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Research Article

The Effects of Lyophilized Euphorbia hirta Linn. Extract on the Function and Morphology of Sprague – Dawley Rat's Platelet subjected to Heparin – Induced Peripheral Platelet Destruction

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Abstract

Peripheral platelet destruction is well documented in diseases like Dengue Hemorrhagic Fever. The use of Euphorbia hirta has been reported to affect the hematological system. This research aims to determine the effect of Euphorbia hirta on platelet function and morphology, as well as other parameters of hemostasis, such as bleeding time and clotting time.

A crude extract was prepared by decoction and was lyophilized. Eighteen female Sprague-Dawley rats were divided into 3 groups (A, B and C). Peripheral platelet destruction was induced by subcutaneous administration of 1 unit heparin per gram of body weight. Group A were treated with 100 mg/kg E. hirta decoction by oral gavage for 7 days. The remaining groups (B, and C) served as time and vehicle controls respectively. Platelet response, platelet count, bleeding time, and clotting time were assessed at baseline, and after induction, 7 days after their assigned treatment. Arachidonic acid response, and ADP response were measured using mutiple electrode aggregometry (MEA). Scanning electron microscopy (SEM) was used to visualize changes in platelet morphology. To compare results, data were subjected to statistical analysis using One-way ANOVA and Tukey HSD post-hoc analysis. Results showed there were a 20% increase in the platelet count, a 58.7% decrease in bleeding time, and an increase in clotting time throughout E. hirta administration. MEA shows that E. hirta significantly increases platelet response to arachidonic acid.

This present work revealed that the E. hirta decoction possesses platelet-increasing property by being able to reverse the platelet destruction caused by heparin. Also, aggregometry results show that this phenomenon may be associated with the effect of E. hirta on arachidonic acid-induced aggregation.

Keywords: whole blood impedance aggregometry; scanning electron microscopy; Euphorbia hirta.

Introduction

Euphorbia hirta Linn. (Euphorbiaceae) has been implicated to be useful anecdotally for several conditions: enema for constipation, anti-emetic, anthelmintic, hemostatic, sedative, anti-asthmatic, and local irritant [1] Several experimental studies on the pharmacological properties of E. hirta have demonstrated that the aqueous and methanolic extracts of the leaves exert anti-bacterial [2], anti-inflammatory [3] antimalarial, anti-amoebic, antifungal, anti-asthmatic, diuretic, antidiarrheal, anti-oxidant, antifertility and galactogenic activity [4].

Of its many uses, E. hirta is more popularly known in the Philippines to be useful in the management of dengue fever (DF) and dengue hemorrhagic fever (DHF), particularly by increasing platelet count. This anti-thrombocytopenic effect, as well as its effect on other

hemostatic parameters (clotting time and bleeding time) had been demonstrated in many animal studies [5,6,7]. The potential use of E. hirta as an antithrombocytopenic decoction is attributable to its effect on platelet distribution and possibly to the platelet protective activity of its antioxidant polyphenolic constituents [5].

Hemorrhage is a significant pathological feature of Dengue fever, a viral inflammatory syndrome. Dengue fever is a severe febrile disease, characterized by abnormalities in hemostasis and increased vascular permeability, which in some cases, results in hypovolemic shock syndrome and Dengue Shock Syndrome (DSS) [8].

Statistics show that dengue is the most common human arbovirus infection and is responsible for thousands of deaths every year. Estimates suggest that 50 million dengue infections occur annually with up to 500,000 cases of dengue hemorrhagic fever (DHF) and at least 22,000 reported deaths [9]. Dengue virus causes dysfunction in normal hemostasis by activating the L-arginine pathway, which ultimately leads to the reduced aggregation of human platelets. This results into the complications mentioned earlier [8]. Currently, neither vaccine nor effective use of therapeutic agent is currently available for dengue viral infection [10]. The management of dengue-infected patients mainly involves supportive care of symptoms and transfusion of plasma to control internal bleeding. A patient is discharged when the absence of bleeding diathesis is established, and when an increasing trend on serial platelet count is observed. This increasing trend of platelet count has been set by the World Health Organization as a criterion for discharge of patients diagnosed with dengue infection [9].

