ORIGINAL ARTICLE

EFFECT OF *JUSTICIA ADHATODA* (MALABAR NUT) LEAF EXTRACT ON DRUG-INDUCED COAGULOPATHY IN MICE AND IN-VITRO PLATELET AGGREGATION OF HUMAN BLOOD

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Background: *Justicia adhatoda* is widely used in traditional medicine for treatment of menorrhagia, piles and bleeding disorders. Oral antiplatelet and anticoagulant drugs are routinely prescribed to patients with cardiovascular diseases. These drugs have one major adverse effect that they can cause spontaneous haemorrhage, which can be fatal. Development of a haemostatic agent can help in effective management of drug-induced haemorrhages. This study was devised to observe the effect of leaf extract of *Justicia adhatoda* on coagulation profile in mice and to evaluate its effect on in-vitro platelet aggregation. **Methods:** The study was divided into two parts. First part was designed to evaluate the effect of *J. adhatoda* leaf extract on coagulation parameters. Three drugs were used to induce coagulopathy viz., warfarin, aspirin and dabigatran. Bleeding time, platelet count, PT and APTT were estimated. Second part of this study was devised to observe the effect of *J. adhatoda* leaf extract on in vitro platelet aggregation of human. Percent aggregation was recorded by light transmission aggregometer for three minutes. **Results** Leaf extract of *Justicia adhatoda* decreased bleeding time from 6.1±2.36 minutes in normal control to 1.9±1.03 minutes in extract treated mice. There was no effect on the coagulation parameters. Platelet count increased significantly only in the aspirin treated group that received the extract to 540±46.8x10^3 /µl from 436.9±37.9x10^3 /µl of aspirin treated group. Platelet aggregation *in vitro* increased in a dose dependent manner. **Conclusion:** *Justicia adhatoda* leaf extract is effective in controlling excessive bleeding *in vivo,* in mice with acquired platelet defect produced by aspirin. This haemostatic effect is probably due to increased platelet aggregation as indicated by the *in vitro* results.

Keywords: Haemostasis; *Justicia adhatoda*; Platelet aggregation; Coagulation; Coagulopathy

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INTRODUCTION

Oral anticoagulant and antiplatelet drugs are commonly used for the prevention and treatment of cardiovascular disorders. They are prescribed commonly in patients with atherosclerotic diseases, deep vein thrombosis, valvular heart diseases and atrial fibrillation. Unfortunately, all the anticoagulants can cause haemorrhagic complications in patients.[1] Until recently, warfarin was the most commonly used drug for venous thromboembolic events. However, constant monitoring and risk of haemorrhage has prompted the introduction of newer drugs such as dabigatran.[2] Although newer agents are safer to use, but their most important drawback is the lack of antidote for reversal of anticoagulation. Hence, increased risk of haemorrhage in patients receiving anticoagulant therapy is a major concern, requiring rapid restoration of hemostasis.[3]

Anticoagulant drugs such as warfarin and dabigatran act by blocking one or more steps of the coagulation pathway, finally preventing the formation of fibrin meshwork and hence, formation of clot. Antiplatelet drugs inhibit the process of platelet adhesion, degranulation or aggregation.[4] Irrespective of the mechanism, all these drugs increase the tendency of bleeding by interfering with the process of hemostasis.[5] The annual incidence of haemorrhage during oral anticoagulant therapy is 2–5% for major bleeding, 0.5–1% in cases of fatal bleeding, and 0.2–0.4% for intracranial bleeds.[6]

Bleeding due to warfarin therapy can be controlled by nonspecific measures such as FFPs (fresh frozen plasma) or PCC (prothrombin complex concentrates). However, these are expensive and sometimes not readily available. Vitamin K acts as an antidote to warfarin but it takes at least 4–6 hours for reversal.[7] Dabigatran is a relatively safer drug as compared to warfarin

and has a lower incidence of bleeding, but currently there is no effective antidote present to reverse its effects in cases of emergency.[8] Similarly, there is no antidote available for aspirin. In case of accidental injuries in patients on aspirin therapy, platelet transfusion, desmopressin and other factors have been tried to reverse the antiplatelet effect, but with moderate success. This strongly supports the need to develop an effective agent to reliably reverse the anticoagulant and antiplatelet effects of drugs in case of emergencies.[9]

Justicia adhatoda belongs to the Family Acanthaceae. It is a perennial, evergreen and highly branched shrub with an average height of 1–2.5 meters. It has opposite ascending branches with pink, white or purple flowers. It has an extremely unpleasant taste and a bitter smell. The plant grows in abundance in the Pothohar region of Pakistan. It is also grown in various regions of India, Nepal, Sri Lanka and Malaysia.[10,11]

