

Anti-platelet effect of *Bryophyllum Pinnatum* Aqueous extract in human blood

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ABSTRACT

Background: *Bryophyllum pinnatum* has demonstrated anti-inflammatory effect through arachidonic acid pathway. Blockage of arachidonic acid pathway causes reduced production of thromboxane A₂, which may be responsible for its possible anti-platelet effect.

Aim: To compare the effect of *Bryophyllum pinnatum* aqueous extract on platelet aggregation in vitro, by using arachidonic acid, thrombin and ADP as agonists.

Study design: Experimental Study

Setting: This study was conducted in Hematology Laboratory of Children Hospital, Lahore and Pharmacology Department of Post Graduate Medical Institute, Lahore.

Method: Twenty healthy volunteers aged between 24-35 years from both genders, were selected from patient's attendants and staff of Children Hospital. Their blood samples were evaluated for platelet count. Platelet rich plasma was prepared and platelet aggregation was checked with three different concentrations of *Bryophyllum pinnatum* by using ADP, arachidonic acid and thrombin. The data collected was processed by using Statistical Package for Social Sciences (SPSS 20), using one way ANOVA followed by post hoc Tukey's test. A *p* value of ≤ 0.05 was considered significant.

Results: This study demonstrated *in vitro* anti-platelet effect of *Bryophyllum pinnatum*, which was not dose dependent. Inhibition of platelet aggregation was marked with arachidonic acid, minimal with thrombin and negligible with ADP.

Conclusion: The possible mechanism of anti-platelet effect of *Bryophyllum pinnatum* is mainly through arachidonic acid pathway and partially through thrombin inhibition.

Key words: *Bryophyllum pinnatum*, Platelet aggregation, Arachidonic acid, Thrombin, ADP

INTRODUCTION

Normal hemostasis of blood depends on formation of primary platelet plug at site of endothelial injury. Collagen is exposed at sites of endothelial injury, which initiates platelet plug formation, leading to formation of fibrin-containing thrombi. In certain pathological conditions, blood vessels are occluded by this platelet plug leading to disorders like cerebro-vascular accidents, cardiac ischemia and peripheral vascular diseases.¹ In view of this sequence of events, the incidence of acute ischemic cardiovascular accidents can be prevented by inhibition of platelet aggregation². For this purpose, different agents are being used, of which aspirin is the most important and frequently used. Other agents like Cangrelor, clopidogrel, prasugrel, ticagrelor, and vorapaxar have been compared with aspirin to check benefits versus side effects of these agents in patients with coronary atherosclerosis³. Even after discovery of multiple anti-platelet agents there is always room for newer, safer and more efficient agents. In this background, herb called *Bryophyllum pinnatum* was evaluated for its effect on platelet aggregation.

Bryophyllum pinnatum is a small erect plant that belongs to family Crassulaceae.⁴ Its other common names are *Kalanchoe pinnata*, "pathar chat" in Urdu, air plant and

life plant in English⁵. This plant attains a height of 1- 1.5 meter. The thick leaves are 5 to 6 cm wide with curved margin⁶.

Anti-inflammatory effects of *Bryophyllum pinnatum* are known for centuries. It is used traditionally, as hemostatic, topical preparation for migraines and headaches, and given orally for urethritis, fever, arthritis, internal bruises, fractures, and respiratory inflammations.⁷ In a recent study, ear edema was induced in Swiss mice by using different irritants like croton oil, capsaicin, arachidonic acid and phenol. The results showed that *Bryophyllum pinnatum* leaf extract effectively reduced edema, epidermal hyperplasia, vasodilatation and inflammatory cell infiltration possibly by inhibition of arachidonic acid pathway.⁴ Non-steroidal anti-inflammatory drugs act by same mechanism and inhibit platelet aggregation. So, it was postulated that *Bryophyllum pinnatum* may also inhibit platelet aggregation and to check this hypothesis, effect of *Bryophyllum pinnatum* leaf extract was studied on platelet aggregation, using light transmission aggregometer (LTA).

MATERIALS AND METHODS

Leaves of *Bryophyllum pinnatum* were collected and identified by the Botany Department of University of Punjab, Lahore. Leaves of *Bryophyllum pinnatum* were washed with tap water then dried under shade. They were crushed manually and immersed in distilled water in 1:10

Received on 26-08-2018

Accepted on 02-01-2019

(w/v) for 24 hours.⁸ Whatman filter paper no.1 was used to filter supernatant, which was dried in air to powder form. The dry extract was weighed and kept at 4°C. Different concentrations of *Bryophyllum pinnatum* i.e. 30mg/ml, 100 mg/ml and 300 mg/ml were prepared daily using normal saline⁹.

