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Total phenolic, flavonoid, and antioxidant capacity of bajakah (*Spatholobus littoralis* Hassk)

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ABSTRACT

The bajakah (*Spatholobus littoralis* Hassk.) is one of the typical plants from Kalimantan Island, which has potential as a drug, one of which using as an antioxidant is due to the secondary metabolites contained therein. The aims of the research are to estimate the total phenolic content, total flavonoid content, and antioxidant capacity of the bajakah by different methods. Extraction was carried out by reflux method using a solvent gradient of n-hexane, ethyl acetate, and ethanol 96%. Phenols and flavonoid content were calculated using spectroscopy. Antioxidant capacity was measured against ascorbic acid and Trolox using the CUPRAC, DPPH, and FRAP methods and reported as mg Trolox equivalent (TEAC) and ascorbic acid equivalent (AEAC) per gram extract. The ethanol extract showed the highest antioxidant capacity for the CUPRAC and FRAP methods, the n-hexane extract for the DPPH method on both varieties of bajakah tampala. Each method provides significantly different antioxidant capacity values. The CUPRAC and FRAP methods found the highest antioxidant capacity in the white variety of bajakah ethanol extract. Meanwhile, in the DPPH method, saw the highest antioxidant capacity in the red variety of n-hexane bajakah extract. DPPH and CUPRAC give a positive correlation with TPC, where CUPRAC assay produced higher values than FRAP and DPPH assays.

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1. Introduction

Antioxidants play an important role in food by inhibiting oxidation processes and contributing to health promotion rendered by many dietary supplements, nutraceuticals, and functional food ingredients. Various assays can monitor antioxidant activity with different mechanisms, including hydrogen atom transfer (HAT), single electron transfer (ET), reducing power, and metal chelation. Therefore, understanding the principal mechanisms, advantages, and limitations of the measurement assays is important for properly selecting method(s) for valid evaluation of antioxidant potential in desired applications (Shahidi and Zhong, 2015). Antioxidants can be found naturally in plants, animals, and microorganisms or can be synthesized by chemical means. Higher plants and their constituents provide a rich source of natural antioxidants, such as phenolics and flavonoids, which are widely found in spices, herbs, fruits, vegetables, cereals, grains, seeds, teas and oils (Shahidi and Zhong, 2015; Aryal et al., 2019). Natural phenolic and flavonoid compounds are plant secondary metabolites that present an aromatic ring containing at least one hydroxyl group (Tungmunnithum et al., 2018). Phenolic compounds are good electron donors as their hydroxyl groups can directly contribute to the antioxidant action (Bendary et al., 2013). According to several reports in the literature, phenolic compounds exhibit free radical inhibition, peroxide decomposition, metal inactivation or oxygen scavenging in biological systems and prevent oxidative disease burden (Babbar et al., 2015).

The search for new bioactive compounds from natural sources can be made through ethnobotany and chemotaxonomic approaches. One species that can be developed as a medicinal ingredient is the bajakah (*Spatholobus littoralis* Hassk.). This plant comes from the inland areas of Kalimantan Province and has not spread to other regions (Saputera and Ayuchecaria, 2018). However, this plant is widely used empirically by rural communities in Central Kalimantan for various diseases (Novanty et al., 2021). Bajakah tampala is one of the plant species of the genus *Spatholobus*, which is widely distributed and lives in mainland Asia, of which 29 species grow and live in the forests of Southeast Asia (Fitriani et al., 2020).

Fig. 1. Bajakah tampala. White variety (a), red variety (b)

2. Materials and methods

2.1. Materials

In this research, we use two varieties of bajakah plant, red and white varieties that were collected from Nunukan District, North Kalimantan (Fig. 1). Ascorbic acid, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), neucoproine, and 2,4,6-tripyridyltriazine (TPTZ) were supplied by Sigma-Aldrich (MO, USA). All other chemicals were of analytical grade.

2.2. Extraction

Both bajakah stem (500 g) were extracted by reflux employing three solvents with increasing polarity. Extraction was started with n-hexane and repeated three times for each of them. The residue was subsequently refluxed by a similar method using ethyl acetate and ethanol. Total volume of each solvent is 5 l. The extract was evaporated at 50°C under vacuum.

2.3. Total phenolic content

The total phenolic content was evaluated with an adaptation of Folin-Ciolcalteu reagent test (McDonald et al., 2001). Each extract and gallic acid of 0.5 ml was piped into 5 ml Folin-Ciolcalteu reagent 10% and 4 ml of sodium carbonate 1 M and incubated the mixtures for 15 min. The absorbance was measured at wavelength 765 nm using spectrophotometer UV-Vis (Thermo scientific Genesys 10s). The results were reported as mg gallic acid equivalents (GAE) per gram extract (mg GAE/g extract) according to the standard calibration curve of gallic acid 10-70μg/ml.

