Development and validation of TLC-densitometry method for quantification of tetraprenyltoluquinone in the stem bark hexane extract of *Garcinia cowa* roxb

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**ABSTRACT**

A thin layer chromatography (TLC) method coupled with densitometry was optimized and established to quantify tetraprenyltoluquinone (TPTQ) in the hexane extract of *Garcinia cowa* roxb stem bark. The stationary phase was TLC plates of silica gel 60 F254, with a thickness of 250 μm, while a mobile phase was the mixture of hexane: chloroform: ethyl acetate: formic acid in the ratio of 20:70:9:1 was selected for the chromatographic system. TLC separation of the components was followed by densitometric analysis at 230 nm. The chromatogram revealed a tight spot for TPTQ at the Rf ¼ 0.41/C60.03). The value of the regression equation (r2 ¼ 0.992) demonstrated an excellent linear association between peak area and TPTQ amount in the 250–750 μg.spot-1 range. The precision, specificity, and accuracy of the approach were all validated. The 72.65 and 242.15 μg.spot/C0 were found as the limit of detection and quantification, respectively. The proposed TLC method can be considered for identifying and quantifying TPTQ in *Garcinia cowa* Roxb bark hexane extracts.

1. Introduction

*Garcinia cowa*. R is locally known as asam kandis, and has long been used as traditional medicine. The various part of this plant has been utilized as antipyretic agents, cough mixture, stomachache, and blood thinners [1]. For instance, the ethanolic extract of *G. cowa*. R has been reported to suppress the growth of exhibited T47D cancer cells line [2]. On the other hand, the dichloromethane fraction of the rinds could induce apoptosis of HeLa cervical carcinoma [3]. In addition, the leaves and the root of *G. cowa* R contained several cytotoxic compounds, while from the bark was isolated anti-inflammatory compounds [4, 5, 6]. Two isolated compounds, cowanin and tetraprenyltoluquinone (TPTQ) revealed cytotoxic activity against T47D human breast cancer and H-460, respectively. Furthermore, cowanin could decrease cell mobility and stimulate cell cycle arrest on T47D cancer cells line [7, 8, 9, 10].

In earlier research, HPLC and HPTLC techniques have been developed to analyze the levels of rubraxanthin compounds in *G. cowa*. R extracts [11, 12, 13]. Besides, the HPLC method determined the level of rubraxanthin in blood plasma [14]. Hexane fraction contained TPTQ compound and showed the potential cytotoxic activity toward lung cancer cells. Therefore, determining the quantity of TPTQ compound in this fraction is necessary. As far as we concern, there are no reports published on the method for determining the content of TPTQ in *G. cowa*. R extract.

2. Material and methods

2.1. Chemicals

All of the chemicals and reagents used were analytical grade. Reference standard TPTQ was provided from the previous studies [10].

2.2. Plant collection

*Garcinia cowa* stem bark was gathered in September 2020 in Lembah Harau, West Sumatra, Indonesia. Dr. Nurainas (botanist) identified it and kept it at the Herbarium Universitas Andalas (ANDA). The bark was sliced and left to dry in a oven at 50 °C for 72 h. The bark sliced was then ground and sieved to obtain a fine powder (20 meshes).

2.3. Extraction conditions

A pulverized powder of *G. cowa* R bark (100 g) was extracted with hexane using soxhlet method. A rotary evaporator then concentrated the extract.
2.4. Instrumentation

TLC analysis was performed on the TLC system (CAMAG, Switzerland). Precoated silica gel 60 F254 TLC plates (20 x 20 cm) with a layer thickness of 0.2 mm served as the stationary phase (E. Merck KGaA, Darmstadt, Germany). Prior to chromatography, TLC plates were wetted with methanol and then heated at 110 °C for 30 min at 110 °C. About 0.5 μl standard TPTQ and sample solution were spotted on the TLC plates using the semi-automatic TLC sampler Nanomat 4. The TLC plate was ascending eluted in the glass box saturated with the mobile phase.

The mixture of Hexane, chloroform, ethyl acetate, and formic acid (20: 70: 9: 1 v/v/v/v) was used as the mobile phase. CAMAG TLC Scanner 4 in absorption mode at 230 nm with a slit dimension of 5 mm × 0.3 mm was used for detection and densitometric scanning. The scanning speed of the sample track was 20 mm/s, and the scanning speed of the spot spectrum was 100 nm/s. The entire procedure was carried out using the winCATS planar chromatographic manager software (CAMAG, Muttenz, Switzerland, version 1.4.7.2018). All analyses were performed in a laboratory at ambient temperature (25 °C).

2.5. TPTQ standard stock solution preparation

The TPTQ reference standard stock solution (1000 g/ml) was made by precisely weighing 10 mg of TPTQ, transferring it to a volumetric flask, and adding methanol. It was followed by the dilution to produce standard working solution (250–750 g.spot⁻¹).

On the TLC plate, each concentration was applied six times. The previously described mobile phase was then used to develop the plate. The calibration graphs were created by plotting the peak areas versus concentrations. The least-squares linear-regression analysis was used to generate linear calibration curves.

