Advance Sustainable Science, Engineering and Technology (ASSET)
Vol. 6, No.3, July 2024, pp. 0240307-01 ~ 0240307-08
ISSN: 2715-4211 DOI: https://doi.org/10.26877/asset.v6i3.598

Phytochemical and Antioxidant Activity of Akway (*Drymis piperita* Hook f.) Stem Bark Ethanol Extract

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Abstract. The amount of solvent affects the effectiveness of the extraction, so the chemical components are extracted entirely from the plant. This study investigated the best ratio of Akway bark : solvent (ethanol) to produce Akway extract with the highest antioxidant activity. Extraction with the treatment of Akway skin: ethanol ratio of 1:2; 1:4; 1:6 (b/v) was carried out and then the results were tested for total phenolic and flavonoid content and antioxidant activity. The best Akway bark:ethanol ratio was obtained at 1:4 (b/v) ratio with 9.54% extract yield, 289.57 mg GAE/g total phenolic content, 185.47 mg eq quercetin/g total flavonoids. Akway bark extract has potential as a source of antioxidants as indicated by its high antioxidant activity with DPPH free radical scavenging and ferric reducing activity of 50% (IC50) at extract concentrations of 16.59 µg/mL extract and 60.93 µg/mL extract, and carotene bleaching inhibition activity of 85.76%. There are 4 types of phenolic compounds and 4 types of flavonoid compounds identified with the highest percentage of components, namely Pereniporin B (57%).

Keywords: Akway bark, bioactive extraction, antioxidant activity, bioactive compound

(Received 2024-05-15, Accepted 2024-06-08, Available Online by 2024-06-11)

1. Introduction

Traditional medicine has been used for prevention, treatment, and immune enhancement since ancient times. Many indigenous plants in an area are often used for medicinal purposes [1]. Akway (*Drimys piperita* Hook f.) is an endemic Papuan plant with woody, thick-leaved aromatic characteristics and is related to winteraceae, which is used to treat malaria and increase vitality and endurance [2]–[4]. This plant grows in the Arfak Mountains of West Papua at an altitude of 1200-2400 meters above sea level [5].

Akway is reported to be able to act as an antioxidant and antimicrobial. Ethanol extract of Akway bark contains alkaloid, saponin, triterpenoid, flavonoid, and tannin compounds [6]. The total phenolic and total flavonoid contents of the ethanol extract of Akway bark amounted to 148.8 mg gallic acid

equivalents/g and 82.14 mg quercetin equivalents/g, with free radical scavenging activity at a concentration of 800 μg/mL of 89.35% [7].

Maceration is one of the extraction methods to separate organic compounds in plants using organic solvents [8]. The ratio of solids to solvent affects the yield and antioxidant components extracted, but the excessive use of solvents can reduce the yield and quality of the extract [9]. The effect of solid-solvent ratio on Kedawung leaf extract by microwave-assisted method has a significant effect, where the solid-solvent ratio of 1:40 g/ml produces yield, total phenolic content, flavonoids and antioxidant activity [10].

The most widely used solvent for the extraction of bioactive compounds is methanol, but excellent results can be obtained with ethanol. In addition, ethanol is non-toxic; hence, some authors recommend using this solvent to extract bioactive compounds [11]. Studies on the solid-solvent ratio and phytochemical identification of the ethanol extract of Akway bark have not been identified. Therefore, this study aims to determine the effect of the material-solvent ratio on the total phenolic content, flavonoids, and antioxidant activity of ethanol extract of Akway bark and identify active components.

2. Methods

2.1. Materials and instrument

The materials used in this study include Akway bark obtained from the Arfak Mountains, Manokwari, West Papua. Chemical reagents used in this study include ethanol, distilled water, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, BHT, ascorbic acid, 30% hydrogen peroxide, 0.2 M phosphate buffer pH 6.6, natrium carbonate, folin-ciocalteau, standard gallic acid, potassium ferricyanide, trichloro acetate, ferric chloride, β-carotene, linoleic acid.

