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Comparison on Total Phenolics and Flavonoids and Antioxidant Activities of Methanol Extract of Horseshoe Crab (*Tachypleus gigas*) Eggs

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Abstract: The marine environment can be a source of abundant bioactive compounds. One of the horseshoe crab species scattered in Indonesian sea waters is *Tachypleus gigas*. It was reported that the eggs of *T. gigas* contained flavonoids, saponins, alkaloids, and steroids. Flavonoids are polyphenol compounds that have the ability as natural antioxidants. In this study, total phenolics, flavonoids, and antioxidant activity tests were carried out on the methanol extract of *T. gigas* eggs. The total phenolics content used the Folin Ciocalteu method, the total flavonoids used the aluminum chloride colorimetric method, and the antioxidant activity test used the FRAP and DPPH methods. The test results showed that the total phenolics and flavonoids were 0.53506 ± 0.001335 mg GAE/g extract and 0.52067 ± 0.000731 mg QE/g extract, respectively. Meanwhile, the results of the antioxidant activity test with the FRAP method obtained a total antioxidant capacity of 29.85 µmol Fe^{2+}¿/gDW¿ in the medium category and antioxidant activity with the DPPH method obtained an IC_{50} value of 597.0397 µg/mL in the very weak category.

Keywords: Antioxidant activity, phenolic, flavonoid, *Tachypleus gigas*

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1. INTRODUCTION

In the body, oxidation reactions can be the initial cause of most diseases. These reactions can cause continuous and cumulative oxidative damage to essential macromolecules, especially DNA (1). Oxidative damage occurs as a consequence of the excessive production of free radicals, which cannot be processed caused of the insufficient availability of antioxidants, so they accumulate (2). Free radicals are produced during normal metabolic activity in the body and trigger the emergence of various diseases, such as neurodegenerative diseases, cataracts, rheumatoid arthritis, asthma, and others (3). Related to that, an antioxidant is a necessary agent that can reduce the occurrence of oxidative processes and the harmful effects caused by free radicals (4).

Antioxidants are substances that can protect body cells from damage because of free radicals as unstable molecules (5). Antioxidants can slack down the lipid oxidation process by capturing or deactivating free radicals; initiation and propagation reactions can be inhibited (6). The antioxidant activity of a compound works through a series of reaction mechanisms, including the ability to transfer single electrons, release hydrogen electrons, or chelate transition metals (7).

Antioxidants based on the sources are distinguishable into two types endogenous and exogenous (8). Endogenous antioxidants are a type of antioxidant as a natural defense produced by the human body (9). The body also needs exogenous antioxidants to overcome the excess free radicals generated by oxidative stress (10). Exogenous antioxidants are obtained from food or supplements consumed, which consist of natural antioxidants like vitamins A, C, E, phenolic acids, flavonoids, and carotenoids, for synthetic antioxidants are like butylhydroxytoluene, octyl gallate, propyl gallate, and tertiary-butylhydroquinone (11). However, synthetic antioxidants have side effects that are bad for health. Therefore, natural antioxidants can be an alternative to antioxidant agents that are safer to consume than synthetic antioxidants (12).

The horseshoe crab is an aquatic animal known as a living fossil from the Limulidae family (13). Here are four types of horseshoe crab animals scattered worldwide, three of which are found in Indonesia*: Carcinoscorpius rotundicauda, T. tridentatus,* and *T. gigas* (14)*.* It has been discovered that coastal communities consume lots of this part of the horseshoe crab egg (15).

T. gigas eggs contain flavonoids, steroids, saponins, and alkaloids (16). Flavonoids are phenolic compounds with essential biological abilities, their activity both as free radical scavengers, namely antioxidants (17).

This study tested the antioxidant activity of the methanol extract of *T. gigas* eggs using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) methods. In the DPPH method, antioxidant compounds act as proton donor compounds to DPPH free radicals, reducing DPPH free radicals to form stable compound molecules (DPPH-H). The DPPH free radical becomes a stable molecule characterized by a color change from purple to yellow as a non-radical compound (reduced diphenylpicrylhydrazine, DPPH-H) (18). Meanwhile, the mechanism in the FRAP method is electron donation, where the antioxidant compounds in the sample act as reducing agents (19). In the FRAP method, antioxidant compounds will reduce the yellow Fe^{3+}-TPTZ complex to blue Fe^{2+} (20). Based on the description above, it is necessary to know about the potential and efficacy as an antioxidant from the methanol extract of *T. gigas* eggs using the DPPH and FRAP methods, which begins with testing the total phenolic and total flavonoid levels.

