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Comparison and evaluation of the methods for measuring hemolytic activity of *Stomolophus meleagris* jellyfish tentacle extract

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Abstract

Hemolytic activity assessment is a widely used method to evaluate the toxicity of marine organisms, including jellyfish. However, there are some challenges associated with testing hemolytic activity. In this study, four methods were employed to compare the hemolytic activity of jellyfish tentacle extract (TE). Firstly, a microplate reader was used to compare the mouse hemoglobin solution at three different wavelengths (415 nm, 541 nm, and 576 nm), and the most sensitive wavelength was selected for further experiments. Secondly, photomicrograph counting was used to determine the number of complete red blood cells in the field of view. Thirdly, a microplate reader was used to test hemolytic activity in a 96-well plate at 415 nm. Fourthly, a Bicinchoninic Acid (BCA) kit was used to test the concentration of hemoglobin in the solution. Finally, a UV-Vis Spectrophotometer was used to test hemolytic activity at 415 nm. Among the three wavelengths tested, the absorption value was most sensitive at 415 nm. The photomicrograph counting method was able to reflect changes in the shape of Red Blood Cells (RBCs). The microplate reader method may exhibit deviations when the solution concentration is high, while external factors could influence the BCA kit when the toxin concentration in the experimental group is low. The spectrophotometer method was found to be relatively accurate and sensitive to changes. When optimizing the method, it is important to consider the applicability of the Beer-Lambert law and the concentration of solutions.

Introduction

The cannonball jellyfish (*Stomolophus meleagris*) is a venomous marine organism that belongs to the class Scyphozoa and is widely distributed in the Atlantic

and Pacific. The jellyfish are common in the coastal waters of Zhejiang, Jiangsu, and Liaoning provinces in China, so jellyfish stings are often reported.[1-3] The nematocysts hidden on its body surface contain a highly toxic venom which can cause skin redness, swelling, intense pain, tissue necrosis, inflammation, and hemolysis. In severe cases, it can even cause shock and death.[1,4] With global warming and increasing seawater eutrophication, the frequency of offshore jellyfish outbreaks has increased, hurting local tourism and capture fisheries.[5,6]

A survey showed that people do not have sufficient knowledge about jellyfish stings.[7,8] When a human unintentionally touches the body surface of a jellyfish, the barbed wire in the nematocysts is ejected due to external stimuli and penetrates the human skin, injecting the venom into the human body.[9] The venom diffuses into the subcutaneous capillaries and will be transported to various tissues and organs in the body through the blood. Therefore, studying the hemolytic activity of toxins is an important part of the toxicity evaluation of toxins.

At present, the evaluation methods commonly used in laboratories to measure the hemolysis effect of toxins include the microplate reader method, spectrophotometer method, Bicinchoninic Acid (BCA) kit method, and photomicrograph counting method. The first three methods are all based on the Beer-Lambert law and use specific absorption wavelengths to measure the properties of hemoglobin. The photomicrograph counting method is based on dropping several experimental samples on the cell counting plate and measuring them using photo imaging and manual counting of intact Red Blood Cells (RBCs) in the field of view. The above methods have their advantages, disadvantages, and scope of application.[10,11] This study used the above four methods to determine the hemolysis induced by the jellyfish Tentacle Extract (TE). By comparing the effect of four kinds of methods, the optimal method for the determination of TE hemolysis activity in different concentration conditions was obtained, providing references and improvement directions for the laboratory to evaluate toxin-induced hemolysis.

Materials and Methods

Preparation of jellyfish toxin samples

The study utilized jellyfish TE as its samples, which were sourced from the sea of Shengsi Island, Zhejiang Province in September 2020. After species identification, the sample was confirmed to be *Stomolophus meleagris* jellyfish. To obtain the TE, we

removed the tentacles from the sampled jellyfish and placed them in a dialysis bag, which was then dialyzed in a 1×PBS solution at low temperature. The resulting jellyfish TE had an osmotic pressure equal to 1×PBS, and was aliquoted and stored in an ultra-low temperature refrigerator at -80℃.

