THE EFFECTS OF STONE FRUIT EXTRACT ON THE PROCESS OF PLATELET AGGREGATION IN VITRO

A Senior Scholars Thesis

by

PETER M. DELEEUW

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Major: Biomedical Sciences

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Approved by:

Research Advisor	Luis Cisneros-Zevallos
Director for Honors and Undergraduate Research	Sumana Datta

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ABSTRACT

The Effects of Stone Fruit Extract on the Process of Platelet Aggregation in Vitro.
(July 2011)

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This project attempts to examine the impact stone fruits can have on the clotting of blood in the human body. Plum rich anthocyanin extract (RAE) was added in different concentrations (1-25µg/ml) to rabbit platelets suspended in Tyrode's buffer and mixed in microplate wells. Platelet aggregation was then induced by 5 µM ADP (Adenosine Diphosphate), and the percent of transmittance was measured by a microplate reader. A change in aggregation of over 10% was recorded. The results suggest that the extract inhibits the aggregation caused by ADP. This would also lead one to believe that there are compounds in stone fruits that could lower the chances of cardiovascular disease by limiting aggregation and subsequently inflammation in arterial walls.

ACKNOWLEDGMENTS

I would like to say thanks to Dr. Cisneros-Zevallos for being my advisor and never losing hope on my research. I would not have been able to finish this project without his funding and support. I would also like to thank Freddy Ibanez, who taught me how to work in the lab, and always gave me a hand. Paula Simons was able to help create the phenolic extracts for the research, so a big thanks goes out to her. I am indebted to Dr. Walkowiak, who started me on the right track with his advice on platelets, and to Dr. Gresham, who helped me acquire the rabbit blood I needed. Finally, thanks to my lab mates (Congmei Cao, Huiyong Ma, and Paula Castillo), my family, and my girlfriend for giving me support when I needed it.

NOMENCLATURE

A	Absorbance
ADP	Adenosine Diphosphate
C	Degrees Celsius
GP	Glycoprotein
PRP	Platelet Rich Plasma
RAE	Rich Anthocyanin Extract
Resv.	Resveratrol
T	Transmittance
TFA	Triflouroacetic Acid
TXA_2	Thromboxane A_2
vWF	von Willebrand Factor

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CHAPTER I

INTRODUCTION

Cardiovascular diseases are one of the leading causes of death in the United States. Many factors play a role in the onset of these diseases, but recent research has shown that platelets are a critical component in the inflammatory response of arterial walls (*1*). This inflammation can lead to atheroprogression and atherosclerotic lesions. In fact, heart attacks and strokes are caused due to the platelets main function - blood clots. In previous studies, peaches and plums were proven to contain antioxidants that had anticancer capabilities (*2*). Similar stone fruits were now tested to see if their phenolic compounds also have anti- platelet properties. It has already been discovered that Resveratrol, a phenolic compound found in grapes has an inhibitory affect on the aggregation process (*3*).This research project hopes to prove that certain plum varieties contain anthocyanins that inhibit platelet aggregation similar to the drugs on the market like Aspirin and Plavix. The human body retains compounds that bind to receptors on the surface of platelets. This binding causes a cascade of events including shape change and the release of more agonists. **Figure 1** (*4*), illustrates the biochemical pathways involved when inducing platelet aggregation. When blood vessel walls in the body are damaged, collagen is exposed to blood. Platelets are able to bind to the collagen directly

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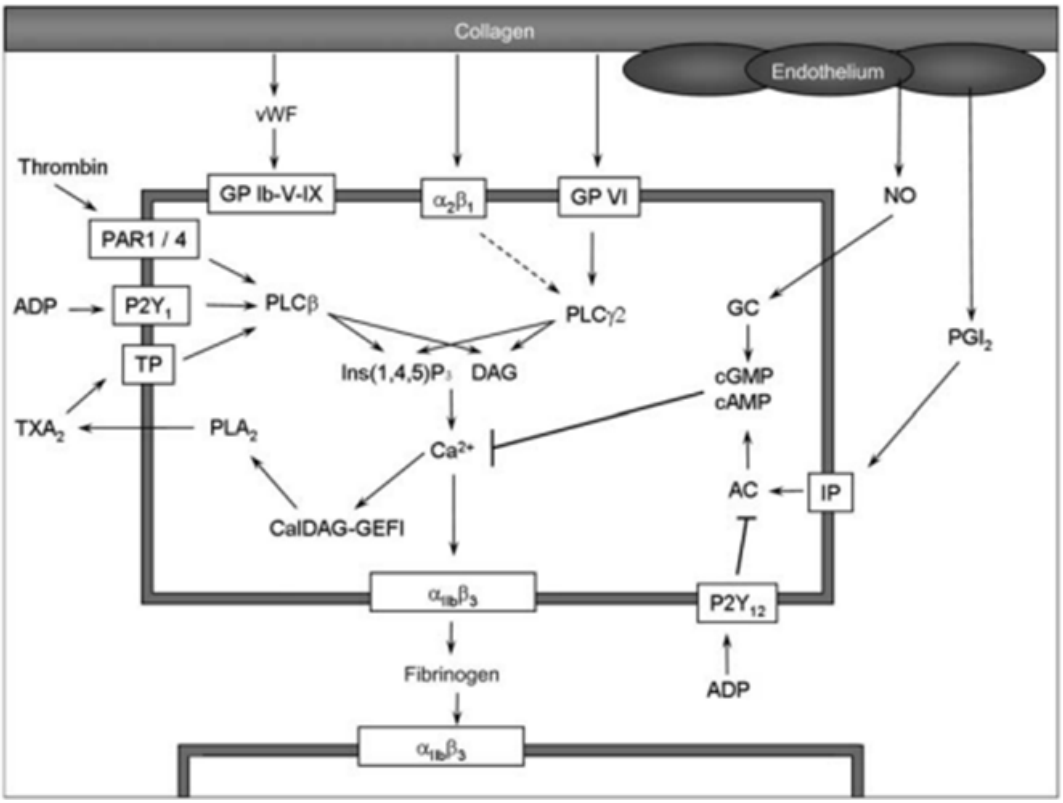

