Identification of active compounds and antioxidant activity of teak 
(Tectona grandis) leaves

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Abstract. Suryanti V, Kusumaningsih T, Marliyana SD, Setyono HA, Trisnawati EW. 2020. Identification of active compounds and antioxidant activity of teak (Tectona grandis) leaves. Biodiversitas 21: 946-952. Teak (Tectona grandis) grows in tropical area and the teak wood is widely used for building construction and furniture. Young teak leaf contains red pigment and often are used for natural food coloring. The research objectives were to isolate the young teak leaf chemical compounds and to investigate their antioxidant activities. The compounds identification was carried out by UV-Vis Spectroscopy, GC-MS and LC-MS. The antioxidant activity determination was carried out by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method. The total phenolics content was determined using Folin-Ciocalteu method and total anthocyanins content was examined using the H-differential method. Two fractions were observed further by UV-Vis Spectroscopy, GC-MS and LC-MS. The UV-Vis spectrum of F3 confirmed the presence of anthocyanins which has maximum absorbance at 208 and 492 nm. The GC-MS spectrum of fraction A (F3a) revealed 4-hydroxy-4-methyl-2-pentanone, glycerin monacetate, glycerin diacetate and 1-eicosanol. The UV-Vis spectrum of Fc also confirms the presence of anthocyanins which has maximum absorbance at 489 nm. The LC-MS spectrum of fraction C (Fc) shows anthocyanins, namely malvidin-3-o- (6-o-acetyl)-5-o-diglutoside. The F3 and Fc teak leaf fractions have strong antioxidant activities as their IC50 values are less than 50 ppm. Total phenolic of teak leaf extract was 6.17 mg GAE/g and total anthocyanins of teak leaf extract were 67.5 mg/L.

Keywords: Antioxidant activity, teak leaves, Tectona grandis, total anthocyanins, total phenolics

INTRODUCTION

Tectona grandis (teak) belongs to Eukaryota domain, Plantae kingdom, Spermatophyta phylum, Angiospermae subphylum, Dicotyledonae class, Lamiales order, Verbenaceae family and Tectona genus. Teak is a tropical plant species distributed in the countries of Indonesia, India, Thailand, Nigeria, and Myanmar (Ogunmefun et al. 2017). Teak cultivation in Indonesia has a total area of 923.92 ha (Hermawati et al. 2015). Teak wood is extensively used for building construction and furniture and teak leaves are widely used as food wrapping and as natural food dye. Teak leaf is also widely used in Asia for the treatment of diabetes, inflammation, bronchitis, cancer, skin diseases, malaria and TBC (Rajuri et al. 2010).

Teak leaves contain secondary metabolites such as flavonoids, alkaloids, tannins, anthraquinones, naphthoquinone, anthocyanins and cyanidins which have various activities, such as antibacterial, antitoxic and antioxidant activity (Murukan and Murugan 2018a, Murukan and Murugan 2018b, Murukan and Murugan 2018c and Arief et al. 2014). The green young teak leaves turn to brownish-red color when they are ripped. The released color indicates the young teak leaves contain anthocyanins since anthocyanins give red, orange, purple, blue to black colors (Du et al. 2015). The green young teak leaf also contains tannins as indicating by sticky sap of leaves. Tannin and phenolic compounds, such as anthocyanins possess antioxidant activities (Kutlu et al. 2011 and Arief et al. 2014). The use of compounds rich in antioxidant activities is growing rapidly both for food and medicines (Hanani et al. 2005). The common antioxidants used in food are synthetic antioxidants. The use of synthetic antioxidants should be very limited and be avoided because they can be carcinogenic. Therefore, the use of natural antioxidants is growing for safety reasons (Ghany 2015).