Twenty normal healthy volunteers of both genders were selected from volunteer blood donors at blood bank of Children Hospital, Lahore. Individuals of age group 18-35 years, with normal platelet count ($150-400 \times 10^3/\mu\text{L}$) were included in study after taking informed consent. Subjects were asked to refrain from coffee, caffeinated beverages and exercise at least two hours before blood sampling. Exclusion criteria were history of bleeding disorder, pregnancy, any existing illness; and intake of any drug affecting platelet aggregation in past two weeks.

After a loose tourniquet, 10 ml of blood was drawn from the antecubital vein. Two ml was put into EDTA vacutainer and rest was taken in four 3.2% sodium citrate vials. All blood samples were processed within two hours of collection. Complete blood count of sample in EDTA vacutainer was performed by using automated hematology analyser. Samples with haemoglobin greater than 10 g/dl and normal platelet count were selected for further processing. Clotted or hemolysed samples were discarded. Platelet rich plasma (PRP) was prepared centrifuging citrated whole blood at 1500 rpm for 5 minutes at room temperature. Aim was to concentrate platelets from $150-400 \times 10^9 /\text{L}$. Platelet count was confirmed by automated hematology analyser.

Light transmission aggregometer (LTA) was used to check platelet aggregation *in vitro* by using arachidonic acid, thrombin and ADP as agonists. The concentration of arachidonic acid, thrombin and ADP were 0.5 mM, 1 unit/ml and 10 μM respectively.¹⁰ Four microcuvettes were taken and were labelled as C, BL, BM and BH for Control, *Bryophyllum pinnatum* low, medium and high concentration respectively and 245 μL PRP was then taken in all the microcuvettes. Normal saline 2.5 μL was added in control, whereas 2.5 μL of different concentrations of *Bryophyllum pinnatum* were added in BL, BM and BH¹¹. All microcuvettes were incubated for 30 minutes at 37°C¹². Aggregation was induced by adding arachidonic acid 2.5 μL , making final concentration 0.5mM (per chrono log USA arachidonic acid kit leaflet). Reading was then taken for 3 minutes and resulting percentage aggregation was recorded on graph papers.¹³ This process was repeated using 2.5 μL of thrombin making final concentration 1 unit/ml and 2.5 μL of ADP for a final concentration of 10 μM .

Statistical analysis: The data collected was processed by using SPSS 20. It was checked for normal distribution by using Shapiro-wilk test of normality. Platelet aggregation and inhibition was presented as mean \pm standard deviation (SD). One way ANOVA was used to test significance of difference between effects of three concentrations of *Bryophyllum pinnatum* leaf extract as well as between different agonists, followed by post hoc Tukey's test p value of ≤ 0.05 was considered significant, ≤ 0.01 highly significant and ≤ 0.001 very highly significant.

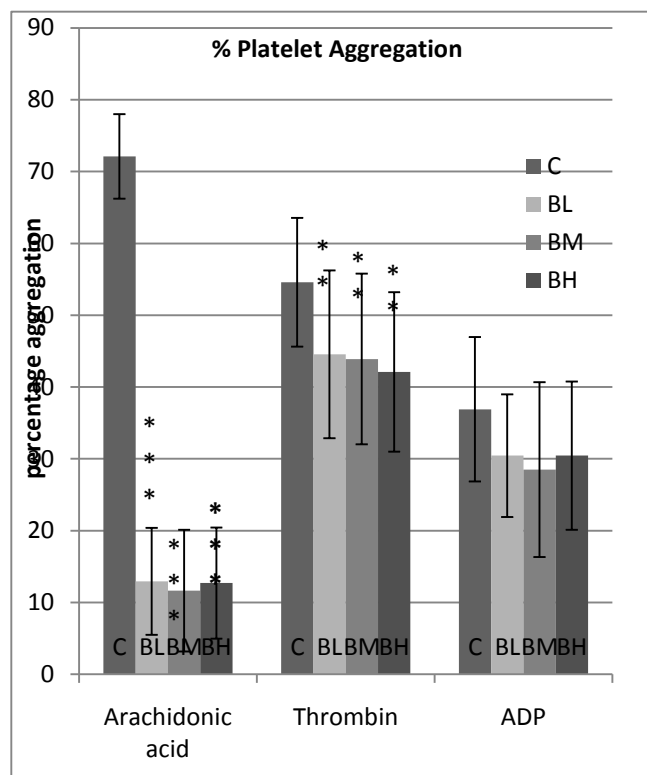
RESULTS

Blood sample from 20 subjects were collected. Male to female ratio was 1:1. Mean \pm SD age of the subjects was

28.85 ± 3.29 years with range of 24 to 35 years. Mean \pm SD haemoglobin level was 13.29 ± 2.0 g/dl and mean \pm SD platelet count was $263.2 \pm 45.18 \times 10^3/\mu\text{L}$.

