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ARTICLE

HPLC–UV analysis of sample preparation influence on flavonoid yield from *Cirsium japonicum* var. *maackii*

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Abstract This study was conducted to optimize the extraction conditions of flavonoids from *Cirsium japonicum* var. *maackii* (ICF-1). The effects of sample material ratio, solvent concentration, extraction time, solid-to-solvent ratio, and number of extractions on flavonoid extraction efficiency were analyzed. Three flavonoids were specifically investigated: cirsimarin (**1**), hispidulin (**2**), and cirsimaritin (**3**). In single-factor experiments, each variable had a significant effect on the determination of content of compounds **1**–**3**. The optimal conditions for extraction were found to be: mass, 15 g; ratio of spring and fall leaves, 4:1; extraction solvent, 70% ethanol; extraction time, 4 h; solid-to-solvent ratio, 1:20; and number of extractions, 1. The results of the study were used to maximize the potential of ICF-1 samples and optimize the efficiency of the extraction process.

Keywords Cirsimarin · Cirsimaritin · *Cirsium japonicum* var. *maackii* · High-performance liquid chromatography-UV · Hispidulin

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Introduction

The perennial herb *Cirsium japonicum* is widely distributed in many Eurasian countries and cultivated in China, Japan, and Korea. It is commonly known as the Japanese thistle and has been used in oriental medicine for its antihypertensive [1], anti-hemorrhagic, and diuretic effects [2]. Moreover, owing to its many uses in traditional medicine, numerous studies have been performed to determine the various bioactive metabolites found in *C. japonicum,* and their importance in medical and pharmacological applications. Recently, it has been shown that various secondary metabolites isolated from *C. japonicum* exhibit bioactive properties, such as anticancer [3, 4], antidiabetic [5], antioxidant [4, 6], antibacterial [7], and antitumor effects [8].

Among the myriad phytochemical constituents derived from *C. japonicum,* flavones are commonly isolated from this plant [5]. Some of the biologically important flavones reported from *C. japonicum* include cirsimarin, hispidulin, and cirsimaritin. These flavones are known to possess beneficial pharmacological effects: hispidulin promotes the suppression of cancer cells and hepatoprotection [9–11]; cirsimarin has antilipolytic and antioxidant activity [12]; cirsimaritin has antibacterial, anti-inflammatory, and antioxidant properties [13–15]. The extraction of these flavone compounds from their plant sources is a necessary step in the phytochemical and pharmaceutical applications involving the use of such compounds. Several studies have been conducted on the extraction and analytical determination of such flavones from *C. japonicum.* However, to the best of our knowledge, no studies on the optimization of the extraction process of cirsimarin, hispidulin, and cirsimaritin have been conducted [16]. Hence, the aim of this study was to investigate how the sample preparation

and alterations in the extraction variables (i.e., solvent concentration, sample weight, extraction time, number of extraction, and raw sample preparation) affected the yield of the aforementioned flavone compounds [17]. Ultimately, this study aimed to develop an optimized method for the extraction process of cirsimaritin, cirsimarin, and hispidulin from *C. japonicum* var. *maackii* (ICF-1). An additional experiment was done to replicate a production process that involves larger amounts of samples using factors that are efficient, economical, uses less hazardous solvents and materials, and suitable for production in large industry which aims to recover possible high content of compounds while considering their potential application as a nutraceutical.

Materials and methods

Plant materials and chemicals

The dried leaves (CJL) of ICF-1 were obtained from Imsil Herbal Medicine, Republic of Korea, during spring and fall of 2015. A sample voucher was deposited at the herbarium of the Department of Integrated Plant Science, Chung-Ang University, South Korea. Ethanol (EtOH) and high-performance liquid chromatography (HPLC) grade solvents (acetonitrile, methanol, and water) were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea). Standard compounds were acquired from Sigma (St. Louis, MO, USA). Lab Partner model PDO-150 drying oven (Namyangju, Korea) was used.