The research instruments used include a vacuum pump (Rocker 300), rotary evaporator (IKA RV 10), UV Vis spectrophotometer (Thermo Scientific Genesys 10S), HR-MS (Thermo Scientific Hybrid Quadrupole Orbitrap Mass Spectrometer), water bath shaker (Memmert).

2.2. Extraction Akway bark

The extraction of active components of Akway bark follows the method of Cepeda et al. (2018). Akway bark was dried until the moisture content reached 10-12%, then ground and sieved using a 40 mesh sieve. The extraction process was carried out using the maceration method, with the treatment of the ratio of Akway bark powder and solvent (ethanol) 1:2, 1:4, and 1:6 (b/v). The extraction was conducted at room temperature for 72 hours, with continual stirring at 100 rpm. The extract was passed through filters and the solvent was then evaporated using a rotary evaporator at 40°C and 60 rpm until all the solvent evaporated.. The resulting dry extract was stored in a dark colored bottle and kept at 4ºC. The extract yield is determined based on the percentage of dry Akway bark weight to the resulting extract.

2.3. Total Phenolic and Flavonoid content

Total phenolic content was determined following Hung et al. (2002) [12]. 0.1 mg extract was dissolved in 0.1 mL of distilled water plus 0.1 mL of 50% Folin- Ciocalteu reagent and then vortexed. After 3 minutes, 2 mL of 2% Na_2CO_3 solution was added and kept in a dark room for 30 minutes. The absorbance was obtained using a spectrophotometer at 760 nm. A standard curve was created using gallic acid. The results are presented in mg equivalent gallic acid per g extract.

Total flavonoid content was determined according Roy *et al* (2009) [13]. 50 μL of extract, 4 mL distilled water and 0.3 mL of 10% $NaNO_2$. After incubating for 6 minutes, add 4 mL of 10% NaOH and distilled water to a volume of 10 mL, vortexed for 1 minute, and left for 15 minutes. Absorbance was measured at a wavelength of 510 nm. The blank used distilled water. Flavonoid content was calculated using quercetin standard as mg equivalent of quercetin/g extract.

2.4. Antioxidant activity determination

Free radical scavenging capacity of Akway bark extract was obtained based on the ability of the extract to scavenge DPPH free radicals. 1 mL of various extract concentrations was added with 2 mL of DPPH solution in 0.08 mM methanol, then it was vortexed and left for 30 minutes at room temperature under dark conditions. The absorbance was detected at a wavelength of 517 nm, with methanol as a

blank. Free radical scavenging capacity was calculated according to the equation 1. The values were presented as IC_{50} (ppm), indicating the effective concentration at 50% free radical scavenging activity.

$$\text{Free radical scavenging capacity (\%)} = [\ 1 - \frac{sample\ absorbance}{blank\ absorbance}\ ] \times 100\% \quad (1)$$

Ferric reducing capacity of the extract is determined by the extract capability to reduce Fe^{3+} to Fe^{2+}. 2.5 mL extracts at various concentrations were dissolved with phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide ($K_3Fe[CN]6$). The mixtures underwent incubation at 50°C for 20 minutes before mixing 2.5 mL of 10% TCA and centrifuged at 3000 rpm for 10 minutes. 2.5 mL top layer fraction was added with 2.5 ml of distilled water and 100 µl of 0.1% ferric chloride. The absorbance was measured at 700 nm. The results were presented as IC_{50} (ppm), representing the effective concentration at 0.5 absorbance. Higher absorbance implies more reducing power [14].