While this practice is popular and effective, there is a fraction of people throughout the world who prefer the advantage of the natural effects that alternative medicine offer. Considering the effect of increasing platelet count, E. hirta aqueous extract has been deemed to be a possible treatment option for patients with DF and DHF.

Despite the widespread use of E. hirta, however, most of the involved molecular mechanisms of how it exactly promotes hemostasis, other than increasing the platelet count, have not been set clear. Platelet count reduction was observed in the study [11]. Platelet count determination at 1 hour after administration revealed 36.1% platelet count reduction at 100 mg/ml dose of the methanolic extract, and 19.2% reduction for 100 mg/ml of the aqueous extract. At lower doses, no significant change in platelet reduction was achieved. The authors suggested that the reduction in platelet count possibly indicates an aggregation of platelet at the site of injury forming a temporary loose platelet clumping.

In another study, aqueous extract of E. hirta exerted an inhibitory effect on platelet aggregation [12]. The effect of aqueous extract of E. hirta on platelet aggregation in Sprague-Dawley rats was measured using arachidonic acid (AA) as the inducer of aggregation. Results obtained show a significant dose dependent inhibition of platelet aggregation at a dose of 10, 30 and 100 µL (5 g dry herb/150 ml). This effect was attributed to the cyclooxygenase inhibition by E. hirta, as supported by the reduction in carrageenan-induced paw edema upon administration on mice, and the strong reduction of the release of prostaglandins I2, E2, and D2 in rat stomach. The inhibitory effect on release was comparable to that of indomethacin at a concentration of 1 µg/ml.

Another observation that brings conflict to the establishment of E. hirta's mechanism of action is the ability of polyphenols to inhibit platelet aggregation. E. hirta has been found to contain numerous polyphenolic compounds. The methanolic extract of E. hirta was found to be rich in polyphenol content (gallic acid equivalent (GAE) using the Folin-Ciocalteu method was 1496.54 ±4.03 mg per 100 g of fresh sample) and had a low IC50 value for DPPH (78.33µg) and hydroxyl scavenging (662.62 µg) [13]. In a review [14] , E. hirta was found to be rich in flavonoids (euphorbianin, leucocyanidol, camphol, quercitrin and quercitol), polyphenols (gallic acid, myricitrin, 3,4-di-O-galloylquinic acid, 2,4,6-tri-O-galloyl-Dglucose, 1,2,3,4,6-penta-O-galloyl-β-D-glucose), tannins (euphorbins A, B, C, D, E), triterpenes and phytosterols: β-Amyrin, 24-methylenecycloartenol, and β-Sitosterol. The lyophilized E. hirta extract mainly contains cathecins [5].

Catechins protect platelets from peroxidative stress and their aggregation. Platelet aggregation induced by arachidonic acid, adenosine diphospate (ADP) and epinephrine were inhibited in human subjects treated with catechin or epicatechin. The inhibitory effect was dose dependent at concentrations of 20-200 µg/mL [15].

These findings of polyphenol-mediated inhibition of platelet aggregation and the phytochemical constituents of E. hirta being mainly polyphenolic compounds, seem to be a conflict to the belief that E. hirta is effective in the management of DF and DHF patients. The antithrombocytopenic effect of E. hirta is possibly due to the platelet protective activity of its antioxidant polyphenolic constituents [5].

The feasibility of inducing dengue virus to the animal models was the limitation of this research. Heparin-induced thrombocytopenia was

used as a disease model on the Sprague-Dawley rats into which the Euphorbia hirta extract was administered.

The study was conducted to determine the effect of Euphorbia hirta (Linn.) lyophilized aqueous extract on platelet aggregation and morphology when administered for 7-day treatment by oral gavage to female Sprague-Dawley rats.

Methodology

Acquisition and Preparation of Plant Extract

The leaves of Euphorbia hirta Linn were collected from its natural habitat in San Luis, Pampanga. The plant material was brought to the National Museum for authentication.