Figure 1. Platelet Aggregation Biochemical Pathways. This diagram shows the metabolites in the body that initiate platelet aggregation, the receptors on the platelet surface, and the cascade of events that occur. It includes ADP binding to the $P2Y_1$ and $P2Y_{12}$ sites on the platelet's surface.

through receptors α2β1, and Glycoprotein (GP) VI. GP Ib-V-IX complex also adheres to collagen, but requires a ligand called von Willebrand Factor (vWF) to complete the process. After adhesion has taken place, platelets will then release the contents of their dense and α granules, which include ADP. Activated platelets will also create thrombin and Thromboxane A_2 (TXA_2.) ADP, thrombin, and TXA_2 all bind to specific receptors on the platelet's surface, causing signal transduction events that lead to GPIIb-IIIa entering an active state. GPIIb-IIIa is responsible for binding to fibrinogen and subsequently other platelets. ADP was chosen because it is involved in many stages of the aggregation process. In the body, ADP can initiate platelet activation and aggregation. It is also involved in the second wave of aggregation, as well as ADP release from platelet granules (*5*). A compound that inhibits ADP activated aggregation would halt the whole aggregation process, rather than just the beginning or end. In the past, platelet aggregation was measured by a machine called a platelet aggregometer. Recent research has been able to reproduce the same results by using a microplate reader (*6*). This gives the ability to run multiple samples at the same time, with very controlled conditions. Depending on the results of this project, plums could be proven to be essential to the diet of many individuals. If the results show a strong inhibitory effect, then further research could be done to isolate the specific anthocyanins responsible.

CHAPTER II

METHODS

Rich anthocyanin extract preparation

The stone fruit solutions were extracted using a protocol from Dr. Cisneros' Food Science Lab, which was created by Paula Simons. Stone fruit extract was taken from Black Splendor Plums grown in California. Once the plums arrived they were pitted and stored in -80C until used. 250 grams of fruit were homogenized with 580 ml of methanol, and stored in 4C for 24 hrs. These samples were then centrifuged at 29000 x g for 15 min, and the supernatant was evaporated at 35C using a rotavapor (Büchi, Switzerland). Remaining aqueous solution was re-diluted into 0.1% TFA and loaded into a SEP Pack c18 cartridge (10g, Waters Corp, Milford, MA). The cartridge had previously been conditioned to a pH below 2, with 20ml of 0.1% TFA in methanol and 20 ml 0.1% TFA. The cartridge was washed with 100 ml of 0.1% TFA, and the RAE from the plums were collected with 0.1% TFA in Methanol. The methanol was evaporated using the same rotavapor, and the remaining aqueous solution was freeze dried at -50C and 200mmHg of pressure. The powder was stored at -20C until use.

