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Research Article

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Extraction technique to separate kaempferol from Soursop (*Annona muricata*) leaves

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ABSTRACT

Soursop leaves contain various compounds that have biological activity such as kaempferol as anticancer. Twelve extraction techniques were performed to obtain the best extraction technique to get high kaempferol content on Soursop leaves extracts. The dried Soursop leaves powder was extracted with different solvent and different technique such as maceration, sonication, reflux, and soxhletation. The yield of extract, toxicity against Artemia salina larvae, total phenols, total flavonoids, total tannin, and thin layer chromatography profile of all extracts were determined. Total phenol and total flavonoids content were determined by spectroscopy, while total tannin content was determined by titration method. The results showed that the yields of extracts werevarying from 4.09 – 18.64%. All extracts were toxic since they showed LC_{50} value less than 1000 ppm. Tannin content on the extracts was varying from 3.78 – 7.59%, phenolic content from 6.16 – 16.44%, and flavonoid content from 0.63 – 10.25%. The extract with high content of total phenols and total flavonoids, low of tannin content, and low spot intensity on thin layer chromatograph was selected for high performance liquid chromatography analysis. Sonication extraction of n-hexane residues was chosen as the best extraction technique for kaempferol isolation from the Soursop leaves with kaempferol content of 1.22%. In addition to high content of kaempferol, sonication was chosen due to the highest yield of extraction, the shortest extraction time and the least impurities.

Keywords: kaempferol, extraction, Soursop leaves.

INTRODUCTION

Soursop (*Annona muricata*) is one of the plants that are found in tropical countries, such as Indonesia. This plant is grown commercially to collect the fruits as food ingredient. Beside it, Soursop fruit also used to treat dysentery, ulcers, hemorrhoids, and anticonvulsants [1]. Soursop leaves also reported have several activities, such as to lowering the blood sugar level, improve immune system, and treat cancer [2-4]. Several studies have reported that Soursop leaves extract containing flavonoid and othe phenolic compounds such as quercetin, catechin, and kaempferol [5]. The flavonoid from *Annona dioca* reported had activity to inhibit the Ehrlich cancer cells [6].

Kaempferol in nature are in the glycoside form which have many biological activities such as anti-inflammatory, anti-fungi, antioxidant, and anti diabetics [7-9]. Kaempferol can be separated by extraction from natural resources using ethanol or methanol [10]. Several extraction techniques have been developed to extract kaempferol from natural resources such as by sonication, soxhletation, and reflux [9, 11-12]. Different technique and different solvent used on extraction process is resulting different yield and purity of kaempferol extracted. Therefore, the research to determine the best technique to extract kaempferol from Soursop leaves which is simple, cheap, fast and has high yield are needed.

EXPERIMENTAL SECTION

Materials

All solvents used were analytical grade or HPLC grade and obtained fromm Merck (Darmstadt, Germany), kaempferol and quercetin standard from Nacalai (Tokyo, Japan), and Soursop leaves from Conservation and Cultivation of Biopharmaca Resources Unit, Darmaga Campus, Bogor Agricultural University, Indonesia. The samples were sieved, dried on oven 50 ^{o}C for 72 hours, and grinded prior to use. The species name was identified by Research Center for Biology Indonesian Institute of Science, Cibinong, Indonesia

Methods

The research was focus on selection the extraction method from different sources of Soursop samples. The first group of samples was using the dried Soursop leaves, the second group (*n*-hexane residue) was using the residue of dried Soursop leaves after extracted with *n*-hexane, and the third group (EtOAc residue) was using the *n*-hexane residue after extracted with ethyl acetic. Each group of samples was extracted by solvent (1 g:5 mL) in different methods. The methods used were maceration by ethanol for 1 week [10], maceration with ultrasonic wave by water-methanol (85:15) for 3 hours [11], reflux by methanol 70% on 60–70ºC for 3 hours [9], and soxhletation by methanol 70% [12]. Each extract was dried by rotary evaporator.

The yield, tannin content, total phenolic content, total flavonoid content, and toxicity of all extractswere determined to select the prospective extract. The prospective extracts is the extract which has high yield, high flavonoid content, high tocixity, low tannin content, and low total phenolic content. Beside that the prospective extract also determined by thin layer chromatogram profile. The extract which had high intensity of kaempferol spot and only limitted number of other spots are the prospective extract.

The kaempferol content was determined by high performance liquied chromatography (HPLC) on the selected extract. The highest kaempferol content was reported as the best method to separate the kaempferol from Soursop leaves.