Preparation of mouse red blood cell suspension

The experiment utilized Institute of Cancer Research (ICR) male mice, aged 6 to 8 weeks, which were procured from the Teaching Support Office of Naval Medical University, Shanghai. To collect the blood sample, a blood needle was inserted into the mouse's retro-orbital plexus, and at least 500 μL of blood was collected using a 1.5 mL Eppendorf tube soaked in heparin sodium. The collected blood was then rinsed slowly with 700 μL of 1× PBS solution and centrifuged at 2000×g at room temperature for 5 minutes. After centrifugation, the blood was repeatedly washed with 1× PBS solution until the supernatant was clear and transparent. Subsequently, 100 μL of the RBC pellet at the bottom was aspirated and mixed with 22.2 mL of 1× PBS solution at a ratio of 1:222 to form a mouse RBC suspension (0.45% RBC). The suspension was gently blended and mixed before use. All experiments were conducted according to the guidelines of the Committee on Ethics of Medicine of Naval Medical University.

Suitable wavelength screening

The RBCs of the mouse were suspended and subjected to pressure filtration through a 0.45 μm filter membrane to obtain the mouse hemoglobin solution. According to the proportion of TE and PBS, the solution was divided into 0%, 1%, 3%, 10%, 15%, 20%, 30%, 60%, and 100%, in which 0% group was without TE.

The experimental group used RBCs as the hemolysis object, and the control group used PBS as the hemolysis object. All groups were determined by a microplate reader. The hemoglobin absorption peaks at 415 nm, 541 nm, and 576 nm were measured for each sample.

Detection of hemolytic activity

Experiment grouping and sample addition

The experiment consisted of 10 experimental groups and 10 control groups (n=4).

Saponin was used as a positive control because of its hemolysin, which could destroy RBCs. The groups were designated as either experimental (E) or control (C), and the sample was added according to Table 1.

Sample processing

After adding the sample, each group was placed in a water bath at 37 ℃ for 30 minutes. Then the mixture was shaken thoroughly to ensure even distribution.

Microphotograph counting method

This method utilized a microscope camera to capture images of the RBC suspension after adding TE. The hemolytic activity of TE was evaluated by counting the number of intact RBCs within the field of view. At the concentration of 0%, the field of view was filled with normal RBCs. To begin the cell counting process, 20 μL of the experimental group's cell suspension was carefully placed into the COUNTSTAR cell counting plate. A high-resolution image of the cells was captured, and the cells were meticulously counted using a microscope set at 400× magnification.

Microplate reader

The experimental and control groups were subjected to centrifugation at 26℃ and 2000×g for 5 minutes. Following centrifugation, 100 μL supernatant was added to the 96-well plate. The absorbance was measured at 415 nm and the data was recorded.

BCA kit

The BCA kit is a protein quantification kit that utilizes the coloration method of sodium quinolinate chelating cuprous ions.[7,12] Given that both hemoglobin and the main active components in TE are proteins, this method can be used to detect hemoglobin in samples. The BCA kit contained Buffer A solution and Buffer B solution. Buffer A solution and Buffer B solution were composed of a 50:1 working solution. The protein standard was diluted with PBS to 0.5 mg/mL. Protein standard was separately taken 0, 1, 2, 4, 8, 12, 16, and 20 μL to the 96-well plate. When the volume of the standard product was less than 20 μL, the diluent of the standard product was added to 20 μL. To perform the assay, the sample was centrifuged, and 4 μL supernatant was diluted in 16 μL 1× PBS solution in a 96-well plate. Next, 200 μL

BCA working solution was added, and the plate was incubated at 37 °C for 30 minutes. The absorbance was measured at 562 nm wavelength after subtracting the absorbance of the TE group from that of the experimental group. The resulting data could be used to measure the hemolysis of blood cells caused by toxins.

