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Exploration of the flavonoid content of *Ziziphus spina-christi* leaf extract and antioxidant activity assay through in vitro and *in silico* methods

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ABSTRACT

The human body naturally has an antioxidant system to counteract free radical reactivity in a sustainable manner, but if the number of free radicals in the body is excessive, additional antioxidants are needed from food intake, namely vitamin C, vitamin E, carotenes and flavonoids. One plant that has the potential as an antioxidant is *Ziziphus spina-christi* (ZSC) because it contains phenolics and flavonoids. This study aims to determine the flavonoid content both qualitatively and quantitatively and to test the antioxidant activity of ZSC leaf extract using in vitro and *in silico* attenuation methods. Determination of the total flavonoid content of ZSC leaf extract using a comparison of quercetin. In vitro the antioxidant activity assay of ZSC leaf extract was carried out by measuring the reducing activity of ZSC leaf extract against the radical DPPH using ascorbic acid as a comparison, while the *in silico* method used QSAR and pharmacophore modelling techniques. The results showed that the total flavonoid content obtained from ZSC leaf extract was 0.2515 ± 0.0013 mg QE/g D.W with an IC_{50} of 58.9296 ppm. Furthermore, from the *in silico* method using pharmacophore modeling and QSAR techniques, 8 hit compounds were obtained from the content of ZSC with IC_{50} QSAR ranging from 6.57 μM to 0.0004 μM, which was thought to be the metabolite that had the most role in its antioxidant activity. This value indicates that ZSC leaf extract has potential as a very strong antioxidant. ZSC leaf extract can be used as an antioxidant candidate in drug and cosmetic preparations through the oral or percutaneous route. It also proves that QSAR and pharmacophore modelling techniques can be used as confirmatory tests for in vitro results in determining the antioxidant activity of natural materials.

Keywords : antioxidant, flavonoid, *in silico*, in vitro, *Ziziphus spina-christi*

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INTRODUCTION

Indonesia is known as a mega biodiversity country, a nation with has a lot of biodiversities in Indonesia's tropical forests, there are about 30,000 plants, it is estimated that around 9,600 species are known to have medicinal properties, and about 200 species are important medicinal plants for the traditional medicine industry (Isnawati et al., 2019). Currently, many people are returning to using natural materials, in practice are accustomed to living by avoiding synthetic chemicals and preferring natural ingredients. One of them is the use of medicinal plants (Afrianto et al., 2020).

On the other hand, in everyday life, we cannot be separated from exposure to free radicals, such as cigarette smoke, fried, burned foods, excessive sun exposure, motor vehicle fumes, certain drugs, toxins and air pollution (Albaigés, 2014). Free radicals are molecules that have one or more unpaired electrons. These unpaired electrons cause free radicals to become highly reactive compounds against body cells by binding to the cell's molecular electrons through oxidation reactions (Nasrollahzadeh et al., 2020). Compounds that are able to inactivate the development of free radical reactions by preventing the formation of radicals so that they become unreactive and relatively stable are called antioxidants (Nasrollahzadeh et al., 2020). The human body naturally has an antioxidant system to counteract free radical reactivity in a sustainable manner, but if the number of free radicals in the body is excessive, additional antioxidants are needed from food intake, namely vitamin C, vitamin E, carotenes and flavonoids (Ergüder et al., 2007).

One plant that has the potential as an antioxidant is ZSC because it contains phenolics and flavonoids (El Maaiden et al., 2019; Elsadig Karar, 2016). ZSC is also rich in other biological benefits, such as anti-inflammatory, antimicrobial, antifungal and prevents the formation of tumours (Bahmani et al., 2020). Parts of the plant ZSC are widely used in traditional medicine ranging from roots, stems, leaf, fruits and seeds (Asgarpanah, 2012; Alnahdi, 2017). ZSC leaves are a rich source of phenolic compounds including chlorogenic acids, proanthocyanidins, simple and highly glycosylated flavonoids. Fifty-seven components were tentatively detected and characterized on the basis of reported analytical data from three to four pieces of independent methods including retention time, UV/Vis spectroscopy, high resolution mass spectrometry (HR-MSn), and tandem mass spectrometry (MSn) (Elaloui et al., 2021).

Flavonoids are formed in plants from the aromatic amino acids phenylalanine, tyrosine and malonic (Agati et al., 2012). In plants, flavonoid aglycones (flavonoids without bound sugars) exist in various structural forms. They all contain 15 carbon atoms in their basic nucleus, which are arranged in the C6-C3-C6 configuration, i.e. two aromatic rings linked by a three-carbon unit which may or may not form a third ring (Brunetti et al., 2018). Flavonoids in plants are rarely found singly but in mixed forms. Flavonoids are a class of water-soluble compounds. Flavonoids in plants are bound as glycosides and aglycones. Therefore the analysis of flavonoids is better by examining aglycones (Ferraz et al., 2020). The classification of flavonoids in plant tissues was initially based on the study of solubility properties and color reactions. The flavonoid group, namely anthocyanins, proantocyanins, flavonols, flavones, glycoflavones, biflavonils, chalcones, aurons, flavones, and isoflavones. Flavonoids contain a conjugated aromatic system showing strong absorption bands in the UV and visible spectrum regions (Chang et al., 2002; Paula et al., 2017).

