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Original Research Article

Determination of Reaction Time on Antioxidant Assays of Duck, Hen, and Quail Egg Whites

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ABSTRACT

Natural antioxidants such as egg white are complex compounds that prevent cell damage from oxidative stress. Typically, the antioxidant confirmatory tests are carried out using a predetermined reaction time without kinetics. Therefore, this study was aimed at comparing the antioxidant activities of hen egg white (HEW), duck egg white (DEW), and quail egg white (QEW) over time using three different antioxidant activity determinative assays. Purebred hen, duck, and quail eggs were obtained for the study. The protein content of the egg white was measured. The antioxidant activity of the egg white was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power, and metal (Fe^{2+}) chelating assays. The results showed that the maximum level of antioxidant activity was attained at various reaction times. With a concentration of 4.3% after 90 minutes, HEW had the highest antioxidant activity, followed by DEW with 1.8% after 100 minutes and QEW with 1.3% after 40 minutes. Based on the reducing power assay, only DEW showed the ability to reduce Fe^{3+} with an increase in absorbance from 0.098 to 0.207 and 0.128 to 0.223 at concentrations of 0.0625 and 0.125 mg/mL, respectively. Given the variations in their antioxidant activities, the chelation ability of HEW (60%) was higher than that of DEW (35%) and QEW (36%). The findings of this study showed that antioxidant evaluation of HEW, DEW, and DEW exhibit kinetic reactions.

Keywords: Antioxidant, DPPH, Egg white, Metal chelating, Reducing power

Introduction

Antioxidants protect the body from oxidative stress and neutralize or minimize the impact of free radicals in the body.[1] A molecule with an unpaired electron in an atomic orbital that results in instability and high reactivity is referred to as a free radical. These radicals initiate chain reactions by attacking other molecules to destroy cells and cause chronic disease. The primary role of antioxidants is to scavenge free radicals, breaking the chain of free radicals and preventing their production. Antioxidants may also function as reducing agents and transition metal chelators.[2-4] They scavenge free radicals, function as reducing agents, and reduce the oxidation caused by transition metals like Fe. Plants,[5] fruits,[6] and animal product derivatives like fish by-products,[7] and eggs,[8] can all be sources of natural antioxidants. They contain various components of complex antioxidant compounds that can protect cell damage from oxidative stress and reduce the risk of chronic disease. Furthermore, the natural antioxidant also shows no long-term health risk to the consumer.[3,9,10]

Studies on natural antioxidants have focused primarily on plant sources. However, animal derivatives such as eggs also contain antioxidant substances. The proteins, such as ovalbumin, ovotransferrin, lysozyme, and cystatin contained in egg white exhibit antioxidant properties. The presence of the SH (thiol) groups in ovalbumin plays a role in redox regulation and shows the ability to chelate transition metals.[11] Hen, quail, and duck eggs are the major animal protein sources and become the most consumed poultry eggs.[12]

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The phylogenetic analysis based on egg white proteome of quail and chicken had a much closer relationship than duck.[13] Consequently, the difference in their protein patterns may have contributed to their significance in biology. Similar protein amounts may be found in egg whites from hens, quail, and ducks, with respective percentages of 71.92, 71.31, and 72.36. The hydrophobic amino acids (Pro, Gly, Ala, Val, Ile, Leu, Phe) contained in those egg whites are 34.54, 34.94, and 34.95%, respectively.[12] The antioxidant content in egg whites from various types of local poultry has various antioxidant capacities.[10] Therefore, understanding animal-derived antioxidants, like egg white, can explain how food industries or human health can be assisted.[14] The study related to antioxidant activity assay is conducted at a single time. In contrast, the previous assay was determined at the end of the reaction.[15] For these reasons, some preliminary measurements should be performed to determine the ideal conditions for reaction time and DPPH concentration.

