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Standardization of Semanggi (*Marsilea crenata* C. Presl.) Leaves from Benowo District, Surabaya for Standardized Herbal Raw Material

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ABSTRACT

Semanggi (*Marsilea crenata* C. Presl.) is a unique plant that grows in East Java, Indonesia. Its leaves are widely used as ingredients for traditional food. Semanggi leaves contain phytoestrogen compounds that can be used for their antineuroinflammatory, antiosteoporosis, and antioxidant properties. This effect is believed to be caused by kaempferol as an active marker. This study aims to determine the specific and non-specific parameters of semanggi leaves from Benowo District of Surabaya. The standardization results for specific parameters revealed the macroscopic characteristics of the leaves in radius ± 2 cm, pale green to yellowish-green color, smooth surface, obdeltoid shape, and four leaves. The microscopic features are stomata on the epidermis, crystal sand calcium oxalate, bone leaves with vascular bundle, and no space between the epidermis. The organoleptic characteristics are yellowish-green color, tasteless, astringent smell. The physicochemical of water-soluble content result was 7.4566 ± 0.1173% and soluble ethanol was 7.7466 ± 0.4083%. The phytochemical positive test results for the identification of alkaloids, flavonoids, and terpenoids. Based on UHPLC-HRMS analysis, semanggi leaves contain 0,41% kaempferol with a retention time of 6.88 ± 0.2 minute. The results for non-specific parameters indicated that the content of total ash, acid-insoluble ash, moisture, and drying shrinkage content was 0.8136 ± 0.0171%; 7.9700 ± 0.1044%; 6.2547 ± 0.2864%; and 9.0936 ± 0.1305% respectively. The results indicate that the semanggi leaves sample have been assessed and found to meet the established standards.

Keywords: *Marsilea crenata* C. Presl.; standardization; specific and non-specific parameters

INTRODUCTION

Plants have been widely used as medicine by humans since ancient times. It can be said that our ancestors used and utilized plants as medicine to treat various diseases during that time. The science related to medicinal plants is one of the oldest scientific fields, dating back to ancient civilizations such as Egypt, China, India, and Greece (Jamshidi-Kia et al., 2018). The use of medicinal plants in Indonesia has a long history, characterized by the prevalence of traditional healing places and the widespread use of plant-based medicinal products in the community (Emilda et al., 2017). Semanggi (*Marsilea crenata* C. Presl.) is one of the many plants that can be processed into natural medicine. Its leaves are widely used as ingredients for traditional food (Ma'arif et al., 2018; Ermawati & Supeni, 2022). Semanggi grows abundantly in the Benowo District, particularly in Kendung Village where the majority of residents are semanggi farmers. This

village is therefore known as Semanggi Village (Humaidi et al., 2021).

Semanggi has the potential to be used as a raw material for herbal preparations due to its many benefits, as shown by research that has been conducted. Studies have found that semanggi leaves contain phytoestrogen compounds, which can be effective as anti-neuroinflammatory (Ma'arif et al., 2019; Vijayalakshmi et al., 2015), anti-osteoporotic (Aditama et al., 2022; Ma'arif et al., 2019), and anti-oxidant agents (Bhanukiran et al., 2022). Studies on Semanggi from Benowo village have been conducted at various stages, including in vivo, in vitro, and in silico, with the aim of establishing Semanggi as a viable herbal raw material. In silico studies have identified 19 compounds (Ma'arif et al., 2022) in Semanggi ethanol extract and 7 compounds in ethyl acetate fraction (Agil et al., 2020) that exhibit anti-neuroinflammatory activity similar to 17 β-estradiol. In vitro studies have shown that the n-hexane fraction of Semanggi at a concentration of 62.5 ppm can increase osteocalcin expression by 457.35 (Aditama et al., 2020). In vivo studies have demonstrated that Semanggi extract at a concentration of 2.5mg/ml in 96% ethanol can increase zebrafish-induced rotenone motility (Ma'arif et al., 2022). From all the compounds found, kaempferol was believed to be an active marker in semanggi leaves. Kaempferol was an isoflavon that was included in phytoestrogens due to structural similarity. However, in order to make semanggi a standardized herbal medicine that is acknowledged to have activity in reducing inflammation, and to ensure its efficacy and safety, it needs to undergo not only preclinical testing but also standardization.

