

The Best Extraction Technique for Kaempferol and Quercetin Isolation from Guava Leaves (*Psidium guajava*)

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Abstract. Guava leaves contain various compounds that have biological activity such as kaempferol and quercetin as anticancer. Twelve extraction techniques were performed to obtain the best extraction technique to isolate kaempferol and quercetin from the guava leaves. Toxicity of extracts was tested against *Artemia salina* larvae. All extracts were toxic (LC₅₀ value less than 1000 ppm) except extract of direct soxhletation on guava leaves, and extract of sonication and soxhletation using *n*-hexane. The extract with high content of total phenols and total flavonoids, low content of tannins, intense color of spot on thin layer chromatogram was selected for high performance liquid chromatography analysis. Direct sonication of guava leaves was chosen as the best extraction technique with kaempferol and quercetin content of 0.02% and 2.15%, respectively. In addition to high content of kaempferol and quercetin, direct sonication was chosen due to the shortest extraction time, lesser impurities and high toxicity.

Keywords: guava leaves, high performance liquid chromatography, kaempferol, quercetin

1. Introduction

Guava (*Psidium guajava*) is a tropical fruit and guava leaves are often used as a traditional medicine to treat wounds, diarrhea, cough, mouth sores, and dengue fever. The guava leaf extract is reported to have antioxidant [1], anti-reaction nonenzimatic reducing sugar in patients with hyperglycemia [2], anti-inflammatory [3], and anticoagulants [4] activities. Some studies show that guava leaves extract contains phenolic compounds such as Ferulic acid, Gallic acid and flavonoids such as catechins, quercetin, and kaempferol [2].

Research on kaempferol and quercetin in guava has not been done, whereas kaempferol known to have many pharmacological activities such as antioxidant, antimicrobial [5], antidiabetic [6], anti-inflammatory and analgesic [7], whereas quercetin is known to have antioxidant activity [8], antibacterial [9], and antiviral [10]. Activities kaempferol and quercetin can be tested by Brine Shrimp Lethality Test (BSLT). This test is used as a preliminary test to determine the potential pharmacological activity of natural ingredients. It has also been perceived as an inexpensive alternative method for cytotoxicity assay. The combination of kaempferol and quercetin content of extracts reported a synergistic effect of antiproliferative in culture Human cancer cell lines [11].

A wide variety of extraction techniques to isolate kaempferol and quercetin has been developed. According to Tang et al. [12], kaempferol and quercetin have been isolated from the leaves of *Ginkgo biloba* with a yield respectively 0.0204 mg/g sample and 0.0371 mg/g sample, respectively [12].



Extraction technique used is macerated with the aid of sonication using methanol-water (85:15). Extraction of kaempferol (0.0007 mg/g sample) of *Sideroxylon foetidissimum* leaves according Erosa-Rejon et al. (2010) was performed by extracting the leaves with ethanol with maceration method for 1 week [13]. Extraction of kaempferol (613 mg/g sample) of soybean leaves by Zang et al. (2011) conducted by refluxing methanol leaves with 70% for 3 hours [6]. Loizzo et al. (2007) has succeeded in extracting kaempferol (12:12 mg / g sample) and quercetin (0.085 mg / g sample) from the leaves of *Ailanthus excelsa* with Soxhletation using 70% methanol [14]. The variety of extraction techniques result in yield kaempferol and quercetin produced also varies. Therefore, it is necessary to do research using a variety of extraction techniques to determine the best extraction technique of the most simple, cheap, and fast and produce maximum yield. This study aims to determine the best extraction techniques for the isolation of kaempferol and quercetin in guava leaf and determine its activity with the method BSLT.

2. Materials and Methods

The research was started by collecting the guava leaves from Conservation and Cultivation Station Unit of Tropical Biopharmaca Research Center at Darmaga Campus, Bogor. The leaves were powdered prior to extraction. The toxicity by BSLT toxicity test method, the total tannin content, total phenolic content, the total flavonoid content, and thin layer chromatography profile of extracts were determined. The kaempferol and quercetin content of the selected extract, then determined by high performance liquid chromatography to select the best extraction method.

