Journal of Materials and Environmental Sciences
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2020, Volume 11, Issue 4, Page 609-614

http://www.jmaterenvironsci.com

Phytochemicals, Total Flavonoid Content and Antioxidant Activity of Methanol Extract of *Diospyros discolor* Seeds

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Received 22 October 2019,
Revised 14 March 2019,
Accepted 16 Match 2019

Keywords
- ✓ *Diospyros discolor*,
- ✓ Seeds,
- ✓ Antioxidant activity,
- ✓ Total flavonoid,
- ✓ Phytochemical.

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Abstract

Diospyros discolor fruit can be found as a local fruit of Bogor, Indonesia, which is sold in traditional markets. Phytochemicals, total flavonoid content and antioxidant activity of *D. discolor* seeds has been studied. Maceration and soxhlet techniques were used in extracting *D. discolor* seeds. The antioxidant activity of methanol extracts of *D. discolor* seeds has been investigated by employing the radical scavenging activity using the DPPH• method. The total flavonoid content was measured by a UV-Vis spectrophotometer based on Zou method. *D. discolor* seeds extract maceration has a higher antioxidant capacity with an IC_{50} value of 18.9 µg / mL, with IC_{50} of quercetin as a positive control was 6.38 µg / mL. Total flavonoid content of the maceration extraction technique about 335.40 ± 8.62 mg CAE/g dry powder.

1. Introduction

Degenerative diseases are mediated by free radicals with high reactivity such as cancer, cardiovascular, cataract, and inflammation. Oxidative stress in cells caused by an increase in the number of free radicals [1]. Antioxidants are compounds that, in small quantities, are capable of inhibiting the oxidation process and helps the endogenous defense system to reduce oxidative damage [2].

Every plant produces a secondary metabolism which is a natural antioxidant [3]. Antioxidant activity in a plant can be caused by the content of phenolic compounds [4]. A flavonoid is a group of phenolic compounds that are most commonly found in plants [5]. The potential for phenolic antioxidants in plants is related to their ability to donate electrons, reduction power and the ability to chelate metal ions [5,6].

Bioactive compounds from plant materials can be extracted by various conventional extraction techniques including soxhlet extract, maceration, and hydrodistillation. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing [7]. This conventional technique is a popular and inexpensive technique for obtaining bioactive compounds from plants.

Diospyros discolor is a tropical plant that belongs to the Ebenaceae family. In Indonesia, *D. discolor* (Figure 1a) is known as "bisbul" and is an endemic plant in Bogor [8]. *D. discolor* plants including fruit, bark, and leaves was investigated to have antioxidant activity beside it also has antidiarrhoeal and anticancer properties [1,9]. Analgesic activity [10] and metal adsorption capacity [8]

of *D. discolor* seeds (Figure 1b) have also been studied and have received a great deal of attention to be developed.

In the present study, the comparisons of the methanol extract of *D. discolor* seeds between maceration and soxhlet extraction techniques were examined for antioxidant activity. The method of antioxidant activity used in this study was 2,2-diphenyl-1-picrylhydrazyl (DPPH). Total flavonoid levels were determined to see the correlation with antioxidant activity.

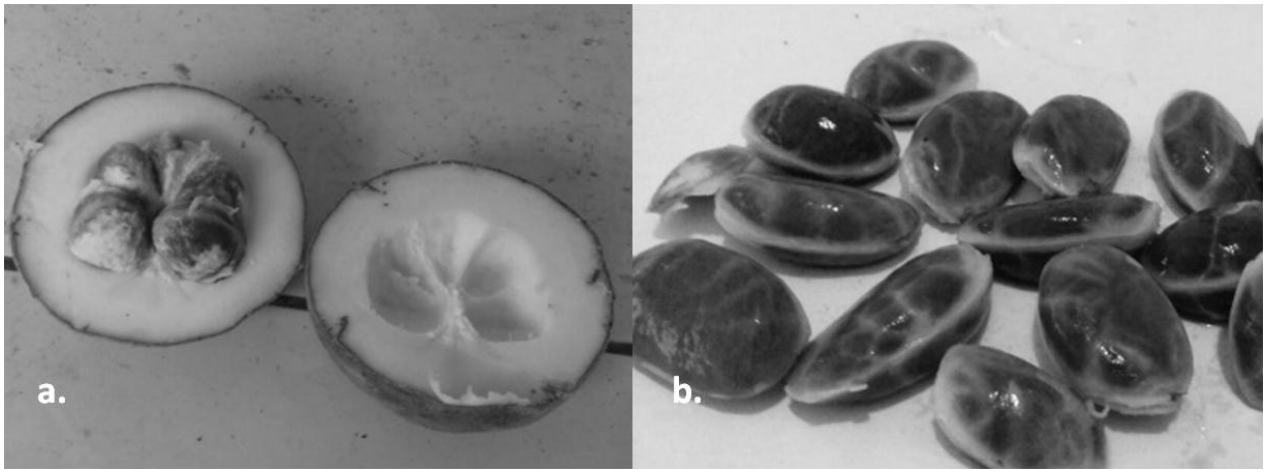

Figure 1. *Diospyros discolor* (a) and seeds (b)