The regulation established by the head of the Food and Drug Supervisory Agency (BPOM) in Indonesia states that in order to develop herbal preparations into standardized herbal medicines, a standardization process is required (BPOM, 2019). The difference in standardization levels gives standardized herbal medicines a higher level of evidence compared to herbal medicines that do not undergo standardization. To develop semanggi as a biopharmaceutical plant or medicine raw material, it is necessary to standardize the plant. Standardization is a process that aims to ensure stability and safety, as well as to maintain the content of secondary metabolites that have pharmacological effects in plants. Standardization is a series of parameter tests carried out using references related to the safety and quality of preparations. Standardization aims to obtain a product whose quality and stability can be guaranteed (Jannah et al., 2021). Standardization involves quantitatively and qualitatively homogenizing the properties of medicinal plants to ensure the quality, safety, efficacy, and reproducibility of their raw materials for herbal preparations (Nafiu et al., 2017). Parameters in standardization include specific and non-specific parameters. The specific parameters aim to estimate compounds and groups of compounds that have pharmacological benefits in plants, through qualitative and quantitative analysis. Non-specific parameters focus on chemical, physical, and microbial aspects that may impact the safety and efficacy of medicinal plants (Wardani, 2022). The purpose of this study was to determine the specific and non-specific parameters of semanggi leaves from Benowo District, Surabaya.

METHODS

Plant Material

The plant material used in this study are semanggi leaves that were cultivated in Benowo District at December 2021, and identified in the Materia Medica Batu at February 2022 with the specimen number 1a-17b-18a: Marsileaceae-1: *Marsilea crenata* C. Presl.

Chemical Materials

The chemical materials used in this study include reagents such as chloralhydrate, $FeCl_3$, Mayer, Liebermann-Burchard, and HCl 1N, which were purchased from LP. Kimia Jaya Labora, and solvents such as 95% ethanol (Smartlab), aquadest, Whatman filter paper No. 42, chloroform (Merck), 96% ethanol (Merck), and methanol pro LCMS (Merck), which were

purchased from the Central Laboratory of Life Science at Universitas Brawijaya.

Determination of Specific Parameter

Microscopic Examination

Microscopic examinations were conducted using a computer-integrated trinocular microscope. The sample in the form of semanggi leaves sample powder was spread on a glass slide and dripped with chloralhydrate. Furthermore, the sample that has been dripped with chloralhydrate was covered with a cover glass from the slide and then observed under a microscope.

Macroscopic Determination

Macroscopic examinations were conducted to observe the visible characteristics of semanggi leaves such as their shape, size, and texture. A ruler was used as a measuring tool to determine the radius of the leaves and the length of the herbs. The results of the examinations were documented and displayed the physical characteristics of the semanggi plant.

Organoleptic Test

The test was conducted using the five senses on the semanggi leaves sample sample to be observed. Testing other than the taste of sample was carried out using the questionnaire listed in the attachment to the respondents. The results of observations are descriptive of the characteristics of sample.

Determination of Water-Soluble Content and Ethanol Soluble Content

Determination of water-soluble content was conducted using 2 g of semanggi leaves sample powder macerated using 40 ml of chloroform-saturated aquadest (for water-soluble content) and using 40 ml of 95% ethanol (for ethanol-soluble content). The sample sample was put into a dark glass bottle and filled with chloroform-saturated aquadest. The glass bottle containing the sample was shaken for the first 6 hours and then allowed to stand for the next 18 hours. After that, the sample was transferred to a porcelain cup which had been weighed and heated using a water bath to remove residual solvent. The sample was then put into the oven at 105^{o}C for 15 minutes and then allowed to stand in a desiccator for 5 minutes. After that, the sample was weighed until constant. The test was carried out with 3 replications.

Identification of Phytochemical Compound

The identification of phytochemical compounds was conducted by preparing a 2-g sample of semanggi leaves sample macerated for 3x24 hours in 96% ethanol, which was covered with aluminum foil in a measuring cup. After the maceration process, the extract was filtered, resulting in a liquid extract of semanggi leaves. The extract was then dripped onto a drip plate and subjected to Mayer's reagent (for identifying alkaloids), a maximum of 20 drops of $FeCl_3$ reagent (for identifying flavonoids), and Liebermann-Burchard reagent (for identifying terpenoids). Colour changes were observed and documented.

