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ANTIOXIDANT PROPERTIES OF ETHYL ACETATE FRACTION OF SUNGKAI LEAVES (*PERONEMA CANESCENS* JACK): EXPERIMENTAL AND COMPUTATIONAL APPROACHES

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Abstract

This research aimed to evaluate the antioxidant activity of the ethyl acetate fraction of Sungkai leaves experimentally and computationally. The secondary metabolites were analyzed using LC-MS/MS. Experimentally, antioxidant activity was analyzed using the DPPH method and theoretical antioxidant properties were determined using the density functional theory (DFT)/B3LYP/6-31G method. The antioxidant activity of the ethyl acetate fraction of Sungkai leaves has an IC_{50} 62.879 ± 0.43 mg/L. The total phenolic content obtained was 99.00 ± 2.96 mgGAE/gram sample. Characterization using LC-MS/MS obtained five compounds with the highest composition: (3R)-sophorol, physcion, isorhamnetin, pilosin, and takakin. Calculation of global reactivity value shows that the isorhamnetin compound is the most reactive with a bandgap is 3.6838 eV, chemical potential of 3.6416 eV, hardness of 1.8419 eV, and softness of 0.5429 eV. The mechanism of proton and electron transfer in all test compounds is easier through the HAT mechanism because it has a smaller BDE energy compared to the total energy of IP + PDE and PA + ETE. The conclusion is that the ethyl acetate fraction has quite good antioxidant activity, especially the isorhmanetin compound

Keywords

Antioxidants, Sungkai leaves, ethyl acetate, DPPH, density functional theory

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Introduction. Reactive oxygen species (ROS) and reactive nitrogen species (NOS) such as superoxide radicals ($^{\circ}O_2$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\circ}OH$), $^{\circ}NO$, and $^{\circ}NO_2$ are the main causes of oxidative stress. This condition contributes to protein denaturation, mutagenesis, and lipid peroxidation in aerobic cells that cause many degenerative diseases in humans such as cancer,

heart disease, and cerebrovascular disease [1]. Active antioxidant compounds are needed to avoid oxidative stress due to free radicals. Antioxidants play an important role because of their ability to prevent oxidative reactions by free radicals [2]. In addition, antioxidants also help maintain healthy immunological cells [3]. Various plants have been widely used as natural antioxidants, one of which is Sungkai leaves (*Peronema canescens* Jack) [4]. Sungkai is a plant that grows in tropical areas with abundant rainfall such as Indonesia. Indonesian people use this plant as a medicine for fever, malaria, as mouthwash to prevent toothache, and to maintain health [5]. Experimentally, antioxidant activity in Sungkai plants using the method of compound interaction with free radicals 2,2-diphenyl-1-pyrrylhydrazyl (DPPH) has been commonly carried out.

In 2021, Peni Pindan N. *et al.* conducted phytochemical and antioxidant activity tests on hexane, ethyl acetate, and ethanol fractions of Sungkai leaves. The results showed that Sungkai leaves have various components such as alkaloids, flavonoids, phenolics, steroids, and saponins with IC_{50} values of 607.475 ppm, 12.986 ppm, and 15.766 ppm respectively [6]. Using the same method, research conducted by Maigoda T. *et al.* obtained the antioxidant activity of methanol extract of Sungkai leaves with an IC_{50} value of 116.7865 ppm [7].

Experimental studies show that the antioxidant activity of the ethyl acetate fraction of Sungkai leaves has strong antioxidant activity, but there is no theoretical explanation for the antioxidant properties of the ethyl acetate fraction using a computational approach. Therefore, in this study, the antioxidant properties were analyzed experimentally using the DPPH method and computationally using the density functional density (DFT) method. With this study, the antioxidant activity of compounds contained in Sungkai leaves can be known experimentally and computationally. Furthermore, information is obtained on whether the compounds contained in Sungkai leaves have the potential as drug candidates.

Materials and methods. *Material.* Sungkai leaf samples (*Peronema canesces* Jack) were obtained from the Salak River area, Saruaso Village, Tanjung Emas District, Tanah Datar Regency. The solvents used for extraction were hexane, ethyl acetate, and methanol. DPPH and ascorbic acid were used as antioxidant tests, and 20 % sodium carbonate, Follin — Ciocalteu reagent, gallic acid, and distilled water were used as total phenolic tests.

Sample preparation. A total of 2 kg of Sungkai leaves were dried and then ground using a grinder. A total of 500 g of sample was macerated using methanol three times 24 hours in a row. The resulting solution was filtered, and the methanol extract was concentrated using a rotary evaporator. This process was repeated until the filtrate became clear [8].

Fractionation of methanol extract. A total of 20 g of concentrated methanol extract was fractionated using a separating funnel with 3 solvents, namely hexane, ethyl acetate, and methanol. The methanol extract was put into a separating funnel, water and hexane were added in a ratio of 1 : 2, then shaken and left to stand until 2 layers were formed. The methanol fraction was separated from the hexane fraction by opening the funnel valve and collecting the hexane fraction in an Erlenmeyer flask. This process was repeated until the hexane layer became clear. Fractionation was continued using ethyl acetate solvent so that at the end of the process the methanol-water fraction was obtained [9]. All fractions obtained were concentrated with a rotary evaporator.

