4. Conclusion

Phytochemical investigation of the aerial parts of *L. indica* has resulted in the isolation of three sesquiterpene lactones, lactuside B (**1**), 11β,13-dihydrolactucin (**6**), cichorioside B (**7**); two phenylpropanoids, caffeic acid (**2**), caffeic acid methyl ester (**3**); and two quinic acid derivatives, 3,4-di-*O*-caffeoylquinic acid methyl ester (**4**), 3,5-di-*O*-caffeoylquinic acid methyl ester (**5**). To the best of our knowledge, this study was the first to isolate compounds **1-3** from *L. indica*, and compounds **4** and **5** from the *Lactuca* genus.

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DETERMINATION OF CHLORPHENIRAMINE ADULTERATED IN HERBAL PRODUCTS USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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Summary

Determination of Chlorpheniramine Adulterated in Herbal Products Using High Performance Thin Layer Chromatography

A high-performance thin layer chromatographic (HPTLC) method was established for the identification, qualitative, and quantitative analysis of chlorpheniramine adulterated in herbal products. The samples were extracted with methanol, followed by TLC separation with cyclohexane-acetone-ammonia as mobile phase and UV scanning detection at 254 nm. The method was validated according to AOAC 2016 guidelines, which showed high specificity, good precision, and accuracy. The validated method was applied to 60 herbal products collected from the market via online purchase, at private pharmacies, or from traditional healthcare practitioners. Chlorpheniramine was detected in 18/60 products.

Keywords: *Chlorpheniramine, Adulteration, Herbal products, High performance thin layer chromatography.*

1. Introduction

In Vietnam, about 20% of the population is involved with chronic allergy and urticaria. This percentage kept increasing due to environmental pollution, resulting in high demand for pharmaceuticals and nutraceuticals for the treatment of allergies and irritation. Besides chemical drugs, people with allergies often prefer

herbal products because they believe herbal products are "harmless" or have fewer side effects [1]. Being aware of this tendency, some manufacturers created falsified herbal products by illegally adding synthetic drugs into their products to increase pharmacological activities. Among the anti-histamines, chlorpheniramine was commonly found in adulterations, thanks to its effectiveness and low cost. Several studies in Vietnam have revealed the presence of anti-histamines including chlorpheniramine in herbal products, using high performance liquid chromatography (HPLC) [2], high performance thin layer chromatography (HPTLC) [2], and liquid chromatography coupled to tandem mass spectroscopy (LC-MS/MS) [3]. HPTLC is an advanced technique of TLC with several advantages namely auto sampling, auto developing chamber with temperature and humidity control. Most importantly, UV scanning and automatic data analysis in HPTLC enable much higher precision in the quantitation of pharmaceuticals. Apart from that, when compared to other quantitative methods, HPTLC is a simple method that enables the analysis of many samples in a single experiment, saving time and money for analysis. In this study, an HPTLC method was developed for fast screening and determination of chlorpheniramine adulterated in herbal products collected from the market.

2. Materials and methods

Materials and chemicals

Reference standards: Chlorpheniramine (100.0%, Lot No. C0522032) was purchased from the National Institute of Drug Quality Control.

Placebo: Liquid extract was obtained from 18 herbal medicines selected based on traditional Oriental remedies or commonly used for the treatment of allergic symptoms: *Fructus Forsythiae suspensae* (weeping forsythia) 10 g, *Herba Menthae* (mint) 8 g, *Radix Paeoniae lactiflora* (Chinese peony) 8 g, *Radix et Rhizoma Glycirrhizinae* (licorice) 8 g, *Radix Platycodi grandiflori* (platicodon root) 10 g, *Gardenia jasminoides* (cape jasmine) 12 g, *Semen Sofae Praeparatum* (fermented soybean) 10 g, *Cortex Paeoniae suffruticosae* (free peony) 8 g, *Rhizoma et Radix Notopterygii* (notopterygium root) 10 g, *Flos Lonicerae* (Japanese honeysuckle) 10 g, *Herba Elsholtziae ciliatae* (Vietnamese balm) 16 g of, *Fructus Arctii lappae* (burdock root) 12 g, *Radix Saposhnikoviae divaricatae* (fangfeng) 12 g, *Fructus Xanthii strumarii* (rough cocklebur) 8 g, *Radix Bupleuri chinensis* (thorowax root) 12 g, *Cimicifuga foetida* (creeping barberry) 10 g. Placebo samples were prepared as follows: Medicinal herbs are washed, drained, and dried at 60°C, then sliced thinly, and chopped (if necessary). Weigh 5 times the medicinal herbs according to the above ratio. Put them in a pot, and add water to cover the medicinal herbs by about 3 cm. Boil for 3 hours, occasionally adding water to ensure the water is above the medicinal herbs, then decant the liquid extract into another pot. Repeat once more, mix the two liquid extracts and filter. Evaporate in the water bath to obtain a 5:1 concentrated extract sample.