Preparation of standard solution

Standard solutions for the compounds, cirsimarin (**1**), hispidulin (**2**), and cirsimaritin (**3**), were prepared. To prepare the calibration curve, 1 mg/mL of each compound was dissolved in HPLC methanol and subjected to serial dilution. The solution was filtered through a 0.45-μm membrane filter prior to injection to the HPLC system. The peak areas of the individual compounds were compared with those of a standard curve prepared from the appropriate standard compounds. The peak area (Y), concentration (X, mg/mL), and mean values ($n = 5$) of the calibration curves of the compounds were calculated.

Sample preparation of CJL samples

CJL were ground to a powder and placed in tea bags before extraction. Single-factor experiments of the sample ratio, solvent concentration, sample weight, extraction time, number of extractions, solid-to-solvent ratio, and raw sample preparation were determined to evaluate their effects on the yields of compounds **1–3**. All extractions were performed under reflux.

Sample material ratio

The material ratio was determined by mixing spring and fall of CJL. The following spring:fall ratios; 3:2, 1:4, 1:1, 2:3, and 4:1 were used to determine the maximum yield of compounds **1–3**. Fifteen grams of mixed spring and fall of CJL was extracted with EtOH (300 mL) for 3 h at 78.3 °C (3×). The supernatant was collected and concentrated *in vacuo*. The concentrated sample was oven-dried at 50 °C for 24 h and used for HPLC analysis.

Solvent concentration

To determine the appropriate concentration of EtOH, 15 g of starting material at the previously determined maximum ratio was extracted using different concentrations of EtOH (30, 50, 70, and 95%) and distilled water. Samples were prepared using 300 mL of these solvents. All other conditions for extraction were the same as described above.

Extraction time

The optimal extraction time was investigated by evaluating the contents of compounds **1–3** after 1, 2, 3, and 4 h of extraction. Ground CJL at the previously determined maximum ratio (15 g) was extracted with 30% EtOH (300 mL) at 78.3 °C under reflux for different lengths of time (3×).

Solid-to-solvent ratio

The contents of compounds **1–3** were evaluated in terms of solid-to-solvent ratio. CJL (spring:fall = 3:2) was extracted with 30% EtOH (300 mL) for 4 h at 78.3 °C (3×) using the following ratios: 1:10 and 1:20. After extraction, the samples were concentrated *in vacuo* and oven-dried at 50 °C. The dried sample was used for HPLC analysis.

Number of extraction

Fifteen grams of CJL (spring:fall = 3:2) was extracted with 30% EtOH (300 mL) for 4 h at 78.3 °C. The extraction process was repeated twice. Other conditions were the same as previously described.

Large-scale extraction

The contents of compounds **1–3** were also determined after large-scale extraction using the predetermined conditions. Powdered sample (100 g) was extracted with 30% EtOH

(2 L) at 78.3 °C for 3 h. The extract was filtered and concentrated *in vacuo* at 78.3 °C for 3 h. The total solid content was determined by transferring 50 ml of aliquot to a measured container and oven-dried at 105 °C for 24 h. The percent solid content was calculated by subtracting the oven-dried sample in the container and the initial weight of the container and dividing by the initial volume of the aliquot, multiplied by 100. Lyophilization was performed for 48 h. The aliquots of the powdered sample, extracted sample, and concentrated sample were subjected to HPLC.

HPLC analysis

A Waters 1525 HPLC system, coupled with a Waters 2489 UV/VIS detector, and an autosampler, was used for the quantification of compounds **1–3**. The concentrations of these compounds were determined from the concentrations of a standard compound used as external standard. The quantitative analysis was performed using a reverse-phase system. Separation was conducted on an INNO C18 column (25 cm × 4.6 mm, 5 μm). The column temperature was maintained at 30 °C, and the flow rate was set at 1 mL/min. The mobile phase comprised: (A) acetonitrile and (B) 0.5% acetic acid, and the elution was conducted in gradient system, 83% (A)–17% (B) for 10 min; reduced to 70% (A) for 10 min and maintained for 15 min; reduced to 20% (A) for 5 min; reduced from 20 to 0% for 5 min and maintained for 5 min; and increased from 0 to 83% (A) for 10 min and maintained for 5 min. The injection volume was 10 μL, and the UV absorbance was recorded at 270 nm.

