# Quantitative Analysis of Chemical Compositions in *Boesenbergia rotunda* (L.) Mansf. Cultivated in Thailand using TLC Densitometry and HPLC Analysis

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## Abstract

Fingerroot was used as a spice and medicinal plant, including in dietary products. The plant raw materials were cultivated in many areas of Thailand, and 33 fingerroot samples were studied. This study quantitatively analyzed pinostrobin using thin layer chromatography (TLC) and RP-HPLC. TLC fingerprint provided a distinct pattern of bands after the development, while TLC densitometry was a technique to measure the density of separated bands on the developed TLC plates and provided both qualitative and quantitative information on the separated components. Standard pinostrobin and fingerroot samples were developed on a silica gel $G_{60}$ $F_{254}$ and developed with toluene: acetone: methanol: formic acid (90: 5: 2: 1). The developed plates were detected on 366 nm and sprayed with derivatization reagents. The pinostrobin content and relative percent peak areas with RP-HPLC analysis were also determined in the Fingerroot ethanolic extract. Pinostrobin contents were in the range of 0.5 – 1.7 %w/w and 0.2-1.5 %w/w with TLC and HPLC, respectively. All samples showed pinostrobin content lower than 2.0% w/w according to the acceptance criteria in the Thai Herbal Pharmacopoeia. Among the four main compounds, the relative percent peak area showed pinostrobin was the highest amount in most fingerroot samples and subsequently was the pinocembrin amount in some fingerroot samples from central areas of Thailand. Therefore, the landlaces, environmental conditions, and post-harvesting conditions should be considered to improve the quality and content of the active components.

***Keywords***: *Boesenbergia rotunda, fingerroot, pinostrobin, HPLC, TLC*

## 1. Introduction

*Boesenbergia rotunda* (L.) Mansf. or fingerroot, was used in spice and herbal medicine in Thailand and Southeast Asia, including Malaysia, Indonesia, and China. There were several parts of Thailand cultivating fingerroot. The active compounds were variable depending on cultivation areas, harvesting time, and post-harvesting process. Fingerroot or commonly named krachai (white krachai or yellow krachai) in Thailand, its rhizome was cylindrical, aromatic, and finger-like in appearance. It was typically golden-yellowish to brownish on the exterior and yellowish on the interior. Meanwhile, krachai dum (Thai black ginger) is a distinct black or dark purple color inside the rhizome. Its scientific name was *Kaempferia parviflora* Wallich ex Baker, and its bioactive compounds were methoxyflavones, phenolic compounds, and volatile oils.

Roots and rhizomes were the main parts of fingerroot that accumulated the bioactive compounds. Flavones (e.g., alpinetin, pinocembrin, and pinostrobin) and chalcones (e.g., boesenbergin A, cardamonin, panduratin A, 4-hydroxypanduratin A) were main compounds found in fingerroot (Jaipetch et al., 1982; Tan et al., 2015). They showed anti-oxidant, anti-microbial activities, and cell cytotoxicity in cholangiocarcinoma (Sopitthummakhun et al., 2021). Panduratin A was reported for potential inhibition of the SARS Co-V2 virus and prevention of viral replication at pre-entry and post-infection (Kanjanasiriart et al., 2020). Pinostrobin was reported to exhibit antibacterial activity against *Streptococcus mutans* and *Streptococcus pyrogens* and

suppress adipocyte differentiation recently (San et al., 2022). The amount of phytoconstituents was prominently affected by environmental conditions rather than genetic variations (Thomya et al., 2023). This study focused on the quantitative analysis of pinostrobin rather than other flavonoids due to the fact that the highest amount is found in plant materials and is a relatively stable compound. Most of the studies had reported the pharmacological activities of the fingerroot extracts, not the isolated compounds; some activities were the combination of more than one active compound. This study quantified pinostrobin content as it showed relatively high content compared with other compounds.