Kopa et al. (2014) isolated compounds from teak leaves by extraction using methanol, water, and ethyl acetate, followed by purification by Sephadex LH-20 column chromatography. Murukan dan Murugan (2017) isolated teak leaves compounds by extraction using methanol containing 0.5% HCl, followed by liquid-liquid extraction using ethyl acetate and column chromatography using Amberlite XAD-7. Murukan dan Murugan (2018b) isolates teak leaves compounds by extraction using methanol containing 1% HCl then continued by liquid-liquid extraction with ethyl acetate and chloroform, and finally by Amberlite XAD column chromatography. Baharuddin et al. (2015) isolate the teak leaves compounds by extraction using methanol which was then followed by Liquid Vacuum Chromatography (LVC). Arief et al. (2014) had extracted teak leaves compounds with ethanol which was applied for sausage meat. Lismawening et al. (2013) had extracted young teak leaves with water and applied it to the glass. Qadariyah et al. (2018) extracted teak leaves by
ultrasonic method with ethanol 60% and water as solvents and applied it to the fabric. Although the isolation of teak leaf compounds has been performed, however, information on diversity of the teak leaves compounds is still limited. Therefore, this study focuses on the identification of antioxidant active compounds from teak leaves.

MATERIALS AND METHODS

Materials
The first three young leaves were harvested from teak plant in Boyolali, Jawa Tengah, Indonesia. Gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Aldrich. The other chemicals used were purchased from E-Merck and the solvents used were analytical grade without further purification.

Sample preparation
Young teak leaves were cut into small pieces and dried by aerating at room temperature. Then, the dried young teak leaves were ground to fine powder. The dried powder of young teak leaves (1 kg) was macerated using methanol (5 L) with presence of 1% HCL solution. The extract was then evaporated using rotary evaporator to get methanol extract (20 g).

Fractionation by Liquid Vacuum Chromatography (LVC)
The methanol extract (15 g) was separated into fractions based on the polarity of the compounds. Impregnation sample was carried out before the separation process by LVC using silica gel 60 (0.2-0.5 mm) Merck as a stationary phase. The eluent used was a mixture of hexane and ethyl acetate with a ratio of 4:6, 2:8 and 0:10 in total volume of 300, 450 and 1500 mL respectively.

Fractionation by Gravity Column Chromatography (GCC)
The extract obtained from LVC was introduced into the packed column of 20 g silica gel 60 (0.063-0.200 mm) Merck. A polarity gradient was used for mixtures of hexane and ethyl acetate with the ratio of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6 and 0:10 in total volume of 50 mL. Eluent was collected in a test tube and analyzed by thin-layer chromatography (TLC). The eluted fractions with similar spots or Rf values were collected and concentrated using rotary vacuum evaporator.

UV-Vis Spectroscopy
The UV-Vis analysis with wavelength of 800-200 nm was performed for fraction A (F_A) and fraction C (F_C) using acetone as solvent with Perkin Elmer Precisely Lambda 25 UV-Vis.

Gas Chromatography-Mass Spectroscopy (GC-MS)
The F_A was analyzed by Shimadzu QP2010S GC-MS. The GC-MS was run using EI 70 Ev ionizing type, Rtx 5 Ms column type which has column length of 30 m and diameter 0.25 mm, column temperature of 70°C, injector temperature of 300°C, splitless injection method, detector temperature of 300°C and carrier gas He of 13.7 kPa.

Liquid Chromatography-Mass Spectrometry (LC-MS)
The F_c was analyzed by LC-MS Waters 2489 with UV-Vis detector. The column temperature was 35°C and the solvent used was a mixture of solution A (aquadest: formic acid = 9: 1) and solution B (aquabident: acetonitrile: formic acid = 6: 3: 1) with a flow rate of 1 mL/min for 25 minutes. The solvents gradient used for the initial 5 minutes was 75% solution A and 25% solution B, the next 5 minutes was 71% solution A and 29% solution B, the next 5 minutes was 66% solution A and 34% solution B, the next 5 minutes was 62% solution A and 38% solution B, 57% A and 43% solution B, last 5 minutes was 100% solution B. The absorbance of ultraviolet was measured at 520 nm. MS analysis was used ESI ionization.