Spectrophotometer

Spectrophotometry is a widely used method for determining protein concentration.[13] Due to the small sample volume, a 10-fold dilution of the sample in 1× PBS solution is necessary before measurement. The samples were placed into the quartz cuvette with a 1 cm optical path after combing 300 μL supernatant with 2700 μL 1× PBS solution from the aforementioned groups. The absorbance was measured at 415 nm and the resulting data was recorded.

Result data processing

The statistical analysis and mapping of data were conducted using Microsoft Office and Origin 2018. All data was presented as mean ± standard deviation (SD). The Least Significant Difference (LSD) method was employed to demonstrate significant differences between groups ($p<0.05$).

Results

Measurement of the concentration of hemoglobin using different wavelengths

The proposed method relies on data obtained from three key absorption wavelengths of mouse hemoglobin. Of these, the 415 nm peak exhibited the highest absorbance value and was the most sensitive to changes in gradient concentration. The OD value of 415 nm was about ten times higher than the other two wavelengths, making it particularly responsive to variations in hemolytic activity (Figure 1A). Conversely, the percentage of absorbance at 541 nm and 576 nm were nearly identical (Figure 1B), so we utilized the 415 nm wavelength in subsequent experiments.

Photomicrograph, count, and record the number and morphological changes of RBCs

In the experimental groups with low concentrations of TE, the number of cells significantly increased within the field of view in the 1% and 3% groups compared

with the 0% group. As the concentration of toxin increased, the RBCs lost water and shrank, causing the cell membrane to become fragile and producing blood shadows within the field of view (Figure 2A). Ultimately, in the 60% and 100% groups, almost all blood shadows were visible within the field of view. The number of RBCs increased first and then decreased (Figure 2B). The microphotograph counting method provided a general trend of the hemolytic activity of TE and allowed for the observation of changes in RBCs at different concentrations of TE on a small scale.

Measurement of the optical density (OD) using microplate reader

After centrifugation, the sample's supernatant was added to a 96-well plate and measured using a microplate reader.[14] Beer-Lambert law is the theoretical basis of colorimetric analysis and spectrophotometry. Beer-Lambert law is finite and established under the condition that the substance to be measured is a homogeneous dilute solution, gas, etc., without scattering caused by solute, solvent, or suspended matter. The incident light is monochromatic parallel light. The TE hemolysis curve was calculated by subtracting the background value and the absorbance of the control group's supernatant after centrifugation. When the TE concentration was below 30%, complete hemolysis was not achieved. However, when the TE concentration reached 30%, the experimental group underwent complete hemolysis, as indicated by the slower change in absorbance. Nevertheless, when the TE concentration reached 60%, the hemolysis rate was higher than the 100% concentration group, indicating a deviation in hemolytic activity (Figure 3). When the TE concentration was less than 20%, the hemolysis and toxin concentration showed a linear change. However, when the TE concentration exceeded 20%, it approached complete hemolysis and its color effects began to show up. In the experimental group with a TE concentration over 30%, subtracting the color of TE resulted in a high hemolysis rate, making the Beer-Lambert law inapplicable to the experimental group with a higher TE concentration. At this point, the hemolysis rate did not change regularly with the concentration as the independent variable. The absorption of the microplate reader was only applicable in the low concentration range.

BCA reagent quantitative determination of protein concentration

The experimental results indicated that when TE concentration exceeded 30%, both the absorbance and hemolytic activity exhibited a downward trend. When TE concentration was low, there was an obvious deviation between the measured value and the theory. When TE concentration was lower than 30%, the correlation between RBC hemolysis and TE concentration was stronger (Fig.4.B). However, despite these limitations, the overall trend of the BCA method was consistent with the theoretical hemolysis situation (Figure 4A).