Therefore, this study was conducted to compare the antioxidant activity of hen egg white (HEW), duck egg white (DEW), and quail egg white (QEW) using the 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power, and metal (Fe^{2+}) chelating assays in relation to the reaction time.

Materials and Methods

Sources of egg

Three eggs, each from purebred hen, duck, and quail were obtained from farms at the University of Muhammadiyah Malang, the Wendit duck farm, and the Karangploso poultry farm located in Malang Regency, East Java, Indonesia. All selected eggs, which were under two weeks old, were further processed following the earlier study.[16]

Determination of egg white protein content

The protein content of the egg white was measured using the UV-spectrophotometric method. The protein content of the egg white was determined at wavelengths of 260 nm and 280 nm.[17] Meanwhile, the protein concentration was calculated using the following equation:

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Protein concentration (mg/mL) = 1.55 (A_{280}) – 0.76(A_{260})

Radical scavenging activity by DPPH assay

The DPPH radical scavenging assay with some modifications was used to determine antioxidant activity.[18] Ascorbic acid was used as the control for this assay. Briefly, 4 mL of the DPPH (0.24 mg/mL) was added to 400 µL of the sample solutions. The antioxidant test used a UV-Vis spectrophotometer at a particular wavelength to create a colour, indicative of a transition. The DPPH assay was measured at 517 nm every 10 minutes for 120 minutes to evaluate the optimal reaction time. Finally, the radical scavenging activity was calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{absorbance of control - absorbance of sample}}{\text{absorbance of control}} \text{ x } 100$$

Reducing power assay

A previously reported reducing power assay with some modifications was used.[19] Ascorbic acid was employed to develop the standard curve (control). After 10 and 20 minutes of reaction time, the absorbance was measured at 700 nm, and the absorbance indicated that the reducing power had increased.

Metal chelating assay

The chelation of ferrous ions was determined according to a previous study with some modifications.[19] Ethylenediaminetetraacetic acid (EDTA) was used as a control. The absorbance was measured at 562 nm every 10 minutes for 120 minutes to evaluate the reaction. Furthermore, the percentage of the chelating activity or the inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following equation:

$$\% \text{ Chelating Activity} = \frac{\text{absorbance of control - absorbance of sample}}{\text{absorbance of control}} \text{ X } 100$$

Statistical analysis

All the collected data were analyzed descriptively following the previous study that was carried out.[20]

The reducing power data were analyzed using three-way ANOVA ($p < 0.05$) and the percent chelation was analyzed using two-way ANOVA ($p < 0.05$), followed by Tukey HSD tests. All measurements from the experiments were repeated in triplicate, and the data were presented as mean ± standard deviation. The results were presented in graphical form in Microsoft Excel with the x-axis as the reaction duration and the y-axis as percentage inhibition for the DPPH assay, absorbance for the reducing power assay, and percentage chelating activity for the metal chelating assay.

Results and Discussion

Reaction time of antioxidant activity by DPPH assay

The results of the present study demonstrated that all the three egg white samples had increased percent inhibition with time, which indicated strong scavenging activity up until the reaction period. Figure 1D shows that ascorbic acid exhibited continuous inhibition with a prolonged reaction time. Every sample reached the optimal reaction time at different time, but HEW reached an optimal time in 90 minutes with the highest inhibition at 14.9% as illustrated in Figure 1A. DEW achieved the optimal time of 100 minutes with the highest inhibition of 9.8% (Figure 1B). Meanwhile, QEW, as shown in Figure 1C, reached the optimal duration of 40 minutes with the highest inhibition of 10.7%. Compared to egg-white samples, ascorbic acid attained its peak concentration at 80.6–96.9% in 10 minutes (Figure 1D). The antioxidant capacity of ascorbic acid is obtained from a limited number of electrons, and it happens due to its simple molecular structure. The antioxidant mechanism of ascorbic acid is a type of hydrogen atom (HAT) reaction and singlet oxygen inactivation.[13] The protein in egg whites serves as a comprehensive free radical scavenger. The hydrophobic or aromatic amino acids in egg white protein contribute to the large number of electrons transferred to DPPH. In addition, egg white protein can also accept excess electrons from an atom to prevent the formation of new radicals.[22] The egg white sample at the four concentrations of 0.0625, 0.125, 0.375, and 0.625 mg/mL did not exhibit a change in colour from purple to yellow but rather a reduction in intensity. It is suspected that the sample's poor antioxidant activity contributed to the ability to decrease DPPH radicals. In comparison to other series, HEW, DEW, and QEW displayed the highest percentage inhibition at a dose of 0.125 mg/mL.

