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Research Article

In Vitro Platelet Aggregation Inhibition Activity of *Psophocarpus tetragonolobus* (L.) Dc Pod Extract

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ABSTRACT

The study aimed to investigate antiplatelet activity of the pods of *Psophocarpus tetragonolobus in vitro* in search for a new botanical source of a platelet aggregation inhibitor for the prevention of stroke, the second leading cause of mortality in the Philippines. This study utilized maceration with hexane as the extraction method. Four concentrations (4mg/mL, 2mg/mL & 0.5mg/mL) of *Psophocarpus tetragonolobus* pods were used in the study. Aspirin (2mg/mL) and NSS were the positive and negative controls, respectively. The methods employed in the study were Giemsa Microplate Assay for qualitative analysis and UV-Vis spectrophotometric analysis for quantitative analysis. Giemsa microplate assay findings revealed that the 4.0mg/mL test solution had the least violet gels present to no violet gels visible at all. Furthermore, the UV-Vis Spectrophotometric Analysis corresponded with the results and found that the 4mg/mL concentration contained the least mean absorbance reading (among the pod extracts) of 0.409, the highest average percent platelet aggregation inhibition of 69.58% (± 2.93) and the greatest average percent antiplatelet activity of 75.49% (± 3.07), among the pod extracts. In conclusion, the pod extract, at 4.0mg/mL concentration, was able to inhibit platelet aggregation.

Keywords: platelet aggregation inhibition, *Psophocarpus tetragonolobus*, Giemsa microplate assay.

INTRODUCTION

Worldwide, nearly six million die and another five million are left permanently disabled from stroke every year[1]. In the Philippines, the Department of Health recognized stroke as a huge threat to health as the leading cause of mortality, and concluded that in every nine minutes, one stroke mortality occurs[2]. Stroke, or cerebrovascular accident, involves the loss of blood supply to the brain when one or more blood vessels burst or clog due to a clot. There are two types of clots which cause stroke, the thrombus and embolus. A thrombus is a blood clot that forms in a vessel and remains in the vicinity, while an embolism is a clot that travels from the site where it formed to another location in the body[3]. With recruitment of additional platelets and the incorporation of fibrin meshwork, the hemostatic plug grows into a thrombus, which under normal physiologic conditions is regulated and eventually undergoes fibrinolytic dissolution once its hemostatic function is fulfilled[4]. However, under pathologic conditions such as rupture of an atherosclerotic plaque, the very same life-sustaining hemostasis turns to an aberrant, amplified process leading to an occlusive thrombus that can result in fatal conditions such as an acute myocardial infarction and ischemic stroke[5-8]. Currently, there are three categories of drugs affecting the blood which can help prevent stroke: anticoagulants; thrombolytics; and antiplatelets. Anticoagulants prevent clot formation by inhibiting the activation of the clotting factors. Thrombolytics induce fibrinolysis by activation of plasminogen to plasmin which lyse the fibrin and ultimately dissolves the clot. Antiplatelet drugs or platelet aggregation inhibitors prevent platelet aggregation. However, platelet aggregation inhibitor drugs such as Aspirin and other nonsteroidal anti-inflammatory drugs cause gastrointestinal and central nervous system adverse effects. Furthermore, the glycoprotein IIb/IIIa inhibitors are associated with thrombocytopenia and ticlopidine may cause thrombotic thrombocytopenic purpura (TTP)[9]. These data led the researchers to investigate new possible sources of antiplatelet drugs. Fortunately, the Philippines has many natural resources and many have potential for medical uses. Among the entire natural flora in the Philippines we shall look more closely on the *Psophocarpus tetragonolobus* locally known in the Cebuano dialect as *Sigarilyas*. There have been many studies conducted on the Fabaceae family of plants such as *Spatholobus suberectus*, *Euchresta formosana*, *Lonchocarpus sericeus*, *Peltophorum pterocarpum*, *Clotoria ternatea* and have shown to possess antiplatelet activity. However, studies for the antiplatelet activity of *Psophocarpus tetragonolobus* have not been carried out yet. *Sigarilyas* has been found to possess plant secondary metabolites like alkaloids, steroids, sterols, indoles and phenols and show a good potential for platelet aggregation[10-12]. This study aimed to determine the presence or absence of gel clots using Giemsa Microplate Assay, determine the percent platelet aggregation inhibition based on the increase or decrease of absorbance

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readings of proteins released by platelets upon aggregation and the median effective concentration of the pod extract.

