/MeOH | |
| (9/1) | 5 | (9/1) | 10 |
| (8/2) | 7 | (8/2) | 10 |
| (7/3) | 8 | (7/3) | 8 |
| (6/4) | 7 | (6/4) | 7 |
| Chloroform/EtOH | | Ethyl acetate/EtOH | |
| (9/1) | 11 | (9/1) | 9 |
| (8/2) | 9 | (8/2) | 7 |
| (7/3) | 11 | (7/3) | 6 |
| (6/4) | 12 | (6/4) | 10 |
| Petroleum ether/MeOH | | Ethyl acetate/acetone | |
| (9/1) | 1 | (9/1) | 9 |
| (8/2) | 2 | (8/2) | 7 |
| (7/3) | 2 | (7/3) | 8 |
| | | (6/4) | 9 |
| Petroleum ether/EtOH | | Petroleum ether | 1 |
| (9/1) | 4 | Chloroform | 5 |
| (8/2) | 3 | Ethyl acetate | 8 |
| (7/3) | 4 | Acetone | 9 |
| (6/4) | 5 | Ethanol | 7 |
| | | Methanol | 5 |

dimensional TLC. Although pure chloroform or methanol has less spots rather than ethyl acetate, acetone, and ethanol, the mixture of chloroform and methanol is the best elution system in this case. On the other hand, the mixture of ethyl acetate and acetone that expected to be the best elution system, as acetone or ethyl acetate gave the best separation, gave the least quality of separation compared to the mixture of chloroform and methanol.

The next step was the search for the second mobile phase for the two dimensional TLC. To make the different characteristic of the second mobile phase, it can be done by changing the selectivity and the polarity. The adding of ethyl acetate will change the electricity field and the adding of methanol will increase the polarity. As shown in Table 2, the separation using ethyl acetate/chloroform/methanol (28/28/44) gave the best quality of separation compared to the other composition. Therefore, this mobile phase can be applied for the second non-polar/semi polar mobile phase in two-dimensional TLC.



## Search for Polar Mobile Phase

To find the polar mobile phases, the present composition of the polar mobile phases were compared and then the compositions were modified. The basic compositions were ethanol/methanol (1/9), ethyl acetate/formic acid/ acetic acid/water (100/11/11/27), and water/methanol/butanol (1/1/9). Based on these three mobile phases the modification of the composition was made. As shown in Table 3, the elution of the sample using chloroform/methanol/formic acid/acetic acid/water (80/20/10/15/5) resulted in the best separation compared to the elution using the other mobile phases. This mobile phase was appropriate to use as the first mobile phase in two-dimensional TLC for the polar system.

The consideration to choose the second mobile phase was based on the mobile phase that has a different selectivity but results in a good separation. Water/ethanol /butanol (1/1/9) was chosen for this reason, as it is the

**TABLE 2** The number of spots detected in TLC after developed using mobile phase that was modified by adding of ethyl acetate to chloroform/methanol. The spots were observed under UV with $\lambda = 254$ and 366 and after spraying anisaldehyde solution in sulfuric acid

| Elution System | Number of Spots |
|---|---|
| Ethyl acetate/chloroform/methanol (2/2/1) | 12 |
| Ethyl acetate/chloroform/methanol (1/2/1) | 11 |
| Ethyl acetate/chloroform/methanol (1/9/1) | 11 |
| Ethyl acetate/chloroform/methanol (28/28/44) | 13 |
| Ethyl acetate/chloroform/methanol (1/1/4) | 9 (tailing) |

**TABLE 3** The number of spots detected in TLC after developed using several polar mobile phases. The spots were observed under UV with $\lambda = 254$ and 366 and after spraying with anisaldehyde solution in sulfuric acid

| Elution Systems | Number of Spots |
|---|---|
| ethanol/methanol | |
| (1/9) | 8 |
| (3/7) | 6 |
| (4/6) | 5 (tailing) |
| ethyl acetate/formic acid/acetic acid/water | |
| (100/11/11/27) | 12 |
| acetone/formic acid/acetic acid/water | |
| (100/11/11/27) | 5 |
| chloroform/methanol/formic acid/acetic acid/water | |
| (90:10:10:15:5) | 13 |
| chloroform/methanol/formic acid/acetic acid/water | |
| (80/20/10/15/5) | 17 |
| ethanol/formic acid/acetic acid/water | |
| (100:10:15:5) | 3 (tailing) |
| water/ethanol/butanol | |
| (1/1/9) | 13 |
| (5/5/9) | 5 (tailing) |
| (3/3/9) | 9 |
| water/methanol/butanol | |
| (1/1/9) | 11 |
| Chloroform/methanol/water | |
| (90:10:1) | 10 |



mixture of the solvents that are different from the first mobile phase and the separation is quite good.