2. Material and Methods

2.1. Materials

1,1-diphenyl-2-picrylhydrazyl (DPPH), Quercetin, Catechin were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, chloroform, sodium nitrite, sodium hydroxide, Aluminum chloride hexahydrate was bought from Smart-Lab (Tangerang, Indonesia). *D. discolor* fruit was bought from a traditional market in Bogor.

Spectrophotometric assays were performed on a single beam UV-Vis spectrophotometer (Optizen POP, Mecasys, Korea). Each of the *D. discolor* samples were analyzed in triplicate.

2.2. Extract preparations for analysis and antioxidant assay

D. discolor seeds were cleaned, ground and sieved. The powder was dried in an oven at 40ºC for 8 h.

Maceration samples. The powder (150 g) was soaked in 450 mL of 98% methanol for 3 days, stirring every 12 h and replaces the solvent every 1 day. The final extracts were filtered using Whatman filter paper (Whatman Ltd., UK). The solvent of the filtrate was removed by rotary evaporator 40ºC and stored at 4ºC for further use.

Soxhlet samples. The powder (20 g) was extracted with 200 mL of 98% methanol using a Soxhlet extractor. After the solvent of the filtrate was removed by a rotary evaporator, it was stored at 4ºC for further tests.

2.3. Phytochemical Screening

Preliminary qualitative phytochemical analysis was made by following standard procedures by using the following reagents and chemicals[11].

2.4. Determination of total flavonoid

The total flavonoid content was determined by the use of a slightly modified colorimetric method[12]. A 1-mL aliquot of appropriately diluted sample solution was mixed with 4 mL of distilled water and subsequently with 0.3 mL of a 5% $NaNO_2$ solution. After 6 min, 0.3 mL of a 10% AlCl3 solution was added and allowed to stand for 6 min, then 2 mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 10 mL, then the mixture was thoroughly

mixed and allowed to stand for another 15 min. The absorbance of the mixture was determined at 502 nm versus a prepared water blank. Total flavonoid contents were expressed in terms of catechin equivalent (CAE) (standard curve equation: $y = 0.002x + 0.002$, $R^2 = 0.998$), mg of CAE/g of dry extract.

2.5. DPPH radical scavenging assay

A 2-mL solution of DPPH$^{\bullet}$ 0.1 mmol/L was mixed with 1 mL of extract in methanol at different concentrations[13]. The reaction mixture was vortexed thoroughly and incubated in a dark room for 30 min. The absorbance of the mixture was measured at 515 nm. Quercetin was used as a reference. Percentage DPPH radical scavenging activity was calculated by the following equation:

$$\% \text{ DPPH radical scavenging activity} = [(A_0-A_1)/A_0 \times 100\%] \quad (1)$$

where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extracts/standard. The half-maximal inhibitory concentration (IC_{50}) was calculated from the graph between the percent of inhibition against concentration.

2.6. Statistical Analysis

Statistical comparisons were assessed with one-way analysis of variance (ANOVA) using SPSS Statistics (IBM SPSS Statistics) for Windows program, when differences were significant ($p<0.05$).

3. Results and discussion

3.1. Sample extraction

The yields of *D. discolor* seeds extract of maceration technique were worth 38.67% which showed the highest extract yield, while soxhlet extraction 14.70% as presented in Table 1. The difference in extract yield is due to the high temperature used in the soxhlet technique, volatile organic compounds are degraded or decomposed during the extraction process[14].

Table 1. Total flavonoid of methanol extract of *D. discolor* seeds

Extraction Technique	Yield (%)	Total Flavonoid (mg CAE/g of dry extract)	Antioxidant activity IC_{50} (µg/mL)
Maceration	38.67	335.40 ± 8.62	18.91
Soxhlet	14.70	54.65 ± 0.63	76.87

Values are mean ± S.E.M, n=3; and IC_{50} of quercetin as a positive control was 6.38 µg/mL

3.2. Phytochemical Screening

Preliminary qualitative phytochemical analysis that has been carried out consists of alkaloids, flavonoids, saponins, steroids, triterpenoids, and tannins can be seen in Table 2. Alkaloid compounds found in *D. discolor* seeds extract were shown from the precipitate formed by adding Mayer reagents.

