The influence of extraction methods on rutin yield of cassava leaves (Manihot esculenta Crantz)

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Abstract

Rutin, a well-known bioflavonoid, was found abundantly in cassava leaves. In the present study, extraction techniques including maceration, boiling, reflux, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) were optimised to increase the yield of rutin. Extraction parameters such as solvents, solid-liquid ratio, temperature, and time were optimised to give better extraction yields for each method. HPLC analysis showed a high content of rutin which is up to 2.4% per dry weight of cassava leaves. The extraction yields under optimised condition were found to be 16.00 ± 0.21, 20.38 ± 0.66, 22.33 ± 2.3, 24.49 ± 0.41, and 23.37 ± 1.00 g rutin per kg dry weight for maceration, boiling, reflux, UAE and MAE methods, respectively. Specifically, UAE reduced the extraction time to 90 min, using only 40–60% of aqueous ethanol. Meanwhile, MAE completed the extraction under 5 min and no significant differences in output was observed between the use of water and aqueous ethanol. Accordingly, with the extraction efficiency of up to 99 and 94%, respectively, both processes provided better results. The subsequent green purification using chilling method produced a typical cassava bioflavonoid containing 82% of rutin and 17% of nicotiflorin. This study informs a new abundant source of rutin and provides the optimum condition of extraction methods for high yield of rutin from cassava leaves.

1. Introduction

Indonesia is currently known as the top third of the world cassava producing countries which has produced more than 21 million ton of cassava tubers annually and gained 7.5% of the world production share (FAO, 2018). The cassava tuber is the prominent part commonly consumed by local people as the energy source due to the high content of starch. Also, it is used as a raw material industry to produce tapioca flour or its derivatives for multipurpose. The fresh tuber contains water and carbohydrates as the abundant components ranging from 60 to 70% and 12 to 33%, respectively. Other constituents such as protein, fibres, minerals, cyanide, and fat are present in a small amount (Rodriguez-Sosa et al., 1976; Emmanuel et al., 2012). Another part, leaves are also consumed as vegetables in various dishes which is also found in people of Africa, Asia, and Latin America (Kubo et al., 2006; Latif and Müller, 2015). However, the leaves have not been fully utilised since only the young leaves are used for vegetables and more of them are going to waste after cassava tuber harvesting. Interestingly, cassava leaves are reported to have bioactivities such as anti-oxidant, anti-tyrosinase, anti-inflammatory, and hepatoprotective (Harini et al., 2019; Kubo et al., 2006; Tsumbu et al., 2011; Tao et al., 2015). It is believed that the high content of flavonoids is responsible for those activities. A recent study showed that at least seven glycoside flavonoids are present in the cassava leaves which are clovin, myricetin-3-0-rutinoside, robinin, rutin, hyperoside, nicotiflorin, and narcissin. Rutin was found to be the major flavonoid in cassava leaves (Tao et al., 2019).

Accordingly, aside from the known economic value of the cassava tubers, the leaves may add the economic value of the cassava plants due to its potency to be used as an alternative source of dietary flavonoids for nutraceutical products. Many nutraceutical products containing high flavonoids have been released to the market. However, stabilising and increasing the flavonoid content is still a challenging process in food or nutraceutical industries. Moreover, the significant loss of flavonoids still occurs during...
storage and processing of flavonoids-based products. Therefore, the effort to provide excellent methods that could be used to enhance the yield of flavonoids from plants might eventually improve the flavonoid-based products. Selecting plants rich in flavonoids and developing of extraction methods which are highly efficient to produce the maximum yield of flavonoids, non-toxic, safe for the environment, and affordable are major factors that should be considered for industrial-scale (Kelly et al., 2019).

Solid-liquid extraction is the most widely used method for flavonoid extraction. However, this conventional method lacks efficiencies such as the use of huge amounts of solvents, low yield, the loss of some compounds, less specificity, long time required, and high energy consumption due to the prolonged heating. The new emerging extraction technology, however, might overcome such serious issues. The technology includes ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), high-pressure extraction (HPE), and supercritical-fluid extraction (SFE). Numerous studies have shown the efficiency of these methods for the extraction of polyphenol-rich plants (Maroun et al., 2018).