Calibration curves

Standard solutions of compounds **1–3** were dissolved in HPLC methanol to prepare concentrations in the range of 0.1–1000 μg/mL. The calibration curves of compounds **1–3** were calculated by using the peak area (Y), concentration (X, μg/mL), and mean values ($n = 3$) ± standard deviation (Table 1). Linear regression was used to determine the linearity of the calibration curve. The contents of compounds **1–3** were determined from the corresponding calibration curves.

Results and discussion

Single-factor experiments of the sample material ratio, solvent concentration, extraction time, number of extraction, and solid-to-solvent ratio were determined to evaluate their effects on the content of compounds **1–3**. These variables are known to be critical for the extraction efficiency. The influence of these variables on the content of flavonoids from CJL was studied. HPLC analysis was used to simultaneously quantify and identify the flavonoids studied. Compounds **1–3** were detected at retention times of 16.49, 30.03, and 32.11 min, respectively (Fig. 1). The single-factor experiments showed that each variable had significant effects on the recovery of compounds **1–3** which suggests that the determination of the appropriate variable will reveal the most optimal and reliable method for the sample preparation and extraction of CJL. The sample material ratio of spring and fall leaves had the most significant effect on the recovery of compounds **1–3** (Table 2). Compound **1** had the highest yield at a 1:1, whereas the lowest yield was observed at a 4:1 ratio. Recovery of compounds **2** and **3** was highest at the ratio of 4:1. Zhishen et al. [18] studied the flavonoid content of mulberry leaves collected during spring and fall and reported that spring leaves contained higher amount of flavonoids than fall leaves. Similar results were shown in a study by Olszewska (2007), who reported that the aerial parts of *Prunus serotina* had a higher total flavonoid content in spring. Limited studies have been investigated on considering the seasonal dynamics of the flavonoid content of *C. japonicum* [18]. Previous investigations have suggested that the leaves in flowering season contained a significantly higher level of secondary metabolites, which decreased after flowering [19].

The effect of EtOH concentration on the content of compounds **1–3** was determined based on the extraction of CJL with different concentrations of EtOH (Table 3). Compounds **1** and **2** exhibited the same pattern, in which the recovery increased with an increase in the EtOH concentration. However, the highest content of compound **3** was recovered at 70% EtOH and lowest content was in the water extraction. As shown in Table 4, the extraction time and content were significantly interrelated. The use of EtOH in the extraction of flavonoids is preferential, and it

Table 1 Calibration curves for compounds **1–3**

Compound	t_R	Calibration equation [a]	Correlation factor, r^2 [b]
1	16.49	$Y = 2000000X + 72170$	0.999
2	30.03	$Y = 2000000X + 424411$	0.996
3	32.11	$Y = 1000000X + 438652$	0.991

[a] Y = peak area, X = concentration of standard (mg/mL)

