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METABOLITE PROFILE AND ANTIOXIDANT CAPACITY OF *Centella asiatica* LEAVES EXTRACT WITH DIFFERENT EXTRACTION METHODS

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Abstract

Centella asiatica known as pegagan in Indonesia, belongs to the Apiaceae family and is widely used as salad, cosmetics ingredients, and herbal medicine for improving memory. This research aimed to determine the total phenolics content (TPC), antioxidant capacity (AC), and FTIR spectrum of *C. asiatica* leaves extracts using different extraction methods. *C. asiatica* extracts were prepared using maceration, reflux, and ultrasonication. The Folin-Ciocalteu method was used to determine TPC, and we found a higher level of TPC when using maceration, about 9,96 ± 0,20 mg GAE g^{-1} dry powder. Antioxidant capacity from the three extracts was measured using DPPH and FRAP methods. The highest antioxidant capacity using DPPH and FRAP methods was found in C. asiatica leaves extract using maceration with a value of 29,60 ± 0,71 and 14,04 ± 0,38 μmol TE g^{-1} dry powder, respectively. The FTIR spectrum of each extract indicates the presence of vibration from several functional groups such as O-H, C-H, and C=C, likely from phenolics and C-O. The pattern of FTIR spectrum from the three *C. asiatica* leaves extracts gives different spectrum profiles and characteristics. Principal component analysis using FTIR spectrum data shows good clustering for each extract with 89% data variability (PC1 77% and PC2 12%). It can be concluded that TPC, AC, and FTIR spectrum profiles are different when using different extraction methods.

INTRODUCTION

Centella asiatica, known locally as *pegagan* and belonging to the Apiaceae family, can grow and is widely found in tropical and subtropical regions. Several phytochemical compounds have been identified in *C. asiatica*, which belongs to the triterpene [1] alkaloids [2], and phenolics group, including flavonoids, are known to have certain biological activities in this plant [3,4]. *C. asiatica* also has essential oil compounds such as farnesol and caryophyllene [5]. The known biological activity of *C. asiatica* is wound healing [6], cardiovascular [7], memory improvement [8], anti-inflammatory, anticancer, antioxidant, and anxiolytic [9]. The presence of phenolic compounds, including flavonoids and phenolic acids, plays an essential role in the antioxidant activity of *C. asiatica* extract [10].

Phenolic compounds are known to have antioxidant activity because their redox potential could act as metal chelators, reducing agents, singlet oxygen quenchers, and hydrogen donors [11]. The composition and concentration of metabolites depend on several factors, one of which is the use of the extraction method. Various extraction methods can be used to extract phytochemical compounds, including cold extraction, such as maceration, with the addition of heat, such as reflux, and with the help of ultrasonic waves, known as the ultrasonication method. Differences in extraction methods will determine the results of differences in the composition and levels of a compound and its biological activity because the level of antioxidant activity in *C. asiatica* depends on the composition and amount of antioxidant compounds [9].

Metabolomics analysis could determine differences in the overall composition and concentration of metabolites in a sample [12]. So far, no reported paper has evaluated changes in the composition and concentration of metabolites in

C. asiatica based on different extraction methods. The extraction method was chosen based on its wide use, such as maceration, reflux, and ultrasonication, which have their respective advantages. Metabolite profile evaluation was done by determining total phenolics content, FTIR spectrum fingerprint analysis, and antioxidant capacity using the DPPH and FRAP methods. The grouping of extracts obtained by different extraction methods was performed using multivariate analysis, namely principal component analysis (PCA). Therefore, this study aims to evaluate the effect of different extraction methods on *C. asiatica* on total phenolic content and antioxidant capacity.

EXPERIMENT

This research was conducted at the Tropical Biopharmaca Research Center (TropBRC), International Institute of Food, Nutrition, and Health, IPB University. This study compels references to the methods used in the study and shows information about whether the method has been modified.

Material

In this study, we used four months post-planting of *C. asiatica* leaves and obtained the sample from TropBRC with the voucher specimen number BMK0506072022. Ethanol absolute, Folin-Ciocalteu reagent, $FeCl_3$ $.6H_2O$, $NaCH_3COO$, Na_2CO_3, and concentrated HCl were obtained from Merck (Darmstadt, Germany). 2,2-dipenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), and gallic acid were purchased from Sigma-Aldrich (St Louis-USA).

