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IN VITRO THROMBOLYTIC ACTIVITY OF THE ETHANOLIC FRUIT PULP EXTRACT OF *Crescentia cujete* (CALABASH) ON HUMAN WHOLE BLOOD

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Abstract: Cardiovascular diseases (CVD) are a prominent cause for mortality worldwide. These conditions often increase the risk of blood clot formation (thrombosis) and may result in death. Due to the financial difficulties of people from lower income countries such as the Philippines, patients have difficulty in complying with the expensive medications prescribed to be taken and opt for alternative remedies from among locally available resources. This study aims to identify the in vitro thrombolytic activity of the ethanolic fruit pulp extract of *Crescentia cujete* (calabash) on human whole blood. The Streptokinase solution (positive control) showed a mean absorbance of 1.22±0.790. However, the two concentrations of the *Crescentia cujete* ethanolic fruit pulp extract, namely, 35 mg/mL and 17.5 mg/mL, showed a mean absorbance of 0.980±0.752 and 0.787±0.818, respectively. The normal saline solution (negative control) showed a mean absorbance of 0.716±0.885. The results of one-way repeated measures ANOVA showed that the absorbance of hemoglobin released from clotted blood was significantly different among the treatment and control groups with F (2.149, 40.825) = 20.541, $p < .05$, $n^2 = 51.9\%$. Analysis of the results show that the 35 mg/mL ethanolic fruit pulp extract of *Crescentia cujete* has thrombolytic activity on human whole blood.

Keywords: *Crescentia cujete* (calabash) thrombolytic activity, streptokinase, in vitro thrombolytic activity

I. INTRODUCTION

For the past 15 years, cardiovascular diseases (CVD), such as ischemic heart disease and stroke, accounted for 17.9 million deaths, making it a leading cause of

mortality on a global basis (WHO, 2019; WHO, 2020). According to the Philippine Statistics Authority, in 2020, ischemic heart disease, also known as coronary heart disease, was responsible for 105,114 deaths, comprising 17.14% of all deaths (Philippine Statistics Authority, 2021). Manifestations include chest pain or discomfort, which is often due to the formation of blood clots in the coronaries (Healthgrades, 2020).

The formation of these blood clots causes partial or complete blockage of blood vessels predisposing to most CVDs (Ashorobi et al., 2021). The World Health Organization (WHO) suggests minimizing salt intake, increasing consumption of fruits and vegetables, regular physical activity, along with avoidance of tobacco and alcohol to reduce the risk of cardiovascular disease (WHO, 2019).

According to the World Bank, the Philippines is considered as one of the low- and middle-income countries [LMIC] ("Data for Philippines, Lower middle income | Data", 2021; "Top 25 Developed and Developing Countries", 2021). The impact of stroke as measured by disability-adjusted life-years (DALYs) and mortality rates is more than three-fold higher in low-income countries compared to that of high- and middle-income countries and approximately 87% of all stroke deaths worldwide occur in LMICs (Addo et al., 2012). Medication and treatment are often expensive, limited, and non-accessible to those living in a low- and middle-income countries ("Top 25 Developed and Developing Countries", 2021).

Over the years, many thrombolytic medications have been discovered such as tissue plasminogen activator (tPA), urokinase, and streptokinase. The mechanism of these drugs is to dissolve or lyse the blood clot (Klabunde et al., n.d.). However, these drugs have limitations which include bleeding, the need for large doses to be maximally effective, and limited fibrin specificity (Sayeed et al., 2015). Because of these limitations, there is a need to develop new drugs from natural sources.

Crescentia cujete is an accessible plant primarily found in the Visayas and Mindanao. Flavonoids are part of *Crescentia cujete*'s phytochemical components that are said to have thrombolytic activity (Ejelonu et al., 2011; Pasicolan et al., 2014; Olaniyi et al., 2018; Nwogwugwu et al., 2016). The benefits of *Crescentia cujete* (calabash) are found in its wood, ribs, and gourd. However, there is less attention focused on the clot lysing activity of the fruit pulp. Hence, the researchers evaluated the presence of phytochemical components and the thrombolytic activity of *Crescentia cujete* fruit pulp extract on human whole blood.