The leaves of the plant are rich in several alkaloids such as vasicine, adhatodine, adhavasinone, saponins, flavonoids, tannins, poly-phenols, sterols, glycosides and metabolites such as carbohydrates, steroids and alkanes.[12] Leaf juice and extract has been used for many years, in folk medicine, for treatment of conditions like asthma, bronchitis, tuberculosis, external or internal bleeding such as peptic ulcers, piles, bleeding gums and menorrhagia.[13]

Currently, there are very limited options available for management of bleeding emergencies due to drugs or other causes. Also, the available treatments are either non-specific or extremely expensive. Besides this, *J. adhatoda* is a component of various herbal formulations used for cough remedies. If the haemostatic effect of this herb is demonstrated, it could lead towards awareness of a potential adverse interaction for patients taking anticoagulant or antiplatelet drugs.

The aim of this experimental study was to establish the effect of the leaf extract of *Justicia adhatoda* on coagulation profile and platelet aggregation.

MATERIAL AND METHODS

Study was conducted after approval of ethical committee of PGMI, Lahore for animal study. For in-vitro platelet aggregation of human blood, ethical approval was obtained from Children Hospital, Lahore.

Justicia adhatoda leaves (260 grams) were collected from Bagh-e-Jinnah, Lahore. They were identified from the Department of Botany, GCU, Lahore. After washing thoroughly with water, the leaves were dried at the room temperature. The weight of dry leaves became constant (60 grams) after three days. Coarsely powdered leaves were immersed in ethanol (1:10 w/v ratio) for a week. The supernatant was filtered with the filter paper (Whatman) twice. The filtrate was allowed to be dried at room temperature. The crude form of extract was then weighed (9 grams) and stored at 4 ºC temperature.[14]

Swiss albino mice within the weight range of 25–35 grams were chosen and housed in the animal house of Postgraduate Medical Institute, Lahore. They were provided with optimum nutrition and hygiene. The animal handling criteria given in 'Guide for the care and use of laboratory animal'[15] was duly followed.

Coagulopathy in mice was induced using three different drugs viz warfarin, aspirin and dabigatran. Powdered warfarin (Shaigan Pharmaceuticals (Pvt) Ltd) was dissolved in distilled water. It was administered by oral route, once daily, at a dose of 20 mg/kg for three consecutive days.[16] Aspirin powder (Schazoo Pharmaceuticals (Pvt) Ltd), dissolved in distilled water, was administered orally as a single daily dose (120 µg/day) for three consecutive days.[17]

Dabigatran capsules (Boehringer Ingelheim Pharmaceuticals, Inc.) were opened. Granules were ground to powder form. The powder was mixed in distilled water to form a suspension. Single dose of 30 mg/kg was administered by the oral route.[16]

J. adhatoda leaf extract was dissolved in normal saline. It was administered to the mice intraperitoneally at a dose of 200 mg/kg. This dose was selected after conducting a pilot study to determine the dose-response relation of three different concentrations of extract on bleeding time in mice. The leaf extract was administered twenty-four hours after the last doses of warfarin and aspirin, and 75 minutes after single dabigatran dose.[16]

Sixty-four animals were divided into eight groups, with eight animals in each group. Group A (normal control) was given distilled water orally for three days, followed by intraperitoneal injection of normal saline on fourth day. Group B was experimental control, orally receiving distilled water for three days, followed by intraperitoneal leaf extract administration on fourth day. Group C and D received warfarin by oral route for three days. Group E and F were given oral aspirin solution for three days, while Group G and H received single dose of dabigatran, orally. On the fourth

day, Group C, E and G were injected with normal saline intraperitoneally, whereas Group D, F and H received intraperitoneal leaf extract.

After one hour of administration of leaf extract or normal saline, animals were anesthetized. Bleeding time was estimated by tail transection. Blood sample was collected by cardiac puncture. Platelet count was calculated manually with improved Neubauer's chamber.[18] Prothrombin time and activated partial thromboplastin time were estimated with the Sysmex CA-660 coagulation analyser.

Six healthy human subjects, within the age group of 24–37 years, from both genders were randomly chosen from staff of Children Hospital, Lahore. Informed consent was obtained from the participants. History was taken and general physical examination was performed according to inclusion and exclusion criteria.