Spectrophotometric method for measuring optical density (OD)

TE concentration measured with a visible-ultraviolet spectrophotometer was compared with the theoretical concentration, and the results were generally consistent (Figure 5A). The data showed that the hemolysis rate increased with the increase of TE concentration. After subtracting the background absorbance with 1× PBS solution, the results showed a generally increasing trend in hemolytic activity (Figure 5B). However, the microplate reader detected a deviation in the absorbance of 60% concentration samples, while the spectrophotometer showed good sensitivity after dilution. Therefore, it can be concluded that the spectrophotometer was a more sensitive and accurate method than the microplate reader. Calvaresi *et al.* also demonstrated the accuracy of the spectrophotometer.[15] As the solution was diluted, the concentration of the measured sample did not exceed the applicable range of Beer-Lambert law, making the spectrophotometer a more accurate method.

Discussion

There are four methods to evaluate hemolysis, and each of them has its advantages and disadvantages.[10,11] In this study, we analyzed the absorption peaks of hemoglobin and found that the wavelengths of 541 nm and 576 nm had similar absorbance values, while the wavelength of 415 nm had the most sensitive response to hemoglobin, with the absorbance value 10 times higher than the other two wavelengths. Therefore, we used the 415 nm wavelength for subsequent experiments that did not involve the addition of other substances for color development. The photomicrograph and

counting methods are not widely used for accurate hemolytic activity evaluation, but it can intuitively see the changes in cell morphology as evidence for other methods. It is possible to explain some experimental errors by observing the changes of RBCs in the microscopic field of view.

We observed that in samples with a lower TE concentration, the number of RBCs in the field of view was significantly higher than that of the non-te groups. Previous studies have shown that lipid peroxidation damage and metalloproteinases are the main harm caused by jellyfish TE to the human body.[16] Lipid peroxidation produces oxygen free radicals that can destroy the phospholipid bilayer structure of the cell membrane, leading to damage to the cell membrane.[17] Therefore, antioxidants usually have a good effect on the treatment of jellyfish stings. The slight lipid peroxidation produced by low concentrations of TE neutralizes the electric potential of the RBC membrane surface, resulting in the aggregation of RBCs.[18,19] After jellyfish stinging, the RBCs in the patient's blood are more likely agglutinated and form thrombus, greatly increasing the incidence of cardiovascular and cerebrovascular diseases.[20] The destruction of the extracellular matrix by metalloproteinases affects RBC permeability and makes the cell membrane fragile, weakening, or even lossing protection.[21]

RBCs are susceptible to changes in external conditions that cause cell damage and hemolysis. Therefore, many blood shadows of different sizes are observed in the samples with higher TE concentrations. Although the use of a microplate reader simplifies the sample measurement process, the difference in absorbance of different high-concentration TE samples is still noticeable. We believe that in the case of high TE concentration, it is beyond the scope of application of Beer-Lambert law, and light scatters slightly in the passing through the solution, resulting in a deviation in absorbance.[22] There is also the possibility that part of the blood shadows is not completely precipitated after centrifugation, which affects the value of absorbance in the solution.

In addition to using hemoglobin to absorb specific wavelengths of light, we used the BCA protein quantification kit to quantitatively measure hemoglobin in the supernatant. We found that with TE concentration greater than 30%, subtracting the absorbance of the TE measured protein concentration, the hemolytic activity appears to decrease. The BCA method was also used to determine hemolytic activity. Among the methods mentioned above, three of them are based on the Beer-Lambert law, which involves absorbance measurement. However, during the actual measurement

process, the color of the test sample can interfere with the results, especially when hemoglobin is present. This can cause deviations in the experimental results, which can exceed the limit of the Beer-Lambert law. Additionally, errors can occur due to sample loading mistakes and the characteristics of blood cells.[23] For example, when measuring the hemolysis rate of TE with a microplate reader, the hemolysis rate at a TE concentration of 60% could exceed the hemolysis rate at a TE concentration of 100%, which was unreasonable. The color of TE affected the absorbance and influenced the results. To obtain the more accurate results, the spectrophotometer was used for measurement. Diluting the sample in a cuvette could keep it within the applicable range of the Beer-Lambert law. Although this method may be more time-consuming, it can result in smaller errors caused by the sample addition process and more accurate results.