Sample collection: 60 herbal products (9 liquid and 51 solid) including herbal drugs and herbal dietary supplements were purchased online or at the pharmacies, by private traditional healthcare practitioners. These products are claimed for the treatment of allergy, urticaria, and irritation.

Chemicals: Methanol, cyclohexane, acetone, triethylamine, ethyl acetate, and ammonia were of analytical grade (AR, Merck, Germany). Thin layer plates of TLC *silica gel* 60 F254 were purchased from Merck (Germany).

Instrumentation

High performance thin layer chromatographic analysis was performed on the Camag HPTLC system (CAT No 027.6200, Camag, Switzerland) with autosampler, automatic developing chamber, TLC visualizer (photo scanning), TLC scanner (UV-Vis absorption or fluorescence measurement) and controlled by winCATS. Other equipment included HERMLE Z306 centrifuge (Hermle, Germany), Helma ultrasonic (Helma, Germany); Labinco BV L46 vortex (Labinco, Netherlands)

Methods

Standard solution: Chlorpheniramine standard solution at 100 µg/mL was prepared in methanol as a stock standard solution. Working standard solutions were obtained from this solution by dilution in methanol.

Spiked sample: To 1 g of the placebo extract, add 800 µL of 1.25 mg/mL chlorpheniramine standard solution. Gently shake and leave for 15 minutes. Add 25.00 mL methanol, vortex for 5 minutes, sonicate for 15 minutes, and centrifuge at 6000 rpm for 10 minutes. The supernatant solution was filtered through a 0.45 µm membrane.

Method validation: The analytical method was validated according to AOAC [4] in terms of specificity, system suitability, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy.

3. Experimental results

Sample treatment

The sample preparation was adapted from [2] as follows: Before sampling, the homogenization step should be done: shake thoroughly for liquid herbal products; grind and mix thoroughly for solid herbal products. An amount of 1.0 g of liquid product or 0.5 g of solid product was transfered into a centrifuge tube where 25.00 mL of methanol was added. Vortex for 5 minutes, sonicate for 15 minutes at room temperature, and centrifuge at 6000 rpm for 10 minutes. The clear supernatant solution was filtered through 0.45 µm membrane.

Chromatographic conditions

To select the mobile phase, a standard solution, and a spiked sample was used to ensure good separation of chlorpheniramine from all natural compounds from the herbal materials. The spiked sample was prepared by adding chlorpheniramine standard solution to the placebo sample (liquid extract of 18 herbal materials). This sample was introduced to the TLC plate together with chlorpheniramine standard solution in methanol for mobile phase screening. Four different mobile phases were investigated based on previous studies: (1) chloroform-methanol (97:3, v/v) [4]; (2) ethyl acetate-methanol-ammonia (8:2:0.5, v/v/v) [5]; (3) ethyl acetate-methanol-ammonia (85:10:5, v/v/v) [6]; (4) cyclohexane-acetone-ammonia (8:2:0.5, v/v/v) [2]. Among the 4 mobile phases, system (4) with cyclohexane-acetone-ammonia (8:2:0.5) showed the best results in separating chlorpheniramine from interfering traces from herbal extract, with retention factor R_f of 0.19 (Fig. 1A). The UV spectrum of chlorpheniramine trace in the spiked sample was illustrated in Fig. 1B, showing a maximum absorbance wavelength at 254 nm. Therefore, 254 nm was chosen as the quantitation wavelength.

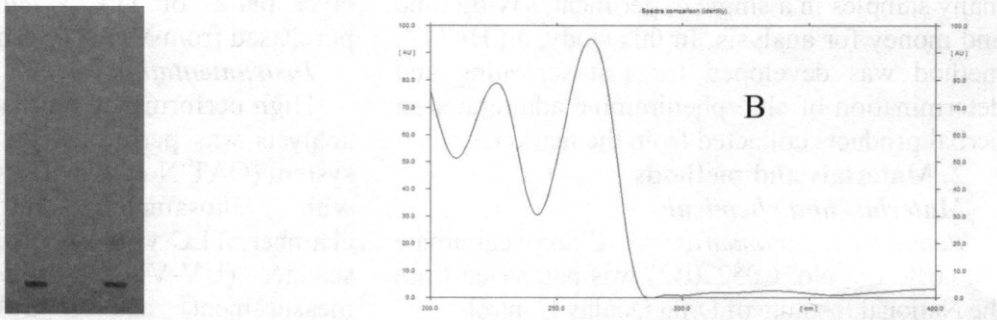

Fig. 1. TLC separation was obtained with mobile phase (4) with traces of placebo, standard and spiked sample from left to right (A), and UV spectrum of chlorpheniramine trace in the spiked sample (B).