Kaempferol Measurement using UHPLC-HRMS

Semanggi leaves sample were extracted with 96% ethanol using the ultrasonic assisted extraction method (Sonica, Italy). The filtrate was collected and concentrated using a rotary evaporator (Heidolph, Germany) to obtain a concentrated extract of 96% ethanol. The concentration of the extract and standard compound of kaempferol was weighed at 0.75 mg and then dissolved in 1.5 mL of methanol per LCMS. The standard compound of kaempferol and the extract were prepared into a 10 ppm solution and then prepared in the Dionex Ultimate 3000/Q-Exactive UHPLC System (Thermoscientific, USA) coupled to the Quadrupole-Orbitrap HRMS System (Thermoscientific, USA) autosampler. Each sample will be entered into the Hypersil Gold aQ 50 x 1 mm x 1.9 µm column with a flowrate of 40 µL/minute for 15 minutes. The analysis mode used for the UHPLC-HRMS system is PRM in the range m/z 284.000–286.000 to target the presence of kaempferol in the sample. The results of the analysis were interpreted using

ThermoScientific XCalibur 4.1 and mzCloud (ThermoScientific, USA).

Determination of Non-specific Parameter

Determination of Drying Shrinkage

The samples were placed in the tray of a moisture analyzer and kept until the weight stabilized at approximately 2 g, as indicated by the green light. The analyzer was then set at 105°C, and the samples were dried until completion. The final result was determined by measuring the weight loss after drying at 105°C. The test was conducted in triplicate.

Determination of Water Content

The method used in this study is gravimetry, which is a quantitative method that measures the mass of a liquid that evaporates into a gas (Cavaniol et al., 2022). An empty porcelain cup was ignited in an oven at 105°C for 30 minutes, then cooled for 5 minutes in a desiccator and weighed to a constant weight. A porcelain cup whose weight is known is included in a sample of 10 g of semanggi leaves sample. The porcelain cup containing the sample was put into the oven at 105°C for 5 hours. After 5 hours, the cup containing the sample was removed and allowed to stand using a desiccator for 5 minutes. The result of ignition is weighed until constant. The test was conducted in triplicate.

Determination of Total Ash Content

The first step is to ignite the porcelain crucible in a furnace at 600°C until the weight is constant. After obtaining a constant weight of the porcelain crucible, 2 g of semanggi leaves sample is placed into the porcelain crucible. The porcelain crucible containing the sample is then ignited to ashes in an electric furnace. The sample that has turned into ash is left for 15 minutes in a desiccator, and then the test results are weighed to a constant weight. The test was conducted in triplicate.

Determination of Acid Insoluble Ash Content

The previously obtained total ash content was further processed by dissolving it in 25 ml of 1N dilute HCl for 5 minutes and filtering it using ash-free filter paper. The ash residue on the filter paper was then rinsed with hot water and placed back into the porcelain crucible. The porcelain crucible with the filter paper containing the ash was ignited again at 600°C until it became completely ash. The resulting ash was allowed to cool for 15 minutes in the desiccator, and the test results were weighed until a constant weight was achieved. The test was performed in triplicate.

RESULTS AND DISCUSSION

Result of Specific Parameter Determination

The results of microscopic observations (Figure 1) show that the most important microscopic feature of semanggi leaves is the presence of anisocytic type stomata and spirally thickened vascular tissue found in the bone leaf. Additionally, the examination of the semanggi leaves sample powder revealed the abundance of leaves bones. The macroscopic observations of Semanggi leaves belonging to the *M. crenata* species revealed several characteristics. The leaves are composed of four leaflets with numerous bone leaf on each side, and the shape of the leaflets is obdeltoid. The color of the leaves ranges from green to yellowish green, and the surface is smooth with a radius of approximately 2 cm. Macroscopic examination also revealed that Semanggi leaves resemble those of Creeping Woodsorrel (*Oxalis corniculata*) in appearance (Figure 2). Semanggi leaves from the *M. crenata* species are characterized by four leaflets at the end of the petioles, as well as leaf veins that form a fan-like pattern and obdeltoid shape. The difference between *M. crenata, M. quadrifolia,* and *M. schelpiana* is in the length of the petiole, leaf dissection, and the total stomatal index (Wu & Kao, 2011).

