Jurnal Ilmiah Farmasi (Scientific Journal of Pharmacy) 18(1) Januari-Juli 2022, 55-63
ISSN: 1693-8666
available at http://journal.uii.ac.id/index.php/JIF

Combination of ethanolic extract on total flavonoid *Centella asiatica L.* leaves and *Imperata cylindrica L.* roots with UV-Vis spectrophotometric method

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Abstract

Background: Flavonoids are widely employed as phytomedicines and as secondary metabolites generated by plants, where they serve key roles in plant physiology. Antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal, and antiviral activities are only a few of the potential biological effects of flavonoids. The discovery of medicinal plants containing flavonoid chemicals is potential as supportive and preventative treatment, notably for COVID-19 which has caused a pandemic in several countries, including Indonesia.
Objective: This study aimed to determine the total flavonoid content in the ethanolic extracts of *C. asiatica* leaves and *Imperata cylindrica* roots to find the potential of flavonoid-rich plants as an alternative source of COVID-19 treatment.
Methods: The ethanolic extracts from the combination of *C. asiatica* leaves and *I. cylindrica* roots (with five combination ratios) were tested for total flavonoid content using the UV-Vis Spectrophotometry method. The total flavonoid content of the extract combination was analyzed using a one-way ANOVA test.
Results: The total flavonoid contents of the combination of *C. asiatica* leaf and *I. cylindrica* root extracts at a ratio of 1:1, 2:3, 3:2, 4:1, and 1:4 were 45.88 ± 0.08, 42.14 ± 0.08, 40.52 ± 0.08, 66.28 ± 0.08, and 40.88 ± 0.13 mg/g EQ, respectively. The homogeneity with Levene's test obtained a *p*-value of 0.303. The one-way ANOVA exhibited the p-value of *F*-test statistics < 0.001.
Conclusion: The total flavonoid contents of ethanolic extracts from *C. asiatica* leaves and *I. cylindrica* roots are at a ratio of 4:1 > 1:1 > 2:3 > 1:4 > 3:2. The high total flavonoid content plays a role in increasing anti-inflammatory and immunomodulatory activities in COVID-19 patients.
Keywords: Total flavonoid content, *C. asiatica*, *I. cylindrica*, UV-Vis Spectrophotometry, COVID-19

1. Introduction

Coronavirus disease 2019 (COVID-19) was first confirmed in Wuhan, People's Republic of China, and has caused a pandemic in various countries globally, including in Indonesia (Setiati & Azwar, 2020; Liskova *et al.*, 2021; Nguyen *et al.*, 2012; Saakre *et al.*, 2021; Solnier & Fladerer, 2021). Based on the data from WHO on July 28, 2021, the Government of Indonesia reported as many as 3,287,727 (47,791 new) confirmed cases of COVID-19 with details of 88,659 (1,824 new) deaths and 2,640,676 recovered cases originating from 34 provinces with 510 districts (WHO, 2021). Several long-standing drugs are generally used as antivirals such as favipiravir and ribavirin, anti-HIV protease inhibitors such as ritonavir and lopinavir, and anti-inflammatory agents such as tocilizumab or dexamethasone are used to treat COVID-19 (Mandal *et al.*, 2021). Besides the therapeutic options of antiviral drugs, natural plant products play a role in supportive and prophylactic care may be an option (Mandal *et al.*, 2021; Solnier & Fladerer, 2021).

Indonesia is one of the countries with tropical forests that are rich in various medicinal plants (Al Manar, 2018). *Centella asiatica* and *Imperata cylindrica* are plants that are commonly found in Indonesia. The leaves of *C. asiatica* are usually used as green vegetables and salads, while the roots of *I. cylindrica* are used as herbal drinks. One of the secondary metabolites content of *C. asiatica* and *I. cylindrica* is flavonoid compounds (Bhattacharya *et al.*, 2017; Suhendra *et al.*, 2019).