A decoction was made by boiling 100 grams of plant material in 500 mL of water for 15 minutes. The decoction was vacuum filtered, stored in glass vials, and frozen for subsequent lyophilization at De La Salle University Chemistry Department. The percent yield was computed with the lyophilized extract. The lyophilized plant extract was then prepared for the treatment of the rat platelets subjected to heparin-induced peripheral destruction.

Methods and Materials

Heparin, and NSS were purchased from a local drugstore with a prescription. Whatman filter paper #9 from Whatman Internation Ltd. (Maidestone, England). EDTA Microtainer Tubes were purchased from medical supplies stores available in Bambang, Hirudin Double Wall Vacutainer Tubes were purchased from Germany.

Test Animals

Eighteen (18) female Sprague – Dawley rats, 6 to 8 weeks old, weighing approximately 150 to 200 grams were used in this study. The experimental animals were purchased from the Food and Drug Administration (FDA) in Alabang, Muntinlupa City.

Female Sprague-Dawley rats were used in this study since they are the preferred test animals for hematologic studies [16]. Furthermore, a larger amount of blood for sampling is obtained when this animal model is used. The rats were housed in the UST-RCNAS Animal House at Thomas Aquinas Research Complex in University of Santo Tomas, España, Manila.

The animals were acclimatized for the duration of seven (7) days. All animals were kept in a room with temperature set about 25ºC (±2ºC) and humidity at 75% (±3%). Ventilation rate will be maintained between 10-20 air changes per hour by mechanical means. Lighting is artificial (fluorescent light) and will be scheduled 12 hours of light and 12 hours of dark cycle.

After acclimatization, the test animals were divided in three groups (A, B and C) based on their assigned drug treatment. Each group was comprised of 6 rats. The table below summarizes the group assignments:

Group		Days treatment	Drug treatment
A	Experimental group	7	E. hirta (100mg/kg)
B	Vehicle control group	7	NSS
C	Time control group	7	---

Table 1: Test Animal Group Assignment

Group A was administered with lyophilized E. hirta extract. Group B was given NSS and was designated as the vehicle control group. Group C will serve as a time control and will not be given any kind of treatment to assess the platelet function of a normal rat in the absence of heparin-induced platelet destruction.

The volume to be administered by oral gavage was within the range of 20-50 mL/kg body weight per dose.

Group A: Experimental Group (Treatment with Plant Extract)

E. hirta extract 100 mg/kg was administered by oral gavage for 7 days. Concurrently, heparin 1U/g via subcutaneous (SC) administration was given to the rats for 7 days to induce platelet peripheral destruction. Blood was extracted from the group for the determination of platelet count, clotting time, platelet aggregrometry at Day 0 (baseline), Day 7 of treatment. The bleeding time was assessed by the modified Duke's method at Days 0 and 7.

Group B: Vehicle Group (Treatment with Normal saline solution)

Normal saline solution (NSS) 0.1 mL/kg was administered to the test animals by oral gavage for 7 days. Concurrently, heparin 1U/g via subcutaneous (SC) administration was given to the rats for 7 days to induce platelet peripheral destruction. Blood was extracted from the group for the determination of platelet count, clotting time, platelet

aggregometry at Day 0 (baseline) and Day 7 of treatment. The bleeding time was assessed by the modified Duke's method at Days 0 and 7.

Group C: Time Control Group

The rats in Group C will not be given any treatment for 7 days. Blood was extracted from the group for the determination of platelet count, clotting time, and platelet aggregometry at Day 0 (baseline) and Day 7 of treatment. The bleeding time was assessed by the modified Duke's method at Days 0 and 7.

Blood collection

Blood was collected from the rats by tail tipping/clipping method at the start of the experiment and on the seventh day of the treatment. No anesthesia was employed for tail tipping method (based on UST RCNAS-IACUC recommendation). Approximately 1.5 - 2 mL of blood was collected by clipping no more than 1cm off the tip of the tail with sharp scissors. No more than two tail tippings were done on the animal. The collected blood was then allowed to flow into a sterile microtainer. After extraction, a piece of sterile gauze was applied on the tipped area of the tail to allow for hemostasis.