The final chosen chromatographic conditions included: TLC *Silica gel* 60 GF_{254} plates with dimensions of 20 cm x 10 cm; mobile phase of cyclohexane-acetone-ammonia (8:2:0.5, v/v/v); detection at 254 nm; quantitation wavelength for UV scanning 254 nm; sample volume 10 µL.

Method validation

Selectivity

Introduce simultaneously 4 samples on one TLC plate: blank solution (methanol), chlorpheniramine standard solution, liquid extracts from placebo sample, and the liquid extracts spiked sample. After the development of TLC analysis, the traces with R_f equivalent to chlorpheniramine were scanned at 254 nm, and results obtained from WinCATs software were illustrated in Fig. 2.

With *standard solution,* a symmetric sharp peak was detected as chlorpheniramine atretention factor R_f of 0.19. No peaks corresponding to chlorpheniramine were found with the *blank solution* and *placebo sample.* With the *spiked sample*, a chlorpheniramine peak was detected, with a peak purity of 0.9963 and a matching ratio of 0.9916 when overlapping the spectrum of this peak with the corresponding peak from the *standard sample.* Therefore, the HPTLC method has high selectivity.

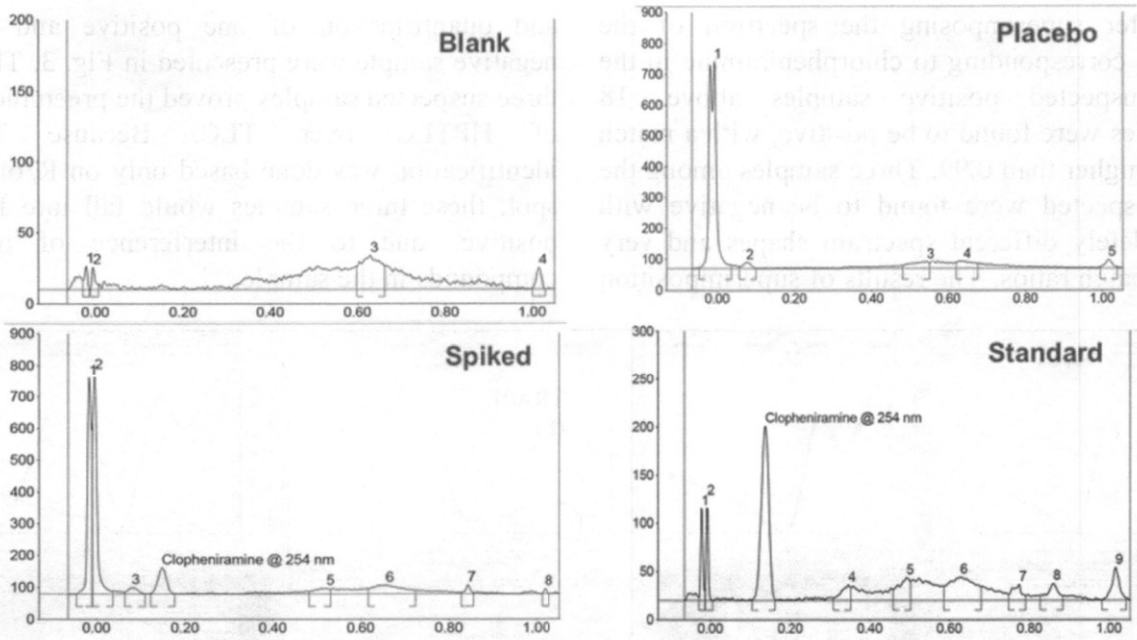

Fig. 2. Chromatograms for selectivity test: blank solution, placebo sample, spiked sample, and standard solution. Horizontal axe stands for retention factor R_f and vertical axe stands for UV absorbance at 254 nm

System suitability

Analyze 6 times of 100 µg/mL chlorpheniramine standard solution. The results showed the repeatable retention factor R_f with RSD=0% (≤ 1.0%) and repeatable peak area with RSD of 1.11% (≤ 2.0%), meeting AOAC 2016 requirements [7]. The system is suitable for analyzing chlorpheniramine.