2.6. Analytical wavelength selection

The wavelength was selected in the range of 200–400 nm.

2.7. Sample preparation for TLC analysis

The hexane extract of stem bark was accurately weighed (20.2 mg) and added 10 mL of methanol in volumetric flask. The dilution was performed with methanol to achieve a 202 μg/mL concentration.

2.8. Validation of the method

The analytical method was validated according to the International Conference on Harmonization (ICH, 1995). The parameter included Linearity, precision, accuracy, specificity, Limit of quantitation (LOQ), and Limit of detection (LOD) [15].

2.9. Quantification of TPTQ in hexane extract of G. cowa R stem bark

For quantification purposes, the obtained chromatogram was identified the Rf value for the respective peak and then followed by quantification using a linearity curve and regression equation.

3. Results

3.1. Mobile phase optimisation

The experimental parameters such as mobile phase and scanning wavelength were optimized to ensure an accurate, precise, selective, and repeatable results. The best solvent system to get the best separation of TPTQ in the extract was hexane: chloroform: ethyl acetate: formic acid (20: 70:9:1, v/v/v/v). The best wavelength that produced the most excellent sensitivity was 230 nm. Figure 3 illustrate the TLC method's specificity by showing the perfect separation of TPTQ and other peaks. The resolution was 1.47, and it was considered an excellent separation. The Rf values of TPTQ were found to be 0.41 ± 0.03 (Table 1).

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Table 1. System suitability data of the proposed method.

| Parameters       | TPTQ | Retardation factor | Resolution | Tailing Faktor |
|------------------|------|-------------------|------------|---------------|
|                  |      | 0.41 ± 0.03       | 1.47       | 1             |

Table 2. Linear regression data for the calibration curves (n = 6).

| Parameter          | Data    | Linearity range μg.spot⁻¹ | Regression Correlation Y² | Slope ± Standard error | Intercept ± Standard error | Confidence limit of slope⁺ | Confidence limit of Intercept⁺ |
|--------------------|---------|--------------------------|--------------------------|------------------------|---------------------------|----------------------------|-------------------------------|
|                    |         | 250–750                  | 0.992                    | 11.034 ± 30.182        | 1386.334 ± 46.698         | 4.672 to 6.008              | 1308.697 to 1395.078          |

⁺ 95% confidence intervals.
⁻ Y = a + bc, where c is the concentration of the substance in μg.spot⁻¹ and Y is the peak area.

Table 3. Limit of detection (LOD) and limit of quantification (LOQ).

| Concentration Range (μg.spot⁻¹) | slope       | Standard deviation of y intercept | LOD μg.spot⁻¹ | LOQ μg.spot⁻¹ |
|---------------------------------|-------------|----------------------------------|---------------|--------------|
| 250–750                         | 2.755       | 115.972                          | 72.50         | 241.65       |

Table 4. Recovery studies.

| TPTQ added μg.spot⁻¹ | TPTQ found μg.spot⁻¹ | Recovery (%) |
|----------------------|----------------------|--------------|
| 366.041              | 352.199              | 99.74        |
| 457.55512            | 451.103              | 98.59        |
| 549.06144            | 540.386              | 98.42        |

Table 5. Results of intra-day and inter-day precision (n = 6).

| Actual concentration μg.spot⁻¹ | Intra-day | Inter-day |
|-------------------------------|-----------|-----------|
|                               | Measured concentration μg.spot⁻¹ | % RSD | Measured concentration μg.spot⁻¹ | % RSD |
| 250                           | 248.349 ± 8.522 | 1.03 | 236.881 ± 8.299 | 1.801 |
| 500                           | 510.322 ± 9.346 | 1.51 | 458.123 ± 11.261 | 1.123 |
| 750                           | 763.111 ± 12.487 | 1.44 | 730.462 ± 22.559 | 1.591 |

RSD: Relative Standard Deviation.

Figure 1. Structure of tetraprenyltoluquinone (TPTQ).
Figure 2. TLC densitograms of standard TPTQ (0.5 μg spot$^{-1}$); (a) G. cowa R extracts (1μg spot$^{-1}$); (b) and G. cowu R extracts (1.32 μg spot$^{-1}$) which added TPTQ as reference standard (0.16 μg spot$^{-1}$); (c).
3.2. Validation

3.2.1. Linearity

By examining a series of different concentrations of TPTQ solution, the TLC methods for identifying TPTQ were verified for linearity. Seven TPTQ concentrations were chosen for this study because they demonstrated reasonable linearity in the range of 250–750 μg spot⁻¹. The correlation coefficient value \( r^2 \) of 0.992 validates the linearity of each calibration graph (Figure 5, Table 2).

3.2.2. LOD and LOQ

LOD and LOQ were calculated as \( 3.3 \sigma \) and \( 10 \sigma \) respectively. Where \( \sigma \) is the standard deviation of the response (y-intercept) and \( S \) is the mean of the slope of calibration plot \([15]\). 72.5 μg spot⁻¹ and 241.65 μg spot⁻¹ were the LOD and LOQ, respectively (Table 3).

