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Phytochemical Analysis and Antioxidant Activity of Brotowali (*Tinospora crispa* L. Mier) Stem

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ABSTRACT. Free radical in the body could cause degenerative diseases such as diabetes mellitus and hyperlipidemia, which could be prevented by the supplementation of antioxidant whether it is synthetic or natural. Flavanoids is a phenolic compound, was extracted from natural sources is known for its antioxidative potential. This research aimed to investigate the active compounds in brotowali (*Tinospora crispa*) stem to find new antioxidative potential using its ability to bind 2, 2'-diphenyl-1-picrylhydrazyl (DPPH). Brotowali stem was extracted using ethanol and fractioned using *n*-hexane, ethyl acetate, and water. Water fraction was hydrolyzed by chloric acid. The antioxidant activity of ethanol extract, water fraction, and hydrolized water fraction (subfraction I and subfraction II) were determined using spectrophotometry (DPPH methods). The compound of flavonoid was identified using KLT with $AlCl_3$ reagent spray. The results showed that ethanol extract and water fraction of brotowali stem contained flavonoid glycoside of flavonol group. Antioxidant activity, quantified using IC_{50}, of ethanol extract, water fraction, subfraction I and subfractiont II were 49.92 µg/mL, 38.25 µg/mL, 36.12 µg/mL, and 16.18 µg/mL, respectively. In addition, this research was found that hydrolysis of water fraction using chloric acid like in subfraction I and subfraction II was able to improve its antioxidant activity.

Keywords: *Tinospora crispa,* acid hydrolyzed, antioxidant, phytochemical analysis, flavonoid glycoside

INTRODUCTION

Brotowali (*Tinospora crispa*) is a type of medicinal plant from the genus Tinospora Miers and belongs to the Menispermaceae tribe. *T. crispa* plants are classified as medicinal plants that grow wild in the forests or planted on the fence of the yard (Pradhan, Ojha & Pandey, 2013). The *T. crispa* plant is empirically used as a traditional medicine to cure various diseases. The content of medicinal compounds is found in all parts of the plant, (roots, stems and leaves). *T. crispa* plants contain alkaloide compounds, soft resin, starch, glycosides, picroretosides, harsa, bitter substances picroretine, tinocrisposide, berberine, palmatine, columbine and picrotoxin (Ahmad, Jantan & Bukhari, 2016). Based on these compounds there are known several pharmacological effects such as analgesics, antipyretics, anti-inflammatory, anti-coagulants, tonic, antiperiodic, stomatic and diuretic (Sinku & Sinha, 2018). Chronic diseases such as atherosclerosis, diabetes mellitus, rheumatoid arthritis can be caused by oxidative stress (Ahmad et al., 2016). Oxidative stress is closely related to systemic inflammatory processes, endothelial cell proliferation, apoptosis, and vasoconstriction and contributes to various degenerative diseases such as cancer, diabetes mellitus, atherosclerosis which are the causes of coronary heart disease or heart failure (Yosie, Effendi, Sifzizul, & Habsah, 2011). Oxidative stress can cause free radicals and can be inhibited by administering antioxidants. At present, the use of synthetic antioxidants such as Butylated Hydroxy Toluene (BHT) is starting to be limited because it is carcinogenic. There are side effects on synthetic antioxidants, so we need to look for alternative antioxidants from natural resources (Yosie et al., 2011).

Medicinal plants such as *T. crispa* can be used as a source of antioxidants because of their flavonoid content (Irianti, Puspitasari, & Suryani, 2011). Extraction of active compounds from natural sources usually begins with extraction followed by fractionation to separate each compounds of polarity (Tiwari, Kumar, Kaur, Kaur, & Kaur, 2011). Previous research found that the *T. crispa* fraction produces lower antioxidant activity than the water fraction compared to its ethyl acetate fraction (Irianti et al, 2011; Choundary, Siddique, Azmat, & Khatoon, 2013). The water fraction