Linearity

Prepare two series of standard solutions of chlorpheniramine with concentrations at 20-40-50-60-80-100 µg/mL, one *blank* series (in methanol), and one *placebo* series (in *placebo sample* extracts). Analyze the two series of samples and calculate the matrix effect (ME%) to evaluate the influence of the placebo matrix on the calibration curve. The results showed that the *placebo sample matrix* had a negligible effect, with the obtained ME=6.79% (< 15%). Therefore, the calibration curve for the next experiments was prepared in methanol.

Precision and accuracy

Intraday accuracy and precision were determined by adding the exact amount of stock standard solution to 1.0 g *placebo* matrix at 3 concentration levels (low, medium, high) so that chlorpheniramine concentrations in the final sample would be 20 - 50 -80 µg/mL respectively. Samples were shaken well and left for 15 minutes before sample treatment was done according to protocol. Repeat 8 times at each concentration level. Interday precision and accuracy were performed similarly but on a different day of analysis. At concentrations of 20 - 50 - 80 µg/ mL, good accuracy was observed with recovery from 90.9% - 102.3% (within the required range of 80 - 110%) with RSD% of 1.1% - 5.4% (< 7.3% as recommended by AOAC). Thus, the developed method showed good precision and accuracy as validated according to AOAC 2016 guidelines [7].

Limit of detection (LOD), limit of quantitation (LOQ) of the method

Add to the *placebo* sample exact amounts of standard solution to obtain gradually decreasing concentrations. Process samples and analyze them according to the developed method. The LOD and LOQ were found for chlorpheniramine concentrations at 1.5 and 4.5 ppm.

Real sample analysis

The developed HPTLC method was applied to 60 collected samples of traditional medicinal or herbal products collected from pharmacies, traditional healthcare practitioners, and online purchases. Results showed that 21 samples were suspected to be adulterated with chlorpheniramine. In the chromatograms obtained from these samples, traces were detected at the R_f of chlorpheniramine.

After superimposing the spectrum of the traces corresponding to chlorpheniramine in the 21 suspected positive samples above, 18 samples were found to be positive, with a match ratio higher than 0.99. Three samples among the 21 suspected were found to be negative with completely different spectrum shapes and very low match ratios. The results of superimposition and quantification of one positive and one negative sample were presented in Fig. 3. These three suspected samples proved the preeminence of HPTLC over TLC. Because TLC identification was done based only on R_f of the spot, these three samples would fall into false positive, due to the interference of other compounds in the sample.