Ten ml of blood was drawn from ante cubital vein of each subject. Two ml was transferred to the EDTA vacutainers for estimation of complete blood count by haematology analyser. Two ml each was put in four vials containing 3.2% sodium citrate.[19] Platelet rich plasma (PRP) was prepared by centrifuging citrated whole blood at 500 rpm for duration of 15 minutes at 37 ^{o}C temperature, according to the standard laboratory practice at Children Hospital. Platelet count of PRP was measured again by haematology analyser. Obtained PRP was transferred to capped plastic tubes and kept at room temperature for half an hour before further processing.[20]

Platelet rich plasma was then incubated with normal saline and three different concentrations of *Justicia adhatoda* leaf extract for 30 minutes. The respective concentrations (30, 100, and 300 µg/ml)[21] were labelled as low dose (JL), medium dose (JM) and high dose (JH). Arachidonic acid was used to induce platelet aggregation. Percent aggregation was recorded by light transmission aggregometer (LTA) for three minutes.

All the obtained data was transcribed into GraphPad Prism 5 for statistical analysis. Shapiro Wilk test was applied to check the normality. The numerical data was represented as descriptive statistics, i.e., Mean±SD in tables and Mean±SE in graphs. It included bleeding time, platelet count, PT, APTT and platelet aggregation. Analysis of Variance (ANOVA) and *post hoc* Tukey's test were applied. p-value of <0.05 was taken as significant.

RESULTS

There was an increase in the bleeding time in groups that received drug treatment only (C, E and G) while a decrease was observed in drug + extract-treated groups (B, D and F), as compared to normal control. Bleeding time decreased significantly after extract administration in mice treated with warfarin and aspirin (C vs. D and E vs. F), having a p-value of <0.001 and <0.01, respectively (Figure-1).

Numerically, the platelet count was higher in the extract-treated groups in comparison to the groups that were not given the extract. However, platelet count raised significantly in group treated with aspirin + extract (F), as compared to the group that was given aspirin only (E), having a p-value <0.05 (Figure- 2).

Prothrombin time increased significantly in groups that received warfarin and dabigatran, in comparison to the normal control, with a p-value <0.001, each. However, no significant difference was observed between the means of groups that received the leaf extract (Figure-3).

Activated partial thromboplastin time raised significantly in dabigatran treated groups (G and H), with respect to normal control, having a p value < 0.001 each. However, no significant decrease in APTT was seen in extract treated groups B, D, F and H (Figure-4).

There was no significant increase in platelet aggregation in low dose extract group (JL) with respect to the control group. Percent aggregation raised significantly in medium dose (JM) and high dose (JH) groups in comparison to normal control and JL (p-values < 0.001 each). It also increased significantly in JH as compared to JL and JM, having p-value <0.001 and <0.01, respectively (Figure-5).

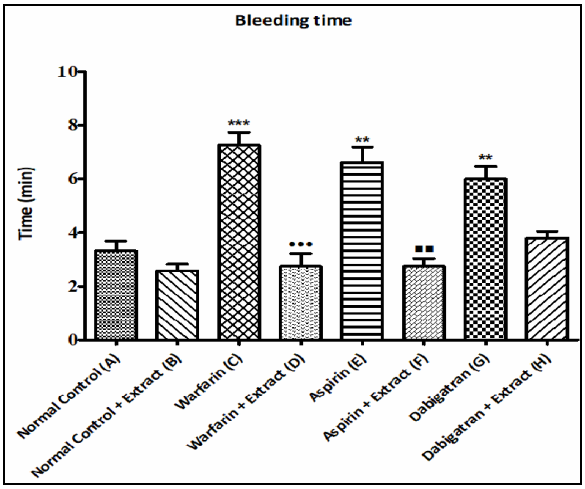

Figure-1: Effect of *Justicia adhatoda* leaf extract on bleeding time (mean ± SE) in mice (n=8)

** = p<0.01 (vs. normal control), *** = p<0.001 (vs. normal control). ●●● = p<0.001 (vs. warfarin alone), ■■ = p<0.01 (vs. aspirin alone)