The ability of the extract to inhibit carotene bleaching was determined according to the method of Nyanyiru *et al.* (2020) [15]. 10 µl of linoleic acid, 100 µl of Tween 80, and 1 ml of β-carotene solution (2 mg of β -carotene in 10 ml of chloroform) were mixed. After that, 25 mL of 30% hydrogen peroxide was added slowly to the β -carotene emulsion mixture and the solution was shaken vigorously to form a stable emulsion. 2.5 mL of the emulsion solution was added 100 µL of extract at a concentration of 10 mg/mL, while the control contained disstiled water. The mixture was stirred vigorously and the absorbance was measured at 470 nm. Next, the sample was placed in a water bath at 50°C for 180 minutes and absorbance was measured. The antioxidant activity of the samples was evaluated with the inhibition of β -carotene bleaching by the formula :

$$\text{Inhibition (\%)} = \frac{abs\ \ sample\ at\ 180\ min - abs\ control\ \ at\ 180\ min}{abs\ control\ at\ 0\ \text{min} - abs\ control\ at\ 180\ min} \times 100\% \quad (2)$$

2.5. Analysis of experimental data and determination of the best extract ratio

The experimental design used a single-factor complete randomized design (CRD) with a variable ratio of Akway bark: ethanol 1:2, 1:4, an 1:6 (b/v) with 3 treatment replicates. The parameters analyzed (TPC, TFC, DPPH radical scavenging activity, ferric reducing activity, and β-carotene bleaching inhibiton) were repeated 3 times, and the data collected from each independent variable were analyzed by Statistical Product and Service Solution (SPSS) software with the One-way Analysis of Variance (ANOVA) method at the 5% significance level. The presence of significant differences ($p < 0.05$) between treatment means was tested by the Duncan Multiple Range Test (DMRT) method. The best solid-to-solvent ratio was determined based on the lowest IC_{50} value of DPPH free radical scavenging activity parameters and reducing power, as well as the highest ability of the extract to inhibit carotene bleaching.

2.6. Component identification of Akway bark extract

The best solid to solvent ratio was identified using HR-LCMS (Thermo Scientific™ Dionex™ Ultimate 3000 UHPLC coupled with Thermo Scientific™ Q Exactive™). Phytochemical separation of sample extracts was carried out with a Hypersil Gold aQ analytical column (100 mm x 1 mm x 1.9 mm). Mobile phase A consisted of distilled water and 0.1% formic acid), while mobile phase B consisted of acetonotrile + 0.1% formic acid. Flow rate 40 µL/min, injection volume 5 µL. Gradient profile 5-90 %B in 16 minutes, 90 - 90 %B up to 18 minutes, 90 - 5% B in 25 minutes. Mass instrument has a resolution setting of 70,000 FWHM, MS2 data at 17,500 FWHM for positive and negative. Compound Identification with Thermo Scientific™ Compound Finder Software including mzVault, ChemSpider, and mzCloud libraries [16].

3. Results and Disscussion

3.1. Yield Extract

The yield of Akway bark extract is presented in Table 1. The ratio of material to solvent had a significant effect on the yield of the extract produced. The more solvents used, the higher the extract yield. Noviyanty et al. (2019) reported that the yield of dragon fruit peel extract increased from 22.66 -

to 25.92% with a higher ratio of solvent material [9]. The higher the quantity of solvent used, the difference in the concentration of the solution inside and outside the plant cell decreases, thus promoting the diffusion rate of particle solutes and extracting more components [17].

3.2. Total Phenolic and Flavonoid Extract

Phenolic and flavonoid compounds confirmed their role as anticancer, antimicrobial, antioxidant, antimalarial, neuroprotective, antitumor, and attenuation or prevent neurodegenerative diseases [18], [19]. The total phenolic and total flavonoid contents of the ethanol extract of Akway bark obtained ranged from 232-289 mg GAE/g and 163-185 mg eq quercetin/g. Cepeda et al. (2018) reported the total phenolic and total flavonoid contents of Akway bark ethanol extracts of 148.40 mg GAE/g and 82.14 mg eq quercetin/g [20]. The difference in these results is due to differences in the analytical methods used and the location where it grows, affecting the content of secondary metabolites contained in these plants [21].