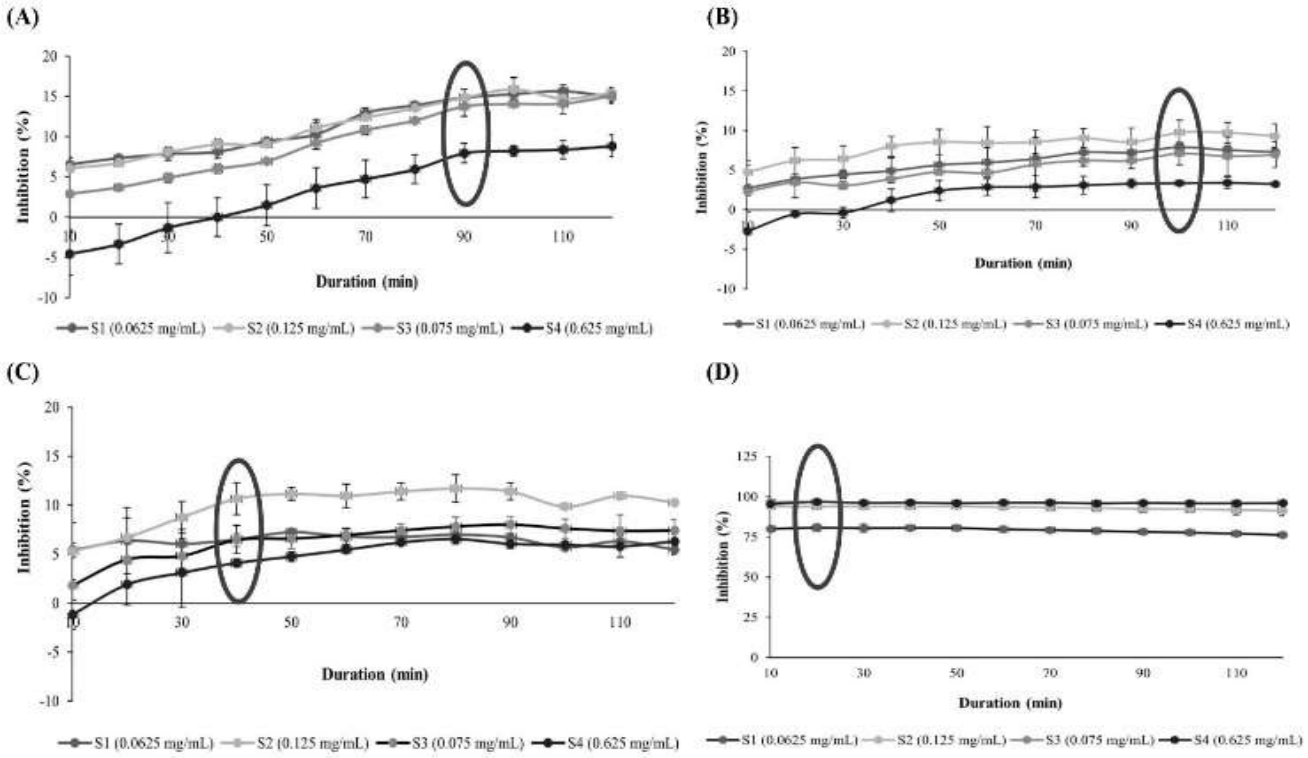

Figure 1: Percentage inhibition of the DPPH radical based on reaction duration. A: HEW at 90 min; B: DEW at 100 min; C: QEW at 40 min; D: Ascorbic acid at 20 min; HEW: Hen egg white; DEW: Duck egg white; QEW: Quail egg white; DPPH: 1,1-diphenyl-2-picrylhydrazyl

