# Simultaneous determination of four neuroprotective compounds of *Tilia amurensis* by high performance liquid chromatography coupled with diode array detector

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## ABSTRACT

**Background:** *Tilia amurensis* consists of various compounds, such as flavonoids and terpenoids. **Objective:** A simple and reliable high performance liquid chromatography (HPLC) coupled with the diode array detector (DAD) method has been established for simultaneous determination of epicatechin, nudiposide, lyoniside, and scopoletin isolated from *Tilia amurensis*. **Materials and Methods:** Optimum separations were obtained with a SHISEIDO $C_{18}$ column by gradient elution, with 0.1% Trifluoroacetic acid (TFA) water-methanol as the mobile phase. The gradient elution system was completed within 40 minutes. The flow rate and detection wavelength were 1 mL/minute, 205 nm, 250 nm, and 280 nm, respectively. **Results:** Validation of the analytical method was evaluated by linearity, precision, and the accuracy test. The calibration curve was linear over the established range with $R^2 > 0.997$. The limit of detection (LOD) and limit of quantification (LOQ) ranged from 0.01-15.20 μg/mL and 0.03-46.06 μg/mL. The method exhibited an intraday and interday precision range of 96.25-105.66% and 93.52-109.92%, respectively (RSD <2.80%). The recoveries and relative standard deviation (RSD) of the four compounds in *Tilia amurensis* were in the range of 90.42-104.84% and 0.2-2.58%. **Conclusion:** This developed method was accurate and reliable for the quality evaluation of the four compounds isolated from *Tilia amurensis*.

**Key words:** High performance liquid chromatography/diode array detector, quality control, simultaneous determination, *Tilia amurensis*

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## INTRODUCTION

In many countries, herbal medicines have been generally medicines used for thousands of years. Increased side effects, lack of healing treatment for several chronic diseases, expenses of new drugs, microbial resistance, and emerging diseases are some of the reasons for renewed public interest in complementary and alternative medicines.[1] At present, there are many studies that have been performed to explain the bioactive compounds of herbs and demonstrated their mechanism of remedy and prevention of various disease containing neurodegenerative disorders.[2-6] The trees of the *Tilia* species are used around the world for their medicinal properties. *Tilia amurensis* is commonly known as a bee tree and widely distributed in various countries, including Korea, China, and Japan. In Korea, the flowers of this tree have been applied as a therapeutic agent for alleviating a fever and its leaves have also been traditionally used to treat cancer. Previous chemical studies of this species have demonstrated the presence of coumarin, flavonoid, lignin, and triterpene. In recent times, the anti-tumor, anti-inflammatory, and topoisomerase I and II inhibitory activities of this plant have been reported.[2,7]

Herbal medicines consist of numerous compounds, which showed pharmacological activities, such as phenols, flavonoids, alkaloids, and terpenoids.[8-10] These various compounds indicate diverse therapeutic effects and the quality control of each compound is difficult. With the expanding resources and for controlling the quality of

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herbs from different areas, it is very essential to develop a simple and effective HPLC-DAD method. In the past decades, a large number of analytical strategies have been designed to evaluate the quality of medicinal herbs or herbal preparations. These include quantification of a single compound or multiple compounds, as well as fingerprint analysis. Single marker compound quantification is simple and convenient, but it does not provide sufficient quantitative information for the other active compounds in complex natural products. In the process, techniques such as HPLC, (gas chromatography) GC, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS) are often used. However, HPLC is simple, reliable, and inexpensive, and has been widely used for quantitative analysis of herbal medicine.[11-15]

There are no reported simultaneous analyses for the quality control of *T. amurensis*. In this study, we aim to develop a method of simultaneous determination by HPLC-DAD for qualitative and quantitative analysis of four active compounds, (-)-epicatechin, nudiposide, lyoniside, and scopoletin in *T. amurensis*. These compounds have been isolated from *T. amurensis* recently.