The analysis of variance revealed that the ratio of Akway bark : ethanol had a significant influence on total phenolic and total flavonoid levels. The total phenolics and flavonoids of Akway extract increased from 1:2 to 1:4 ratio treatment, and then they were relatively stable at 1:6 ratio (Table 1). A higher solute-solids ratio increases the concentration gradient, which leads to an increase in the diffusion rate of compounds from the extracted solid material into the solvent so that more active components are extracted, which can increase the total phenolic and flavonoid levels. The solvent-solvent ratio of 1:4 showed the best extraction condition based on the extract's highest total phenolic and flavonoid content. The addition of solvents up to a ratio of 1:6 has no change in total phenolics and flavonoids, which is thought to be due to the extract solution having passed the saturation point so that there is no increase in total phenolics and flavonoids [9].

Table 1. Yield, total phenolics and total flavonoids of Akway bark extract.

Akway : ethanol ratio (b/v)	Yield (%)	Total phenolic contents (mg eq gallic acid/ g)	Total flavonoid contents (mg eq quercetine/g)
1:2	5.08 ± 0.85^{a}	232.86 ± 6.34^{a}	163.60 ± 5.54^{a}
1:4	9.54 ± 1.01^{b}	289.57 ± 10.59^{b}	185.47 ± 6.41^{b}
1:6	15.00 ± 0.46^{c}	268.67 ± 26.26^{b}	176.67 ± 10.86^{ab}

*Values with the same letter in a column indicate not significantly different at $\alpha<0.05$

3.3. Antioxidant activity of Akway Extract

A comparison of the antioxidant activity of the ethanol extract of Akway bark against the antioxidant activity of ascorbic acid and BHT is presented in Table 2. The capability of the extract to scavenge DPPH radicals was significantly affected by the solvent-ingredient ratio ($p<0.05$). The IC_{50} values of DPPH inhibition and ferric reducing power of Akway bark extract were 16.43-19.05 ppm and 60.93-63.32 ppm. The antioxidant activity of Akway bark ethanol extract increased from 1:2 to 1:4 ratio treatment; there was no significant difference between the antioxidant activity of extracts from 1:4 and 1:6 treatments.

The increase in antioxidant activity of the extract was proportional to the increase in total flavonoid content (Table 1). These results are in accordance with Montoro et al. (2006), who stated that black *M. communis* berries have high radical scavenging activity, which can be related to their high flavonoid content [22]. Akway bark is a potential antioxidant with higher total phenolic and flavonoid content and antioxidant activity than extracts of Kedawung leaf, *Parkia speciosa* Peel, and Buton Bajakah root [8], [10], [23].

The IC_{50} value of Akway bark extract DPPH inhibition was 64% higher than the IC_{50} of ascorbic acid, and 20% higher than BHT. These results indicate ascorbic acid has the highest DPPH free radical scavenging activity. The antioxidant activity of ascorbic acid is higher than that of extract because ascorbic acid has a free polyhydroxy group that acts as a free radical scavenger that can increase antioxidant activity [24].

The reducing power of extracts is often used as an indicator of potential antioxidant activity. Extracts with a reducing ability indicate that the extract is an electron donor that can reduce metal ions, accelerating the oxidation process to function as a secondary antioxidant [25]. The material:solvent ratio did not affect the IC_{50} value of reducing power of Akway extract, but the IC_{50} value of Akway extract was higher than the IC_{50} value of ascorbic acid and BHT. The IC_{50} value of reducing power of ascorbic acid is the lowest compared to BHT and Akway extract because ascorbic acid accelerates metal ion-dependent reactions such as Fe^{2+} and Cu^{+} ions and the reaction is faster than Fe^{3+} and Cu^{2+} [26].

Table 2. Antioxidant Activity of Akway bark extract.