2. MATERIAL AND METHODS

2.1. Materials

Horseshoe crab (*T. gigas*) eggs were taken from south coast of Madura Island, aquades, methanol p.a. (Merck, Germany), Folin-Ciocalteu (Merck, Germany), Na_2CO_3 (Merck, Germany), gallic acid (42649, Merck), quercetin (Sigma Aldrich, USA), L(+)-ascorbic acid (Merck, Germany), $AlCl_3$ (Merck, Germany), CH_3COOK (Merck, Germany), $CH_3COONa.3H_2O$ (Merck, Germany), glacial acetic acid (Sigma Aldrich, USA), HCl p.a. (Merck, Germany), TPTZ (Sigma Aldrich, USA), $FeCl_3.6H_2O$ (Merck, Germany), $FeSO_4.7H_2O$ (Merck, Germany), and DPPH (Himedia, India).

2.2. Extract Preparation

As much as 200 g of *T. gigas* egg simplicia which had been dried and mashed, was then macerated with 400 mL methanol p.a. Maceration was carried out in a closed container for 24 hours with three repetitions. The maceration results were then filtered, the sample filtrate was obtained, then concentrated using a vacuum rotary evaporator, and a thick methanol extract of *T. gigas* eggs was obtained. Then calculated the amount of extract yield using the calculation as Equation (1) (21):

$$\text{Extract yield (\%)} = \frac{Wa}{Wb} \times 100 \quad (1)$$

with Wa being the mass of the extract obtained (g), and Wb being the mass of the simplicia powder (g).

2.3. Total Phenolic Content Test

Total phenolic levels were defined with the Folin Ciocalteu (FC) method (22). As much as 100 mg extract was dissolved in 10 mL of methanol p.a., and a concentration of 10,000 µg/mL was obtained. Next, 0.4 mL of the extract was added with 2 mL of 10% Folin-Ciocalteu and 1.6 mL of 7.5% Na_2CO_3 and vortexed for 1 minute, then the sample was incubated at room temperature for half an hour. The absorbance of the sample was read using UV-Vis spectrophotometry against a blank (sample extract was replaced with only methanol p.a.) at the maximum wavelength, namely 754.5 nm. The calibration curve uses gallic acid with a concentration of (2.5-40) µg/mL. The absorbance of the methanol extract sample obtained was interpolated in the standard curve linear regression equation. The total phenolic content will be represented in mg of gallic acid equivalent per g of extract (mg GAE/g extract).

2.4. Total Flavonoid Content Test

The total flavonoid content of the methanol extract of *T. gigas* eggs was measured utilizing the aluminum chloride colorimetric method with quercetin as a standard (23). 100 mg of extract dissolved in methanol p.a. 10 mL to obtain a concentration of 10,000 µg/mL. Quercetin was used as a standard calibration curve with a concentration range of 5-80 µg/mL. 0.5 mL (sample, standard, and methanol as blank) was added with 1.5 mL methanol p.a., 0.1 mL $AlCl_3$ 10%, 0.1 mL CH_3COOK 1 M, and 2.8 mL distilled water. Then, the mixture was vortexed for 1 minute and incubated at room temperature for 30 minutes. The absorbance was read by UV-Vis spectrophotometry on a blank at a maximum wavelength of 429.5 nm. Total flavonoid content is represented in mg quercetin equivalent per g of extract (mg QE/g extract).

2.5. Antioxidant Activity Test

The antioxidant activity test of the methanol extract of *T. gigas* eggs was carried out using FRAP and DPPH.

2.5.1. FRAP Method Antioxidant Activity Test

An antioxidant activity test by the FRAP method on a sample is performed as a procedure (24,25). FRAP method is used to calculate the total antioxidant capacity (26). The FRAP reagent was prepared to consist of 300 mmol acetate buffer solution (pH 3.6), 10 mmol/L TPTZ solution in 40 mmol/L HCl, and 20 mmol/L $FeCl_3.6H_2O$ with a ratio of 10:1:1 respectively. The FRAP reagent was heated in a water bath at 37°C for 10 minutes. Samples of egg methanol extract of *T. gigas* were prepared by dissolving 100 mg of the extract in 10 mL of methanol p.a. The standard for the calibration curve uses $FeSO_4.7H_2O$ (200-700) µmol/L. A total of 100 µL (sample, standard, and methanol as blank) was mixed with 300 µL of distilled water and 3 mL of FRAP reagent. Then, the

mixture was vortexed for 1 minute and incubated in the dark at 37 °C for 4 minutes. The absorbance is read at the maximum wavelength of 595 nm. The antioxidant activity of a sample using the FRAP method will be expressed as µmol Fe^{2+}/g DW.