contains several glycoside flavonoid that are able to maintain free radicals (Niah & Helda, 2017). Flavanoids were found in *T. crispa* include cathecin, luteolin, morine, and routine (Choudhary et al., 2013). Flavonoid glycosides have lower antioxidant activity compared to their aglycone form (Bajpai, Singh, Chandra, Negi, Kumar & Kumar, 2016). Quercetin, one of the known aglycons, shows a higher antioxidant activity compared to the form of glycosides (Kim & Jang, 2010). Conversion of aglycones from glycoside forms can be done by acid hydrolysis (Tiwari et al., 2011). Previous studies reported that the antioxidant activity of Mulberry leaves on hydroxyl and peroxy radicals increased after hydrolysis (Kim & Jang, 2010; Ghafar, Prased, Weng, & Ismail, 2010). Phytochemical screening research is important to provide a strong and rational basis for the use of *T. crispa* plant parts preparations as antioxidant agents. Such research needs to be supported and accelerated, given the potential of Indonesia's natural wealth which has empirically revealed its potential as a medicine. This is supported by the reason that degenerative diseases are the dominant diseases and are the highest causes of death. The discovery of compounds in *T. crispa* as an antioxidant is a break through alternative therapy for degenerative diseases with low cost and smaller side effects.

EXPERIMENTAL METHODS

Samples and Reagents

The sample for this research is the stem of Brotowali (*Tinospora crispa* L.) which collect from Kedungbanteng, Banyumas district at March 2018, the specimen determined at Taksonomi laboratory, Faculty of Biology, Jenderal Soedirman University with certificate number 754/UN.23.028/TA.00.01/2018. Solvents and reagen such as 96% ethanol, *n*-hexane, and ethyl acetate, methanol (Merck), quercetine (Sigma Aldrich), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), $AlCl_3$ stain, aquadest, HCl_2N, TLC plat, and spectrophotometer.

Simplisia making and extraction

Stems of brotowali were sorted and washed, chopped, dried, and powdered. Five hundred gram of powder were maserated for 3x24 hours, then filtrated every 24 hours. The filtrate was collected and evaporated until all the solvent was completely evaporated. The yield of extract was determined.

Fractionation

Fractionation was done using partition method. Extract was parted into liquid-liquid from polar solvent to non-polar solvent orderly. 70% ethanol was added to the 7.5 gram ethanol extract until it completely solved. Then 50 mL n-hexane was added. Partition was done continuously until clear n-hexane was produced using separatory funnel. *n*-hexane filtrate was then evaporated using evaporator. Insoluble hexane phase was solved in aquadest, then subjected to a partition using 50mL ethyl acetate using the same method as in the separation of n-hexane. Ethyl acetate fraction or insoluble ethyl acetate were then evaporated until thick solvent free extract were produced.

Hidrolysis

Hydrolysis was done using HCl 2N (Apriandanu & Yulizar, 2017). Hydrolysis step was only done on water fraction because it is assumed that flavonoid glycoside is water soluble. Five grams water fraction was dissolved in 50 mL of the mixture of ethanol and HCl 2N (1:1 v/v), refluxed for 90 and 180 minutes and cooled in room temperature. The result of hydrolysis was fractionated by partition using mixture of water: ethyl acetate (1:1, v/v) until produced ethyl acetate subfraction I (reflux for 90 minute) and ethyl acetate subfraction II (reflux for 190 minute). Natrium sulphate anhydrate was added to remove water trace. Then evaporation was done in room temperature without heating.

Thin layer chromatography

Thin layer chromatography (TLC) was done to observe chromatogram profile of ethanol extract and other fractions. The author used silica gel 60 F254 as stationary phase and mobile phase of ethyl acetate: dichloromethane: acetic acid: aquadest (100:25:10:10:11 v/v) and chloroform: methanol: formic acid (44: 3.5: 2.5 v/v) with retention factor of 8 cm to determine flavonoid. Plate was observed under visible spectrum of UV 254, and UV 366. Quercetin was used as control. Sample was then sprayed using $AlCl_3$ to detect the flavonoid groups. Determination of flavonoid contains of ethyl acetat subfraction was evaluated with TLC use toluen: ethyl acetate: methanol: formic acid (32:14:12:5 v/v) and chloroform methanol: formic acid (44: 3.5: 2.5 v/v) as mobile phase.

Antioxidant activity measurement using DPPH (Scherer & Godoy, 2009)

Methanol 0.1 mL and 3.9 mL DPPH 0.08 mM solution in reaction tube and used as blanko. Into other reaction tube containing 3.9 mL DPPH 0.08 mM and samples in methanol 0.1 mL was added. Solution was then vortexed to help the mixing process and incubated in the dark for 30 minutes. Absorbation of the solution was then measured using spectrophotometer with wavelength of 517 nm. 0.1 mL of samples diluted in methanol was added into 3.9 mL DPPH 0.08 mM. Incubation was done for an hour and absorbation was measured in the maximum wavelength of DPPH.