Instrumentation

The instruments used in this work are an FTIR spectrophotometer Tensor 37 (Bruker Optik GmBH, Karlsruhe, Germany), an ultrasonicator (Drawell, Chongqing, China), an Epoch microplate reader (BioTek, Winooski, USA), and a rotary evaporator (Heidolph GmbH & Co. KG, Schwabach, Germany).

Procedure

Preparation and Extraction

Firstly, *C. asiatica* leaves were cleaned and dried in the oven at 45 ℃ for five days. After that, we pulverized it until 80 mesh. Extraction was performed using maceration, reflux, and ultrasonication using 70 % ethanol as extraction solvent with six replications. Maceration was done by adding 100 mL of 70 % ethanol to 20 g of *C. asiatica* leaves powder at room temperature for 3 × 24 hours and filtered every 24 hours. After that, we added another 100 mL of 70 % ethanol for the second and third. For reflux extraction, *C. asiatica* leaves powder was weighed 20 g, and then added 100 mL of 70 % ethanol at 65 ℃ for 90 minutes. After filtering, the residue was added again with 100 mL of 70 % ethanol and reflux returned for the second and third time with a 60-minute extraction. Ultrasonication extraction was conducted by mixing the *C. asiatica* leaves powder (20 g) with 100 mL of 70 % ethanol. The mixture was extracted at 25 KHz, 95 % power for 90 minutes at 25 ℃. We replaced the water in ultrasonic tanks every 15 minutes to maintain the temperature in the bath ultrasonicator. After ultrasonication, filtrate was obtained by filtering, and the residue was reextracted two times using the same solvent but with a time extraction of 60 minutes for each test. The filtrate obtained from each method was different extractions, then evaporated with a rotary evaporator until the extract was thick. For the yield calculation, crude extracts from the three extraction methods were dried using freeze-dried until dry for 3 × 24 hours. The dried extract will then be used to determine the total phenolic content and measurements of the FTIR spectrum.

Measurement of FTIR Spectrum

Two mg of *C. asiatica* extract was weighed and mixed with 200 mg of potassium bromide (KBr) until homogeneous. Pellets are made using a hand press. FTIR spectrum was recorded using an FTIR spectrophotometer Tensor 37 with a deuterated triglycine sulfate detector in the mid-infrared region (400−4000 cm^{-1}) with a resolution of 4 cm^{-1} and a scanning speed of 32 operated with OPUS software version 4.2 (Bruker Optik GmbH, Karlsruhe, Germany). The FTIR spectrum data is then stored in data point table format. The original FTIR spectrum has been preprocessed with standard normal variate (SNV), baseline correction, Savitzky Golay smoothing, and Savitzky Golay derivative using Unscrambler X software version 10.4 followed by grouping the extracts based on the extraction method using PCA.

Determination of Antioxidant Capacity by DPPH and FRAP Method

Determination of antioxidant capacity using the DPPH method refers to the procedure used by Salazar-Arandra et al [13]. *C. asiatica* leaf extract was made with a concentration of 125 mg L^{-1}. 40 µL of the sample solution was pipetted into a 96-well plate, 120 µL of 125 µM DPPH solution was added, and incubated in the absence of light and at room temperature for 30 minutes. Absorbance was measured using a microplate reader at a wavelength of 515 nm. Determination of antioxidant capacity was calculated based on linear regression of trolox and measurements were carried out three times at trolox concentrations of 2, 4, 8, 16, 31, 63, and 125 mg L^{-1}. Antioxidant capacity was expressed as trolox equivalent (TE) in µmol trolox g^{-1} dry powder.

The determination of antioxidant capacity using the FRAP method was carried out using the procedure described by Yahia et al [14]. The test was performed by placing 20 µL of sample and 280 µL of FRAP reagent into the well, and the plate was incubated for 30 minutes. Absorbance was measured at 630 nm. A calibration curve was prepared using Trolox as a standard and measurements were carried out in three repetitions. The results were expressed as TE in µmol g-1 dry powder. FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM 2,4,6-tripyridyl-2-triazine (TPTZ) in 40 mM HCl, and 2.5 mL of $FeCl_3$ 20 mM.