Platelet Function Analysis

Platelet Aggregation

After blood extraction, 1.5 mL of blood was stored in a sterile double wall vacutainer for analysis of platelet aggregation using hirudin as the anticoagulant. The determination of the platelet-aggregating activity was done through the use of whole blood impedance aggregometer (Multiplate™, DynaByte) using ASPItest and ADPtest.

ASPItest

Approximately 300 µL saline 0.9%, was preheated at 37°C, to which 300 µL of the whole blood sample with the anticoagulant was added and subsequently incubated for 180 seconds. After incubation, 20 µL of ASPItest reagent (0.5 mM arachidonic acid, Dynabyte) was added. Simultaneously, the machine was activated and set to run for six minutes. Data was recorded using the provided software.

ADPtest (for hirudinized blood)

Approximately 300 µL saline 0.9%, was preheated at 37°C, to which 300 µL of the whole blood sample with the anticoagulant was added and subsequently incubated for 180 seconds. After incubation, 20 µL of ADPtest reagent (6.5 mM adenosine diphosphate, Dynabyte) was added. Simultaneously, the machine was activated and set to run for six minutes. Data was recorded using the provided software.

Platelet Count

0.5 mL of the blood collected was placed in an EDTA microtainer. The blood was analyzed at HI-PRECISION Diagnostics, Corner Del Monte/Banawe, Quezon City using a Coulter Counter.

Bleeding Time

Bleeding time was determined by using a modified Duke's method [5]. The exposed tail tip after collection of blood was utilized for the determination of bleeding time. The bleeding tail was patted with filter paper every 15 seconds until no blood is transferred to the paper. Bleeding time is defined as the time from initial spot on filter paper to the last visible spot. If necessary, the bleeding time was manually stopped after 20 minutes to prevent animal death.

Clotting Time

Clotting time was determined as follows: one drop of blood from each animal was placed on a glass slide. The blood was rubbed with a lancet every 30 seconds. Clotting time is defined as the time from rubbing to the formation of thread-like structures that cling to the lancet

Statistical Analysis

Data obtained from platelet aggregation, platelet count, bleeding time and clotting time were analyzed using One-Way Analysis of Variance (ANOVA), Tukey HSD, Box Plot Analysis and Pearson Correlation.

Results

Platelet Count Results

The plotted averages of platelet count of the treatment group against the time and vehicle controls are shown in Figure 1. After seven day induction with heparin, the vehicle control group showed a decrease in mean platelet count (742±5.42) compare to baseline (897.8±58.38). This shows that treatment with heparin at 1 IU/g was able to decrease platelet count.

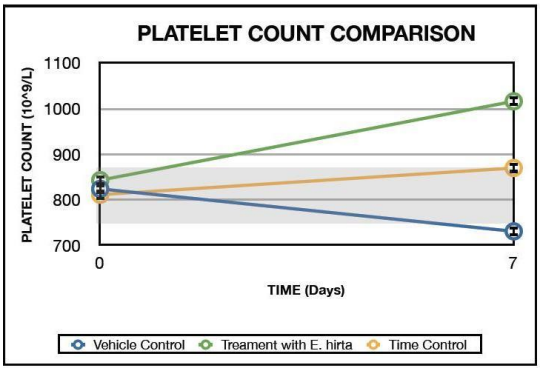


Figure 1: A line graph on the platelet count comparison between the different groups. The gray area represents the confidence limits based from the platelet count measured during baseline determination.

After seven day induction with heparin, the vehicle control group showed a decrease in mean platelet count (742±5.42) compare to baseline (897.8±58.38). This shows that treatment with heparin at 1 IU/g was able to decrease platelet count.

After seven day treatment of E. hirta with heparin, the mean platelet count (1056.47±57.14) of the treatment group showed a statistically significant increase (p= 0.02491). Furthermore, comparing this mean value from the vehicle control group (742±5.42), and time control group(830±29.44) through post hoc analysis. Tukey HSD Test for Differences Between Means, shows that the differences in the mean platelet count was statistically significant with a p value of 0.00012, and 0.00255 respectively.

Bleeding Time Results

The plotted averages of the bleeding time of treatment group against the time and vehicle controls are shown in Figure 4.2. After the seven-day subcutaneous administration of heparin, the bleeding time (823.6±18.73 seconds) increased significantly (p=0.00012) from baseline (88.33 ±18.73 seconds) for the vehicle group.