MATERIALS AND METHODS

Collection and Preparation of the Test Plant

Fresh *Psophocarpus tetragonolobus* L. pods were purchased from Borongan City Public Market, Borongan City, Eastern Samar, Philippines. The pods were authenticated by the Department of Biology, University of San Carlos. The pods were properly washed to remove dust, dirt and other impurities that may have been present, then wiped dry, and cut into small pieces using a clean chopping knife.

Preparation of the Plant Extract

Fifty grams of the cut pods were placed in an Erlenmeyer flask and were added with two hundred milliliters of hexane. The flask was then stoppered and the plant material was allowed to soak for 72 hours with occasional agitation. The extractive was filtered through a Buchner funnel, the filtrate was kept, and the marc was discarded. The filtrate was then heated at 50°C on an evaporating dish under a fume hood until all of the solvent has evaporated. The residue was saved and labelled as the hexane-free extract.

Preparation of the Test Solutions

Forty milligrams of plant extract was added with three drops of Tween 80 and Normal Saline Solution to make a 10 mL solution for Giemsa Microplate Assay. For UV-Vis Spectrophotometric Analysis, 40 mg plant extract was added with three drops of Tween 80 and distilled water q.s. to make a 10 mL solution. These were the first drug concentrations (4mg/mL). From the first drug concentrations, two one mL aliquots were obtained and diluted further with Normal Saline Solution (for Giemsa Microplate Assay) and the other with distilled water (for UV-Vis Spectrophotometric Analysis) until 2 mL. These were the second drug concentrations (2 mg/mL). From the 2 mg/mL concentration, two 0.5 mL aliquots were obtained and diluted with Normal Saline Solution (for Giemsa Microplate Assay) or with distilled water (for UV-Vis Spectrophotometric Analysis) until 2 mL. These were the third drug concentrations (0.5 mg/mL).

Preparation of Controls

In order to make a 10 mL solution of Aspirin, 20 mg of Aspirin USP was dissolved in a sufficient volume of Normal Saline Solution (for Giemsa Microplate Assay) and distilled water (for UV-Vis Spectrophotometric Analysis) with the use of volumetric flasks. The resulting concentration of the positive control used in this experiment was 2 mg/mL. Normal Saline Solution was then used as the negative control for Giemsa Microplate Assay and distilled water was the negative control for UV-Vis Spectrophotometric Analysis. One 10 mL set up was left untreated for each.

Giemsa Microplate Assay[13]

Platelet suspension (160 µL) of blood type O^+ was transferred using a micropipette to the wells of a 96-well microtitre plate. Eighteen wells have been allotted for the platelet suspension and were incubated at 37°C for five minutes in order to mimic the normal human body temperature. The plant extract (40 µL) has been added in three wells each, with the three corresponding extract concentrations (4mg/mL, 2mg/mL and 0.5 mg/mL). In the same amount, the positive control (Aspirin) and negative control (Normal Saline Solution) were added in three wells each, while three wells were filled and were left untreated. They were incubated again at 37°C for 30 minutes. Platelets were induced to aggregate with the addition of 0.25M calcium chloride (4 µL). The microplate was incubated again at 37°C for 30 minutes. Giemsa dye (0.4%, 4µL) were added to each well and incubated further for 5 minutes. The microtitre plate was inverted, tapped and washed with distilled water. Each well was observed for the presence or absence of gels, which was easily visualized by the violet color imparted by the giemsa dye, which indicates platelet aggregation response.

Spectrophotometric Analysis

Six sets of platelet suspensions (3.08mL each) were transferred to test tubes and they were incubated at 37°C for five minutes. The three test concentrations (0.77mL each) and the positive (aspirin) and negative (distilled water) controls, in the same amounts, were added to each of the test tubes and one setup was left untreated. The test tubes were incubated for 30 minutes at 37°C. Platelets were then induced to aggregate with additions of 0.25M calcium chloride (0.08mL). The test tubes were incubated once more for 30 minutes. After the platelets have been induced to aggregate, 1mL of the remaining liquid portions of each test tube were obtained and transferred to another set of test tubes. Each of the liquid portions was diluted with 40mL of distilled water to make 41mL solution with slight agitation. From the 41mL solutions, only 4mL of each was used for analysis. They were transferred in cuvettes and placed in the spectrophotometer. Proteins such as multimerin, thrombospondin and platelet factor 4, which are released by the aggregated platelets, were quantified at 280 nm[14].