2.4. Total flavonoid content

The total flavonoids content was measured using Chang's adapted method (Chang et al., 2002). Each extract and quercetin of 0.5 ml was piped into 1.5 ml ethanol, 2.8 ml aquadest, 0.1 ml aluminium chloride 10% and 0.1 ml sodium acetate 1 M. The mixture was incubated at 15 min, and absorbance was absorbance read at 415 nm. The results were presented as mg equivalents of quercetin (QE) per gram extract (mg QE/g extract) according to the standard calibration curve of quercetin 10-70 μg/ml.

2.5. Antioxidant activities

2.5.1. DPPH assay

Calep et al. (2015) method was slightly modified based on DPPH radical-scavenging activity (Celep et al., 2015). First, 0.5 ml of samples was mixed with 3 ml of 0.1 mM DPPH solution prepared in methanol. Then, the test solution ware incubated in the dark at room temperature for 30 min, and the absorbance of the mixture was measured at 515 nm. Trolox (12-20 μg) and ascorbic acid (20-100 μg) were used as standards, and results were reported as mg Trolox equivalent antioxidant capacity (TEAC) and ascorbic acid equivalent antioxidant capacity (AEAC) per gram of extract.

2.5.2. CUPRAC assay

The extract's CUPRAC ion reducing capacities were determined according to the method of Özyürek (Ozyürek et al., 2008). As much as 3 ml CUPRAC (CuCl2 0.01 M, Neucoproin 0.0075 M in ethanol p.a, and buffer NH4Ac pH 7.0 (1:1:1)) solution were added to 1,1 ml of extract (0,5 ml extract in methanol mixture destilled water until 1,1 ml). The absorbance of the final solution (of 4.1 ml total volume) at 450 nm was read against a reagent blank after 30 min left standing at room temperature. The results were reported as TEAC and AEAC per gram of extract. Each antioxidant's calibration curves (concentration vs. CUPRAC capacity) were constructed based on Trolox and ascorbic acid as a standard. TEAC and AEAC coefficients were calculated as a ratio of molar absorptivity of each extract to that of ascorbic acid and Trolox in this assay.

2.5.3. FRAP assay

The extract's FRAP ion reducing capacities were determined according to the method of Özyürek (Ozyürek et al., 2008). As much as 3 ml of the FRAP reagent was added to 1 ml (0.05 ml sample and 0.95 ml aquadest) sample. The absorbance at 595 nm was read against a reagent blank at the end of 30 min. Trolox and ascorbic acid were used as standard, and the total antioxidant capacity of bajakah was measured as TEAC and AEAC per gram of extract.

3. Results and discussion

Bajakah was separated by organic solvents and resulted three extracts: n-Hexan extract, ethyl acetate extract, and ethanol extract. The total phenols' content of three extracts ranged from 84.57±1.26 to 505.50±11.57 mg GAE/g extract, and the flavonoids content was from 5.83±0.10 to 28.91±1.97 mg QE/g extract for red varieties and 172,49±3,35 to 601.96±16.23 mg GAE/g extract, and 20.07±1.26 to 34.25±0.36 mg QE/g extract for white varieties. The phenolic and flavonoid content (relative percentage) determined are reported in Table 1. As shown in Table 1, the highest total phenolic and flavonoid contents are ethanol extract 505.50±11.57 g GAE/g extract and 28.91±1.97 g QE/g extract for red varieties respectively. The highest total phenolic content are ethanol extract 601.96±16.23 g GAE/g extract, the highest total flavonoids are ethyl acetate extract 34.25±0.36 g QE/g extract for white varieties. The flavonoid of bajakah is higher than the flavonoid content of *Spathalobus subeructus* (444.38 mg/g and 198.25 mg/g), which is from the same genus (Fu et al., 2017).

Table 1. Total phenolic and flavonoid content of bajakah.

Extract	Red varieties of bajakah		White varieties of bajakah	
	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	TPC (mg GAE/g extract)	TFC (mg GAE/g extract)
n-hexane	84.57±1.26	5.83±0.10	172.49±3.35	20.07±1.26
Ethyl acetate	217.98±7.95	15.17±0.17	653.53±6.00	34.25±0.36
Ethanol	505.50±11.57	28.91±1.97	601.96±16.23	20.45±0.66

DPPH values of bajakah extract given range from 10.85±0.0 to 14.37±0.0 mg AEAC/g extract and 0.21±0.0 to 0.76±0.0 mg TEAC/g extract for red varieties and 10.36±0.0 to 14.06±0.0 mg AEAC/g extract (Table 2) and 0.13±0.0 to 0.72±0.0 mg TEAC/g extract were higher than those of bajakah white varieties with (Table 3). DPPH values of bajakah for red varieties were higher than those of bajakah white varieties with range extract from 10.85±0.0 to 14.37±0.0 AEAC/g extract and 0.21±0.0 to 0.76±0.0 TEAC/g extract. A significant difference between extraction solvents was found ($p<0.05$) for both red and white varieties, as shown in Table 2 and Table 3.