## The Two Dimensional TLC Pattern Comparison to Find the Active Spots

The ethanol extract that was active in the tracheospasmolytic assay was fractionated by means of CPC using the two-phase solvent system that had been developed by a previous researcher.[15] The fractionation was carried out using *n*-heptane/ethyl acetate/methanol/water (6/1/6/1) as the two-phase solvent system as shown in Figure 1.

As shown in Figure 2, after developing of the ethanol crude extract in two-dimensional TLC using the non-polar/semi polar mobile phase, 30 spots were observed. Based on the 2D TLC comparison of all fractions of CPC, the suspected active spots in tracheospasmilytic assay can be determined. The spot was considered to be the active one if it was present in the TLC chromatogram of the active fraction and vice versa. If the spot appeared in the TLC chromatograms of both active and non active fraction, it was considered as a non active spot.

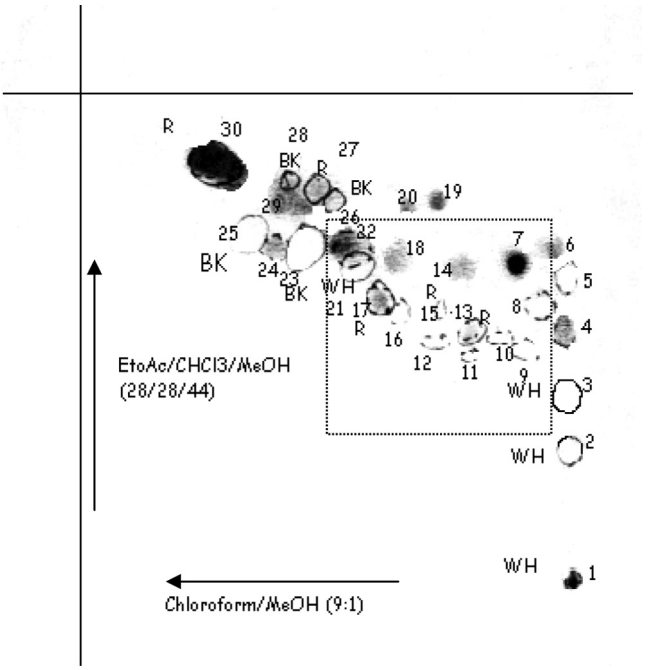


**FIGURE 2** The non-polar/semi polar spots of the ethanol extract that were detected in two dimensional TLC using chloroform/methanol (9/1) as the first mobile phase, ethyl acetate/chloroform/methanol (28/28/44) as the second mobile phase and silica gel as a stationary phase. As much as 30 spots were observed under UV with $\lambda = 254$ (⁘) and $\lambda = 366$ (○) and after spraying with anisaldehyde solution in sulfuric acid. The suspected active spots via the tracheospasmolytic assay are in the dot box. Spots under $\lambda = 366$ were noticed in different colors: white (WH), red (R), and black (BK).

There are several spots (spot 7–22) that seem to correlate with activity. In Figure 2, these spots were shown inside the dot frame. As spot 1–6 (the polar spots) were not considered in terms of the activity, the 2D TLC comparison using the polar mobile phase was not necessary. Thus, it can be concluded that the semi polar compounds are responsible for the activity in the tracheospasmolytic assay.

The pattern of these active spots on 2D TLC can be considered as a fingerprint of this active extract. This approach is very helpful in order to choose the best extraction method. In this case, for example, supercritical fluid extraction using supercritical $CO_2$ could be the best choice. Extraction using ethanol could be another choice as it can dissolve the semi-polar compounds. The parameter can be developed based on the optimum condition which can extract all the estimated active compounds. This 2D TLC system can be used to check whether all expected spots appear when the extract was produced.

This is the first report on the application of this combination of CPC, TLC, and bioassay to identify the active compounds from plant extracts.

It was done by manual handling of the chromatograms, however, this method can be further developed by using densitometric measurements of the TLC-plates and analyze all the results by means of multivariate data analysis.

## CONCLUSION

The estimation of the active compounds possessing anti-tracheospasmolytic activity in the ethanol extract of *Vitex trifolia* Linn was possible by using a 2D TLC chromatogram pattern comparison between the CPC fractions. The best non-polar/semi polar mobile phases for the 2D TLC using silica gel as the stationary phase were chloroform/methanol (9/1) as the first mobile phase and ethyl acetate/chloroform/methanol (28/28/44) as the second mobile phase.

It can be concluded that the semi polar compounds are responsible for the activity. This approach can be used for fingerprinting of the plant extracts, which is very necessary to be used to assure their quality.



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