Sample	IC_{50} (ppm)	
	Free radical scavenging capacity	Ferric reducing capacity
Ekstrak Akway ratio 1:2	19.05 ± 1.80[a]	62.61 ± 0.73[a]
Ekstrak Akway rasio 1:4	16.43 ± 0.18[b]	60.93 ± 0.29[a]
Ekstrak Akway rasio 1:6	16.93 ± 0.20[b]	63.32 ± 1.61[a]
Asam Askorbat	10.09 ± 0.09[c]	28.74 ± 0.69[b]
BHT	13.73 ± 0.02[d]	33.51 ± 0.64[c]

*Values with the same letter in a column indicate not significantly different at $\alpha<0.05$

3.4. β-carotene bleaching inhibition capacity of Akway Extract

One of the determinations of antioxidant activity uses the β-carotene bleaching method. The principle of this method is the presence of free radicals derived from the oxidation process of linoleic acid which acts as a free radical that attacks β-carotene so that it undergoes a degradation process characterized by blanching the color of β-carotene. Adding samples with antioxidant activity will neutralize the free radicals of linoleic acid, thus preventing the degradation of β-carotene (color bleaching). The higher the inhibition percentage, the stronger the antioxidant activity of the extract.

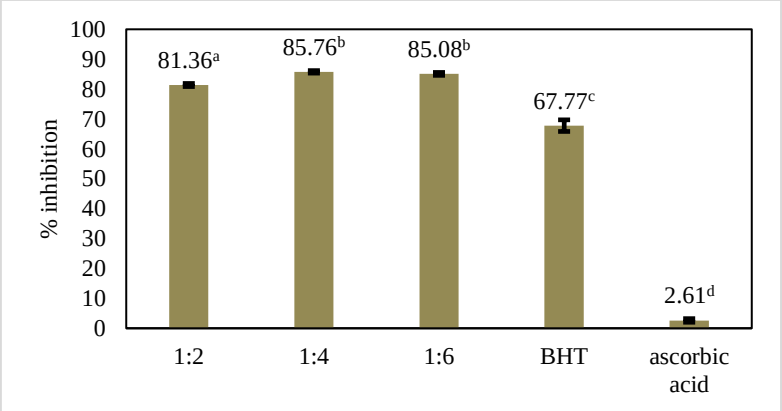

Figure 1. β-carotene bleaching inhibition of Akway extract

The inhibitory activity of carotene degradation of Akway bark extract was higher than ascorbic acid and BHT (Figure 1). The lowest carotene degradation inhibition activity was shown by ascorbic acid because ascorbic acid is a water-soluble (polar) antioxidant which less effective in protecting β-carotene linoleic acid emulsion [27]. The percentage inhibition of carotene degradation of Akway extract is higher than BHT because Akway extract contains fatty acids (Table 3), so solubility in the linoleic acid emulsion phase of β-carotene is high and can inhibit β-carotene degradation. Based on Table 2, the optimum Akway : ethanol ratio is 1:4 based on the IC_{50} value of DPPH free radical scavenging and the percentage inhibition of carotene degradation.

3.5. Components of Akway Extract

The results of compound component identification analysis of ethanol extract of Akway bark (*Drymis pipperita* Hook f.) are presented in Table 3. There are 4 types of phenol compounds identified, such as neochlorogenic acid, 5-p-coumaroylquinic acid, chlorogenic acid, dan 2,6-di-tert-butylphenol. The

Flavonoid compounds that have been identified are pereniporin B, kaempferol, 5-(5,7-Dihydroxy-3-methoxy-4-oxo-4H-chromen-2-yl)-2- hydroxy phenyl-beta-D-xylopyranoside,dan 4-Hydroxy-3-(3-methyl-2-buten-1yl)-phenyl-6-O-[(2R,3R,4R)-3,4-dihydroxy-4-(hydroxy methyl) tetrahydro-2-furanyl]-beta-D-glucopyranoside. The significant component in Akway bark extract is Pereniporin B, reportedly an anti-inflammatory and anticancer [28].

