Quality control standardization of Indonesian noni fruit (Morinda citrifolia) extract and evaluation of their angiotensin-converting enzyme inhibitory activity

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Abstract

Morinda citrifolia fruit (Noni) has been used as a folk medicine in several countries. Noni possessed various pharmacological activities such as; anticancer, antidiabetic, antihypertensive, antarthritic, and antioxidants. The present study evaluated pharmacognostic properties, profiling of active constituent through High-Performance Thin Layer Chromatography (HPTLC) and Liquid Chromatography-High Resolution Mass Spectrometer (LC-HRMS) run for quantitative and qualitative phytochemical analysis and determining angiotensin-converting enzyme (ACE) inhibitor activity of Noni from three different locations. The physicochemical parameters of crude drugs and extracts met the requirement of Indonesian Herbal Pharmacopeia. Total phenol content was 2.16-3.08 mg GAE/g extract and total flavonoid content was 0.11 - 1.58 mg QE/g extract. HPTLC analysis revealed that scopoletin content in Noni was in the range of 0.44 - 0.51%. The results were also corresponding well with LC-HRMS fingerprint analysis. In addition, Noni fruit extract from Bogor potential exhibited activity in inhibiting ACE with an IC50 value of 206.26 µg/mL.

Keywords

Angiotensin-converting enzyme, herbal medicine, Morinda citrifolia, scopoletin, standardization

Introduction

It has been known that traditional medicine has played a crucial role in preventing and treating several diseases of humankind since ancient times due to lesser side effects related to their use. The use of herbal medicine continued to increase and reported many people restored to traditional medicine to treat various health challenges in different national healthcare settings (Ekor 2014). It is estimated that more than 80% of people, particularly in developing countries, rely on natural products as a primary source of their medication (Dias et al. 2012). Therefore, the use of this herbal medicine must be supported by documentation and stringent quality control to be accepted in medical
systems worldwide (Shreedar et al. 2013). Standardization of herbal medicine through pharmacognostic, phytochemical analysis, and preliminary phytochemical studies are globally carried out to verify, authenticate genuine ingredients, and essential for considering the pharmacological effect of the natural product and endorsing the efficacy and safety of the crude drug. Correct identification and quality assurance of components are indispensable to ensure reproducible herbal medicinal results, which will support their safety and efficacy (Shreedar et al. 2013). Moreover, it is necessary to validate the quality of traditional medicine to avoid any adverse effects (Amir et al. 2019). Pharmacognostic standardization of plant materials includes morphological, anatomical, and biochemical properties (WHO 2004).

*Morinda citrifolia*, belongs to family Rubiaceae, has been used as traditional medicine in several countries including Indonesia. This plant possessed several pharmacological activities such as immunomodulator, anti-inflammation, anticancer, anti-diabetic, hepatoprotective and anti-hypertension (Hirazumi et al. 1999; Liu et al. 2001; Wang et al. 2002; Wigati et al. 2017). Several phytochemical compounds have also been isolated from the fruit of this plants, for instance; 3-chloro-8-methylthio11H-indol-3-β-ol, 5-Pregnen-3β-ol-20 onefluoroacetate, α-amyrin, pinene, (2E) -3, 7, 11, 15-tetramethyl-2- hexadecen-1-ol, stigmasterol, lignan Americanin A, quercetin 3-O-β-D-glucopyranoside and ursolic acid (Sang et al. 2001; Singh et al. 2012; Kovenda-na et al. 2014).

