Total Phenolic, Flavonoid, Tannin Content and DPPH Scavenging Activity of *Caesalpinia sappan* Linn. Bark

Brandon Aristo Verick Purba, Rini Pujiarti, Masendra, and Ganis Lukmandaru

Abstract

Caesalpinia sappan is a shrubby Fabaceae tree commonly found in Indonesia, traditionally utilized as natural dye and herbal drink. However, in the making of traditional herbal drink, the bark is often discarded as residues. This research aimed to investigate total phenolic (TPC), flavonoid (TFC), and tannin (TTC) content as well as the antioxidant activity (DPPH scavenging activity) of the bark successive extracted with *n*-hexane, ethyl acetate, methanol, and hot water as well as analyzed it with GC-MS. The result showed the highest amount of TPC (824.16±62.28 mg GAE/g), TFC (185.03±1.91 mg QE/g), and TTC (987.07±30.98 mg TAE/g) in the methanol extract of the bark. GC-MS analysis resulted hydroquinone as a major constituent in the methanol extract. Further, antioxidant activity was found the strongest on methanol extract (IC_{50}=63.48), while correlation between antioxidant activity and TFC was found the highest (R^2=0.93). These findings suggest that bark of *C. sappan* is a suitable source of natural antioxidant with strong activity to DPPH radical.

Keywords: *Biancaea sappan,* bark extract, antioxidant activity, colorimetric, GC-MS.

Introduction

Caesalpinia sappan Linn. is a small-medium sized shrubby tree member of Fabaceae found in South East Asian countries including Indonesia, Vietnam, Myanmar, Philippine, and Malaysia. It is a fast-growing species which able to attain 3.6 m height in its 1st year with proper exposure to sunlight (Mathew *et al.* 2007). Due to its thorny bark, *C. sappan* is often utilized as hedgerow plant to protect farms against vermin such as wild boar (Najiyati *et al.* 2005). Its compact wood can also be utilized for carpentry and musical instrument material (Mathew *et al.* 2007). Moreover, *C. sappan* wood is utilized as source of red dye for textiles and also made into traditional herbal drink by mixing it with other spices, locally called *wedang uwuh* in Yogyakarta, Indonesia (Winarsi *et al.* 2018).

C. sappan is traditionally utilized as herbal drink ingredient in regard to its health benefits. Several bioactivities such as antibacterial, anti-inflammatory, wound healing, and antioxidant has been reported from *C. sappan* wood extract (Zhao *et al.* 2008; Nirmal and Panichayupakaranant 2015; Sucita *et al.* 2019). These bioactivities might be also attributed by several phenolics in the extract which include flavonoids, chalcones, xanthone, and tannin (Sucita *et al.* 2019; Chen *et al.* 2008). Phenolics ability as antioxidant is well reported. Their antioxidant activity is attributed to their ability to donor hydrogen atoms to reactive oxygen and nitrogen species radicals while still being stable due to their structure (Pereira *et al.* 2009). The accumulation of radicals in human body can cause oxidative stress, which is suspected to lead into various diseases including cancer (Adwas *et al.* 2019).

Bark is the outermost part of the tree and acts as a protective layer against environmental and pathogenic threats. One of their defense mechanisms against pathogens and pest are attributed to their accumulation of secondary metabolites (Pásztory *et al.* 2016). Due to this reason, extractives in bark were found in larger quantities compared to wood in general (Sjöström 1993). However, in wood processing, bark is often discarded as residues. In fact, secondary metabolites from bark might be utilized and beneficial for human health. Moreover, extract from the bark of *C. sappan* received less scientific attention compared from its wood and in its traditional utilization for herbal drink in Indonesia, its wood and small part of inner bark often mixed together while the outer bark discarded as a waste. Previous research has reported the antioxidant activity of *C. sappan* wood extract (Setiawan *et al.* 2018). The objectives of this research were to investigate total phenolic, flavonoid, and tannin content, antioxidant activity, as well as compounds identification through GC-MS analysis in *C. sappan* bark.