Mean Absorbance Reading

Mean absorbance readings were obtained for each test concentration/control per trial. This is to determine the average absorbance readings of each test solution and control

Mean absorbance reading=

$$\frac{\text{Sum of absorbance readings of test solution or control}}{\text{Number of trails performed}}$$

Percent Inhibition of Platelet Aggregation

The mean absorbance readings were then used to calculate the percent antiplatelet activity with the succeeding formula:

% Inhibition of Platelet Aggregation =

$$\frac{\text{Absorbance}_{\text{untreated}} - \text{Absorbance}_{\text{treated}}}{\text{Absorbance}_{\text{untreated}}}$$

The percent inhibition of platelet aggregation values compares the absorbance readings among treated (with plant extract or aspirin) controls and the untreated control.

Percent Antiplatelet Activity

Table 1: Giemsa Microplate Assay Results Summary.

	Presence or Absence of Violet Gels		
Test Solutions	Trial 1	Trial 2	Trial 3
4.0 mg/ml	(+)	(-)	(+)
2.0 mg/ml	(+)	(+)	(+)
0.5 mg/ml	(+)	(+)	(+)
Positive Control (Aspirin)	(+)	(-)	(-)
Negative Control (Distilled Water)	(+)	(+)	(+)
Untreated	(+)	(+)	(+)

[(-) No violet gels visible; (+) Violet gels present].

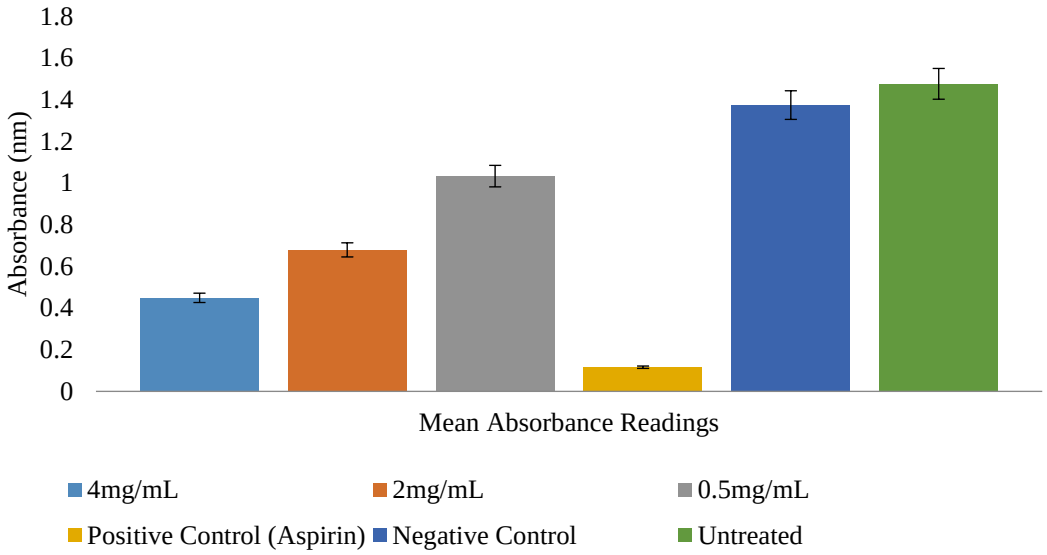

Figure 1: Mean Absorbance Readings of Test Solutions & Controls.