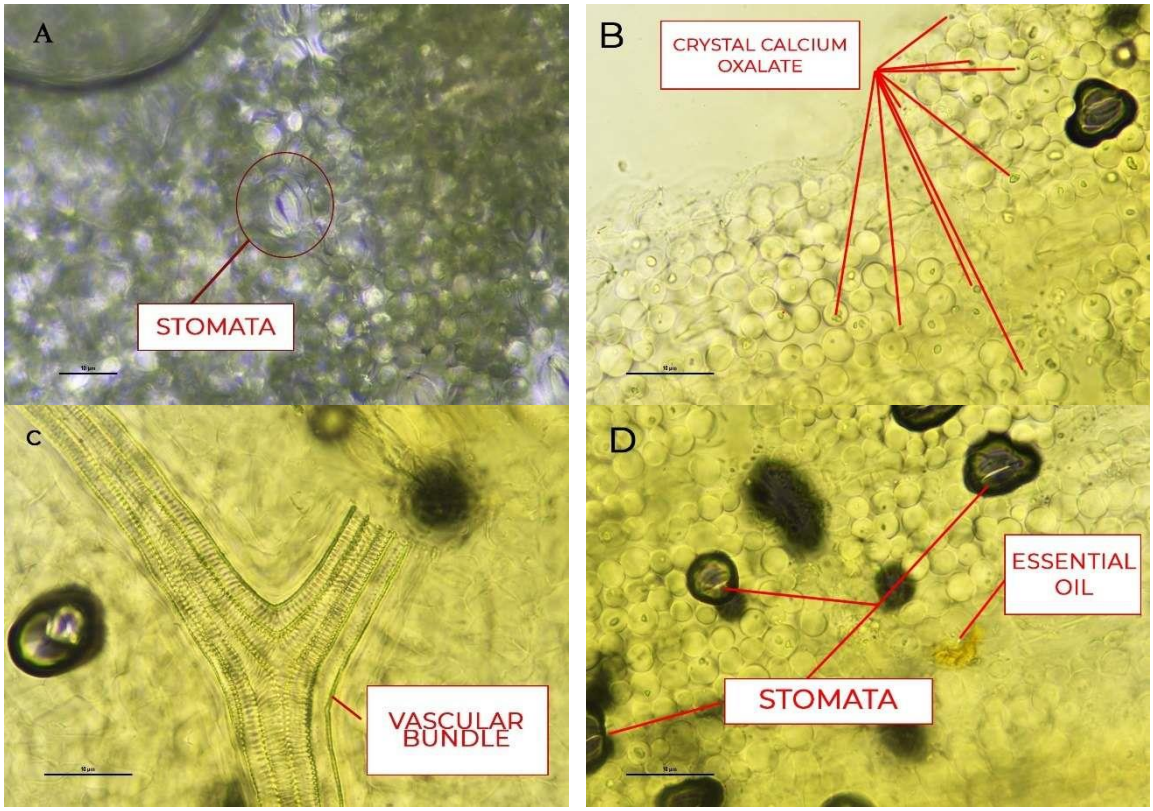

Figure 1. Powder microscopy: (A) presence of stomata on the surface of semanggi leaves, (B) the presence of calcium oxalate in the form of sand crystals, (C) leaves veins with spirally thickened vascular bundle, and (D) fragments of stomata, epidermis, and essential oil were observed.