Only one study has reported the optimisation of the flavonoid extraction from cassava leaves. This study has only used UAE technique for the extraction and yielded about 6.37 g rutin per kg dry weight of cassava leaves at the optimised condition (Tao et al., 2019). We believe that the yield could be increased by optimising other parameters or using other extraction techniques. Moreover, the rutin content could be higher when using plant materials natively grow at their origin area. The present works were aimed to provide simple, robust and affordable extraction methods by adapting conventional and advanced extraction methods for cassava leaves to obtain a high yield of flavonoids. Also, a simple purification using the chilling method was tested to purify rutin or flavonoids from cassava leaves extract that could be applied in food or nutraceutical industries.

2. Material and methods

2.1. Chemicals

HPLC-grade methanol, ortho-phosphoric acid, ethanol, and TLC-silica gel 60 GP254 were purchased from Merck (Darmstadt, Germany). Rutin was purchased from Sigma-Aldrich.

2.2. Plant material

Fresh cassava leaves sold as vegetables were purchased from the local market in Bandung. The specimen was botanically identified and deposited in School of Life Sciences and Technology, Bandung Institute of Technology. The leaves were separated from their petioles, air-dried in an oven at 40 °C, and grounded to obtain the dried powder with a particle size around 0.2–1 mm. The powder was kept away from the light and stored in an air-tight container at room temperature until use.

2.3. Extraction methods and experimental design

Several extraction methods were optimised to obtain a high yield of rutin from cassava leaves. Extraction variables such as solvents (water, ethanol, and their combination), solid-liquid ratios, temperature, and extraction time were independent variables. The optimum condition of the extraction was determined using single factor experiment in which the level for each independent variable was chosen based on the rutin yield. In the design, the first variable was set with different ranges whereas other variables were constant. Subsequently, variation of the second variable was studied using the best condition of the first variable from the initial step, and the rest variables were kept constant. The optimum condition of the rest variables was continually investigated according to the previous stages.

After all extraction processes, the plant extracts were collected, filtered, centrifuged, and stored at −20 °C for the subsequent analysis. Conventional extraction and advanced extraction methods were performed in this experiment. The use of conventional extraction including maceration, boiling, and reflux and the use of advanced extraction methods including ultrasonic-assisted and microwave-assisted extraction were adopted and described in details as follows.

2.3.1. Conventional extraction methods

2.3.1.1. Maceration. Maceration was performed using ethanol (solid-liquid ratio, 1:20, w/v) with different extraction time as the variable. Briefly, 50 ml of ethanol was added into 2.5 g of plant material in an Erlenmeyer flask with lid. The extraction occurred at room temperature away from the light for 1, 4, 8, 16 and 24 h.

2.3.1.2. Water boiling. The boiling procedure was performed using water as the solvent, where solid-liquid ratios and extraction times were used as extraction variables. Firstly, 2.5 g of plant material was added into 25, 50, and 75 ml of boiling water to give solid-liquid ratios of 1:10, 1:20, and 1:30, respectively. The extraction time was performed for 15, 30, 60, 90, and 120 min.

2.3.1.3. Reflux. For the reflux method, water and ethanol were used for the extraction either as single solvents or as aqueous ethanol. Variables including solid-liquid ratio, percentage of ethanol/water, temperature, and time were optimised in this method. Briefly, 2.5 g of plant material was weighed in a 250 ml Erlenmeyer flask equipped with a condenser apparatus. Extraction was performed on a hot plate with continuous stirring at 300 rpm. The percentage of ethanol used was 0, 20, 40, 60, and 80%. The solvent used was 25, 50, and 75 ml to obtain solid-liquid ratios of 1:10, 1:20, and 1:30, respectively. Variation of extraction temperature was 30, 40, 50, and 60 °C, and extraction time was 1, 2, 3, and 4 h.

2.3.2. Advanced extraction methods

2.3.2.1. Ultrasonic-assisted extraction (UAE). The ultrasonic-assisted extraction was performed in an ultrasonic bath which provides indirect contact to the sample to minimize damage compared to a direct ultrasonic probe. An ultrasonic cleaner (SkyMen, China) was set to 50 °C with frequency 40 kHz and the water bath was allowed to reach the desired temperature. Then, 2.5 g of each plant material was placed in 50 ml polypropylene tube with lid and immersed in the ultrasonic bath. The extraction was optimised with the following variables: extraction time (15, 30, 60, 90, and 120 min), percentage of aqueous ethanol (0, 20, 40, 60, and 80%), and solid-liquid ratios (1:5, 1:10, 1:20, 1:30 w/v).

