

Antioxidant activity of *Tinospora crispa* extracted with different ethanol solvents



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ABSTRACT

Introduction: *Tinospora crispa* (*T. crispa*) is an herbaceous plant that commonly grows wild in tropical regions of South East Asian countries such as Indonesia, Malaysia, and Thailand. In Indonesia, this plant is well known to be used as a traditional medicine to treat gout, diabetes, hypertension, rheumatic, fever, and appetite stimulant. Researches worldwide indicate that *T. crispa* poses several pharmacological properties. One of those is the antioxidant activity, acting as a free radical scavenger. The objective of this study was to determine the antioxidative properties of *T. crispa* and to compare the different ethanol solvents used for extraction.

Methods: The amount of 300 g *T. crispa* powder was extracted using 70%, 80%, and 96% ethanol. The spectrophotometry method is used to assess the total flavonoid and polyphenol contents as well as the DPPH assay.

Results: The 80% ethanol had the highest flavonoid content $0.090 \pm [0.453\%]$, while 96% ethanol indicated the lowest $0.038 \pm [3.090\%]$. In the case of phenolic content, 96% ethanol showed the highest result $0.521 \pm [11.341\%]$. However, this value was relatively comparable with the other solvents. The highest DPPH activity was shown by 80% ethanol $6.46 \text{ mg} \pm [3.04 \text{ mg}]$.

Conclusion: Despite low in the concentration, flavonoid and polyphenol content was successfully determined from *T. crispa* by using different ethanol solvents. Based on the result of antioxidants concentration and activity, 80% ethanol is the most ideal solvent to be used for extraction of *T. crispa*.

Keywords: *Tinospora crispa*, antioxidant activity, flavonoid, phenolic.

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INTRODUCTION

The development and progression of several chronic diseases including cancer, neurodegenerative, cardiovascular, diabetes mellitus, and aging have been suggested due to the involvement of oxidative/nitrosative stress.^{1,2} This condition occurs due to the presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydroxyl, superoxide, and nitric oxide radicals in the body.³ These molecules can cause not only DNA damage but also oxidation of lipid and proteins in the cell.⁴ Antioxidant system in the human body normally has the ability to counterattack these radicals resulting in the balance between oxidation and anti-oxidation. However, the amounts of these radicals are also affected by factors such as lifestyle and environment. Excessive ROS and RNS production can be induced by smoking and alcohol intake as

well as radiation and environmental toxins. Hence the balance between oxidation and anti-oxidation was disrupted leading to disease development.⁴

The risk of oxidative stress can be reduced by taking exogenous antioxidants. Medicinal plants are known as valuable sources of exogenous antioxidants.^{4,5} Many medicinal plants which have been used worldwide were reported that have an antioxidant activity such as *Lamiaceae* (rosemary, sage, oregano, marjoram, basil, thyme, mints, balm), *Apiaceae* (cumin, fennel, caraway), and *Zingiberaceae* (turmeric, ginger).⁵ Antioxidants derived from medicinal plants are mainly polyphenols (phenolic acids, flavonoids, anthocyanins, lignans, and stilbenes), carotenoids (xanthophylls and carotenes), and vitamins (vitamin E and C). These natural antioxidants in general pose a broad spectrum of biological effects such as antibacterial, antiviral, anticancer, anti-

inflammatory, and anti-aging.⁴

Tinospora crispa (*T. crispa*) is one of the traditional medicinal plants which has been reported to have antioxidant activity. *T. crispa* is an herbaceous plant that commonly grows wild in tropical regions of South East Asian countries such as Indonesia, Malaysia, and Thailand.⁶ *T. crispa* is commonly known as *Brotowali*, *Antawali*, and *Andawali* in Indonesia. The leaves of *T. crispa* have heart-shaped 6-12 cm long and 7-12 cm wide. The stems are brownish and fleshy with protruded blunt tubercles.⁶ This plant is well known to be used as a traditional medicine to treat gout, diabetes, hypertension, rheumatic, fever, and appetite stimulant. The habitat of *T. crispa* is in the rainforest and deciduous forest with an elevation up to 1000 m. The suitable soil for growth is moist or dry with sufficient sunlight exposure.^{6,7} In this study we cultivated, extracted, and analyzed *T. crispa* to determine the

antioxidative properties including the total flavonoid content, the phenolic content, and antioxidant activity.

METHODS

Cultivation of *Tinospora crispa*

Cultivation of *T. crispa* was done by using stem cutting material. Watering was done every day until initial growth which was then altered to once a week. Organic fertilizer from cows and goat manure was applied. Plants were harvested when they reached 6 months to 1 year old. Approximately 5 cm of the old stem was cut and dried at 50° C for 7 days. The dried stem was subjected to a milling machine to make it into powder.

Extraction

The powder was then used for extraction using the maceration method. Three different ethanol solvents including 70%, 80%, and 96% were used. The amount of powder for extraction was 300 g. The process of evaporation was in 1.5 hour.