Platelet rich plasma preparation and data collection

Rabbit blood was acquired from the Texas A&M Comparative Tissue Department. Rabbit blood has been used in other papers for platelet aggregation tests, and it is comparable to human blood (*7*). The blood is drawn into tubes with 3.8% sodium citrate added at a 9:1 ratio. The blood must be used within 4 hours of being drawn; otherwise the platelets no longer aggregate effectively. Once the blood is obtained, it is centrifuged at 200 x g for 20 minutes. Platelet Rich Plasma (PRP) is then taken from the initial sample and centrifuged at 1000 x g for 20 min. The pellet of PRP created by the centrifugation is suspended in Tyrode's Buffer (140mM NaCl, 10mM glucose and 15mM Tris-HCl, pH 7.4), and then washed with the same buffer. The platelet count of the PRP is then taken with a hemocytometer just to confirm there is a good concentration of platelets (about 5x10^8 platelets/ml). The wells of a plate are then filled with 110µl of PRP, and different concentrations of extract from 1-25µg/ml. Some wells will just have 120ul of PRP to be controls for zero aggregation respectively. The plate then sits at 37C for 30min before the test is run to incubate. Right before the plate is read by the microplate reader, 10µl of 5µM ADP (the agonist) is added to each well, (except the wells with 120µl of PRP only.) The plate reader (Biotek Synergy HT), runs at 37C, and measures absorbance at 595nm. The plate reader takes readings every 30 seconds, and shakes the plate at 20 second intervals in order to stimulate aggregation. The data is then turned into aggregation curves with certain calculations.

CHAPTER III

RESULTS

The results obtained seem to suggest that the plum rich anthocyanin extract did inhibit the effects of ADP and the platelet aggregation process. The science behind the use of the microplate reader for aggregation testing is fairly simple. The shaking of the plate along with the agonist (ADP) causes the platelets in the solution to clot. This clotting allows more light to pass through the wells of the plate, decreasing the absorbance (*8*). Absorbance (A) and transmittance (T) are inversely related through the equation $T = 10^{-A}$. If an effective inhibitor is added to the reaction, aggregation will decrease, along with transmittance. The data obtained directly from the run in the microplate reader is shown in **Figure 2.** It would be expected for the wells with PRP to have the highest absorbance, the wells with PRP and ADP to have the lowest absorbance, and the wells with the inhibitors to be somewhere in between. This is not the case in the present study. When looking at the plates after the trial runs, a small layer of sediment was detected on the bottom of the wells. This means that the shaking of the plate reader and Tyrode's buffer did not keep the clots evenly dispersed throughout the solution. Although some papers have shown to have success with plate readers (*6, 9*), it seems that not all plate readers are directly compatible in keeping the platelet aggregates in suspension. In previous experiments with pig blood, this exact plate reader produced similar results (**Figure 3.**) suggesting that there is a discrepancy in how the plate reader acquires data. **Figure 4** aids in explaining why the clots sinking to the bottom of the

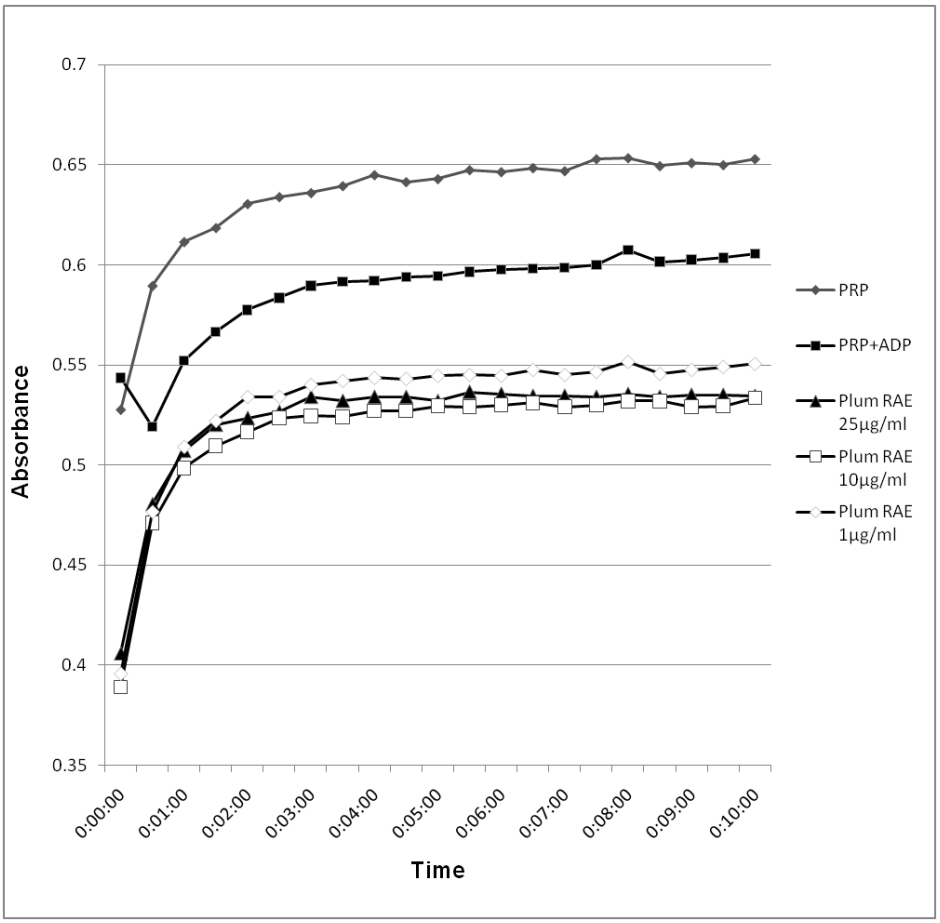

Figure 2. Absorbance vs. Time - Raw Data. This is the raw data acquired from the plate reader. The wells with the potential aggregation inhibitors have lower absorbances than PRP+ADP due to the mechanics of the plate reader and the clots sitting on the bottom of the wells.