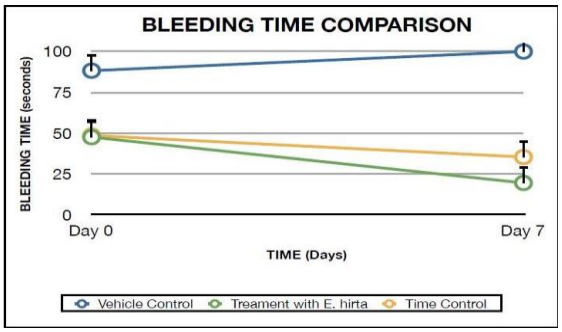


Figure 2: A line graph on the bleeding time comparison between the different groups.

After seven day treatment of E. hirta with heparin, there was a decrease in the mean bleeding time (19.67±0.63 seconds) of the treatment group from baseline (47.67±2.38 seconds).

While the time control group, which received no treatment had a decrease in mean bleeding time of (35.5±1.25 seconds) from baseline (48.67±3.71 seconds). Post-hoc analysis showed there was a significant difference in the mean bleeding time of the treatment group and the vehicle control group after treatment (p=0.0001) at day 7. While there was no significant difference (p=0.99) in the mean bleeding time of the treatment group and the time control group.

Clotting Time Results

The plotted means of the clotting time of the treatment group against the time and vehicle controls are shown in Figure 3. After induction with 1 IU/g body weight for 7 days, the mean clotting time of the vehicle control group (556.67±107.40) increased significantly (p=0.0001) compared to baseline (68.33±4.61). This mean clotting time is statistically significant compared to the time control group (p=0.00027).

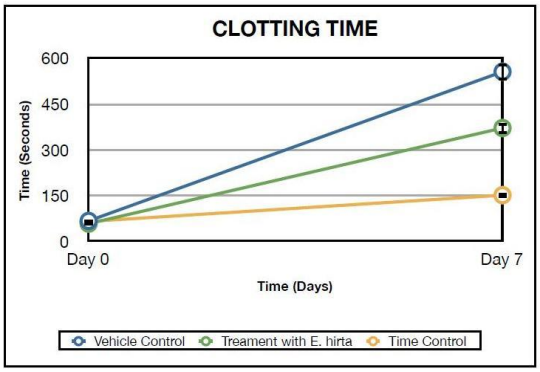


Figure 3: A line graph of the clotting time results between the different groups.

After the seven-day treatment with E. hirta and heparin, there a significant increase in mean clotting time (372±80.22 seconds) from baseline (59.33±4.29 seconds) was observed. However, post-hoc analysis, comparing the mean clotting time of the treatment group, to the vehicle control group (p=0.20) and the time control group (p=0.08), shows that there was no significant difference.

Platelet Aggregometry

ADPTest Results

From Figure 4, Seven day administration of 100 mg/kg of E. hirta causes a significant (p=0.00001) increase of the mean area under the

curve of the treatment group (68.67±2.73) from baseline (40.89±4.14). While the time control group (45.67±4.6), and the vehicle group (46.67±0.44) also had an increase of mean AUC from baseline, this increase is not statistically significant with a p value of 0.75, and 0.63 respectively.

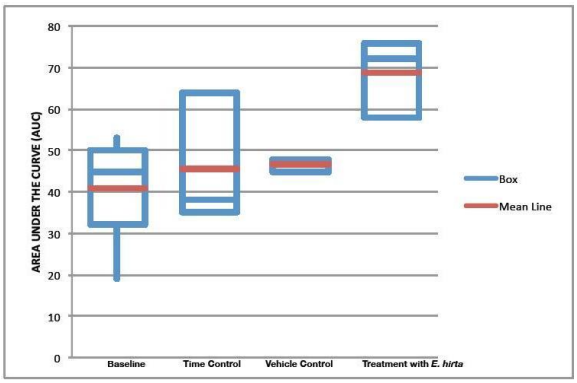


Figure 4: A box plot diagram showing the distribution and mean values of the area under the curve (AUC) of the different groups from ADPTest.