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The results of the three egg white samples were different from those of ascorbic acid based on the DPPH assay's kinetics. Meanwhile, the antioxidant activity of HEW was higher than DEW and QEW. The physicochemical properties of egg whites, such as viscosity, influenced the antioxidant properties of the proteins. The stability of the egg white protein may have been disrupted by the DPPH solution, which further reduced their antioxidant activity. The lowest and greatest values of viscosity were found in QEW and HEW, respectively. The total protein content of egg whites varies depending on the type of poultry species. The concentration and make-up of the protein have a positive correlation with the egg white gel's strength.[23] The strength of the egg-white gel positively correlates with the concentration and composition of the protein.[23]

Reaction time of antioxidant activity by reducing power assay

The reducing capability of the antioxidant sample was measured by the transformation of Fe^{3+} to Fe^{2+}. The reduction ability in the sample of egg white and ascorbic acid increased with increasing concentration and reaction time, as shown in Figure 2.

The antioxidant activity of HEW and QEW in reducing Fe^{3+} was insufficient. On the other hand, the reducing capability of DEW was significantly higher than HEW and QEW, but it was still somewhat low when compared to ascorbic acid. At a concentration of 0.0625 mg/mL, it was increased from 0.098 (10 minutes) to 0.207 (20 minutes). Furthermore, at a concentration of 0.125 mg/mL the absorbance increased from 0.128 (10 minutes) to 0.223 (20 minutes). The standard solution, or ascorbic acid, used as a positive control, changed colour and indicated increased absorption. As shown in Figure 2, the absorbance of ascorbic acid increased from 0.507 (10 minutes) to 0.588 (20 minutes) at a concentration of 0.0625 mg/mL, and from 0.664 (10 minutes) to 0.695 (20 minutes) at a concentration of 0.125 mg/mL. The results can be attributed to the complex molecular structure of the globular proteins in egg white that preserve reducing power for a longer period as opposed to the short-term reduction ability of ascorbic acid.

The observation of the egg white reducing power can be explained by DEW having lower ovalbumin, ovotransferrin, and lysozyme concentrations than HEW. Ovalbumin makes up 54 and 40%, respectively, of the total egg white proteins in HEW and DEW (Table 1). Different SH groups are present in different poultry species. Hen albumin contains the OVAs of Cys11, Cys30, Cys367, and Cys382, whereas duck albumen contains Cys11 and Cys331.[24,25] This can be presumed because HEW ovalbumin has more cysteine residues with free SH groups than DEW and QEW. Furthermore, the quantity of free SH groups in ovalbumin presumably affects its antioxidant capabilities. Ascorbic acid has the highest antioxidant activity compared to the three samples tested. The previous study reported that ascorbic acid was weakly affected by the reaction time.[26]

However, only DEW significantly increased absorbance on the reducing power assay (Figure 2D). The reducing power of a compound can be used as an indicator of potential antioxidant activity. For example, its ability to reduce Fe^{3+} to Fe^{2+} is often tested as an indicator of electron donor activity.[27] Increased absorbance of the reaction mixture indicates high reducing power.[28] As a result, DEW may have a superior single electron transfer (SET) mechanism for its antioxidant abilities than the hydrogen atom (HAT) and the chelation of transition metals (Fe^{2+}) mechanisms. In contrast, the antioxidant activity of HEW is superior to the SET mechanism in terms of HAT and chelation of transition metals (Fe^{2+}). The antioxidant activity of QEW was shown to be lower than that of HEW and DEW due to the different mechanisms of interaction with free radicals. The reducing ability of DEW was not as good as ascorbic acid; hence, the use of TCA can affect the protein in egg whites. TCA is known as an effective protein-precipitating agent and a relatively weak acid that maintains an acidic pH in water. As a result, its addition to proteins in an aqueous solution can cause the water molecules that are hydrogen-bonded to a protein to break apart. The proteins lose their secondary structure and become denatured because TCA disrupts hydrogen bonding.[29]