Total phenolic content test. The total phenolic content test followed the procedure of Salim E. *et al.* and Santoni A. *et al.* with the Folin — Ciocalteau method. A total of 10 mg of ethyl acetate fraction was dissolved in 10 mL of methanol, to obtain a stock solution with a concentration of 1000 mg/L. Galic acid is then diluted to concentrations of 20, 40, 60, 80, and 100 mg/L. An aliquot of 0.5 mL of the 500 mg/L test solution was mixed with 0.5 mL of Folin — Ciocalteu reagent in a 10 mL volumetric flask and shaken. After 5 min, 1 mL of 20 % Na_2CO_3 solution was added. The mixture was diluted to the right with distilled water and incubated for 120 min at room temperature. Absorbance was measured at a wavelength of 765 nm using a UV-Vis spectrophotometer (ThermoFisher Scientific, GENESYS 30 Visible Spectrophotometer) under room temperature. After that, a calibration curve of gallic acid solution is made. The statistical processing of the results was performed using *Microsoft Excel*, where we applied (e.g., mean calculation, standard deviation, and regression analysis). The results were expressed as a percentage (%) with gallic acid equivalent (GAE, mg GAE/g dry extract) [10, 11].

Antioxidant activity test of Sungkai leaf ethyl acetate fraction using DPPH method. The antioxidant activity test was determined by following the modified procedure of Ammaji S. *et al.* [3]. Aliquots of 3 mL of 0.1 mM DPPH solution were added to 2 mL of test solution with various concentrations. For control, 3 mL of DPPH solution was mixed with 2 mL of methanol. The solution was left for 30 min in the dark. Furthermore, the absorbance of each concentration of test solution and control was measured at a wavelength of 517 nm.

Based on the absorbance obtained, the % inhibition was calculated using the equation

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \cdot 100\,\%.$$

Here *Ac* is control absorbance value; *As* is absorbance value of the sample.

After obtaining the % inhibition value from the calculation, the IC_{50} value of each test sample was determined using the regression equation obtained.

LC-MS/MS analysis. The ethyl acetate fraction of Sungkai leaves was characterized using LC-MS/MS, and then the types of secondary metabolites found in Sungkai leaves were determined. The analysis process began using an ultra-performance liquid chromatography (UPLC) unit (*LC: ACQUITY UPLC® H-Class System,* Waters, USA) and a high-resolution mass spectrometer (*Xevo G2-S QTof,* Waters, USA). The sample was loaded into a C18 chromatography column with a particle size of 1.8 μm (2.1 × 100 mm) at a column temperature of 50 °C and a room temperature of 25 °C. The mobile phase used consisted of water containing 5 mM ammonium formate (phase A) and acetonitrile with the addition of 0.05 % formic acid (phase B). Measurements were carried out with a gradual gradient at a flow rate of 0.2 mL/min for 23 min, with an injection volume of 5 μL previously filtered through a 0.2 μm needle filter. After chromatographic separation, sample components were determined using a mass spectrometer with electrospray ionization (ESI) in positive mode. Mass spectrometry was performed in the mass range of 50–1200 m/z, with the source and desolvation temperatures set at 100 and 350 °C, respectively. In addition, the cone and desolvation gas flow rates were 0 and 793 L/h, respectively. The collision energy was varied between 4 and 60 eV to produce ion fragmentation. The entire process of instrument control, data acquisition, and analysis was performed using *Masslynx* software version 4.1 [12].

Molecular structure optimization with DFT method. After the main molecular structure contained in Sungkai leaves is known, molecular optimization is carried out. The molecular structure is created in *Gauss View 6.0* and optimized using *Gaussian 16W* software with the DFT calculation method on the B3LYP/6-31G basis set [13]. The most stable structural state is characterized by the lowest total energy. The output data is in the form of optimal structure, highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) contours, HOMO energy, LUMO energy, and formation enthalpy (ΔH_f). From the ΔH_f value, the bond dissociation enthalpy (BDE), ionization potential (IP), proton dissociation enthalpy (PDE), proton affinity (PA) and electron transfer enthalpy (ETE) values are determined using equation below [13].

Antioxidant molecules function to inhibit free radicals through three main reaction mechanisms [14–16].

1. Hydrogen atom transfer (HAT) with BDE energy:

$$ArOH + R^{\bullet} \rightarrow ArO^{\bullet} + RH$$

2. Single electron transfer-proton transfer (SET-PT).
   2.1. Single Electron Transfer (SET): $R^{\bullet} + ArO^{\bullet\bullet}H \rightarrow R^{-} + ArO^{\bullet}H^{+}$
   2.2. Proton transfer (PT): $ArO^{\bullet}H^{+} \rightarrow ArO^{\bullet} + H^{+}$
3. Sequential proton loss electron transfer (SPLET).
   3.1. Sequential proton loss (SPL): $ArOH \rightarrow ArO^{-} + H^{+}$
   3.2. Electron transfer (ET): $ArO^{-} + R^{\bullet} \rightarrow ArO^{\bullet} + R^{-}$

The reaction enthalpy is calculated using the following formula:

$$BDE = \Delta H_f(ArO^{\bullet}) + \Delta H_f(H^{\bullet}) - \Delta H_f(ArOH);$$

$$IP = \Delta H_f(ArOH^{\bullet+}) + \Delta H_f(e^{-}) - \Delta H_f(ArOH);$$

$$PDE = \Delta H_f(ArO^{\bullet}) + \Delta H_f(H^{+}) - \Delta H_f(ArOH^{\bullet+});$$

$$PA = \Delta H_f(ArO^{-}) + \Delta H_f(H^{+}) - \Delta H_f\left(ArOH\right);$$

$$ETE = \Delta H_f(ArO^{\bullet}) + \Delta H_f(e^{-}) - \Delta H_f(ArO^{-}).$$

Determination of global reactivity analysis parameter values. Global descriptive parameter values indicate molecular reactivity [17]. The parameters used are bandgap ΔE, electronegativity χ, chemical potential μ, hardness η, softness σ, electrophilicity ω, nucleophilicity ε, ionization energy I, and electron affinity A.