2.1. Extractions

Guava leaves are divided into two parts; the first part, directly extracted with 4 kinds of techniques, namely maceration (M1), maceration by sonication (SN1), reflux (R1), and Soxhlet (S1). The second part was extracted with n-hexane (Soxhlet method), and the resulting residue is further divided into two parts. The first part, directly extracted with 4 kinds of techniques, namely maceration (M2), maceration by sonication (Sn2), reflux (R2), and Soxhlet (S2). The second part was extracted further with ethyl acetate (Soxhlet method); the residue was then extracted with 4 kinds of techniques, namely maceration (M3), maceration by sonication (SN3), reflux (R3), and Soxhlet (S3). The whole extract is obtained (M1, M2, M3, SN1, Sn2, SN3, R1, R2, R3, S1, S2, and S3) after concentrated by rotary evaporator

2.1.1. Maceration. The extraction process is based on Erosa-Rejon et al [13]. Leaf samples were extracted with ethanol at room temperature for 1 week. Extraction is done 3 times.

2.1.2. Maceration by sonication. The extraction process is based on Tang et al [12]. Leaf samples were extracted with methanol-water (85:15) with the aid of sonication for 3 hours. The extraction was repeated 3 times.

2.1.3. Reflux. The extraction process is based on Zang et al [6]. Leaf samples were added with 70% ethanol and then refluxing at a temperature of 60-70°C for 3 hours.

2.1.4. Soxhlet. The extraction process is based on Loizzo et al [14]. Leaf samples were added with methanol 70% and the Soxhlet process was performed.

2.2. Total Phenolic Content Determination

Each about 25 mg diluted with methanol: water (1: 1) into a 25 ml flask. About 0.9 mL of standard solution (gallic acid) and extract was added by 4.5 mL reagent Folin-Ciocalteu, and shaken with a vortex. After 3 minutes, each solution was added to 3.6 mL of Na₂CO₃ 7.5%, shaken and incubated for 1 hour. The absorbance of the standard solution and the sample was measured by UV-Vis

spectrophotometer at a wavelength of 765 nm. The total phenol content of samples is determined using a regression equation of standard gallic acid.

2.3. Tannin Content Determination

Extract of 0.5 g was dissolved in DMSO, added to 10 ml of distilled water and heated at a temperature of 40-60 ° C for 30 minutes. The solution was filtered and diluted with distilled water until 50 mL and 5 mL of the solution was then added by 5 mL indigo carmine. The solution was subsequently titrated with 0.1 N KMnO₄.

2.4. Total Flavonoid Content Determination

The extracts about 200 mg was added with 1 mL hexamethylenetetramine (HMT) 0.5%, 20 mL acetone, and 2 ml of HCl. The mixture was hydrolyzed by refluxed for 30 minutes. The result was filtered and added with acetone to 100 ml. About 20 mL of solution was added to 20 ml of water and 15 ml ethyl acetate. Ethyl acetate fraction was collected in a 50 mL volumetric flask. Extraction was repeated by adding 10 mL ethyl acetate. The total ethyl acetate fraction was added with ethyl acetate until 50 ml. Furthermore, 10 ml of the mixture was added to 1 mL of 2% AlCl₃ and 5% glacial acetic acid in methanol. The mixture was homogenized and allowed to stand 15-30 minutes. The absorbance value at a wavelength of 425 nm was measured with a UV-VIS spectrophotometer

2.5. Brine Shrimp Lethality Test

Ten larvae of *Artemia salina* were added to 1 mL extract with varying concentrations of 200-14000 µg/ml. Control without the addition of extract was used as negative control. After 1 day (24 hours) the number of dead shrimp larvae was determined and the LC₅₀ value was determined by probit analysis.