Determination of total phenolics
The total phenolics determination was performed according to Doymaz and Karasu (2018) with slight modification. Gallic acid solution (1 mL of 10 ppm) was put into a 5 mL volumetric flask, then was added 0.5 mL of Folin-Ciocalteu reagent and left for 1 minute. The mixture was then added 3.5 mL of 7.5% Na_2CO_3 and was left for 1 minute. The samples were measured using UV-Vis Spectroscopy at 10 minutes intervals until reaching equilibrium state. Standard curves of gallic acid with concentrations of 2, 4, 6, 8, 10 ppm were treated with the same method to determine the quantitative of total phenolics. Total phenolics test was carried out for 100 ppm extract samples. The extract sample (1 mL) in 5 mL volumetric flask was added Folin-Ciocalteu (0.5 mL) and left for 1 minute. The mixture was then added with 3.5 mL of 7.5% Na_2CO_3 and left for the maximum time before the absorbance value was measured. Total phenolics was calculated using equation (1), where C = concentration of gallic acid established from the calibration curve (mg/ml), V = volume of extract (mL) and m = weight of the plant extract (g).

\[
\text{Phenolics Total} \left( \frac{\text{mg GAE}}{\text{g}} \right) = \frac{C \times V}{m} \quad \ldots \quad (1)
\]

Determination of total anthocyanins
Total anthocyanin test was completed according to Lee et al. (2005). Teak leaf extract (50 mg) in HCl-KCl buffer solution pH 1 (4 mL) and teak leaf extract (0.05 g) in CH_3COOH-CH_3COONa buffer solution pH 4.5 (4 mL) were left for 2 hours. The absorbance was measured at 520 nm and 700 nm. Total anthocyanins was calculated using equation (2), where A = (A_520nm - A_700nm;pH 1.0) - (A_520nm - A_700nm;pH 4.5), MW = molecular weight of the cyanidin-3-glucoside (449.2 g/mol), DF = dilution factor, E = molar extinction coefficient (26,900 L/mol.cm) and l = cell path length (1 cm).

\[
\text{Total Anthocyanins} \left( \frac{\text{mg/L}}{} \right) = \frac{A}{\varepsilon \times 1} \times \text{MW} \times \text{DF} \times 1000 \ldots \quad (2)
\]
Determination of antioxidant activity

The antioxidant activity determination was performed according to Salazar-Aranda et al. (2011). Teak leaf extract (5 mg), FA (5 mg), FC (5 mg) or vitamin E (5 mg) was diluted with methanol in a 50 mL volumetric flask to give the concentration of 100 ppm. Each extract and fraction solution was made at concentrations of 5, 10, 15, 20 and 25 ppm in a 5 mL volumetric flask, then added 1 mL of a 0.2 mM DPPH solution in methanol. The solution was left for 30 minutes before they were analyzed at $\lambda_{\text{max}}$ wavelength. The $\lambda_{\text{max}}$ wavelength was determined by measuring DPPH 100 ppm solution at wavelength of 800-400 nm. Antioxidant activity was calculated using equation (3), where $A = $ absorbance of the negative control and $B = $ absorbance of the sample. The correlation between each concentration and its percentage of scavenging was plotted, and the IC$_{50}$ was calculated by interpolation. The antioxidant activity was quantified as IC$_{50}$ that represents the effective concentration of each extract which scavenges 50% of DPPH radicals.

$$\text{Antioxidant activity} = \frac{(A-B)}{A} \times 100 \quad \text{(3)}$$

Data analysis

Isolated compounds were identified and analyzed using UV-Vis Spectroscopy, GC-MS and LC-MS. Total phenolics, total anthocyanins, and antioxidant activity are shown in the form of figures that quantitatively stated their content in teak leaves.

RESULTS AND DISCUSSION

The resulting fractions from LVC were collected into four falcon tubes based on the same TLC spots using a mixture of hexane and ethyl acetate (1: 5) as eluent, which are fractions 3-5, fractions 6-8, fraction 9-20 and fractions 21-30. The fractions 3-5 and fractions 9-20 were then fractioned further by GCC. Fractionation of fractions 3-5 was completed by GCC for two times to obtain fraction A (FA). Fractionation of fraction 9-20 was also performed for two times by GCC to obtain fraction C (FC). The UV-Vis Spectroscopy spectra of fraction FA and FC are shown in Figure 1.