The calculation of this parameter value is determined from the formula below:

$$\Delta E = E_{LUMO} - E_{HOMO};\ \chi = (-E_{HOMO} - E_{LUMO})/2;$$

$$\mu = (E_{HOMO} + E_{LUMO})/2;\ \eta = (E_{HOMO} - E_{LUMO})/2;$$

$$\sigma = 1/\eta;\ \omega = \chi^2/(2\eta);\ \varepsilon = 1/\omega;\ I = -E_{HOMO};\ A = -E_{LUMO}.$$

Results and discussion. *Total phenolic content of ethyl acetate fraction.* Phenolic is an antioxidant molecule that functions to inhibit free radicals by donating hydrogen atoms to free radicals [18]. The total phenolic content test using the Folin — Ciocalteu reagent is based on its ability to oxidize hydroxyl groups ($^{-}$OH). The total phenolic test is measured spectroscopically using gallic acid as a standard. Gallic acid compounds are used as standards because they are included in the benzoate hydroxyl derivative group, which is part of phenolic acid. The ability of phenolic acids to inhibit free radicals depends on the number and position of hydroxyl and methoxy groups in their molecules. Gallic acid, 3,4,5-trihydroxybenzoic acid is the strongest antioxidant in the phenolic acid group [19]. In addition, gallic acid is one of the stable and relatively inexpensive

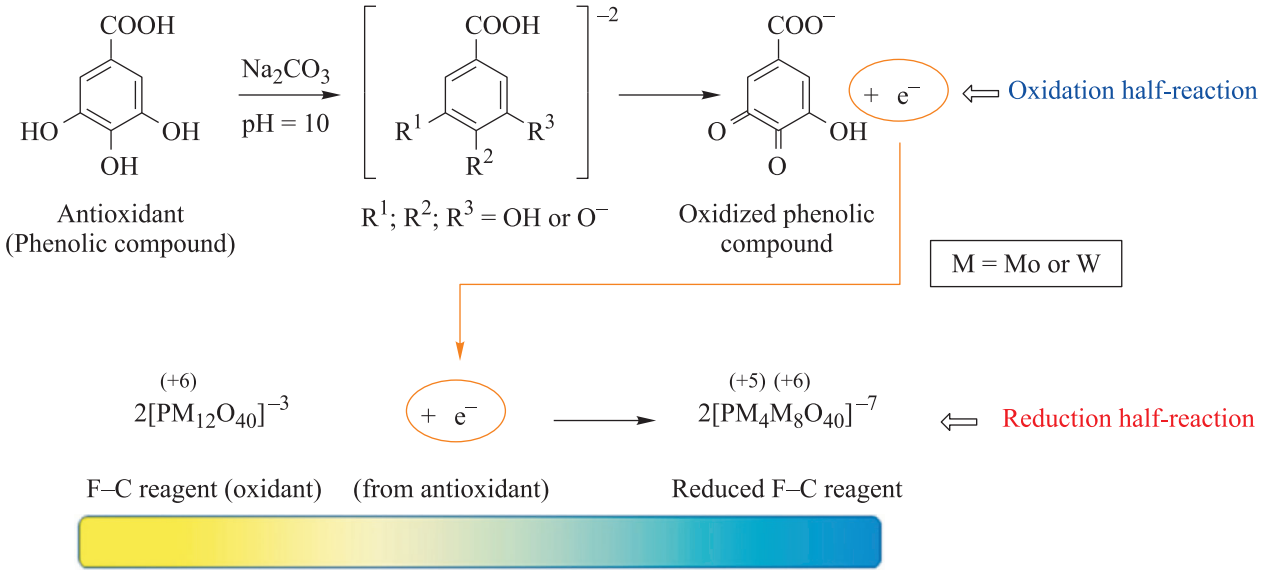

Fig. 1. Reaction mechanism of Folin — Ciocalteu reagent with gallic acid [19]

natural phenolic compounds [20]. Phenolic compounds reduce the phosphotungstic-phosphomolybdic complex in the Folin — Ciocalteu reagent to form a blue molybdenum component [21]. This reaction causes a color shift from yellow to blue, and the magnitude of this shift is directly proportional to the number of phenolic compounds present in the sample [19] as shown in the calibration curve in Fig. 1. Higher concentrations of gallic acid result in higher absorbance values due to the increased formation of phenolic ions.

Gallic acid reacts with Folin — Ciocalteu in basic conditions which is indicated by a color change from yellow to blue. Sodium carbonate is added to the mixture containing the sample and Folin — Ciocalteu reagent to increase the pH to 10 and avoid excessive alkalinity. The mixture is incubated at room temperature for 2 hours to ensure complete reaction and stable blue color. The blue complex can be detected at a wavelength of 760 nm. The reducing capacity of phenolic compounds is measured as GAE [19].

The total phenolic content was calculated using a regression equation derived from the standard calibration curve of gallic acid in Fig. 2. The linear regression equation obtained was $Y = 0.006x + 0.066$ and the correlation coefficient (R^2) was 0.9987. Based on the results of this study, the total phenolic content of the ethyl acetate fraction was 99.00 ± 2.96 mgGAE/gram.

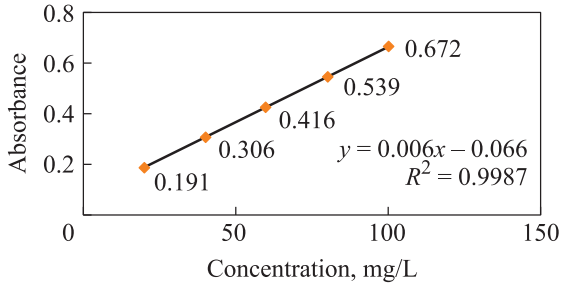

Fig. 2. Calibration curve of gallic acid standard

Antioxidant activity of ethyl acetate fraction of Sungkai leaves. The antioxidant activity of the ethyl acetate fraction of Sungkai leaves was tested using the DPPH method. This method is used to determine the ability of antioxidants to inhibit free radicals. The DPPH method has been widely used because it is a fast, simple, and inexpensive method.