In research based on natural materials, one of the crucial factors is the place of growth and the variable factors of each sampling location. Therefore, the sampling location was determined from three location points around the research area, which is an area that grows a lot of noni fruit so that it can be used sustainably and developed into valuable downstream areas. Many studies report the potential of noni fruits to be developed as a standardized herbal medicine. However, there is few information available about standardization and quality control of Noni fruit collected from several locations in Indonesia. In this study, we chose three locations: Sukabumi, Bogor, and Tangerang, with different altitudes, temperatures, and rainfall. Sukabumi is located in the highlands (300 – 1,000 m); the climate tends to be wet, with temperatures from 18 °C to 29 °C and an average annual rainfall of 2,805 mm/year; Bogor has a minimum elevation of 190 m and a maximum of 330 m above sea level, a wet tropical climate with high rainfall, and a temperature of 21 °C to 32 °C. Meanwhile, Tangerang is located in the lowlands (≤ 44 m), the air temperature ranges from 23.4 °C to 34.2 °C, the average humidity is 80.0%, and the rainfall is moderate (https://www.bps.go.id/). Therefore, the present study to evaluate pharmacognostic properties such as macroscopical, microscopical, and physicochemical characterization and profiling of active constituent of Noni fruits. Moreover, angiotensin-converting enzyme (ACE) inhibitors were also determined.

### Materials and methods

#### Chemicals

Scopoletin, gallic acid, quercetin, Folin Ciocalteu’s, gallic acid, HEPES and Rabbit lung by acetone dehydrated were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Ethanol 96%, methanol, ethyl acetate, n-hexa-ne from JT Baker. Substrate hippuryl-glycyl-glycine (Hip-Gly-Gly) was from Bachem (Torrance, CA, USA), and captopril (Cat: 1091200 US Pharmacopeial).

#### Plant collection and extraction

The ripen fruits of *Morinda citrifolia* (Noni) were collected from three growing different locations in Indonesia such as Tangerang, Bogor, and Sukabumi, in September 2020. Plants were identified by Botanist in the Herbarium Bogoriense, Research Center for Biologi-BRIN. Samples were washed with running tap water, drained, and chopped into small pieces. Samples were dried in an oven at temperature of 45 °C for three days and ground into powder for further analysis. Each the dried powdered of fruit (500 g) were extracted using 70% ethanol under macerati-on condition. The organic solvents were evaporated using rotary vacuum evaporator to produce crude extract.

#### The quality control standardization of crude drugs and crude extracts

The assessment including macroscopic/microscopic studies, water content, ash content, acid insoluble ash content, water soluble extract content, ethanol-soluble content, contaminant, water soluble extract content, ethanol-soluble contaminant, pathogen-microbial contaminant, and heavy metal contaminant were referred to the Indonesian Herbal Pharmacopeia (PHI)14 (Depkes 2008).

#### Phytochemical analysis

Phytochemical analysis to detect the presence of alkaloid, flavonoids, triterpenes/steroid, and saponin in the extract using standard methods was carried out using colorimetric methods according to previous study with slight modification (Yadav et al. 2011). The total phenolic content was estimated according to Folin-Ciocalteu method and total flavonoid was determined according to aluminums trichloride method using quercetin as the reference compound (Dewi et al. 2019).

#### High Performance Thin Layer Chromatography (HPTLC) analysis

#### Preparation of standard compound and extract

Stock solution of standard scopoletin was prepared in methanol. The working solution of standard compound was subsequently diluted in methanol to afford a series
of scopoletin solution of 10, 50, 100, 200 and 500 μg/mL. Next, each Noni crude extracts (100 mg) were dissolved in 10 mL methanol.