[b] r^2 = correlation coefficient for three data points in the calibration curve

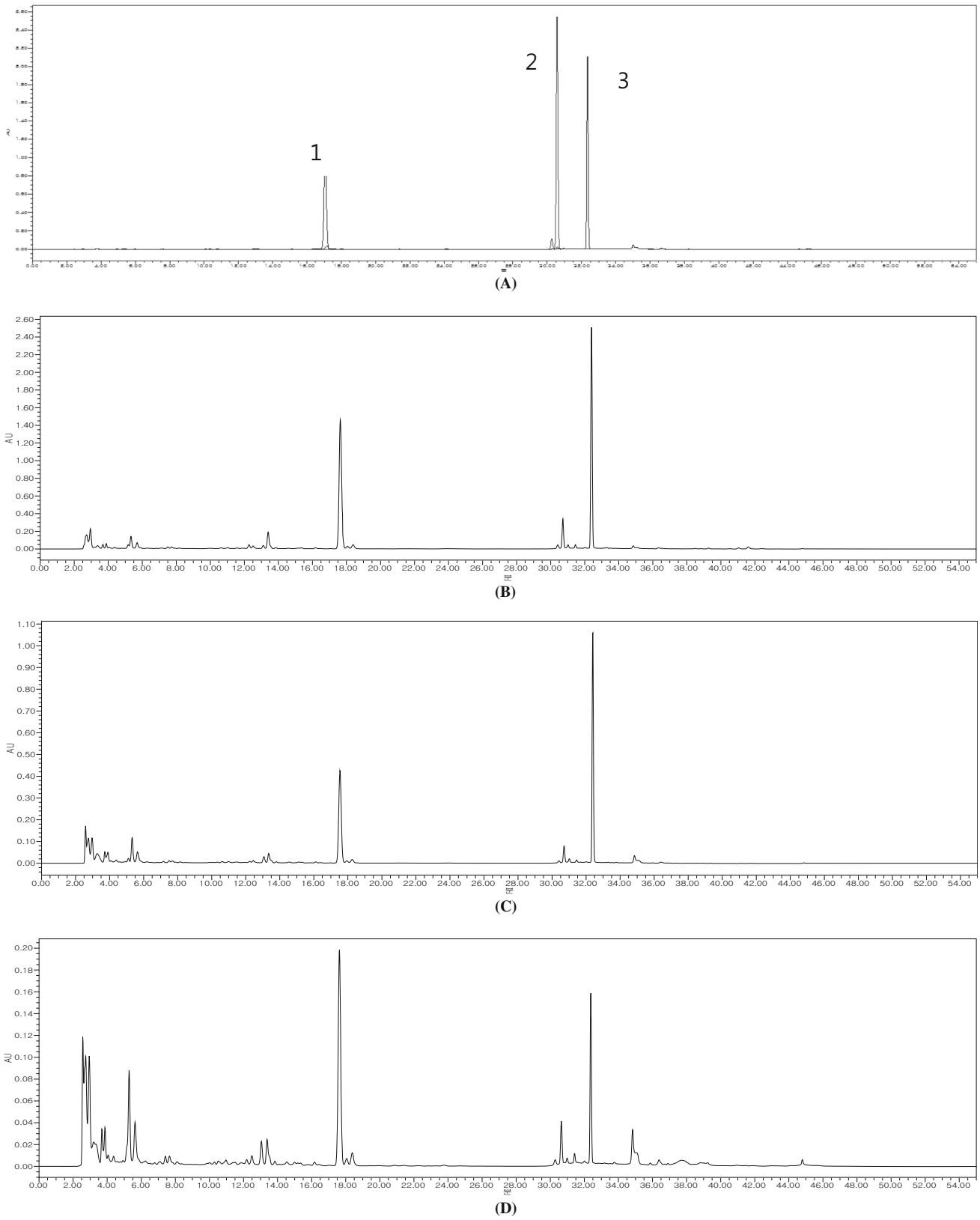
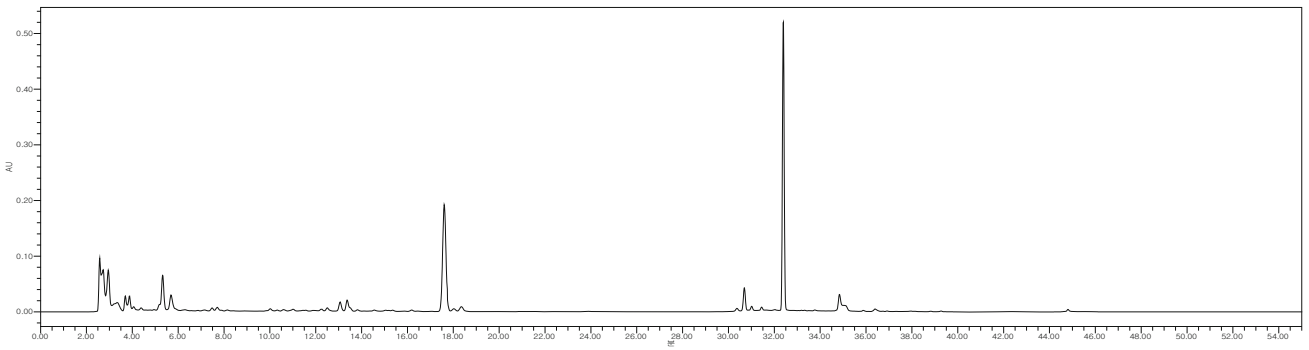
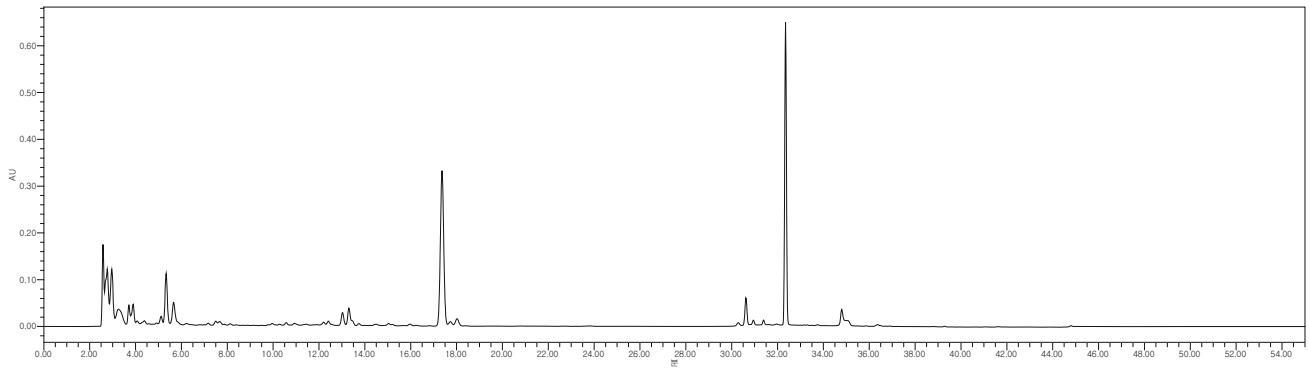