The quality of plant raw materials was a major concern in medicinal products. Thin layer chromatography (TLC) densitometry was a rapid technique to identify and quantify compounds. The method was recommended in Pharmacopoeia including Thai Herbal Pharmacopoeia (THP, 2021), Thai Herbal Preparation Pharmacopoeia (THPP, 2022), and the United State Pharmacopoeia (USP) in the dietary supplement volume (USP 41/NF36, 2018). TLC was able to analyze crude samples containing multiple components. In addition, several samples can be analyzed simultaneously on the same plate, resulting in high throughput, time-saving, and low cost. TLC densitometry was combined TLC with densitometry to measure the concentration of substances by analyzing the intensity of spots on a TLC plate. It was adapted to quantitative analysis of several samples compared with the standard. HPTLC (high-performance thin layer chromatography) was a chromatographic technique using small and uniform particle plates, automated sample applicators, and automated development chambers offering high sensitivity. Some articles reported that TLC and HPTLC techniques were used in the quality control of herbal medicines (Liang et al., 2004; Suphrom et al., 2014; Pratiwi et al., 2023). This research determined the pinostrobin content of fingerroot from various sources in Thailand.

## 2. Objectives

The objectives of the study were to determine pinostrobin content in fingerroot plant materials with TLC densitometry and RP-HPLC and to compare the flavonoid profile of fingerroot samples growing in Thailand.

## 3. Materials and Methods

Standard pinostrobin was bought from Biopurify phytochemicals, China. Working standard pinostrobin was courteously isolated according to the procedure reported by Pattamadilok and Sakpetch (2021) in the laboratory. The standard was analyzed with HPLC, and the molecular weight was confirmed with mass spectrometry. Acetone, ethanol, methanol, and toluene were analytical grade and bought from Carlo Erba. Methanol and acetonitrile HPLC grade were bought from Duksan, Korea. Formic acid was purchased from Fischer Chemicals. Ultrapure water was prepared on the Merck water purification system. NP (diphenylboric acid β-aminoethyl ester complex) and vanillin were bought from Sigma Aldrich. PEG (polyethylene glycol 4000) was bought from Tinnakorn Chemical and Supply Co., Ltd.

Fingerroot samples were purchased from cultivated areas (33 sources) in Thailand. It categorized into six regions: north (N1-N5), northeast (NE1-NE10, east (E1-E2), center (C1-C9), west (W1-W3), and south (S1-S4) of Thailand (Table 1). Fresh samples were cleaned, dried in the oven (50 °C), and grind to powder for the tests (sieve no. 80).

### *Pinostrobin Content using TLC Densitometry*

Standard pinostrobin was prepared at the concentration of 1,000 µg/mL in methanol and diluted to 500 µg/mL. The standard solution was applied 2 to 6 µL on the plate (n=3), which was equal to 1,000 to 3,000 ng/band. Fingerroot samples were accurately weighed 5 g each and extracted in 100 mL ethanol using a shaker for 6 hours and stand for 18 hours. The extracts were centrifuged (4,000 rpm and 10 minutes), and then the solutions were analyzed with TLC and RP-HPLC. Pinostrobin content was calculated by a calibration curve of standard pinostrobin. TLC method validation was in the supplement document.

TLC was developed on a TLC aluminum sheet silica gel $G_{60}F_{254}$ (Merck). The mobile phase was toluene – acetone – methanol – formic acid (90-5-2-1) (Sueree & Ruangrungsi, 2016) and the TLC tank was

equilibrated for at least 30 minutes. The standard and sample solutions were applied on the plate 5 µL each for identification using Linomat (Camag). The band length was 6 mm, and the distance between bands was 10 mm. For quantitative analysis, each sample was applied to two tracks (n=2). The developing distance was 80 mm. TLC scanning was performed by TLC scanner at slit dimensions (4.0 x 0.3 mm, micro), and the scanning speed was 20 mm/s. The plates were detected by Visualizer at 254 and 366 nm. The densitogram of samples was calculated corresponding to standard curve of pinostrobin. NP (1% in methanol) and PEG TS (5% in ethanol) were sprayed reagents on a warm plate (80 °C, 10 minutes). Vanillin (1%) sulfuric acid in methanol was also used as derivatization for comparison.