## MATERIALS AND METHODS

### Plants material and materials

The woods of *Tilia amurnesis* (Tiliacea) were collected in the Kyungdong traditional herbal market (Seoul). The origin of *T. amurnesis* was identified by Dr. Young Bae Seo, a professor of the College of Oriental Medicine, Daejeon University, Korea. The voucher specimen (CJ022M) was deposited at the Kangwon National University in Chuncheon, Korea. Four standard compounds, (-)-epicatechin, nudiposide, lyoniside, and scopoletin were isolated from the woods of *T. amurnesis* by various chromatographic techniques. Their chemical structures were confirmed based on $^{1}$H- NMR and$^{13}$ C- NMR data and compared with the reported data [Figure 1]. The HPLC solvent for the gradient elution system, water, and methanol were purchased from J.T. Baker (USA). Trifluoroacetic acid (TFA) was of analytical grade, from Dae-jung, in Korea.

### Conditions for high-performance liquid chromatography and instrument

The HPLC (Dionex Ultimate 3000 system, Germany) instrument was equipped with a model series LPG 3X00 pump, ACC-3000 auto sampler, TCC-3000SD column oven, and DAD-3000(RS) diode array UV/VIS detector. Separation of *Tilia amurensis* was done on an SHISEIDO $C_{18}$ column (S-5 μm, 4.6 mm I.D. ×250 mm). The separation was carried out using the gradient elution

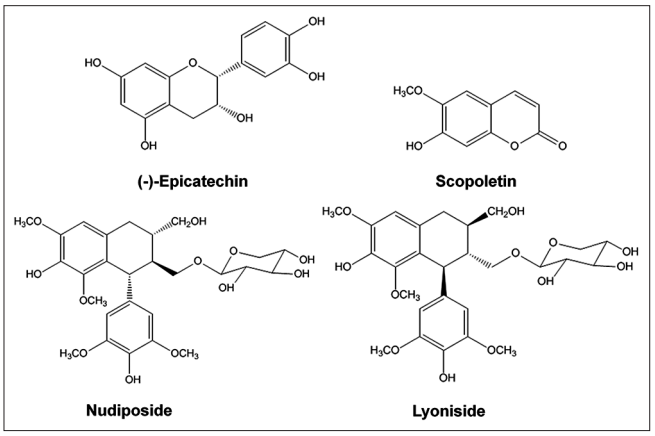


**Figure 1:** Chemical structures of (-)-epicatechin, nudiposide, lyoniside, and scopoletin

procedure with mobile phase A (water containing 0.1% TFA) and B (methanol). The linear gradients changed as follows: 0-5 minutes, isocratic 5% B; 5-20 minutes, linear gradient 5-30% B; 20-25 minutes, isocratic 30% B; 25-40 minutes, linear gradient 30-80% B. The total run time was 40 minutes at a flow rate of 1 mL/minute. The peaks of the compounds were monitored by a diode array detector, and the detection wavelength was set at 205 nm. The sample injection volume was 20 μL, and the column temperature was 35°C.

### Preparation of standard solutions

Standard stock solutions of four compounds were prepared in methanol and stored below 4°C. Working standard solutions were prepared by serial dilution of stock solutions with methanol and water. The stock solutions were prepared with a concentration of 100 μg/mL for (-)-epicatechin, 433 μg/mL for nudiposide, 100 μg/mL for lyoniside, and 100 μg/mL for scopoletin, respectively. The solutions were filtered through a 0.45 μm filter.

### Preparation of sample solution

The wood of *Tilia amurensis* was extracted by ultrasonication extraction, in 80% methanol. The extracted methanol solution was evaporated till the residue was obtained. To obtain the powder, freeze-drying was conducted. The powder was accurately weighed (131 mg) was dissolved in 5 mL of methanol. The sample solution was filtered through 0.45 μm membrane filters before injection into the HPLC-DAD.