**Chromatographic condition**

Scopoletin and Noni crude extracts were separated and analyzed using a HPLC system (CAMAG Linimoat 5, Switzerland). Chromatographic development was carried out on 10×10 cm precoated silica gel 60 F254, aluminium plate (E. Merck) chamber with ethyl acetate–n-hexane (3:2, V/V) as mobile phase. Two (2) μL of standard compound and extracts were applied to the plate using of an automatic specimen applicator (CAMAG Linimoat 5, Switzerland), fitted with a Hamilton microliter syringe (Bonaduz, Switzerland). The conditions were set at band length 8 mm. HPTLC plates: 20×10 cm, 0.2 mm thickness pre-coated with silica gel 60 F254; Merck. Band size: 6 mm, slit dimension: 5.00 × 0.45 mm. Scanning speed: 10 mm/s. Experimental conditions: temperature was 28±2 °C; relative humidity was 40%. After developing, the TLC plate was dried using an air dryer and for post-chromatographic treatment sulfuric acid in methanol (5%) reagent was used as visualization agent. Quantification measurements were analyzed using UNIFI software version 1.5 and the peaks were tentatively assigned with the comparison to Waters build-in library, while scopoletin was assigned by comparison to authentic standard of scopoletin (Sigma, S2500). MS conditions were as follow: column temperature 40 °C, mass range: 100–1200 Da, cone voltage 30 V, capillary 2kV, source temperature 120 °C, desolvation temperature 500 °C, cone gas flow 50 L/h, desolvation gas flow 1000 L/h, collision energy (ramp: 10–40 eV). Leucine enkephaline was used as an internal mass correction, infused every 10 s during the whole run.

**Angiotensin-Converting Enzyme (ACE) inhibitory activity**

This assay was performed as previously described with minor modifications (Sera et al. 2004; Endringer et al. 2014) by measuring the cleavage of the substrate hippuryl-glycyl-glycine by angiotensin converting enzyme (ACE) and subsequent reaction with trinitrobenzene sulfonic acid to form 2,4,6-trinitrophenyl-glycyl-glycine. Its Absorbance of the final product was read at 415 nm in microplate reader (Multiscan GoThermoscientific). The readings were compared against a blank solution (DMSO), which was prepared in a similar manner except for sodium tungstate and sulfuric acid solutions which added before enzyme. Captopril was used as a reference standard. Assay was performed in triplicate, and the results were expressed as percentages of inhibition (% inhibition = (AB – AS)/AB) × 100%, where AB is the absorbance of the blank solution and AS is the absorbance of sample.

**Statistical analysis**

Each experiment was performed in triplicate. To calculate standard error and mean, Microsoft Office Excel 2019 (Microsoft Corp, USA) was used.

**Results and discussion**

**The quality control standardization of crude drugs and crude extracts**

It has been known that pharmacognostic evaluation of medicinal plant is an important step in quality control of herbal medicine. Macroscopic and microscopic characterization as well as phytochemical analysis have been performed to identify and detect some adulteration and substitution that may be reduce purity and quality of raw materials. In this study, Noni crude drugs and its extract have been standardized based on its pharmacognostic and phytochemical analysis.

**Macro- and microscopic description of Noni’s fruit crude drug**

As shown in Fig. 1A, the macroscopic characterization of Noni fruit was ovoid, ellipsoid or roundish (3–10 × 3–6 cm) with an embossed appearance, slightly wrinkly, waxy, semi-translucent skin, and turns from green to yellow and to almost white as it ripens; the fruit surface is faintly patterned with 4- to 6-sided outlines, each with a central “eye”; the pulp is fleshy and juicy, dull-yellow or yellowish white and gelatinous when the fruit is ripe; it has numerous hard oblong-triangular...
reddish-brown pits, each containing 4 seeds about 3.5 mm long. In the form of a cross-section of the fruit, the shape of a flat slice, the outer surface is smooth with remnants of the seed burrows, the inner surface is rough, there are 4–5 fruit compartments, each partition with 2–3 seeds, the protrusions of the seeds are clearly visible, brown in color, characteristic odor, slightly bitter taste. Furthermore, for its microscopic characteristic, the crude drugs contain thin-walled parenchyma cells, exocarp and sparsely distributed thin strands of vascular tissue containing tracheary elements with annular, spiral and rarely, pitted secondary walls; acicular calcium oxalate crystals, starch granules and oil globules (Fig. 1B). The macroscopic and microscopic observations revealed that noni fruit crude drug was in agreement with the Indonesian Herbal Pharmacopeia.