II. METHODOLOGY

Before data collection, the study underwent ethical review by the Cebu Doctors' University Institutional Ethics Review Committee.

Mature and fresh fruits of *Crescentia cujete* (calabash) were collected from Minglanilla, Cebu. The plant's taxonomy was identified and verified by the Department of Agriculture Region VII office located at Mandaue City, Cebu. The fruit pulp of *Crescentia cujete* was scooped out and then placed in an Erlenmeyer flask. The *Crescentia cujete* fruit pulp was extracted based on the method used in the study conducted by Pasicolan et al. and Honculada and Mabasa, with slight modifications wherein, 500 grams of the *Crescentia cujete* fruit pulp was immersed in 500 mL of 95% ethanol for 72 hours (Pasicolan et al., 2014; Honculada et al., 2016). After 72 hours, the preparation was then filtered using filter paper and a glass funnel before rinsing with fresh portions of 95% ethanol.

A rotary evaporator set at 50°C was then used on the filtrate to obtain a dark crude extract with watery consistency. This ensured that the flavonoids of interest were

not destroyed during the process as they are only thermally stable up to 70°C. This was then labeled and stored as the Crude Ethanol Extract.

The Crude Ethanol Extract was then subjected to standard tests for qualitative determination of phytochemical compounds.

Two hundred fifty milliliters of an initial 70 mg/mL concentration of ethanolic fruit pulp extract of *Crescentia cujete* (calabash) was prepared. Portions of this solution were diluted to obtain 50 mL of 35 mg/mL concentration and 50 mL of 17.5 mg/mL concentration. The solvent used to dissolve and dilute the extract was normal saline solution (NSS). These two solutions served as the experimental groups.

A commercially available vial of lyophilized Streptokinase (1,500,000 IU) was reconstituted with 5 mL of normal saline solution (NSS). A volume of 100 µL (30,000 IU) from the stock solution was then used for the determination of in vitro thrombolytic activity (Zamanlu et al., 2017). This solution served as the positive control group. Normal saline solution was used as the negative control.

Volunteers were recruited from staff members and students currently enrolled in any of the colleges of Cebu Doctors' University. A thorough medical history was obtained from the volunteers. The World Health Organization Blood Donor Screening Form and standard COVID-19 screening questionnaires were utilized. Vital signs were then taken from the volunteers. Inclusion criteria included age of at least 18 years old, with no history of bleeding disorders or recent viral infections, and with a prothrombin time of 8-13 seconds that was subsequently determined. Excluded from the study were those who were sick of any acute illness on the day of the blood extraction, those who had a history of hematologic disorders, those whose prothrombin time results were abnormal during a subsequent determination (Kamal et al., 2007), those under anticoagulant therapy for the past three months (Kearon & Akl, 2014), and those who had used oral contraceptives or nonsteroidal anti-inflammatory drugs (NSAIDs) within the two weeks prior to blood extraction. There were a total of twenty volunteers who fulfilled the inclusion criteria and who had none of the exclusion criteria. Informed consent was then obtained from these volunteers.

Blood samples were collected from the twenty volunteers. A registered medical technologist performed the blood extraction on the volunteers. Five mL of blood were drawn from each volunteer and transferred into buffered sodium citrate tubes. From each of the samples, four 50 µL portions were transferred into individual red top blood tubes using a calibrated micropipette for a total of 80 tubes, each labelled accordingly, A volunteer's four portions were allotted in the following manner: one for each of the two experimental groups, one for the positive control group, and one for the negative control group.

To determine the in vitro thrombolytic activity of *Crescentia cujete* (calabash), the following were performed: one hundred microliters (100 µL) of each of the two concentrations of *Crescentia cujete* (35 mg/mL and 17.5 mg/mL), the positive control (Streptokinase), and the negative control (NSS) were separately added to 50 µL of the volunteers' citrated plasma samples. Afterwards, 100 µL of PTT reagent and 100 µL of calcium chloride were added to each of the mixtures. The mixtures were then incubated for 30 minutes at 37°C. After incubation, the resultant liquid lysed clot was aspirated using a semi-automated micropipette and transferred to a test tube. A sufficient amount of normal saline was carefully added to obtain a 3 mL solution before the test solution was transferred into a cuvette. The absorbance reading was measured at 405 nm using a UV-Vis spectrophotometer.

