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Research Article

In Vitro Antiplatelet Aggregation Activity of Aqueous and Alcoholic Leaf Extracts of *Azadirachta indica*

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Abstract

Azadirachta indica considered as 'kalpavriksh of kalyuga' has diverse pharmacological properties. The present study was carried out to assess the *in vitro* antiplatelet aggregation activity of *Azadirachta indica* leaf extracts by platelet aggregation assay using platelet rich plasma (PRP). Aqueous and alcoholic leaf extracts (AILE) were prepared and the percentage inhibition of platelet aggregation was examined and compared with standard drug aspirin. The studies revealed that both the extracts dose dependently inhibited platelet aggregation. The effect was more pronounced in alcoholic extract (45.8%) than aqueous extracts (25%). These results suggest that phytoconstituents of leaf extract exerted antiplatelet aggregation activity. However further studies to identify the active ingredient and their molecular mechanism needs to be explored.

Keywords: *Azadirachta indica; AILE; Antiplatelet Aggregation Activity; Platelet Rich Plasma*

Introduction

Circulatory disorders are implicated in the pathogenesis and progression of atherosclerosis, cardiovascular diseases and other vascular complications. Of the various circulatory disorders, thrombosis represents the major cause of death globally, affecting millions of people with annual incidence rates varying from 1 per 10,000 young adults to 1 per 100 elderly persons [1]. It is a fatal disease characterized by the development of a thrombus in the circulatory system due to the failure of homeostasis. Arterial and venous thrombosis have an important impact on worldwide morbidity and mortality.

Anticoagulants, antiplatelets, and thrombolytic drugs are the therapeutic modalities currently available to treat thrombotic diseases. Of these, antiplatelet aggregation drugs are the first line of medicine for prevention and cure of arterial thrombus diseases [2]. However, all the categories of antithrombotic drugs exerts diverse side effects such as injury to gastric mucosa, decrease the number of platelets and white cells and induces asthma [3]. Thus, developing antithrombotic drugs with little side effects is still an demanding task for the scientific community. Plant based drugs have been considered as an alternative to synthetic drugs. The World Health Organization (WHO) estimates that about 80% of the population in the developing countries depends on traditional medicine for their primary health care.

Azadirachta indica, commonly known as neem, or Indian lilac, is a tree native to the Indian subcontinent and belongs to the mahogany family Meliaceae. In sanskrit it is considered as 'kalpavriksh of kalyuga' as it has diverse pharmacological properties. Neem based medicines are used since antiquity and they are the major components in different alterative practices of medicine such as Siddha, Ayurvedha and in Unani [4]. In this context, the present study was designed to investigate the antiplatelet aggregation activity of aqueous and alcoholic leaf extracts of *Azadirachta indica* on ADP induced *in vitro* platelet aggregation.

Materials and Methods

Collection and extraction

The leaves of *Azadirachta indica* was collected from Bhuvanagiri, Cuddalore district, and was further identified and authenticated by a botanist. The plant leaves were washed with distilled water and air dried in darkness at room temperature and blended into a uniform

dry powder. About 2g of plant leaf powder was mixed with 100 ml of distilled water and ethanol and extracts were prepared separately using soxhlet apparatus. Extracts were filtered through Whatman No.1 filter paper and evaporated to dryness in a vacuum evaporator and stored until use.

Preparation of platelet rich plasma (PRP)

It was prepared by centrifuging whole blood mixed with acid citrate dextrose at 1000 rpm for 5 minutes.

Platelet poor plasma (PPP)

After the removal of PRP, the remaining blood was centrifuged at 3000 rpm for 5 minutes to get a platelet poor plasma which served as a control.

Chemicals

ADP, Aspirin, ethanol and all chemicals used were of analytical grade.

Anti-platelet aggregation activity

PRP and PPP were taken into siliconized glass cuvettes. The cuvettes were incubated at 37°C for 5 minutes. The aggregation was initiated by adding 20 µl of ADP (10 µM) to 1 ml of PRP. The aggregation was recorded for 5 min at 600 nm. The effect of different concentrations (200 - 800 µg) of *Azadirachta indica* leaf extracts (AILE) was studied by incubation with PRP at 37°C for 5 minutes before the addition of ADP. Commercial Aspirin was used as reference standard. The maximal aggregation was recorded. The aggregation is expressed as % inhibition (X) calculated by using the following equation: $X(\%) = (A-B)/A \times 100$. where A=maximal aggregation of the control, and B=maximal aggregation of drug-treated PRP.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) software was used to analyze the data. Throughout this study mean ± SEM of means were used to describe the data in figures. Statistical analysis were assessed using one-way ANOVA. A p-value < 0.05 was considered as significant.