Materials and Methods

Bark Material and Extraction

The bark sample of *C. sappan* was collected from Forest Research and Education of Wanagama I, Gunung Kidul District, Yogyakarta, Indonesia. Collection was done trees with 9 cm stem diameter. Successive extraction was done to the milled bark sample (100 g). Extraction were done using reflux apparatus with *n*-hexane (6 h), methanol (MeOH) (6 h), ethyl acetate (EtOAc) (6 h), and hot-water (3 h). Each extract was dried with rotary evaporator and stored in a flask in room temperature. The extract yield from each solvents were calculated as percentage of oven-dried weight.

Total Phenolic Content

Briefly, 0.5 ml of extract diluted in dimethyl sulfoxide (DMSO) was added to 2.5 ml of Folin-Ciolcateu reagent (10% v/v) (Merck, Germany) and incubated for 2 mins. As much as 2 ml of 7.5% (w/v) Na_2CO_3 solution was then added to the mixture and sample was incubated for 30 mins under room temperature. Absorbance was measured with UV-Vis spectrophotometer in 765 nm wavelength. Gallic acid in various concentrations were also subjected to the same procedure and calibration curve was made ($y=0.1333x + 0.0042$; $R^2 = 0.99$). The results are expressed as mg gallic acid equivalent (GAE)/g of the sample (Baba and Malik 2015).

Total Flavonoid Content Assay

Extracts were diluted with DMSO and 2 ml of the solutions were added to 2 ml of 2% aluminum chloride ($AlCl_3.H_2O$). The mixture was then shaken and incubated in 22°C temperature for 30 mins. The absorbance of each mixture was then measured using UV-Vis spectrophotometer in 415 nm wavelength. A calibration curve using quercetin was also prepared with the same procedure ($y=0.0388x - 0.0001$; $R^2 = 0.99$) and the results were expressed as mg quercetin equivalent (QE)/g of the sample (Diouf *et al.* 2009).

Total Tannin Content Assay

Preparation; 0.1 ml of extract sample (1000 ppm) was diluted with distilled water (7.5 ml). To the solution, Folin-Denis (0.5 ml) and 1 ml of sodium carbonate (35%) was reacted. The solution was added with distilled water until 10 ml volume. The final reaction was stood at ambient temperature for 30 min and the sample absorbance was read at 760 nm. To calculate total tannin content, the standard of tannic acid was used for calibration ($y=0.694x-0.0079$; $R^2=0.99$), therefore the unit of total tannin content was mg tannic acid equivalent (TAE)/ g dried extract sample (Padmaja 1989).

DPPH Radical Scavenging Activity Assay

DPPH radical scavenging activity was measured by mixing 0.1 ml of diluted extract in four concentration (25, 50, 100, and 200 µg/ml) with 3 ml of 0.1 mM diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA). The mixture was shaken and kept for 30 mins under 22°C temperature in dark. Blank also prepared with the addition of solvent only. Absorbance was measured at 512 nm wavelength with UV-Vis spectrophotometer and radical scavenging activity was calculated with the following formula:

$$\text{Radical scavenging activity (\%)} = 100 \times (A_0 - A_1)/A_0 \quad (1)$$

Where A_1 is sample absorbance and A_0 is blank absorbance. Antioxidant activity was then expressed as IC_{50} or the concentration needed to inhibit DPPH by 50% in µg/ml.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The MeOH extract of *C. sappan* bark was silylated according to literature (Wijayanto *et al.* 2015). Firstly, 1 mg of sample was dissolved into TMCS (15 µl) and BSA (85 µl). Secondly, the reaction was incubated for 1 hour at room temperature. Then, the sample was diluted with 1 ml of MeOH. For analysis, 1 µl of silylated sample was injected to GC-MS machine. The GC condition: Rtx-5MS capillary column (30 m x 0.25 mm I.D. and 0.25 µm; GL Sciences, Tokyo, Japan); column temperature from 70 °C (1 min) to 290 °C at 5 °C/min; injection temperature of 270 °C; detection temperature of 290 °C; acquisition mass range from of 50-800 amu using helium as the carries gas. GC-mass spectrometry (GC-MS) data were collected with a GCMS-QP 2010 (Shimadzu, Japan). The mass spectrum of sample was compared to NIST library. In this study, peak relative method was applied for calculation of *C. sappan* bark constituents GC-MS analysis.