The GC spectrum of fraction FA is presented in Figure 2 and LC-MS spectrum of fraction FC is displayed in Figure 3. Analysis of GC chromatogram of fraction FA revealed that this fraction contains 76 compounds. However, MS data of the fraction FA revealed that only 15 compounds were able to identify (Table 1).

![Figure 1. UV-Vis spectra of fraction FA (A) and fraction FC (B)](image-url)
Figure 2. GC Spectrum of fraction FA

Figure 3. LC Spectrum of fraction FC

Table 1. Identified compounds of fraction FA by GC-MS analysis.

| Retardation time (mins) | Peak number | % area | Molecule formula | Compound |
|-------------------------|-------------|--------|------------------|----------|
| 3.433                   | 1           | 1.40   | C₆H₁₂O₆         | butyl acetate |
| 4.008                   | 2           | 8.50   | C₆H₁₂O₂         | 4-hydroxy-4-methyl-2-pentanone |
| 16.592                  | 8           | 4.28   | C₅H₁₀O₄         | Glycerin monoacetate |
| 19.433                  | 9           | 0.91   | C₆H₁₂O₅         | glycerin diacetate |
| 31.508                  | 23          | 0.34   | C₆H₁₀O₂         | isopropyl myristate |
| 33.592                  | 33          | 0.86   | C₆H₁₂O₂         | 1-eicosanol |
| 33.708                  | 34          | 2.72   | C₅H₁₀O₄         | methyl decanoate |
| 35.092                  | 35          | 0.56   | C₁₉H₃₆O₂        | 1-octadecene |
| 37.100                  | 41          | 1.11   | C₁₀H₁₈O₂        | 9,12-dimethyl octadecadienoate |
| 37.233                  | 42          | 3.81   | C₁₇H₃₆O₂        | 11-methyl octadecenoate |
| 38.975                  | 48          | 0.62   | C₁₇H₃₆Cl        | 1-chloro hexadecane |
| 44.267                  | 62          | 0.20   | C₁₉H₃₄          | hexadecane |
| 47.425                  | 67          | 0.34   | C₁₉H₄₂          | eicosanoid |
| 48.775                  | 69          | 0.50   | C₁₀H₃₄NO        | 9-octadecanamide |
| 48.900                  | 70          | 0.36   | C₁₇H₃₆          | heptadecane |

Table 2. Retardation time (Rt) and mass-to-charge ratio (m/z) of compounds of fraction FC eluded by LC-MS.

| Rt (Retardation time) | m/z     | Compound                                      |
|-----------------------|---------|-----------------------------------------------|
| 0.77                  | 699.35  | -                                             |
| 1.70                  | 700.35  | -                                             |
| 3.17                  | 700.45  | -                                             |
| 4.39                  | 700.35  | -                                             |
| 6.11                  | 700.55  | -                                             |
| 6.45                  | 697.35  | -                                             |
| 8.36                  | 683.34  | petunidin-3-O- (6-O-acetyl)-5-O-diglucoside   |
| 9.00                  | 694.35  | -                                             |
| 10.99                 | 698.35  | -                                             |
| 12.81                 | 691.79  | -                                             |
| 13.19                 | 698.35  | -                                             |
| 15.01                 | 700.35  | -                                             |
| 17.24                 | 697.35  | malvidin-3-α- (6-O-acetyl)-5-O-diglucoside    |
| 21.07                 | 695.35  | -                                             |

Table 3. IC₅₀ values of the samples

| Sample                | IC₅₀ (ppm) | Antioxidant activity |
|-----------------------|------------|----------------------|
| Methanol extract      | 21.95      | Very strong          |
| Fraction FA           | 29.73      | Very strong          |
| Fraction FC           | 32.71      | Very strong          |
| Vitamin E             | 24.12      | Very strong          |

Total phenolics and anthocyanins contents of teak leave methanol extract was 6.17 mg GAE/g and 67.5 mg/L, respectively. The DPPH method using methanol as solvent was applied for determination of antioxidant activity (Lung and Destiani 2017) and vitamin E was used as a positive control. The antioxidant activity of the samples was confirmed by decreasing the absorbance and changing sample solution color from purple to yellow. The
antioxidant activity of samples was quantified as IC$_{50}$ values which are presented in Table 3.