Aggregative curves from Table 2 shows that the treatment group after seven day administration of E. hirta resembles a bell-shaped curve similar to baseline, but with a larger AUC, and a higher peak when compared to the vehicle control, and time control group. Incidentally, the vehicle control group had a relatively smaller AUC, while the vehicle control had a similar AUC from baseline.

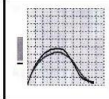
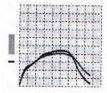
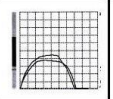
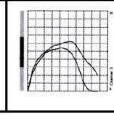
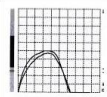
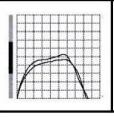
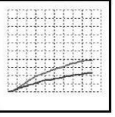
	Treatment (*E. hirta* + Heparin)	Vehicle Control (Heparin)	Time Control (w/ 80 mg ASA)	(+) Control (w/ 80 mg ASA)
Day 0				
Day 7				

Table 2: Comparison of the ADPTest aggregation curves from different treatment group using Multiplate™ Whole Blood Impedance Aggregometer.

In Table 3, E. hirta treated blood samples produced a tracing which resembles a bell-shaped curve. In contrast, the positive control clopidogrel treated blood samples produced a tracing which resembles a line, with a smaller area under the curve.

ASPITest Results

From Figure 5, Seven day administration of 100 mg/kg of E. hirta causes a significant (p=0.00017) increase of the mean area under the curve of the treatment group (82±1.5) from baseline (40.89±4.14) in the ASPITest. The vehicle control group (54.67±3.17) also had an increase of mean AUC from baseline, however this increase is not statistically significant with a p value of 0.84.

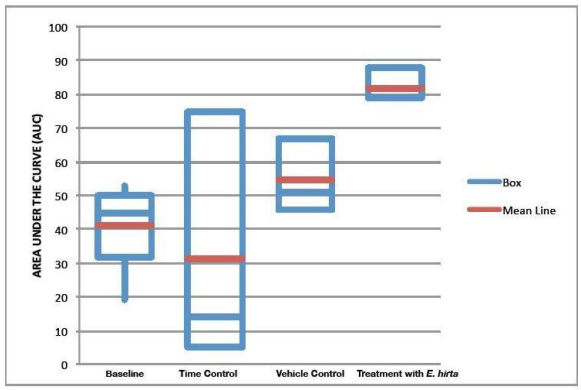


Figure 5: A box plot diagram showing the distribution and mean values of the area under the curve (AUC) of the different groups from ASPITest.

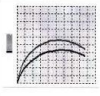
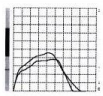
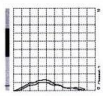
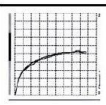
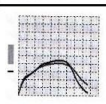
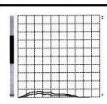
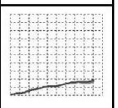
	Treatment (*E. hirta* + Heparin)	Vehicle Control (Heparin)	Time Control (w/ 80 mg ASA)	(+) Control (w/ 80 mg ASA)
Day 0				
Day 7				

Table 3: Comparison of the ASPITest aggregation curves from different treatment group using Multiplate™ Whole Blood Impedance Aggregometer.

Aggregative curves from Table 3 shows that a representative sample from the treatment group after seven day administration of E. hirta resembles a half parabola similar to baseline but of less curvature. Also, the aggregation curve of the treatment group at day 7 has larger AUC, and a higher AU when compared to a representative vehicle control, and a representative time control group. Incidentally, the representative from the vehicle control group had a relatively smaller AUC, while the representative from the time control group had a distinctly smaller curve throughout.

Blood samples treated with aspirin demonstrate a near-linear curve characterized by a low AUC, and AU. In contrast, the aggregation curves of E. hirta treated blood samples have the hallmarks of a half parabola.

Discussion

The results obtained from the platelet count comparison demonstrate that E. hirta is able to increase platelet count even with the concurrent treatment with heparin, which was able to decrease the platelet count in the vehicle control group. This shows that E. hirta was able to increase platelet count by reversing the peripheral platelet destruction initiated by heparin in Sprague-Dawley rats. The significance of these results can be directly applied to mitigate the pathophysiology of DHG. E. hirta, theoretically, can alleviate the hallmark symptom dengue, thrombocytopenia, due to decrease platelet production, and increased destruction of platelet through immune-mediated response.