Flavonoids are reducing compounds that can inhibit many oxidation reactions. Flavonoids have the ability as antioxidants because they are able to transfer an electron to free radical compounds, where R• is a free radical compound, Fl-OH is a flavonoid compound, while Fl-OH• is a flavonoid radical (Li et al., 2020).

A simple and fast method to determine antioxidant activity is to use the free radical 2.2-diphenyl-1-picrylhydrazyl or DPPH (Olszowy and Dawidowicz, 2018). This method is often used to test compounds that act as free radical scavengers or hydrogen donors and evaluate their antioxidant activity, as well as quantify the amount of radical-antioxidant complex formed. The DPPH method can be used for samples in the form of solids or liquids (Kedare & Singh, 2011).

Determination of activity using an *in silico* approach is currently a way to be taken in the discovery and development of drugs from synthetic compounds and natural materials, which can reduce costs and time. The isolation of natural compounds for further testing of extracts can take a long time and cost a lot, and it is not sure that the isolated compounds can provide the desired effect. Thus, finding the active compound with an *in silico* computational approach to determine which compounds have the most potential for isolation in the future, becomes something relevant. QSAR (Quantitative Structure-Activity Relationship) and pharmacophore modeling techniques are *in silico* methods to predict the metabolite compounds contained in natural materials, which have the most role and have the most potential in providing antioxidant activity.

This study aims to determine the flavonoid content both qualitatively and quantitatively and to test its antioxidant activity in ZSC leaf extract in vitro using the DPPH and *in silico* methods using QSAR and pharmacophore modeling techniques. Furthermore, in the future can select flavonoid compounds that have the potential as antioxidants.

MATERIALS AND METHODS

Plant materials

ZSC leaf were collected in December 2019 from Kebun Quran Foundation, West Bandung District, West Java, Indonesia. The plant sample was identified in Jatinagor Herbarium, Plant Taxonomy Laboratory, Biology Departement, Padjadjaran University, West Java, Indonesia, under the code plant identify number 051/HB/02/2019. Plants were dried at room temperature for two weeks in a dry and airy environment. The dried samples were powdered and stored in the dark at a dry place until further use.

Reagent materials

Quercetin (Sigma Aldrich, UK), Ascorbic acid (Sigma Aldrich, UK), 1.1-difenil-2-pikrihidrazil (DPPH) (Sigma Aldrich, UK), Sodium acetate (Sigma Aldrich, UK), Aluminium chloride (Sigma Aldrich, UK), Hydrochloric acid (Sigma Aldrich, UK), Magnesium metal (Sigma Aldrich, UK), Methanol (Sigma Aldrich, UK) and Ethanol (Sigma Aldrich, UK). All other chemical reagents used were of analytical grade.

Methods

Extraction

The 50 gram sample of ZSC leaf was weighed and extracted using the infundation method in 1 liter of water until the simplicia was submerged at 90°C for 15 minutes. Then the resulting liquid extract was evaporated using a rotary vacuum evaporator to obtain a concentrated extract.

Screening of relative molecular mass

ZSC leaf extract was made at a concentration of 1 mg/L (total volume ± 3 mL) in methanol. Relative molecular mass measurements were carried out using Ultra Performance Liquid Chromatography mass spectrometry and tandem mass spectrometry (UPLC MS/MS H-Class) with a spectrum range from 1.91 to 1301.91 m/z (Elsadig Karar, 2016).

Flavonoid qualitative analysis

ZSC leaf extract 30 mg was put into a test tube, then a little magnesium metal powder and a few drops of concentrated HCl were added. A positive reaction containing flavonoids is indicated by the formation of an orange-red color (Chang et al., 2002).

Flavonoid quantitative analysis

Quercetin standard curve generation

Quercetin was weighed as much as 25 mg and put into a 25 mL volumetric flask, then ethanol was added to 25 mL (main solution 1000 µg/mL). Then a series of standard solutions of 60 µg/mL, 80

µg/mL, 100 µg/mL, 120 µg/mL and 140 µg/mL were made. Each 0.5 mL of the standard solution was pipetted with 1.5 mL of ethanol, 0.1 mL of 10% aluminium chloride ($AlCl_3$), 0.1 mL of 1 M potassium acetate and 2.8 ml of distilled water was added. After that, it was incubated for 30 minutes at 25°C. The absorption was measured at a wavelength of 431 nm using a UV-Vis spectrophotometer. Then a calibration curve is made by connecting the absorption value as coordinates (Y) and the concentration of the standard solution as abscissa (X).