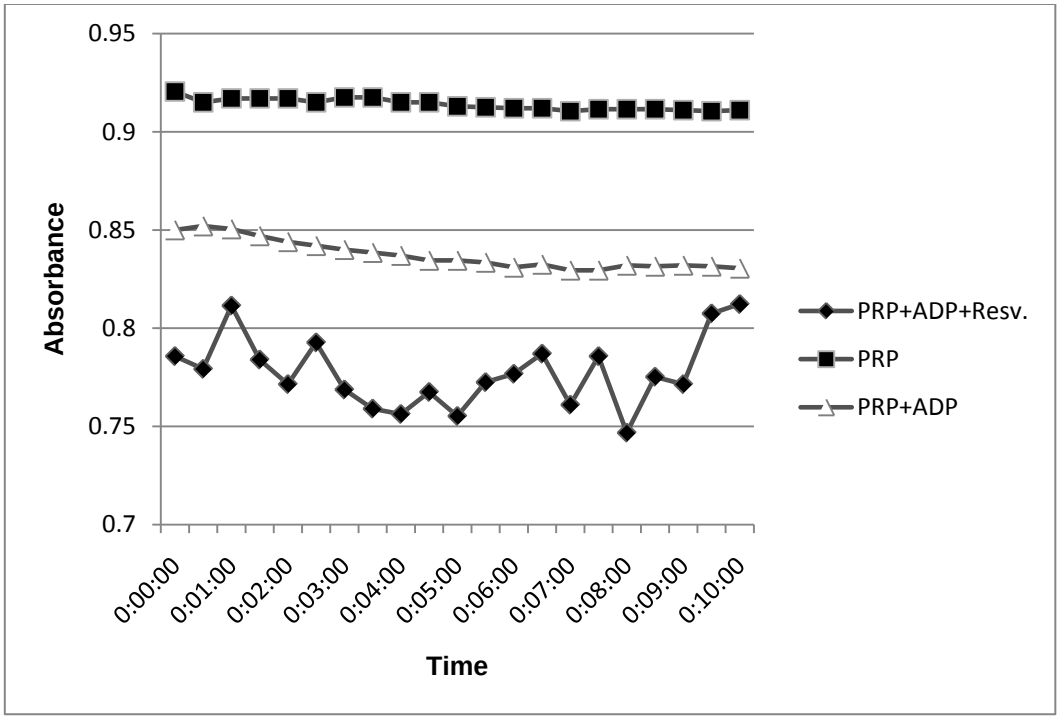

Figure 3. Absorbance vs. Time - Pig Blood. Shows similar results to Figure 2, except pig blood was used and resveratrol was tested as an inhibitor.