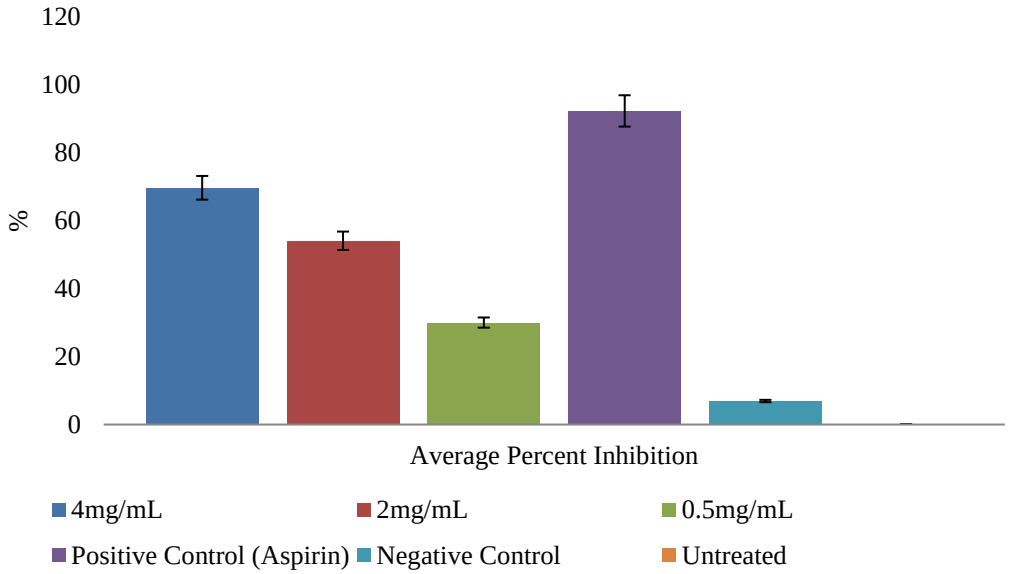

Figure 2: Percent (%) Inhibition Activity of Test Solutions & Controls.

The percent antiplatelet activity of the test solutions and controls were computed and values were compared.

Percent (%) Antiplatelet Activity =

$$\frac{\%\ \text{Inhibition of TS}}{\%\ \text{Positive Control}} \text{ X } 100$$

The percent antiplatelet activity values compare the percent inhibition values among the test solutions and positive control.

Median Effective Concentration (EC50)

Half maximum effective concentration (50%) was used in the study to determine the concentration of the extract to

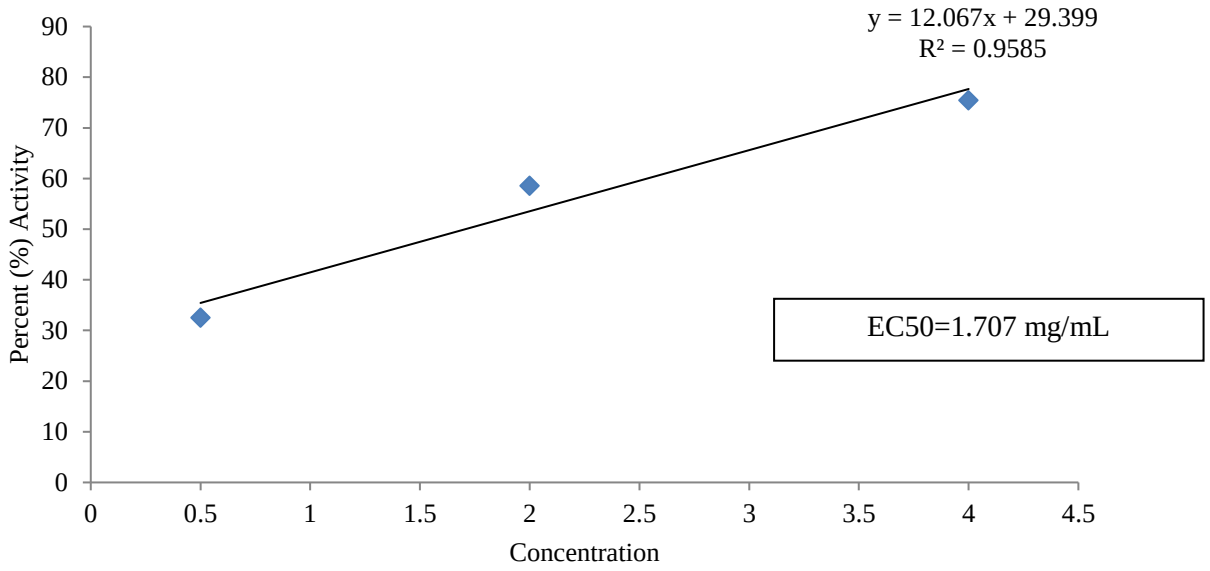

Figure 3: Percent (%) Antiplatelet Activity and Median Effective Concentration (EC50) of Test Solutions.