***Pinostrobin Content using HPLC Analysis***

Standard pinocembrin and pinostrobin were prepared at the concentration of 1,000 µg/mL in methanol and diluted to 200 µg/mL. Then, they were further diluted to the concentration of 100 to 0.31 µg/mL with methanol. The standard solutions and the fingerroot sample solutions from ethanolic extract were filtered through PTFE 0.22 µm and injected 5 µL with an auto-injector in duplicate. RP-HPLC [Agilent 1260, US] was equipped with a Zorbax $C_{18}$ extended column (4.6 x 250 mm, 5 µm) and 25 °C column temperature. The mobile phase was 0.02% formic acid in water (A) and acetonitrile (B) with gradient run (30-99 %B in 27 minutes, stayed for 1 minute and returned to 30%B and re-equilibrated for 5 minutes) and flow rate of 1 mL/min. The chromatogram was monitored at the wavelength of 290 nm. The Flavonoid profile was compared from the relative percent peak area of four main flavonoids (alpinetin, pinocembrin, pinostrobin, and panduratin A, respectively). HPLC method validation was in the supplement document.

**Table 1** *Boesenbergia rotunda* (L.) Mansf. samples in Thailand

| Sample no. | Province name | | | | | |
|---|---|---|---|---|---|---|
| | North (N) | Northeast (NE) | East (E) | Center (C) | West (W) | South (S) |
| 1 | Chiang Rai | Nongbualumpoo | Prachinburi | Sukhothai | Tak | Chumporn |
| 2 | Chiang Mai | Chaiyapoom | Chachengsao | Pitsanulok | Kanchanaburi | Suratthani |
| 3 | Payao | Khallasin | - | Pichit | Prachoukirikan | Nakhonsrithammarat |
| 4 | Lumpang | Roiet | - | Petchaboon | - | Songkhla |
| 5 | Uttaradit | Yasothorn | - | Uthaithani | - | - |
| 6 | - | Nakhonratchasrima | - | Chainart | - | - |
| 7 | - | Surin 1 | - | Lopburi | - | - |
| 8 | - | Srisaket | - | Saraburi | - | - |
| 9 | - | Ubonratchathani | - | Nakhonpathom | - | - |
| 10 | - | Surin 2 | - | - | - | - |

## 4. Results

Fingerroot samples were purchased from various sources categorized by geography according to National Geography Commission of Thailand (Table 1). All fingerroot sample showed light yellowish to light brownish color with characteristic odor. Pinostrobin contents were quantified correlated to the calibration curve of pinostrobin and calculated to percent on dried weight (Table 2). The contents (0.54-1.69% w/w) were in comparable with the results obtained from HPLC analysis (0.20-1.49 %w/w). Independent t-test showed that pinostrobin content analyzed with these two techniques was not statistically different ($p$-value = 0.615). Four major flavonoid compounds in fingerroot samples from various parts of Thailand were reported as relative percent peak areas, which is shown in the bar graph (Figure 1). Most of them showed similar profile, in which pinostrobin was the highest flavonoid content, except that some samples from the central area showed comparable pinocembrin and pinostrobin contents. Pinostrobin content was in the range of 64-69%, while pinocembrin was in the range of 21 – 38% among these four compounds. In addition, those samples from the central area showed as high as 59% pinocembrin content. Panduratin A was about 10-13%, and samples from the northeastern area showed a relatively high amount (16%).