Flavonoids are responsible for plant's color, taste, and pharmacological activity as a secondary metabolite found naturally in plants (Liskova *et al.*, 2021; Sapiun *et al.*, 2020; Solnier & Fladerer, 2021). The structure of flavonoids consists of a diphenyl propane ring with two benzene cores joined by an oxygen-containing ring. Furthermore, they are bound in a pyran ring (Amić *et al.*, 2007; Sapiun *et al.*, 2020). The flavonoids in the body are antioxidant, anti-inflammatory, antiallergic, antihypertensive, anticancer, anti-asthma, anti-bronchitis, antihemorrhagic, antimutagenic, antineoplastic, hepatoprotective, and antiviral. The activity of flavonoids as an antiviral, especially against SARS and MERS coronaviruses is through the mechanism of inhibition of protease enzymes such as 3-chymotrypsin-like protease (3CLpro), papain-like protease (PLpro), and helicase by suppressing the activity of an angiotensin-converting enzyme (ACE), increasing the body immunity against viral infection, and suppressing the inflammatory process associated with COVID-19 infection (Liskova *et al.*, 2021; Solnier & Fladerer, 2021). Several types of flavonoids that play a role in COVID-19 include apigenin, luteolin, quercetin, amentoflavone quercetin, daidzein, puerarin, epigallocatechin, epigallocatechin gallate, gallocatechin gallate herbactin, rifolin, and pectrininin (Jo *et al.*, 2020; Mrityunjaya *et al.*, 2020; Saakre *et al.*, 2021). Castilliferol and castillicetin are two types of flavonoids extracted from the complete *C. asiatica* plant. Using 2,2-diphenyl-1-picryl hydrazyl radical solution, both demonstrated good antioxidant activity, with IC_{50} values of 23.10 and 13.30 µg/mL, respectively (Subban *et al.*, 2008). In addition, the ethyl acetate fraction extracted from *I. cylindrica* contains a high concentration of flavonol compounds (a type of flavonoid) (Khaerunnisa *et al.*, 2020).

The determination of flavonoid content in the combination of extracts of *C. asiatica* leaves and *I. cylindrica* roots were carried out by absorption of the sample in the ultraviolet-visible (UV-Vis) region because this technique is widely used with several advantages such as simplicity, low operating costs, and reliable results. Aluminum chloride ($AlCl_3$) was added to the sample, and its absorption was measured using a UV-Vis spectrophotometer to obtain the flavonoid spectrum (Fernandes *et al.*, 2012). The quantification of flavonoids after complexation with $AlCl_3$ showed satisfactory performance according to several studies. Several previous research had determined the flavonoid content of several medicinal plant species such as *Eugenia uniflora, Fibraurea*

choroleuca, Sargassum polycystum, Melicopelunu ankeda, Polygonum minus, Murraya koenigii, Eugenia polyantha, Amomum compactum, and *Sedum sarmentosum* using a UV-Vis spectrophotometric method (Chang & Othman, 2014; Chen *et al.*, 2010; Nurcholis *et al.*, 2021). The procedure allowed estimation of the total flavonoid content with a specificity of the majority of aglycones (free flavonoids and O-glycosylation), increases the analysis representativeness, and minimizes deviation possibility. In addition, the complex formed between flavonoid-Al exerts a bathochromic effect which plays a key role in the method (da Silva *et al.*, 2015). This research was conducted to determine the total flavonoid content of the combination of the ethanolic extracts of *C. asiatica* leaves and *I. cylindrica* roots.

2. Methods

2.1 Chemical reagents

The materials and reagents used included ethanol, aluminum chloride, sodium acetate, and standard quercetin. All chemicals were provided by Sigma Aldrich Corp. (USA). The *C. asiatica* leaves and *I. cylindrica* roots were collected from Balai Materia Medika in Batu, Malang, Indonesia.

2.2 Sample extraction

The dry powder of *C. asiatica* leaves and *I. cylindrica* roots were weighed as much as 50 g respectively and each put into a maceration vessel. A total of 250 mL of ethanol was added to each vessel, macerated overnight in a closed container, and protected from light. On the second day, the simplicia was filtered, and the pulp was added with 150 mL of ethanol and macerated overnight. The treatment on the third day was the same as the second day by adding 100 mL of ethanol. The extraction results were collected and evaporated using a rotary evaporator to obtain a viscous extract.

2.3 Determination of the maximum wavelength of quercetin

A standard solution of 20 ppm quercetin was pipetted 1.0 mL and put into a test tube. Then, 1.0 mL of 2% $AlCl_3$, 0.1 M CH_3COONa, and 2.0 mL of distilled water were added into the test tube. The solution was vortexed for 1 minute and incubated for 30 minutes. Absorption was measured in the wavelength range of 200–500 nm using a UV-Vis spectrophotometer. The blanks used were a mixture of 1.0 mL of ethanol, 1.0 mL of 2% $AlCl_3$, 1.0 mL of 0.1 M CH_3COONa, and 2.0 mL of distilled water.

