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The Effect of Cultivation Site Altitudes on The Quality Parameters of Meniran (*Phylanthus niruri* L.)

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ABSTRACT

Meniran *(Phyllanthus niruri* L.) is known as a medicinal plant for immunomodulatory, hypoglycemic, diuretic treatment, and kidney disorders. It has phenolic and flavonoid as the active compounds. Several commercial products utilize meniran as the main ingredient for immunomodulators. However, the standard raw material for production has not been well reported. The study aimed to standardize the quality of meniran's raw materials from different locations and altitudes. The phenolic compounds as the active component and their antioxidant activity were further studied. The raw materials were collected from Sukoharjo, Jember, and Karanganyar which are located from 104 masl, 243 masl, and 722 masl. The specific and non-specific standardization based on Indonesian Herbal Pharmacopoeia were investigated. The antioxidant activity was investigated. It resulted in the specific and non-specific parameters that were according to standard except for the acid soluble ash test from the Jember sample which was much higher (6.333% ± 0.969) than the standard (<1.2%). In addition, the flavonoid (1.529 ± 0.167%w/w) and phenolic (1.65 ± 0.006 %w/w) content showed that the sample collected from Jember was significantly higher than other locations. However, the strongest antioxidant activity was in the sample collected from Sukoharjo (IC_{50} 101.84µg/mL). On the other hand, there was no correlation between the total flavonoid and the total phenolic to antioxidant activity in this plant. It suggested that the antioxidant activity in these plants did not depend on the flavonoid and phenolic compounds. In conclusion, the raw material of meniran (*Phyllanthus niruri* L.) collected from 3 different altitudes showed the quality of herbal medicine of mainly non-specific parameters and met the requirements of Indonesian Herbal Pharmacopoeia. The total phenolic and flavonoid as the active substances varied through all 3 different altitudes. It was in line with the antioxidant activity which varied to all 3 different altitudes due to the phytochemical profile variety.

Keywords: antioxidant; flavonoid; phenolic; *Phyllanthus niruri*; standardization

INTRODUCTION

Meniran (*Phyllanthus niruri* L.) is well-known as a traditional medicine in Indonesia. It comes from the whole plant including roots, stems, leaves, and seeds. It has been reported to have anti-bacterial properties (Gunawan *et al.*, 2008), anti-cancer (Ifandari *et al.*, 2013), immunomodulators (Aldi *et al.*, 2014), hypoglycemic (Chairul *et al.*, 2000), diuretic treatment (Rosadi and Marini, 2016), kidney disorders (Winarti *et al.*, 2014), antigout (Fariz *et al.*, 2018), antipyretics (Jansen *et al.*, 2015) and bile disorders (Alegantina *et al.*, 2015). It is one of the herbal products that are in most demand during the COVID-19 pandemic to support the immune system.

Known as an herbal medicine, the study of this plant goes in several ways that correlate with the immune system. One of the most studied is the antioxidant that can balance free radical activity. It correlates with damage to membranes which contributes to one associated with dysregulation of immune (Hajian, 2014). Besides, antioxidant plays an important role in enhancing the healthy system by preserving an immune cell against oxidative stress (De la Fuente, 2002). Therefore, the study of antioxidants related to supporting the immune system is still needed to investigate mainly herbal medicine which is proven commercially as an immunomodulator.

As commercial products, there are external and internal issues that influence the quality of herbal medicines. The former includes contamination which covers non-specific parameters such as lost on drying, total ash and insoluble acid ash. It is affected by, one of them is manufacturing i.e. post-harvesting step which determine the quality of herbal medicines. The less lost on drying the better the herbal is. It produced the dried raw material stayed longer during

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storage. Moreover, the higher ash content the less quality of herbal products due to the high content of mineral. The excess of mineral, including toxic metals, might be harmful to human.

