# Quantitative Analysis of Some Phenolic Compounds in Ayurved Siriraj Wattana (AVS073) Using HPTLC

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## ABSTRACT

Ayurved Siriraj Wattana Recipe (AVS073) has long been used for healing in Thai traditional medicine treatment for health promotion, appetite inducement and retardation of health degeneration. The formula comprises 18 herbal components. Phenolic compounds are commonly found in different kinds of plants and show considerable pharmacological activities. Therefore, AVS073 and its 18 components were assessed for some presence of phenolic compounds. High performance thin layer chromatography (HPTLC) technique was used with the developing solvent mixture (hexane : ethyl acetate : acetic acid, 31:14:5). A mixture of caffeic acid, ferulic acid, gallic acid, kojic acid, p-coumaric acid and vanillic acid was used as a reference marker to identify the presence of individual phenolic compounds. By this method, caffeic acid and gallic acid were detected in AVS073 in the range of 0.0022-0.0065 and 0.0051-0.0058 % w/w, respectively. Of the 18 components of AVS073, the most commonly found phenolic acid was caffeic acid (0.0021 - 0.0695 % w/w). Gallic acid was detected in the highest amount in *Ferula assa-foetida*. However, ferulic acid, kojic acid and p-coumaric acid were found in some components, but not in AVS073. The chromatogram fingerprints of caffeic acid and gallic acid might be useful for the quality assessment of AVS073 production.

**Keywords:** High performance thin layer chromatography (HPTLC), fingerprint analysis, quantitative analysis, chromatographic fingerprinting

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## INTRODUCTION

According to Thai traditional medicine theory, illness is caused by an imbalance of four elements i.e., earth, water, wind and fire, and so the principle of the treatment is focused on the balancing of these elements. Using herbal based ingredients, several formulations were established for many treatments of conditions.[1]

Ayurved Siriraj Wattana (AVS073) is one of the Thai traditional formulations which comprises numerous herbal ingredients. It has long been used for health promotion, as an appetizer and for retardation of health degeneration. The formula comprise. *Boesenbergia rotunda* (L.) Mansf.; *Saussurea lappa* C.B. Clarke; *Liqusticum sinense* Oliv. cv. Chuanxiong; *Cinnamomum ilicioides* A. Chev.; *Carthamus tinctorius* L.; *Mal-*

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*lotus pandus* (Willd.) Muell. Arg.; *Cladogynos orientalis* Zipp. ex Span.; *Derris scandens* (Roxb.) Benth; *Cryptolepis buchanani* Roem.& Schult.; *Tinospora crispa* (L.) Hook.f. & Thomson *Caesalpinia sappan* L.; *Piper nigrum* L.; *Ferula assa-foetida* Regel; *Drypetes roxburghii* (Wall.) Hurusawa; *Aegle marmelos* (L.) Correa; Citrus sinensis (L.) Osbeck; *Terminalia chebula* Retz. and *Cyperus rotundus* L. A wide range of pharmacological properties of AVS073's components have been demonstrated in several studies.[2-6] The mechanism of action may be associated with the presence of phenolic compounds which are well-known phytochemicals found in all plants.[7-8] One of the main phenolic classes within plants are phenolic acids. However, environments such as cultivation, harvesting and season can affect plants.[9-11] Thus, the analysis of the main chemical constituents in each plant is needed to ensure the consistency of the components. Using thin layer chromatography (TLC), the chromatographic fingerprinting of phenolic acid in AVS073 was established and compared to that of phenolic markers (caffeic acid, ferulic acid, gallic acid, kojic acid, p-coumaric acid and vanillic acid).

TLC is the common chromatographic method which provides a pattern of fingerprint usually used for herbal analysis[12]. It is a flexible and uncomplicated chromatographic method that can simultaneously determine many samples at the same time. The information regarding polarity allows the identification of the constituents in the samples. The normal phase such as silica gel is the most commonly used stationary phase to analyse nearly all substances. TLC method was used in this study to screen and quantify phenolic compounds in AVS073 and its 18 herbal components. High performance thin layer chromatographic (HPTLC) has been finally developed for assessment of the quality of herbal drug and AVS073 production.