Following the successful completion of the aforementioned experiments, we proceeded to conduct hemolysis evaluation experiments. To ensure the accuracy of our results, measures were implemented to control the precision of sample addition and minimize the potential for errors resulting from human operations. To achieve this, we exercised great care when measuring and adding samples, taking care to dilute them as much as possible. The purpose was to ensure that the samples did not exceed the limit of Beer-Lambert law, which would have compromised the accuracy of our results.

Conclusions

In this study, the hemolysis effect of *Stomolophus meleagris* jellyfish TE was determined by four common laboratory methods: the microplate reader method, BCA kit method, spectrophotometer method, and photomicrograph counting method. The choice of methods depends on what the experimenter is interested in, such as RBCs morphology or concentration. We found that different concentrations of TE need to correspond to detection methods to obtain more accurate results of the hemolysis effect.

The microplate reader method, BCA kit method, and spectrophotometer method are all based on the Beer-Lambert law and use specific absorption wavelengths to measure the properties of hemoglobin. The color and concentration of TE will affect the results of hemolytic activity determination. The microplate reader method can simplify the measurement process. However, in the case of high TE concentration, it

will exceed the scope of the Beer-Lambert law, and the light will be slightly scattered when passing through the solution, resulting in a deviation of absorbance. The BCA kit method is more suitable for qualitative detection. When TE concentration is low, the measured value by the BCA kit has a large deviation from the theoretical value, and the accuracy is insufficient. In contrast, the spectrophotometer method is more sensitive and accurate. At present, the spectrophotometer method has been widely used in scientific research and industrial applications because of its high accuracy and reliability in measuring the concentration of various substances. Since the sample is diluted and the concentration of the sample under test remains within the applicable range of the Beer-Lambert law, this method takes longer, but the results are more accurate. The photomicrograph counting method is not widely used to accurately evaluate hemolytic activity, for example, electrostatic effects can cause the cell counting method used in microscope analysis to be inaccurate. But using the photomicrograph counting method the changes in cell morphology can be visually seen. For example, the slight lipid peroxidation caused by a low concentration of TE neutralizes the electric potential on the surface of the red cell membrane, resulting in the aggregation and increase of RBCs. By looking at changes in RBCs in the microscopic field of view, the results of other detection methods for the hemolysis effect can be supported.

It is important to consider the factors that can affect the measurement process to overcome these limitations and improve the accuracy of experimental results. For instance, when optimizing the experiment, researchers should consider the limitations of the Beer-Lambert law and find ways to reduce the impact of solute interactions on the results. This can be achieved by adjusting the experimental conditions, such as the concentration of the sample or the wavelength of the light used in the measurement. Reliable experimental results can be obtained by choosing suitable experimental methods.

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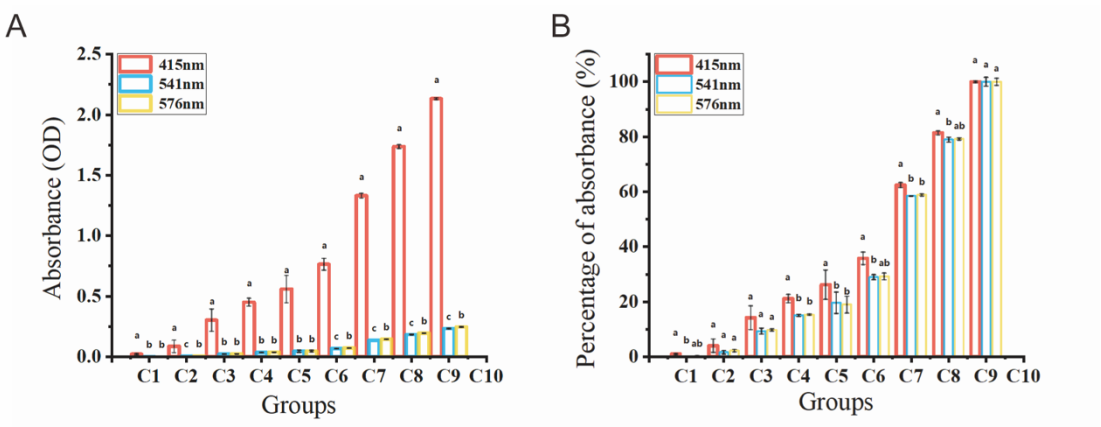