The internal issues influencing the herbal medicines quality are due to the pharmacologically active phytochemicals such as water-soluble extract, ethanol-soluble extract, flavonoid and phenolic content. It might be affected by the cultivation specifically the altitude of growth which somehow are crucial factor related to the abundance of active substances. The total flavonoid content in Sambaing colok (*Iresine herbstii*) depended on the altitude of growth; the higher the plant grow the higher the total flavonoid content is (Safrina *et al.*, 2018). These phytochemicals are associated with the pharmacological activity. Thus, it is necessary to understand the optimum altitude for growing herbal medicines. Therefore, this study aimed to determine the quality of herbal medicine meniran (*Phyllanthus niruri* L.) by measuring the specific and non-specific parameters. Besides, the investigation of total phenolic, total flavonoid and its antioxidant activity were studied.

This study collected meniran (*Phyllanthus niruri* L.) from 3 different altitudes i.e. Sukoharjo (104 masl), Jember (243 masl) and Karanganyar (772 masl). The specific and non-specific parameter of standardize herbal medicine were studied to all samples. The antioxidant activity was investigated afterwards.

METHODOLOGY

Materials

Meniran (*Phyllanthus niruri* L.) has been cultivated by local farmer at Sukoharjo and Karanganyar. Two months old of plants are ready to be harvested to all part of plants. The harvesting and post-harvesting were handled properly as Good Agricultural and Collection Pratices (GACP) in order to maintain the good quality of raw materials. Besides, plant material from Jember was collected from commercial suppliers.

Quercetin standards (*Sigma*), DPPH (*Sigma*), natrium carbonat (*Merck*), dan folin-ciocateu (*Merck*), gallic acid (*Merck*), aquadest, hydrochloric acid (*Merck*), ethanol (95%) (*J.T. Bakker)*, chloroform (*Merck*), hexamethylentetramine (MKR), acetone (*Merck*), ethyl acetat (*Merck*), acetic acid (*J.T. Bakker)*, methanol (*J.T. Bakker)*, aluminum chloride (*Merck*).

Methods

The determination of the plant

The plant was determined in the Biology Laboratory of Mathematics and Natural Science Universitas Sebelas Maret.

Non-specific parameters

Lost on drying test. Two gram of dried material was placed into the moisture balance and measured to drying lost.

Total ash test. Two gram of dried material was placed in the crucibles and processed in the furnace at 600°C. Let it cool at room temperature, weigh and determine the total ash (Departemen Kesehatan Republik Indonesia, 2008).

Insoluble acid ash test. 25 ml of HCl 1 N was added into total ash from the previous step and boiled for five minutes. The acid-insoluble part was collected and filtered. It then was washed with hot water, smelted in a crucible and weighed until a constant weight (Departemen Kesehatan Republik Indonesia, 2008).

Specific parameters

Water Soluble Extract Test. Five gram of dried material was macerated using 100 ml chloroform saturated water for 8 hours. 20 ml of filtrate was filtered and evaporated on a water bath at 105°C until a constant weight. Then, percent of extract was calculated (Departemen Kesehatan Republik Indonesia, 2008).

Ethanol Soluble Extract Test. Five gram of dried material was macerated using 100 ml 96% ethanol solvent for 18 hours. 20 ml of filtrate was filtered and evaporated on a water bath. Then, percent of extract was calculated (Departemen Kesehatan Republik Indonesia, 2008).

Total Flavonoid Quantification

Total flavonoid was analyzed using aluminium chloride colourimetric method (Benítez *et al.*, 2011). Quercetin used as standard. It was diluted to 2, 4, 6, 8, and 10 ppm. Reagen of 1 mL HMT solution, 20 mL aceton, and 2 mL HCl and 200 mg sample were mixed and extracted using reflux extraction method two times. It was then separated by funnel three times using 20 mL aquadest and 15 mL ethyl acetate. Ten mL of each standards solution were mixed with 1 mL $AlCl_3$ 2% and ad 25 mL CH_3COOH 5%. The mixture was incubated at room temperature for 3- minutes then measured at 380 nm using GenesysTM UV-Vis spectrophotometer.