Preparation of extract test solution

A total of 0.1 gram sample of ZSC leaf extract was weighed and dissolved in 5 mL of methanol and centrifuged, then filtered and the filtrate obtained was added to 10 mL of methanol (10.000 ppm).
Determination of total flavonoid content in extract

The blank solution was prepared by replacing the standard solution with 0.5 mL of ethanol, add 1.5 mL of ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate and add 2.8 ml of distilled water. After that, it was incubated for 30 minutes at 25°C. Each absorbance measurement was compared against the blank. The test solution contained 1.0 mL of the test solution, then added ethanol to 10 ml in a volumetric flask. A total of 0.5 mL of the solution was then added with 1.5 mL of 95% ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water was added.

After that, it was incubated for 30 minutes at 25°C. The absorption was measured at a wavelength of 431 nm using a UV-Vis spectrophotometer (Orhan and Üstün, 2011; Gao et al, 2019). The test was carried out in triples. Total flavonoid levels can be calculated using the formula:

$$F = \frac{c \, x \, V \, x \, f \, x \, 10^{-6}}{m} \, x \, 100\%$$

Information:
F : Total flavonoid
c : Quercetin equivalence (µm/mL)
V : Extract total volume
f : Dilution factor
m : Sample weight (g)

In vitro antioxidant activity assay with DPPH attenuation method

Preparation of DPPH solution

A total of 4 mg of DPPH was weighed and put in a 25 mL volumetric flask, added methanol to the mark, homogenized. Then the absorption was measured at a wavelength of 517 nm using UV-Vis spectrophotometry.

Preparation of stock solution

A total of 25 mg of ZSC leaf extract was weighed and put in a 25 mL volumetric flask, added methanol to the mark, homogenized. Then it is centrifuged so that the concentration becomes 1000 ppm as stock solution.

IC_{50} value measurement

The stock solution was diluted in series by pipetting 0; 0.08; 0.16; 0.24; 0.32 and 0.40 mL, then 1 mL of DPPH solution was added and made up to 4 mL of methanol to obtain concentrations of 0, 20, 40, 60, 80 and 100 ppm. The mixture was homogenized and allowed to stand for 30 minutes in the dark at 37°C. The absorbance was measured at a wavelength of 517 nm using UV-Vis spectrophotometry. The absorbance value of each concentration variation was recorded and the IC_{50}

value was calculated. The test was carried out in triples using ascorbic acid as a comparison (Halim et al, 2018; Molyneux, 2004).

In silico antioxidant activity determination

In this study, we use several *in silico* tools, i.e. pharmacophore modeling and QSAR prediction to predict the metabolite compounds contained in ZSC, which have the most role and have the most potential in providing antioxidant activity. The materials used to make pharmacophore and QSAR models are phenolic and flavonoid group compounds with high antioxidants activity through the DPPH method assay.

Pharmacophore modelling

The active compounds selected to make the pharmacophore model were 15 phenolic compounds from the research of (Chen et al., 2020) and 7 flavonoid compounds from the research of (Silva et al., 2002), these compounds were known to have very strong antioxidant activity through the DPPH test method. As for the test compounds, phenolic compounds and flavonoids contained in ZSC plant (Elsadig Karar, 2016). The 3D structure of all compounds was traced through the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database and then prepared using Quantum ESPRESSO v.6.4.1 to optimize the 3D structure of these compounds (Giannozzi et al., 2009, 2020). After that, the training compounds were entered into the Ligand Scout 4.3 (Wolber and Langer, 2005). After that, 5 compounds were selected to be used as training sets and the rest were used as test sets. The selection of compounds was based on different structural variations, i.e. 1 compound from the Hydroxybenzoic acid, Hydroxycinnamic acid, and Hydroxyphenylacetic acid groups, and 2 compounds from the flavonoid groups.

Furthermore, the 5 compounds in the training set were put into a molecular alignment perspective, to align their structures according to the pharmacophore similarities. The same pharmacophore features among the five compounds transferred to a virtual screening perspective to validate the pharmacophore model with the AUC-ROC criteria (Wolber and Langer, 2005). Validation was carried out using test set compounds which were active compounds that were not used in modeling, while decoy set compounds were determined using the Decoy Finder 2.0 (Cereto-Massagué et al., 2012). A pharmacophore model is declared valid if the AUC-ROC value is > 0.5. In further, the pharmacophore model was used to find hit compounds, from phenolic contents in ZSC.

Quantitative Structure–Activity Relationship (QSAR) modelling

The first procedure in QSAR modeling is carried out similar to pharmacophore modeling, is by preparing the same active compound, then the compounds were minimized with the MMFF94s force field in the Molecular Operating Environment (MOE) 2014 (Cereto-Massagué et al., 2012). After that, the training compounds were entered into the MOE 2014 database for descriptor calculations. The descriptors obtained were then determine for statistical processing, to found the descriptor that had the most effect on antioxidant activity. The QSAR equation model obtained was then validated according to Economic Co-operation and Development (OECD) standards, e.g. R2 and Leave-One-Out (LOO) Q2 (Benzidane et al., 2020; Dhumal et al., 2020). The QSAR equation was then used to predict the IC_{50} or antioxidant activity of the phenolic and flavonoid compounds contained in ZSC plant which were previously hits from screening pharmacophores.