Results and Discussion

Abnormal platelet aggregation is one of the precipitating event in the development of thrombotic disorders. Prevention and treatment of arterial thrombosis is essential as it may result in disastrous consequences such as heart attack, pulmonary embolism or stroke. Treatment for established arterial thrombosis includes the use of antiplatelet drugs and thrombolytic therapy [5]. Antiplatelet drugs alter the platelet activation at the site of vascular damage crucial to the development of arterial thrombosis.

Platelets are anucleated cells that originate from the megakaryocytes of bone marrow and circulate within the vascular tree without any significant interaction with the vessel wall. Lesions in the vascular wall or exposure of extracellular matrix components initiates the adherence of platelets to the endothelial cell which subsequently releases granule components such as ADP, serotonin, and thromboxane A2 that amplifies the aggregation process by recruiting circulating platelets.

The ADP-induced platelet activation is autocatalytic, as upon activation by ADP, platelets release other ADP molecules that acts on nearby platelets, amplifying the reaction. ADP acts through G-protein coupled receptors P2Y1 and P2Y12 as both receptors work closely

together to ensure a complete activation and aggregation of platelets. The activity of ADP-induced platelet activation requires the availability of Ca^{2+} and it is inhibited by cAMP. Therefore, increased intracellular Ca^{2+} and a decrease in cAMP levels are crucial for ADP induced platelet activation and aggregation.

In this study, antiplatelet aggregation activities of aqueous and alcoholic extracts of AILE were performed at the concentration of 200, 400 and 800 µg/ml respectively. The effect of extracts at different concentrations were measured and compared with the standard drug aspirin (Table 1). Both the extracts dose dependently inhibited platelet aggregation induced by ADP. The percentage inhibition of aggregation was significantly higher in the alcoholic extract when compared with the same dose of aqueous extract (Figure 1 and 2). Vidhya., *et al.* [6] showed that concentrations of hridya yoga extract exhibited effective antiplatelet aggregation activity induced by ADP.

Groups	Percentage inhibition
Control	0.00
Aspirin (100 µg/ml)	58 ± 0.32^{e}
Alcoholic extracts	
AILE (200 µg/ml)	16.6 ± 0.09^{b}
AILE (400 µg/ml)	25.0 ± 0.16^{c}
AILE (800 µg/ml)	45.8 ± 0.31^{d}
Aqueous extracts	
AILE (200 µg/ml)	8.3 ± 0.22^{a}
AILE (400 µg/ml)	16.5 ± 0.07^{b}
AILE (800 µg/ml)	25.0 ± 0.16^{c}

Table 1: *Comparison on percentage inhibition of different extracts of Azadirachta indica on ADP induced platelet aggregation. Values are shown in percentage inhibition (mean ± SEM) of platelet aggregation with respect to control. n = 6 for each concentration. Letters (a-e) denote homogenous subsets at p < 0.05.*