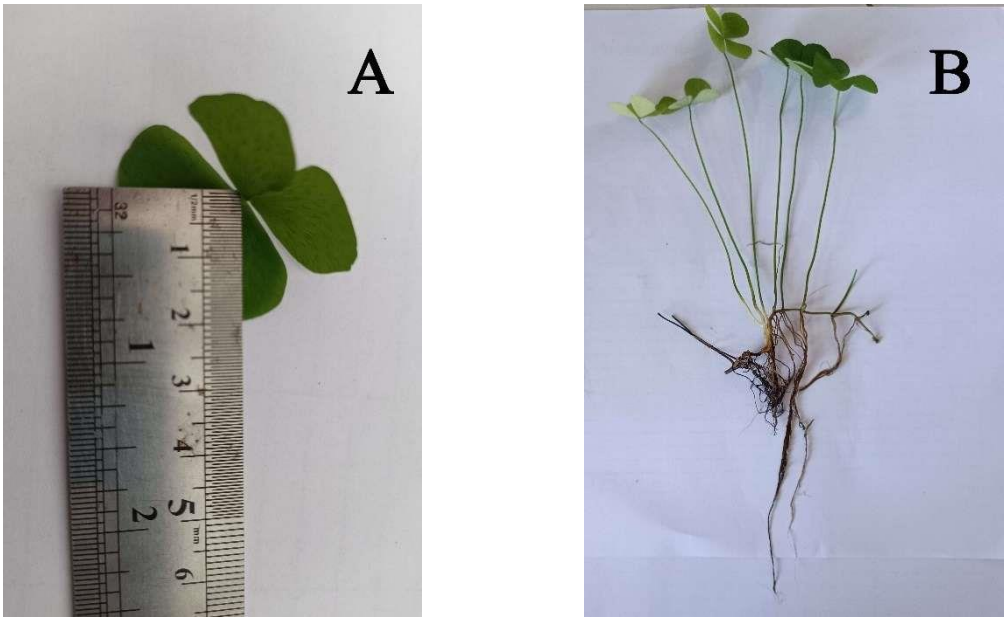

Figure 2. Macroscopic examination of semanggi: (a) The radius of semanggi leaves is approximately ± 2 cm. (b) The length of semanggi herb is approximately ± 31 cm

Table 1. Result of Specific Parameter Determination

Parameter		Result	Conclusion
Water soluble content		7.4566% ± 0,1173	Standard has been determined
Ethanol soluble content		7.7466% ± 0,4083	Standard has been determined
Identification of phytochemical compound	Alkaloid (Mayer)	White precipitate appears, indicating presence of alkaloid	Contains alkaloids
	Flavonoid ($FeCl_3$)	Color changes to dark green, indicating a lot of flavonoids	Contains flavonoids
	Terpenoid (Liebermann-Burchard)	Color changes to light green, indicating presence of terpenoids	Contains terpenoids

The sample of semanggi leaves exhibits a dark green hue and a characteristic aromatic scent with an astringent note. Additionally, it has a unique fresh taste that tends to be bitter. The plant's aroma is attributed to secondary metabolites of the terpenoid group (Guo et al., 2020). The interpretation of organoleptic results based on the Indonesian Herbal Pharmacopoeia Edition II (2017) indicates that expressions such as "aromatic scent" are merely descriptive and do not establish a purity standard for the substance being evaluated.

The determination of the water-soluble essence content revealed (Table 1) a yield of 7.4566% ± 0.1173, while the ethanol-soluble extract content was 7.7466% ± 0.4083. The findings indicated that the Semanggi leaves sample had a greater concentration of compounds dissolved in organic solvents than in water solvents. This may be attributed to the fact that ethanol can dissolve both water-soluble compounds and some compounds that are insoluble in water due to its polar nature as an organic solvent. Additionally, ethanol has the ability to break down more phenolic compounds than water (Kusharyati et al., 2020). Water and ethanol-soluble extracts are needed to determine the amount of solubility of sample in water and organic solvent (Febrianti et al., 2019).

The compound identification test produced positive results for all three tested compound groups, namely alkaloids, flavonoids, and terpenoids (Figure 3, Table 1). The results of the flavonoid compound test were consistent with previous research, indicating that Semanggi leaves contain a high level of phytoestrogens (Ma'arif et al., 2019). Flavonoids are the largest group of phytoestrogens, known to act like genistein. Alkaloid and terpenoid tests resulted in a color change on the drop plate, with positive results indicated by a white precipitate (with Mayer), a color change to blackish-green (with FeCl3), and a color change to green (with Liebermann-Burchard) (Salempa et al., 2019). Semanggi is known to have pharmacological effects in preventing osteoporosis due to its flavonoid and terpenoid compounds (Ma'arif et al., 2019; Tripatmasari et al., 2020). In addition, testing with alkaloids can also be detected as alkaloids can dissolve in organic solvents (Alfiani, 2022).

