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ANTIOXIDANT ACTIVITY OF N-HEXANE, DICHLOROMETHANE, ETHYL ACETATE, AND METHANOL EXTRACTS OF *Litsea cubeba* Lour. BARKS

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ABSTRACT

Free radicals are molecules with unstable unpaired electrons and originate from environmental pollutants and from unhealthy people's lifestyles that reduce the quality of life in the presence of various degenerative diseases from premature aging, stroke, and even cancer. Antioxidants are used to neutralize, reduce and inhibit the formation of new free radicals in the body by becoming electron donors for free radicals so that free electrons in free radicals become paired and stop damage in the body. With the presence of antioxidant compounds, oxidative stress triggered by free radicals can be stabilized and neutralized to reduce the risk of damage to body cells. Indonesia is a country rich in spice plant resources. One type of spice plant that has the potential to be developed is Attarasa (*Litsea cubeba* Lour.) which is widely found in the North Tapanuli area. This plant contains essential oils in the fruit, stems, roots, and leaves. This makes all parts of this plant smell good. This study aimed to determine the antioxidant activity of a fraction of *Litsea cubeba* Lour. barks. The fraction with n-hexane, dichloromethane, ethyl acetate, and methanol as solvent. Antioxidant activities were determined with ABTS, CUPRAC, and O-Phenanthroline method. The data were analyzed using the Principle Component Analysis (PCA) multivariate exploration technique, which was analyzed using the Minitab software. The extracts were obtained IC_{50} values from the ABTS (395.65 ± 0.37; 344.78 ± 0.57; 30.37 ± 0.04; 283.07 ± 0.85); CUPRAC (220.77 ± 0.21; 144.50 ± 0.26; 10.00 ± 0.01; 11.13 ± 0.04); O-Phenanthroline (798.27 ± 0.06; 410.01 ± 0.23; 175.75 ± 0.25; 32.01 ± 0.03) respectively. While the total phenol was analyzed (23.56 ± 0.56; 2.63 ± 0.01; 77.44 ± 0.56; 600.21 ± 0.54) respectively. Based on the results obtained, the ethyl acetate extract of *Litsea cubeba* Lour. barks have antioxidant activity with a very strong category.

Keywords: Antioxidant, Activity, Extracts, *Litsea cubeba* Lour, Bark.

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INTRODUCTION

The degenerative disease occurs in the function of organs, generally occurring in the elderly or advanced age, but can also occur at a young age or still young. This disease can cause the body's immune system gets weaker, followed by various other disorders. The worst consequence of the degenerative disease is that it can end in death.[1] Free radicals are compounds that can damage the immune system or the immune system of the human body. The compounds in question are foreign and not recognized by the body. Radiation of chemical substances, environmental pollution, toxins, fast food, and foods fried at high temperatures can cause the growth of free radicals in the body with complex chemical reactions. In excessive amounts, these compounds will have a pathological effect on the body, both physically and mentally. The formation of free radicals in the body must be blocked, inhibited, and cleaned with a compound that can eliminate free radicals, namely antioxidants.[2] Antioxidants are compounds closely related to the presence of free radicals or Reactive Oxygen Species (ROS), which can occur due to metabolism in the human body. Antioxidants are divided into synthetic and natural antioxidants. Natural antioxidants are compounds produced from the

extraction process of natural materials such as fruits and plants, namely phenolic compounds, tocopherols, carotenes, flavonoids, and so on. Natural antioxidants, if consumed by humans, have good properties and do not cause side effects for the body.[3,4] Indonesia is a country rich in spice plant resources. One type of spice plant that has the potential to be developed is Attarasa (*Litsea cubeba*) which is widely found in the North Tapanuli area. This plant contains essential oils in the fruit, stems, roots, and leaves. This makes all parts of this plant smell good. Attarasa (*Litsea cubeba* Lour.) is a plant from the Lauraceae family, which is rich in essential oil compounds. Traditionally, the essential oil of atta rasa is used for antidepressant, anti-inflammatory, antioxidant, pesticide, antimicrobial, anticancer, and neuropharmacology.[5] One of the mechanisms of action of antioxidant compounds is donating hydrogen atoms or protons to radical compounds so that they can complete the lack of electrons needed by free radicals and inhibit the free radical formation chain reaction, making radical compounds more stable. Based on their function, food antioxidants can be classified into primary antioxidants and secondary antioxidants. Primary antioxidants are chain-breaking by donating hydrogen or electrons to free radicals and turning them into more stable ones to delay the initiation stage and inhibit auto-oxidation in the propagation stage. Meanwhile, secondary antioxidants reduce the rate of lipid autooxidation through various mechanisms, including the binding of metal ions, oxygen capture, and hydroperoxide decomposition into non-radical products.[6,7] In a study to determine the IC_{50} value of antioxidants using the *2,2-azinobis-3-Ethylbenzothiazoline-6-Sulfonic Acid* (ABTS) method, Cupric ion reducing antioxidant capacity (CUPRAC) and O-Phenanthroline.