2.5.2. DPPH Method Antioxidant Activity Test

Antioxidant activity test of the methanol extract of *T. gigas* eggs was carried out using the DPPH method based on references from (27,28). The DPPH method measures free radical inhibition or inhibition (29). DPPH free radicals were prepared at a concentration of 0.1 mM in methanol. Samples of egg methanol extract of *T. gigas* were carried out by designing solutions with various concentrations of 400-2000 ppm. Vitamin C, gallic acid, and quercetin were used as comparisons or positive controls. Vitamin C is dissolved at a concentration of (1 - 3) µg/mL, quercetin (0.5 – 1.7) µg/mL, and gallic acid (0.2 - 1.4) µg/mL. A total of 4.5 mL (sample, standard, and methanol as a blank) was mixed with 0.5 mL of 0.1 mM DPPH and vortexed for 1 minute, then incubated in the dark room at 37 °C for half an hour. The absorbance is read at the maximum wavelength of 515 nm. The percentage of inhibitory activity is calculated as Equation (2):

$$\frac{(|blank|-|sample|)}{|blank|}\times 100 \qquad (2)$$

Information:
Abs blank: Absorbance of DPPH in the absence of sample
Abs sample: Absorbance of DPPH + sample

The IC_{50} value (50% inhibitory concentration) is a concentration of the test sample to inhibit free radicals (DPPH) up to 50%. The IC_{50} value is calculated based on the % inhibition obtained from each test sample concentration. Furthermore, it is substituted in the regression equation where concentration is on the x-axis and % inhibition is on the y-axis. In the regression equation y = ax + b, if the y value is substituted by number 50, the x value will be obtained as the IC_{50} value.

3. RESULTS AND DISCUSSION

3.1. Preparation of *T. gigas* egg methanol extract

Extraction is separating materials or withdrawing a dissolved component by an appropriate solvent (30). Extraction by maceration method is carried out because this method is a simple, straightforward method and does not require heating, so there is less possibility of damage to natural materials. The maceration method, which requires a long time, allows many compounds to be extracted perfectly (31). The maceration method is a safe extraction method and is often used to determine polyphenolic compounds (32).

The solvent used in the above extraction is methanol, a polar solvent. Flavonoids are polyphenolic compounds with many hydroxyl groups, which make them polar. Thus, methanol can be very suitable as a good solvent in extracting flavonoid compounds (33). After being macerated using methanol solvent, the macerate is filtered using a buchner funnel and concentrated with a vacuum rotary evaporator to produce a thick extract. The yield percentage of the resulting viscous extract is 11.74%. The yield value is the weight of the secondary metabolite compounds obtained from the sample (34).

3.2. Total Phenolic Content

The ability of a phenolic compound to form phenoxy radicals which are stable in the oxidation process makes this compound widely used as an antioxidant agent (35). The total phenolic content test is done to determine the number of phenolic compounds contained in the egg extract of *T. gigas*. Measurement of total phenolic levels using the Folin-Ciocalteu (FC) method. The FC method is based on reducing FC reagents by phenolic compounds in an alkaline state (36). The hydroxyl group of the phenolic compound will react with the FC reagent containing phosphomolybdate-phosphotungstate, which will then form a blue tungsten-molybdenum complex (37). Na_2CO_3 7.5% is added to make the atmosphere alkaline so that the protons in phenolic compounds dissociate into phenolic ions (38). The formation of phenolic ions serves to reduce FC reagents so that the molybdenum ion center will receive one electron from a phenolic antioxidant which causes a reduction of the ion Mo^{+6} to Mo^{+5} followed by a color change from yellow to blue (39−40), the reaction as shown in Figure 1.

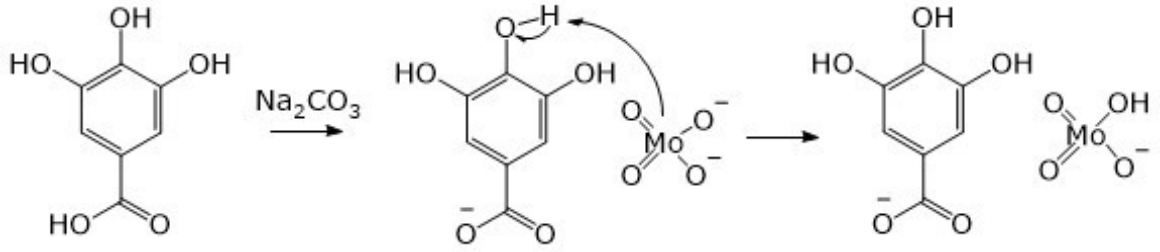

Figure 1. Reaction mechanism between FC reagent and gallic acid.