Reaction time of antioxidant activity by metal chelating assay

According to the results, all three egg white samples were able to chelate Fe^{2+} after a 10-minute reaction time. On a time-dependent basis, all of the egg white samples displayed decreasing chelating activity. The highest chelating activities of 60, 35, and 36% were achieved by HEW, DEW, and QEW, respectively (Figure 3A-C). Maximum chelating activity for EDTA as a positive control persisted for 120 minutes after the reaction (Figure 3D). This suggests that within the first 10 minutes of the reaction, egg white binds to Fe. After more than 10 minutes, the link between the egg white and iron (EW-Fe) becomes unstable. Due to the emission of Fe, ferrozine is affected. The stability of the link between egg white and iron may be less supported by the reaction environment (EW-Fe). The percentage of chelating activity in the three egg white samples with EDTA during the first 10 minutes of the operation varied. The bond between the egg white and Fe (EW-Fe) becomes unstable at a time duration of more than 10 minutes.

Table 1: Comparison of hen and duck egg white major proteins

Source	Ovalbumin	Ovotransferrin	Lysozyme
Duck	40 %	2 %	1.2 %
Hen	54 %	12 %	3.4 %

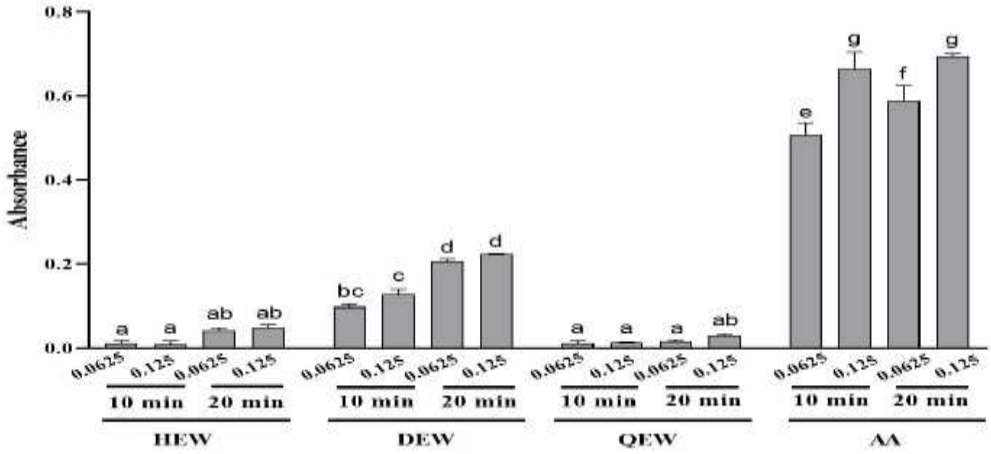

Figure 2: The reducing ability of egg white samples in various concentrations and times based on the reducing power assay. HEW: Hen egg white; DEW: Duck egg white; QEW: Quail egg white; AA: Ascorbic acid; The different letters indicate a significant difference ($p<0.05$) between groups based on Tukey HSD test.