RESULTS AND DISCUSSION

ZSC leaf extraction

Extraction aims to attract chemical compounds contained in a simplicia by using a suitable solvent that can attract these chemical compounds. Leaf simplicia of ZSC were extracted by the infundation method using aquadest as a solvent, because flavonoid in ZSC leaf tend to be polar so they are very soluble in water. Water was chosen as a solvent because it is commonly used, the price is cheap, easy

to obtain, stable, and non-toxic. In addition, this method uses a simple tool, the time required is relatively short which is only 15 minutes.

Relative molecular mass screening of ZSC extract

The relative molecular mass screening of ZSC leaf extract aims to describe the content of flavonoid compounds in crude extract which is confirmed by literature data. According to research on the phenolic profile of ZSC leaf extracts, there are fifty-seven phenolic compounds (fifty-two of them are known) to their regioisomeric level in the methanol/water extract of ZSC leaf. Highly glycosylated flavonoids, proanthocyanidins, and chlorogenic acids were identified as the major components (Karar, 2016). The results of screening the relative molecular mass of ZSC leaf extract in the spectrum range 0-1100 m/z using UPLC MS/MS H-Class can be seen in Figure 1.

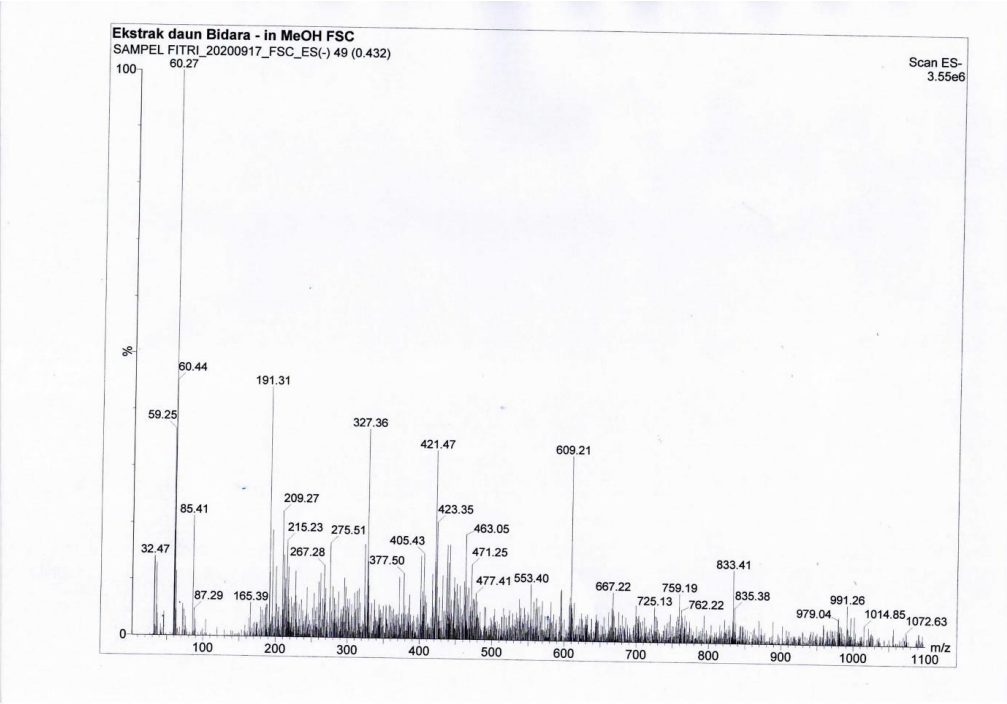

Figure 1. Results of relative molecular mass screening of ZSC leaf extract

The following results is the relative molecular mass data that has a high intensity in the leaf extract of ZSC as a result of the MS/MS H-Class UPLC screening which is compared to literature data, summarized in Table 1.

Qualitative analysis of flavonoids content

Qualitative analysis of flavonoids in the leaf extract of ZSC as a preliminary test was carried out to provide an overview of the class of compounds contained in the extract of ZSC. The flavonoid compound group test was carried out with the addition of HCl and magnesium metal. The purpose of adding magnesium and HCl is to reduce the benzopyrone core contained in the flavonoid structure so that the color changes to orange or red. The addition of HCl resulted in an oxidation-reduction reaction between Mg metal as a reducing agent and flavonoid compounds causing a color change from yellow-orange to red (Chang et al, 2002). The results of the qualitative analysis of the flavonoid content in the leaf extract of ZSC can be seen in Table 2.