Blanko was prepared by dissolving 0.1 mL sample into 3.9 mL methanol. DPPH control solution was consisted of 0.1 mL methanol and 3.9 mL 0.08 mM DPPH.

Data analysis

Data analysis was done by descriptive qualitative method by determining Rf value of thin layer chromatography observation under UV 254, UV 366, visible spectrum, and staining reagents. C_{50} value of water fraction before and after hydrolysis was analysed statistically with t-test paired sample test using SPSS 16.0 to detect data significancy.

RESULTS AND DISCUSSION

In the present study we used phytocemical analysis to determine the chemical compounds of extract and fraction of *T.crispa* stem. Phytochemical screening is a qualitative test of secondary metabolite content in plant parts, such as alkaloids, flavonoids, saponins, tannins, terpenoids and others (Agustina et al., 2017). The advantages of phytochemical analysis in preliminary study are simple, fast, minimal equipment, and semiquantitatative (Banu & Cathrine, 2015).

Phytochemical screening is intended to identify flavonoid compounds thad had antioxidants in the *T. crispa* stem bark. The plant that extracted with different solvent could determined the presence or absence of phytochemicals (Pradhan et al., 2013). The phenol content and antioxidant activity had good relationship in *Tinospora crispa* and *Tabernaemontana corymbosa* extract (Zulkefli, Mohamad, & Abidin, 2013). The yield of ethanol extract was 9.1% and the characterization of this extract was brownish green color and sticky paste. Flavonoid glycosides in *T. crispa* stems had been successfully extracted using 96% ethanol (Irianti et al., 2011). In present study the extract was fractionated with n-Hexane, ethyl acetate and water. The yield and characterization of fractions of T.crispa were shown in **Table 1**. The highest weight fraction is the water fraction. Water or insoluble fractions in ethyl acetate are reported to contain a lot of flavonoid glycosides (Koay & Koay, 2013).

The water fraction is hydrolyzed with acid. The process of hydrolysis in materials depends on acid concentration, hydrolysis time, temperature, and type of solvent (Wach et al., 2007). The chromatogram profile showed that quercetin had one spot on hRf 70. The *T. crispa* stem extract, fraction, and acid hydrolised fraction had a similar chromatogram with quercetin (**Figure 1**). All fractions have the same chromatogram profile but there were different color intensities.

Separation of natural compounds by thin layer chromatography does not produce pure compounds because the presence of pollutant compounds or unwanted compounds still available (Choudhary et al., 2013). A high hRf value indicated less polar compounds, if on TLC method with non-polar mobile phase and polar stationary phase, so polar compounds are more easily eluted. Observation of chromatograms on UV light before and after spraying, showed flavanoid compounds that contain free 3-OH and accompanied or without free 5-OH and isoflavones without free OH (Rasidah, Syahmani & Iriani, 2019). The presence of flavonoid aglycone in this study was shown on chromatogram after spraying with DPPH. All fraction showed yellow stains at hRf 43, but subfraction with diferent time of reflux at 90 minutes and 180 minutes showed yellow stains at hRf 65. The flavonoid glycoside was identified before hydrolysis and after hydrolysis were found aglycone flavanoid. Previous study reported that fractionation and separation of T.crispa could isolate 17 flavonoids (Chang, Ho & Lee., 2015).

According to the results of DPPH test, the activity of free radical scavenging of subfraction II was more potent almost 2 times than the water fraction. Commonly glycoside had less potent antioxidants (Bajpai et al., 2017). In present study, subfraction that contain flavonoid aglycone had better antioxidant activity compared than water fraction and ethanolic ekxtract (**Figure 2**). In this study, subfraction I had no significant antioxidant activity with the water fraction, because in hydrolysis process for 90 minutes had not yet released many flavonoid aglycones.