2.6. Thin Layer Chromatography Profile

Each extract was analyzed by Thin Layer Chromatography on silica gel G₆₀F₂₅₄ and *n*-hexane:ethyl acetate (2:98) as eluent. The detection was observed in the UV lamp 254 and 366 nm. An extract containing a fluorescent and R_f value the same with kaempferol and quercetin standards will be analyzed further.

2.7. Kaempferol and Quercetin Content Determination

About 30 mg of each extract was added to 2 ml of HCl 4 M, shaken and heated for 30 minutes and extracted by 2 mL ethyl acetate. Ethyl acetate fractions were separated and rinsed with 1 mL ethyl acetate 2 times. The collected ethyl acetate fraction evaporated and diluted with methanol till 10 ml. Standard solutions and the sample were then filtered with 0.45 µm membrane and 20 µl was injected in high performance liquid chromatography (HPLC) for analysis.

HPLC Conditions used is C18 column, UV detector at λ 370 nm, oven temperature 30 ° C, mobile phase acetonitrile 30% in KH₂PO₄ 0.025 M buffer pH 2.5 with isocratic elution, and flow rate of 1 ml / min¹⁴. The kaempferol and quercetin content were determined by comparing the peak area with the peak area of standards.

2.8. Statistical analysis

All data obtained were analyzed using the analysis of variance at 95% confidence level (p<0.05) using the Duncan's test with SPSS 16.

3. Results and Discussion

The difference preparation and extraction technique used in this study is to find the highest content of kaempferol and quercetin. The separation process was Soxhlet with *n*-hexane and ethyl acetate and comparison with the leaves without separation step first. The aim of Soxhlet with *n*-hexane is to eliminate the fat attached to the leaf tissue so expect it easier to extract the polar compounds such as kaempferol and quercetin. The continue separation by Soxhlet with ethyl acetate aimed to eliminate

components that are semi-polar. Table 1 shows that the longer the separation step, the smaller % yield got. The highest yield is found in the extraction process of M2.

The highest yield of the extract is not guaranty the highest kaempferol and quercetin content. The screen which extract will be used for the next step, total phenolic, tannin and flavonoid content were determined to each extract. The result is shown in table 1. The highest total phenolic compound is found in extract of S1 while M1 has the highest tannin content. The high phenolic compounds in the extracts S1 indicates that phenolic compounds can be extracted optimally with direct extraction without separation step. According to Guimarães-Beelen et al. (2006) tannins can be optimally extracted by maceration using ethyl acetate and water [16]. M1 extracts were extracted with methanol: water so that the levels of tannins produced the highest. Other extracts have low levels of tannin extraction step because it has experienced a long and residue derived from ethyl acetate. Flavonoids are phenolic compounds that can be extracted using polar organic compounds such as methanol and ethanol [14]. The highest content of the total flavonoid extract is found in M1.

Table 1. Phenolic, Tannin, Flavonoid Contents and LC₅₀ value against *A. salina* of all extracts. Difference letter means significantly different at $p > 0.05$

Extraction technique	Extract name	Yield (%)	Phenolic content (mg/g)	Tannin Content (%)	Flavonoid content (mg/g)	LC ₅₀ (ppm)
Maceration	M1	22.82 ^d	831.13 ^e	15.81 ^a	21.43 ^a	526.24 ^f
	M2	43.38 ^a	758.70 ^g	8.56 ^c	20.54 ^a	281.52 ^e
	M3	13.95 ^e	843.35 ^d	6.21 ⁱ	11.49 ^c	100.03 ^d
Sonication	Sn1	20.05 ^d	753.34 ^g	7.31 ^f	16.22 ^b	41.21 ^b
	Sn2	10.78 ^e	557.95 ⁱ	6.98 ^g	9.75 ^d	3862.62 ^j
	Sn3	6.60 ^f	868.19 ^c	6.47 ^h	5.09 ^e	745.54 ^g
Reflux	R1	14.22 ^e	837.68 ^{de}	8.91 ^b	11.24 ^c	31.22 ^a
	R2	13.49 ^e	877.82 ^c	7.63 ^e	4.44 ^f	36.34 ^a
	R3	14.18 ^e	910.58 ^b	8.20 ^d	3.19 ⁱ	70.76 ^c
Soxhlet	S1	35.44 ^b	957.77 ^a	6.02 ^j	4.67 ^f	1340.81 ⁱ
	S2	24.57 ^c	807.49 ^f	7.78 ^e	3.85 ^g	1095.52 ^h
	S3	14.36 ^e	729.16 ^h	5.75 ^k	3.55 ^h	132.13 ^d