## MATERIALS AND METHODS

### *Chemicals and samples*

All solvents for the analysis were analytical grade. Hexane and acetic acid were purchased from Merck, Germany. Ethanol was purchased from Scharlau while ethyl acetate was obtained from Riedel-deHaen. Caffeic acid, ferulic acid, gallic acid, kojic acid, p-coumaric acid and vanillic acid were purchased from Sigma, USA.

AVS073 and its eighteen components were manufactured as powder under GMP Guidelines by the Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

### *Instruments*

Spotting device: CAMAG Linomat V Automatic sample spotter (Muttenz, Switzer land); Syringe: 100 ul Halmilton (Bonadus, Switzerland); Twin trough glass chamber (20×10 cm); HPTLC plates: silica gel 60 F254 plate 20 × 10 cm, (Merck, Darmstadt, Germany). Documentation: CAMAG Reprostar 3 with WinCATS software; Quantitative evaluation: CAMAG TLC Scanner 3 with WinCATS software.

### *Preparation of standard solution*

One milligram of each phenolic acid (caffeic acid, ferulic acid, gallic acid, kojic acid, p-coumaric acid and vanillic acid), was dissolved in one millilitre of 80 % ethanol and used as a mixture of phenolic reference markers.

### *Preparation of sample solution*

One hundred grams of AVS073 and each component were individually extracted with one liter of 80 % ethanol and evaporated under reduced pressure by rotary evaporator. It was kept frozen overnight prior to lyophilization. One hundred milligrams of the lyophilized powder was redissolved with 1 mL of 80% ethanol and vortex mixed for 10 minutes. After being centrifuged at 11,700 g at 4°C for 10 minutes, the supernatant was filtered through a 0.2 μm filter membrane before being applied on TLC plate for analysis.

### *Chromatogram of phenolic compound in AVS073 and its components*

To obtain the obvious fingerprint, the concentrations of samples were varied to get the optimal amount for applying on HPTLC plate. Three batches of AVS073 and one batch of its eighteen compositions were re-dissolved and

applied in triplicate. The mobile phase was hexane: ethyl acetate: acetic acid (31:14:5, v/v), the saturation time was 20 minutes and the developing distance was 6.0 cm. Visualization of separated compounds was achieved by fluorescent quenching on 254 nm for aromatic rings and conjugated double bond compounds or native fluorescence compounds under 366 nm ultraviolet light (UV). After staining with 0.5 % Fast Blue B salt (FBS) and heating at 110°C, phenolic compounds existing in the samples were detected under visible light. Some known phenolic compounds were identified by comparing retention factor value (Rf-value) and absorption spectrum with that of the phenolic reference markers.

### *Quantification of phenolic compounds in AVS073 and its components*

Five concentrations of each phenolic acid in duplicate, ranging from 25-900 ng, was diluted from each stock solution of phenolic acid (1 mg/ml) and used to establish a calibration curve. Three batches of AVS073 and one batch of its eighteen compositions were re-dissolved and applied in triplicate. The quantifications of phenolic acid in the AVS073 and its components were measured at the maximum absorption wavelength and calculated in terms of % w/w.

## RESULTS

### *TLC fingerprint analysis of phenolic compounds in AVS073 and its components*

Phenolic acid including caffeic acid, ferulic acid, gallic acid, kojic acid, p-coumaric acid and vanillic acid were used as phenolic markers in the study. After the development in the condition of mobile phase, hexane : ethyl acetate : acetic acid (31:14:5), these phenolic markers were screened under UV of 254 nm, 366 nm and detected under visible light after staining with 0.5 % FBS. The chromatogram fingerprint of these markers has been shown in Fig 1, Fig 2. The Rf values, which are identification parameters obtained for each phenolic marker have been described in Table 1. Kojic acid, gallic acid and caffeic acid were found as separated zones at the Rf values of 0.10, 0.18 and 0.33, respectively.