2.3.2.2. Microwave-assisted extraction (MAE). The household microwave (Brabantia, Dutch) was used in this experiment. The microwave was modified and equipped with a glass condenser which fits the round bottom flask as the extraction vessel. The extraction was performed for 2.5 g of plant material in the 250 ml digestion vessel. During extraction, the vessel was attached to the condenser to prevent the loss of solvents. The MAE parameters were optimised according to following variables: microwave power (180, 360, 540, and 720 W), extraction time (1–10 min), percentage of...
aqueous ethanol (0, 20, 40, 60, and 80%), and solid-liquid ratios (1:5, 1:10, 1:15, 1:20 w/v).

2.4. Flavonoid purification

The chilling method was used to purify the flavonoids from cassava leaves extract. Briefly, extraction of 50 g plant material was performed using MAE with water as solvent at the best condition. The extraction was repeated twice to fully extract all flavonoids. The extracts were filtrated and combined in the 1000 ml Erlenmeyer. Flavonoids were obtained by precipitation at 4 °C overnight. The precipitates were collected by filtration and subsequently washed with ethanol to separate flavonoids and impurities. The insoluble impurities were retained on the filter paper, where flavonoids dissolved in ethanol. Then, the flavonoid solution was concentrated under reduced pressure and allowed overnight for the precipitation of flavonoids at room temperature. The precipitate was rinsed with water several times and dried at 50 °C overnight.

2.5. High-performance liquid chromatography

Rutin flavonoid was analysed using LC-20AD liquid chromatography equipped with SPD-20AU UV/Vis detector and CTO-20A oven pump (Shimadzu, Japan). A reversed-phase column, LiChrospher® 100 RP-18 5 μm column (100 mm length, 4 mm diameter, 20 mm pre-column), was used for separation. The HPLC condition was as follows: the mobile phase consisted of 50% (v/v) methanol with 0.01% (w/v) ortho-phosphoric acid, the column temperature was set to 30 °C, the flow rate was 0.5 ml/min, injection volume was 20 μl and UV wavelength was 260 nm (a modified method from Kuntić et al., 2007). The linearity, limit of detection (LOD) and limit of quantification (LOQ) of rutin were determined according to the method described in Baalbaki et al. (2018) and Lim et al. (2018).

2.6. LC-MS analysis

Analysis of the purified flavonoids was performed using a Waters Acquity UPLC system coupled to a XEVO QTof-MS. The separation was carried out on an Acquity UPLC® BEH C18 column (2.1 × 100 mm, 1.7 μm) by injecting 2 μl of samples and the mobile phase was acetonitrile and 0.1% formic acid in water. A linear gradient program was set at 0–30 min from 20 to 80% acetonitrile. The column was maintained at 25 °C and the flow rate was 0.2 ml/min. For mass analysis, the QTOF detector was operated in full scan mode (TOF mode) using the positive ESI mode. Capillary and sampling cone voltages were 3.5 kV and 60 V, respectively. The source and the desolvation temperatures were 100 and 450 °C, respectively. The cone gas flow and desolvation gas flow rate were 50 and 800 L/h, respectively. Nitrogen was used as the collision gas with the collision energy of 10 eV. Compounds were recorded by full-scan mass analysis from m/z 100 to 1000, 1.0 s of scan time with 0.02 s interscan time.

2.7. Statistical analysis

All samples for the extraction and analysis were performed in triplicate and the results were reported as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey post host test was used to analyse the effects of the extraction variables on the rutin yield. The significant differences among treatments were estimated at p < 0.05.

3. Results and discussion

3.1. Identification of rutin in the cassava leaves

HPLC method was developed to identify the presence of rutin in the extract of cassava leaves. The method was able to separate rutin from other substances present together in the extract (Fig. 1). For the quantification, the linear range of rutin was achieved at 1–250 μg/ml with the correlation coefficient (r²) = 1. The LOD and LOQ were 0.06 and 0.21 μg/ml, respectively. HPLC analysis showed that the content of rutin was 24.69 ± 0.72 g/kg or 2.4% dry weight (DW) of cassava leaves. This is higher than the result from Tao et al. (2019) where the rutin content in the cassava leaves was 0.6% DW.