**Table 2** Pinostrobin content analyzed by TLC densitometer and RP-HPLC

| Areas | | %w/w Pinostrobin (Mean ± SD) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Northeast** | | **NE 1** | **NE2** | **NE3** | **NE4** | **NE5** | **NE6** | **NE7** | **NE8** | **NE9** | **NE10** |
| | TLC | 1.05±0.18 | 1.10±0.06 | 0.95±0.04 | 1.19±0.02 | 1.07±0.06 | 1.07±0.04 | 1.11±0.08 | 0.80±0.07 | 0.95±0.23 | 0.60±0.62 |
| | HPLC | 1.49±0.03 | 1.24±0.01 | 1.51±0.01 | 1.27±0.02 | 1.23±0.01 | 1.06±0.01 | 1.28±0.01 | 1.08±0.01 | 1.27±0.01 | 1.41±0.00 |
| **Center** | | **C1** | **C2** | **C3** | **C4** | **C5** | **C6** | **C7** | **C8** | **C9** | |
| | TLC | 0.78±0.10 | 0.59±0.02 | 0.51±0.01 | 0.55±0.00 | 0.76±0.05 | 1.00±0.08 | 0.82±0.11 | 0.88±0.27 | 1.69±0.58 | |
| | HPLC | 1.06±0.00 | 0.19±0.00 | 0.20±0.00 | 0.19±0.00 | 0.24±0.00 | 0.37±0.00 | 0.51±0.00 | 0.37±0.02 | 1.06±0.00 | |
| **North** | | **N1** | **N2** | **N3** | **N4** | **N5** | | | | | |
| | TLC | 0.54±0.11 | 1.46±0.28 | 1.02±0.14 | 1.53±0.08 | 1.20±0.02 | | | | | |
| | HPLC | 1.02±0.01 | 1.19±0.01 | 1.01±0.06 | 1.35±0.01 | 0.74±0.00 | | | | | |
| **South** | | **S1** | **S2** | **S3** | **S4** | | | | | | |
| | TLC | 1.34±0.16 | 1.46±0.17 | 1.06±0.46 | 0.98±0.96 | | | | | | |
| | HPLC | 1.15±0.01 | 1.51±0.01 | 1.13±0.00 | 1.34±0.02 | | | | | | |
| **East** | | **E1** | **E2** | | | | | | | | |
| | TLC | 1.52±0.20 | 1.54±0.21 | | | | | | | | |
| | HPLC | 1.08±0.00 | 1.25±0.01 | | | | | | | | |
| **West** | | **W1** | **W2** | **W3** | | | | | | | |
| | TLC | 1.22±0.36 | 1.31±0.39 | 1.01±0.79 | | | | | | | |
| | HPLC | 0.89±0.01 | 1.19±0.01 | 1.27±0.01 | | | | | | | |

**Table 3** Statistical analysis of pinostrobin content compared between TLC densitometry and RP-HPLC

| | TLC densitometry | RP-HPLC | T-test | *p*-value |
|---|---|---|---|---|
| Mean | 1.05 | 1.00 | 2.000 | .615 |
| Variance | 0.10 | 0.17 | | |
| N | 33 | 33 | | |


**Figure 1** Percent relative peak area of identified flavonoid found in fingerroot samples

Figure 2 shows the representative of the TLC fingerprint of fingerroot samples from center of Thailand detected at 366 nm after being sprayed with NP and PEG TS. All samples showed similar TLC elution pattern, the difference was only the density of band color which was related to the amount of the components. TLC densitogram showed the retention factor ($R_f$) values of pinocembrin, panduratin A, and

pinostrobin were 0.55, 0.61, and 0.81, respectively. All samples showed similar characteristic profile with this mobile phase system. Although pinostrobin eluted last and closed to the solvent front, it was resolved well from other components, as shown in Figure 3. The spectra of standard pinostrobin and fingerroot samples, which were eluted at the same $R_f$, were compared (Figure 4), and the maximum absorption wavelength was 290 nm. The representative chromatograms of eluted compounds in fingerroot samples from northeastern and central areas of Thailand were compared with that of standard pinostrobin. (Figure 5). The elution order in this system was alpinetin, pinocembrin, pinostrobin and panduratin A, respectively (retention time 9.8, 12.8, 16.6, 22.2 minute, respectively). Pinostrobin was the highest content in most samples while some samples from central areas showed relatively high content of pinocembrin.