Spontaneous thrombolysis was prevented by performing the spectrophotometric analysis of thrombolytic activity (SATA) assay immediately after incubation.

The absorbance of a diluent blank, containing all reagents and treatment control except the blood clot, was measured at 405 nm to remove the absorptive interference of the blood clot that may affect the results.

The proper protocol for the disposal of potentially infectious wastes was applied throughout the study.

The mean absorbance of hemoglobin from the spectrophotometric analysis was calculated for the experimental groups treated with the two concentrations of *Crescentia cujete* (calabash), the positive control group treated with Streptokinase, and the negative control group with normal saline solution (NSS).

One-way Repeated-Measures ANOVA was used to determine the significant difference in the mean absorbance of hemoglobin released from clotted red blood cells exposed to each of the four groups at .05 level of significance. Bonferroni post-hoc test was used to determine the significant difference in the mean absorbance of hemoglobin released from clotted red blood cells exposed to each of the four groups. Mauchly's test showed that sphericity assumption was not met, $x2 (5) = 16.611$, $p < 0.05$, so Huynh-Feldt correction was applied.

All data were saved in the personal computers of the researchers, password-protected, and also in the flash drive for printing and file-transferring purposes.

III. RESULTS AND DISCUSSION

Phytochemical screening yielded positive results for saponins, glycosides and flavonoids and negative results for tannins, alkaloids, steroids, and triterpenoids.

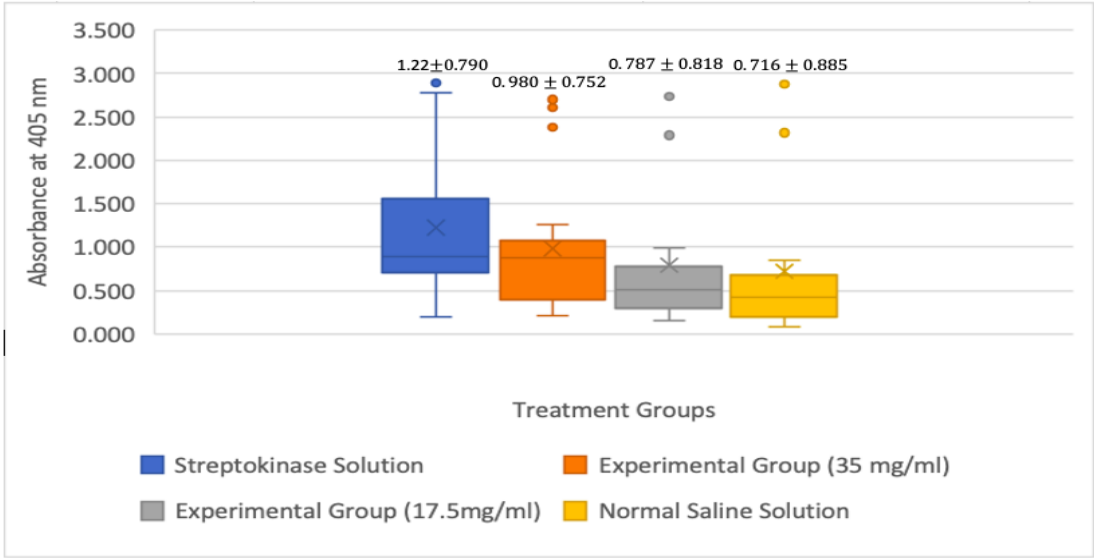

Figure 1. Mean Absorbance of Hemoglobin Released from Each Lysed Clot

Figure 1 shows the mean absorbance obtained from each of the groups. Compared to the positive control group, clotted blood exposed to both the 35 mg/mL and 17.5 mg/mL fruit pulp extract had lower mean absorbance. Clotted blood exposed to 35 mg/mL fruit pulp extract had higher mean absorbance than that exposed to 17.5 mg/mL fruit pulp extract. Therefore, it can be observed that the higher the concentration of the fruit pulp extract, the greater the mean absorbance of the hemoglobin released. When more hemoglobin was released, it meant that the thrombolytic activity was also greater.