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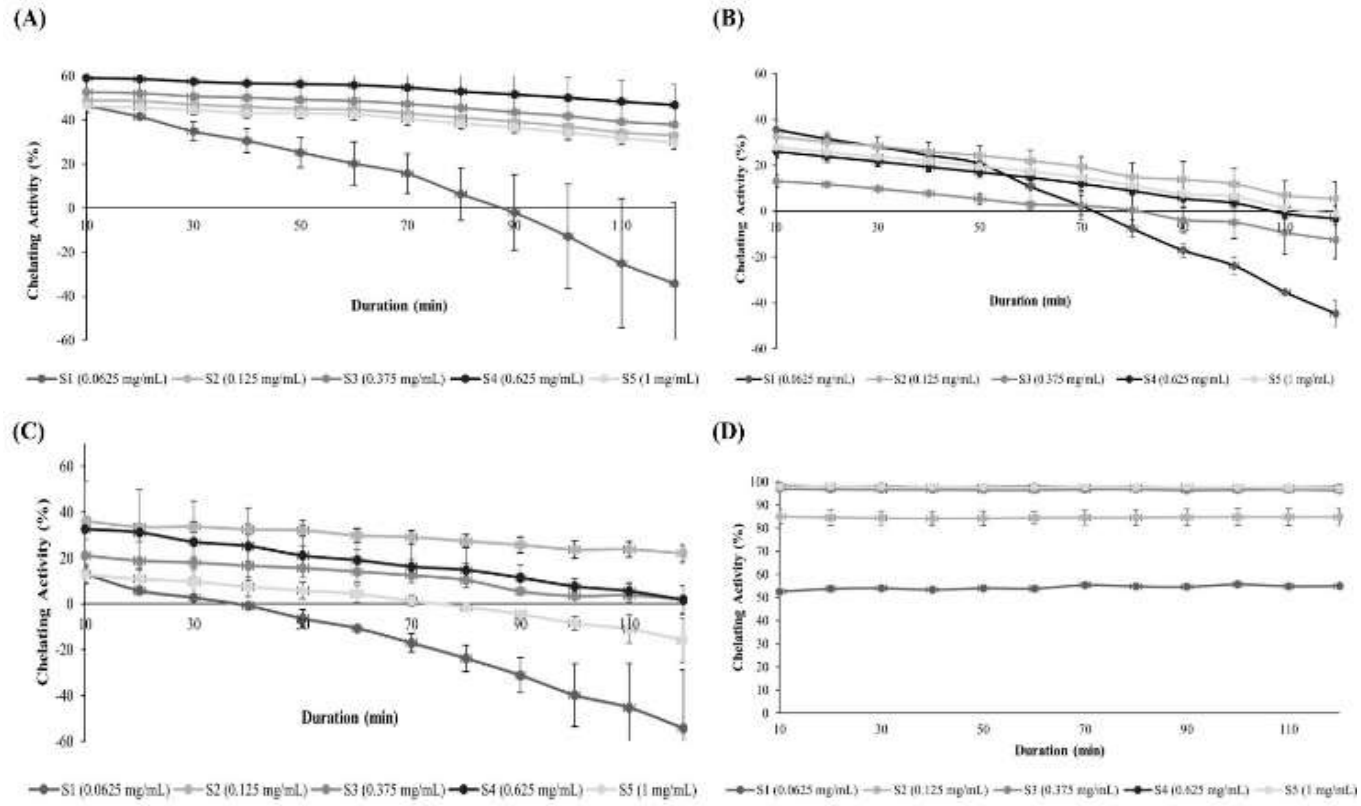

Figure 3: Percentage chelating activities of egg white samples based on metal chelating assay. A (HEW): Hen egg white; B (DEW): Duck egg white; C (QEW): Quail egg white.

Therefore, the Fe is released and interacts with ferrozine. The reaction environment may be less supported in maintaining the stability of the bond between the egg white and Fe (EW-Fe). During the first 10 minutes of the procedure, the three egg white samples with EDTA had different percentage chelating activities.