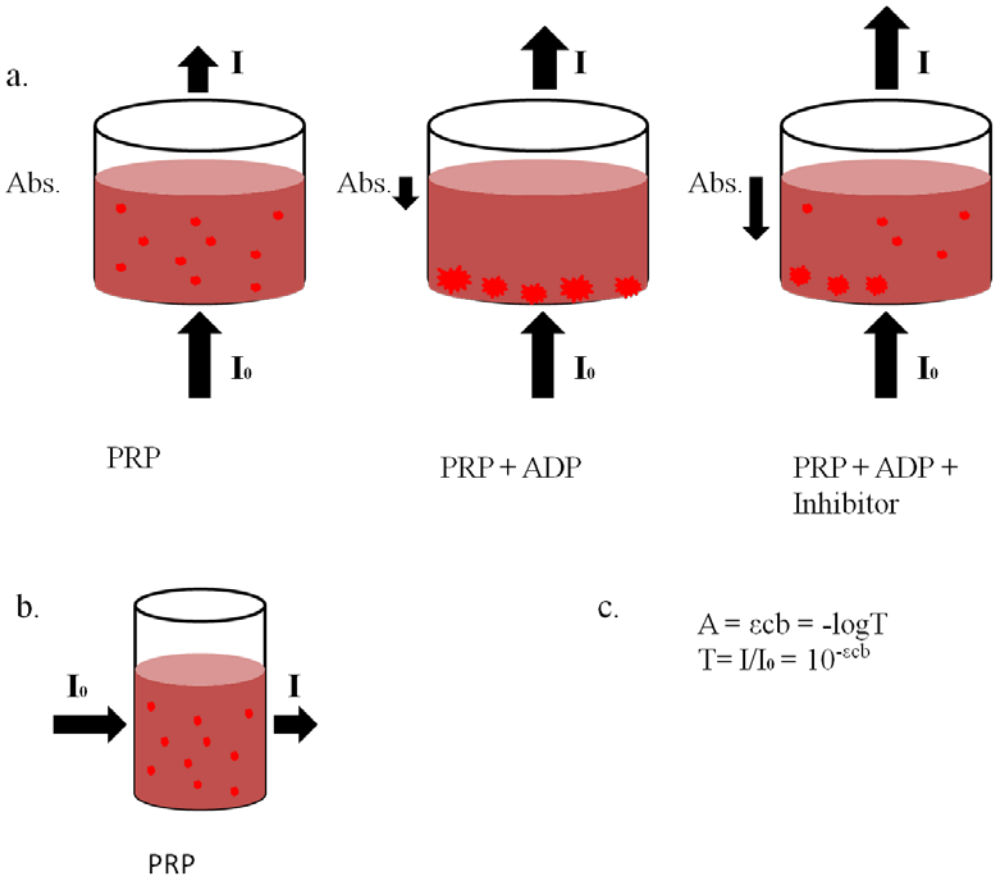

Figure 4. The Mechanics Behind Absorbance Readings. This diagram explains the problem with the data gathering of the plate reader. Light passes vertically in the plate reader (a.), so when clots sink to the bottom they affect the readings. PRP+ADP would have had a much lower absorbance if the reading was taken horizontally like in an aggregometer (b.) The equations to the side of this figure (c.) also help explain the logic behind absorbance and transmittance.

wells cause an issue when using a plate reader. While aggregometers measure optical density in the horizontal direction, plate readers measure optical density in the vertical direction. The vertical pathway of light along with the platelets lying on the base of the well, explain the results seen in **Figure 2**. In order to correct for this problem, all of the different variable wells were subtracted from the control, or PRP. This creates a graph of delta absorbance based on the difference in absorbance of the control (**Figure 5.**), which resembles an expected aggregation test. Many times, researchers will put their data into the % transmittance graphs, so **Figure 6** shows the delta absorbance readings after being converted into transmittance and multiplied by 100.

Accordingly, the results show that extracts seems to drop the percent aggregation by about 10%. This might seem like a small decrease in aggregation, but resveratrol had similar effects when attempting to inhibit aggregation via ADP in previous studies (*3*) as well as in the present study (**Figure 7.**) using pig blood. Platelet aggregation is also crucial in the healing of wounds, meaning an extreme inhibition of aggregation could be dangerous. It is interesting to note that the different concentrations of extract made only minute differences in the ability to inhibit aggregation. It is possible that after a certain concentration of extract, the inhibition reaches a maximum effect (above 10µg/mL). This is promising, because it could mean that taking a less concentrated form of the extract could be almost as beneficial as a stronger concentration. It is also interesting that the 10µg/ml of plum extract inhibited aggregation the most. The difference between the 10ug/ml and the 25ug/ml however, is very small. The major information gathered from