Table 1. Relative molecular mass data with high intensity on ZSC leaf extract results from UPLC-MS/MS screening

Relative Molecular Mass from Screening Results (m/z)	Relative Molecular Mass from Literature Data (m/z) (Elsadig Karar, 2016)	Compound Name from Literature Data (Karar, 2016)
289.31	289	Catechin Epicatechin
433.16	433	Quercetin 3-O-arabinoside
447.24	447	Quercetin 3-O-rhamnoside Kaempferol 3-O-glucoside
505.37	505	Quercetin-O-acetyl hexoside
609.21	609	(Epi)gallocatechin-(4, 8')-(epi)gallocatechin (Epi)gallocatechin-(4, 8')-(epi)gallocatechin (Epi)gallocatechin-(4, 6')-(epi)gallocatechin (Epi)gallocatechin-(4, 8')-(epi)gallocatechin (Epi)gallocatechin-(4, 6')-(epi)gallocatechin (Epi)gallocatechin-(4, 8')-(epi)gallocatechin Quercetin 7-O-(6-O-rhamnosyl-glucoside) Quercetin 3-O-(6-O-rhamnosyl-galactoside) Quercetin 3-O-(6-O-rhamnosyl-glucoside) (Rutin)
623.55	623	Isorhamnetin 3-O-(6-O-rhamnosyl-galactoside) Isorhamnetin 3-O-(6-O-rhamnosyl-glucoside)
901.56	901	Quercetin 3-O-(2, 6-di-O-rhamnosyl-galactoside) 7-O-rhamnoside Quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7-O-rhamnoside
917.97	917	Quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7-O-galactoside Quercetin 3-O-(2, 6-di-O-rhamnosyl-glucoside) 7-O-glucoside

Table 2. Qualitative test of flavonoid leaf extract of ZSC

Group Tested	Reagent	Color	Conclusion
Flavonoid	Magnesium Powder + HCl + amyl alcohol	Orange-Red	+

Determination of total flavonoids content

Determination of total flavonoid content using the $AlCl_3$ method is the formation of a stable complex between aluminum chloride and C-4 keto groups, as well as at C-3 or C-5 hydroxyl groups of flavones and flavonols. In addition, $AlCl_3$ forms stable acid complexes with orthohydroxyl groups on the A- or B- rings of flavonoid compounds. Quercetin was chosen as a comparison solution because it is one of the flavonoid compounds that can react with $AlCl_3$ to form complexes **(**Figure 2**)** (Chang et al., 2002).

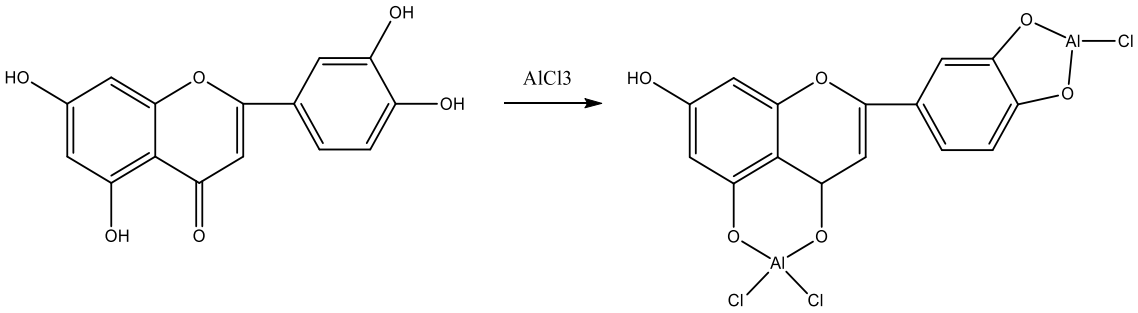

Figure 2. Complex formation reaction of Flavonoid-$AlCl_3$ (Molyneux, 2004)

Maximum wavelength absorption measurements were carried out in the range of about 400-800 nm. The maximum wavelength produced was 431 nm at a concentration of 60 µg/mL, the maximum wavelength was then used to measure the absorption of the calibration curve and sample of ZSC leaf extract. From the calibration curve obtained a linear regression equation, namely y = 0.0062x + 0.0059 with a correlation coefficient (r^2) = 0.9983. The value of r which is close to 1 indicates a linear calibration curve and there is a relationship between the concentration of quercetin solution and the absorption value. In the determination of flavonoid levels, the addition of potassium acetate is to detect the presence of a 7-hydroxyl group, while the incubation treatment for 30 minutes before the measurement is intended to make the reaction run perfectly, thus providing maximum color intensity. The determination of the flavonoid content of the leaf extract of ZSC was carried out in triples, which was 25.145 ± 0.0013 mg QE/g D.W. This result was higher than the previous study, which was 0.15312 mg QE/g D.W, caused by the extract of ZSC in 70% ethanol (Haeria et al., 2016). The results of the quantitative analysis of the total flavonoid content in the leaf extract of ZSC can be seen in Table 3.