3.2. Effect of extraction techniques on the rutin yield

The effect of maceration on the rutin yield was shown in Table 1. The extraction yield was time-dependent, where the highest yield of rutin was found to be 16 g/kg DW after 24 h of maceration. However, statistical analysis showed that no significant different (p < 0.05) on the extraction time of 16 and 24 h (15.48 ± 0.58 and 16.00 ± 0.21 g/kg, respectively). Therefore, 16 h of maceration could be considered enough to extract most of the rutin from cassava leaves. Ethanol was chosen as the main solvent since it is commonly used in herbal or drug preparation and generally recognised as safe (GRAS). Moreover, since the solubility of rutin in ethanol is 5.5 g/l (Krewson and Naghsri, 1952), the use of 1:20 (w/v) solid-liquid ratio during maceration was still below the saturation point where rutin content in the sample is 2.4% DW.

Rutin extraction from plants using water boiling was disclosed in a few reports (Humphreys, 1964; Hsu, 1999; Chang and Muir, 2006). In this experiment, boiling extraction of the cassava leaves was optimised. Table 2 showed the effect of solid-liquid ratio and extraction time on the rutin yield. The higher yield of rutin was obtained using solid-liquid ratio 1:30 (w/v) which resulted in 14.70 ± 0.43 g/kg. Although this was higher than using 1:10 or 1:20 (w/v) of solid-liquid ratio, no significant difference has been observed (p < 0.05). Furthermore, the effect of extraction time was investigated at the solid-liquid ratio of 1:30 (w/v), where 15 min of boiling was found to produce the highest yield of rutin (20.38 ± 0.66 g/kg). The extraction time with longer than 15 min resulted in lower extraction yields which might be affected by thermal degradation. In the aqueous solution, rutin has been reported to be degraded as it was exposed at high temperatures for a long period. Quercetin, isoquercitrin, and their methyl derivatives were major compounds resulted from rutin transformation (Dawidowicz et al., 2016). Quercetin was isolated in quantities from maniocoba, a unique local dish in Brazil prepared by cooking fresh cassava leaves for 4–5 days. The authors suggested that the high amount of rutin contained in fresh leaves was completely hydrolysed after cooking processes (Kubo et al., 2006). In our study, however, the observation of the degradation products of rutin has not been performed yet.

Reflux was the last classical method used for the extraction of rutin from the cassava leaves. This method has been widely used for the extraction of metabolites from various plant samples due to its simplicity in the instrumentation and operation. The extraction involves medium heat and continuous agitation which eventually complete the extraction in hours depending on the several extraction factors. Based on the results from maceration and water boiling, aqueous ethanol was chosen as the solvent for the reflux extraction since the presence a small portion of water has been known to enhance the polarity of the organic solvent. This would increase the extraction efficiency of rutin from plant tissues since

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this flavonoid contains two sugar moieties (Chua 2013). In this study, the effects of four extraction variables on the rutin yield were examined, i.e. extraction time, ethanol concentration, temperature and solid-liquid ratio. Statistical analysis showed that

![HPLC chromatogram from the extract of cassava leaves (above) and rutin as the reference standard (bottom).](image)

**Fig. 1.** HPLC chromatogram from the extract of cassava leaves (above) and rutin as the reference standard (bottom).

### Table 1
Extraction yield resulted from the ethanol maceration.

| Extraction condition | Extraction variable | Extraction yield, g Rutin/kg DW* |
|----------------------|---------------------|---------------------------------|
| Solvent, ethanol     | Extraction time, h  | 10.59 ± 0.35^a               |
| 1:20 (v/v)           | 4                   | 11.74 ± 0.22^b               |
| Temperature, room    | 8                   | 13.05 ± 0.12^c               |
| Solid-liquid ratio,  | 16                  | 15.48 ± 0.58^d               |
| 1:20 (v/v)           | 24                  | 16.00 ± 0.21^e               |

* Data of mean ± standard deviation were from the triplicate experiment. The same superscript letters are not significantly different (p < 0.05).