EXPERIMENTAL

Preparation of Extracts

The simplex powder was extracted with n-hexane, dichloromethane, ethyl acetate, and methanol solvent respectively under reflux conditions.[8]

Phytochemical Screening

Phytochemical screening of secondary extract metabolites was carried out by thin-layer chromatography with standard procedures.[9,10]

2,2-azino-bis(3-etilbenzotiazolin)-6-sulfonic Acid Free Radical Assay

Free radical scavenging from the extracts was reacted with 7mM ABTS and 2.45 mM potassium persulfate, then incubated for 30 minutes and measured at a wavelength of 734 nm.[11]

Cupric Reducing Antioxidant Capacity (CUPRAC)

Sample preparation with various concentrations, then 1mL of each concentration was taken and pipetted to add $CuCl_2$, ammonium acetate pH 7, and neocuproine. Then it was measured at a wavelength of 450nm.[12]

O-phenanthroline Reduction Assay

The sample was dissolved with solvent to be made into various concentrations, then O-phenanthroline, $FeCl_3$, and methanol were added. Incubated for 40 minutes and then measured at a wavelength of 510 nm.[13]

Total Phenolic

The sample was weighed and then diluted according to the desired concentration, then added folin reagent, distilled water, and vortexed for 1 minute. After mixing, sodium carbonate was added and incubated for 90 minutes to be measured at a wavelength of 775 nm.[14]

Statistical Analysis

Data has been presented as mean ± standard deviation (SD) and continues with Principle Component Analysis (PCA) multivariate exploration technique, which was analyzed using the Minitab software.

RESULTS AND DISCUSSION

Results of phytochemicals screening with Thin Layer Chromatography (TLC)

Phytochemical screening of secondary metabolites from, n-hexane, dichloromethane, ethyl acetate, and methanol fractions by thin-layer chromatography is shown in Table-1 below.

Table-1: Results of Phytochemicals Screening

Sample	NELC	DELC	EAELC	MELC
Alkaloids	-	+	+	+
Flavonoids	-	+	+	+
Tannins	-	+	+	+
Saponins	-	+	+	+
Glycosides	-	+	+	+
Steroids/Triterpenoids	+	+	+	+

Description: (+) positive; (-) negative

Antioxidant Activity of *Litsea cubeba* Lour. Bark Extracts with ABTS, CUPRAC, O-Phenanthroline, and Total Phenolic

In order to determine the antioxidant capacity that directly reacts with the ABTS cation radical, the ABTS method's basic tenet is to eliminate the color of the ABTS cation. Antioxidants can decrease the colorless-to-colorless ABTS radical, which has a nitrogen core and is often blue-green in hue, into a non-radical state. The ABTS technique is extremely light-sensitive; even the synthesis of ABTS•- needs an incubation period of 12 to 16 hours in complete darkness.[15,16] The CUPRAC method uses bis(neocuproine) copper (II) (Cu $(Nc)_2^{2+}$) as a chromogenic reagent. The blue Cu $(Nc)_2^{2+}$ reagent will be reduced to yellow (Cu $(Nc)^{2+}$. The Cu (II)-neocuproine (Cu (II)-$(Nc)_2$) reagent is used as a chromogenic oxidizing agent because it reduces Cu (II) ions can be measured. The method of antioxidant capacity using the above reagent is called CUPRAC (cupric reducing antioxidant capacity). The absorption formed by the chelate (Cu(I)-$(Nc)_2$) resulting from the reduction by antioxidant compounds is measured at wavelengths 450 nm.[12] The phenanthroline method uses $FeCl_3$ as a source of Fe^{+2} ions and ortho-phenanthroline as an iron complexing agent. The mechanism that occurs is that Fe^{+3} from $FeCl_3$ will oxidize compounds that are antioxidants; as a result, Fe^{+3} will be reduced to Fe^{+2}, resulting in the formation of a complex orange-red compound caused by the complex cation $[Fe(C_{12}H_8N_2)_2]^{2+}$.[13] The antioxidant results from the extracts of methanol, n-hexane, dichloromethane, and ethyl acetate atta rasa stem bark can be seen in Table- 2.