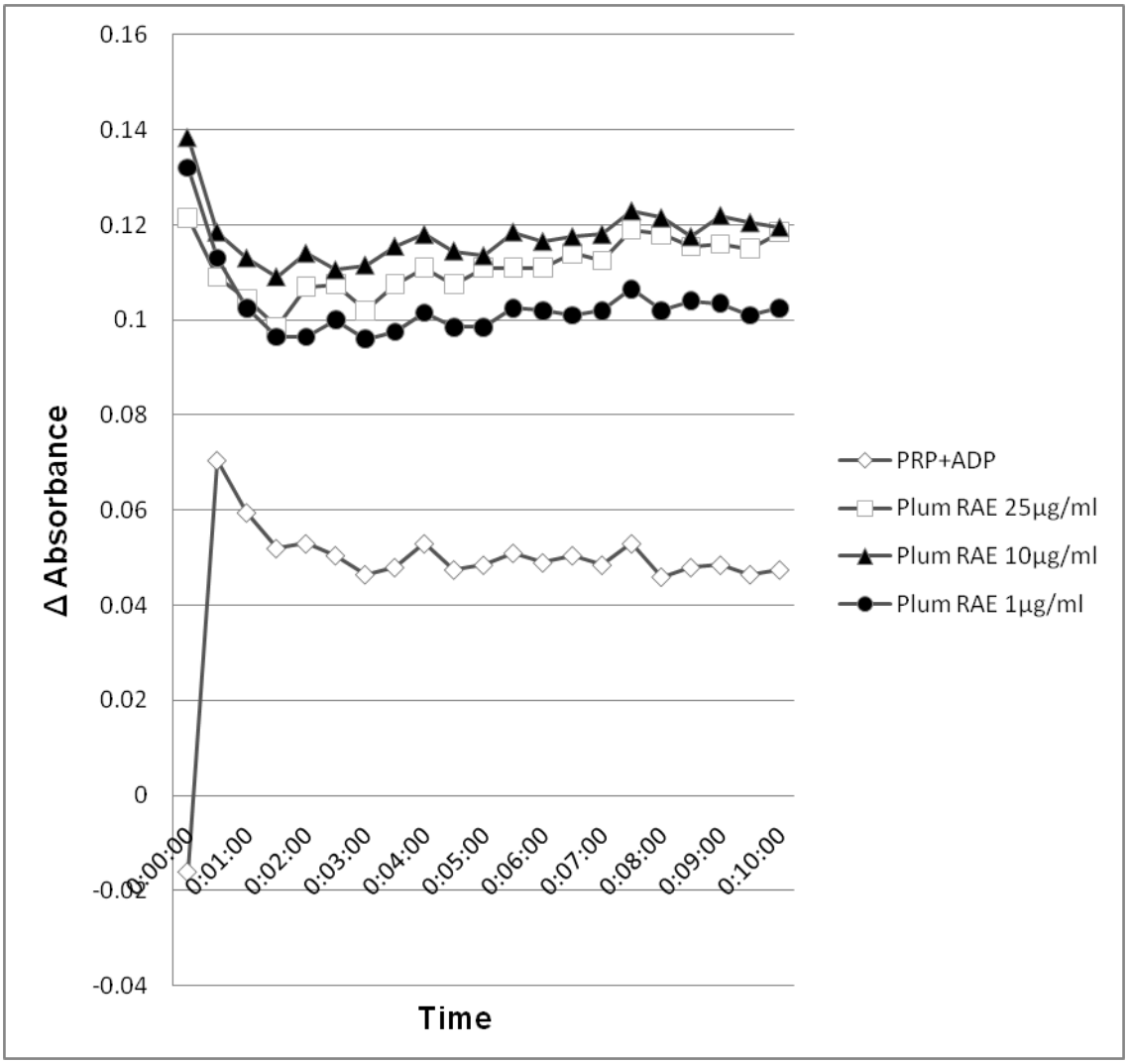

Figure 5. Delta Absorbance vs. Time. Data created by subtracting the different variables from PRP. This corrects values from data gathering.

$A_{PRP} - A_{PRP\ +ADP} = \Delta\ A$ (equation indicating ADP effects)

$A_{PRP} - A_{PRP\ +ADP+Plum\ RAE} = \Delta\ A$ (equation indicating ADP+ inhibitor effects)