DPPH radical scavenging assay is among the most frequently used methods and offers the first approach for evaluating antioxidant activity. It is an ET-based method with the HAT mechanism being only a marginal reaction pathway in the assay (Prior et al., 2005). DPPH is a stable chromogen radical with a deep purple color. The DPPH scavenging assay is based on the electron donation of antioxidants to neutralize DPPH radicals. The reaction is accompanied by the color change of the DPPH measured at 517 nm, and the discoloration acts as an indicator of the antioxidant efficacy.

Contrary to expectations, reaction stoichiometry (together with the initial rate) showed negligible correlation to the number of phenolic –OH groups per antioxidant molecule or redox potential. Steric accessibility to the hindered DPPH radical site may control

the reaction rate to a stronger extent than specific chemical properties of antioxidants (i.e., initial rates decrease with the bulkiness of multiple-ring phenols). Because DPPH scavenging is a mixed-mode (both ET- and HAT-based) assay, antioxidants mainly acting with the HAT mechanism are strongly influenced by kinetic solvent effects, in those differences in the strength of hydrogen bonding of the solvent to phenolic –OH groups and or DPPH interfere with the release of H atoms (e.g., phenols reacted fastest in methanol and slowed dramatically in ethanol or acetone).

FRAP values of bajakah extract given range from 4.77±0.3 to 16.12±1.1 mg AEAC/g extract and 7.17±0.4 to 21.57±1.4 mg TEAC/g extract for red varieties (Table 2) and 1.05±0.3 to 29.09±0.3 mg AEAC/g extract and 2.43±0.4 to 38.04±0.4 mg TEAC/g extract were higher than those of bajakah white varieties with (Table 3). FRAP values of bajakah for white varieties were higher than those of bajakah red varieties with a range extract from 1.05±0.3 to 29.09±0.3 AEAC/g extract and 2.43±0.4 to 38.04±0.4 TEAC/g extract. A significant difference between extraction solvents was found ($p<0.05$) for both red and white varieties, as shown in Table 2 and Table 3.

The FRAP assay is a typical ET-based method that measures the reduction of ferric ion (Fe^{3+})–ligand complex to the intensely blue-colored ferrous (Fe^{2+}) complex by antioxidants in acidic media. Antioxidant activity is determined as an absorbance increase at 593-595 nm, and results are expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard. Unlike other ET-based methods, FRAP assay is carried out under acidic pH conditions (pH 3.6) to maintain iron solubility and, more importantly, drive electron transfer. Therefore, it will increase the redox potential, causing a shift in the dominant reaction mechanism (Shahidi and Ambigaipalan, 2015). The original FRAP assay uses tripyridyltriazine (TPTZ) as the iron-binding ligand. At the same time, alternative ligands have also been employed for ferric binding, such as ferrozine for ascorbic acid reducing power evaluation. As a result, Prussian blue is produced as the end product, quantified spectrophotometrically, indicating the tested antioxidants' reducing power. Production of Prussian blue may be through two different routes with the same outcome. Antioxidants can either reduce the Fe^{3+} in the solution to Fe^{2+}, which binds the ferricyanide to yield prussian blue, or reduce the ferricyanide to ferrocyanide, which binds the free Fe^{3+} in the solution and forms prussian blue. The simplified scheme for these two reactions is given a blow (Berker et al., 2012).

CUPRAC values of bajakah extract given range from 19.94±2.0 to 144.80±8.5 mg AEAC/g extract and 32.4±2.9 to 217.4±12.4 mg TEAC/g extract for red varieties (Table 2) and 30.51 ±0.6 to 351.02 ±1.2 mg AEAC/g extract and 49.9± 0.8 to 518.1 ±1.7 mg TEAC/g extract were higher than those of bajakah white varieties with (Table 3). CUPRAC values of bajakah for white varieties were higher than those of bajakah red varieties with a range extract from 30.51±0.6 to 351.02±1.2 AEAC/g extract and 49.9±0.8 to 518.1 ±1.7 TEAC/g extract. No significant difference between extraction solvents was found ($p>0.05$) for both red and white varieties, as shown in Table 2 and 3.