**Discussion**

As presented in Table 3, methanol extract, fraction F$_A$ and fraction F$_C$ had very strong antioxidant activity. The strong antioxidant activity of methanol extract was confirmed by the total phenolics and anthocyanins content of the methanol extract which is known to have strong antioxidant activities. The strong antioxidant activities of fraction F$_A$ and fraction F$_C$ were also confirmed by the presence of chemical contents of those fractions.

As shown in Fig. 1 (a), the UV-Vis spectrum of fraction F$_A$ has two peaks at 208 and 492 nm. This spectrum feature was similar to the spectrum obtained by Baharuddin et al. (2015), which had two peaks at 220 and 522 nm. The UV-Vis spectrum of fraction F$_C$ has peak at 489 nm (Fig. 1 (b)). Anthocyanins give peak at wavelength of 475-550 nm, indicating that fractions F$_A$ and F$_C$ contain anthocyanins.

As confirmed by GC-MS analysis, fraction F$_A$ contains alkanes (namely hexadecane, 1-chloro hexadecane, heptadecane, and eicosanoid), alcohols (namely 1-icosanol), ketone (4-hydroxy-4-methyl-2-pentanone), esters (namely butyl acetate, isopropyl myristate, methyl decanoate, 11-methyl octadecenoate, 9,12-dimethyl octadecadienoate), amide (namely 9-octadecenamide) and glycerin (namely glycerol monoacetate and glycerin diacetate). These compounds mostly have chromophore groups that give specific colors (Qadariyah et al. 2018) and the color of fraction F$_A$ was orange. Yet, no anthocyanins in fraction F$_A$ were detected by GC-MS analysis because the GC column temperature was 300°C, while anthocyanin’s boiling point was more than 300°C. Among those 15 compounds of fractions F$_A$, 4 compounds have OH groups on their structure which cause antioxidant activity of fraction F$_A$. They are 4-hydroxy-4-methyl-2-pentanone (1), glycerin monoacetate (2), glycerin diacetate (3) and 1-icosanol (4). These compounds force together to reduce free radicals. Murukan and Murugan (2018c) reported that methanolic leaf extracts of young and mature leaf of *T. grandis* has phenolic compounds such as gallic, vanillic, *p*-hydroxybenzoic, ferulic, chlorogenic, sinapic, *p*-coumarate, and cinnamic acids.

The LC-MS analysis of fraction F$_C$ revealed 15 peaks. Two compounds having retention time of 8.36 and 17.24 minutes were able to be identified. The compound which was eluted at 8.36 minutes had m/z 683.34 and was classified as secondary metabolite of anthocyanin, namely petunidin-3-O-(6-O-acetyl)-5-O-diglucoside (5) (Figure 3) Han et al. (2017). The second compound had m/z of 697.35 and was also classified as secondary metabolite of anthocyanin, namely malvidin-3-O- (6-O-acetyl)-5-O-diglucoside (6) (Figure 4). This result is in accordance with the research of De la Cruz et al. (2012) and Han et al. (2017) that malvidin-3-O- (6-O-acetyl)-5-O-diglucoside (6) was determined at a retention time of 18 minutes according to LC-MS result. These anthocyanins have OH groups, heterocyclic rings and conjugated double bonds in its structure that provide very strong antioxidant activity. In conclusion, the research showed that the teak leaves have active compounds which strong antioxidant activities.
Figure 4. Identified compounds which cause antioxidant activity

Figure 5. MS data of the compound with retention time of 8.36 minute

Figure 6. MS data of the compound with retention time of 17.24 minute

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