Bleeding time is a crude of test of hemostasis. It indicates how well platelets interact with blood vessel walls to form blood cots. Also, the test is dependent upon an adequate number of functionally active platelets that can adhere to the endothelium to form aggregates. Thus, this is a useful test can be applied to detect qualitative defect of platelets. With the decrease in mean bleeding time observed in the treatment group despite concurrent treatment with heparin compared to the vehicle group, this reveals that E. hirta increases the ratio of the higher quality platelets compared to the lower quality platelets. Thus, an improved bleeding time resulted which was comparable to the time control values, which was a remarkable finding. This is important in DHF, because it helps increase the count of the functional platelets which are more useful in the control of bleeding episodes, therefore improving the prognosis of the disease.

Clotting time is a measure of the time required for a sample of blood to coagulate in vitro under standard conditions. This value is a reflection of the time required of the coagulation cascade to generate thrombin. Thrombin promotes platelet activation which ultimately leads to platelet plug formation. E. hirta was able to improve the mean clotting time of the treatment group undeterred by the concurrent administration of heparin, an indirect thrombin inhibitor. Since E. hirta decoction is able to improve clotting time, theoretically, it can be applied to improve the defective clotting time observed in DHF.

The combined improvements in platelet count, bleeding time, and clotting time through E. hirta demonstrates its potential application in improving hematological problems encountered in DHF due to thrombocytopenia.

Whole Blood Impedance Aggregometry shows that the E. hirta treated groups showed a mean increase in the AUC in both ADPTest, and ASPITest compared to the negative control and time control group. AUC is a direct measure of platelet activity. Thus, E. hirta was able to increase activity, providing vital details to determine its mechanism of action.

ADPTest results show that E. hirta is not a platelet inhibitor on the ADP-mediated platelet aggregation receptor. Evidence of this is when E. hirta treated blood samples produced a tracing which resembles a bell-shaped curve. In contrast, clopidogrel treated blood samples produced a tracing which is nearly linear, with a smaller area under the curve. This is because clopidogrel is an antagonist on this receptor.

ASPITest results show that E. hirta is not a platelet inhibitor on the thromboxane-mediated platelet aggregation receptor. This is because E. hirta treated blood samples produce semi-parabolic curves similar to baseline. In contrast, blood samples containing aspirin, produced an aggregation curve which is near-linear characterized by a low AUC, and AU. Aspirin, an irreversible COX inhibitor, prevents the conversion of arachidonic to thromboxane, a potent platelet agonist.

Conclusion

Given the list of findings based from the aggregation data, the possible mechanism of action in which E. hirta exerts its effect could be attributed. First, the increase in the AUC could be attributed to the higher concentration of active platelets in the initial blood sample. It has showed that E. hirta increases platelet count in both ethanol-induced, and heparin-induced thrombocytopenia [5].

Next, a possible cause for the increase in AUC could be through the decreased concentration of natural platelet inhibitory agents in samples treated with E. hirta is found in whole blood samples of the control group. Nitric oxide (NO) is a free radical that is a potent inhibitor of platelet aggregation [17] through two mechanisms: (1) the overproduction of cGMP, which desensitizes G-Protein dependent receptors such as TxA2 receptors; (2) the overproduction of cAMP, which hydrolyzes the weak agonist, ADP, to inactive AMP.

Euphorbia hirta contains phenolics that are possibly the active constituents in controlling the hematological manifestations of dengue [5]. In a separate study [18], some of this phenolic compounds includes quercetrin, myricitrin. Myricitrin also seems to be a powerful anti-oxidant, inhibiting NOS [3]. Thus, affecting the conversion of L-arginine to nitric oxide free radicals in the

endothelial cell. Furthermore, through the free radical scavenging action of flavonoids such as quercetrin, the concentration of nitric oxide free radicals in the periphery is decreased. Thus, explaining the cytoprotective mechanism of E. hirta.

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