Measurement of total phenolic levels uses gallic acid as a standard because gallic acid is a pure and stable compound (41). Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic compound with vigorous antioxidant activity. The total phenolic content contained in an extract is expressed as GAE, which

is the amount equivalent to mg of gallic acid in 1 g of the sample (42). The results of the standard calibration curve for gallic acid with a concentration of (2.5-40) µg/mL obtained a linear regression equation, y = 0.0173x + 0.0184, with R^2 = 0.9982. Based on the calculation results, the total phenolic content contained in the methanol extract of *T. gigas* eggs is 0.53506 ± 0.001335 mg GAE/g extract.

3.3. Total Flavonoid Content

Flavonoids have biological activity as antioxidants, where their potency is highly dependent on the number and position of the free-OH group (43). Hydroxyl radicals from flavonoid compounds can inhibit the action of free radicals and mediate the effects of antioxidant activity related to health benefits (44). The total flavonoid content test was carried out to determine the number of flavonoids contained in *T. gigas* egg extract. Measurement of the total flavonoid content is done using the colorimetric method with the addition of an $AlCl_3$ reagent.

Aluminum chloride can react with flavonoid group compounds to produce a stable acid complex with C_4 as a ketone group and C_3 or C_5 as hydroxyl groups of flavones or flavonol compounds, forming yellow compounds (37). The reaction that occurs between flavonoids and $AlCl_3$ is described as follows in Figure 2 (45).

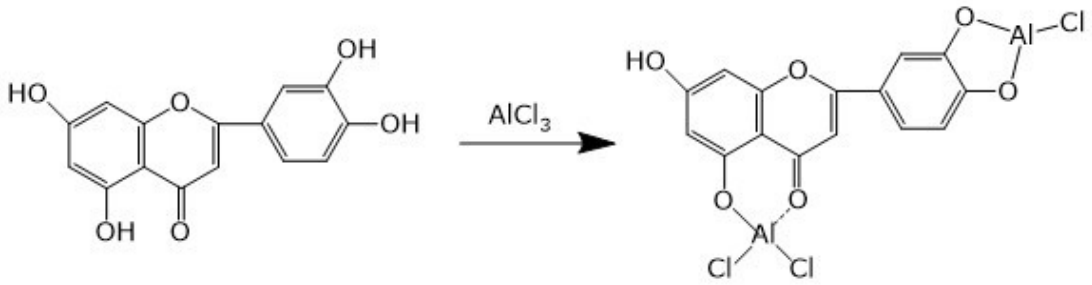

Figure 2. Reaction between flavonoids-$AlCl_3$.

The standard used in measuring total flavonoid levels is quercetin because quercetin is one of the flavonoid compounds of the flavonol group, which has a ketone group at C_4 and a hydroxyl group at C_3 and C_5 (37). Quercetin is the most effective flavonoid compound for capturing free radicals such as superoxide, hydroxyl, and peroxyl radicals. Quercetin can also inhibit various oxidation reactions due to phenolic radicals, which are stabilized by the resonance effect of aromatic rings (46).

The total level of flavonoids in the sample extract is expressed as quercetin equivalents (QE), namely the equivalent amount of mg quercetin in 1 g of the sample using the linear equation of the standard calibration curve. Quercetin standard calibration curve with a concentration of (5-80) µg/mL has obtained a linear equation, y = 0.0079x - 0.0075, with R^2 = 0.9998. Based on sample absorbance calculations, the total level of flavonoids contained in the methanol extract of *T. gigas* eggs was 0.52067 ± 0.000731 mg QE/g extract.

3.4. Antioxidant Activity with FRAP Method

Antioxidant activity using the FRAP method aims to define the total antioxidant capacity contained in the sample. FRAP method can measure the total antioxidant content of a sample based on the principle of the ability of an antioxidant compound to reduce Fe^{3+}-TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) to Fe^{2+}-TPTZ which is blue in acid condition (47). The Fe^{3+}-TPTZ complex compound is an oxidizing agent that may exist in the body and can damage the body's cells (48) Samples that have the ability as antioxidants are thought to reduce Fe^{3+}-TPTZ so that the Fe^{3+}-TPTZ compound cannot react anymore, which causes damage to body cells (49). This Fe^{3+}-TPTZ complex compound is an iron salt from a mixture of TPTZ with $FeCl_3$ in an acidic medium, known as the FRAP reagent (39). The replenishment of $FeCl_3$ aims to form complex compounds Fe^{3+}. The low pH condition 3.6 aims to simplify the reduction process (50).

