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Original Research Article

In Vitro Aggregation Inhibition Activity of n-Hexane Leaf Extract of *Azadirachta indica* of Human Platelet

Linus A. Nwaogu[1]*, Chidi U. Igwe[1], Adaku V. Iwueke[2], Chieme S. Chukwudoruo[1]

[1]*Department of Biochemistry, School of Biological Sciences, Federal University of Technology, P.M.B. 1526, Owerri, Imo State, Nigeria*
[2]*University of Agriculture and Environmental Sciences, Umuagwo, Imo State, Nigeria*

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ABSTRACT

In Nigeria, *Azadirachta indica* leaf is used traditionally in the treatment of various diseases. The present study aims at investigating the science behind *in vitro* aggregation inhibition activity of n-hexane leaf extract of *Azadirachta indica* of human platelet which can be used for the prevention and management of stroke, which has been found to be a common cause of morbidity in Nigeria. The study was carried out using platelet-rich plasma of fifty female student volunteers of blood group O^+ with different concentrations of *Azadirachta indica* leaf extract obtained with n-hexane as extraction solvent. Three concentrations: 0.5, 2.0 and 4.0 mg/mL of *Azadirachta indica* leaf extract were used. Aspirin (2.0mg/mL) and distilled water served as the positive and negative controls respectively. Preliminary phytochemical screening of *Azadirachta indica* leaves revealed the presence of saponins, flavonoids, tannins, steroids, alkaloids, cardiac glycosides, terpenoids and phenols. The *Azadirachta indica* leaf extract showed a dose effect on percentage platelet aggregation inhibition of 55% at 2 mg/mL higher when compared to aspirin which gave 40% at the same concentration. The results obtained revealed that *Azadirachta indica* leaf extract exhibited considerable percentage platelet aggregation inhibition and antiplatelet activity higher than aspirin possibly due to some important phytochemical constituents of the leaf and thus can be used in the prevention and management of stroke.

***Keywords*:** Antiplatelet activity, Phytochemistry, Aggregation, Inhibition,Neemstick, Leaves.

Introduction

Medicinal plants have been used for decades before the advent of orthodox medicine for the treatment of many illnesses. Plants parts such as leaves, flowers, stem barks, roots, seeds and fruits have been used as constituents of herbal medicines. The medicinal values of these plant parts lie in their phytochemical compositions, which produce definite physiological action on human body.[1]Many medicinal plant species contain important nutrients and phytochemicals that could be pharmacologically essential.[2]The ethno-pharmacological importance and application of plants in the treatment of diseases and ailments in traditional setting have been an age long practice. The plant kingdom represents a rich store house of organic compounds, many of which have been used for medicinal purposes and could be used in the development of novel agents with good efficacy in pathological disorders. Plants are the richest source of drug for traditional and modern medicine, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs.[2] Currently, herbal products are preferred by many people because of less testing time, higher safety, efficacy, cultural acceptability and assumed 'lesser' side effects. Furthermore, the phytochemicals present in plant-based products are thought to be compatible with the human body because they form part of the daily chemicals, human ingest with.[3]Aspirin is a common antiplatelet drug that has been reported to have adverse effects. Aspirin inhibits prostaglandin synthesis from arachidonic acid which protects the lining of the stomach thereby predisposing the stomach lining to ulcer. One potential explanation for aspirin failure is variable response of individual patients to aspirin with consequent inadequate platelet inhibition.[4]Hence, it is necessary to explore medicinal plants and their natural constituents with minimal side effects.Platelet aggregation is a process of cell-to-cell adhesion initiated by activation of specific membrane receptors for various types of agonists (e.g. adenosine diphosphate (ADP), 5-hydroxytryptamine or serotonin, collagen, epinephrine, norepinephrine, thromboxane A_2 and thrombin. Activation of specific membrane receptors for these agonists initiate a chain of events such that the shape of platelets changes from discoid to spherical and platelet α- and dense granules within the platelet is activated to secrete their contents. Secretion of, e.g. P-selectin (cell adhesion protein) and glycoprotein IIb/IIIa (GPIIb/IIIa) receptors from α-granules and ADP and 5-hyrdoxytryptamine (5-HT) from dense granules may bind to receptors on adjacent platelets, thereby recruiting additional platelets that form clumps.[5]

Figure 1: *Azadirachta indica* (Neem) Leaves

*Corresponding author. E mail: nwogulinus@gmail.com
Tel: +2348037510952

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Plants can synthesize wide variety of chemical compounds that are used to perform critical biological functions. Many of these plant chemicals have beneficial effects on health when consumed by humans and can be effectively used to treat human diseases. One of such plants used to treat or manage human disease is *Azadirachta indica*.