2.4 Calibration curve preparation

The quercetin standard was weighed at 10 mg. The powder was put into a 100.0 mL volumetric flask, and ethanol was added. The solution was sonicated for 2 minutes until dissolved,

and ethanol was added to mark the limit. The quercetin series solution was made in several concentrations, namely 10, 20, 30, 40, 50 ppm. Each concentration was pipetted at 1.0 mL, and 1.0 mL of 2% $AlCl_3$, 0.1 M CH_3COONa, and 2.0 mL of distilled water were added in a test tube. The solution was vortexed for 1 minute and incubated for 30 minutes. Then, the absorption was measured through data maximum wavelength range of 428.2 nm using a UV-Vis spectrophotometer. The blanks used were a mixture of 1.0 mL of ethanol, 1.0 mL of 2% $AlCl_3$, 1.0 mL of 0.1 M CH_3COONa, and 2.0 mL of distilled water.

2.5 Determination of the total flavonoid content of the combination extract

Samples of the combination ethanol extract of *C. asiatica* leaves, and *I. cylindrica* roots were made in various ratios, including 1:1, 2:3, 3:2, 4:1, and 1:4. Each extract mixture with various ratios was made with a concentration of 0.05% b/v in ethanol solvent. Afterward, 1.0 mL of each solution was pipetted, and 1.0 mL of 2% $AlCl_3$, 0.1 M CH_3COONa, and 2.0 mL of distilled water were added in the test tube. The solution was vortexed for 1 minute and incubated for 30 minutes. The absorption was measured at a maximum wavelength range of 428.2 nm using a UV-Vis spectrophotometer. The blanks used were a mixture of 1.0 mL of ethanol, 1.0 mL of 2% $AlCl_3$, 1.0 mL of 0.1 M CH_3COONa, and 2.0 mL of distilled water. The reading was repeated three times. The determination of total flavonoid content (TFC) was carried out based on the following formula:

$$\text{Flavonoid} = \frac{\mathbf{Y \times N \times V}}{\mathbf{W}}$$

Description: Y = flavonoid concentration from the standard curve equation (mg/g); N = value of dilution; V = volume of extraction (mL); W = weight of mixed powder (g)

3. Results and Discussion

Using the same solvent, namely ethanol, the percentage of *C. asiatica* leaves maceration was 21.4%, and *I. cylindrica* roots was 4.0%. Maceration was chosen because the process is simple and can be used for compounds that are unstable by heating. Plant cells undergo cell wall degradation by ethanol solvents which causes a release of flavonoid compounds. As a result, polar flavonoid compounds will be higher in the extraction (Suhendra *et al.*, 2019).

Determination of the maximum wavelength due to the reaction between a standard solution of quercetin and aluminum chloride obtained a value of 428.2 nm. The maximum wavelength was chosen because it provided the maximum absorption and was used to identify compounds qualitatively. Then, the maximum wavelength testing used quercetin compound. It was because quercetin is a flavonoid type that was discovered in 1930, had a ketone group located at carbon

number 4 (C-4), and had a hydroxyl group on carbon atom number 3 (C-3) or number 5 (C-5) (Mustapa *et al.*, 2019; Shah *et al.*, 2016).

A standard series solution of quercetin with concentrations of 0–50 ppm was measured at the maximum wavelength. The relationship between levels and absorbance was made in the linear regression line equation curve and resulted in the equation y = 0.015X + 0.001 with an R-squared value of 0.998 and a p-value < 0.001. The calibration curve is determined to obtain a relationship between the levels and the absorbance of the quercetin standard solution (Harron, 2013). The relationship was determined from the correlation coefficient parameter, which is expressed by the value of R-squared (da Silva *et al.*, 2015; Puspitasari & Wulandari, 2017). The R-squared value of the quercetin standard calibration curve was 0.998, which meant that the experimental data accuracy value of the calibration curve was 99.8%. R-squared values range from 0.95 to 1 indicating that the method is suitable, so it can be used to determine the total flavonoid content in the extract of *C. asiatica* leaves and *I. cylindrica* roots (Sapiun *et al.*, 2020).

The combination of the ethanolic extracts of *C. asiatica* leaves and *I. cylindrica* roots in various ratios were measured at a maximum wavelength of 428.2 nm. The total flavonoid content of the combination of *C. asiatica* leaf and *I. cylindrica* root extract at a ratio of 1:1, 2:3, 3:2, 4:1, and 1:4 was 45.88±0.08 mg/g EQ, 42.14±0.08 mg/g EQ, 40.52±0.08 mg/g EQ, 66.28±0.08 mg/g EQ, and 40.88±0.13 mg/g EQ, respectively. All test data for flavonoid totals can be seen in Figure 1.