Total Flavonoid Concentration Test

The total flavonoid content was determined spectrophotometrically using 1 g of *T. crispa* extract. Solvent consisting of 1 ml 0.5% (W/V) hexamethylenetetramine, 20 ml acetone, and 2 ml HCl 25% (W/V) was added and then refluxed for 2 h after boiling. The sample was filtered using cotton and topped up to 100 ml using acetone and homogenized. A filtrate of 20 ml was taken and added with 20 ml of water. Ethyl acetate was then added 15 ml and mixed for 10 mins. The sample was set aside to get separation and the ethyl acetate phase was then taken. The extraction was repeated in triplicate using 10 ml ethyl acetate. After that, the sample was washed with 50 ml of water. Ethyl acetate was then added and homogenized. Ten ml of this sample was prepared and mixed with 1 ml AlCl₃ solvent and methanol-glacial acetic acid. The solution was then set aside for 30 mins. After that, it was scanned between 300 – 500 nm. The absorbance was measured in λ maximum (\pm 425 nm). The flavonoid content was calculated using the following formula: % Flavonoid = Absorbance x 1.25/g sample.

Total Phenolic Concentration Test

The total phenolic content was determined as gallic acid equivalent spectrophotometrically. The sample was prepared by pipetting 0.250 ml into a vial and dried using nitrogen gas. For generating the standard curve, gallic acid solution was diluted in water to get concentrations ranging from 5 to 40 ppm. The residue in the vial was then dissolved in 1 ml aquades following the addition of Folin-Ciocalteu 0.5 ml. The sample was set aside for 5 mins before adding 2 ml 10% Sodium carbonate solution. After 10 mins incubation, absorbance was measured in λ 760 nm. The formula from the standard curve was used to calculate phenolic content.

DPPH Radical Scavenging Assay

The free radical scavenging ability of the extracts was examined by DPPH assay. Sample was prepared in ethanol at different concentrations (1 – 4.6 mg/ml). One ml of sample was then mixed with a solution of 40 ppm DPPH in ethanol. The reaction mixture was left at room temperature for 30 minutes. The control was the mixture of 1 ml ethanol and 3 ml 40 ppm DPPH. The absorbance was measured at 519 nm. Percentage of DPPH radical scavenging activity was calculated using the following formula: % inhibition = $\{(A_0 - A_1) / A_0\} \times 100$. Where % inhibition is the DPPH radical scavenging activity, A₀ is the absorbance of the control, A₁ is the absorbance of the sample. The value of % inhibition was then plotted against concentration to generate graph and calculate IC₅₀.

RESULTS

Extraction

From 300 g *T. crispa* powder using maceration method gave liquid yield displayed in Table 1. The highest yield was attained by using 70% ethanol.

Determination of total flavonoid content

Flavonoid contents were analyzed showing that 80% ethanol gave the highest yield. The usage of 70% ethanol indicated slightly lower yield compared to 80% ethanol.

Interestingly, 96% ethanol had the lowest result. it was 2.3 fold lower than the result of 80% ethanol (Figure 1) (Supplementary Table 1).

Determination of total phenolic content

Total polyphenol was determined as gallic acid equivalent spectrophotometrically (figure 2). The detail data was given in Supplementary table 2. The highest to the lowest yield was obtained using 96%, 80%, and 70% ethanol respectively (Figure 2) (Supplementary Table 2).

DPPH Assay

To understand the antioxidant activity, DPPH assay was performed. The highest activity was shown by 80% ethanol. The activity indicated at least 2 fold higher compared to 70% and 96% ethanol (Figure 3) (Supplementary Table 3).

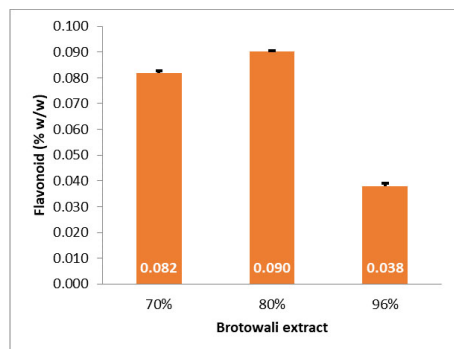
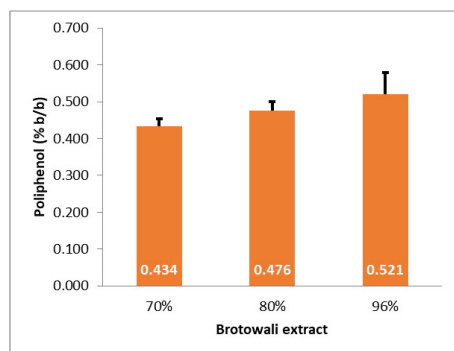
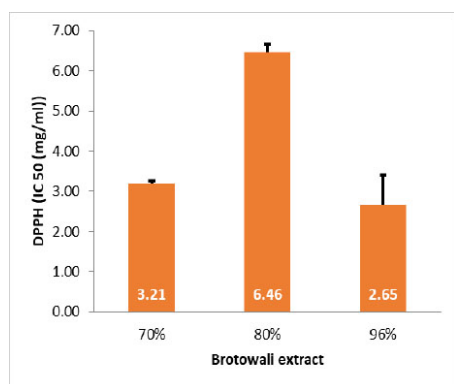
DISCUSSION