Table 3. Total flavonoid content of ZSC leaf extract

Extract weight (gram)	Equivalent Level (ppm)	Total Flavonoid Level (%)	Total Flavonoid Level (mg QE/g D.W)
0.1	25,145 ± 0.1317	2.5145 ± 0.0132	25.145 ± 0.0013

In Vitro antioxidant activity assay

The method used in antioxidant testing is the uptake method of 1.1-diphenyl-2-picrylhydrazyl (DPPH) because it is a simple, fast, easy, and accurate method and uses a small number of samples in a short time to evaluate the antioxidant activity of plant extracts, by comparison with ascorbic acid. The concentration of ascorbic acid was made smaller than the sample concentration because it is a pure compound that has been proven to be a very strong antioxidant compound (Carmona-Jiménez a et al, 2014).

Radical 1.1-diphenyl-2-picrylhydrazyl (DPPH) is an organic compound containing unstable nitrogen with strong absorbance at a maximum absorption wavelength of 517 nm and dark purple in color. The working principle of DPPH is to take hydrogen atoms (electron transfer) contained in an antioxidant compound, for example phenol compounds to make it more stable, or antioxidant compounds will donate hydrogen to DPPH by reacting with antioxidants, the absorption of DPPH will be reduced which is marked by a radical color change DPPH free from purple to pale yellow. Determination of antioxidant activity using the DPPH attenuation method is expressed by the DPPH attenuation value **(**Figure 3**)** (Carmona-Jiménez et al., 2014). Free radical scavenging reactions by flavonoid compounds as shown in the following Figure 4 (Hernández-Rodríguez et al., 2018).

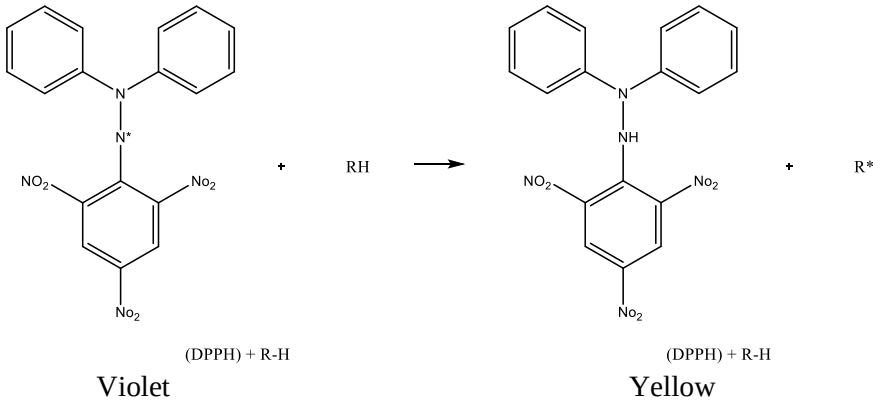

Figure 3. Reaction of DPPH radicals with antioxidants (Carmona-Jiménez et al., 2014)

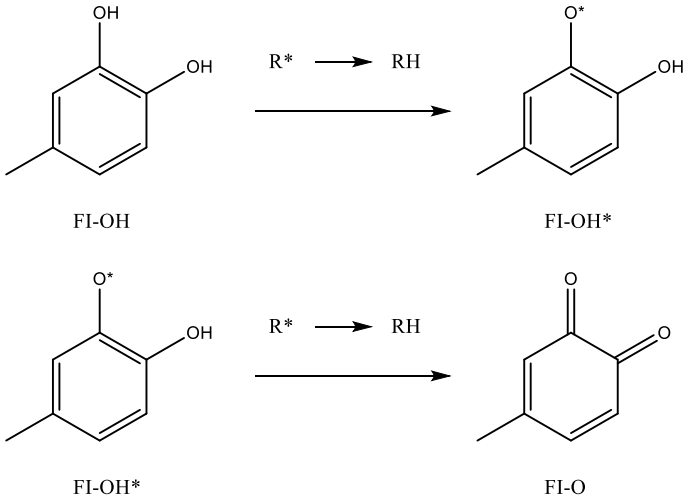

Figure 4. The mechanism of radical attenuation by flavonoids (Hernández-Rodríguez et al, 2018)

The antioxidant potential of a sample is measured by measuring the percentage of immersion against DPPH free radicals which is expressed by the IC_{50} value, which is a number that indicates the concentration of the extract that is able to inhibit 50% oxidation. The IC_{50} value is inversely proportional to the antioxidant activity. The higher the IC_{50} value, the smaller the antioxidant activity, and vice versa. The IC_{50} value of ascorbic acid as a comparison and ZSC leaf extract can be seen in Table 4 and Table 5.