Total Phenolic Quantification

Determination of total phenolic content using UV-Vis spectrophotometer has been described below (Sun, Powers and Tang, 2007). Gallic acid standard was made in series with a concentration of 10-50 µg/m. Determination of the maximum wavelength using additional 5 mL of Folin-Ciocalteu reagent (1:10 in distilled water) and 4 mL of 1 M sodium carbonate which was allowed to stand for 30 minutes. Scanning uses a wavelength of 600-800 nm. Dried material at concentration of 400 µg/mL was weighed and diluted to 10 µg/mL using methanol. The 10 µg/mL test solution was then added with 5 mL of Folin-Ciocalteu reagent (1:10 in distilled water) and 4 mL of 1 M sodium carbonate and scanned at the maximum wavelength.

Antioxidant activity by DPPH scavenging assay

Antioxidant activity test using DPPH 100 µg/mL. The standard solution used is quercetin with a concentration series of 5-15 µg/mL. Determination of the maximum wavelength using DPPH solution with various concentrations of 20, 40, and 80 µg/mL which was shaken for 30 seconds and allowed to stand for 30 minutes for operation time. Scanning was carried out using a UV-Vis spectrophotometer at a wavelength of 400-600 nm. The control absorbance measurement of the DPPH solution used 2 mL of DPPH solution which was diluted in 10 mL of methanol and then allowed to stand for 30 minutes. The meniran test solution was made in series with a concentration of 40-60 µg/mL. The absorbance control solution and the test solution were scanned at the maximum wavelength.

The data analysis

The specific and non-specific parameter data were collected from 3 different altitudes were statistically analyzed using the *one-way* analysis of variance (ANOVA) test. Post-hoc analysis was LSD *post hoc* test with a confidence level of 95%.

RESULT AND DISCUSSION

The plant was determined as *Phyllanthus niruri* L by the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta No. 162/UN27.9.6.4/Lab/2019. It has been known as meniran in local resident (Figure 1). Plants which were cultivated by local farmer were then harvested and processed at the Post-Harvest Processing Center, Public Health Office, Karanganyar District.

Lost on drying test was used to determine the weight percent lost during the drying process. It removed most of volatile compounds, essential oils and water up to less than 10%. It is beneficial for dried material during storage. Lost on drying to all samples exhibited in line with standard Indonesian Herbal Pharmacopoeia requirement (< 14%) (2008) (Table I). It is suggested that manufacturing mainly the drying process has been done properly.

Total ash test was determined to measure the total mineral content in the raw material such as anorganic salts (phosphate, carbonate, chloride, nitrate sulfate and alkali metal) and organic salts (salt from malic acid, oxalate, acetic, pectic and others) (Rakhmawati *et al*., 2014). Besides, it showed the contamination such as soil and sand during post-harvest. The total ash and acid insoluble ash content should be less than 0.2% and 1.2% respectively (Indonesian Herbal Pharmacopoeia, 2008). The result depicted sample collected from Karanganyar and Sukoharjo met the requirement, while those collected from Jember did not (Table 1I). It might be caused by the impurities such as soil or sand which remain after the post-harvesting process.

Soluble extract was determined to measure the compounds dissolved in ethanol and water. The more chemical compounds dissolved in water, the more chemical activity of the plant showed (Paramita *et al.*, 2019). Compounds dissolved in water and ethanol in meniran are alkaloid, saponin, flavonoids and tannin (Krisyanella *et al*., 2013). The results showed that samples collected in Sukoharjo, Jember, and Karanganyar met the requirements which were more than 16.0% for water-soluble extract and more than 8.0% for ethanol-soluble extract (Table I).

Total flavonoid was extracted by reflux based on Indonesian Herbal Pharmacopoeia (2018). It is a method to accelerate an extraction thermally for extended period of time which is shorten the extraction time. However, the weakness of this method is that it requires a large amount of solvent (Susanty and Bachdim, 2016). Hydrochloride acid is used to hydrolyze flavonoid glycosides which is common present in plants (Markham, 1988). It generates flavonoid aglycones which is quercetin and sugars. Liquid-liquid extraction using separating funnel applied to separate flavonoid aglycones afterwards. Ethyl acetate used as solvent to dissolve flavonoid aglycones; and water for dissolving sugar (Zirconia *et al.*, 2015). Quercetin was used as standard for total flavonoid quantification. It is one of the

Figure 1. Meniran (*Phyllanthus niruri* L.)