The CUPRAC assay is a copper reduction assay developed as a variant of the FRAP assay. It uses copper as the oxidant instead of iron in the FRAP assay. The method measures the reducing power of antioxidants to convert cupric (Cu^{2+}) to cuprous (Cu^{+}) ions. Like the FRAP assay, a ligand is employed to form a copper–ligand complex to facilitate absorbance measurement.

Neocuproine (Nc; 2,9-dimethyl-1,10-phenanthroline) is the ligand commonly used in CUPRAC assay. The complex Cu^{2+}–neocuproine can be reduced by antioxidants to Cu^{+}–neocuproine, which is a chromophore with maximum absorption at 450 nm (Shahidi and Zhong, 2015). Another version of the optical sensor-aided CUPRAC assay used a miniature reflectance spectrometer to measure reflectance changes at 530 nm instead of absorbance (Bener et al., 2013).

Overall, the antioxidant capacity of both red and white varieties was lower than that of Novanty et al. (63,141.06 mg/L GAEAC), but the method was not specific and different (Novanty et al., 2021).

Table 2. Antioxidant capacity of bajakah red varieties

Extract	CUPRAC		FRAP		DPPH	
	AEAC/g extract	TEAC/g extract	AEAC/g extract	TEAC/g extract	AEAC/g extract	TEAC/g extract
n-Hexsan	19.94±2.0[a]	32.4±2.9[a]	4.77±0.3[a]	7.17±0.4[a]	14.37±0.0[a]	0.76±0.0[a]
Ethyl Acetate	73.50±2.2[b]	113.4±3.2[b]	14.59±0.4[b]	20.44±0.3[b]	10.89±0.0[*b]	0.22±0.0[b]
Ethanol	144.80±8.5[c]	217.4 ±12.4[c]	16.12±1.1[c]	21.57±1.4[c]	10.85±0.0[*b]	0.21±0.0[c]

The reported value is mean±SD (n=3), values in the same column followed by a different letter (a-c) are significantly different ($p< 0.05$).

Table 3. antioxidant capacity of bajakah white varieties

Extract	CUPRAC		FRAP		DPPH	
	AEAC/g extract	TEAC/g extract	AEAC/g extract	TEAC/g extract	AEAC/g extract	TEAC/g extract
n-Hexsan	30.51±0.6[a]	49.9±0.8[a]	1.05±0.3[a]	2.43±0.4[a]	14.06±0.0[a]	0.72±0.0[a]
Ethyl Acetate	268.89±5.4[b]	398.4±7.8[b]	21.80±0.5[b]	28.78±0.6[b]	10.35±0.0[b]	0.15±0.0[b]
Ethanol	351.02±1.2[c]	518.1±1.7[c]	29.09±0.3[c]	38.04±0.4[c]	10.36±0.0[b]	0.13±0.0[c]

The reported value is mean±SD (n=3), values in the same column followed by a different letter (a-c) are significantly different ($p< 0.05$).

Although different values were found from each antioxidant capacity assay, they still showed similar trends, as evidenced by their significant correlation (**) in Table 4. DPPH and CUPRAC positively correlate with TPC ($P>0.01$). In contrast, there were negative statistical correlations between TFC and antioxidant capacity. The quantity of phenols and flavonoids may not be the only factor affecting the bioactivities of bajakah. Nevertheless, this result could demonstrate that the qualitative profile of phenols and flavonoids in bajakah is also involved in biological activities. Some investigations have proven that the antioxidant effects of phenols and flavonoids are structurally related (Rice-Evans et al., 1996; Shahidi and Ambigaipalan, 2015).

Table 4. Bivariate correlation of results from three antioxidant capacity assays and total Phenolic and flavonoid contents.

Antioxidant parameter	Pearson's coefficient correlation (r)	
	TPC	TFC
DPPH	.923**	.009
FRAP	.195	.712
CUPRAC	.923**	.582

**. Correlation is significant at the 0.01 level (2-tailed).

Based on the phenol and flavonoid content of bajakah plants, pharmacological activities related to degenerative diseases and oxidative stress can be studied. Furthermore, bajakah plants also have the potential to be developed as antioxidant preparations such as supplements, herbal preparations, and cosmetics.

4. Conclusion

The highest antioxidant capacity was shown in the ethanol extract of bajakah white variety for CUPRAC and FRAP methods and n-hexane extract of red variety in the DPPH method. DPPH and CUPRAC have a positive correlation with TPC. CUPRAC assay produced higher values than FRAP and DPPH assays, and CUPRAC assay seemed to be a better method for expressing the antioxidant capacity of phenolic compounds.

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Conflict of interest

There are no conflict of interest.

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