Table 4. Relationship between ascorbic acid concentration and antioxidant activity against DPPH

Concentration (ppm)	Free Radical Scavenging Activity (% inhibition)	Linear Regression Equation
0	0.000	y = 7.4718x + 2.1636 r^2 = 0.9971 IC_{50} = 6.4023 ± 0.030 ppm
2	19.191 ± 0.021	
4	32.261 ± 0,031	
6	47.718 ± 0,034	
8	62.344 ± 0,029	
10	75.622 ± 0,038	

Table 5. Relationship between ZSC leaf extract concentration and antioxidant activity against DPPH

Concentration (ppm)	Free Radical Scavenging Activity (% inhibition)	Linear Regression Equation
0	0.000	y = 0.7882x + 3.5517 r^2 = 0.9926 IC_{50} = 58.9296 ± 0,340 ppm
20	21.473 ± 0.39	
40	36.515 ± 0.37	
60	52.801 ± 0.43	
80	67.531 ± 0.26	
100	79.461 ± 0.52	

The results showed that the strong antioxidant activity of the leaf extract of ZSC with an IC_{50} value of 58.9296 ppm. While the IC_{50} value of ascorbic acid is 6.4023 ppm, indicating a very strong antioxidant activity. The activity of ZSC leaf extract was weaker compared with ascorbic acid due to the purity of ZSC leaf extract is lower than references as pure active compounds.

Specifically, a compound is said to be a very strong antioxidant for IC_{50} value of 50 ppm, strong for 50-100 ppm, moderate for 101-150 ppm and weak for IC_{50} > 150 ppm (Haeria et al., 2016). This study provides evidence that the leaf of ZSC contain flavonoid compounds and have the potential as antioxidants that are good for health.

In silico antioxidant activity determination

Determination of activity using an *in silico* approach is currently a way that can be taken in the discovery and development of drugs from synthetic compounds and natural materials, which can reduce costs and time. Isolation of natural compounds for further testing of extracts can take a long time and cost a lot, and it is not certain that the isolated compounds can provide the desired effectiveness. Thus, finding the active compound with an *in silico* computational approach to determine which compounds have the most potential for isolation in the future, becomes something relevant.

Pharmacophore modelling and virtual screening

The compounds selected as the training set include siringic acid, synaptic acid, homovanillic acid, catechins and quercetin. The pharmacophore model obtained consists of 6 features, including 3 hydrogen bond acceptors, 1 hydrogen bond donor, 1 aromatic, and 1 hydrophobic **(**Figure 5**)**. The validation process is carried out using a test set and a decoy set. The test set was obtained from active compounds that were not used as a training set, a total of 18 compounds, while the decoy set was obtained through a search using the DecoyFinder 2.0, obtained a total of 395 decoy compounds.

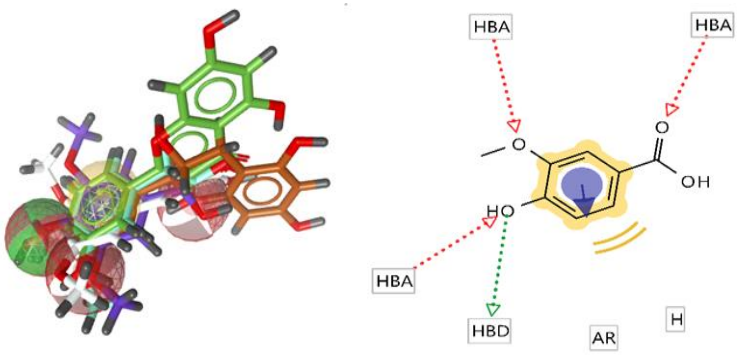

Figure 5. Alignment of the training set molecules and features of the obtained shared pharmacophores; HBA: Hydrogen bond acceptor (3), HBD: Hydrogen bond donor (1), AR: Aromatic (1), and H: Hydrophobic (1)

The previously obtained pharmacophore model was then used to screen the test set and decoy set, then the ROC graph was evaluated. Obtained 100% AUC-ROC value of 0.66 **(**Figure 6**)**, which indicates a valid pharmacophore model or able to properly select active compounds among decoy compounds (Hikmawati et al., 2022).

The pharmacophore model obtained was then used to predict the phenolic and flavonoid active metabolites of the ZSC leaf, where a number of 25 active metabolites were used, whose structure can be obtained through the PubChem database. The 25 compounds were predicted for their pharmacophore using a previously validated pharmacophore model. The search results obtained 8 hit compounds that have appropriate pharmacophore models, these compounds include: quercetin 7-ramnosyl glucoside, isoramnetin 3-O-glucoside, rutin, quercetin 3-O-ramnoside, Epicatechin-(4,8')-epigallocatechin, quercetin 3-O-arabinoside, epicatechin, quercetin-O-acetyl hexoside.