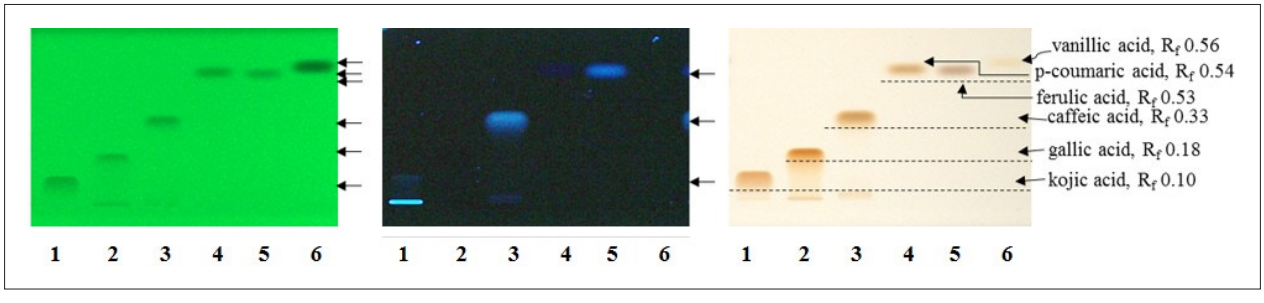


**Fig 1.** HPTLC profile of phenolic markers developed in mobile phase of hexane: ethyl acetate : acetic acid (31:14:5, v/v): track 1 kojic acid; track 2 gallic acid; track 3 caffeic acid; track 4 p-coumaric acid; track 5 ferulic acid and track 6 vanillic acid, visualized under ultraviolet light of 254nm (left), 366 nm (middle) and visible light after spraying with FBS (right).

**TABLE 1.** Summary of HPTLC profile of each phenolic marker developed in mobile phase of hexane: ethyl acetate : acetic acid (31:14:5, v/v)

| Track no | Phenolic markers | visualization under | | | Rf-value |
|---|---|---|---|---|---|
| | | uv 254 nm | uv 366 nm | visible light after FBS stained | |
| 1 | kojic | √ | - | √ | 0.10 |
| 2 | gallic | √ | - | √ | 0.18 |
| 3 | caffeic | √ | √ | √ | 0.33 |
| 4 | p-coumaric | √ | - | √ | 0.54 |
| 5 | ferulic | √ | √ | √ | 0.53 |
| 6 | vanillic | √ | - | √ | 0.56 |