**Preliminary phytochemical screening**

Phytochemical screening provides a general overview of active compounds presence in the medicinal plants. Phytochemical compounds in a plant extract can be affected by several factors, including geographical location, harvest time, and extraction method. The results of preliminary phytochemical screening of Noni fruits obtained from three different locations were presented in Table 1. It showed that several phytochemical constituents such as alkaloids, flavonoids, quinones, and saponins were found in all extracts from different locations. However, terpenoids and saponins were not presented in the extracts from three locations. The presence of wide range of bioactive compounds in aqueous and alcoholic extracts were accordance with earlier studies (Ranvir et al. 2017).

Furthermore, the total phenol (TPC) and flavonoid content (TFC) in the extracts were also determined using colorimetric method. The results were interpreted as gallic acid equivalent (GAE) and quercetin equivalent (QE) in mg/g extract. As shown in Table 1, Noni fruit extract from Bogor contains the highest values of total phenol and flavonoids compared to others extract with TPC and TFC values of 3.08±0.72 mg/g GAE and 1.11±0.35 mg/g QE, respectively. The TPC value of these three Noni extracts is higher than the TPC value of Noni fruit extract from the Semarang area which is around 0.051±1.677 mg gallic acid equivalent per g of extract (Wigati et al. 2017).
Physicochemical, heavy metal contaminant and microbial contaminant analysis

Physicochemical standard gives important information for further investigation and facilitate the identification of formulations in routine industrial production. The results of physicochemical analysis of Noni fruit crude drugs were displayed in Table 2. The result of loss on drying revealed that all crude drugs obtained from different locations were less than 10%. It has been known that the value of loss on drying (LOD) at 110 °C should be less than 10%, to eliminate contamination by mold and growth. Furthermore, determination of total ash content play role in order to evaluate the purity and presence of inorganic material such as sulphated ash, water soluble ash, acid insoluble ash, salt and/or silica in the samples. The results revealed that the total ash and the acid insoluble ash contents were less than 1% for crude drugs and 15% for extracts. Next, the water content refers to the minimum range amount of water content in the extract was determined by toluene distillation. It has been known that water content is associated with quality of extract in which less water content related with less possibility mold contamination. Water content of crude drugs and extracts were between 2.14–3.20%. This result met the standard of PHI (less than 10%). All those parameters meet the requirements of the Indonesian herbal pharmacopeia.

Contamination by heavy metals such as mercury (Hg), copper (Cu), cadmium (Cd), and arsenic (As) in herbal remedies can be attributed with several aspects, including environmental pollution, and can pose clinically relevant dangers for the health of the user and should therefore be limited (WHO 1998). Inductively coupled plasma - optical emission spectrometry (ICP-OES) was used to determine contamination of crude drugs and extracts from heavy metal. As shown in Table 2, there was no heavy metal detected in crude drugs and extracts obtained from three different locations. The results were in agreement to previous study where the maximum limit for Pb ≤ 10 mg/kg, Cd ≤ 0.3 mg/kg, Hg ≤ 0.5 mg/kg, and As ≤ 5 mg/kg (Angelina et al. 2021). The next parameter of herbal quality control is microbial contamination. The result showed that there was no contamination from any bacteria such as Escherichia coli, Staphylococcus aureus; Pseudomonas aeruginosa, Salmonella sp, and Shigella sp., found in all crude drugs and extracts (Table 2). Thus, the results of heavy metal and microbial contamination analysis was in agreement with Indonesian Herbal Pharmacopeia (BPOM RI 2010).