### Table 2
Extraction yield resulted from the boiling method.

| Extraction condition | Extraction variable | Extraction yield, g Rutin/kg DW* |
|----------------------|---------------------|---------------------------------|
| Extraction time, 60 min | Solid-liquid ratio, w/v | 11.82 ± 2.74^a               |
| 1:10                 | 1.05 ± 0.22^b               |
| 1:20                 | 1.27 ± 0.59^c               |
| 1:30                 | 1.40 ± 0.43^d               |
| Solid-liquid ratio, 1:30 | Extraction time, min | 20.38 ± 0.66^e               |
| 15                   | 18.32 ± 0.39^b               |
| 30                   | 18.81 ± 0.54^c               |
| 60                   | 14.59 ± 0.53^d               |
| 120                  | 13.71 ± 1.92^e               |

* Data of mean ± standard deviation were from the triplicate experiment. The same superscript letters are not significantly different (p < 0.05). Statistic results were obtained by multiple comparisons for each extraction condition.
data were almost the same, suggesting the need for factorial designs to get a better result for this method (Table 3). Overall, 2–3 h of the extraction time was considered optimal to extract most of the rutin in a single step extraction. High extractable of rutin was found at the concentration range of 40–80% aqueous ethanol, the solid-liquid ratio at 1:10 (w/v), and the temperature at 40–50 °C. Combination of these extraction variables on the reflux method resulted in the rutin yield up to 22 g/kg, which was 90% of the extraction efficiency. These were higher compared to the ethanol maceration and water boiling. The use of aqueous organic solvent was a critical factor that can increase the extractability of rutin from cassava leaves and other plants (Chua, 2013; Thoo et al., 2013; Habtemariam and Varghese, 2015; Chua et al., 2017; Gullon et al., 2017; Tao et al., 2019).

The two advanced extraction techniques, UAE and MAE, were further optimised to increase the yield of rutin. Extraction variables for UAE were set up by considering the best condition obtained when using reflux. According to the statistic results, the concentration of aqueous ethanol and the extraction time were the main factors affecting the yield of rutin. No significant difference has been observed about the effect of the solid-liquid ratio (p < 0.05). Table 4 showed that the UAE method could increase the rutin yield up to 24 g/kg from cassava leaves when extracted using 40–60% of aqueous ethanol at 50 °C for 90 min. The solid-liquid ratio at 1:10 (w/v) was considered enough for the optimal yield. The use of higher solid-liquid ratios was not found to statistically increase the rutin yield, suggesting that most of the rutin has been completely extracted using 1:10 of solid-liquid ratio. These UAE conditions are relatively similar to the study conducted by Tao et al. (2019) where the highest total flavonoid content of cassava leaves resulted with the extraction conditions at 70 °C with 50% ethanol in 1:20 of solid-liquid ratio for 2 h. These results showed that ultrasound extraction has not only improved the yield of rutin but also shortened the extraction time and reduced the concentration of aqueous ethanol, which was more efficient than using conventional extraction.

As reported in many scientific studies, microwave-assisted extraction (MAE) can reduce the extraction time, the amount of solvent, and energy consumption (Orsat and Routray, 2017). The

| Extraction condition | Extraction variable | Extraction yield, g Rutin/kg DW* |
|----------------------|---------------------|----------------------------------|
| Solvent, 80% aqueous ethanol | Extraction time, h | 18.43 ± 1.02a |
| Temperature, 50 °C | 2 | 19.56 ± 1.09ab |
| | 3 | 22.09 ± 0.16a |
| | 4 | 20.59 ± 0.61ab |
| Solid-liquid ratio, 1:20 (w/v) | Aqueous ethanol, % | 16.71 ± 0.90a |
| Temperature, 50 °C | 20 | 19.16 ± 0.69a |
| | 40 | 21.18 ± 0.25a |
| | 60 | 21.28 ± 0.49a |
| | 80 | 21.88 ± 1.25a |
| Solid-liquid ratio, 1:10 (w/v) | Extraction time, 3 h | 20.53 ± 0.68a |
| Temperature, 50 °C | 40 | 21.87 ± 0.59a |
| | 50 | 21.94 ± 1.41a |
| | 60 | 18.20 ± 0.34a |
| Solvent, 80% aqueous ethanol | Solid-liquid ratio, w/v | 20.35 ± 1.47a |
| Temperature, 50 °C | 1:10 | 22.33 ± 2.23a |
| | 1:20 | 22.02 ± 0.57a |

* Data of mean ± standard deviation were from the triplicate experiment. The same superscript letters are not significantly different (p < 0.05). Statistic results were obtained by multiple comparisons from each extraction condition.