Total tannin content [13]

About 0.5 gram extract was added by 50mL water and heated on 40–60^{o}C for 30 minutes and filtered. The indigo carmine was added to the filtrate and titrated by $KMnO_4$ 0.1 N till the color change into yellowish gold.

Total phenolic content

About 25 mg extract was dissolved with 25mL methanol:water (1:1). A 300 µLthe solution was added by 1.5 mL Folin-Ciocalteu (1:10) and mixed. After 3 minutes, the 1.2 mL Na_2CO_3 7.5% was added to the solution and the absorbance of the solution was measured at 765nm and reported as galic acid equivalent/g samples.

Total flavonoid content [14]

The extract about 200mg was dissolved by aceton and hexamethylenetetramine (HMT) 25%. The solution then hydrolyzed by HCl 25% on 80 ^{o}C for 30 minutes. The hydrolyze product was partition by ethyl acetate and the EtOAc fraction was collected and added by $AlCl_3$2% and the absorbance was measured at 425 nm.

Toxicity by Brine Shrimp Lethality Test [15]

About 10 brine shrimp larvae put on the well consisted of 4.5mL sea water and added by 0.5mL extract solution. The extract concentration was ranging from 1 - 5000µg/mL. The dead larvae number was determined after 1 day (24 hours). The lethal concentration 50% (LC_{50}) was determined.

Thin Layer Chromatography (TLC) Profile

A 25 mg extract was hydrolyzed by HCl 4 N and partition by EtOAc. The EtOAc fraction was dried till 1mL methanol. This methanol solution was spotted on silica gel plate together with kaempferol. The plate then eluted by chloroform:methanol (9.75:0.25). The detection used was ultraviolet on 366 nm.

Kaempferol content by HPLC [16]

The method used was using C18 column with 30% acetonitrileand70% phosphate buffer 0.025 M pH 2.5. The isocratic method was used with 1.0mL/min of flow rate. The detector was UV at 370 nm. The kaempferol content was measured by comparing the peak area of kaempferol by the peak area of same retention time peak on the samples. The samples used was the hydrolyze samples by HCl4M. The sample and the standard injected was about 20 µL

RESULTS AND DISCUSSION

The yield, toxicity, tannin content, total phenolic content and flavonoid content of all extraction method and all groups of the samples are shown in Table 1. The highest yield of extract was found on soxhletation method from the dried Soursop leaves. The soxhletation method gave the highest yield on each group of the samples, because the soxhletation used boiler and reflux which circulates the solvent. The result was high number of extract component moved to the solvent. Based on the group of the samples, the dried Soursop leaves group had the highest yield compare to the other samples. It means that pre-extraction process before the main extraction process decrease the yield because some component are not in the residue anymore. After extraction by EtOAc, the residue only gave small amount of the yield. From all of the extract, the highest yields was found on soxhletation method from the dried leaves.

Table 1. The yield (%), toxicity (LC_{50} in ppm), tannin content (%), total phenolic content (%) and flavonoid content (%) of all extraction method and all groups of the Soursop leaves samples

Group of the samples	Methods	Yields (%)	LC_{50} (ppm)	Tannin (%)	Phenolic content (%)	Flavonoid content (%)
Dried Soursop leaves	Maceration	10.04	209± 13	7.6 ± 0.1	13.9 ± 0.1	10.3 ± 0.0
	Sonication	9.16	128± 3	5.2 ± 0.1	14.3 ± 0.1	4.5± 0.0
	Reflux	6.88	79± 2	4.9± 0.1	10.4 ± 0.1	1.7 ± 0.0
	soxhletation	18.64	46 ± 4	5.6± 0.1	12.9 ± 0.1	7.1 ± 0.1
Residue of *n*-hexane extract	Maceration	9.67	455 ± 14	4.9 ± 0.1	10.4 ± 0.0	5.6 ± 0.1
	Sonication	9.81	150 ± 3	4.6 ± 0.2	11.4 ± 0.1	4.5 ± 0.1
	Reflux	6.11	93 ± 4	6.2 ± 0.1	8.7± 0.1	0.9 ± 0.0
	soxhletation	17.36	152± 3	6.9 ± 0.1	16.4 ± 0.0	2.3 ± 0.0
Residue of EtOAc extract	Maceration	3.18	300 ± 6	4.8 ± 0.1	9.6 ± 0.1	0.8 ± 0.0
	Sonication	4.09	648 ± 11	6.7 ± 0.2	8.6 ± 0.1	1.1± 0.0
	Reflux	4.73	299 ± 7	6.9 ± 0.1	6.2 ± 0.1	0.9 ± 0.0
	soxhletation	12.76	198 ± 8	3.8 ± 0.1	8.2 ± 0.1	0.6 ± 0.0

The toxicity of all extracts reported in LC_{50} value. LC_{50} is the concentration that can kill 50% population of the animal test. Based on the data on Table 1, all of the extracts have the LC_{50} less than 1000 ppm. According to Meyer *et al* , a crude extract is toxic if the crude extract has LC_{50} value less than 1000 ppm [15]. It means all of the extracts are toxic. The mosts toxic extract is soxhletation extract from the dried leaves.