Chromatogram profile of ethanol extract of *T. crispa* stem and its fraction can be seen in **Figure 3**. The water fraction had chromatogram profile wth spots on hRf 7 and 18 which did not appeared in subfraction I, whereas in subfaction I and subfaction II appear 2 stains namely hRf 71 and 78 which do not appear in water fraction. In addition, stains with hRf 18 in the water fraction appear to decrease intensity at subfractions, whereas flavonoid aglycones with higher hRf values. Flavonoid aglycones released from glycosides were identified by DPPH reagents on chromatograms (**Figures 1B5** and **1B6**). The color of the stain is pale yellow of hRf value of 39. The presence of subfraction I and subfraction II showed yellow stains with a value of hRf 71. The presence of yellow stains indicates of free flavonoid aglycones and indicator of the flavonoid group.

In this study, the water fraction had higher free radical activity than the other fractions, because in the water fraction contained flavanoid glycosides. While flavonoid aglycones have higher antioxidant activity than glycosides. The antioxidant activity of subfraction I was not significantly different from the water fraction, because hydrolysis for 90 minutes had not released all the aglycone flavonoids, showed chromatogram profile (**Figure 1A**), the water fraction

had stains with hRf values 7 and 18. While subfraction I and subfaction II had stains with hRf 71 and 78 which did not occur in the water fraction. In this study, the water fraction after being sprayed with the $AlCl_3$ reagent, showed a reduced color intensity (hRf 26) compared to subfraction I and II.

Table 1. Weight of fractions and yield of ethanol extract of brotowali

Weight of ethanol extract (g)	Fraction	Fraction weight	Fraction yield (% w/w)	Colour
50	*n*- Hexane	5.78	22.06	yellow
50	Ethyl acetate	3.60	15.10	pale yellow
50	Water	8.30	31.22	red chocolate

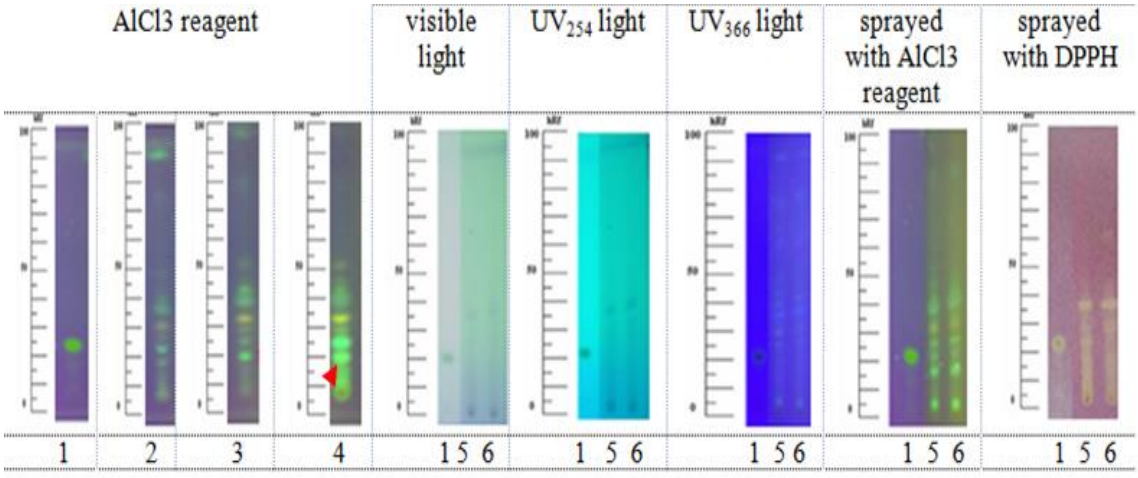

Figure 1. Chromatogram profile of extract, fraction, and acid hydrolised fraction from *T. crispa* stem. Stationary phase: silica gel 60F254; Mobile phase: toluen: ethyl acetate: methanol: formic acid (32:14:12:5). 1. Quercetin standard, 2. Ethanol extract, 3. Ethyl acetate extract, 4. Water extract, 5. Subfraction I, and 6. Subfraction II. Arrows shows the appearance of flavonoid.