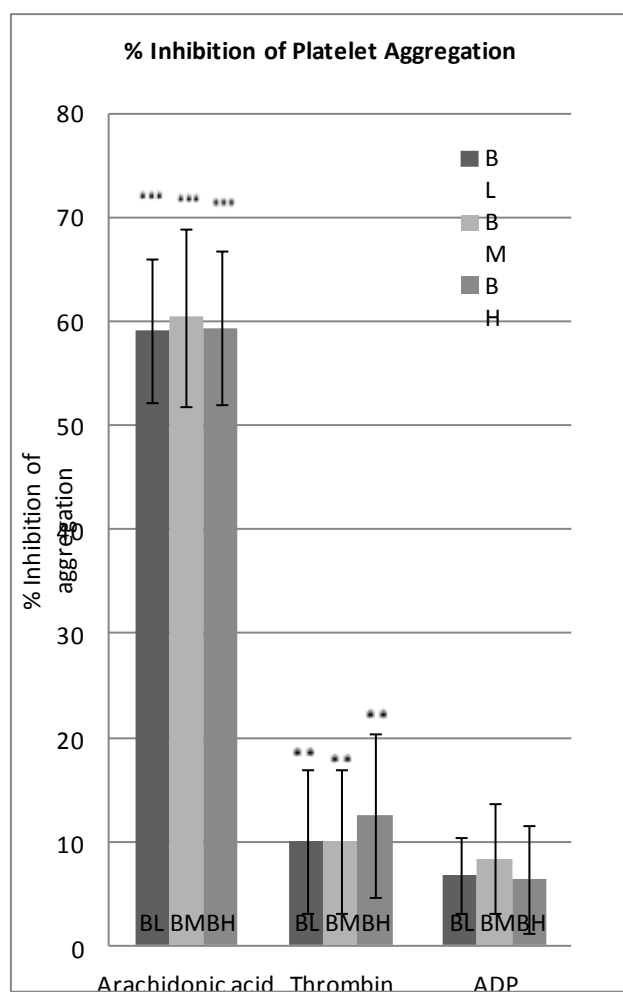
Figure 1 shows effect of different concentrations of *B. pinnatum* on platelet aggregation by using different agonists. One way ANOVA revealed significant difference with arachidonic acid and thrombin with p value of 0.001 and 0.01 respectively, while it was insignificant with ADP as agonist with p value of 0.061. Difference among various concentrations of *B. pinnatum* was insignificant on post hoc analysis.

Inhibition of platelet aggregation was calculated by subtracting value of platelet aggregation with each treatment from control value. Figure 2 Shows bar chart of inhibition of platelet aggregation. By applying one way ANOVA, difference among different concentrations of *B. pinnatum* was not significant with any agonist, while it was significant between different agonists with P value < 0.001 . Multiple comparison revealed that *B. pinnatum* extract inhibited platelet aggregation induced by arachidonic acid more as compared to that induced by thrombin and ADP (p value < 0.001), while inhibition of thrombin induced platelet aggregation was more as compared to that by ADP (p value < 0.01). Effect of different concentrations of *Bryophyllum pinnatum* on platelet aggregation (mean \pm SD) by using different agonists ($n=20$).



*** p value ≤ 0.001 vs control ** p value ≤ 0.01 vs control, C: Control
BL: *Bryophyllum pinnatum* (Low concentration)
BM: *Bryophyllum pinnatum* (Medium concentration)
BH: *Bryophyllum pinnatum* (High concentration)

Effect of different concentrations of *Bryophyllum pinnatum* on inhibition of platelet aggregation (mean \pm SD) by using different agonists ($n=20$)



***p value ≤ 0.001 versus thrombin and ADP C ----- Control
 ** p value ≤ 0.01 versus ADP, C

BL: *Bryophyllum pinnatum* (Low concentration)

BM: *Bryophyllum pinnatum* (Medium concentration)

BH: *Bryophyllum pinnatum* (High concentration)

DISCUSSION

The present study demonstrated the effect of *Bryophyllum pinnatum* on platelet aggregation *in vitro* using arachidonic acid, thrombin and ADP as agonist by use of light transmission aggregometer¹⁴.