Based on the data shown in the graph in Fig. 3. Concentration is directly proportional to the percentage of inhibition. The greater the concentration of the sample, the freer radicals are inhibited by the antioxidant compound. The IC_{50} value of the antioxidant activity of the ethyl acetate and ascorbic acid fractions can be seen in Table 1. Samples are categorized as strong antioxidants if they have

an IC_{50} value < 50 mg/L, medium if between 50–100 mg/L, and weak if > > 100 mg/L [23]. Based on Table 1, the antioxidant activity of the ethyl acetate fraction is classified as medium, because it has an IC_{50} value of 62.879 ± 0.43 mg/L.

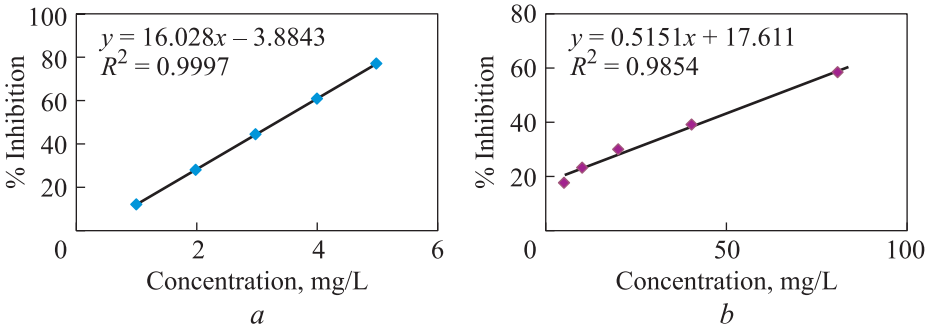

Fig. 3. Standard curve of % inhibition of ethyl acetate fraction (*a*), and ascorbic acid (*b*)

Table 1

IC_{50} values of ethyl acetate and ascorbic acid fractions

Sample	IC_{50} (mg/L) ± SD
Ethyl acetate fraction	62.879 ± 0.43
Ascorbic acid	3.362 ± 0.02

Antioxidant activity testing was carried out with ethyl acetate fraction concentrations: of 5, 10, 20, 40, and 80 mg/L, and ascorbic acid was used as a control with concentrations of 1, 2, 3, 4, and 5 mg/L. Antioxidant activity was calculated based on the % inhibition of DPPH free radicals by antioxidant compounds. This principle is based on the reduction of the number of DPPH free radicals. When reacted with antioxidant compounds, DPPH will be reduced to non-radical diphenyl-picrylhydrazyl (DPPH-H), resulting in a color change from purple to colorless or pale yellow [22]. Antioxidant activity is determined based on the IC_{50} value, the smaller the IC_{50} value, the greater the antioxidant activity of a sample [6].

In general, the content of Sungkai leaf extract has been identified, such as flavonoids, tannins, phenolics, and alkaloids [6]. In previous research, Santoni A. *et al.* found that ethyl acetate extract contains flavonoids, phenolics, saponins, and steroids [11]. Several groups of compounds such as flavonoids, tannins, and other phenolic compounds have the potential to be antioxidants. The antioxidant activity of these compounds will increase with the increasing number of hydroxyl groups. Phenolic compounds donate hydrogen atoms or electrons to free

radicals to stabilize radical compounds [5]. Phenolic compounds and their derivatives, such as flavonoids and tannins, have been reported as potential free radical quenchers and inhibitors. If the number of flavonoids in the extract increases, the antioxidant activity will also be higher. Flavonoid and tannin compounds are groups of phenolic compounds and have high antioxidant activity. These two compounds will provide a synergistic effect that strengthens each other [24, 25].

Composition analysis using LC-MS/MS. Phytochemical analysis using LC-MS/MS aims to identify various compounds contained in the ethyl acetate fraction of Sungkai leaves.

LC-MS/MS analysis is an analytical method that combines the ability to separate compounds from liquid chromatography and is supported by mass spectroscopy. Liquid chromatography separates compounds in a sample based on their physicochemical properties, while mass spectrometry detects charged ions to produce data related to molecular weight, chemical structure, identity, and quantification of compounds. The results of LC-MS/MS measurements are presented in the form of chromatograms in the form of high peak plots, which allow the identification of the molecular weight of compounds in the extract and the determination of accurate amounts in each sample [26]. Based on Fig. 4, and Table 2, five main compounds were identified in the ethyl acetate fraction