### Table 1. Physicochemical, microbial contaminant and heavy metal contaminant analysis of Morinda citrifolia crude drugs and extracts obtained from different location.

| Parameter                  | PHI  | Bogor            | Tangerang        | Sukabumi         |
|----------------------------|------|------------------|------------------|------------------|
| Loss on drying             | < 10 | 7.64±0.31        | 5.93±0.66        | 4.28±0.69        |
| Water content              | < 10 | 2.67±0.71        | 2.00±0.63        | 3.20±0.80        |
| Total Ash (%)              | < 7  | 6.47±0.72        | 5.06±0.27        | 4.80±0.37        |
| Acid insoluble ash (%)     | < 2  | 0.91±0.05        | 0.51±0.03        | 0.21±0.10        |
| Water soluble extractive value (%) | > 21 | 44.61±3.56       | 42.77±1.37       | 47.85±0.71       |
| Ethanol soluble extractive value (%) | > 9.8 | 18.95±1.42       | 12.96±1.27       | 14.59±1.54       |
| Scopoletin content (%)     | > 0.02 | 0.03±0.001       | 0.02±0.002       | 0.02±0.001       |
| Microbiological test *     | Absent | Absent           | Absent           | Absent           |
| Heavy metal determination ** | ND   | ND               | ND               | ND               |

**PHI: Pharmacopoeia Herbal Indonesia**

* Salmonella spp.; Escherichia coli; Staphylococcus aureus; Pseudomonas aeruginosa

** Hg; Pb, As, Cd

ND: Not detect

### Table 2. Phytochemical analysis of Morinda citrifolia fruit extract obtained from different location.

| Parameters                     | Bogor            | Tangerang        | Sukabumi         |
|--------------------------------|------------------|------------------|------------------|
| Alkaloids                      | ++               | ++               | ++               |
| Flavonoids                     | ++               | ++               | ++               |
| Triterpenoids/steroids         | –                | –                | –                |
| Tannins                        | –                | –                | –                |
| Saponins                       | ++               | +                | +                |
| Quinones                       | +                | +                | +                |
| Total phenolic content (mg GAE /g) | 3.08±0.72       | 2.26±0.91        | 2.16±0.23        |
| Total Flavonoid content (mg QE /g) | 1.11±0.35       | 0.76±0.16        | 1.58±0.81        |
Analysis of scopoletin content in the extract by HPTLC

HPTLC has been used as analytical instrument in herbal medicine quality control and fingerprint of plant drugs. Quality control of crude drugs and is based on plant characteristics and morphology and the analysis of marker compounds. It has been reported that the main secondary metabolites of noni fruit are anthraquinones, scopoletin, quercetin, and ursolic acid, as well as some flavonoid compounds (Almeida 2019). According to Indonesian herbal monograph, scopoletin has been used as a biomarker for the standardization of noni fruit extract (PHI 2008). Scopoletin is a coumarin compound which shows a unique spot-on TLC plate under ultraviolet irradiation at of 365 nm which emits blue fluorescence (Fig. 2). Furthermore, the optimum eluent system to achieve TLC profile results showed spots at Rf 0.33. Was hexane: ethyl acetate (2:3). A shown in Fig. 2, scopoletin presence in all Noni extracts from different location is in accordance to standard marker. Based on this result, all those Noni crude drugs and extracts met the requirements of Indonesian Herbal Pharmacopeia. The scopoletin content in crude drugs and extract were 0.02% and 0.38%, respectively. However, only crude and Noni fruit extract from Bogor has scopoletin content higher than the levels that are stated in the monograph, were 0.03% and 0.51%, respectively. It has been known that, one of key factor that affected the quality of herbal medicine in term of its active constituents is location. Differences in growing location and plant age generally cause significant differences in primary and secondary metabolites (Yang et al. 2018), including the marker compound scopoletin in three Noni fruit extracts.