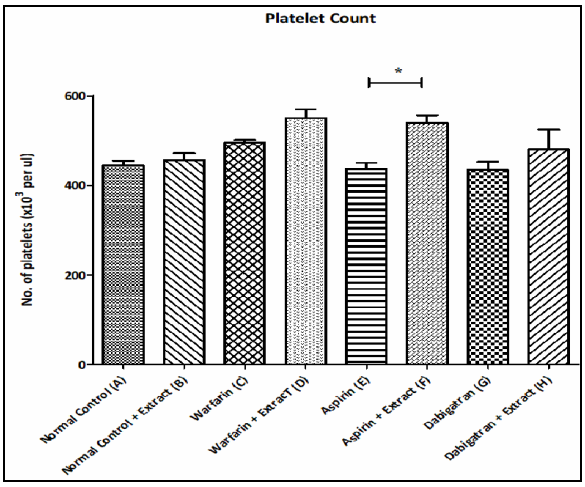

Figure-2: Effect of *Justicia adhatoda* leaf extract on platelet count (mean±SE) in mice (n=8)

* = $p<0.05$ (aspirin + extract vs. aspirin alone)

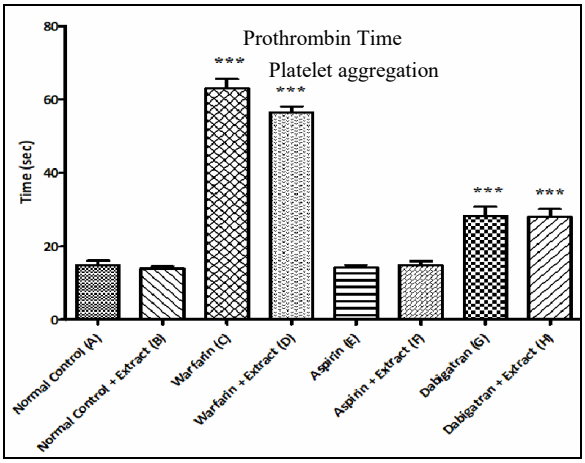

Figure-3: Effect of *Justicia adhatoda* leaf extract on prothrombin time (mean ± SE) in mice (n=8)

***= $p<0.001$ (vs. normal control)

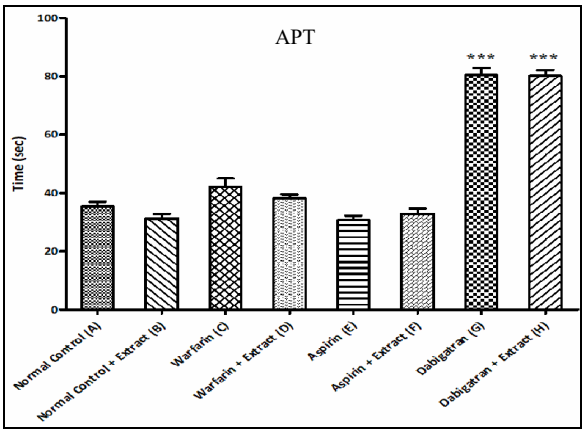

Figure-4: Effect of *Justicia adhatoda* leaf extract on APTT (mean ± SE) in mice (n=8)

*** = $p<0.001$ (vs. normal control)

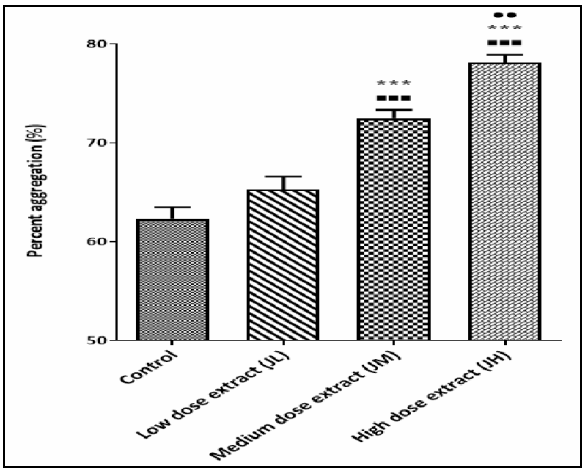

Figure-5: Effect of three different concentrations of *J. adhatoda* on in-vitro platelet aggregation (mean ± SE) in human subjects (n=6)

***= $p<0.001$ (vs. control), ■■■ = $p<0.001$ (vs. JL), ●● = $p<0.01$ (vs. JM)

DISCUSSION

Justicia adhatoda is commonly used for management of bleeding in folk medicine.[13] This experimental study was devised to scientifically demonstrate the effect of *J. adhatoda* leaf extract on coagulation and platelet aggregation.

There was a dose dependent increase in platelet aggregation with increasing concentrations of the extract.