Table 2. Preliminary qualitative phytochemical analysis of *D. discolor* seeds extract

Phytochemical Screening	Reagents/ chemicals	Methanol Extract
Alkaloids	Mayer	++
Flavonoids	Willstatter cyaniding test	++
Saponins	Forth test	++
Triterpenoids	Liebermann-Burchard	+
Steroids	Liebermann-Burchard	++
Tannins	Ferric chloride	++

++: highly present, +: Low, -: absent

Flavonoids were carried out using the Willstatter cyaniding test and a reddish-orange solution was produced. The fourth test was used in saponins identification in the presence of foams. Steroids/triterpenoids were using the Liebermann-Burchard test and Tannins ware using ferric chloride reagent. All identification shows positive results.

3.3. Total flavonoid contents

Flavonoid content in plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Flavonoid content of the different extraction techniques *D. discolor* seeds, maceration and soxhlet were studied. Results revealed that different extraction techniques consisted of different amounts of total flavonoid content. There were statistically significant deferences ($p<0.05$) between the two extraction techniques. The total flavonoid of *D. discolor* seeds maceration technique was higher (335.40 ± 8.62 mg CAE/g dry powder) than soxhlet extraction (Figure 2). The low value of the total flavonoid content on the soxhlet extraction was due to the thermal effect on the extraction process [14].

The comparisons of the total flavonoid content from various parts of *D. discolor* shown in Table 3. Extract of *D. discolor* seeds had a total flavonoid content value that higher than the leaves. However, these seeds extract are still less than stem and root bark [4].

Table 3. The comparisons of the total flavonoid content from various parts of *D. discolor*

Extracts of *D. discolor*	**Total Flavonoid (mg CAE/g of dry extract)**	**Antioxidant activity IC_{50} (µg/mL)**
Leaves	268.000 ± 6.557[1]	72.50[4]
Root bark	382.000 ± 12.097[1]	45.78[4]
Stem bark	412.000 ± 16.700[1]	
Seeds(present)	*335.400 ± 8.620*	*18.91*

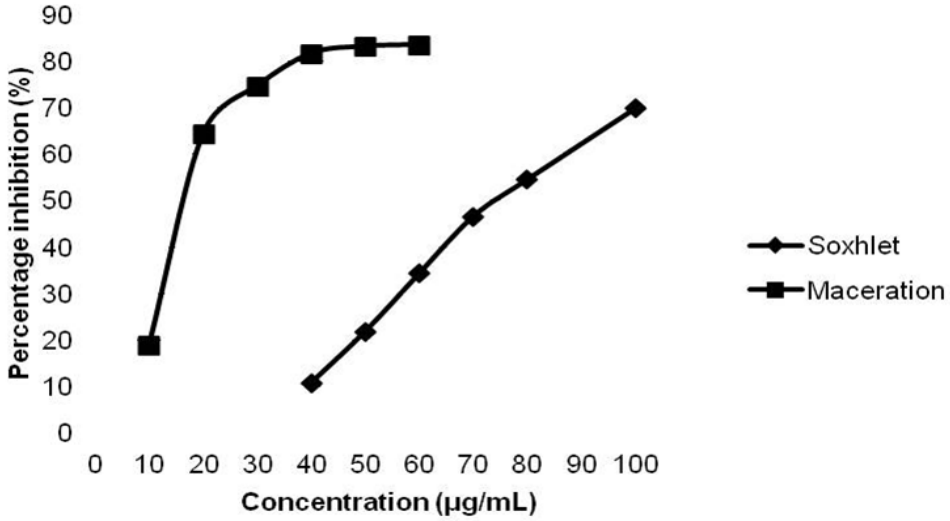

Figure 2. Antioxidant activity of *D. discolor* seeds extract from different technique

3.4. Antioxidant activity

The DPPH radical had been used to determine antioxidant activities which associate with bioactive compounds. Radical scavenger capacities on bioactive compounds are able to reduce DPPH radical using donor hydrogen atom[15]. Antioxidant activities of *D. discolor* seeds with different extraction techniques showed differences significantly. The maceration technique extract had higher antioxidant activity (IC_{50} 18.91 µg/mL) than the soxhlet extract (76.87 µg/mL), with IC_{50} of quercetin as a positive control was 6.38 µg/mL in **Table 1**. *D. discolor* seeds (maceration) had higher antioxidant activity than other parts shown in **Table 3**.

This study confirmed that there is a strong correlation between total flavonoid content and antioxidant activity [16-20]. The option of extraction techniques will also affect the value of total flavonoid content and antioxidant activity. A low IC_{50} value means high antioxidant activity and correlates with high total flavonoid content.

Conclusion

The present study reveals that the difference extraction techniques of *D. discolor* seeds possess significant total flavonoid content and antioxidant activity. The maceration extract of *D. discolor* seeds had a higher total flavonoid content and antioxidant activity. This study provides scientific evidence that the content of flavonoid compounds influences significant scavenging properties of free radicals.

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