VALIDATION AND DEVELOPMENT OF TLC-DENSITOMETRY METHOD FOR STANDARDIZATION OF SOURSOP LEAF EXTRACT (ANNONA MURICATA LINN.) WITH QUERCETIN

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**ABSTRACT:** Standardization is a process involving various chemical analysis methods based on pharmacological data involving general physical and microbiological analyzes aimed at the safety of a natural extract. To obtain the correct result, the standardization step is preceded by method validation. Quercetin can be obtained from the leaves of soursop (Annona muricata Linn.), which use for treatment fever, diarrhea, anti-convulsive, antifungal, antimicrobial, gout, itching, and flu. Determination of quercetin in soursop leaf extract using TLC-Densitometry must be validated by testing selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, and range. Densitometry scanning of the plates silica gel 60 F254 directly at 265 nm was used for analysis of quercetin. The result of selectivity test was 1.53 (>1.5) with mobile phase toluene: ethyl acetate: formic acid (4: 3: 0.4; v/v/v), limit of detection (LOD = 0.018 µg), limit of quantification (LOQ = 0.060 µg), linearity y = 9387.3x – 767.39 (r = 0.9996 and Vxo = 1.9%), Coefficient of Variation (C.V.) of precision 1.906525% and the percent recovery of 96.6% ± 8.26%. The validated and development method was applied for standardization of quercetin in soursop leaf extract and resulted in the quercetin assay in soursop leaf extract was 1.288% ± 9.996% (w/w).

**INTRODUCTION:** Standardization is a process involving various chemical analysis methods based on pharmacological data involving general physical and microbiological analyzes aimed at the safety of a natural extract. To have a good result, there must be a relationship between the quality of the raw materials, in the process materials and the final product; it becomes necessary to develop reliable, specific and sensitive quality control methods using a combination of classical and modern instrumental analytical methods.

Standardization is an important measure to ensure quality control of herbal medicine. Standardization of herbal medicines is the process of determining the levels compared to the specific standards or characteristics, constant parameters, qualitative and quantitative values that bring true quality assurance, efficacy, safety, and reproducibility. Therefore standardization is a tool in the quality control process.

Standardization of drugs means the confirmation of identity and the determination of quality and purity. Standardization here emphasizes the determination of the compound content responsible for pharmacological effects, which is a process of ensuring good quality and standardization for medicines and herbal products. To obtain the correct result, the standardization step is preceded by method validation.
Validation of analytical methods is the assessment of an analytical method based on laboratory experiments to demonstrate that the parameters of accuracy, precision, specificity/selectivity, detection limits (LOD), quantification limits (LOQ), linearity, and range meet requirements for users. In the quantitative analysis of components and substances of medicinal materials derived from biological samples such as those in plants must meet the parameters of accuracy, precision, specificity/ selectivity, detection limits (LOD), quantification limits (LOQ), linearity, and range. Quercetin is an active compound that can be obtained on soursop leaves (Annona muricata, Linn.) and widely used as an alternative treatment for fever, diarrhea, anti-seizure, anti-fungal, antiparasitic, antimicrobial, lumbago, gout, itching, anticancer, anticonvulsant, antidiabetic, anti-inflammatory, anti-arthritis and flu traditionally.

The determination of quercetin content can be determined by Spectrophotometric, HPLC, RP-HPLC method. The TLC-Densitometry method is the primary choice for the analysis of chemical components derived from plants because it has the flexibility to detect almost all compounds, even some inorganic compounds. Systematic approaches and well-designed methodologies for standardization of raw materials and herbal preparation formulations are now widely developed. Given the growing interest in herbal medicine, standard methods of herbal medicine are developed and used in different formulations.

MATERIALS AND METHODS:

- **Instrument**: Analytical Scales (O Haus Pioneer), Rotary Vacuum Evaporator (Heidolph Laborota 4000), 20 x 10 x 5 cm³ chromatography chamber (CAMAG), Densitometer (Shimadzu Dual Wavelength Chromato Scanner CS 930), UV CAMAG Lamp, Capillary Pipes, and Common Glass Tools used in the chemical laboratory analysis.

- **Chemicals**: The soursop leaves (Annona muricata, Linn.) were collected from the Purwodadi Botanical Garden, East Java and were determined by the Indonesian Institute of Sciences UPT of the Purwodadi Botanical Gardens Conservation Center. Quercetin (pharmaceutical grade, Sigma-Aldrich), ethanol pa (Merck), technical acetone, ethyl acetate pa (Merck), toluene pa (Merck), formic acid pa (Merck), methanol pa (Merck), and Silica Gel TLC 60 F254 (Merck).