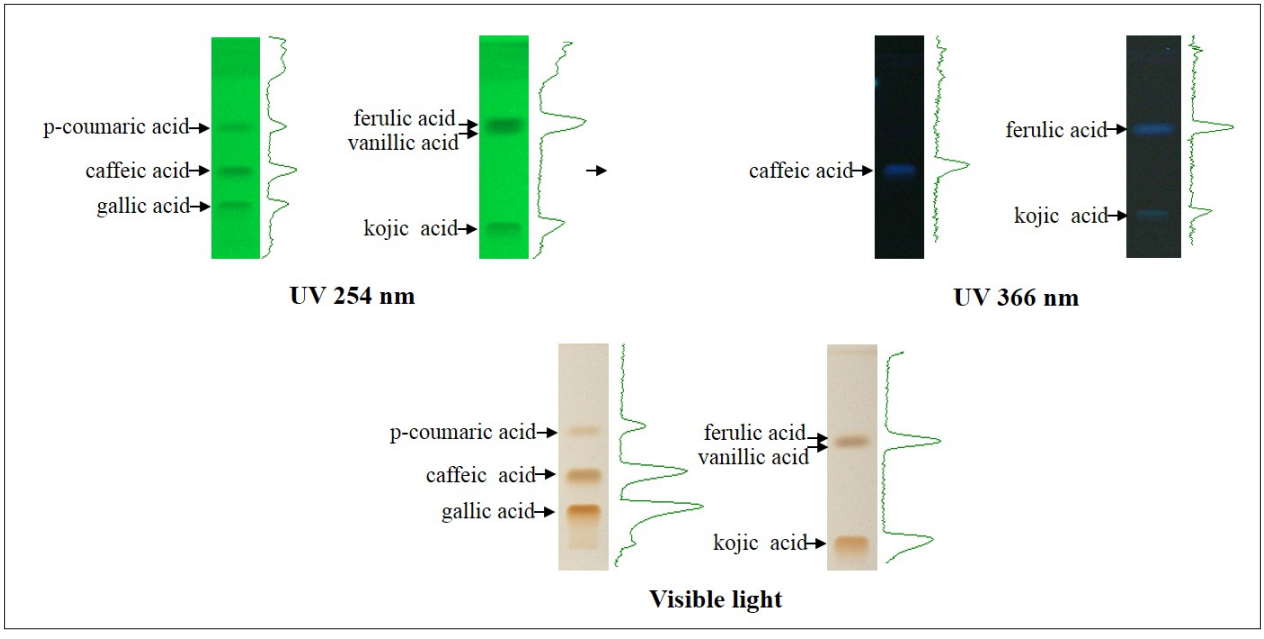


**Fig 2.** Densitometric profile of 6 phenolic markers

Ferulic acid, p-coumaric acid and vanillic acid were nearly detected at the Rf value of 0.53, 54 and 56 respectively. Ferulic acid might be further distinguished by its fluorescence expressed under UV 366 nm. In this condition, caffeic acid, gallic acid and kojic acid were distinguished from each other by their Rf values under UV 254, UV 366 as well as under visible light after spraying with 0.5% FBS except gallic acid which could not fluoresce under UV366. The extraction of AVS073 in 80% ethanol, developed by the same method, expressed several bands under UV 254 nm as well as under visible light after spraying with 0.5% FBS (Fig 3). Based on Rf values of phenolic markers, there were at least, two bands in AVS073 extract which could be possibly identified as gallic acid and caffeic acid (Fig 4). Thirteen components of AVS073 expressed bands of caffeic acid, five of which namely, *C. tinctorius, T. crispa, C. sappan, A. marmelos* and *C. rotundus* were low in intensity, while 5 components expressed gallic acid, two of which namely, *B. rotunda* and *D. roxburghii* were present in low intensity. There were five components which expressed ferulic acid which could be detected and quantified and three components expressed kojic acid, two of which namely, *C. tinctorius* and *A. marmelos* were expressed in low intensity. Also, there was one component which expressed p-coumaric acid with low intensity. The existing phenolic compounds were detected have been summarized in Table 2.

### *Quantification of phenolic compounds in AVS073 and its components*

Five concentrations of each reference marker, caffeic acid and gallic acid, were plotted against their area. The polynomial regression calibration curves were created with r value not less than 0.98 and 0.99, respectively. The maximum absorption wavelength after spraying with FBS for caffeic acid was 440 nm and gallic acid was 485 nm. The quantification of gallic acid content in three batches of AVS073 was in the range of 0.0051- 0.0083% w/w while that of caffeic acid were in range of 0.0022-0.0065% w/w. Moreover, both caffeic acid and gallic acid could be quantified in *Cinnamomum ilicioides*, 0.0027 and 0.0075% w/w and *Ferula assa-foetida*, 0.0695 and 0.2328% w/w, respectively. The other components in which only caffeic acid was detected and quantified were *Mallotus repandus, Derris scandens, Cryptolepis buchanani, Piper nigrum, Citrus sinensis* and *Terminalia chebula*, while only gallic acid was detected and quantified in *Liqusticum sinense* (Table 2).