MAE method used in this experiment was really powerful among other tested methods for rutin extraction. Using MAE method, the yield of rutin obtained was up to 23 g/kg DW, where the extraction occurred for 5 min at 540 W with 60% of aqueous ethanol and 1:10 of the solid-liquid ratio (Table 5). However, according to the statistic results from the extraction variables such as power, extraction time, and solvent concentration, no significant difference was shown (p < 0.05). These results confirmed that even at the lowest power, shortest extraction time and no organic solvent used, the yield of rutin obtained using MAE method has already achieved its optimum level. Therefore, increasing the variables

| Extraction condition | Extraction variable | Extraction yield, g Rutin/kg DW* |
|----------------------|---------------------|----------------------------------|
| Solvent, 60% aqueous ethanol | Extraction time, min | 20.31 ± 1.62a |
| Solid-liquid ratio, 1:10 | 0.5 | 20.31 ± 1.62a |
| Extraction time, 5 min | 1 | 23.24 ± 0.73a |
| | 2 | 22.42 ± 0.47a |
| | 3 | 22.55 ± 0.70a |
| | 4 | 23.38 ± 0.25a |
| Solvent, 60% aqueous ethanol | Air, °C | 18.59 ± 1.17a |
| Power, 540 W | 20 | 19.59 ± 1.17a |
| Extraction time, 5 min | 40 | 19.99 ± 1.82a |
| | 60 | 19.80 ± 1.13a |
| | 80 | 23.05 ± 1.15a |
| | 120 | 21.66 ± 0.87a |
| Solvent, 60% aqueous ethanol | Solid-liquid ratio, w/v | 21.86 ± 1.04a |
| Power, 540 W | 1:5 | 22.63 ± 0.79a |
| Extraction time, 5 min | 1:10 | 21.54 ± 0.46a |
| | 1:15 | 23.15 ± 0.10a |
| | 1:30 | 23.38 ± 0.25a |

* Data of mean ± standard deviation were from the triplicate experiment. The same superscript letters are not significantly different (p < 0.05). Statistic results were obtained by multiple comparisons from each extraction condition.
such as higher power or longer extraction time had no significant effect on the rutin yield. Moreover, the use of higher solid-liquid ratio tends to reduce the yield of rutin since a high amount of solvent might hinder the contact between microwave radiation and the sample matrix as explained by Kala et al. (2016). In comparison to the UAE results, the rutin yield from MAE was a little bit lower which could be explained by the thermal degradation since the temperature of the modified household microwave cannot be adjusted. However, by considering the shortest time as well as the lowest amount of solvent used, the MAE method is likely the best method for optimal extraction of rutin from cassava leaves.

As a summary, data obtained in this work demonstrated how extraction methods gave different results on the rutin yield. As expected, the advanced extraction methods, UAE and MAE, were more efficient than the conventional methods since they had more than 94% of extraction efficiency. Not only the higher yield of rutin was obtained by these two methods but also the shortest time and lowest amount of solvent needed. Fig. 2 provided the optimal yield and extraction efficiency of rutin obtained from each of extraction methods at their best conditions. These data are not only for comparison but also providing us with the best condition of each method for rutin extraction of cassava leaves. Therefore, one can choose which methods will be used and more suitable for different purposes. For example, both UAE and MAE are the optimal method with higher extraction efficiency. However, these methods need expensive equipment and currently cannot be used for large scale extraction. It is preferable to use these methods to extract a small number of samples for research and analysis in the lab scale. In contrast, conventional extractions need less expensive equipment and can be scaled up for industrial purposes. Their low extraction efficiency can be compensated by performing two or more extraction steps such as ethanol maceration. Rufflex extraction can be a choice for scale-up if the use of aqueous ethanol is more preferable, or water boiling can be chosen for the solvent-free extraction. Production cost, time, equipment and subsequent purification steps are the main consideration when choosing the initial extraction methods.

In this study, rutin content in cassava leaves was found up to 24 g/kg or 2.4% of dry weight. This finding revealed that the rutin contents in cassava leaves from Bandung are significantly higher than from China as previously reported by Tao et al. (2019). The origin, growth environment, plant age, and the type of cassava plants might play pivotal factors on the rutin content. Hence, further works will be very interesting to study such factors since cassava plants are widely distributed and growing well in all region of Indonesia. Moreover, there are several types of cassava plant currently cultivated in Indonesia that have not been studied regarding their rutin content yet.