Qualitatively, the total amount of antioxidants can be seen from the intensity of the blue color Fe^{2+}-TPTZ complex compound formed. The darker the color, the greater of antioxidant capacity of the material being tested (51). The reaction that occurs between antioxidant compounds and the Fe^{3+}-TPTZ complex is as follows in Figure 3 (39).

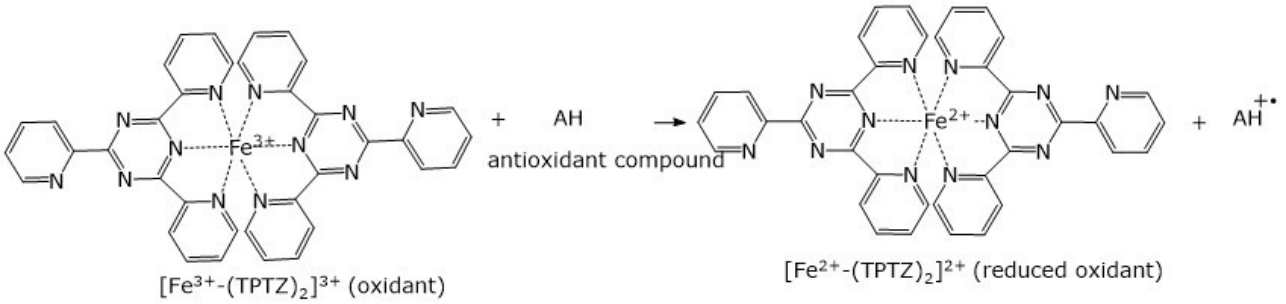

Figure 3. The reaction between FRAP reagents and antioxidant compounds.

In this study, the data measured to determine the total antioxidant capacity was in the form of absorbance measurements. The capacity of the total antioxidant content in the sample extract will be expressed in µmol Fe^{2+}/g DW. Absorbance measurements are done at the maximum wavelength derived from a standard solution of 1000 µmol/L $FeSO_4.7H_2O$ added with FRAP reagent. The absorbance of the sample obtained was then substituted in the standard $FeSO_4.7H_2O$ calibration curve equation at a concentration range of 200-700 µmol/L. A linear equation obtains the standard calibration curve, $y = 0.0015x - 0.0953$, with $R^2 = 0.9976$, where y is the absorbance and x is the concentration.

The positive control comparators used were vitamin C, gallic acid, and quercetin. Vitamin C acts as a secondary antioxidant that can catch free radicals and prevent chain reactions from occurring. Vitamin C has free hydroxyl groups capable of being free radical scavengers (52). As a non-enzymatic antioxidant, vitamin C breaks free radicals chain reaction by trapping peroxyl and other reactive radicals (53). Based on the results of sample absorbance calculations, the amount of total antioxidant capacity contained in the positive control compound and the methanol extract of *T. gigas* eggs) is in Table 1.

Table 1: FRAP value results.

Sample	FRAP Value (µmol Fe^{2+}/g DW)
Methanol extract of *T. gigas* eggs	29.85
Gallic Acid	20899.47
Quercetin	11559.11
Vitamin C	8098.35

There is a difference in the change in the intensity of the color formed in each sample, indicating a difference in the composition of the antioxidants. The FRAP value of a sample is categorized based on its antioxidant activity as follows: very high (>500 µmol Fe(II)/g), high (100-500 µmol Fe(II)/g), moderate (10-100 µmol Fe(II)/ g), and low (<10 µmol Fe(II)/g) (54). The antioxidant activity of the methanol extract of *T. gigas* eggs has been reported with a FRAP value was 29.85 µmol Fe^{2+}/g DW, so this extract has moderate antioxidant activity. Meanwhile, the positive control comparators of gallic acid, quercetin, and vitamin C had very high antioxidant activity.