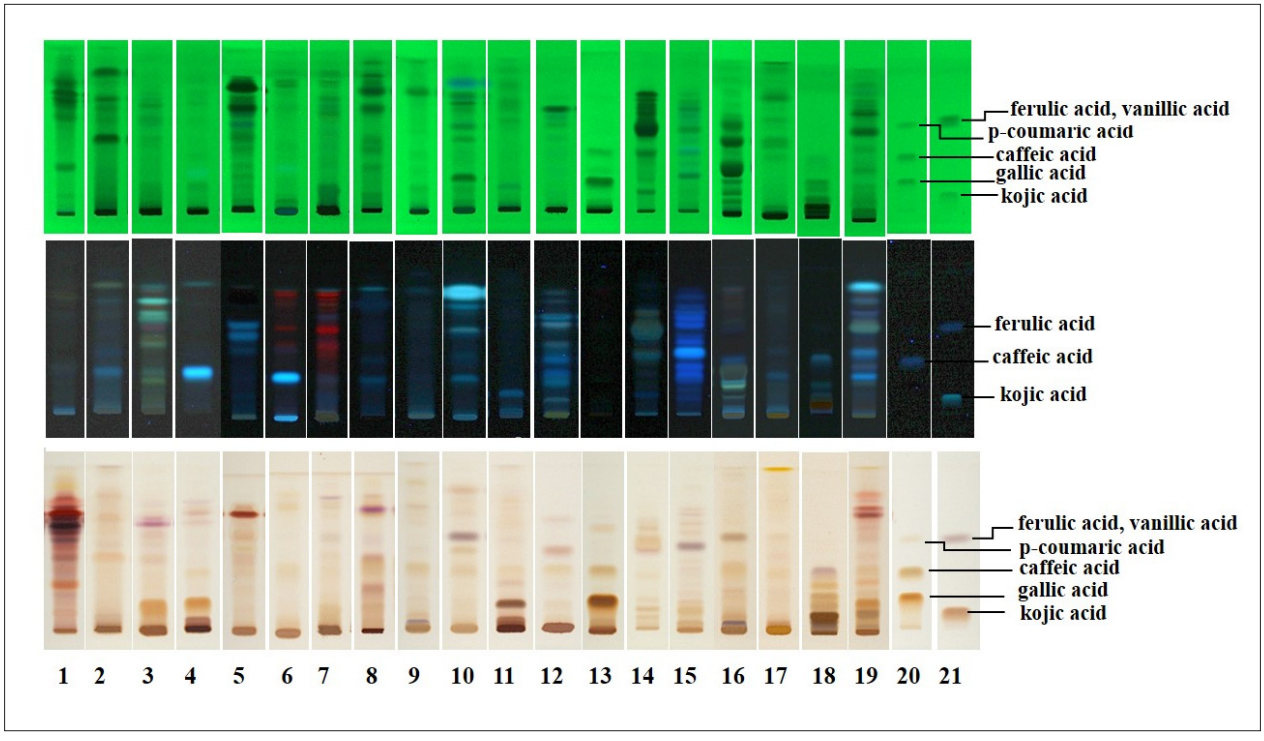


**Fig 3.** HPTLC fingerprints of AVS073 and its 18 components developed in mobile phase of hexane: ethyl acetate : acetic acid (31:14:5, v/v) and visualized under ultraviolet light of 254 nm (top), 366 nm (middle) and visible light after spraying with FBS (bottom). Track 1-18 showed component no 1-18; track 19 showed AVS073; track 20-21 showed phenolic standard mixture from bottom to top: gallic acid, caffeic acid and p-coumaric acid; and kojic acid, ferulic acid and vanillic acid.

## DISCUSSION

Due to their pharmacological activities and their existence in most plants,[13-17] the phenolic compounds were focused on in this experiment. Eighty percent ethanol extract of AVS073 and its components, developed with the same method, expressed several bands under UV 254 nm as well as under visible light after spraying with 0.5% FBS. Bands extending from that of the reference markers including, caffeic acid, ferulic acid, gallic acid, kojic acid, p-coumaric acid and vanillic acid, probably indicated other existing compounds. Two phenolic compounds, gallic acid and caffeic acid were detected and quantified by this method in the range of 0.0051-0.0058 and 0.0022-0.0065 % w/w, respectively. In spite of quantifying ferulic acid in *T. crispa, B. rotunda,*