Table 1. Results of One-way Repeated Measures ANOVA for the Absorbance of Hemoglobin of Red Blood Cells (RBCs) Released from Each Lysed Clot

Source	Type III Sum of Squares	df	Mean Square	F value	Sig.	Conclusion
Treatment	3.113	2.149	1.449	20.541	0.000	Significant

As shown in Table 1, results of one-way repeated measures ANOVA showed that the absorbance of hemoglobin released from each clotted blood sample was significantly affected by the type of treatment group, F (2.149, 40.825) = 20.541, $p < .05$, $n^2 = 51.9\%$. Ratnasooriya et al. (2008), had already shown that streptokinase solution evokes significant clot lysis activity.

Bonferroni post-hoc test was done to compare group pairs, revealing the significant difference between the mean absorbances. At the same time, this test made adjustments before the comparison to prevent data from incorrectly appearing as significant. Thereby, false positives were prevented.

Table 2. Bonferroni Post-Hoc Test for the Hemoglobin Absorbance of Red Blood Cells

Group Pairs	Mean Difference	Sig.	Conclusion
Streptokinase solution & Normal Saline Solution	0.509	0.000	Significant
Streptokinase solution & 35 mg/mL *Crescentia cujete* fruit pulp extract	0.245	0.029	Significant
Streptokinase solution & 17.5 mg/mL *Crescentia cujete* fruit pulp extract	0.438	0.000	Significant
Normal Saline Solution & 35 mg/mL *Crescentia cujete* fruit pulp extract	0.264	0.012	Significant
Normal Saline Solution & 17.5 mg/mL *Crescentia cujete* fruit pulp extract	0.071	0.696	Insignificant
35 mg/mL *Crescentia cujete* fruit pulp extract & 17.5 mg/mL *Crescentia cujete* fruit pulp extract	0.193	0.011	Significant

In comparison, the group pairs in Table 2 showed significant results except for the fifth treatment group pair which consisted of normal saline solution and the 17.5 mg/mL *Crescentia cujete* fruit pulp extract.

The Bonferroni post hoc test showed that on average, the Streptokinase solution had a significantly higher mean absorbance than that of NSS, and 35 mg/mL and 17.5 mg/mL *Crescentia cujete* ethanolic fruit extract, showing that it is an effective thrombolytic agent. Also, 35 mg/mL *Crescentia cujete* ethanolic fruit extract had significantly higher mean absorbance than that of NSS, showing that it had significant thrombolytic activity than that of NSS. This however, was not true for the 17.5 mg/mL fruit extract, which had an average mean absorbance not significantly different from NSS. The 35 mg/mL fruit extract was shown to have a significantly higher average absorbance rate than that of the 17.5 mg/mL fruit pulp extract, showing that the thrombolytic activity of *Crescentia cujete* fruit extract increases as its concentration increases.

The highest mean difference of 0.509 was noted to be on the first treatment group pair which consisted of the positive control group consisting of Streptokinase and the negative control group consisting of NSS. As an isotonic solution, NSS had a low mean absorbance reading. The fifth treatment group pair, which consisted of normal saline solution and the 17.5 mg/mL *Crescentia cujete* fruit pulp extract had the lowest mean difference of 0.071. This means that the 17.5 mg/mL *Crescentia cujete* fruit pulp extract had a similar absorbance reading to that of normal saline solution. The high mean absorbance of Streptokinase is accounted for by the presence of thrombolytic activity initiated by its complex formation with plasminogen, which is then converted to proteolytic enzyme plasmin (Mahmud et al., 2015). This leads to the activation of the cascade that results in fibrin clot lysis. The least mean absorbance observed using normal saline solution could be attributed to its isotonicity or absence of net movement of fluid across the cell, which preserves the cell membrane of the red blood cell. Hence, almost none to minimal clot lysis (Goodhead & MacMillan, 2017).

The hemoglobin released upon the addition of NSS can be attributed to spontaneous thrombolysis due to incubation at 37°C, not because it produces thrombolytic activity.

IV. CONCLUSION

Based on the findings, the 35 mg/mL ethanolic fruit pulp extract of *Crescentia cujete* had thrombolytic activity on human blood although not to the same extent as that of streptokinase, whereas the 17.5 mg/mL ethanolic fruit pulp extract of *Crescentia cujete* had no thrombolytic activity.

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