In comparison to DEW and QEW, HEW demonstrated the maximum activity at each concentration, at 0.625 mg/mL; it was superior to the other series. On the other hand, DEW and QEW were able to achieve the maximum chelating activities at doses of 0.0625 and 0.125 mg/mL, respectively. The concentration and chelating activity of EDTA increased (Figure 4). HEW showed the highest activity at each concentration compared to DEW and QEW, and at 0.625 mg/mL, which was above the other series. The antioxidant properties of a sample can be seen from the ability to chelate transition metal ions, especially Fe^{2+} and Cu^{2+}. The chelating agent can bind to Fe^{2+} to inhibit the formation of the Ferrozine-Fe^{2+} complex. In addition, the presence causes a decrease in absorbance,[30] and the metal chelating assay results are unique to the Fe transition metal. However, these results cannot be generalized since the transition metals require a different measurement method. The results of this study demonstrated that HEW contained higher ovalbumin and lysozyme than QEW,[31], and ovotransferrin concentrations in QEW and HEW were statistically similar (Table 2). Ovotransferrin is a protein derived from the transferrin group, which has two lobes with an iron-binding site in each.[32] The indirect role of preventing iron-induced lipid peroxidation is done by ferrous metal binding. The antioxidant mechanism was measured by its ability to chelate transition metals.[33] Ovalbumin and ovotransferrin found in high concentrations in HEW may be linked to a more significant antioxidant capability than DEW and QEW activities. Ovotransferrin chelates transition metals, indicating the presence of antioxidant capabilities, while ovalbumin with the SH group can act as an antioxidant through the HAT mechanism. In the reducing power assay results, HEW and QEW did not show the ability to reduce, and only DEW significantly increased absorbance, indicating reduction ability.

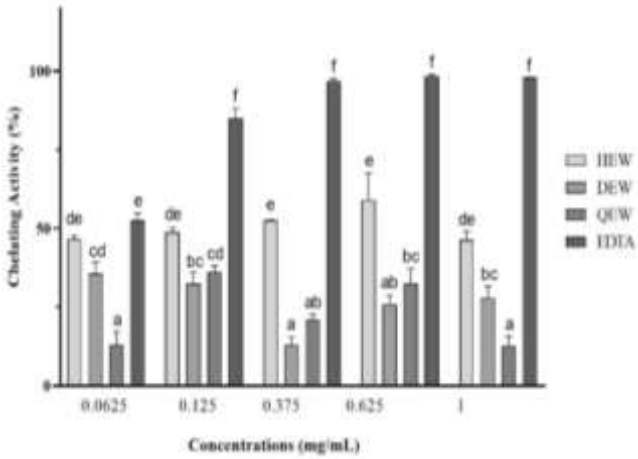

Figure 4: Percentage chelating activity after 10 minutes' reaction time. A (HEW): Hen egg white; B (DEW): Duck egg white; C (QEW): Quail egg white; EDTA: Ethylenediaminetetraacetic acid; The different letters indicate a significant difference ($p < 0.05$) between groups based on Tukey HSD test.

Table 2: Comparison of hen and quail egg white major proteins

Source	Ovalbumin (g/100g EW)	Ovotransferrin (g/100g EW)	Lysozyme (g/100g EW)
Quail	47.63	1.73	3.63
Hen	52.37	1.7	4.23

EW: Egg white

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Conclusion

The findings of this study reveal that in the egg white antioxidant activity assay, the reaction kinetics and sample concentration need to be considered. The optimal reaction times for the DPPH assay on the HEW (4.3%), DEW (1.8%), and QEW (1.3%) are 90, 100, and 40 minutes, respectively. The absorbance value on the reducing power assay of HEW, DEW, and QEW can be compared between 10 and 20 minutes at concentrations of 0.0625 and 0.125 mg/mL. Furthermore, 10 minute reaction time yielded the maximum chelation ability assays of HEW (60%), DEW (35%), and QEW (36%). This indicates that HEW, DEW, and DEW have kinetic reactions regarding antioxidant activity assays.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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