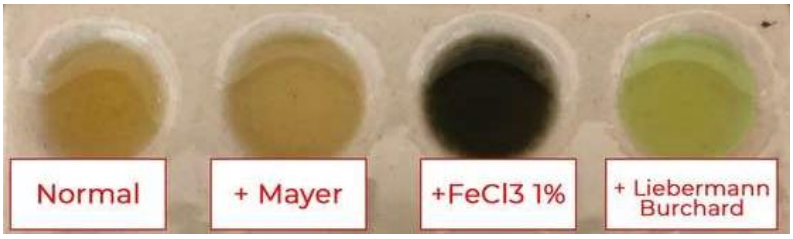

Figure 3. Identification of phytochemical compound result

Table 2. Retention Time Analysis

Sample	Retention Time-Peak (minutes)
Standard compound of kaempferol	2.86 and 7.87
96% ethanol extract of semanggi leaves	0.88 and 6.88

The results of the analysis of the UHPLC-HRMS with PRM mode will theoretically show a single peak of the kaempferol, both the standard compound of kaempferol and the extract of 96% ethanol of semanggi leaves. However, the results of both TICs showed not only the kaempferol peak but also other peaks and different retention times (RT) (Figure 4). The results of the RT observations meant that the TIC could not be directly overlaid to determine the presence of kaempferol (Table 2).

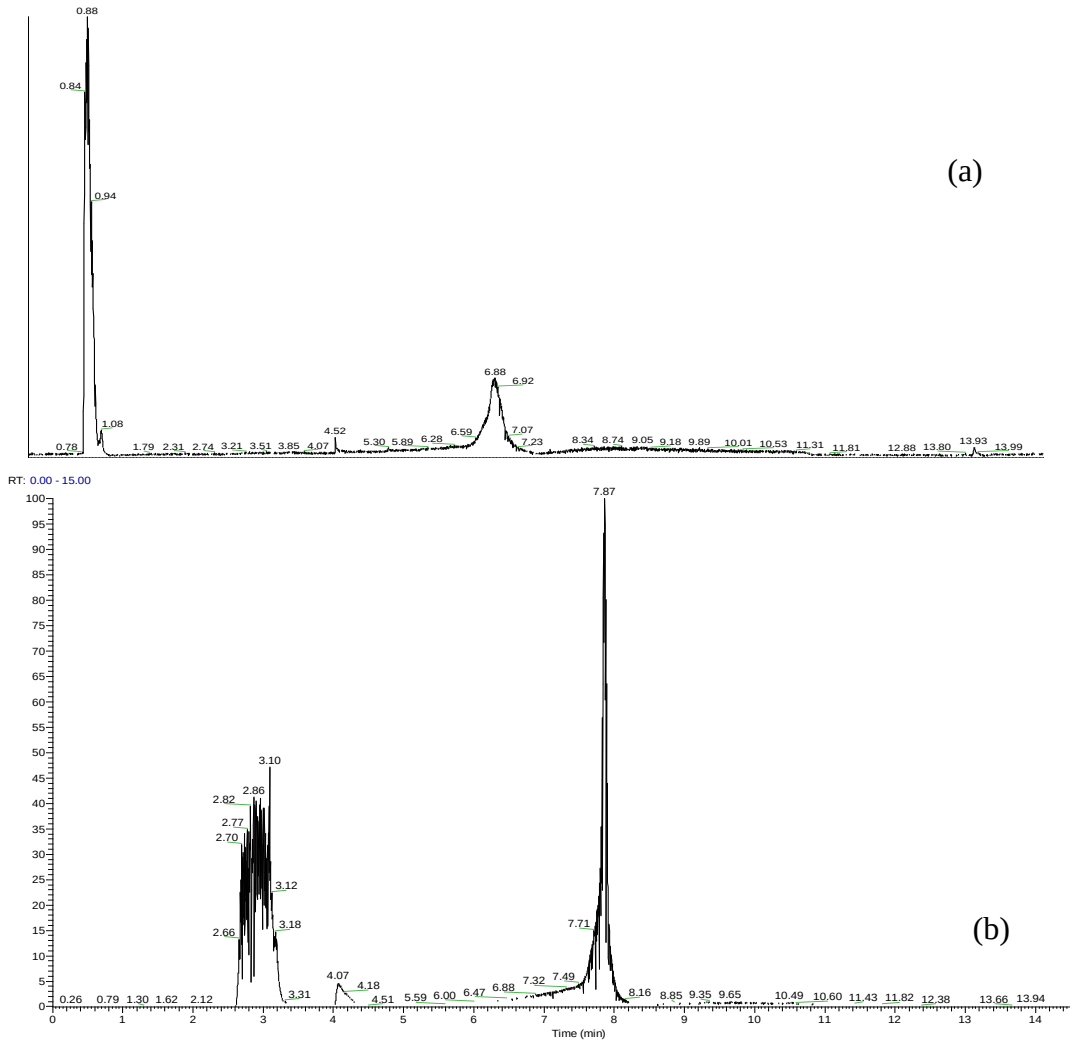

Figure 4 Analysis of UHPLC-HRMS TIC
(a) 96% ethanol extract of semanggi leaves
(b) Standard compound of kaempferol