Phytochemical analysis using LC-HRMS

Almost 200 phytochemicals were reported until 2012 from different parts of Noni fruit (Singh 2012); however, the complete chemical constituent has not been fully reported. Some of the known phytochemicals detected...
in Noni fruit was scopoletin (Mahanthesh et al. 2013), deacetyl asperulosidic acid and asperulosidic acid (Yang et al. 2009) and rutin (Lewis Lujan et al. 2014). Analysis of three standardized crude extracts using LC-HRMS revealed the presence of scopoletin, which was compared to the authentic standard of scopoletin (m/z = 193.0527 [M+H]+, t_R = 3.87 min) shown in Fig. 3. Extracted ion chromatogram of rutin ([M+H]+ = 611.1606) on three samples showed a peak (t_R = 3.67 min) tentatively assigned as a rutin. This assignment made on the basis of MS fragmentation pattern which were showed fragmentation at m/z = 463.1784 [M+H]+, m/z = 487.2463 [M+Na]+ and m/z = 303.0509 [M+H]+, similar to observation from Keki et al (2001). In the other hand, extracted ion chromatogram for asperulosidic acid (m/z = 433.1340 [M+H]+), deacetyl asperulosidic acid (m/z = 391.1234 [M+H]+) and morindin (m/z = 565.1551 [M+H]+) showed no presence for those compounds in the standardized crude extract (Table 3).

The standardized Noni crude extracts obtained from Tangerang, Bogor, and Sukabumi showed no difference in the BPC chromatograms profile. The relative intensity Fig. 4, showed the highest scopoletin content was present in extract obtained from Bogor (563.050; 6.40 % area) compared to Tangerang (306.956; 3.4 % area) and Sukabumi (441.291; 5.29 % area), which was corroborated the result from the HPTLC analysis.

### Determination of in vitro ACE inhibitory activity

Noni has been reported to possess several pharmacological properties, including decreasing high blood pressure, lowering blood sugar, and antioxidants. (ACE) plays important role in the renin-angiotensin system to controls blood pressure. ACE catalyzes the conversion of inactive decapeptide angiotensin I to active octapeptide angiotensin II. The use of ACE
inhibitors is well established as one of the therapeutic principles in the treatment of hypertension (Sera et al. 2005). In Indonesia, Noni has long been used as herbal medicine to help lower blood pressure; as reported by Yanti et al. 2020, Noni is used with other herbal mixtures as a traditional antihypertensive medicine. Therefore, the present study was also performed to evaluate the pharmacological effect of Noni fruit extract on ACE. In this study, the ACE inhibition assay was carried out using a colorimetric method using hippuryl-glycyl-glycine (Hip-Gly-Gly) as a substrate and TNBS as a color indicator which will form the yellow color TNP-Gly-Gly, detected at 415 nm (Sera et al. 2005). As presented in Table 4, showed Noni fruit extract from Bogor exhibits strong activity in inhibiting ACE with an IC$_{50}$ value of 206.26 µg/mL, when compared to extracts from Tangerang and Sukabumi with IC$_{50}$ value of 232.95 and 220.91 µg/mL, respectively. This finding was supported by a previous study in which the IC$_{50}$ value of antihypertensive herbal extracts containing noni was around >200 µg/mL (Yanti et al. 2020). In addition, the ethanolic extract of M. citrifolia leaves and fruit also displayed a significant antihypertensive activity in dexamethasone-induced hypertensive rats (Wigati et al. 2017).

**Table 4.** The ACE Inhibitory activity of *Morinda citrifolia* crude extracts obtained from different locations.

| Sample    | IC$_{50}$ (µg/mL) |
|-----------|-------------------|
| Bogor     | 206.26±13.28      |
| Tangerang | 232.95±8.94       |
| Sukabumi  | 220.91±11.71      |
| Captopril | 52.26±3.46        |

**Conclusion**

It can be concluded that the Noni crude drug and extract in this study fulfilled the requirements as raw material for herbal medicines and showed ACE inhibitor activity. Further, the isolation of active compounds and their characterization from the extract and in vivo antihypertensive activity will be evaluated and reported soon.

**Author contribution**

RTD: Conceptualization, methodology, investigation, formal analysis, supervision, writing original draft; GP: Methodology, investigation, formal analysis, writing-review; AWS: Validation, formal analysis, supervision, writing original draft; MA: Methodology, investigation, formal analysis, writing-review; LM: Investigation, formal analysis, validation; SF: Validation, formal analysis; GFS: Validation, formal analysis

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