**Procedure**:

- **Making Simplicia**: The soursop leaves (Annona muricata, Linn.) that have been washed, drained and dried by air-dried in the open air with no direct sunlight, approximately at room temperature of 25-30 ºC for 1 week. Samples that have dried in a blender and sieved to form a rather coarse powder.

- **Making Extract**: The 0.65 kg soursop leaf Simplicia was extracted using technical acetone (1:10) in the macerator for 24 h with an occasional shake. Remaceration is at least twice, with the same type and amount of solvent. The extracts were concentrated using a rotary vacuum evaporator at temperatures between 60 - 70 ºC and concentrated extracts.

- **Determination of Water Content**: Weigh carefully 1-2 grams of simplicia and extract in a covered porcelain crust that has been preheated at 105º C for an hour and has known the constant weight. The material in the exchange rate is leveled by shaking the exchange rate, dried at 105 ºC for five hours, weighed. The step is repeated until the weight of a constant heating product is obtained (the difference of weighing 0.25% or not exceeding 0.5 mg).

- **Preparation of Quercetin Standard Solution**: Standard solution of parent quercetin was prepared in ethanol p.a 1000 μg/ml. A standard solution of quercetin (60 - 600 μg/ml) was prepared in an ethanol solvent p.a.

- **Determination of Selected Wavelength**: In the TLC plate, bottles of soursop leaf extract (Annona muricata Linn.), quercetin solution, soursop leaf extract (Annona muricata Linn.) were added to quercetin solution. Next observed the spectra in the wavelength region of ultraviolet.

**Validation Method**:

- **Selectivity**: Bottled two μl quilled standard quercetin solution quercetin 300 μg/ml, a solution of 300 mg soursop leaf extract (Annona muricata, Linn.) in 10.0 ml ethanol p.a, 1.0 ml solution of sample solution which has been additions with 1.0.
ml of standard quercetin 300 μg/ml on 60 silica gel TLC plate F254. Eluate of TLC plate with mobile phase toluene: ethyl acetate: methanol (4:0.5:0.5), toluene: ethyl acetate: formic acid (7:3:1), toluene: ethyl acetate: formic acid (4:3:0.4) then observed its chromatogram with Densitometer and calculated its degree of resolution (R<sub>s</sub>).

**Limit of Detection (LOD) and Limit of Quantitation (LOQ):** Bottled two μl quercetin solution of 60 - 500 μg/ml and bottle some ethanol p.a. on the silica gel TLC plate 60 F<sub>254</sub>. Elution the TLC plate with mobile phase toluene: ethyl acetate: formic acid (4:3:0.4) and then observed the area by Densitometer at selected wavelengths. Calculate the Standard Deviation (SD) area of the spot ethanol p.a., and slope regression equation of quercetin standard solution of 60 - 500 μg/ml. Calculation of detection limits (LOD) and quantity limits (LOQ) with the following formula<sup>10</sup>:

\[ Q = k \times S_b / S_l \]

- Q = LOD or LOQ
- k = constant value, (LOD = 3, LOQ = 10)
- S<sub>b</sub> = SD blank
- S<sub>l</sub> = slope of regression equation quercetin

**Linearity:** Bottled two μl standard solution of quercetin 90-500 μg/ml on plate 60 silica gel TLC 60 F<sub>254</sub>. Elution of the TLC plate with mobile phase toluene: ethyl acetate: formic acid (4:3:0.4) and then observed the area at a selected wavelength with Densitometer.

**Precision:** Bottled two μl standard solution of 200 μg/ml quercetin some of the bottling points on the 60 F<sub>254</sub> silica gel TLC plate. Elution of the TLC plate with mobile phase toluene: ethyl acetate: formic acid (4:3:0.4). Observed area of the selected wavelength using Densitometer then calculated the mean area, Standard Deviation (SD), and Coefficient of Variation (KV).

**Accuracy:** One series of quercetin with a weight increase of 80-120% (2.4 mg, 3.0 mg and 3.6 mg), added 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 4, 0 ml of 1.5 grams of soursop leaf extract (*Annona muricata* Linn.) in 50.0 ml ethanol p.a. It is then bottled on the silica gel 60 F<sub>254</sub> TLC plate of two μl. Elution of the TLC plate with mobile phase toluene: ethyl acetate: formic acid (4:3:0.4). The measured area at a selected wavelength and determined (%) Recovery.