Several sesquiterpenes have been found in *Drymys* species, such as polygodial, isopoligodial, drimenol, confertifolin, and isodrimenin [29]. Polygodial is an unsaturated sesquiterpene dialdehyde with various potential biological applications, including antibacterial and antifungal. Besides, polygodial also has potential as an anticancer [30]. The components of Akway bark extract contain DL-carnitine which plays a role for improve stamina and endurance.

Several types of fatty acids in extract indicated that Akway bark extract is still very complex, so it needs to be purified for further research. Research on exploring Akway bark extracts can still be expanded by trying different extraction methods, such as ultrasonic and microwave-assisted extraction.

Table 3. Components of Akway extract

Compounds group	Compound name	Mw	RT (min)	m/z	Area (%)
Fenolik	Neochlorogenic acid	354.09	5.75	353.0870	3.90
	5-p-coumaroylquinic acid	338.10	5.99	337.0921	3.83
	Chlorogenic acid	354.09	9.72	353.0869	1.01
	2,6-di-tert-Butylphenol	206.17	18.05	205.1588	0.38
Flavonoid	Pereniporin B	266.15	17.16	265.1437	56.95
	Kaempferide	300.06	15.79	301.0691	2.57
	5-(5,7-Dihydroxy-3-methoxy-4-oxo-4H-chromen-2-yl)-2-hydroxyphenyl beta-D-xylopyranoside	448.10	11.20	447.0924	1.55
	4-Hydroxy-3-(3-methyl-2-buten-1-yl)phenyl 6-O-[(2R,3R,4R)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydro-2-furanyl]-beta-D-glucopyranoside	472.19	10.32	471.1861	0.70
Fatty acid	5-hydroxy-1,7,7-trimethyl-1,2,3,5,6,7,8-octahydrocyclopenta pentalene-4-carboxylic acid	250.16	17.82	249.1487	17.35
	Linoleidic acid	280.24	22.58	279.2322	2.59
	2,2,3-Trifluoro-3-(1,1,2,2,3,3-hexafluoro-3-trifluoromethoxy-propoxy)-propionic acid	377.97	5.34	379.9677	0.01
	Tetradecanedioic acid	258.18	5.49	239.1643	0.02
	13-hydroxyoctadeca-9,11,15-trienoic acid	294.22	5.47	295.2259	0.01
	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	330.24	14.69	329.2325	1.60
	Oleic acid	282.26	18.35	281.2478	0.82
	9-hydroperoxyoctadeca-10,12-dieonic acid	312.23	18.1	311.2220	0.72
	8-hydroxy-8-(3-octyloxiran-2-yl) octanoic acid	314.24	17.93	313.2377	0.68
	16-Hydroxyhexadecanoic acid	272.23	21.36	271.2271	0.52
	Corchorifatty acid F	328.22	14.21	327.2169	0.44
	12,13-dihydroxyoctadec-9-enoic-acid	314.25	17.67	313.2376	0.33
Asam amino	DL-Carnitine	161.10	5.34	162.1119	0.96
	1-Tetradecylamine	213.25	5.41	214.2521	0.01

4. Conclusion

The best akway bark:ethanol ratio was obtained at 1:4 (b/v) ratio with 9.54% extract yield, 289.57 mg GAE/g total phenolic content, 185.47 mg quercetin/g total flavonoids with DPPH free radical scavenging activity (IC_{50}): 16.59 µg/mL extract. The component with the highest percentage in Akway extract is Pereniporin B, which has a percentage of 57%. This information on the components of Akway extract offers an excellent opportunity for future research with exploration of extraction methods of

Akway bark extract, purification of compounds in the extract, and in vivo studies to determine its health effects, which need to be carried out.

Acknowledgements
The authors would like to precise our appreciation to the Lembaga Pengelola Dana Pendidikan Indonesia for funding this research.

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