Determination of Total Phenolic Content

Determination of total phenolic content refers to the procedure used by Rafi et al [15]. *C. asiatica* extract was made with a concentration of 1000 mg L^{-1}. The extract solution was pipetted as much as 10 µL into the well. Then 160 µL of distilled water and 10 µL of 10% Folin–Ciocalteu reagent was added and incubated for 5 minutes. After that, 20 µL of 7.5 % Na_2CO_3 was added. The mixture was then homogenized and incubated for 30 minutes at room temperature. The absorbance of the mixture was measured at 750 nm. The calibration curve was made by diluting gallic acid in ethanol with eight different concentrations, namely 8, 16, 31, 63, 125, 250, 500, and 1000 (mg L^{-1}). The total phenolic content of each extract was expressed as milligram gallic acid equivalent per gram dry weight of the sample (mg GAE g^{-1} dry powder).

Data Analysis

Data analysis using analysis of variance (ANOVA); if there is a significant effect, further testing will be carried out using Duncan's multiple range test (DMRT) at a significance level of α = 5%. FTIR spectrum absorbance data is displayed using Origin 2019 and grouping of *C. asiatica* leaves extract based on the extraction method by PCA using absorbance data from 1654−790 cm^{-1} as the variable.

RESULT AND DISCUSSION

Yield Percentage of C. asiatica Leaves extract

C. asiatica leaves were extracted using three different methods by evaluating the yield, metabolite profile, and antioxidant activity. The average yield of *C. asiatica* leaves extract ranged from 20.78 % to 26,39 % (**Table 1**). The highest extract yield was obtained from the reflux method, followed by ultrasonication, and the lowest was maceration. Differences in extract yield values can be caused by several factors, one of which is extraction temperature. Temperature greatly influences the properties of the solvent. In the extraction process, temperature contributes to extraction efficiency. Usually, increasing temperature causes an increase in extraction [16]. The results of this study follow previously reported research that *C. asiatica* produces high yields when using a heating process during extraction [17]. The extraction method, adding heat, will produce a higher yield than the cold extraction method. The higher the extraction temperature, the looser the distance between solid molecules will be, and the attractive force of water molecules will easily separate the bonds between solid substances. This will result in faster movement of molecules and solvent so that the rate of transfer of compounds from plant cells into the solvent will increase. The results of the analysis of variance showed that the maceration method gave a significantly different yield from the reflux and ultrasonication methods ($p<0.05$).

Total Phenolic Content and Antioxidant Capacity of C. asiatica Leaves Extracts

Total phenolic content was determined according to the Folin–Ciocalteu method and found that the content was higher by extraction using maceration > reflux > ultrasonication (**Table 1**). Differences in total amount of phenolics can be

caused by factors such as climate and region variations and extraction procedures [18]. The total phenolic content of *C. asiatica* leaves extract was significantly different using the three extraction methods in this study ($p<0.05$) and ranged from 6.54 ± 0.08 to 9.96 ± 0.20 mg GAE g-1 dry powder. The highest total phenolic content was obtained by the maceration extraction method. Phenolic compounds are secondary metabolites that are thermolabile to excessive heating exposure, so temperature control is necessary. The total phenolic content of *C. asiatica* extract was higher than previously reported [19].

The antioxidant capacity of *C. asiatica* leaves extract was determined using the DPPH and FRAP methods. Determination of antioxidant capacity using the DPPH method shows the antiradical capacity of the extract, while the FRAP method measures the ability of the extract to reduce iron ions. The antioxidant capacity of *C. asiatica* leaves using the two methods is shown in **Table 1**. The DPPH and FRAP antiradical activities of *C. asiatica* extract showed significant differences in values from the three extraction methods used ($p < 0.05$). The total antioxidant capacity of DPPH ranged from 18.42 ± 0.50 to 29.60 ± 0.71 μmol TE g^{-1} dry powder, while FRAP was 4.17 ± 0.23 to 14.04 ± 0.38 μmol TE g^{-1} dry powder. The extract obtained from the maceration method provided the highest antioxidant capacity compared to the reflux and ultrasonication methods for the DPPH and FRAP methods. These results indicate the influence of the extraction method for extracting more compounds and their concentration level, causing differences in the level of biological activity of *C. asiatica*.