3.2.3. Accuracy

The proposed method was used to estimate TPTQ from extract after spiking a pre-analyzed sample with 80%, 100%, and 120% of additional TPTQ standard. TPTQ recovery was identified to be 99.74%, 98.59%, and 98.42% on TLC in extract, respectively (Table 4).

3.2.4. Precision

For intra-day precision, three different concentration of TPTQ were analyzed for six times on the same day, while the single concentration of TPTQ was evaluated for thrice on the three subsequent days for inter-day precision. The RSD for the TPTQ assay in the precision study was below 2.0%, confirming that the method was highly precise. The precision result as seen in Table 5.

3.2.5. Specificity

The specificity of the TLC method was assessed by examining the densitograms of \( G. \) cowa Roxb bark extract, \( G. \) cowa Roxb extract plus TPTQ as an internal standard, and the densitogram of standard TPTQ. From the results (Figure 2), the TPTQ standard has an \( R_f = 0.41 \) which also appears in the other 2 densitograms. The three points were also tested for their UV spectrum profile. The Spectrum measurements were done at a wavelength of 230 nm, which is the wavelength that gives the maximum absorption for TPTQ compounds \([3]\). The test results have shown that the three points have identical spectral profiles with TPTQ spectra (Figure 3). The parameter included peak start, peak apex, and peak end positions of the spot were evaluated to determine the peak purity of TPTQ. TPTQ spectra were also shown to exhibit a strong association \( r = 0.9992 \) with the spectra of standard and hexane extract of \( G. \) cowa. R stem bark (Figure 1).

3.3. Quantification of TPTQ in the hexane extract of \( G. \) cowa. R stem bark

The TPTQ in the hexane extract of \( G. \) cowa. R stem bark was identified by comparing its single spot at 0.41 ± 0.03 values with standard peak

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Table 6. TPTQ content in Hexane extract of \( G. \) cowa Stem bark determined by TLC methods.

| Sample ug.ml⁻¹ | Area     | TPTQ in sample (ug.ml⁻¹) | Content (% b/b) |
|----------------|----------|--------------------------|-----------------|
| 2000           | 4175.642 | 505.599                  | 25.279          |

3.2. Overlay UV absorption spectra of TPTQ standard (a), TPTQ in the extract of \( G. \) cowa R (b) and TPTQ in the extract of \( G. \) cowa which added TPTQ as reference standard (c).

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Figure 3. Overlay UV absorption spectra of TPTQ standard (a), TPTQ in the extract of \( G. \) cowa R (b) and TPTQ in the extract of \( G. \) cowa which added TPTQ as reference standard (c).
(Figure 2). Otherwise, the quantity of TPTQ was determined using the linear regression equation, and the amount was presented in Table 6.

4. Discussion

TPTQ was a marker compound of G. cowa. R. This compound has specific cytotoxic activity against H640 lung cancer cells. Thus, it was necessary to determine its quantity in G. cowa. R. The TLC-densitometry method was established and validated in this study. All chromatographic parameters were optimized to obtain the optimum resolution and the best peak shape for TPTQ. The result revealed that the best wavelength for TPTQ detection was 230 nm (Figure 4). In addition, the mobile phase containing the mixture of hexane, chloroform, ethyl acetate, and formic acid (20: 70: 9: 1, v/v/v/v) was found to be the best solvent system since it produced the excellent separation of TPTQ (Rf = 0.41 ± 0.03). The peak found with Rf = 0.41 ± 0.03 in hexane fraction was confirmed as TPTQ since it has identical Rf with the TPTQ standard (Figure 3, Table 1).

This approach validated linearity, selectivity, precision, LOD, LOQ, and recovery. Over the concentration range of 250–750 μg spot⁻¹ TPTQ, the linearity (relationship between peak area and concentration) was measured (r² = 0.992) (Table 2). The obtained LOD and LOQ of TPTQ were 72.65 and 242.15 μg spot⁻¹, respectively. The study also found the intraday and inter-day precision was accurate as the percentage of RSD was less than 2 (Table 5). The suggested method was suitable for the determination of TPTQ in extract due to it has high recovery values, low percentage of RSD value (<2) and low standard deviation, as well as the quantity acquired, was closed to the exact amount. It had a low coefficient of variation value. Table 6 shows the amounts of TPTQ in the hexane extract of G. cowa R bark.
5. Conclusions

A simple, precise, validated TLC technique was established to determine TPTQ in the stem bark of G. cowa. R. The reproducibility and selectivity of this developed method could be implemented for quantifying TPTQ in crude drugs and plant extracts. Furthermore, it also can be implemented as an alternative strategy for quality control, for example, in the detection of adulteration of G. cowa. R extracts. TPTQ was discovered to be a G. cowa marker compound.

Declarations

Author contribution statement

Meri Susanti: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Ardeza Renando Pratama, Mesa Irna Suryani: Performed the experiments; Analyzed and interpreted the data.
Suryati: Analyzed and interpreted the data; Wrote the paper.
Dachriyanus: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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