The result of the extraction was relatively high compared to several literatures. Even the lowest yield by 96% ethanol could be considered a high result. Study conducted by Irianti et al in 2015 and 2011 indicated yield of 3.1 % (W/W) and 12.02 % (W/W) respectively using 96% ethanol as solvent.^{8,9} The use of 80% ethanol was also observed giving a lower yield of 16.4% (W/W).¹⁰ Likewise, Mutiah et al who performed 15 macerations using ethanol 80% indicated the highest yield was 15.916 % (W/W).¹¹ The use of ethanol 99.9% showed also relatively lower yield (15.08 % (W/W)).¹² Another study using 70% ethanol indicated a yield which was 19.34 % (W/W).¹³ Comparison to the yield from further fractionation processes such as n-hexane and water solvent showed that our result was relatively higher than the yield from n-hexane fractionation by Irianti and Warsinah that indicating 24.1 % (W/W)⁹ and 22.06 % (W/W) respectively. While water solvent showed a high yield up to 31.22 % (W/W)⁹ and 57.9 % (W/W).¹⁴

The total flavonoid (TFC) and phenolic (TPC) content were determined and expressed in % (W/W) unit. The data indicated that both flavonoid and phenol

Table 1. The extraction yields using different ethanol solvents.

	Ethanol solvent		
	70%	80%	96%
Weight	100 ml	95 ml	56 ml
rendement	33.33%	31.66%	18.66%

**Figure 1.** Comparison of flavonoid contents among different ethanol solvents.**Figure 2.** Comparison of polyphenolic contents among different solvents.**Figure 3.** Comparison of DPPH activity assay result.

content of the sample was relatively low compared to several publications of similar studies.^{12,13,15-20} The extraction of

T. crispa stem among several studies using ethanol with different concentrations gave various flavonoid content, however, overall indicated that the value is greater than 0.1% (w/w).^{12,13,15} Likewise, the yield of total phenol displayed at least higher than 3% (w/w).^{12,13,15} Other studies using methanol as solvent also showed higher yields.¹⁷ Showed that the methanol solvent gave higher flavonoid (0.953% (w/w)) and phenolic (25.533% (w/w)) content compared to the usage of water and chloroform. Similar study reported to attain flavonoid and phenol as high as 55.58% (w/w) and 6.12% (w/w) respectively.²⁰

In this study, the polyphenolic content was higher compared to flavonoid. It was expected since flavonoid belongs to phenolic group. Although our result indicated high yield of *T. crispa* extract, nevertheless the total phenolic and flavonoid content were low. This might be due to the growth condition and the age of *T. crispa*. It was known that plant maturity and the growth condition affect the phenolic content.^{21,22} Several plants such as *Perilla frutescens* L²³, strawberry²⁴, *Ficu deltoidea* Jack²⁵, *Caisalpinia bonduc* L Roxb²⁶ were revealed the different concentration of flavonoid and phenol was associated to the growth period. *T. crispa* was known to be able to grow up to elevation at 1000 m.⁶ The cultivation of *T. crispa* in this study was at 850 m, this might also affect the genes and metabolites expression hence the concentration of flavonoid and phenol.

Another possibility of the low yield might be due to inappropriate handling during sample preparation and extraction. It was reported that methods including sample preparation, hydrolysis, and extraction give impact in phenolic content. Pretreatment procedure cannot be equated since phenol consists of molecules with diverse polarity, acidity, concentration level, and matrix complexity.²¹ In addition, reaction times and acidity level affected the

yield.^{21,27,28} One of the study revealed that acidic condition decreased the different forms of phenolic acids, ranging from 15 to 95% for ocoumaric acid and sinapic acid, respectively.²⁹

The highest radical scavenging activity was shown from 80% ethanol extract. This might be supported by the higher flavonoid and phenolic content of the extract compared to the others. Interestingly, the 70% ethanol extract which had slightly lower yield compared to 80% ethanol extract indicated half scavenging activity of 80% ethanol extract. One of the limitations of this study is that the analysis was replicate 6 times. More examination is required to validate the result with different time and date.

The principle of DPPH assay is single electron transfer reaction. Thus, to get better insight of the activity, It is suggested for prospective study to perform other radical scavenging activity assay with similar mechanism such as TEAC and FRAP.^{5,30}

CONCLUSION

The success of extraction method depended on the use of solvent and its polarity. The highest yield of extract was obtained by using 70% ethanol. Despite low in concentration, flavonoid and polyphenol content were successfully determined from *T. crispa* stem. Based on the result of antioxidants concentration and activity, 80% ethanol is the most ideal solvents to be used for extraction of *T. crispa*.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTION

All authors similarly contribute to the think about from the investigate concepts, information acquisitions, information investigation, factual investigations, changing the paper, until detailing the

consider comes about through publication.

ETHICAL CONSIDERATION

The investigators agreed to conduct this study in full agreement with the principles of the Declaration of Helsinki' and its subsequent related amendments. This study was approved by the Ethics Committee of the Surabaya Islamic Hospital. Letter of exemption Ref. No. 1213/EC.KEPK/UMS/2020.

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