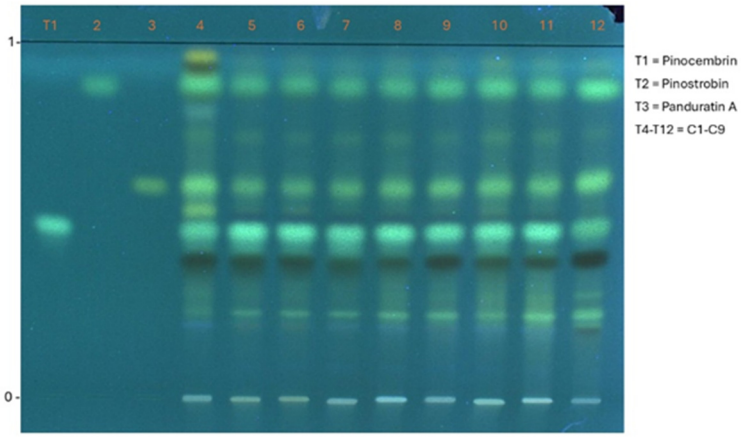


**Figure 2** TLC profile of standard and samples C1-C9 detected at 366 nm after being sprayed with NP-PEG TS

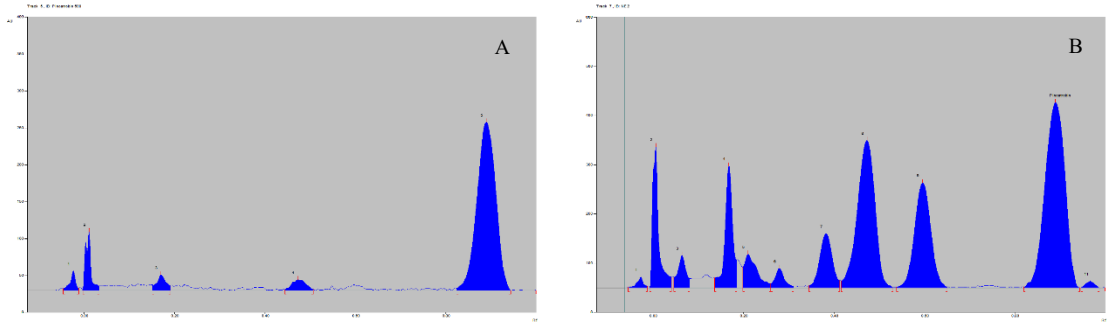


**Figure 3** Densitogram of standard pinostrobin (A) and one of fingerroot sample (B)

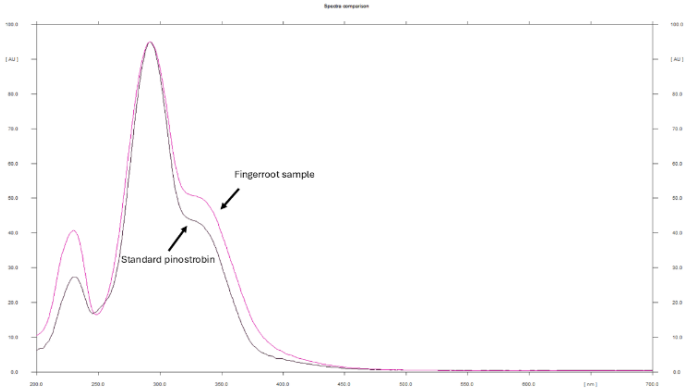


**Figure 4** Overlay spectrum of standard pinostrobin and the fingerroot sample at the same $R_f$ value

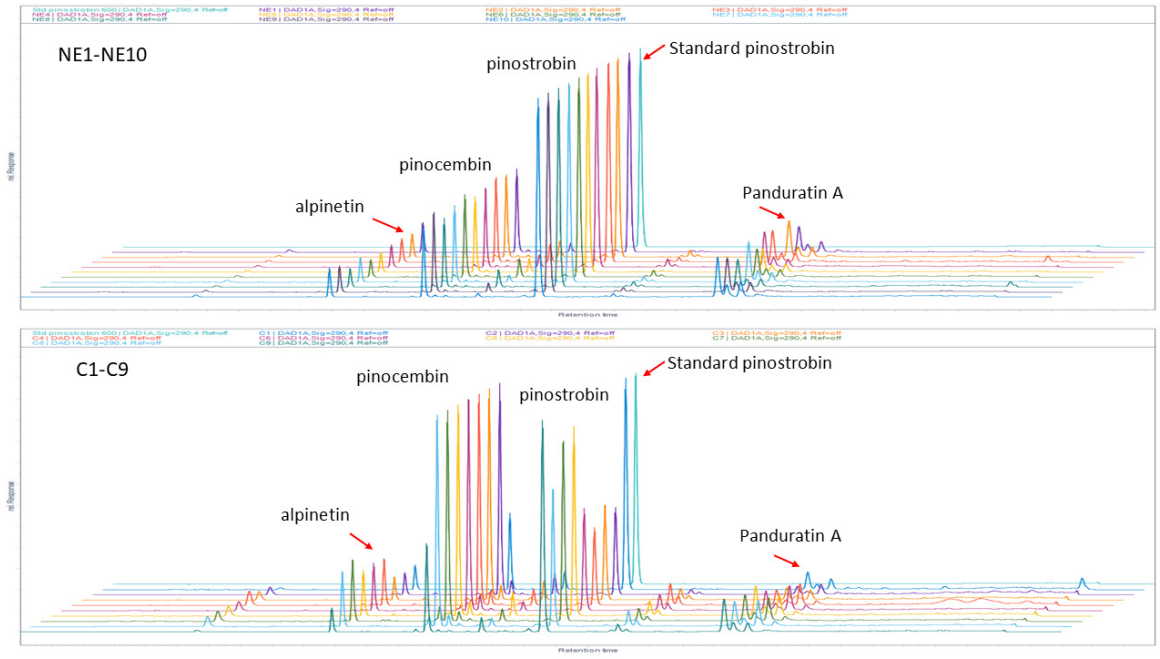


**Figure 5** Overlay HPLC chromatogram of standard pinostrobin and fingerroot samples from northeastern (NE1-NE10) and central (C1-C9) of Thailand

## 5. Discussion

Chromatography techniques were recommended for the identification and quantification of chemical components. The chromatographic fingerprint was a powerful technique to ensure the identity and quality of medicinal plants with multiple components. In this study, TLC densitometry was studied, and the TLC densitogram was calculated as peak areas corresponding to the calibration curve of pinostrobin. The mobile phase which was used in this study (toluene : acetone : methanol : formic acid = 90:5:2:1) resulted the $R_f$ value 0.80-0.82 slightly earlier compared with the mobile phase recommended in THP (dichloromethane: methanol (70: 1), $R_f$ 0.92-0.96. Although the mixture of mobile phase compositions was three organic solvents and one acid, it resulted in more consistency with replicate runs and was well resolved at the bands close to the solvent front. TLC fingerprints (Figure 2) showed similar elution pattern and $R_f$ values compared with those of the results reported by Sueree, and Ruangrungsi (2016). The example of fingerprint of fingerroot samples from the center parts of Thailand also showed the separation of the other two compounds: pinocembrin and panduratin A. In addition, the NP/PEG TS were sprayed for the determination of flavonoid, it resulted yellowish bands, increased sensitivity, and more stable color. The other reagents (e.g. anisaldehyde sulfuric acid and vanillin sulfuric acid TS) were used also. They showed dark purple bands of flavonoids which were fade off later.