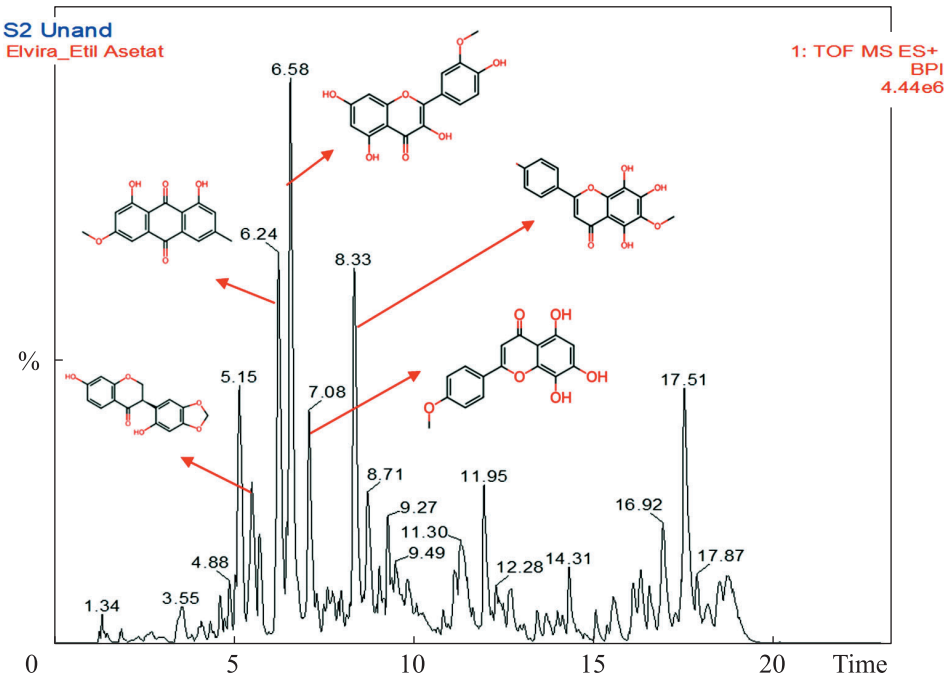

Fig. 4. LC-MS/MS chromatogram of ethyl acetate fraction of Sungkai leaves

of Sungkai leaves, especially flavonoids: isorhamnetin, pilosin, (3R)-sophorol, and takakin. The compound with the highest percentage area is isorhamnetin (12.71 %). In addition, the anthraquinone compound, physcion, was identified with a percentage area of 8.03 %. Previous studies have also identified similar compounds, especially flavonoids and anthraquinones, in Sungkai leaf extract [27].

Table 2

Retention time, area percentage, and molecular weight of the main components of the ethyl acetate fraction of Sungkai leaves

Compounds	Molecular formula	Retention time	Percentage area, %	Molecular weight, g/mol
(3R)-sophorol	$C_{16}H_{12}O_6$	5.52	5.56	300.266
Physician	$C_{16}H_{12}O_5$	6.24	8.03	284.267
Isorhamnetin	$C_{16}H_{12}O_7$	6.58	12.71	316.265
Takakin	$C_{16}H_{12}O_6$	7.1	3.86	300.266
Pilosin	$C_{17}H_{14}O_7$	8.35	7.7	330.292

Optimal geometric structure. Density functional theory calculations are extensively employed to explore the antioxidant characteristics of different compounds. The optimal geometric structure signifies the most stable molecular conformation, which is crucial for accurately forecasting its antioxidant potential. Understanding the structural and electronic attributes of antioxidant compounds is vital for examining the mechanism of free radical inhibition. The parameters for each compound were computed in the gas phase using *Gaussian 16W* software, applying the DFT calculation method and the B3LYP/631G basis set. Compounds in the ethyl acetate fraction possess hydroxyl groups capable of interacting with free radicals. The geometric structures of these compounds are illustrated in Fig. 5. Hydroxyl groups in flavonoids, as depicted in Fig. 5 (3R)-sophorol isorhamnetin, takakin, and pilosin, enhance antioxidant activity by donating hydrogen atoms to stabilize free radicals. Similarly, physcion, an anthraquinone, demonstrates antioxidant properties through its functional groups. These results underscore the significant role of hydroxyl-rich structures in antioxidant mechanisms.

Frontier molecular orbital analysis. Frontier molecular orbital (FMO) analysis is an important approach to studying the chemical properties and antioxidant activity of a compound. FMO consists of HOMO and LUMO [28–30]. According to the FMO theory of chemical reactivity, the transition state in a chemical reaction occurs due to the interaction between the HOMO and LUMO orbitals

of the species involved in the reaction. Based on Koppman's theorem, the negative value of HOMO energy represents *the ionization potential*, while the negative value of LUMO energy represents the electron affinity of the molecule [31]. HOMO energy describes the ability of a molecule to donate electrons, while LUMO energy describes its ability to accept electrons. Therefore, a higher HOMO energy value indicates a better tendency to donate electrons and is associated with better biological potential as the HOMO energy value increases [32].

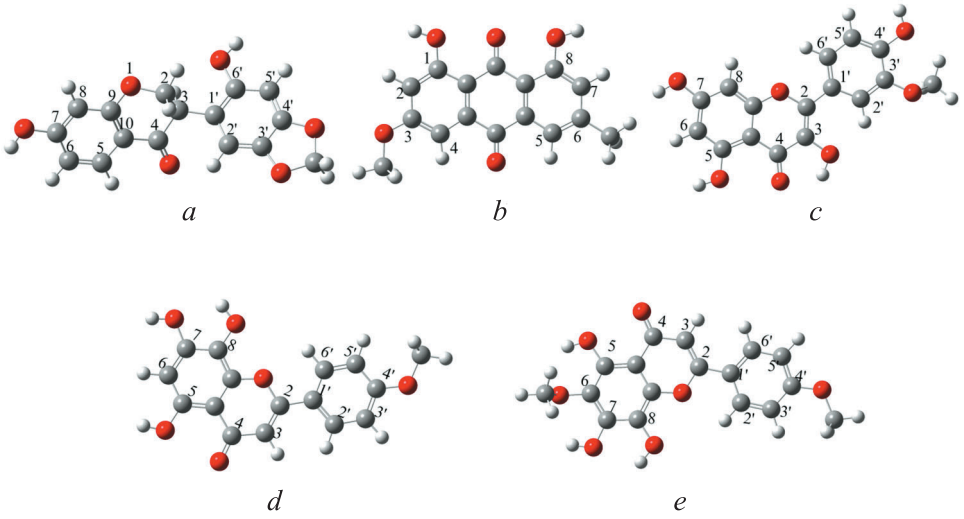

Fig. 5. Optimal molecular structures of (3R)-sophorol (*a*), physcion (*b*), isorhamnetin (*c*), takakin (*d*), and pilosin (*e*) (● oxygen; ● hydrogend; ● carbon)

The electron density of all test compounds can be seen in Fig. 6. The distribution of electron density in the molecule indicates that certain structural parts contribute to the free radical inhibition activity [16]. Based on Fig. 6, the electron distribution of the HOMO contour is seen in the benzene group, while the LUMO contour is distributed in the bicyclic group, especially the C–C and C=C bonds in the benzene ring. Thus, it can be concluded that the benzene ring acts as an electron donor, while the bicyclic group is an electron acceptor. The oxygen atom in the OH group shows significant electron density in the LUMO contour, making the oxygen atom the most likely reaction site to be attacked by free radicals. Furthermore, from Fig. 6, *b*–*e*, the HOMO contour is evenly distributed throughout the molecular structure. The wide spread of the HOMO contour indicates that more parts of the molecular structure participate in chemical reactivity. This is very important for the antioxidant activity of the molecule because the even distribution of electron density increases the potential of the molecule to capture and neutralize free radicals [33].