Fig. 1 HPLC chromatograms of compounds **1**–**3** (**A**), 4:1 spring-to-fall ratio of CJL (**B**), 70% EtOH extraction (**C**), 4 h extraction time (**D**), 1:20 solid-to-solvent ratio (**E**), and one-time extraction (**F**)



(E)



(F)

Fig. 1 continued

Table 2 Effect of sample material ratio on the extraction process

Sample (spring:fall)	Content (mg/g DW)		
	1	**2**	**3**
3:2	5.08 ± 0.06	0.69 ± 0.01	7.82 ± 0.07
1:4	5.06 ± 0.03	0.55 ± 0.00	6.72 ± 0.06
1:1	5.28 ± 0.04	0.65 ± 0.00	7.45 ± 0.04
2:3	5.04 ± 0.03	0.59 ± 0.00	6.95 ± 0.06
4:1	4.66 ± 0.01	0.92 ± 0.00	8.43 ± 0.07

Table 3 Effect of EtOH concentration on the extraction process

Sample	Content (mg/g DW)		
	1	**2**	**3**
Water	0.19 ± 0.00	0.02 ± 0.00	0.64 ± 0.00
30% EtOH	1.34 ± 0.01	0.11 ± 0.00	2.86 ± 0.01
50% EtOH	2.75 ± 0.02	0.29 ± 0.00	8.30 ± 0.06
70% EtOH	3.25 ± 0.02	0.36 ± 0.00	8.74 ± 0.08
EtOH	5.08 ± 0.06	0.69 ± 0.01	7.82 ± 0.07

Table 4 Effect of extraction time on the extraction process

Sample	Content (mg/g DW)		
	1	**2**	**3**
1 h	1.64 ± 0.00	0.15 ± 0.00	2.69 ± 0.00
2 h	1.28 ± 0.01	0.15 ± 0.00	3.73 ± 0.02
3 h	1.98 ± 0.02	0.22 ± 0.00	5.15 ± 0.04
4 h	3.51 ± 0.01	0.27 ± 0.01	5.22 ± 0.07

is safe for the use of humans [20]; for example, the EtOH extracts of *C. japonicum* have been reported to have hepatoprotective, antioxidant, anti-inflammatory, and anxiolytic effects [21–23]. Thus, ethanol was used in this study.

The content of compounds **1**–**3** was increased with an increase in extraction time; the maximum content was determined after 4-h extraction period. An increase in the solid-to-solvent ratio increased the flavonoid content (Table 5). The effect of the number of extractions showed similar pattern as shown in Table 6. However, the recovery of compound **1** was higher when the extraction was repeated twice.