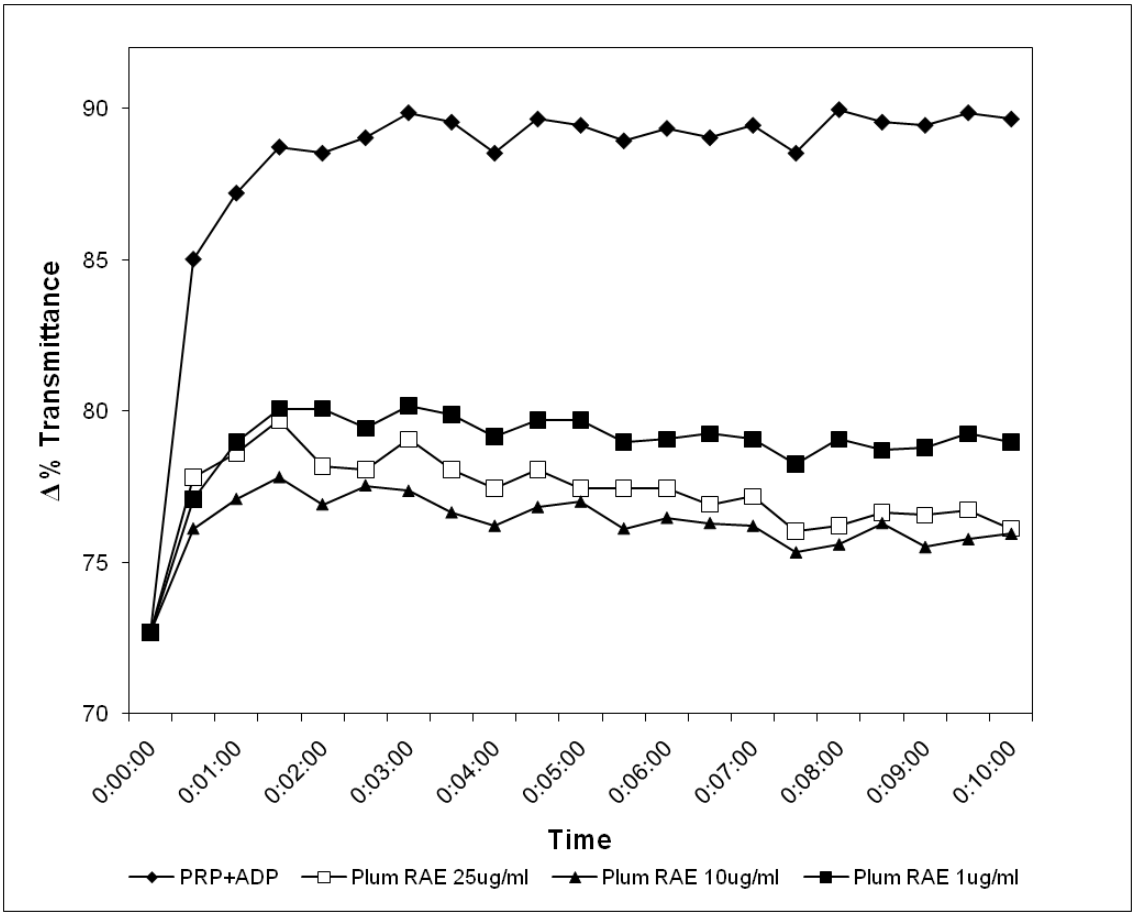

Figure 6. Delta % Transmittance vs. Time. The effect of increasing concentrations of rich anthocyanin extract (1-25µg/ml) on platelet aggregation, agonized by ADP (5µM). Each curve was replicated twice with similar results.

% T = (10^{-A})*100 (equation indicating effects of Δ absorbance values)

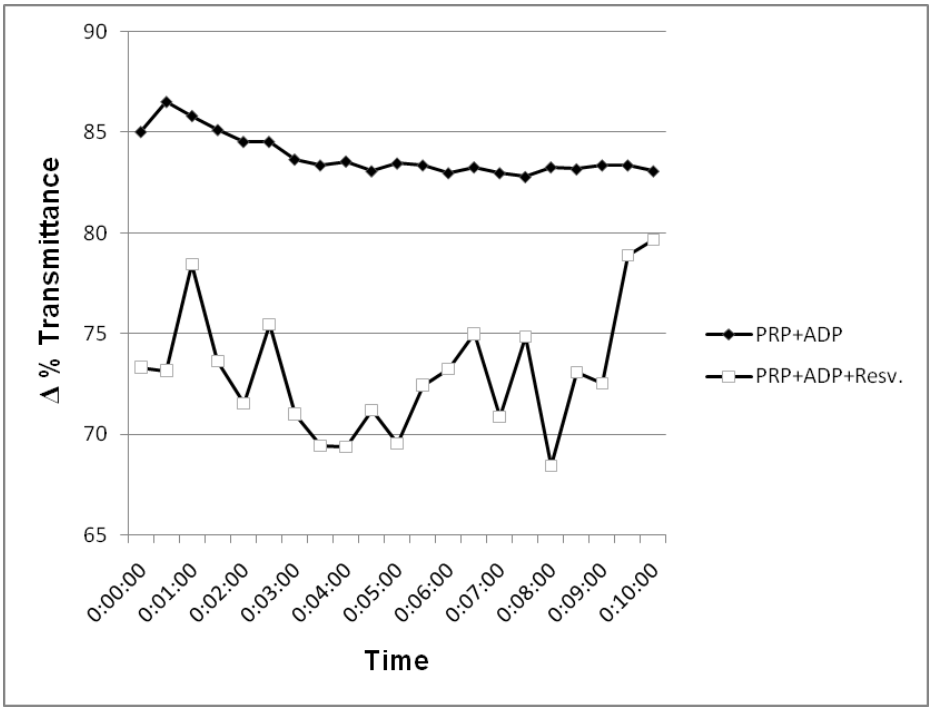

Figure 7. Delta % Transmittance vs. Time – Pig Blood. This graph is similar to Figure 6, except pig blood was used and Resveratrol was the inhibitor being tested. The decrease in transmittance caused by Resveratrol is very similar to the decrease caused by Plum RAE.