**Determination of Quercetin Levels in Extracts:** From one ml of 300 mg soursop leaf extract (*Annona muricata* Linn.) in 10.0 ml ethanol p.a. Then it was added that 1.0 ml of quercetin standard solution with increased concentration (90-500 μg/ml), was bottled on the silica gel 60 F<sub>254</sub> TLC plate of two μl. Elution on the TLC plate with mobile phase toluene: ethyl acetate: formic acid (4:3:0.4). Measured area and determined based on the calibration curve of the quercetin standard solution.

**RESULT:**

**Extract Soursop Leaf (*Annona muricata* Linn.):** From 2.1 kg (wet weight) soursop leaves (*Annona muricata* Linn.) obtained 650 g of dried leaves (31.0%). From 650 g of dried leaves macerated with 12 liters of acetone and after concentration with rotary vacuum evaporator obtained 53.7189 g of soursop leaf extract (*Annona muricata* Linn.) (8.26%).

**Determination of Water Content**: The water content in soursop leaf (*Annona muricata* Linn.) is 13.78 ± 0.2060% (w/w). While the moisture content of soursop leaf extract (*Annona muricata* Linn.) is 8.38 ± 0.0907% (w/w).

**Determination of Selected Wavelength:** From Fig. 1, the selected wavelength on the determination of quercetin levels is 265 nm.

**Validasi Metode:**

**Selectivity:** Selectivity test results determined from the value of the degree of resolution can be seen in Table 1.
FIG. 2: CHROMATOGRAM (A) LEAF EXTRACT OF SOURSOP (B) QUERCETIN SOLUTION (C) SOURSOP LEAF EXTRACT OF ADDED QUERCETIN IN MOBILE PHASE TOLUENE: ETHYL ACETATE: METHANOL (4:3:0.4)

TABLE 1: DEGREE OF RESOLUTION (Rs)

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Rs₁</th>
<th>Rs₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>toluene: ethyl acetate: methanol</td>
<td>0.57</td>
<td>1.57</td>
</tr>
<tr>
<td>(4: 0.5: 0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>toluene: ethyl acetate: formic acid</td>
<td>1.52</td>
<td>1.53</td>
</tr>
<tr>
<td>(7:3:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>toluene: ethyl acetate: formic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4:3:0.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

By looking at the chromatogram Fig. 2 and the degree of resolution Table 1, a toxic toluene phase is chosen: ethyl acetate: formic acid (4: 3: 0.4) because of its resolution degree ≥ 1.527,28.

LOD and LOQ: The regression equation of quercetin solution is 60 - 500 µg/ml, y = 10492x - 152.99, and S.D of ethanol p.a p.a is 63.4825.

\[
\text{LOD} = 3 \times 63.4825/10492 = 0.018 \mu g
\]

\[
\text{LOQ} = 10 \times 63.4825/10492 = 0.060 \mu g
\]

Linearity: Linearity test results obtained \[ y = 9387.3 \times 767.39 \ (r = 0.9996) \], which can be explained there is a linear relationship between quantity quercetin with the area.

FIG. 3: LINEARITY OF QUERCETIN SOLUTION

Precision: The quercetin solution of 213.6 µg/ml was bottled eight times with a two-μl bottling volume with a spacing of 1 cm each on the silica gel 60 F254 TLC plate. Precision test results obtained the average area of quercetin is 3129.575 with Coefficient of Variation (C.V.) = 1.906525% (C.V. requirement <2% 27-28).

Accuracy: The accuracy of the yield (%) recovery of quercetin by adduct can be seen in Table 2. Retrieved (%; w/w) recovery 96.6 ± 7.99 (required for biological samples 80-120%) 27-28.

TABLE 2: ACCURACY

<table>
<thead>
<tr>
<th>Known quercetin (mg)</th>
<th>Replication</th>
<th>Quercetin obtained (mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4 (80%)</td>
<td>1</td>
<td>2.5839</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.7274</td>
<td>94.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.1754</td>
<td>90.6</td>
</tr>
<tr>
<td>3.0 (100%)</td>
<td>1</td>
<td>2.4543</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.7730</td>
<td>92.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.8708</td>
<td>95.7</td>
</tr>
<tr>
<td>3.6 (120%)</td>
<td>1</td>
<td>3.8056</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.6558</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.5781</td>
<td>99.4</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td>96.6</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>7.99</td>
<td></td>
</tr>
<tr>
<td>Coefficient of Variation (C.V.)</td>
<td></td>
<td>8.26</td>
<td></td>
</tr>
</tbody>
</table>

Determination of Quercetin Levels in Extracts: After obtaining optimal validation conditions, it was applied to standardize the determination of quercetin content in soursop leaf extract Table 3.