Statistical Analysis

One-way ANOVA was done using SPSS (IBM, USA) with 95% confidence level. Further, data with significant result was tested with Tukey HSD with 5% significance level. Correlation of colorimetric assay results were correlated with DPPH scavenging activity (100 µg/ml) using linear regression.

Results and Discussion

Colorimetric Assays

The extract yield of each sample were 0.51%, 1.82%, 6.92%, and 7.65% for *n*-hexane, EtOAc, MeOH, and Hot-water soluble extract, respectively. Results of colorimetric assays to measure TPC, TFC, and TTC are showed in Figure 1. In all assays, MeOH extract showed the highest concentration while *n*-hexane showed the lowest. MeOH extract showed the highest value of TPC (824.16±62.28 mg GAE/g), TFC (185.03±1.91 mg QE/g), and TTC (987.07±30.98 mg TAE/g). Further, one-way ANOVA showed significance difference between solvents in all assays ($p<0.01$). Tukey HSD test showed significance difference between all solvents in all assays, where MeOH extract showed significantly the highest amount of TPC, TFC, and TTC.

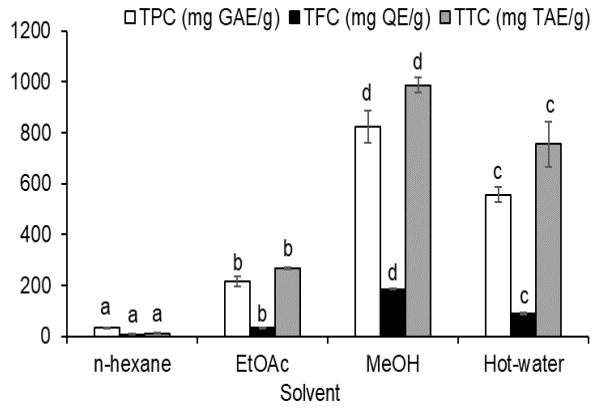

Figure 1. Total phenolic (TPC), flavonoid (TFC), tannin content (TTC) of *C. sappan* extracted with solvents with increasing polarity. Different letters on the histogram (a, b, c, etc.) indicate significant difference with Tukey HSD (5% significance level) between solvents in each individual assay.

Solvent used had significant effect on the results of TPC, TFC, and TTC. Results of TPC indicate that *C. sappan* bark were significantly dominated by phenolic compound in its polar extracts especially in the MeOH soluble extractive, while only a very small amount extracted by *n*-hexane that indicated by some brown colour in the extract. Compared to previous TPC assay on bark of different species, *C. sappan* bark extract can be considered very high in phenolics (Phuyal *et al.* 2020; Wijewardhana *et al.* 2019). Moreover, TPC of *C. sappan* heartwood has been reported in previous research (Febriyenti *et al.* 2018), whereas the amount is slightly lower than the bark from this research. Flavonoid and tannin content were also estimated to be quite high in *C. sappan* bark polar extract by the colorimetric assays. One of flavonoid type compound has been reported in *C. sappan* which is called brazilein, also suspected to give its well-known red colour in the extract (Dapson and Bain 2015). Further, the high amount of TTC might suggest that there are lots of polymeric phenols or flavonoids contained in the extract. Phenolics including flavonoid and tannin is major group of secondary metabolites found in plants and generally bioactive (Miguel-Chávez 2017). The higher amount of TPC in bark indicates its function in bark as a protective measure against pest and disease to its inner tissue. Further, some bioactivities are expected in the polar extract of *C. sappan* bark.

GC-MS Analysis

The detection of MeOH extract of *C. sappan* bark found aromatic compounds as dominant constituents. The compounds can be grouped into phenolic and sugar compounds. The hydroquinone and 2,3-anhydro-d-mannosan were the higher constituents from the phenolic and sugar compounds. The other aromatic compounds were benzoic acid, 1-chloro-2,5-dinitrobenzene, p-diazquinone, 3-methyl-2-nitrophenol, and 2,4-dimethoxyphenol (Table 1 and Figure 2). In comparison, phenolic and sugar compounds such as hydroquinone derivatives were also detected in *Cinamomiun verum* bark (Kankeaw and Masong 2015), benzoic acid in *Terminalia arjuna* bark (Dutta *et al.* 2015), 2,6-dimethoxyphenol and 2,3-anhydro-d-mannosan in *Aesculus chinensis* bark (Li *et al.* 2018).