## RESULTS AND DISCUSSION

### Optimization of high-performance liquid chromatography conditions

We tested various mobile phase compositions (water-acetonitrile, water-methanol, water containing 0.1% acetonitrile, and water containing 0.1% methanol) to optimize

a suitable mobile phase. Various gradient elution proportions of water containing 0.1% methanol were also tested, to achieve the desired separation. Trifluoroacetic acid (0.1%) was added to the water to improve the peak shape and inhibit peak tailing. Based on the maximum UV absorption wavelength of each of the four compounds, the UV wavelength of the DAD detector was selected. The four compounds were identified at 205 nm. A HPLC chromatogram of the four standards is shown in Figure 2a. The peak of each compound was confirmed by comparing the retention time in the HPLC chromatogram and the ultraviolet (UV) spectrum of each marker constituent.

### Validation of high-performance liquid chromatography analytical method

Validation of the HPLC method was performed according to the International Conference on Harmonization (ICH) guidelines by determination of the linearity, precision, and recovery test.

### Linearity, limit of detection, and limit of quantification

Stock solutions of the four compounds were diluted with methanol to provide six concentrations. To establish the calibration graph, mixed standard solutions of six different concentrations were analyzed thrice. The calibration graph was plotted by using the value of the peak area versus the concentration of each compound. The linear regression equation between the concentration of the standards analyzed and the peak area could be given as $Y = Ax + B$, where A was the slope of the calibration curve, B was the intercept of the calibration curve, x was the concentration of the marker compounds, and Y was the peak area. Correlation coefficient ($R^2$) values indicated linearity. The limit of detection (LOD) and limit of quantifi cation (LOQ) were determined for each compound at a signal-to-noise ratio (s/n) of 3:1 and 10:1, respectively. All the standard compounds showed good linearity ($R^2 > 0.997$) in a relatively side concentration range. The LOD and LOQ were measured by the calibration curve. LOD was in the range of 0.01-15.20 μg/mL, LOQ was in the range of 0.03-46.06 μg/mL [Table 1]. This result exhibited a high sensitivity of this established method.

### Precision and accuracy

Intra- and interday variations were chosen to determine the precision of the developed method. For the intraday variability test, calibration sample solutions were analyzed for five replicates within one day, while for the interday variability tests, the solutions were examined in duplicates for three consecutive days. The relative standard deviation (RSD) was taken as a measure of precision and repeatability. The RSD values of the intraday and interday tests were found to be within the ranges of 0.51-2.80% and 0.10-2.26%, respectively, with accuracy ranges of 96.25-105.66% for the intraday test and 93.52-109.92% for the interday test [Table 2]. This result demonstrated good reproducibility of this analytical method.