Table 3. Result of Non-Specific Parameter Determination

Parameter	Result	Requirement	Conclusion
Total ash content	9.0936% ± 0.1305	≤12%	Fulfill the standards
Acid-insoluble ash content	0.8136% ± 0.0171	≤5%	Fulfill the standards
Water content	6.2547% ± 0.2864	≤10%	Fulfill the standards
Drying Shrinkage	7.4566% ± 0.1173	≤10%	Fulfill the standards

It is necessary to determine the presence of kaempferol by conducting TIC crossmatching and confirming the fragmentation pattern of the compound using mzCloud. The results of the analysis showed that kaempferol was detected at RT 7.87 ± 0.02 minutes in the standard compound of kaempferol. On the other hand, it showed that kaempferol was detected as much as 0,41% kaempferol with a retention time of 6.88 + 0.2 minutes in a 96% ethanol extract of semanggi leaves with a similarity value of 68.7%.

The RT shift phenomenon in the 96% ethanol extract of semanggi leaves is known as retention shifting. Retention shifting occurs when the RT of the sample differs > 0.5 minutes from the RT of the standard compound in the UHPLC-HRMS, where one of the contributing factors is the matrix effect of the sample analysis. The risk of the matrix effect increases because of the presence of both amine-derived and carboxylic derivative compounds. As a result of the presence of this group of compounds, the pH of the system changes, resulting in a shift in RT in the sample being analyzed (Lan et al., 2020; Sentkowska et al., 2016). On the other hand, the risk of matrix effects also increases in the presence of compounds that are identical to the target compound. These compounds will change the physicochemical properties of the target compound, and then the RT of the target compound will shift (Sousa et al., 2018).

Result of Non-specific Parameter Determination

The determination of non-specific standardization parameters resulted (Table 3) in a total ash content of 9.0936% ± 0.1305, acid insoluble ash content of 0.8136% ± 0.0171, drying shrinkage of 7.4566% ± 0.1173, and water content of 6.2547% ± 0.2864. Compliance with these non-specific parameter standards ensures that preparations made using standardized medicinal plant raw materials can maintain their safety and stability (Wardani, 2022). The dissolution of total ash in hydrochloric acid is used to determine the presence of light inorganic metals that do not volatilize in air (Xue & Liu, 2021). The previous study, which focused on specific heavy metal detection in semanggi from East Java, showed no excessive limit of mineral concentrations of Pb and Hg (Agil et al., 2021). All tests for specific and non-specific parameters meet the requirements of the Second Edition of the Indonesian Herbal Pharmacopoeia (2017) and the WHO guidelines for the assessment of herbal medicine (1991).

CONCLUSIONS

The standardization results for specific parameters showed that the semanggi leaves sample had a radius of approximately 2 cm, pale yellowish-green color with a smooth surface, obdeltoid shape, and four leaves. Microscopic examination revealed stomata on the upper epidermis, calcium oxalate in the form of sand, numerous leaves veins, and no space between the epidermis. Organoleptically, the sample had a yellowish-green powder color, typical tastelessness, and astringent odor. The water-soluble essence content was 7.4566% ± 0.1173, and it was soluble in ethanol at 7.7466% ± 0.4083. The identification test for alkaloids, flavonoids, and terpenoids gave positive results. Semanggi leaves contain 0,41% kaempferol with a retention time of 6.88 + 0.2 minute and similarity value 68.7%. Non-specific parameter results showed total ash content of 9.0936% ± 0.1305, acid-insoluble ash content of 0.8136% ± 0.0171, water content of 6.2547% ± 0.2864, and drying shrinkage of 7.9700% ± 0.1044. The

results indicate that the semanggi leaves sample have been assessed and found to meet the established standards. Further research is expected to continue the standardization parameters that were not included in this study.

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