TABLE 3: DETERMINATION OF QUERCETIN LEVELS IN SOURSOP LEAF EXTRACTS (ANON MURICATA L.).

<table>
<thead>
<tr>
<th>Replication</th>
<th>Water content (%)</th>
<th>Weight weighed (mg)</th>
<th>Weight gain (mg)</th>
<th>Quercetin levels (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.38</td>
<td>300.1</td>
<td>3.486</td>
<td>1.162</td>
</tr>
<tr>
<td>2</td>
<td>8.38</td>
<td>300.1</td>
<td>3.848</td>
<td>1.282</td>
</tr>
<tr>
<td>3</td>
<td>8.38</td>
<td>300.1</td>
<td>4.258</td>
<td>1.419</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td></td>
<td>1.288</td>
</tr>
<tr>
<td>Standard Deviation (S.D)</td>
<td></td>
<td>0.1287</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient of Variation (C.V.)</td>
<td></td>
<td>9.996</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standardization on the determination of quercetin content in soursop leaf extract obtained 1.288 ± 0.1287% (w/w).

DISCUSSION: The choice of quercetin wavelength was obtained at 265 nm, another researcher reported 266 nm 21. This wavelength difference of one nm can be considered the same. Selectivity test aims to determine the phase of motion selected with the parameter of the degree of resolution.
In this study selected phase of motion was toluene: ethyl acetate: formic acid (4: 3: 0.5), because the degree of resolutions was > 1.5 \(^{27-28}\). Other researchers used the mobile phase toluene: ethyl acetate: methanol (4: 0.5: 0.5) obtained a resolution value of 0.57 and 1.57 \(^{29}\). This use of mobile phase of toluene: ethyl acetate: formic acid is similar \(^{30}\) because the ratio is less precise, it is developed by changing the ratio of the mobile phase system to toluene: ethyl acetate: formic acid (4: 3: 0.5). Different mobile phase systems will also differ the polarity, where the separation process depends on the polarity of the mobile phase system that affects the value of the degree of resolution.

The linearity test was performed by bottling the standard quercetin solution of 90 - 500 μg/ml by two μl (0.12-1.00 μg) on the silica gel 60 F\(_{254}\) TLC plate, eluted and then measured the area. From the calculation results obtained regression equation \(y = 9387.3x - 767.39\) \((r = 0.9996)\). It can be explained that there is a linear relationship between quercetin with bottle and area. The linearity of other researchers in the range of weight of quercetin 0.1-1.00 μg and generated regression equation \(y = 2.8437x + 202.43\) \((r = 0.9881)\) \(^{31}\).

From the results of calculations in this study, obtained limit of detection (LOD) and limit of quantities (LOQ) that is 0.018 μg and 0.060 μg. In research \(^{31}\), the limit of detection (LOD) and limit of quantification (LOQ) for quercetin compounds were 0.05429 μg and 0.16451 μg. The difference of LOD/LOQ depends on the researcher's thoroughness so that if obtained by LOD/LOQ is small it will be obtained more sensitive observation. Precision in this study obtained Coefficient of Variation (C.V.) of 1.91% (C.V. Requirements <2%). Other research \(^{31}\), obtained the C.V. of 6.53%. This difference occurs because of differences in the expertise of researchers, resulting in different precision \(^{10}\).

Accuracy is done by addition method because the matrix of artificial nature sample cannot be made. The accuracy test is used to show the percentage of the proximity of the analysis results with the actual quercetin content \(^{10}\). The accuracy of quercetin in this study obtained the result of 96.6 ± 8.26% (w/w), this result has met the requirements of the accuracy of bioanalysis 80-120% and precision (C.V.) <10% \(^{32}\). The result of accuracy is not much different as reported \(^{19}\), obtained an average percentage recovery of quercetin of 98.6%.

Application of TLC-Densitometry method for determination of quercetin content in soursop leaf extract \((Annona muricata\ Linn.)\) was performed after the optimal optimum condition was obtained. From the result of the determination of quercetin content in soursop leaf extract 1.288 ± 9.996% (w/w). Reported \(^{33}\), the content of the selected flavonoid as a quercetin compound in soursop leaf extract is (9.96 ± 1.53) mg/g. Differences in quercetin levels are caused by differences in sampling sites, genetic factors, growing environment, treatment during growth, and different harvest time \(^{34-35}\).

CONCLUSION: Standardization of soursop leaf extract \((Annona muricata\ Linn.)\) contains quercetin 1.288 ± 0.1287% (w/w).

RECOMMENDATION: The TLC-Densitometric method can be used to standardize soursop leaf extracts containing quercetin.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

REFERENCES:


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