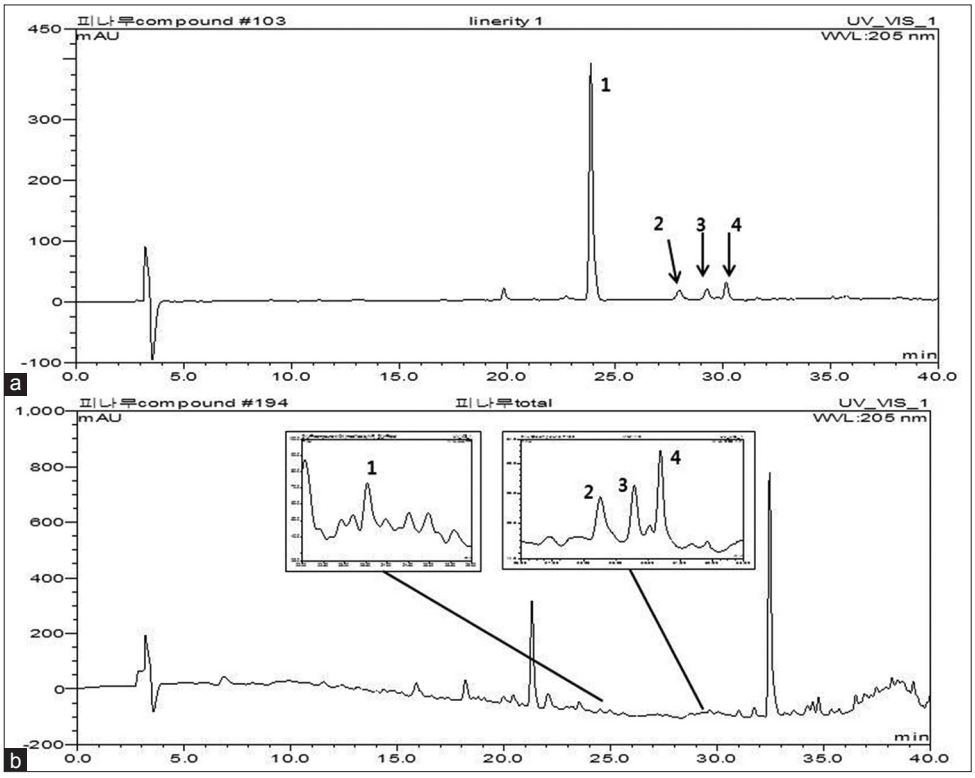


**Figure 2:** The HPLC chromatogram of a standard mixture (a) and the methanolic extract of Tilia amurensis (b) (-)-epicatechin (1), nudiposide (2), lyoniside (3), and scopoletin (4)

**Table 1: Analytical results of calibration curves, limit of detection, and limit of quantification**

| Compound | Regression equation | $R^2$ | Linear range (μg/mL) | LOD (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|---|
| (-)-Epicatechin | y=1.7904x−0.4618 | 0.9992 | 0.50-20.00 | 2.92 | 8.83 |
| Nudiposide | y=0.2314x+0.0242 | 0.9997 | 0.50-20.00 | 0.06 | 0.17 |
| Lyoniside | y=0.2455x+0.0457 | 0.9983 | 0.50-20.00 | 0.01 | 0.03 |
| Scopoletin | [a]y=0.2095x−0.1229 | 0.9978 | 1.09-43.30 | 15.20 | 46.06 |

[a]*Y*: Peak area, *x*: Amount (μg/mL), LOD: Limit of detection; LOQ: Limit of quantification

**Table 2: Analytical results of intra- and interday tests**

| Compound | Intraday | | | Interday | | |
|---|---|---|---|---|---|---|
| Concentration (μg/mL) | Mean (μg/mL) | RSD[a] (%) | Accuracy (%) | Mean (μg/mL) | RSD (%) | Accuracy (%) |
| (−)-Epicatechin | | | | | | |
| 43.3 | 45.61±0.23 | 0.51 | 105.33 | 45.59±0.11 | 0.25 | 105.30 |
| 21.7 | 22.30±0.56 | 2.49 | 102.99 | 23.40±0.29 | 1.22 | 108.10 |
| 10.8 | 11.42±0.20 | 1.72 | 105.49 | 11.28±0.25 | 2.20 | 104.21 |
| Nudiposide | | | | | | |
| 10 | 10.04±0.09 | 0.92 | 100.40 | 10.03±0.07 | 0.72 | 100.34 |
| 5 | 4.81±0.10 | 2.04 | 96.25 | 4.94±0.01 | 0.24 | 98.84 |
| 2.5 | 2.52±0.04 | 1.48 | 100.82 | 2.34±0.02 | 0.75 | 93.52 |
| Lyoniside | | | | | | |
| 10 | 10.66±0.15 | 1.36 | 106.57 | 10.36±0.01 | 0.10 | 103.64 |
| 5 | 5.28±0.12 | 2.19 | 105.66 | 5.07±0.01 | 0.20 | 101.32 |
| 2.5 | 2.49±0.13 | 2.12 | 103.13 | 2.56±0.00 | 0.18 | 102.56 |
| Scopoletin | | | | | | |
| 10 | 10.35±0.29 | 2.80 | 103.48 | 9.84±0.19 | 1.96 | 98.38 |
| 5 | 4.92±0.10 | 2.00 | 98.48 | 5.50±0.12 | 2.26 | 109.92 |
| 2.5 | 2.48±0.04 | 1.73 | 99.18 | 2.75±0.03 | 1.01 | 109.83 |