In comparison, pinostrobin content with HPLC analysis was not exact the same value as those of analyzed by TLC densitometry, there was no significantly difference between these two techniques (Table 3). The content of pinostrobin analyzed by HPLC was slightly lower than those analyzed by TLC densitometry because the higher sensitivity of HPLC and some compounds were well separate as showed small peaks in the chromatogram. However, pinostrobin contents were relatively low and lower than 2.0%w/w as mentioned in THP. One article reported that the fingerroot samples from the northern area showed pinostrobin contents in the range of 1.4 to 4.3 %w/w (or 14.41 – 43.27 mg/g dried sample) analyzed with HPLC (Thomya et al., 2023). In addition, for sample preparation procedure plant materials which were extracted in ethanol by shaker for 6 hour and stand for 18 hours the extract showed relatively high amounts of active components. The limitation was the harvesting time of the tested samples in this study, which were

purchased from various sources. This finding was in agreed with the report of the study "guidelines for transforming Thai medicinal plants through research and innovation" by extraordinary committee, Senate (Ad Hoc committee, Senate, 2023). It also suggested that good agriculture practice was required to facilitate good quality of plant raw materials. The post-harvesting process affected the volatile oil contents of the sample also. Only one sample that yielded a few volatile oil contents after distillation. HPTLC stationary phase, which has smaller particle sizes, would yield clearer bands and much more resolve, resulting in high sensitivity (Jondhale et al., 2022).

## 6. Conclusion

Fingerroot samples, which showed relatively high pinostrobin contents, were from northeastern, northern, and southern areas of Thailand. Pinocembrin showed relatively high content in fingerroot samples from the central area, while panduratin A was found to have high content in fingerroot samples from the northeastern area of Thailand. The results suggested that to improve the quality of plant raw materials, necessary techniques and good practices were required. Although TLC densitometry may not be as sensitive method as HPLC analysis, it can provide rapid analysis of several samples at the same time at a reasonable cost. TLC densitometry can be an alternative method of HPLC analysis for the quality control of medicinal plants.

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