In case of in vivo coagulation study, drugs used to induce coagulopathy were administered orally to mimic routine clinical practice. Intraperitoneal route was selected to administer the extract to assure fast absorption and rapid restoration of hemostasis, as required in emergencies. Platelet count was manually estimated with a hemocytometer because of scarcity of sample. On average, 1.5–2.0 ml of blood was collected from each animal by intra cardiac route. This was transferred to the citrated vacutainers. Therefore, sufficient volume of blood was not available (at least 1.5 ml more) to add sample into EDTA tubes for automated haematology analysis. Due to scarcity of funds and resources, repeated administration of extract, for longer period of time, could not be carried out to assess the time duration for which the haemostatic effect of *J. adhatoda* persists.

The effect of *Justicia adhatoda* on haemostasis has not been demonstrated by any experimental study so far. Therefore, similar studies of other compounds, with proven effects on haemostasis, were used to compare with the results of the current study.

Leaf extract of *Justicia adhatoda* decreased bleeding time in mice. This was comparable with the effect of Ankaferd Blood Stopper (ABS) on tail

bleeding time in rats (p-value =0.001).[22] Similarly, bleeding time decreased after Antarctic krill chitosan powder administration in mice, having p-value <0.005.[23]

A significant increase in platelet count was observed only in the group that was given aspirin+extract (vs. aspirin only group). Scientifically, formation of new platelets from the megakaryocytes cannot take place within an hour of extract administration. Thus, the observed increase in platelet count could be because of release of preformed pro platelets from bone marrow.[24] It may be possible that long term, repeated extract administration produces a statistically significant rise in platelet count, as demonstrated by Atal *et al.*[25]

There was no significant change in PT and APTT. These results are contradictory to the work done by Jiao *et al.* which demonstrates a significant decrease in PT and APTT by *Lagochilus lanatonodus,* as compared to normal control.[26] Similarly, results of the study performed by Lambourne *et al.* are in contrast to this study, demonstrating effective reduction in PT after administration of fresh frozen plasma (FFP) and prothrombin complex concentrates (PCC) in warfarin treated mice, and APTT after administration of recombinant Factor VII in dabigatran treated mice.[16]

A dose dependent increase in platelet aggregation was observed, in comparison to control group. This increase was statistically significant at medium dose (p<0.001) and high dose (p<0.001) as compared to the control group. Moreover, maximum aggregation was demonstrated at the highest dose of extract, with respect to medium (p<0.01) and low concentrations (p<0.001).

Platelet aggregation is a vital part of blood coagulation. During platelet activation, phospholipase A_2 releases arachidonic acid from the platelet membrane phospholipids.[27] Circulating platelets express cyclooxygenase-1 (COX-1) that metabolizes arachidonic acid into unstable intermediates, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2). These are, in turn, converted to several bioactive prostaglandins, including thromboxane A_2. Prostaglandin G_2, prostaglandin H_2 and thromboxane A_2 cause vasoconstriction and promote platelet aggregation, thereby facilitating the process of hemostasis.[28]

Increase in platelet aggregation produced by *J. adhatoda* leaf extract may be attributed to the release of prostaglandins. This is supported by Atal and Chandhoke, who suggested that uterine stimulating and abortifacient effect of leaf extract of *J. adhatoda* might be due to the enhanced production and release of prostaglandins.[29] It is further strengthened by the study conducted by Chandhoke, in which it was demonstrated that administration of aspirin inhibited the abortifacient effect of *J. adhatoda* leaf extract, possibly by inhibition of prostaglandin release.[30]

Summing up the results of all parts of this study, following observations have been made. Firstly, leaf extract of *J. adhatoda* decreases bleeding time. Although this decrease is dose dependent numerically, but it was not significant statistically. Secondly, this reduction in bleeding time is not associated with interference with coagulation cascade as it has not shown any effect on the coagulation parameters. Thirdly, the increase in platelet count was significant only in the aspirin treated group that received the extract (vs. aspirin alone group). Finally, leaf extract has demonstrated dose dependent induction of platelet aggregation.

CONCLUSION

In the light of results of the present study, it can be concluded that leaf extract of *J. adhatoda* has potential to be used as a haemostatic agent. It accelerates haemostasis by inducing platelet aggregation, possibly by increasing the synthesis and release of prostaglandins. Nevertheless, further studies are required to scientifically demonstrate this proposed mechanism.

AUTHORS' CONTRIBUTION

SZ: Concept and design, data acquisition and interpretation, literature search, manuscript writing. MM: Data interpretation, literature search, manuscript writing. AMR: Data acquisition and statistical analysis. NR: Literature search, statistical analysis. SC: Critical revision, proof reading. KZ: Data collection and animal handling.

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