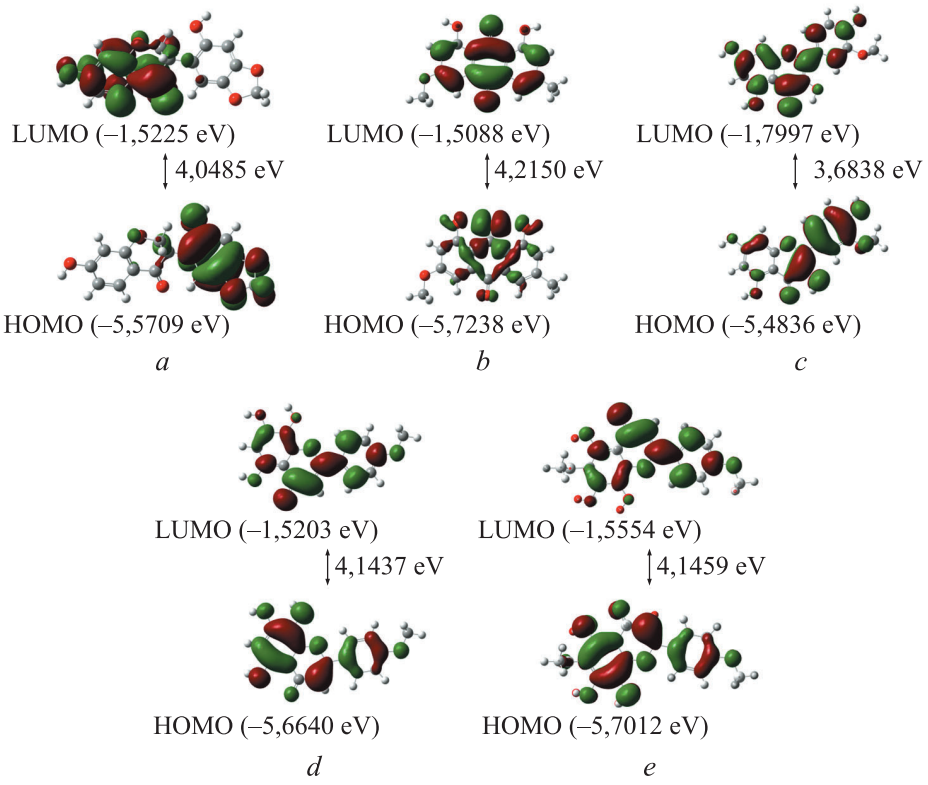

Fig. 6. Contour and energy of HOMO and LUMO (3R)-sophorol (*a*), physcion (*b*), isorhamnetin (*c*), takakin (*d*), and pilosin (*e*)
(• anti-bonding orbital; • bonding orbital)

Electrostatic surface potential analysis. Electrostatic surface potential (ESP) is a mapping of the distribution of electronic charges on the surface of a molecule, which describes the tendency of a region to be electronegative or electropositive based on a color mapping system. In ESP, electronegativity values are ranked according to a color scale: red > orange > yellow > white > green > blue [34]. The red color in ESP mapping indicates electronegative, the redder an area is, the more electronegative it is. Meanwhile, the blue color indicates electropositive, the bluer an area is, the more electropositive it is.

Based on Fig. 7, the most electronegative areas are seen in the C=O and OH groups of all molecules, which are marked in orange to red. Hydrogen atoms bound to carbon atoms and those bound to oxygen atoms tend to be more electropositive, marked in blue. This shows that the hydrogen atoms bound to the oxygen atoms are more easily released.

Global reactivity parameter analysis. The physicochemical properties of organic compounds can be determined based on the stability and reactivity of the molecule, which is based on the difference between the HOMO and LUMO

energies, also called bandgap. Bandgap describes the ability of a compound to transfer electrons. A smaller bandgap value indicates a greater antioxidant potential of the compound because electron transfer becomes easier to form an active state. A small bandgap is also influenced by the length of π-electron conjugation [16, 28, 33].

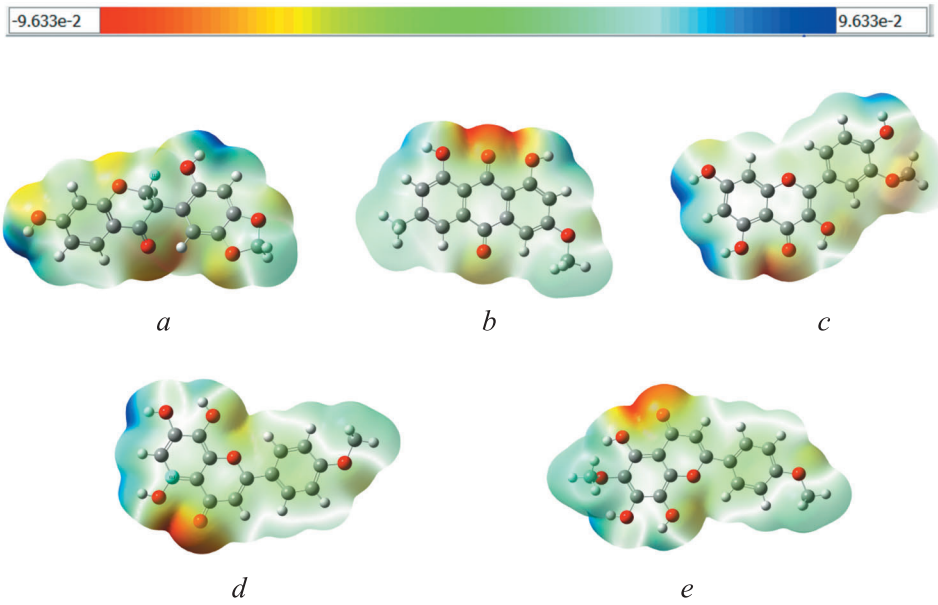

Fig. 7. ESP molecules (3R)-sophorol (*a*), physcion (*b*), isorhamnetin (*c*), takakin (*d*), and pilosin (*e*) (• electronegative; • electropositive)