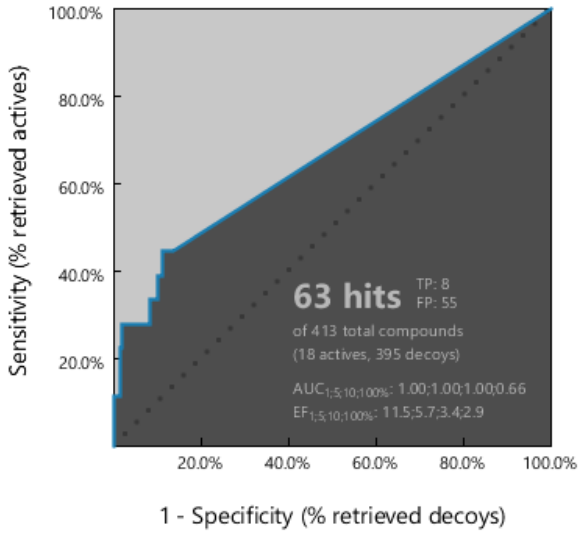

Figure 6. The results of the validation of the pharmacophore model with the AUC-ROC curve

QSAR antioxidant activity prediction

The QSAR equation that can predict the antioxidant activity of phenolic and flavonoid group compounds has been obtained. These equations are: Log IC_{50} = -5.251 + (0.00051 x AM1_dipole) + (-0.00025 x AM1_E) + (0.000028 x AM1_Eele) + (0.00577 x AM1_HF) + (0.31768 + AM1_LUMO) + (0.40226 + LogS) + (0.00276 x vol). The equation consists of 7 descriptors analyzed the most influential which is a type of descriptor that represents electronic, hydrophobic, and steric properties. The descriptors obtained include AM1_dipole, AM1_E, AM1_Eele, AM1_HF, AM1_LUMO, logS, and vol (van der waals value). Validation of the descriptor based on the leave-one out (LOO) Q2 criteria obtained results of 0.84. The value of Q2 > 5 indicates a good QSAR equation model, and the closer to 1 it is stated that the equation model is very strong in predicting activity accurately. In addition, from the analysis of the coefficient of determination (R^2) by making the experimental IC_{50} and predictive IC_{50} values as coordinates, the R^2 value is 0.93 (Figure 7), which shows that the selected descriptor is the most influential descriptor of 93% of phenolic and flavonoid compounds (Hikmawati et al., 2022).

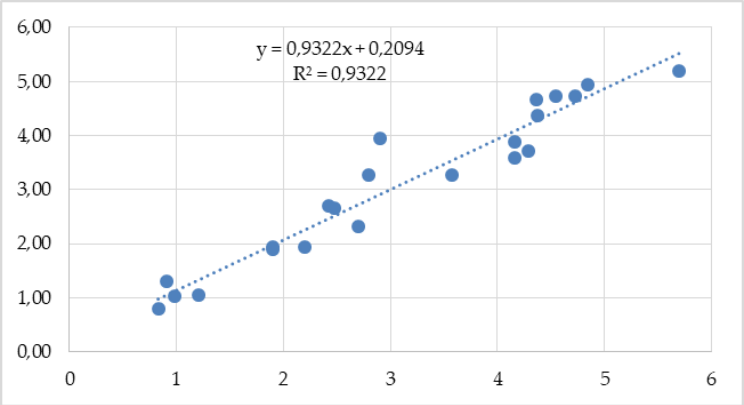

Figure 7. QSAR validation results, comparison between experimental and predictive activity

Furthermore, the obtained QSAR equation was used to predict the antioxidant activity of phenolic and flavonoid compounds contained in ZSC from the calculations, it is predicted that the phenolic and flavonoid compounds contained in the extract of ZSC plant were previously obtained from pharmacophore tracing. Based on the QSAR search, the antioxidant activity of the 8 previous hit pharmacophore compounds was obtained which is represented in Table 6. All compounds showed results with very strong antioxidant activity. In further, Quercetin 3-O-arabinoside with IC_{50} prediction of 0.0004 µM is a compound that provides stronger antioxidant activity with 15 phenolic compounds and 7 flavonoid compounds that have been tested in vitro using DPPH in previous study.

Table 6. Predicted results of antioxidant activity by QSAR model

Compound Name	Predicted IC_{50} (µM)
Quercetin 7-ramnosyl glucoside	2.69
Isoramnetin 3-O-glucoside	2.36
Rutin	1.80
Quercetin 3-O-ramnoside	6.57
Epicatechin-(4,8')-epigallocatechin	1.56
Quercetin 3-O-arabinoside	0.0004
Epicatechin	1.52
Quercetin-O-acetyl hexoside	3.66

CONCLUSIONS

Based on the results of research and data analysis that has been carried out, it can be concluded that the leaf extract of ZSC has a total flavonoid content of 0.2515 ± 0.0013 mg QE/g D.W with strong antioxidant activity at an IC_{50} value of 58.9296 ppm. Furthermore, from the *in silico* assay using pharmacophore modeling and QSAR techniques, 8 hit compounds were obtained from the content of ZSC with IC_{50} QSAR ranging from 6.57 µM to 0.0004 µM, which was thought to be the metabolite that had the most role in its antioxidant activity. This value indicates that ZSC leaf extract is predicted to have potential as an antioxidant which has relatively good activity. ZSC leaf extract can be used as an antioxidant candidate in drug and cosmetic preparations through the oral or percutaneous route. It also proves that QSAR and pharmacophore modelling techniques can be used as confirmatory tests for in vitro results in determining the antioxidant activity of natural materials.

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