Table 2: Analysis of Variance (One-Way).

Descriptive statistics							
Groups	Sample size	Sum	Mean	Variance			
(+)	3	20.7394	6.9131	151.2264			
(-)	3	276.516	92.172	25,488.2799			
0.5 mg/mL	3	89.9246	29.9749	2,700.4211			
2 mg/mL	3	162.0492	54.0164	8,757.5788			
4 mg/mL	3	208.7532	69.5844	14,543.0748			
Untreated	3	0	0	0			
Total	18		42.1101	1,160.1084			
ANOVA							
Source of Variation	d.f.	SS	MS	F	p - level	F crit	Omega Sqr.
Between groups	5	19,686.4265	3,937.2853	1,334.081	0	3.1059	0.9973
Within Groups	12	35.4157	2.9513				
	17	19,721.8422					

exhibit 50% antiplatelet activity. To compute for the EC50, the linear equation, y = mx + b was used.

One-Way ANOVA

In order to identify if the absorbance readings of the test solutions and controls between the test concentrations would show a significant difference, we would be comparing the F-statistical value and the F-critical value. If the F-critical value is less than the F-statistical value, we would reject the null hypothesis (that there is no significant difference between the absorbance readings between the test concentrations in three trials). On the other hand, if the F-statistical value would be less than the F-critical value, we would accept the null hypothesis.

Post-Hoc Analysis

From the ANOVA table, deeper analysis was conducted to strengthen the results. This was done by Tukey HSD test to compare the differences between means. Post-Hoc analysis enables the researchers to identify where the difference lies between test solutions and controls.

RESULTS

Quantitative Analysis

Percent Inhibition of Platelet Aggregation

The percent inhibition of platelet aggregation values were then calculated from individual absorbance readings per trial and the average was obtained: 4mg/mL test solution: 69.58%; 2mg/mL test solution: 54.01%; 0.5mg/mL: 29.97; Aspirin (positive control): 92.17%; Normal Saline Solution (negative control): 6.91% and; Untreated Control: 0%.

Percent Antiplatelet Activity and Median Effective Concentration (EC_{50})

The percent antiplatelet values were computed from the average percent inhibition values and were as follows: 4mg/mL test solution: 75.49%; 2mg/mL test solution: 58.61%; 0.5mg/mL: 32.53%; Aspirin (positive control): 100%; Normal Saline Solution (negative control): 7.51% and; Untreated Control: 0%. Likewise, the median effective concentration was computed. It is evident that distilled water did not contribute to the antiplatelet activity

Table 3: Tukey HSD Test for Differences between Means (Post Hoc Analysis).

Tukey HSD Test for Differences Between Means				
Groups	*Difference*	*Test Statistics*	*P - level*	*Interpretation*
(+) vs (-)	-85.2589	85.9593	0.0001	Significant
(-) vs 0.5 mg/mL	-23.0618	23.2512	0.0001	Significant
(-) vs 2 mg/mL	-47.1033	47.4902	0.0001	Significant
(-) vs 4 mg/mL	-62.6713	63.1861	0.0001	Significant
(-) vs untreated	6.9131	6.9699	0.0037	Significant
(+) vs 0.5 mg/mL	62.1971	62.7081	0.0001	Significant
(+) vs 2 mg/mL	38.1556	38.4691	0.0001	Significant
(+) vs 4 mg/mL	22.5876	22.7732	0.0001	Significant
(+) vs untreated	92.172	92.9292	0.0001	Significant
0.5 mg/mL vs 2 mg/mL	-24.0415	24.239	0.0001	Significant
0.5 mg/mL vs 4 mg/mL	-39.6095	39.9349	0.0001	Significant
0.5 mg/mL vs untreated	29.9749	30.2211	0.0001	Significant
2 mg/mL vs 4 mg/mL	-165.568	15.6959	0.0001	Significant
2 mg/mL vs untreated	54.0164	54.4602	0.0001	Significant
4 mg/mL vs untreated	69.5844	70.156	0.0001	Significant

of the test solutions.