3.5. Antioxidant Activity with DPPH Method

The DPPH method is a method of testing Antioxidant activity utilizing 2,2-diphenyl-1-picrylhydrazyl as a free radical source (55). Measurement of antioxidant activity was carried out when purple DPPH free radicals were mixed with reducing compounds or antioxidants, the absorbance decreased and the formation of reduced DPPH-H color turned pale yellow (39). There is a color change in DPPH (purple) because antioxidant compounds can donate their hydrogen to these free radicals (56). DPPH compounds by antioxidant compounds are converted into DPPH-H (57). The reaction between DPPH and antioxidant compounds can be shown in Figure 4 below.

The antioxidant activity measured by the DPPH method is expressed as an IC_{50} value. IC_{50} is the concentration of antioxidant compound required to reduce DPPH free radicals by up to 50% (58). Lower IC_{50} values indicate a higher ability of antioxidant activity (59). By plotting the concentration (µg/mL) of the test solution on the horizontal axis and the rate of percent reduction value on the vertical axis, the IC_{50} value was determined based on the linear regression equation.

The percentage of inhibition activity (IC_{50}) in the methanol extract of *T. gigas* eggs and the positive controls for gallic acid, quercetin, and vitamin C based on absorbance readings at a maximum wavelength of 515 nm are shown in Table 2.

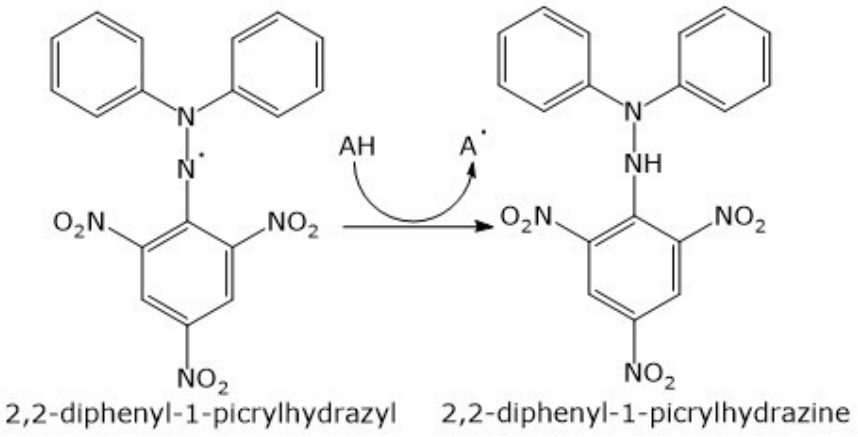

Figure 4: The reaction between DPPH and antioxidant compounds.

Table 2: IC_{50} value of the samples test.

Sample	R^2	IC_{50} (µg/mL)
Methanol extract of *T. gigas* eggs	0.9933	597.0397
Gallic acid	0.9939	0.8329
Quercetin	0.9934	1.5169
Vitamin C	0.9815	2.2095

The strength of the antioxidant activity utilizing the DPPH method can be categorized as follows: very strong (<50 ppm), strong (51-100 ppm), moderate (101-150 ppm), weak (151-200 ppm), and very weak (> 200 ppm) (60). Based on this category, the antioxidant activity of *T. gigas* eggs methanol extract of 597.0397 µg/mL was in the very weak category. In contrast, for the positive control comparison, gallic acid, quercetin, and vitamin C had very strong antioxidant activity.

All results of the determination of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity using the FRAP and DPPH methods in the methanol extract of *T.gigas* eggs are shown in the following Table 3.

Table 3: The total phenolic, flavonoid content, and antioxidant activity of *T.gigas* eggs methanol extract.

Sample	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	FRAP Value (µmol Fe^{2+}/g DW)	IC_{50} DPPH (µg/mL)
Methanol extract of *T. gigas* eggs	0.53506 ± 0.001335	0.52067 ± 0.000731	29.85	597.0397

4. CONCLUSION

The methanol extract of *T. gigas* eggs contained total phenolics and total flavonoids content of 0.53506 ± 0.0013 mg GAE/g extract and 0.52067 ± 0.000731 mg QE/g extract, respectively. Antioxidant activity using the FRAP method obtained a total antioxidant capacity of 29.85 µmol Fe^{2+}/g DW which is included in the moderate category, while antioxidant activity using the DPPH method obtained an IC_{50} value of 597.0397 ppm which indicates its activity as a very weak antioxidant. The results of research conducted by Suwandi et al. (2019) regarding the antioxidant activity of the ethanol extract of *T.gigas* in the very weak category with an IC_{50} of 330.47 ppm. It can be concluded that *T.gigas* eggs have very weak antioxidant activity.

5. CONFLICT OF INTEREST

The authors declare there is nothing conflict of interest

6. ACKNOWLEDGMENTS

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