The antioxidant capacity value obtained by the DPPH method was higher than FRAP. Similar to previous findings, *C. asiatica* extract was reported to have the highest antioxidant capacity value using the maceration method of 57.5 μg mL^{-1} for DPPH and 46 μg mL^{-1} for FRAP at the same sample concentration [20]. This is thought to be caused by differences in testing mechanisms for each capacity determination, as DPPH can reduce compounds with H radical transfer activity and other radicals through electron transfer. The greater the reducing ability, the higher the concentration and capacity value of DPPH.

Antioxidant capacity values align with the higher total phenolic content from all extraction methods. Using Pearson correlation, a positive and significant correlation ($\alpha < 0.05$) was obtained between total phenolic content and antioxidant capacity using the DPPH ($r = 0.95$) and FRAP ($r = 0.98$) methods with $p < 0.01$. The correlation coefficient value shows the strength of the relationship between two variables. The correlation coefficient value is close to 1. Then, the two variables have a very strong relationship [21]. The significant correlation between total phenolic content and DPPH and FRAP provides strong evidence that the primary source of DPPH and FRAP activity is phenolic compounds in *C. asiatica*. In addition, this indicates a very strong relationship between the inhibitory power of free radicals and the reduction potential of polyhydroxy compounds (polyphenols) against iron radicals and ions. In general, these two methods can replace each other.

Table 1. Yield, total phenolic content, and antioxidant capacity of *C. asiatica* leaves extracts.

Extraction Method	Yield (%) ± SD	Antioxidant Capacity of FRAP (μmol TE g^{-1} dry powder) ± SD	Antioxidant Capacity of DPPH (μmol T.E. g^{-1} dry powder) ± SD	Phenolics (mg GAE g^{-1} powder dry) ± SD
Maceration	20.78 ± 3.28 [b]	14.04 ± 0.38 [a]	29.60 ± 0.71 [a]	9.96 ± 0.20 [a]
Reflux	26.39 ± 1.73 [a]	7.22 ± 0.2 4 [b]	26.48 ± 0.75 [b]	8.28 ± 0.11 [b]
Ultrasonication	25.45 ± 2.63 [a]	4.17 ± 0.2 3 [c]	18.42 ± 0.50 [c]	6.54 ± 0.08 [c]

Note: Different letters behind the numbers indicate a real effect using the DMRT test with ($p < 0.05$). Data are the mean of three replicates ± SD (standard deviation). TE: Trolox equivalent, GAE: gallic acid equivalent

Clustering of C. asiatica Leaves Extract

The FTIR spectrum of *C. asiatica* leaves extracted from the three extraction methods showed similar spectral patterns in the wave number range 3439 - 1053 cm^{-1} (**Figure 1**). The similarity of these spectral patterns shows that the composition of the compounds indicated by the identified functional groups is almost similar. Meanwhile, the difference in concentration is related to the peak intensity in the spectrum of the three extraction methods.

The peak that broadens at the wave number around 3400 cm^{-1} is the absorption band of the hydroxyl group (O-H) (**Figure 1**). This band was identified in all *C. asiatica* extracts. Absorption at wave numbers 2962 - 2853 cm^{-1} indicates the presence of the C-H functional group. The next band identified at wave numbers 1650 - 1590 cm^{-1} is the absorption of aromatic C=C. Then the band at wave numbers 1410 - 1310 cm^{-1} is phenol or tertiary alcohol, OH bend, and the band at wave number 1050 cm^{-1} is a peak with strong intensity, primary alcohol C-O stretch [22,23]. Based on research conducted [18], it was reported that plant extracts have high antioxidant properties due to the appearance of hydroxyl groups from phenolic compounds.