One-Way Analysis of Variance (ANOVA)

In the study, the α value used was 0.05. F-statistical value was found to be at 1,334.081, while F-critical was found to be 3.1059. Further analysis was done in order to compare the test concentrations and controls between trials (See Table 2). In the study, the F-critical value was less than the F-statistical value, which indicates that we reject the null hypothesis that the absorbance readings of the test solutions and controls between the three trials showed no significant difference at all in terms of absorbance.

Post Hoc Analysis

All treatment groups had p-levels less than 0.0001 except for the negative control vs. untreated control which had a p-level of 0.0037 (See Table 3). Furthermore, all of the test solutions and controls had significant differences between means. This meant that each test solution had significantly different activities between other concentrations.

DISCUSSION

There are three possible mechanisms of action for antiplatelet activity after calcium levels have been increased. They are the inhibition of the release of platelet granules, inhibition the activation of thromboxane A_2 synthesis, and inhibition of the GP IIb/IIIa receptors (Whalen et al., 2015). Both methods in the study utilized calcium chloride solution as the inducer of platelet aggregation. Aspirin, the positive control in both methods, inhibits thromboxane A_2 synthesis. Aspirin had comparable results with the 4.0mg/mL test solution. *P. tetragonolobus* has been found to possess plant secondary metabolites like alkaloids, steroids, sterols, indoles and phenols[15-17]. Similarly, steroids were also isolated from *Clotoria ternatea* (Fabaceae) and have been found to exhibit platelet aggregation inhibition activity. This was due to significant inhibition of collagen- and ADP-induced aggregation of platelets[18]. In another study, purified steroidal fractions of *Commiphora wightii* (Burseraceae) showed a marked inhibition of ADP, adrenaline and serotonin induced platelet aggregation[19]. Based from results of the previously mentioned studies, it is possible that the presence of steroids in the test solutions, extracted by maceration in hexane, was responsible for the antiplatelet activity. The possible mechanism of action of platelet aggregation inhibition of the *Sigarilyas* pod extract, which most likely contains a significant amount of steroids as identified in their leaves, could be the inhibition of the release of platelet granules. As the platelets were induced to aggregate by calcium level elevation, three effects are possible. Namely, release of platelet granules, activation of thromboxane A_2 synthesis, and activation of the GP IIb/IIIa receptors. The steroids presumably prevent the release of platelet granules. Without these granules, platelets would no longer aggregate with each other, thus leading to the antiplatelet activity. ADP-induced platelet aggregation inhibition is common between the two related studies using plant steroids and could have been the same pathway acted upon by the pod extract. Furthermore, *in vivo* studies may find more significant results as ADP, like thromboxane, is also a chemical mediator bound to collagen in the subendothelium. Therefore, inhibiting ADP will also prevent calcium levels from elevating, and ultimately, prevent platelet aggregation. The relationship between absorbance reading and platelet aggregation inhibition activity is inversely proportional. What is being measured in the study is the amount of proteins, and these proteins are released during platelet aggregation. So the more platelets that aggregate, the more proteins are released. Conversely, if fewer platelets would aggregate, fewer proteins would be released. Hence, if more proteins are released, there is lesser platelet aggregation inhibition activity and goes oppositely if fewer proteins are released. If more proteins are released, the higher the absorbance reading would be (since more proteins would be absorbing light from the spectrophotometer). If this would be the case, then there is lesser antiplatelet activity of the test concentration. On the contrary, if the absorbance reading would be minimal, then fewer proteins have been released, denoting greater antiplatelet activity (inhibited platelet aggregation). The negative control, distilled water, was only used to determine if the solvent, would contribute to

the antiplatelet activity of the plant solution. Furthermore, the untreated control was only used as a basis to for the maximum amount of proteins that could be released by the platelets without inhibition by any test solution or control. As results indicate, the 4mg/mL concentration had the least absorbance reading among all of the test concentrations. This means that it had the highest antiplatelet activity, second to the positive control overall. Meanwhile, the 0.5mg/ml had the highest absorbance reading among test concentrations. This signifies that it had the least antiplatelet activity. While the 2mg/ml showed intermediate absorbance reading and showing intermediate antiplatelet activity. However among all of the test concentrations, it was only the 4mg/mL test solution where both Giemsa Microplate Assay and Spectrophotometric Analysis results coincided and confirmed potential.

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