The tannin content of all extract are varied. The highest tannin content was found on soxhletation extract from *n*-hexane risudue, while the lowest content was found on soxhletation extract from EtOAc residue (Table 1). Tannin is the phenolic compound which found in Soursop leaves, the extraction process that consider as a good process to separate kaempferol is the process which resulted the lowest tannin content.
The total phenolic content also determined to all the extracts. The extracts from EtOAc residue have lower phenolic content compared to other samples type. The highest phenolic content was found on the soxhletation method from *n*-hexane residue (Table 1). The high phenolic content on the extract is consider as prospective method to separate the kaempferol.

The total flavonoid content is determined because kaempferol is one of the flavonoid compound (Fig 1). The highest flavonoid content was found on maceration extract from the dried leaves (Table 1). The high flavonoid content could related to the kaempferol content.

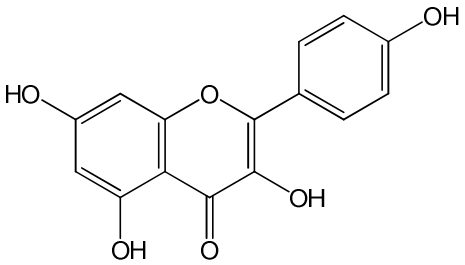

Figure 1. Structure of Kaempferol

The highest yield, phenolic content, and flavonoid content, and the lowest tanin content on the extract are different extract. It made difficult to select the prospective extract, so the thin layer chromatography (TLC) profile is needed. TLC profile could give information about what extract is consisted of kaempferol by comparing the spot with the kaempferol standard. In the nature, flavonoid is not on the free form. Most of the flavonoid is found on glicoside form. To separate the flavonoid from the sugar, the hydrolysis process is needed. To hydrolize the flavonoid

glycoside on the extract the HCl is added to the extract prior to TLC process. The TLC profile of all of the extract is shown in Fig 2.

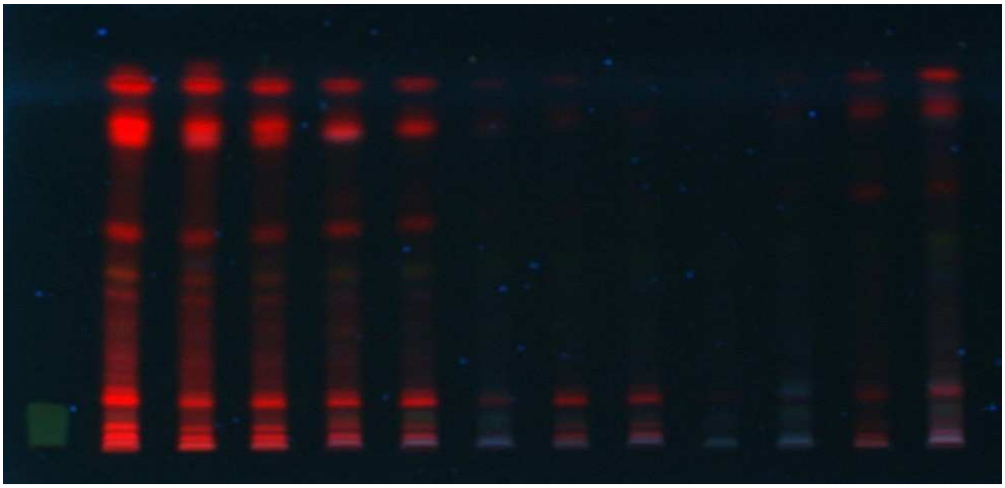

Figure 2. TLC chromatogram of extract of Soursop leaves (from left to the right): kaempferol standard, maceration extract of dried leaves, maceration extract of *n*-hexane residue, maceration of EtOAc residue, sonicaton of dried leaves, sonication of hex residue, sonication of EtOAc residue, reflux extract of dried leaves, reflux extract of hex residue, reflux of EtOAc residue, soxhletation of dried leaves, soxhletation of hex residue, soxhletation of EtOAc residue

Based on the TLC chromatogram on Fig 2, the spot of kaempferol was found with Rf of 0.10, and all of extract are consisted of kaempferol with different amount. From the color of spot on Rf 0.10, the extract from EtOAc residue consisted less kaempferol compared to the other type of samples. The other spots beside spot with Rf 0.10 is undesirable spots because that spot is act as contaminant. To the next step of research, extract from dried material by maceration, sonication,a nd soxhletation method and extract from *n*-hexane residue by maceration and sonication method are used.