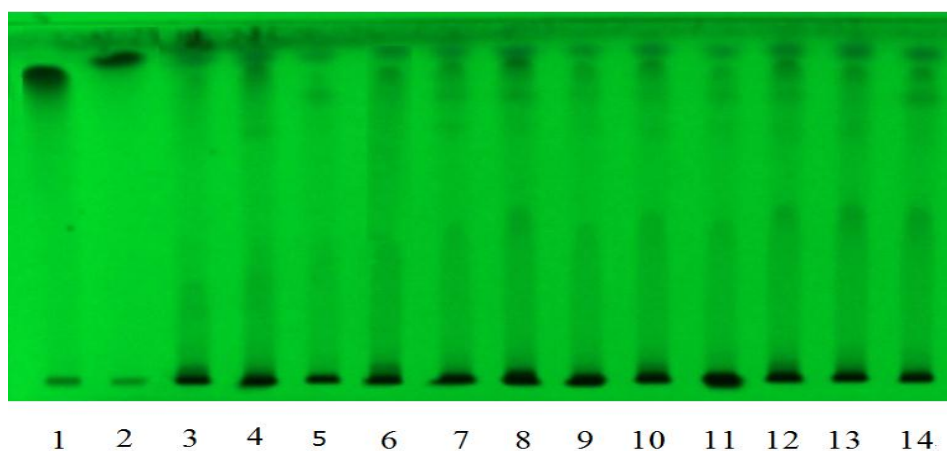


Figure 1. Chromatogram of extract and standard on UV 254 nm; from 1-14: quercetin standard, kaempferol standard, M1, M2, M3, Sn1, Sn2, Sn3, R1, R2, R3, S1, S2, and S3.

The combination of flavonols such as quercetin and kaempferol will give high potency as anticancer especially as antiproliferative and cytotoxic against cultured human cancer cell lines¹¹. One of easy methods to determine the cytotoxic properties of the active compound is based on the number of deaths *Artemia salina* larvae at certain concentrations. The data are usually expressed in LC₅₀ (Lethal Concentration 50%), which is a value that indicates the concentration of toxic substances that can cause 50% of the death test animals [17]. Based on the LC₅₀ values shown in table 1, extract of Sn2, S1, and S2 which has a LC₅₀ value is higher than 1000 ppm is less toxic, while other extracts is potent as a candidate for an anticancer agent.

In addition to the data of phenolic, tannin, and flavonoid content, TLC chromatogram is used to determine the extraction process to isolate kaempferol and quercetin. Figure 2 shows that all extracts generate almost the same bands. Kaempferol standard was found in R_f value of 0.87 whereas quercetin standard of 0.83. The structure of kaempferol and quercetin is shown in figure 2. Detection by UV 366 nm in figure 3 shows that both the standard kaempferol and quercetin had a green fluorescent. Different with the standard, the same R_f band in the extracts did not give green fluorescent but blue. The absence of green fluorescent at the same R_f can be assumed that kaempferol compounds in the extract were blocked by a blue fluorescent compound that has similar polarity with kaempferol.

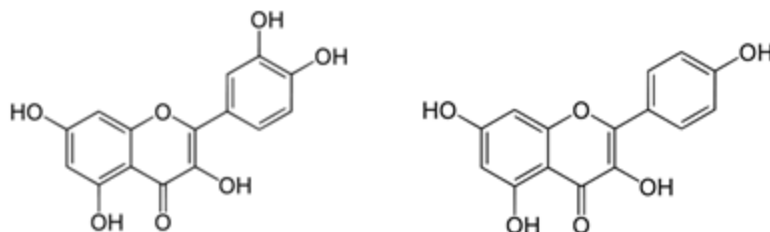


Figure 2. Structure of quercetin (left) and kaempferol (right).