LTA is used to check platelet function *in vitro*. Microcuvettes filled with platelet rich plasma (PRP) are placed between the light source and photocell at 37^o C. Addition of an agonist leads to glycoprotein IIb/IIIa mediated platelet to platelet aggregation, changing shape from discoid to tiny spheres. This causes reduction of absorption of light, resulting increase in light transmission is detected by a photocell. Agents that cause physiologically activation of platelets *in vivo* include thromboxane A2 (TXA2), thrombin, adenosine diphosphate (ADP), epinephrine, serotonin and collagen. Platelets have receptors for each of these agonists and a major integrin receptor $\alpha 2\beta 3$ integrin, also known as glycoprotein IIb/IIIa¹⁵.

Arachidonic is the key agent required for platelet aggregation. It produces thromboxane A (TXA2) which in

turn mobilizes calcium and induces platelet aggregation.¹⁶ Platelet aggregation with arachidonic acid as agonist was 12.95% with BL, 11.65% with BM and 12.70% with BH. It was statistically significantly lower as compared to control which was 72.10%. In our study, platelet inhibition by using arachidonic acid as agonist was 59.15% with BL, 60.45% with BM and 59.40% with BH. In similar study by Miroslava *et al.*, (2014), mean platelet aggregation was reduced to 37% with aspirin in a study group, which consisted of 30 people with documented ischemic stroke at least over a month.¹⁵ Zhao *et al.*, in 2017 used atorvastatin caused inhibition of platelet aggregation in 126 healthy individuals by similar mechanism¹⁷.

Thrombin was another agent used in our study, which causes platelet aggregation. Hung *et al.* (1992) used polyclonal antiserum to inhibit thrombin-induced platelet activation.¹⁸ They demonstrated that there is a potential thrombin receptor on platelets as a potential target for use of anti-platelet therapy. Ku *et al.*, 2015 inhibited platelet aggregation by using aspalathin and nothofagin by inhibiting thrombin. Vascular injury induces generation of α -thrombin which in turn causes platelet aggregation and thrombus formation.¹⁹ Platelet aggregation by α -thrombin is mediated by protease activated receptors (PARs). A glycoprotein (Iba) is the most numerous and most common thrombin binding site on platelets²⁰.

In our study, platelets aggregation with use of thrombin as agonist was 44.5% with BL, 43.9% with BM and 42.1% with BH, which was statistically significantly lower as compared to 54.6% in control. Similarly, platelet inhibition by using thrombin as agonist was 10.05% with BL, 10.07% with BM and 12.5% with BH.

Third agonist used in our study to induce platelet aggregation was ADP. Platelet aggregation mediated by ADP is via two G-protein-coupled purinergic (P2) receptors, namely P2Y1 and P2Y12. The P2Y1 receptor activates phospholipase C and cause platelet aggregation through calcium mobilization.²¹ Platelet aggregation was 30.45% with BL, 28.5% with BM and 30.45% with BH, which were not statistically significantly lower as compared to 36.9% with control. Similarly, platelet inhibition by using ADP as agonist was 6.45% with BL, 8.40% with BM and 6.45% with BH.

To the best of our knowledge, this is the only *in vitro* study; conducted on human blood using *Bryophyllum pinnatum*. This study has demonstrated important aspects regarding *Bryophyllum pinnatum*. First, that *Bryophyllum pinnatum* has anti-platelet effect. Secondly, we checked effect of three different concentrations of *Bryophyllum pinnatum* on platelets and found that its anti-platelet effect is not concentration dependent. Third, the anti-platelet effect is highest against arachidonic acid as compared to thrombin and ADP and lowest with ADP. In future phytochemicals should be isolated and studied for anti-platelet effect. Ex vivo studies will be helpful in determining optimum dose and duration of action.

CONCLUSION

This study demonstrates *in vitro* anti-platelet effect of *Bryophyllum pinnatum*, which is not dose dependent. The possible mechanism of anti-platelet effect of *Bryophyllum pinnatum* is mainly through arachidonic acid pathway and partially through thrombin inhibition.

Acknowledgement: Funding was provided by PGMI, Lahore. Cooperation of donors and laboratory staff is appreciated.

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