Figure 1. Measurement difference of gradient hemoglobin solution under different absorption peaks. A) OD value of three absorption wavelengths (n=3); B) Percentage of absorbance of three absorption wavelengths (n=3). If there was one identical letter between the groups, the difference was not significant. If there was no identical letter, the difference was significant.

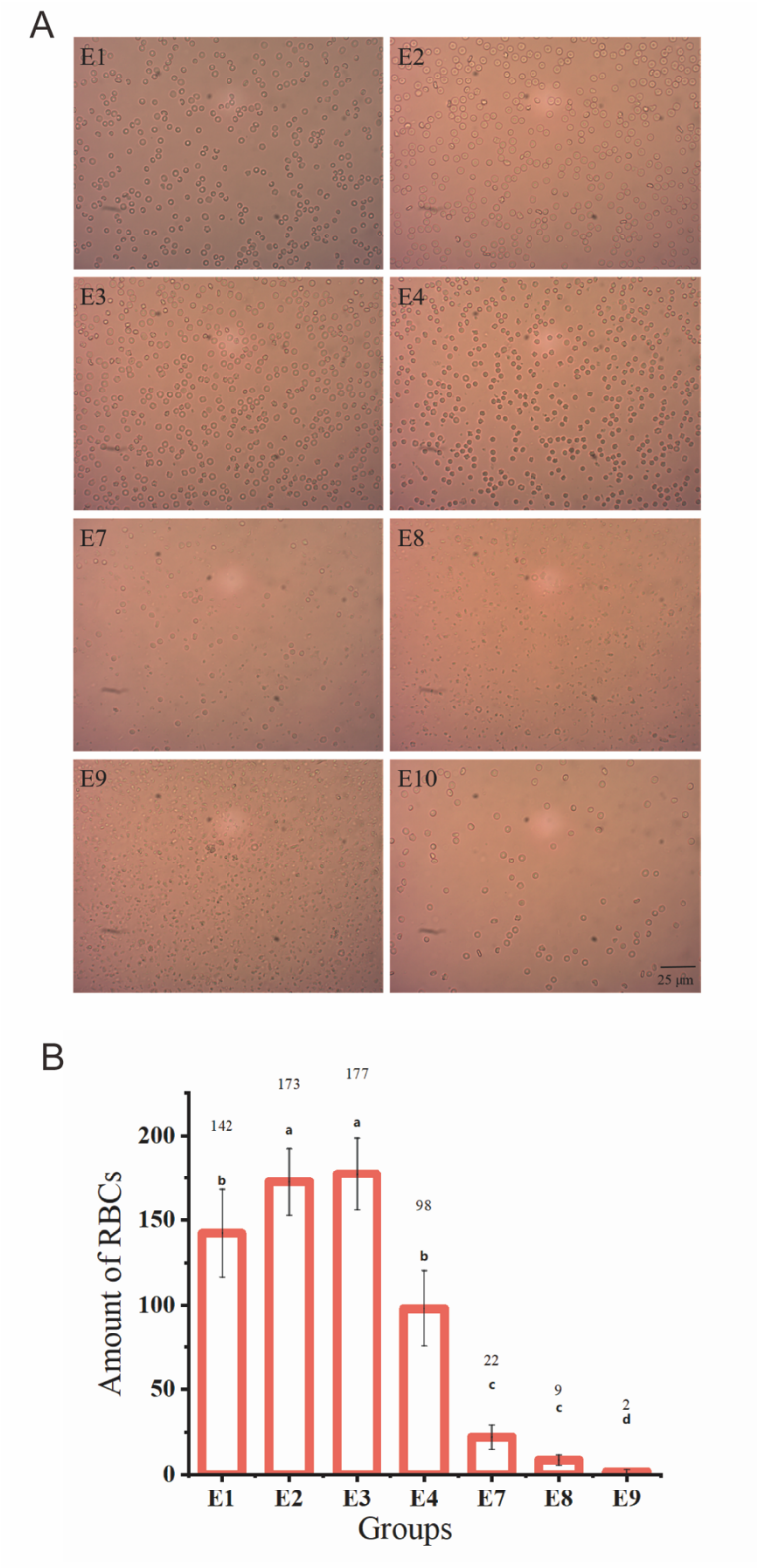

Figure 2. Photomicrograph counting method to measure the hemolysis induced by jellyfish samples. A) Photos of experimental groups “E1”, “E2”, “E3”, “E4”, “E7”, “E8”, “E9”, “E10”, scale bar, 25 