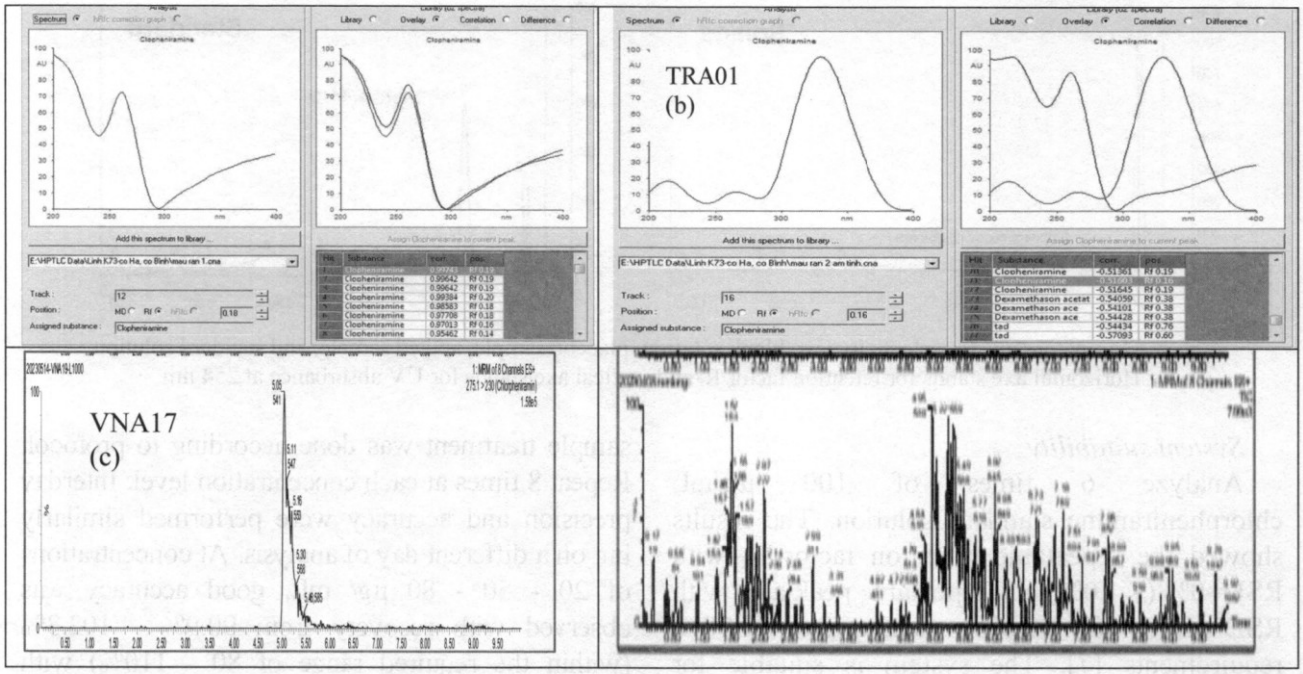

Fig. 3. Spectral overlap results for the positive sample VNA17(a) (match ratio 0.9974) and the negative sample TRA01(b) (match ratio -0,615), Chromatograms for VNA17 (c), TRA01 (d) were detected by LC-MS/MS

With 18 positive samples, quantitation was done on the calibration curve obtained within the same day, and the amount of chlorpheniramine determined was presented in Table 1. Among the positive samples, one VNA19 sample was adulterated with chlorpheniramine at a very high concentration (34.1 mg/dose per day), exceeding the maximum concentration (24.0 mg/dose per day).

Table 1. Chlorpheniramine content adulterated in herbal products determined by HPTLC

Sample code	**VNA02**	**VNA03**	**VNA04**	**VNA05**	**VNA17**	**VNA19**	**VNA35**	**VNA36**	**BA01**
Content (mg/daily dose)	2.04	8.89	7.72	2.53	8.46	34.1	4.18	9.48	2.50
Sample code	HCH03	HCH04	HCH094	HCH99	HCH164	HA26	HA29	HA32	BNH93
Content (mg/daily dose)	7.41	5.02	4.71	2.57	15.4	18.0	4.45	3.79	11.1

Note: VNA-capsule; BA-inhaling powder; HCH and HA- pill; BNH-drinking powder.

To confirm the results from HPTLC, all 60 herbal samples were analyzed in parallel using LC-MS/MS [3]. All 18 positive samples detected by HPTLC were confirmed positive by LC-MS/MS. However, one negative sample by HPTLC (CLA24) showed positive results by LC-MS/MS. Too low content of chlorpheniramine in this sample might be the reason for false negative detection by HPTLC.

4. Discussion

Comments on the selection of analytical conditions

Although there was only one target analyte of interest for this analysis, there were a large number of interfering compounds from herbal extracts. Therefore, the mobile phase for TLC separation was chosen so that most of the interfering compounds were highly retained on silica (as observed in Fig. 2, chromatogram of placebo).

Comments on the results of real sample analysis

100% of positive samples were solid samples (8 capsule samples, 8 tablet samples, and 3 powder samples) possibly because it is easier to mix chemical drugs into solid preparations than liquid preparations.

Comments on the use of this HPTLC in the detection of adulteration in herbal products

HPTLC is an advanced technique that upgraded the applications of TLC to many extents. With simple sample treatment and easy implementation, HPTLC has been used increasingly for qualitative and quantitative analysis [8]. Within one simple 20×10 cm TLC plate, multiple analyses can be done, enabling low-cost and less time-consuming analysis. However, HPTLC has a low detection limit compared to HPLC or LC-MS/MS and the traditional medicine sample matrix is relatively complex with many components, so false positives or false negatives may occur. The false negative sample CLA24 may be due to the large amount of dose taken, so the adulteration content of chlorpheniramine was low. HPTLC's sensitivity was not high enough, so the positive sample could not be detected. Therefore, the study has developed a sample treatment process according to dosage. To facilitate routine implementation, it is necessary to refer to the same amount of weight, but care should be taken when applying to samples that have a large amount of dosage. in 1 large dose. The HPTLC method had better specificity than TLC because detection was done not only based on R_f but also on peak purity and match ratio of the UV spectrum. Therefore, the HPTLC method was chosen to be applied for quick and reliable screening of adulteration in traditional medicinal products collected on the market.

5. Conclusion

In this study, an HPTLC method for the determination of chlorpheniramine adulterated in herbal products was developed. This method offered fast and simple sample preparation and quantitation with high specificity and good sensitivity. The self-made placebo with herbal extracts from a variety of herbal medicinal materials used in the method development and validation also proved the specificity of the detection method. The application of the developed HPTLC method in the analysis of 60 real samples collected from the market gained a significant result. A large number of adulterated samples were revealed: 18 out of 60 samples were found positive with chlorpheniramine.

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