In Table 3, the molecule with higher reactivity is the isorhamnetin molecule because it has a smaller bandgap value, which is 3.6838 eV. The isorhamnetin molecule also has a larger E_{HOMO} indicating a higher polarity of the compound [33]. This implies that charge transfer between electron acceptors and donors is more likely, making it easier for the isorhamnetin molecule to donate electrons to neutralize free radicals. This indicates that isorhamnetin is more reactive as an antioxidant than other molecules.

Table 3

Frontier energy, bandgap, and dipole moment

Molecules	E_{HOMO}, eV	E_{LUMO}, eV	Bandgap, eV	Dipole moment (Debye)
(3R)-sophorol	– 5.5709	– 1.5225	4.0485	0.8165
Physicion	– 5.7238	– 1.5088	4.2150	2.2231
Isorhamnetin	**– 5.4836**	– 1.7997	**3.6838**	6.2200
Takakin	– 5.6640	– 1.5203	4.1437	**7.2516**
Pilosin	– 5.7012	– 1.5554	4.1459	6.2529

The electric dipole moment is a measure of the separation of electric charges in a system. The value of the dipole moment is calculated as the product of the distance between the charges and the magnitude of the charges [35]. The dipole moment is closely related to the polarization of the molecule, molecules with larger dipole moment values tend to be more easily polarized compared to molecules with smaller dipole moment values [36]. According to Table 3, the most easily polarized molecule is the takakin molecule because it has the largest dipole moment value compared to other molecules followed by pilose and isorhamnetin.

Global reactivity parameter analysis is a parameter that functions to estimate the reactivity of antioxidant molecules. Global reactivity parameters are very helpful in comparing the characteristics of different compounds and their reactivity. The easier the reaction between antioxidant compounds and radicals, the better the antioxidant activity of the compound. Global reactivity parameters include hardness, softness, electrophilicity index, ionization energy, and electron affinity [37].

Bandgap is generally related to hardness and softness. Molecules with large bandgaps have higher hardness and are more difficult to polarize, while molecules with small bandgaps are softer and easier to donate their electrons. Data from Table 4. shows that isorhamnetin has the smallest hardness, indicating that it is more easily polarized and has higher reactivity [38]. Soft molecules can easily deliver electrons to acceptors and the ease of electron donation is also affected by electronegativity [29].

Table 4

Values global reactivity parameters of molecules

Molecules	Reactivity parameter, eV						
	χ	μ	η	σ	ε	*A*	*I*
(3R)-sophorol	3.5467	– 3.5467	2.0242	0.4940	0.3218	1.5225	5.5709
Physicion	3.6163	– 3.6163	2.1075	0.4745	0.3223	1.5088	5.7238
Isorhamnetin	**3.6416**	**– 3.6416**	**1.8419**	**0.5429**	**0.2778**	**1.7997**	**5.4836**
Takakin	3.5921	– 3.5921	2.0718	0.4827	0.3211	1.5203	5.6640
Pilosin	3.6283	– 3.6283	2.0729	0.4824	0.3149	1.5554	5.7012

Electronegativity refers to the ability of a molecule or atom to attract electrons toward itself in a bond. The higher the electronegativity value, the easier it is for the molecule to attract electrons from its surroundings [39]. Isorhamnetin has the highest softness and electronegativity values, which are 0.5429 and 3.6416 eV, respectively. These values indicate that isorhamnetin can act as a good electron donor and withdrawing molecule. Soft molecules effectively donate electrons

to neutralize free radicals while molecules with high electronegativity can attract electrons from free radicals, resulting in more stable products.

Electron affinity (EA) is the amount of energy released when a neutral molecule accepts an electron. According to Janak's theory, EA is equal to $-E_{LUMO}$. A positive EA value indicates that the molecule releases energy when accepting an electron, indicating a high preference for accepting electrons [40]. Based on data from Table 4, the isorhamnetin molecule has the highest EA value, which is 1.7997 eV. This shows that the isorhamnetin molecule is more effective in neutralizing free radicals than other molecules. An electrophile is a molecule or atom that lacks electrons and tends to accept electrons. Electrophilicity measures how strongly a molecule attracts electrons from a nucleophile. The greater the electrophilicity value, the more reactive the molecule is as an electro-phile. The electrophilicity value depends on the chemical potential and chemical hardness. Molecules with low chemical potential and small hardness are the most reactive electrophiles [40].

As revealed by the analysis of global reactivity parameters, the isorhamnetin molecule exhibits the most reactive characteristics compared to other molecules. This molecule has low hardness, high softness, high electronegativity, and the most positive electron affinity. The combination of these properties makes isorhamnetin very effective in neutralizing free radicals, so it can be considered a superior antioxidant molecule. Isorhamnetin has a catechol group in the B ring that enhances the reducing capacity through the formation of intramolecular hydrogen bonds, which stabilize free radicals. The 2,3 double bonds conjugated with the 4-oxygen in the C ring allows electron delocalization, strengthening its interaction with radicals and facilitating their capture. The $^{-}$OH groups at positions 3 and 5, combined with the 4-oxygen, further support electron delocalization. Meanwhile, the hydroxyl group at position C7 also enhances the reducing ability. The presence of methoxy at position C3′ increases lipophilicity and molecular stability, enhances electron delocalization, and reduces steric resistance, which facilitates interaction with free radicals. This combination of structures makes isorhamnetin very effective in reducing free radicals [19].