Table 5 Effect of solid-to-solvent ratio on the extraction process

Sample (sample/solvent)	Content (mg/g DW)		
	1	**2**	**3**
1:10	1.31 ± 0.01	0.14 ± 0.00	2.80 ± 0.01
1:20	1.38 ± 0.01	0.16 ± 0.00	4.04 ± 0.02

Table 6 Effect of the number of extraction on the extraction process

Sample	Content (mg/g DW)		
	1	**2**	**3**
1×	1.15 ± 0.01	0.11 ± 0.00	2.41 ± 0.00
2×	1.88 ± 0.01	0.15 ± 0.00	2.51 ± 0.01

An increasing demand for nutraceuticals leads to a greater need on search for new sources of bioactive compounds. Thus, there is an increasing interest on the investigation of plants with potential pharmacological importance that could be used in the food and cosmetic industry, with the goal to discover new drugs to prevent and treat various illnesses. ICF-1 has been regarded as a traditional medicine, and previous studies have supported these claims. Therefore, an additional experiment was done to replicate a production process that involves larger amounts of samples (Table 7). The extraction of bioactive compounds from plant materials depended on various factors including solvent, extraction method, extraction time, and the nature and pharmacological potential of compounds to be extracted [24]. These factors should be simple, fast, economical, uses less hazardous solvents and materials, and suitable for production in large industry which aims to recover possible high content of compounds while considering their potential application as a nutraceutical. Results show that by using CJL-1 and -2 in a ratio of 3:2 (100 g) extracted in 30% EtOH for 3 h, the contents of powdered extracts for compounds **1–3** were 35.35, 1.21, and 59.77 mg/g, respectively. According to the content analysis of the various parts of ICF-1, both CJL-1 and -2 showed higher contents of compounds **1–3** and accordingly they were mixed with higher ratio from CJL-1 which showed the highest content (Table 7). In industry production, the usage of leaves collected both seasons could be of economically advantage and could promote efficiency for the consumption of both leaves. The use of 30% EtOH as the extraction solvent makes it more economical and uses lesser alcohol which promotes better safety to the workers for the industry. For the choice of extraction solvent, a recent study showed that 30% extract has biological activity than other concentrations of EtOH [25]. The mixture of CJL-1 and -2 and 30% EtOH for extraction solvent chosen for them showed good antioxidant activity on streptozotocin-induced apoptosis in pancreatic beta cells [25]. The experiment time used was 3 h instead of 4 h as determined in Table 4 due to time efficiency reasons and the content of compound **3**. As shown in Table 4, the content of compound **3** is almost the same in 4 h. In this reason, 3 h was chosen.

In the present study, the key variable for sample preparation of CJL was studied. An efficient, reliable, and economically suitable variable was suggested to optimize the yield of flavonoids, as determined via HPLC–UV analysis. This is the first report on the optimization of the extraction process of compounds **1–3**. The optimal

Table 7 Production process of CJL from ICF-1

(1) Production process	(2) Process, food and food additive	(3) Content (mg/g)			(4) Yield (g)
		1	**2**	**3**	
Raw materials	CJL (spring:fall = 3:2)	7.85 ± 0.03	0.27 ± 0.01	13.27 ± 0.03	100
↓					
Extraction	30% EtOH (2000 mL)/20 times/ 78.3 °C (3 h)	0.454 ± 0.000	0.016 ± 0.000	0.767 ± 0.002	1730
↓					
Filtration	Filter paper				
↓					
Concentration	78.3 °C/3 h/11.53% solid content	3.49 ± 0.03	0.119 ± 0.001	5.90 ± 0.01	225
↓					
Drying	Lyophilization				
↓					
Sample	Powder	35.35 ± 0.01	1.21 ± 0.01	59.77 ± 0.15	22.2

conditions for the extraction of CJL were found to be: ratio of spring and fall leaves, 4:1; solvent, 70% EtOH; extraction time, 4 h; solid-to-solvent ratio, 1:20; and one time extraction.

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