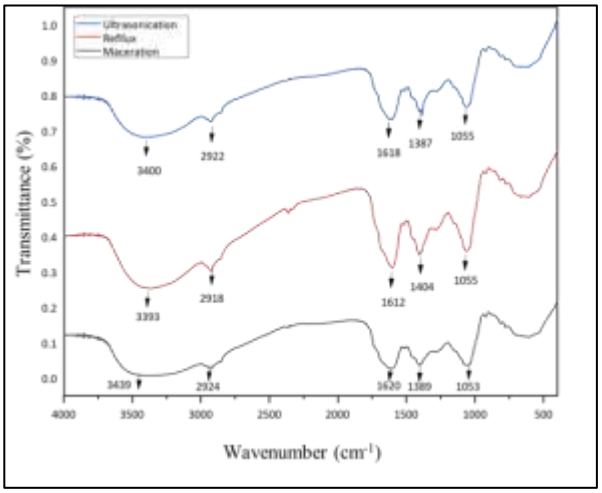

Figure 1. Representative FTIR spectrum of C.asiatica leaves extract based on different extraction methods.

C. asiatica metabolite profiles from each extraction method are grouped using PCA. PCA will simplify the variables resulting from FTIR spectrum analysis into several variables called principal components (PC), which can still represent the structure and variance in the data. Before PCA is carried out, preprocessing aims to eliminate noise, increase the resolution of overlapping spectra, or improve data information. This process is carried out to get good results because data quality greatly influences the results of extract grouping. The preprocessing carried out is SNV to correct the scaling of all variables or normalize the data into an orderly scale so that it can be compared more accurately. Before PCA is carried out, preprocessing aims to eliminate noise, increase the resolution of overlapping spectra, or improve data information. This process is carried out to get good results because data quality greatly influences the results of extract grouping. The preprocessing carried out is SNV to correct the scaling of all variables or normalize the data into an orderly scale so that it can be compared more accurately. A baseline is used to avoid shifts in the spectrum baseline that deviate from the ideal value, which can be caused by temperature and equipment incompatibility [24]. Then, Savitzky Golay smoothing was used to smooth the spectrum obtained, and the Savitzky Golay derivative was used to increase slightly spectral differences.

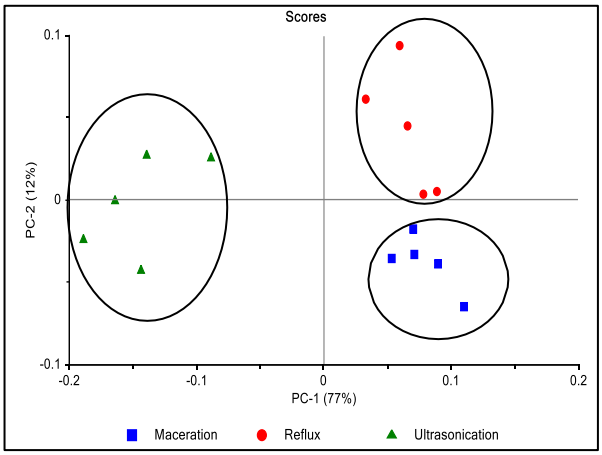

Figure 2. PCA score plot after pre-processing using absorbance in the fingerprint region 1654-790 cm^{-1} from the FTIR spectrum of *C. asiatica* leaves extract with different extraction methods.

PCA using FTIR spectrum data with 449 variables in the fingerprint area 1654−790 cm^{-1} displayed in a two-dimensional plot using two PCs (**Figure 2**), had a variance of 89% (PC1 77% and PC2 12%). Based on the PCA grouping results, *C. asiatica* extracts from different extraction methods were grouped into their respective groups. However, the low PC scale value shows similarities in the composition and concentration of the extracted *C. asiatica* metabolites. The different characters and amounts of components can be seen from the differences in the grouping of *C. asiatica* extracts between extraction methods. The distance between samples shows the similarity between samples [25].

CONCLUSION

The extraction method influences the antioxidant and total phenolic potential of *C. asiatica*, where different extraction methods produce different extraction results. Extracts using the maceration method had the highest values for total phenolics and antioxidant capacity in the DPPH and FRAP methods and the lowest in the

ultrasonication method. Our results show that maceration is an effective and simple method to extract phenolic compounds from *C. asiatica* leaves. The group of phenolic compounds based on Pearson correlation analysis significantly contributes to antioxidant levels because the antioxidant capacity obtained is directly proportional to the value of total phenolic content.

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