this graph is that the extract does inhibit platelet aggregation, and the inhibition is significant. The degree of inhibition is dose dependent, where extreme levels of inhibition are unnecessary when it comes to platelet aggregation. A 10 percent decrease in aggregation in the present study concludes that the results are important enough to encourage more research in this area. **Figure 8** (*10*), shows the precise pathway of ADP. The two nucleotide receptors shown are also GP-coupled receptors. The binding of ADP to receptor $P2Y_1$ causes Ca^{2+} release, shape change, and also aides in the creation of TXA_2. Activation of $P2Y_{12}$ inhibits adenylyl cyclase, starts the secretion of dense granules, and aids in thromboxane and collagen mediated aggregation. The results suggest that the plum RAE contains compounds that bind to these receptor sites, and stop the aggregation process. It is interesting to note that Plavix, a very successful anti-platelet drug, functions by binding to the $P2Y_{12}$ receptor site. This site is preferred over the $P2Y_1$ site because it is found almost solely in platelets, so it doesn't affect the rest of the body. The $P2Y_1$ receptor does have the potential to be a good site for an antiplatelet drug, but currently no clinical research has been done on specific inhibitors for the site. Some of the current inhibitors for these two sites must metabolize in the body before becoming effective, and sometimes their irreversible qualities create health risks. The fact that the polyphenols worked in vitro, suggests that the polyphenols are competitive inhibitors, and are reversible. This a great benefit of the polyphenols. If the compounds in the plum RAE are inhibiting only the $P2Y_{12}$ site, they might have the potential to become critical anti-platelet drugs.

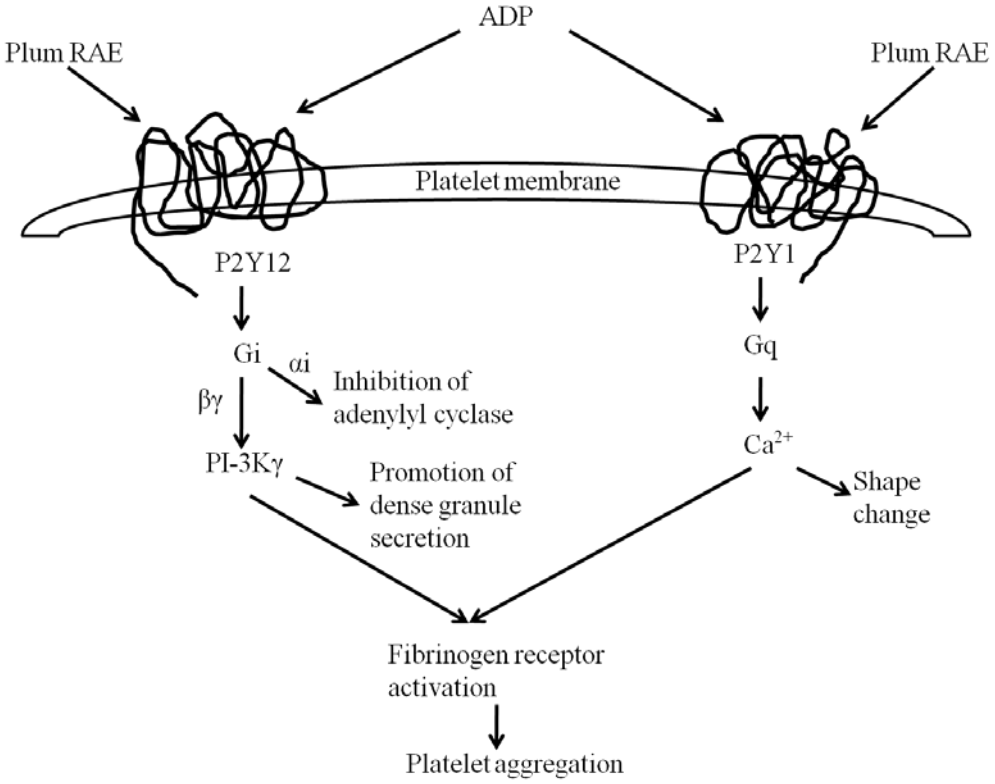

Figure 8. ADP Receptors. ADP binds to the receptors causing shape change and granule release. Our results suggest that compounds in the RAE also bind to these sites, inhibiting ADP and the aggregation process.

CHAPTER IV

SUMMARY AND CONCLUSIONS

This study showed that platelet aggregation can work in certain plate readers as long as a simple modification of data is conducted. This is something that has not previously been reported. After looking at the results of both the rabbit and pig blood with Plum RAE and Resveratrol, it is confirmed that the modification of data is successful, and that the Plum RAE has a significant inhibitory affect. The polyphenols from the plum are somehow blocking the ADP receptors on the surface of the platelets. This study also confirmed that the polyphenols are dose responsive. With all this new information, Plums have the potential to be a dietary source of platelet inhibition. More research needs to be completed on which individual polyphenols are blocking the receptor sites, and which ADP receptor sites are being blocked. Future research could also look into different plum or stone fruit varieties. The goal of this experiment from the beginning was to also test peach extract, but the extraction process did not give adequate samples. Another path of research could test the effect of stone fruit extract on other receptors and pathways mentioned earlier in this paper. Continuing studies might try to use flow cytometry as a more accurate way to test platelet action. Although a good preliminary study, more research can be done on the ability of stone fruits to inhibit aggregation, and the compounds responsible.

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