Table-2: The Results of IC_{50} Values from Various Antioxidant Methods Against the Extracts of *Litsea Cubeba* Lour Barks

No	Sample	ABTS (µg/mL)	CUPRAC (µg/mL)	O-phenanthroline (µg/mL)	Total phenolic (mg/GAE)
1.	NELC	395.65 ± 0.37	220.77 ± 0.21	798.27 ± 0.06	23.56 ± 0.56
2.	DELC	344.78 ± 0.57	144.50 ± 0.26	410.01 ± 0.23	77.44 ± 0.56
3.	EAELC	30.37 ± 0.04	10.00 ± 0.01	32.01 ± 0.03	600.21 ± 0.54
4.	MELC	283.07 ± 0.85	11.13 ± 0.04	175.75 ± 0.25	2.63 ± 0.01

Based on Table-2 *Litsea cubeba* extract with methanol as solvent by ABTS method (283.07 ± 0.85); CUPRAC (11.13 ± 0.04); O-Phenanthroline (175.75 ± 0.25), n-hexane extract ABTS (395.65 ± 0.37); CUPRAC (220.77 ± 0.21); O-Phenanthroline (798.27 ± 0.06), ABTS dichloromethane extract (344.78 ± 0.57); CUPRAC (144.50 ± 0.26); O-Phenanthroline (410.01 ± 0.23), ABTS ethyl acetate extract (30.37 ± 0.04); CUPRAC (10.00 ± 0.01); O-Phenanthroline (32.01 ± 0.03). While the total phenol from the methanol extract (2.63 ± 0.01); n-hexane extract (23.56 ± 0.56); dichloromethane extract (77.44 ± 0.56); ethyl acetate extract (600.21 ± 0.54). The ethyl acetate extract of *Litsea cubeba* barks was carried out in stages to accumulate polar, semi-polar, and non-polar compounds, which caused the components of the compound to separate. From the results, the IC_{50} value in the ethyl acetate extract was very strong compared to methanol, n-hexane, and dichloromethane from the barks of atta rasa. A compound can be called a very strong antioxidant if the IC_{50} value is ≤ 10 ppm, strong if the IC_{50} value is in the 10-50 ppm range, while if the IC_{50} value is in the 50-100 ppm range, weak if the IC_{50} value is in the 100-250 ppm range, and inactive if IC_{50} is more than 250 ppm. Factors that cause high and low antioxidant activity, among others, are their properties that can be damaged when exposed to light, oxygen, high temperatures, and drying.[17,18] Because the CUPRAC reagent is a selective reagent with a low reduction potential value, it was possible to determine the antioxidant activity of the fraction with the smallest IC_{50} value using this method. The Cupric ion-reducing antioxidant capacity (CUPRAC) reagent, which is quick enough to reduce antioxidant thiols, is

one benefit this method offers over other methods for measuring antioxidants. In comparison to other chromogenic reagents, the Cupric ion-reducing antioxidant capacity (CUPRAC) reagent is also more stable and available (e.g., ABTS, O-Phenanthroline). This method does not call for complicated machinery or trained operators and is simple to use in normal laboratories utilizing common colorimeters. This technique can assess an antioxidant's hydrophilic and lipophilic properties.[12] The content of phenolic compounds in the sample fraction played a role in determining the presence of antioxidants. The ethyl acetate fraction owned the highest total phenol content. The low total phenolic content in the n-hexane fraction could be due to this solvent's non-polar nature, and the n-hexane fraction did not change color to blue. The intensity of the blue color is used as an indicator of the presence of phenolic compounds in the sample, where the more concentrated the color intensity, the greater the total phenol content. This blue color occurs because of the oxidation-reduction reaction using Folin-Ciocalteu reagent and sodium carbonate, where phenolic compounds will turn into phenolic ions in alkaline conditions. The phenolic ion formed will reduce phosphomolybdic acid phosphotungstate in the Folin-Ciocalteu reagent during the phenol oxidation process to form a blue tungsten molybdenum complex compound.[19-23]