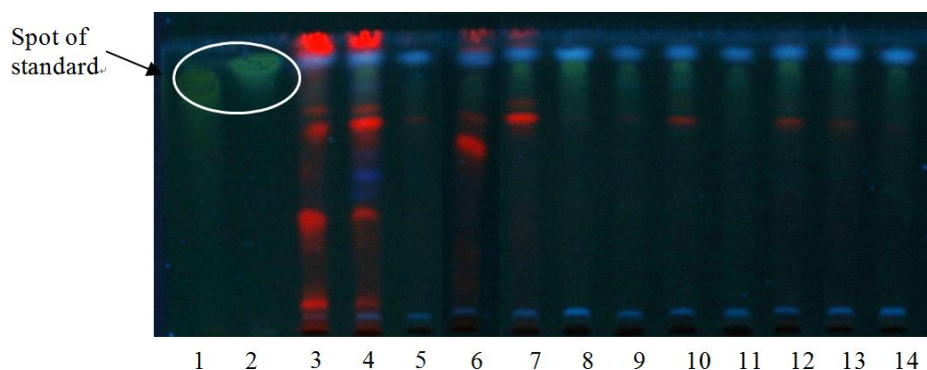


Figure 3. Chromatogram of extract and standard on UV 366 nm; from 1-14: quercetin standard, kaempferol standard, M1, M2, M3, Sn1, Sn2, Sn3, R1, R2, R3, S1, S2, and S3.

In figure 2 showed that the extract of M1, M2, Sn1, and Sn2 has many bands with high intensity. The reflux and Soxhlet from original leaf and residues of *n*-hexane (R1, R2, S1, S2) showed a smaller number of bands and lower intensity of bands, while all extracts derived from residues of ethyl acetate (M3, SN3, R3, S3) show the lowest number and intensity of bands. It can be concluded that more separation step before extraction may be causing the loss of other compounds.

Table 2. Kaempferol and quercetin content on selected extract

Extract/standard name	Concentration (%)	
	Kaempferol	Quercetin
Sn1	0.02 ^b	2.15 ^c
Sn2	0.02 ^b	1.97 ^b
S1	0.03 ^b	1.98 ^b
S3	0.01 ^a	1.14 ^a

Difference letter means significantly different at $p > 0.05$

Based on the fluorescent intensity on TLC, total phenolic content, tannin content, and flavonoid content, extract of Sn1, Sn2, S1 and S3 were selected to determine the kaempferol and quercetin content using HPLC. Selected extracts have high fluorescent intensity, low tannin and phenols content and high flavonoids content. Table 2 shows that the highest kaempferol content was found in extract of S1, while the highest quercetin content was found in Sn1 extract. Kaempferol content of the extract Sn1 is only small different with the S1, but has much higher LC₅₀ value. Extract Sn1 had LC₅₀ values of 41.21 ppm, whereas S1 extracts 1340.81 ppm. So Sn1 extract is considered better because it has a lower LC₅₀ value. The results showed that the guava leaf sonication using methanol-water (85:15) directly from the sample was the best extraction techniques for the isolation of kaempferol and quercetin in guava leaves. The kaempferol and quercetin content on guava leaf extract with the Tang et al (2001) methods on this study is higher (0.04 mg/g sample and 4.30 mg/g sample respectively) compared with *Ginko biloba* leaf extracts reported by Tang et al [12].

4. Conclusion

Best extraction techniques to isolate kaempferol and quercetin from guava leaves are sonication using methanol-water (85:15) with a content of 0.02% by kaempferol and quercetin at 2:15%. This technique had yield about 20.05% and BSLT LD50 of 41.21ppm.

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