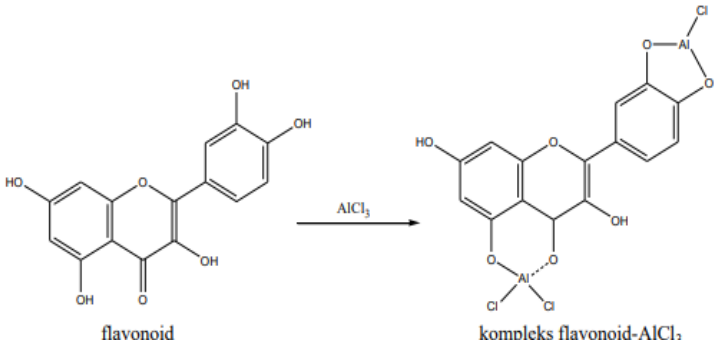

Figure 2. Quercetin and $AlCl_3$ complex reactions (Markham, 1988).

Table I. The result of determination non-specific and specific parameters of herbal simplisia meniran samples Sukoharjo, Jember and Karanganyar. The different letters showed a significant difference at the 95% confidence level compare to Indonesian Herbal Pharmacopoeia (2018).

Sample Origin	Indonesian Herbal Pharmacopoeia	Sukoharjo (104 masl)	Jember (243 masl)	Karanganyar (772 masl)
Non-specific parameters				
Lost on drying test (%)	< 14,00	10.84 ± 0.939 [a]	11.121 ± 0,357 [a]	10,935 ± 0,553 [a]
Total Ash test (%)	< 7,20	6.709 ± 0.100 [b]	14.983 ± 0.190 [b]	7.172 ± 0.020 [b]
Acid Soluble Ash test (%)	< 1,20	0.199 ± 0.050 [b]	6.333 ± 0.969 [b]	0.430 ± 0.028 [b]
Specific parameters				
Ethanol-Soluble Extract (%)	> 8,00	13.71 ± 1.040 [a]	12.822 ± 0.695 [a]	10.327 ± 0.580 [a]
Water-Soluble Extract (%)	> 16,00	23.815 ± 0.275 [a]	22.965 ± 1.490 [a]	22.987 ± 1.009 [b]

flavonoid derivates in the flavonol group (Dewi *et al*., 2018). Linier regression equation for total flavonoid was as follow: y = 0.0689x + 0.0598.

To quantify total flavonoid, the study applied the standard quercetin based on colorimetric complex formation using UV-Vis spectrophotometry (Figure 2). The principle is to form a quercetin complex with $AlCl_3$ so that the absorption band shifts to a longer wavelength. $AlCl_3$ was used as a complex builder due to quercetin has an -OH group neighbors to the carbonyl group and 2 -OH groups in the ortho position. The -OH group complex neighbors to the carbonyl group will be stable with the addition of an acid, while for the 2 -OH group complex in the ortho position is unstable (Markham, 1988). So

Table II. Results of determination of total flavonoid levels herbal simplisia meniran samples Sukoharjo, Jember and Karanganyar. The different letters showed a significant difference at the 95% confidence level within each parameter.

Sample Origin	Sukoharjo (104 masl)	Jember (243 masl)	Karanganyar (772 masl)
Total Flavonoid Levels (%w/w)	0.973 ± 0.105 [a]	1.529 ± 0.167[b]	1.180 ± 0.041 [a]
Total Phenolic Content (% w/w)	1.67 ± 0.01[a]	1.65 ± 0.006[a]	0.90 ± 0.006[b]
Antioxidant Activity (µg/mL)	101.84±2.23[a]	132.35±1.44[b]	174.06±1.92[c]

that the measurement of the test solution is carried out on the complex formed between $AlCl_3$ and quercetin which binds to the -OH group which is neighbors to the carbonyl group. The complex being measured is a complex that is stable with the addition of an acid. The acid used is acetic acid dissolved with methanol.