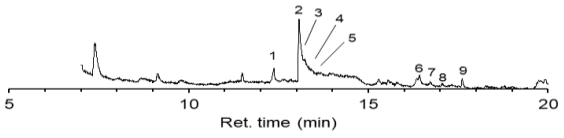

Figure 2. Chromatogram of GC-MS of MeOH extract of *C. sappan* bark; 1. Benzoic acid, 2. hydroquinone, 3. 2,3-anhydro-d-mannosan, 4. 1-chloro-2,5-dinitrobenzene, 5. p-diazoquinone, 6. 3-methyl-2-nitrophenol, 7. 2,6-dimethoxyphenol, 8. 3,4-O-isopropylidene-d-galactose, 9. 2-pentenyl acetate

Table 1. Composition of *C. sappan* bark detected by GC-MS

No.	Ret. time (min)	Constituents	Concentration (% of dried extract)	Similarity index (%)
1	12.4	Benzoic acid	5.4	87
2	13.1	Hydroquinone	51.4	87
3	13.2	2,3-Anhydro-d-mannosan	14.9	60
4	13.3	1-Chloro-2,5-dinitrobenzene	7.1	60
5	13.4	p-Diazoquinone	5.6	60
6	15.4	3-Methyl-2-nitrophenol	2.3	60
7	16.3	2,6-Dimethoxyphenol	2.4	60
8	16.4	3,4-O-Isopropylidene-d-galactose	3.7	65
9	17.6	2-Pentenyl acetate	2.9	80

In this study, some known polyphenolic compounds such as brazilin, brazilein (Dapson and Bain 2015), and sappanol (Uddin *et al.* 2015) were not detected in the GC-MS analysis. Furthermore, the highest detection of hydroquinone in this study indicates hydroquinone as a precursor of polyphenolic in *C. sappan* bark. The high concentration of hydroquinone also suggests that *C. sappan* bark can be utilized as hydroquinone source as well as in pharmaceutical industry.

Antioxidant Activity

Concentration to inhibit DPPH radical by 50% (IC_{50}) value of each extract are shown in Figure 3. Lower IC_{50} value indicates stronger antioxidant activity. The result showed that MeOH extract showed the strongest antioxidant activity, where *n*-hexane extract showed the weakest. Further, gallic acid was used as positive control, where its IC_{50} was slightly lower than MeOH extract.

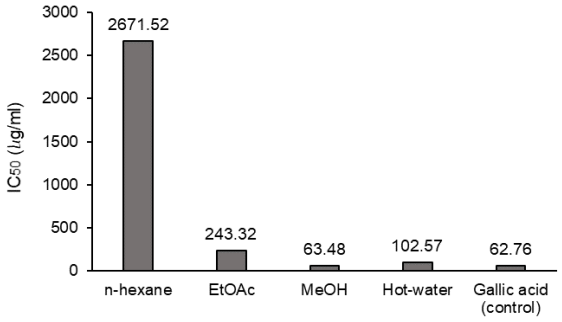

Figure 3. IC_{50} to DPPH radical of each extract.

DPPH is a stable free radical model with dark violet colour that lose its chromophore and turns yellow upon receiving hydrogen atom from antioxidant compounds (Sanchez-Moreno 1999). The result of DPPH scavenging assay suggests that the polar extracts of *C. sappan* bark, especially the MeOH extract, were effectively neutralize DPPH radical with low concentration. This high activity might be attributed by its high concentration of phenolics, flavonoid, and tannin measured in the colorimetric assay. MeOH extract effectiveness to neutralize DPPH radical was comparable to the positive control of gallic acid. By comparing the DPPH IC_{50} of the control, crude MeOH extract of *C. sappan* bark had higher DPPH radical scavenging activity compared to *Albizia adianthifolia,* bark and two well-known antioxidants butyl hydroxytoluene (BHT) and ascorbic acid (Vitamin C) (Brighente *et al.* 2007; Tamokou *et al.* 2012). Further, the detection of aromatic compounds such as hydroquinone and its other derivatives may exhibit antioxidant activity in *C. sappan* bark. Previously, hydroquinone derivative compounds were showed activity against DPPH radical (Kankeaw and Masong 2015). This result indicates that the MeOH extract of *C. sappan* bark is a suitable source of natural antioxidant with strong activity.