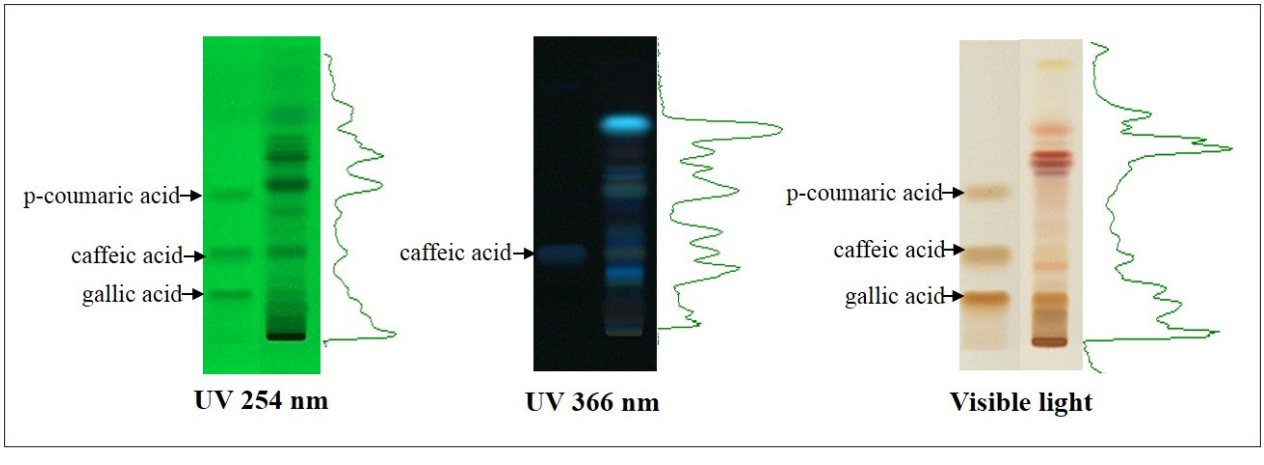


**Fig 4.** Densitometric profile of AVS073 showed the existing of gallic acid and caffeic acid in the recipe.

**TABLE 2.** Summary of each detected and quantified phenolic acid in AVS073 and its 18 components

| Sample name | Detected phenolic acid (% w/w) | | | | | |
|---|---|---|---|---|---|---|
| | Kojic- | Gallic- | Caffeic- | p-coumaric- | Ferulic- | Vanillic- |
| AVS073 | - | 0.0051-0.0058 | 0.0022-0.0065 | - | - | - |
| *B.rotunda* | - | low intensity | - | - | 0.007 | - |
| *S.lappa* | - | - | - | - | 0.009 | - |
| *L.sinense* | - | 0.0025 | - | - | - | - |
| *C.ilicioides* | - | 0.0075 | 0.0027 | - | - | - |
| *C.tinctorius* | low intensity | - | low intensity | - | - | - |
| *M.repandus* | - | - | 0.0021 | - | - | - |
| *C.orientalis* | - | - | - | - | - | - |
| *D.scandens* | - | - | 0.0041 | - | - | - |
| *C.buchanani.* | - | - | 0.0049 | - | - | - |
| *T.crispa* | - | - | low intensity | - | 0.0486 | - |
| *C.sappan* | - | - | low intensity | low intensity | - | - |
| *P.nigrum* | - | - | 0.0063 | - | - | - |
| *F.assa-foetida* | - | 0.2328 | 0.0695 | - | - | - |
| *D.roxburghii* | 0.0042 | low intensity | - | - | - | - |
| *A.marmelos* | low intensity | - | low intensity | - | 0.0154 | |
| *C.sinensis* | - | - | 0.0111 | - | 0.0338 | - |
| *T.chebula* | - | - | 0.0036 | - | - | - |
| *C.rotundus* | - | - | low intensity | - | - | - |

low intensity means unable to quantified due to the intensity is out of the scale

*S. lappa, A. marmelos* and *C. sinensis* as well as kojic acid in *D.roxburghii*, these two phenolic compounds could not be found in the recipe. This may be because the contents of kojic acid and ferulic acid, if existing in the recipe, were too low to quantify by this method. Even though ferulic acid was found in a large amount (0.0486 %w/w), it was in just only one plant component (*T. crispa*). The other remaining 13 compositions may cover up its existence after adding altogether 18 plant components. In contrast, gallic acid was quantified in only 3 plant components, but still exists in the recipe. This may be due to the quite large number of gallic acid (0.2328 %w/w) in *F. assa-foetida* plus some amount in *L. sinense* and *C. ilicioides* including low intensity in *B. rotunda* and *D. roxburghii*. Caffeic acid was also found in the recipe as well, although its content was low content in the component. This may be due to the number of components existing caffeic acid that was as high as 13 plants from a total of 18 plants. Chemical interaction may be another explanation of why some compound was not found in the recipe in spite of existing in the components. However, in this study, caffeic acid and gallic acid could be used as markers for the quality assessment of AVS073.

Since the chromatographic fingerprint is accepted or even recommended as an effective tool to assess quality control,[18] It may be used to monitor the stability of the herbal drugs as well.[19] By using the chromatographic fingerprint of the AVS073 in combination with quantification of these markers, quality control of the different batches of AVS073 can be assessed.

## CONCLUSION

HPTLC fingerprint was used in this study to establish the method for quality control assessment of AVS073. It was viewed under UV 254 nm, 366 nm and visible light after spraying with FBS combined with the quantification of some phenolic compounds. This analysis condition could simultaneously determine 4 phenolic acids (kojic acid, caffeic acid gallic acid and ferulic

acid) in which caffeic acid and gallic acid were selected as markers to quantify in the quality control assessment. Both chromatographic fingerprint and bioactive content could be used to evaluate the consistency and stability of the recipe and its components. Thus the simple, rapid, inexpensive and simultaneous determination method like HPTLC should be, an optimal choice in daily routine work for quality assessment of medicinal herbs, components and the recipe.

## ACKNOWLEDGMENTS

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