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

Brandon Aristo Verick Purba, Rini Pujianti, 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 (IC50=63.48), while correlation between antioxidant activity and TFC was found the highest (R²=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 (Pásztor 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-Ciocalteu reagent (10% v/v) (Merck, Germany) and incubated for 2 mins. As much as 2 ml of 7.5% (w/v) Na₂CO₃ 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 \((y=0.1333x + 0.0042; R² = 0.99)\). 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 (AlCl₃·6H₂O). 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 \((y=0.0388x - 0.0001; R² = 0.99)\) 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 \((y=0.694x-0.0079; R²=0.99)\), therefore the unit of total tannin content was mg tannic acid equivalent (TAE)/g of 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:

\[
\text{Radical scavenging activity (\%)} = 100 \times \frac{(A_0 - A_n)}{A_0}
\]

Where \(A_0\) is sample absorbance and \(A_n\) is blank absorbance. Antioxidant activity was then expressed as IC₅₀ 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. It was applied then 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 (<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.
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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-diazoquinone, 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 Cinamomum 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](image)

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

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

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).

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 TTC, TFC, and TPC 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.

References


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