### 3.3. Simple purification of rutin

Methods for rutin purification are diverse and have been described in several scientific papers or patents. At the moment, rutin has been produced commercially from buckwheat, Sophora plants, fava d’anta, Uncaria elliptica, and eucalyptus (Humphreys, 1964; Chang and Muir, 2006; Minami et al., 2012). Although the purification methods used in factories were not available or described in details, in general, purification of rutin involves aqueous extraction and precipitation. Each plant may have different extraction and purification techniques to obtain rutin in efficient ways. To our knowledge, no one has reported the purification of rutin flavonoid from the cassava leaves. Tao et al. (2019) has only used macroporous resins to obtain flavonoids-enriched extracts from cassava leaves but did not continue the purification.

In this work, the classical method of rutin purification was adopted. The water extract of cassava leaves obtained with MAE was incubated at 4 °C overnight to precipitate rutin. After a few steps of non-chromatographic purification, the purified flavonoids were obtained. This method yielded about 30% flavonoids from water extract. Therefore, the optimization or developing other methods for rutin purification are needed to increase the yield.

HPLC analysis of the purified flavonoids (Fig. 3) showed two compounds, where compound 1 has been confirmed as rutin based on the similar retention time with the authentic standard and compound 2 was unknown. Further confirmation with the liquid chromatography-tandem mass spectrometry resulted in [M+H]+ ions at m/z 611 and 595 for compound 1 and 2, respectively (Fig. Suppl. 1-2). Based on the authentic standard, the parent ion at m/z 611 corresponds to rutin (C_{27}H_{30}O_{16}) which is typically fragmented into two daughter ions at m/z 465 due to the loss of the rhamnosyl (m/z 146) and at m/z 303 as its aglycon ion (Fig. Suppl. 1). Compound 2 has a similar fragmentation pattern to rutin which also has two sugar moieties. It is identified as nictitoflorin (C_{22}H_{30}O_{15}) or kaempferol-3-O-rutinoside (Fig. Suppl. 2).
which has been reported its occurrence in cassava leaves (Tao et al., 2019).

HPLC analysis revealed that the simple purification method can provide the flavonoids with purity >99% of the percentage area. The purified flavonoids, however, consist of 82% of rutin and 17% of nicotiflorin. The results confirm that rutin and nicotiflorin are major flavonoids present in cassava leaves as described by Tao et al. (2019). Unlike the existing plants used for rutin production which usually yield rutin as the sole component, the purified flavonoids from cassava leaves tend to contain rutin and nicotiflorin which might not be separated without subsequent extensive chromatography. Such a result could be the typical rutin obtained from cassava leaves (Fig. 4).

Results provide a new alternative source of bioflavonoids with a high content of rutin. Cassava leaves have several advantages that cannot be found in the existing sources of rutin such as buckwheat, fava d’anta (Dimorphandra mollis), Uncaria elliptica, and pagoda (Sophora japonica) (Balz and Das, 1979; Gevrenova et al., 2007; Lucci and Mazzafera, 2009; Bai et al., 2015). Buckwheat, a short-season crop, cannot be grown throughout the year and the seeds where the highest content of rutin found are edible, hence, limit its use for the rutin production. The last three sources are tree plants that need longer periods to cultivate and the seeds, pod, and buds/flowers where the highest content of rutin found are seasonal and therefore considered as the limitations. As a comparison, cassava is a crop plant that extensively cultivated in the tropical region of Asia and Africa for its edible tuber as carbohydrate sources. Although a perennial plant, the cultivation of cassava is not seasonal. Currently, cassava leaves can be considered free as a by-product after harvesting the tuber and therefore might be economically competitive for rutin production. The plant is seasonal independent that will provide the leaves throughout the year as the raw material. The world cassava production is reaching more than 20 million ton annually, however, no data provide the number of cassava leaves resulted as a by-product from harvesting. Hence, more studies are needed to evaluate the economic feasibility of rutin production from cassava leaves.

4. Conclusions

The choice of extraction methods determines the yield of flavonoids extracted from plants. As proven in numerous studies, the advance extraction methods, such as UAE and MAE, can increase the yield of targeted compounds. In cassava leaves, UAE and MAE
showed a higher yield for rutin compared to the conventional extraction methods. The extraction conditions resulted in rutin yield up to 24 g/kg dry weight where the extraction efficiency reached 99% of the rutin total. Subsequent steps for rutin purification by using chilling method resulted in high purity of flavonoids. Moreover, the characteristic of purified flavonoids from cassava leaves are the presence of rutin and nicotiflorin in a certain ratio. Results inform a new promising source of bioflavonoids.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary material

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