The total flavonoid content of samples collected from Jember (243 masl) (1.529 ± 0.167% w/w) was significantly higher than those in Karanganyar (772 masl) (1.180 ± 0.041% w/w) and Sukoharjo (104 masl) (0.973 ± 0.105% w/w) (Table II). The total phenolic showed significant lower in Karanganyar (772 masl) (0.90 ± 0.006% w/w) than those in Sukoharjo (104 masl) (1.67 ± 0.01% w/w) and Jember (243 masl) (1.65 ± 0.006% w/w). As comparison, the total phenolic content of methanolic extract meniran was 1.2824% (PUTRI, 2018). The differences occurred due to different location and environment which lead to different phytochemicals profile and abundance. Both total flavonoid and total phenolics did not exhibit the linearity due to altitude. The current result was in line with the previous study by Priti et al (Priti *et al.*, 2021) reported that total phenolic and total flavonoid were not related in microgreens of mungbean and lentil grown in plain-altitude and high-altitude. Moreover, similar study indicated that total phenolic and flavonoid in *Valeriana jatamaansi* were not correlated with altitudes (Jugran *et al.*, 2016). However, a study by Pandey et.al (Pandey *et al.*, 2018) exhibited that total flavonoid and phenolic in *Thalictrum foliolosum* were increased at higher altitudes. This implied that phytochemicals in each plant species reacts differently to the environmental regarding with probably the defense mechanism (Wink, 2008). It resulted the variation of profiling and abundance in secondary metabolites.

Plant collected from Sukoharjo (104 masl) exhibited highly significant higher (IC_{50} 101.84 µg/mL) of antioxidant activity than those in Jember (243 masl; 132.35± IC_{50} 1.44 µg/mL) and Karanganyar (772 masl; IC_{50} 174.06±1.92 µg/mL). Tambunan dkk (Tambunan, Swandiny and Zaidan, 2019) reported that the antioxidant activity of ethanolic extract showed IC_{50} 17.55 µg/ml. In previous studies, the IC_{50} results for the activity test of the methanol extract of meniran leaves were 37.18 µg/mL (Sandrasari, 2011). The variation among the study of meniran might be due to the variation of phytochemicals which lead to variation of antioxidant activity.

Current study showed no correlation either total flavonoid or total phenolic to antioxidant activity in this plant. This was similar to previous study that the correlation between total flavonoid and total phenolic did not significantly correlate in mungbean and lentil, respectively (Priti *et al.*, 2021). While total flavonoid was highly correlated to antioxidant activity in lentil (Priti *et al.*, 2021). Similarly, higher flavonoids in onion skin exert higher antioxidant activities (Sagar, Pareek and Gonzalez-Aguilar, 2020). This implied that plants which did not correlate well between the total phenolic and flavonoid regarding to antioxidant activity might have another mechanism in a certain way to do the bioactivity.

The raw material of meniran (*Phyllanthus niruri* L.) collected from 3 different altitudes showed the quality of herbal medicine mainly non-specific parameter met the requirements of Indonesian Herbal Pharmacopoeia (2018). Unless the total ash and acid soluble as test of sample collected from Jember which was beyond the standard. Regarding to specific parameter that the total phenolic and flavonoid as the active substances varied through all 3 different altitudes. All of them met the requirement of Indonesian Herbal Pharmacopoeia (2018). It was in line with the antioxidant activity which varied to all 3 different altitudes due to the phytochemical profile variety.

CONCLUSION

Meniran (*Phyllanthus niruri* L.) as a commercial product of herbal medicine requires good quality of raw material. This build upon the specific and non-specific parameters based on

Indonesian Herbal Pharmacopoeia. Raw material of meniran showed variety of total flavonoid and total phenolic which did not depend on the altitudes. Moreover, the antioxidant activity in these plants did not depend on the flavonoid and phenolic compounds. There are other compounds synergize with those to produce antioxidant activity.

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