Theoretical analysis of antioxidant activity. Each antioxidant compound shows different antioxidant activity and plays a role in neutralizing free radicals. The inhibition of radical reactions is determined by the ease of formation of RH and $ArO^{\bullet}$. The easier the formation of $ArO^{\bullet}$, the better the antioxidant activity of the molecule. The ease of reaction is further determined by the reaction enthalpy, which should be as low as possible [14]. The mechanism of cleavage of OH bonds, both homolytic and heterolytic, can be predicted by calculating the BDE, IP, and PA parameters of the OH group in the antioxidant compound.

The mechanism is considered suitable for antioxidant compounds if it has a smaller parameter value [41]. One of the main mechanisms in which phenolic compounds show their antioxidant capacity is the HAT mechanism. In this mechanism, the BDE parameter value of cleavage of OH bonds is an important factor in measuring the antioxidant activity of the compound. The smaller the BDE value, the easier the hydrogen extraction reaction will occur [30]. The BDE values for the hydroxyl groups of the five antioxidant compounds are shown in Table 5. The lowest BDE value was found in the isorhamnetin molecule at the C4′ atom position, which was 1601.5445 kJ/mol. This indicates that the hydroxyl group at the C4′ position in the isorhamnetin molecule is more likely to donate hydrogen atoms to neutralize free radicals. While other molecules have almost identical BDE values: 1609.7859 kJ/mol for (3R)-sophorol, 1603.2117 kJ/mol for takakin, and 1602.2691 kJ/mol for pilosin. However, the BDE value for physcion is significantly higher, which is 1645.1278 kJ/mol. Therefore, proton donation in the physcion molecule is more difficult compared to other molecules.

Table 5

Energy values of BDE, IP, PDE, PA, and ETE molecules (kJ/mol)

OH position	IP	PDE	BDE	PA	ETE
(3R)-sophorol					
C7	684.3261	970.8920	1651.0115	**1400.4474**	254.7706
C6′	**684.3261**	**929.6664**	**1609.7859**	1415.6517	**198.3408**
Physicion					
C1	720.0355	935.4399	1651.2688	1409.8415	245.6339
C8	**720.0355**	**929.2988**	**1645.1278**	**1407.3997**	**241.9346**
Isorhamnetin					
3	653.2428	995.4640	1644.5003	1446.6111	**197.8892**
C5	653.2428	989.6801	1638.7163	1373.6537	265.0626
C7	653.2428	997.8533	1646.8895	**1365.2259**	281.6636
C4′	**653.2428**	**952.5082**	**1601.5445**	1390.2653	211.2792
Takakin					
C5	675.0633	947.8795	1618.7363	1413.4857	209.4571
C7	**675.0633**	**932.3549**	**1603.2117**	**1358.2268**	249.1915
C8	675.0633	950.1427	1620.9995	1445.7084	**179.4976**
Pilosin					
C5	**674.3833**	**932.0924**	**1602.2691**	1407.3210	258.1208
C7	674.3833	932.1212	1602.2980	**1348.3549**	**171.3218**
C8	674.3833	948.9901	1619.1669	1435.1828	188.1906

In addition, to the HAT mechanism, the SET-PT mechanism is also considered an alternative pathway for polyphenolic compounds to neutralize free radicals. In this mechanism, the IP and PDE parameters serve as important indicators to evaluate antioxidant activity. A low IP value indicates that the antioxidant compound has a good ability to donate electrons, while a low PDE indicates that hydrogen protons are easily dissociated. Therefore, molecules with low IP and PDE values have better antioxidant activity. The IP and PDE values of the five antioxidant compounds are listed in Table 5. The IP values for all molecules are in the range of 653.2428–720.0355 kJ/mol, with the lowest value shown by the isorhamnetin molecule. Meanwhile, the lowest PDE value was also found in the isorhamnetin molecule at the C4′ atom position, with a value of 929.2988 kJ/mol. The C4′ position in the isorhamnetin molecule is the most active in the deprotonation process. Based on the values of these two parameters, the isorhamnetin molecule shows a strong tendency to release protons after the formation of cationic radicals [30].

Proton and electron transfer in the SPLET mechanism can be evaluated using the PA and ETE parameter values, which are presented in Table 5. Lower PA and ETE values indicate better antioxidant capacity [42]. Based on the data from Table 5. Pilosin molecule shows the lowest PA and ETE values, which are 171.3218 and 1348.3549 kJ/mol at the C7 position, respectively. These values indicate that pilosin is more efficient in transferring protons and electrons via the SPLET mechanism compared to other mechanisms. Overall, the bond dissociation mechanism that produces H-radicals and ArO radicals is more compatible with the HAT mechanism, because the BDE energy is lower than the total energy values derived from IP + PDE and PA + ETE [43].

Conclusion. Based on the results of the research that has been done, the antioxidant activity of the ethyl acetate fraction of Sungkai leaves showed moderate activity with an IC_{50} value of 62.879 ± 0.43 mg/L and a total phenolic content of 99.00 ± 2.96 mgGAE/g sample. Characterization using LC-MS/MS identified five main compounds in the fraction, namely (3R)-sophorol, physcion, isorhamnetin, pillosine, and takakin. Computational evaluation of antioxidant activity using the DFT/B3LPY/6-31G method showed that the isorhamnetin compound had high reactivity among other compounds. The mechanism of dissociation of the OH bond of isorhamnetin and 4 other compounds is the HAT mechanism.

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