μm; B) Number of RBCs in the photos of experimental groups “E1”, “E2”, “E3”, “E4”, “E7”, “E8”, “E9” (n=4). If there was one identical letter between the groups, the difference was not significant. If there was

no identical letter, the difference was significant.

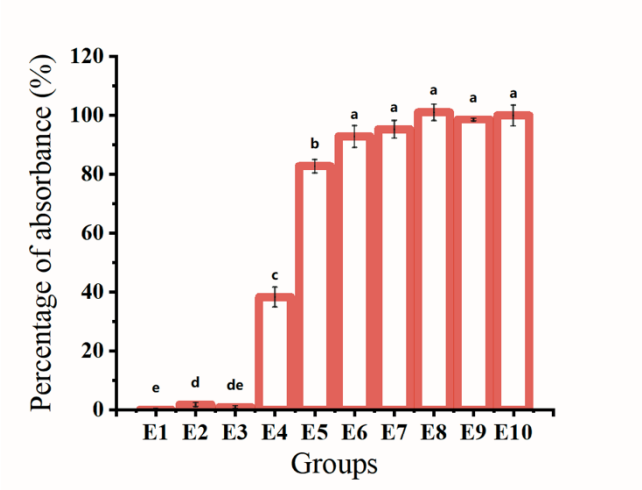

Figure 3. Absorbance of the hemolysis samples measured by microplate reader. Percentage of absorbance of hemolysis samples at 415 nm wavelength (n=4). If there was one identical letter between the groups, the difference was not significant. If there was no identical letter, the difference was significant.