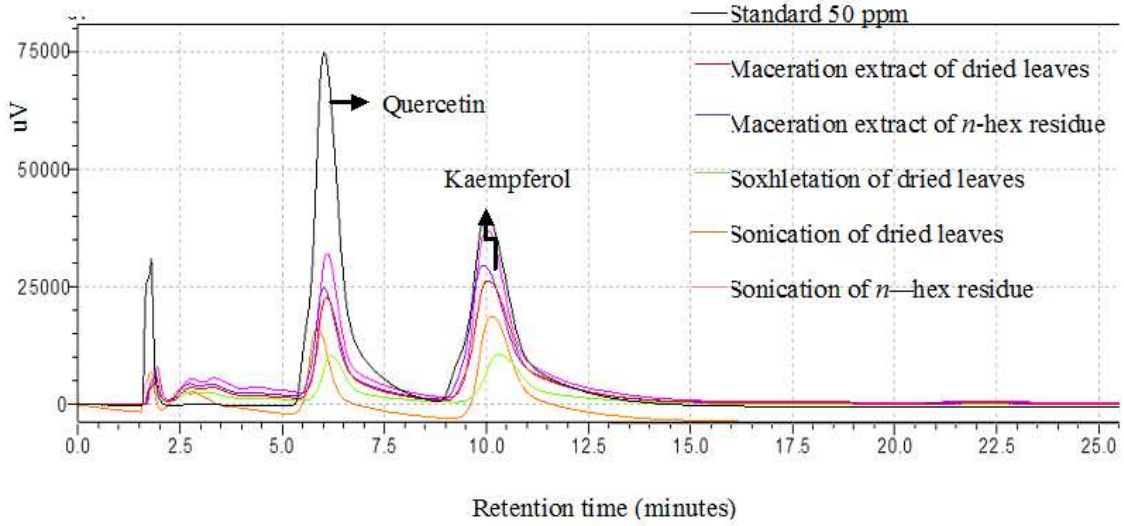

Figure 3. HPLC Chromatogram of selected extract

HPLC method used to determine the kaempferol content on the extract. The chromatogram of HPLC analysis from 5 extracts is shown in Figure 3. Beside kaempferol peak, quercetin peak also appear on all of extract with different peak area. Quercetin appear first before kaempferol on the reverse phase HPLC method because quercetin (Figure 4) is more polar compare to kaempferol [16].

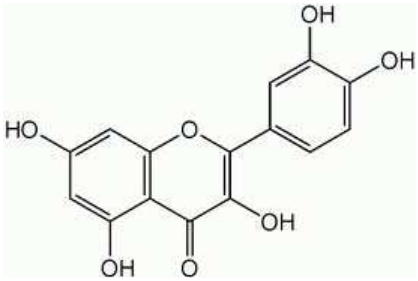

Figure 4. Structure of Quercetin

The kaempferol content and quercetin content on selected extract is reported on Table 2. Kaempferol content on all of the extracts are higher than the quercetin content. The highest kaempferol and quercetin content was found on the sonication extract from *n*-hexane residue. The sonication extract from n-hexane residue is consider as the best extraction method because it has the second high yield compare to othe extract, it is also only need 3 hours to complete the extraction process, and it has less impurities on TLC chromatogram.

Table 2. Kaempferol and quercetin content from selected extract

Type of samples	Extraction method	Kaempferol (ppm)	Quercetin(ppm)
Dried materials	Maceration	1.02 ± 0.01	0.42 ± 0.01
	Sonication	0.84 ± 0.01	0.36 ± 0.01
	Soxhletation	0.43 ± 0.01	0.19 ± 0.01
n-hexane residue	Maceration	1.11 ± 0.01	0.44 ± 0.01
	Sonication	1.22 ± 0.01	0.50 ± 0.01

CONCLUSION

In conclusion, the best extraction method to isolate kaempferol from Soursop leaves is sonication extraction method from *n*-hexane residue. The sonication method from *n*-hexane has kaempferol content of 1.22%, the yield of 17.36%, the shortest extraction time and the least impurities.

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