[a]RSD: [a]Relative standard deviatio

The recovery test was used to evaluate the accuracy of this method. Three different concentration levels of the marker compounds were added to the *Tilia amurensis* sample solution. The solutions were injected thrice. The contents of the marker compounds were calibrated from the corresponding calibration graph. Recovery (%) was calculated by the equation (amount found - original amount)/amount spiked × 100%. The recovery of the selected marker compounds was 90.42-104.84% with an RSD less than 2.58% [Table 3].

The results of the recovery test showed that the established method was reliable and accurate.

## Analysis of *Tilia amurensis*

The HPLC-DAD method established was applied for the analysis of four compounds in the *Tilia amurensis* sample. The peaks of each compound in *Tilia amurensis* were identified by comparison of the retention time and UV spectra with those of the standards. Figure 2b showed that the peaks of each compound were separated successfully within 40 minutes. The contents of the four compounds in *T. amurensis* were calculated from the calibration curves of each standard. Table 4 exhibits the results of the sample analysis. Of the four compounds,

**Table 3: Recovery test of four compounds from *Tilia amurensis***

| Compound | Spiked (μg/mL) | Found (μg/mL) | RSD (%) | Recovery (%) |
|---|---|---|---|---|
| (−)-Epicatechin | 21.65 | 21.32±0.04 | 0.21 | 98.47 |
| | 10.83 | 10.41±0.11 | 1.09 | 96.14 |
| | 5.41 | 4.89±0.07 | 1.38 | 90.42 |
| Nudiposide | 5.00 | 5.09±0.15 | 2.91 | 101.80 |
| | 2.50 | 2.66±0.02 | 0.67 | 106.50 |
| | 1.25 | 1.27±0.02 | 1.72 | 101.62 |
| Lyoniside | 5.00 | 5.24±0.05 | 0.90 | 104.84 |
| | 2.50 | 2.43±0.01 | 0.36 | 97.06 |
| | 1.25 | 1.27±0.01 | 0.93 | 101.73 |
| Scopoletin | 5.00 | 4.80±0.12 | 2.58 | 96.07 |
| | 2.50 | 2.44±0.07 | 2.98 | 97.80 |
| | 1.25 | 1.28±0.02 | 1.28 | 102.58 |

[a]Recovery (%)=(amount found-original amount)/amount spiked×100%, RSD: Relative standard deviatio

**Table 4: Contents of active compounds in *Tilia amurensis***

| | Compounds | | | |
|---|---|---|---|---|
| | (−)-Epicatechin | Nudiposide | Lyoniside | Scopoletin |
| Content (μg/mg) | 0.64±0.05 | 3.21±0.25 | 0.07±0.02 | 2.16±0.08 |

nudiposide had the highest content (3.21 μg/mg) in *T. amurensis* and lyoniside had the lowest content (0.07 μg/mg). These results indicated that this HPLC-DAD method might be used to evaluate the quality of *T. amurensis*.

## CONCLUSION

We established a simple and accurate HPLC-DAD method for simultaneous determination of four marker compounds, epicatechin, nudiposide, lyoniside, and scopoletin, isolated from *Tilia amurensis*. Validation for evaluation of this method was accomplished by using the linearity, precision, and accuracy test. Results of the validation exhibited that the developed method was sensitive, rapid, and reliable. The proposed method could be used to improve the quality control of *Tilia amurensis*. This method could also be useful in controlling the quality of other related pharmaceutical preparations.

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