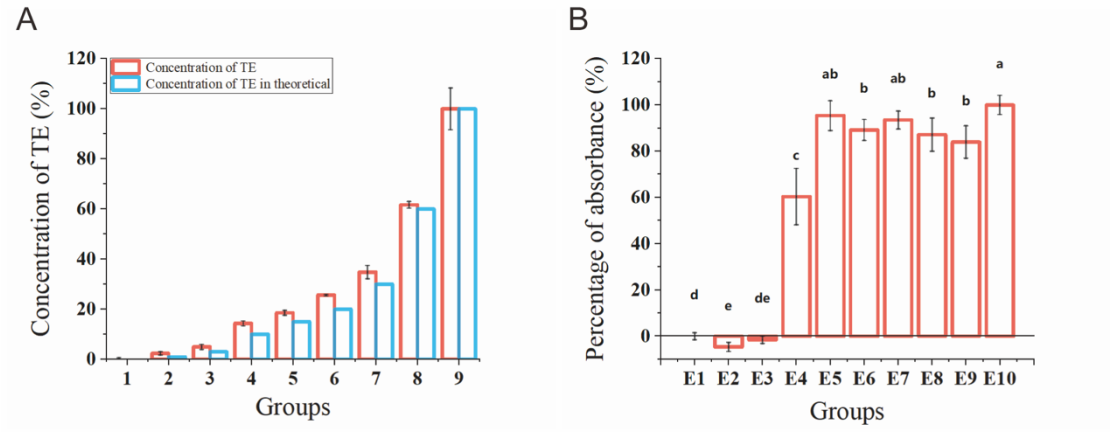

Figure 4. Measurement of the TE samples and hemolysis samples with a BCA kit. A) Comparison between the concentration of TE samples measured with a BCA kit and the theoretical concentration (n=2); B) Percentage of absorbance of hemolysis in the experimental groups. (n=4).

If there was one identical letter between the groups, the difference was not significant. If there was no identical letter, the difference was significant.

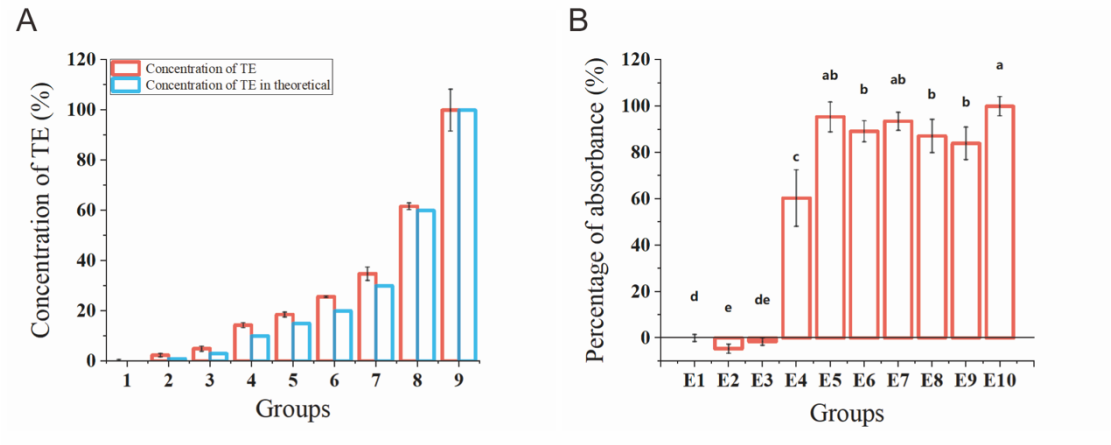

Figure 5. Measurement of the TE samples and hemolysis samples with a visible-ultraviolet spectrophotometer. A) Comparison between the concentration of TE samples measured with a visible-ultraviolet spectrophotometer and the theoretical concentration (n=4); B) Percentage of absorbance of hemolysis in the experimental groups (n=4). If there was one identical letter between the groups, the difference was not significant. If there was no identical letter, the difference was significant.

Table 1. Sample addition table for experimental groups (n=4).

Number	Groups (%)	1× PBS (μL)	TE (μL)	0.45% RBC (μL)	Saponin 25 μg/mL (μL)
E1	0 (negative)	250	0	250	0
E2	1	247.5	2.5	250	0
E3	3	242.5	7.5	250	0
E4	10	225	25	250	0
E5	15	212.5	37.5	250	0
E6	20	200	50	250	0
E7	30	175	75	250	0

E8	60	100	150	250	0
E9	100	0	250	250	0
E10	positive	0	0	250	250
C1	0 (negative)	500	0	0	0
C2	1	497.5	2.5	0	0
C3	3	492.5	7.5	0	0
C4	10	475	25	0	0
C5	15	462.5	37.5	0	0
C6	20	450	50	0	0
C7	30	425	75	0	0
C8	60	350	150	0	0
C9	100	250	250	0	0
C10	positive	250	0	0	250