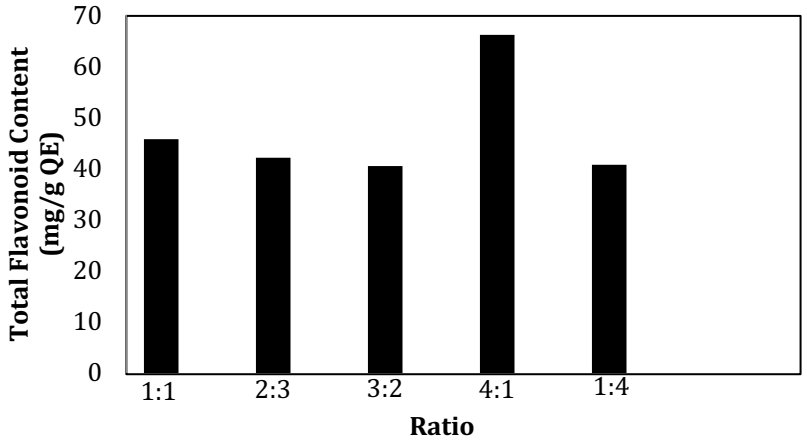

Figure 1. Total flavonoid content from combination *C. asiatica* leaves and *I. cylindrica* roots extract

The total flavonoid content in the extract combination was analyzed by one-way ANOVA statistical test. The homogeneity test with Levene's test obtained a p-value of 0.303. The results of one-way ANOVA test showed that the p-value of F-test statistics was 0.000. A one-way ANOVA test

is used to determine the variation between the five total flavonoid content of the extract combination. Based on the statistical analysis of homogeneity with Levene's test, a p-value of 0.303 > 0.05 was obtained, which means that the five-extract combinations have homogeneous variations in total flavonoid content data. Furthermore, from the results of one-way ANOVA test, it was known that the p-value of F-test statistics was 0.000 < 0.05, which indicates that the five total flavonoid contents of the combination have a significant difference. The total flavonoid content of the extract of *C. asiatica* leaves and *I. cylindrica* roots had a ratio of 4:1 > 1:1 > 2:3 > 1:4 > 3:2. Because the statistical value of the F test was significant at 5% alpha, further comparison tests were carried out between two ratios with a post-hoc multiple comparison test using the LSD method. Based on the results of statistical tests, it was known that the difference in the total flavonoid ratio between 4:1 and 1:1 was 20.16 mg/g EQ in 95% range, between 20.16–20.64 mg/g EQ, and significant at 5% alpha (p-value < 0.001). At a ratio of 4:1 and 2:3, the difference was 14.15 mg/g EQ in 95% range, between 23.91–24.39 mg/g EQ, and significant at 5% alpha (p-value = 0.000). At a ratio of 4:1 and 1:4, it was 25.40 mg/g EQ in 95% range, between 25.16–25.64 mg/g EQ, and significant at 5% alpha (p-value = 0.004). At a ratio of 4:1 and 3:2, the difference was 25.76 mg/g EQ in 95% range, between 25.52–25.52 mg/g EQ, and significant at 5% alpha (p-value < 0.001). These results reveal that the total flavonoid content at a ratio of 4:1 is better than at a ratio of 1:1, 2:3, 1:4, and 3:2 with the same significance value (<0.05).

Centella asiatica contain flavonoids such as kaempferol, quercetin, apigenin, rutin, and naringin (Vasavi *et al.*, 2016), whereas *I. cylindrica* contain tricin, caryatin, jaceidin, flavones, and maritimin (Jung & Shin, 2021). The role of flavonoids has been reported in previous studies as having antiviral and immunomodulatory activity against the coronavirus (Liskova *et al.*, 2021; Ngwa *et al.*, 2020; Zakaryan *et al.*, 2017). Flavonoids as an anti-inflammatory in COVID-19 can inhibit inflammation and reduce the production of pro-inflammatory cytokines. In addition, the increase in flavonoid content will increase immunity. Another mechanism of flavonoids involves inhibiting dipeptidyl peptidase 4 (DPP4) and neutralization of 3CLpro against COVID-19 (Liskova *et al.*, 2021). The greater a plant extract's total flavonoid content, the greater its potential of anti-COVID-19 activity.

4. Conclusion

This research concludes that the total flavonoid content of *C. asiatica* leaf and *I. cylindrica* root extract is at a ratio of 4:1 > 1:1 > 2:3 > 1:4 > 3:2. This finding emphasizes *C. asiatica* and *I. cylindrica* as potential sources of flavonoids with the ability to combat COVID-19.

Acknowledgment

We would like to express our deepest gratitude to the Universitas Islam Malang, Indonesia, for providing research funding through the 2020 UNISMA Institutional Grant (HI-ma) program.

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