Correlation of Total Phenolic, Flavonoid, and Tannin Content to Antioxidant Activity

Correlation between TPC, TFC, and TTC to radical scavenging activity (RSA) are shown in Figure 4. All assays showed positive interaction by linear regression. The highest correlation was showed between TFC and RSA (R^2=0.93), followed by TTC (R^2=0.87), then TPC (R^2=0.84).

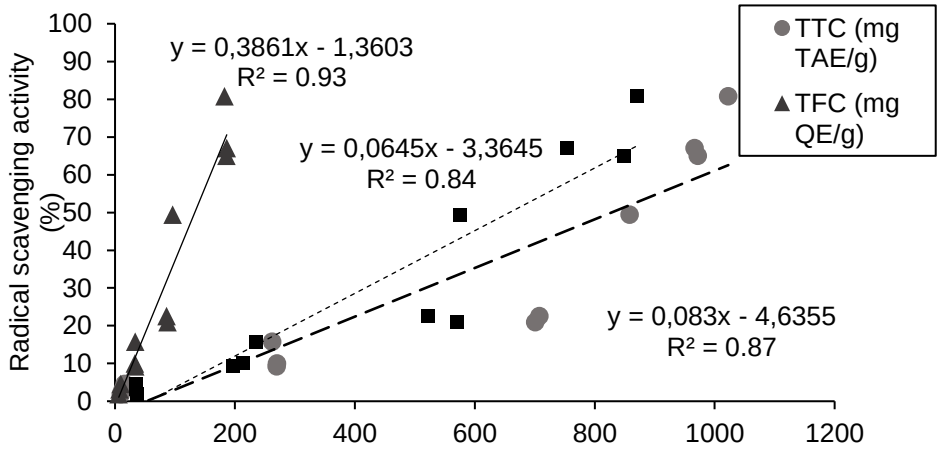

Figure 4. Correlation between colorimetric assays result (TPC, TFC, and TTC) to antioxidant activity (DPPH RSA) in 100 µg/ml concentration.

Phenolic compounds including flavonoid and tannin are well known antioxidant component which is able to neutralize free radicals due to its ability to donate hydrogen atom while still being stable due to its ideal structure characteristic (Amarowicz *et al.* 2004). Linear correlation between phenolic and antioxidant activity has been reported in previous literatures (Shrestha *et al.* 2006; Esmaeili *et al.* 2015). In this research, flavonoid had the highest correlation to antioxidant activity. This result indicates that flavonoid type compounds are more responsible to the antioxidant activity. Similar result

of higher correlation between total flavonoid and antioxidant activity was also reported in previous research on several wild vegetables from western Nepal (Aryal *et al.* 2019). Flavonoid is one of the major parts of compound found in *C. sappan* wood and various flavonoid type compounds has been identified from its wood (Namikoshi *et al.* 1987; Shu *et al.* 2008; Zhao *et al.* 2013). Further, the higher correlation of TTC-DPPH might suggest that long chained polyphenols, including that with flavonoids monomer, are more responsible to the antioxidant activity of *C. sappan* bark extract.

Conclusions

Colorimetric assays to measure TPC, TFC, and TTC, as well as antioxidant activity assay by DPPH scavenging activity method, and GC-MS analysis on successively extracted *C. sappan* bark have been conducted. The results showed the highest amount of TPC, TFC, and TTC in the MeOH extract. Further, the strongest antioxidant activity was exhibited in the MeOH extract. The detection of hydroquinone as a major constituent by GC-MS analysis supported the highest result of antioxidant activity. In this study, high correlation between TFC and DPPH RSA indicates that this antioxidant activity is more attributed to flavonoid type compounds. The results of this study indicates that the MeOH extract of *C. sappan* bark is a suitable source of natural antioxidant with strong activity against DPPH radical.

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