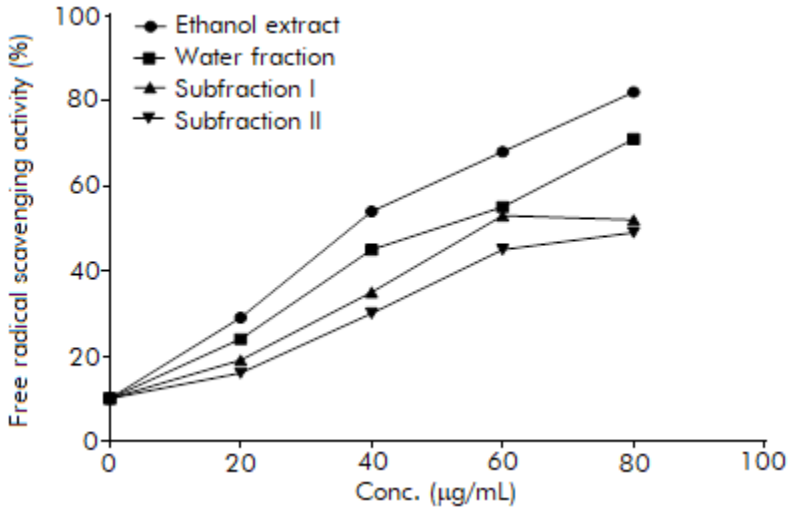

Figure 2. Free radical scavenging activity by DPPH of ethanol extract, water fraction, Subfraction I and subfraction II of Brotowali (*T. crispa*) stem

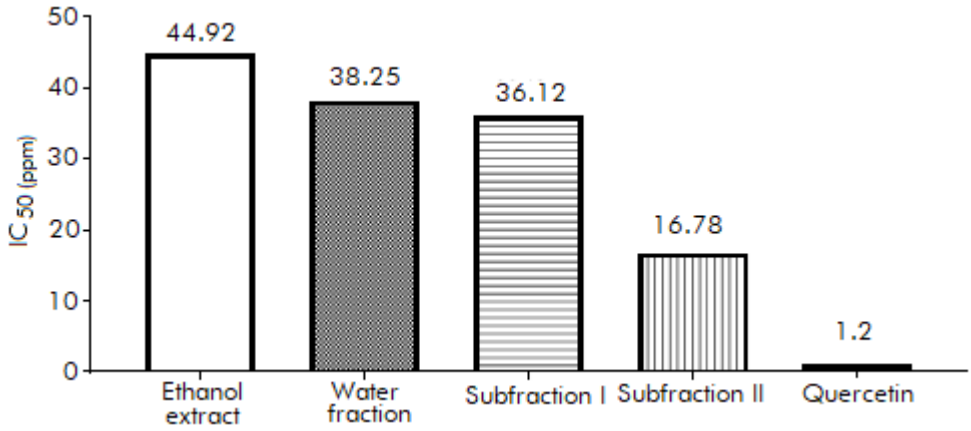

Figure 3. IC_{50} of ethanol extract, water fraction, subfraction I and subfraction II of brotowali (*T.crispa*) stem.

Free radical scavenging activity was determined by in vitro method with DPPH. This method could be used to evaluate the antioxidant activity of certain compounds, estimate antioxidant activity in vivo, and to estimate doses in vivo studies (Mohamad et al., 2017) . IC_{50} values can be determined in the DPPH method to interpret the antioxidant activity of certain compounds. IC_{50} value is defined as substrate concentrations that cause DPPH activity to decrease by 50% (Molyneux, 2004). Lower IC_{50} values indicate higher compound activity in free radical scavenging activity (**Figure 3**).

In previous study, reported that the aqueous crude extract of *T. crispa* stem had high antioxidant activity (Koay & Koay, 2013). Quercetin as a control showed the highest antioxidant activity compared to extracts and fractions of *T. crispa*, because quercetin is a phenolic compound with orthohydroxyphenolic groups in ring B and phenolic groups on ring A that are stable to capture free radicals (Wach, Pyrzynska & Biesaga, 2007; Bhawya and Anilakumar, 2010). Most antioxidant compounds from natural sources form a defense against free radicals that work synergistically with each other (Wang et al., 2002; Koay & Koay, 2013). Fractionation from ethanol extract of *T.crispa* stems could separate compounds with lower free radical scavenging activity and produce synergistic effects. The flavonoid aglycone that extracted from water fraction by HCL 2N of the *T. crispa* stem was able to increase the activity of free radical scavenging. Based on the discussion, we found that subfraction of *T. crispa* had antioxidant activity, so it can be developed as phytotherapy in degenerative disease.

CONCLUSIONS

Ethanol extract and water fraction of *T.crispa* contained flavonoid glycosides compound from the flavonol group. Hydrolysis process of the water fraction using chloric acid increased antioxidant activity. Subfraction II of *T.crispa* had the highest antioxidant activity with the IC_{50} of 36.12 µg/mL.

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