Results of PCA (Principal Component Analysis) Analysis

Using a linear transformation to create a new coordinate system with the greatest variance, principal component analysis is a method for decomposing data into its simplest components. Data dimensions can be decreased by PCA analysis without dramatically affecting the data's features. The purpose of PCA analysis is to use a linear combination of variables to explain the structure of the covariance variance. This PCA analysis is frequently conducted after the conclusion of data processing and as a stage or step in the majority of larger and broader studies. The eigenvalues (Fig.-1) demonstrate that PC1, PC2, PC3, and PC4 have, respectively, contributed 83.3%, 99.2%, and 0.8% of the variation of the variables; the link between each PC and the eigenvalue is shown in Fig.-1. In this analysis, 99.2% of the variables were represented by just PC1 and PC2. The First Principal Component (PC1), which calculates the largest variation of all variables, and the Second Principal Component (PC2), which calculates the second largest variation of all variables, are used to classify and display the closeness of samples using the score plot.[24] The atta rasa bark extracts can be divided into 4 groups according to the score plot in Fig.-2. N-hexane, dichloromethane, ethyl acetate, and methanol, extract are in separate groups and, as the score plot demonstrates, have various antioxidant properties. The more similar the antioxidant activity, the closer the score plot value.

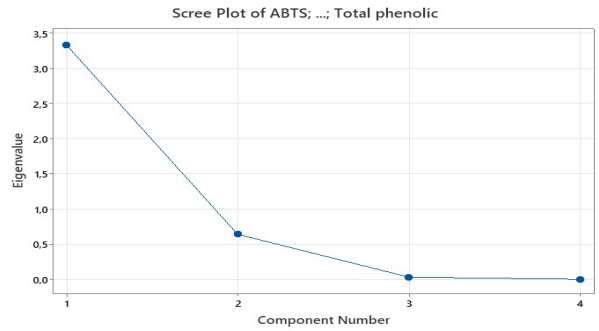

Fig.-1: Scree Plot the Relationship of Each PC with the Eigenvalue

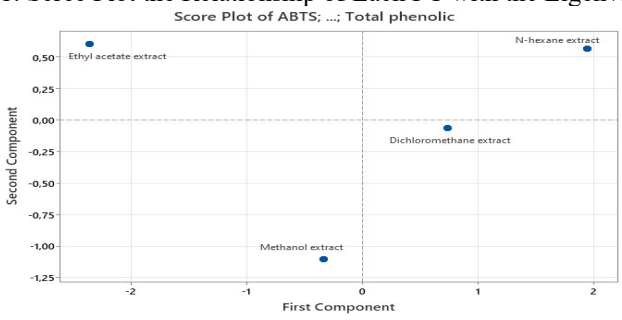

Fig.-2: PCA Score Plot by First Principal Component (PC1) and Second Principal Component (PC2) from *Litsea cubeba* Lour. Extracts

The correlation between variables ABTS and FRAP loading plot can be used. The loading plot represents each variable as a vector, indicating the magnitude of its impact on the PC. If the angle between the two vectors is less than 90 degrees, the two variables are positively connected. The two variables are not likely to be connected if they make an angle of roughly 90 degrees. The two variables, however, exhibit a negative connection if the angle is broader (greater than 90° or close to 180°).[25] ABTS and FRAP form a narrow angle of less than 90° (Fig.-3), thus showing a strong positive correlation between the two variables. The correlation between total phenolic variables with IC_{50} ABTS, CUPRAC, and O-Phenanthroline showed a moderate negative correlation (not significant) because the two vectors in Fig.-3 formed less than 180°.

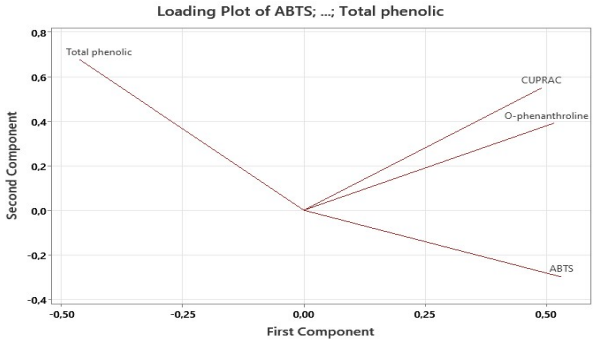

Fig.-3: Loading Plot Curve For Variable Total Phenolic Content And Antioxidant Activity (IC_{50} DPPH, ABTS, and O-Phenanthroline)

CONCLUSION

Based on the results obtained, the ethyl acetate extract of *Litsea cubeba* Lour bark has antioxidant activity with a very strong category.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID numbers given below:

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