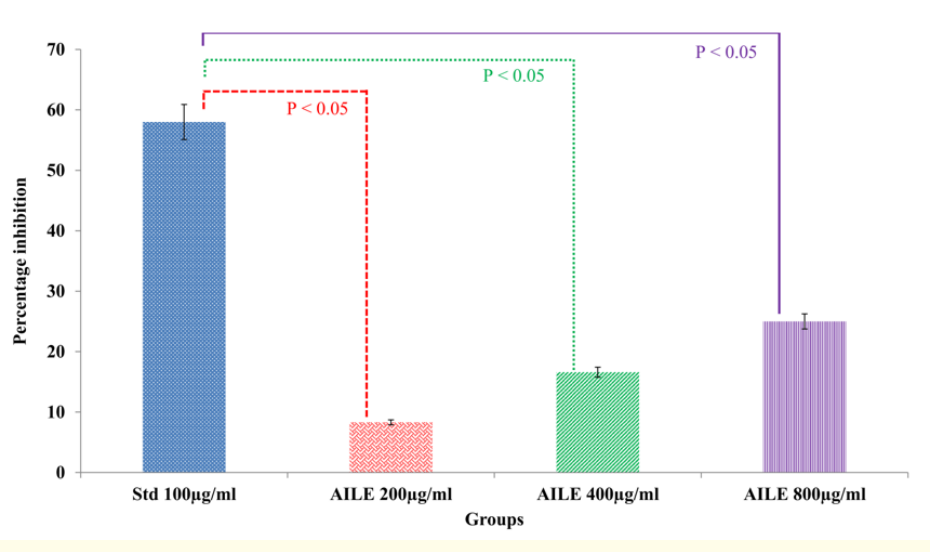

Figure 1: *Dose dependent inhibitory effect of aqueous leaf extracts of Azadirachta indica on ADP induced aggregation of human platelets. Results are expressed as means ± SE (n = 6). Values significant versus std group, p < 0.05.*

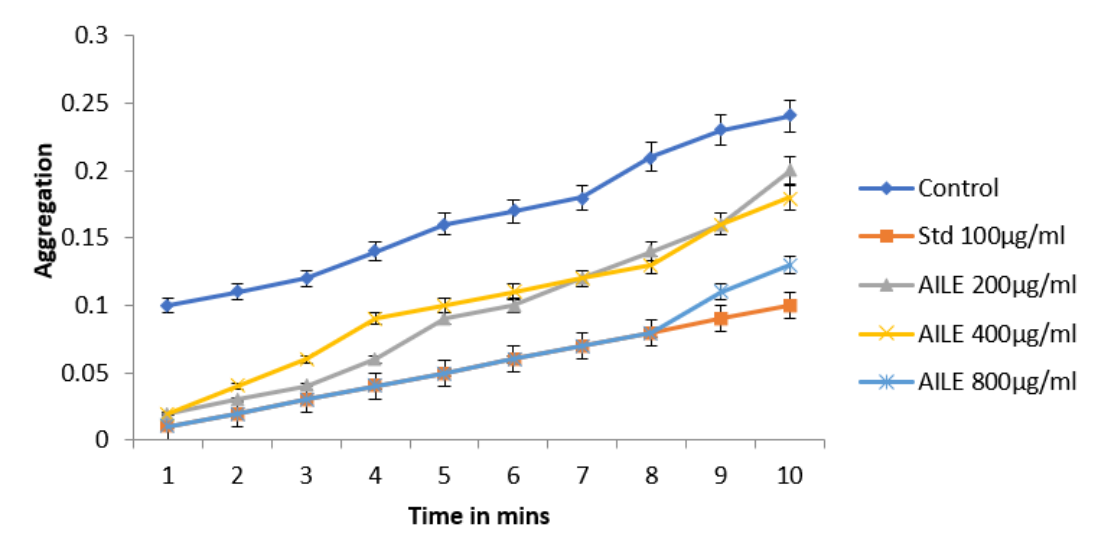

Figure 2: *Effect of alcoholic extract of Azadirachta indica against human platelet aggregation induced by ADP. Results are expressed as means ± SE (n = 6).*

Phytochemical analysis of *Azadirachta indica* leaves has revealed the presence of various flavonoids, saponins and sapogenin [7]. Thus, the anti-platelet aggregation activity of AILE could partly be attributed to their relatively high tannin, phenolic and/or flavonoid contents. These bioactive compounds present in extract might have prevented the adhesion and aggregation of platelets. The anti-platelet aggregation activity of phenolic compound and flavonoids are reported previously [8]. These results confirms that AILE can be considered as herbal remedy for thromboembolic disorders. However, further molecular studies are warranted to confirm the *in vivo* antiplatelet aggregation activity of these extracts.

Conclusion

From this we conclude that aqueous and alcoholic extracts of *Azadirachta indica* inhibited *in-vitro* platelet aggregation induced by ADP in a dose dependent manner. These results support the hypothesis that the intake of AILE may be beneficial in normalizing platelet hyper activation and in prevention of cardiovascular diseases. Therefore, they are good candidates for further *in-vitro* and *in-vivo* studies to find potential lead compounds for antiplatelet aggregation.

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