Azadirachta indica, known as Neem stick, belongs to the family Meliaceae and is widely distributed in Asia, Africa and other tropical parts of the world.[6]Neem is a versatile medicinal plant; almost every part of it is being used in traditional systems of medicine for the treatment of a variety of human ailments. Traditionally, most people take neem juice as a tonic to increase appetite and fever and to remove intestinal worms. Neem oil, bark and leaf extracts have been therapeutically used to control diseases like intestinal helminthiasis, respiratory disorders, constipation, and skin infections.[7]The Neem tree is also considered as a natural insecticide/pesticide plant and the quality of pesticide and pharmacological products depend upon the contents of *azadirachtin* in the plant.[8]*Azadirachtin*, a major compound of neem has potent anti feedant, growth and reproductive regulating properties.[8] Therefore, its quantification is important to understand the insecticidal and pesticide property of the neem tree. Also, the quantity of *azadirachtin* and content in the neem leaf using different solvents should be estimated.The secondary metabolites isolated from this plant are the chemical compounds which occur naturally in plants. These active compounds are generally called phytochemicals. These phytochemicals play protective role in plants and in different ways. Some have shown to be more efficient than others in fighting diseases and illnesses in humans. Researchers have found that phytochemicals have the potential to stimulate the immune system and prevent toxic substances in the diet from becoming carcinogenic, reduce inflammation, prevent DNA damage and aid DNA repair, reduce oxidative damage to cells, slow the growth rate of cancer cells, trigger damaged cells to self-destruct (apoptosis) before they can reproduce, help regulate intracellular signalling of hormones and gene expression, and activate insulin receptors.[9] This is the reason why neem leaf extract is used as hypoglycaemic agent.[10-12]*Azadirachta indica* or Neem is of 15–20 metres (49–66 ft) high. It is evergreen, but in severe drought it may shed most of its leaves. It thrives well in various parts of the country irrespective of soil type. The various parts of neem tree have one or more medicinal applications, it is mainly known in Nigeria for its use in the treatment and management of malaria.[7]*Azadirachta indica* leaves, flowers, fruits, seeds, seed oil, root and bark however, have been employed medicinally for various ailments.[13]

In the case of malaria treatment, the leaves of *Azadirachta indica* is boiled in most cases with lemon grass are boiled in water and the patient is usually asked to inhale the aromatic vapour/steam usually with a blanket covered all around, to induce profuse sweating. The patient is simultaneously asked to drink part of the herbal extract. After that, he/she will bath with the decocted mixture. The procedure is one of the fastest ways to reduce fever episodes caused by malaria.[7]

The Neem tree is one of those plants in nature where every part of the tree has health benefits from the leaves right down to the roots. Neem oil can be produced from its seeds which are of natural health remedies and for other uses.[13]*Azadirachta indica* is a fast-growing tree; the branches are wide and spreading. The fairly dense crown is roundish and may reach a diameter of 20–25 metres (66–82 ft). The neem tree is very similar in appearance to its relative, the Chinaberry (*Melia azedarach*).The opposite, pinnate leaves are 20–40 centimetres (7.9–15.7 in) long, possessing 20 to 31 medium to dark green leaflets about 3–8 centimetres long. The petioles are short. The (white and fragrant) flowers are organised in more-or-less drooping auxiliary panicles which are up to 25 centimetres (9.8 in) long. The inflorescences, which branch up to the third degree, bear from 150 to 250 flowers. An individual flower is 5–6 millimetres (0.20–0.24 in) long and 8–11 millimetres (0.31–0.43 in) wide. Protandrous, bisexual flowers and male flowers exist on the same individual tree. The fruit is a smooth (glubrous), olive-like drupe which varies in shape from elongate oval to nearly roundish in colour. It has thin exocarp with a bitter-sweet pulp. The mesocarp is yellowish-white and very fibrous. The mesocarp is 0.3–0.5 centimetres thick. The white, hard inner shell (endocarp) of the fruit encloses one, rarely two, three, elongated seeds (kernels) having a brown seed coat.[14]

Plants' phytochemical components are commonly used in preventive medicine are flavonoids, polyphenols, saponins, liquids and vitamins. Also, a knowledge of the chemical constituents of plants are desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in discovering new sources of such economic materials as tannins, oils, gums, which are precursors for the synthesis of complex chemical substances. Plants provide sources of medicines, which are helpful in the management and treatment of various categories of human diseases .[15] The present study aims at investigating the science behind *in vitro* aggregation inhibition activity of n-hexane leaf extract of *Azadirachta indica* of human platelet.

Materials and Methods

Plant sample collection

The fresh leaves of *Azadirachtan indica* were collected in September, 2019 from the campus of the Federal University of Technology, Owerri, Imo State, Nigeria, at N5°23'33.6876" longitude and E6°59' 10.5504" latitude and were identified by a taxonomist of the Department of Forestry and Wildlife Technology, School of Agriculture and Agricultural Technology, Federal University of Technology, Owerri**,** Nigeria. A voucher specimen (DFWLT/92) of the plant was deposited at the Herbarium of the Research Development and Conservation Program (RDCP) research center, Department of Forestry and Wild Life, Federal University of Technology, Owerri, Nigeria, for reference purposes.

Preparation of plant sample

The leaves were destalked, washed with potable water and shade-dried at room temperature with constant turning to prevent fungal growth. The leaves were later powdered using an electric blender and stored in air-tight containers at room temperature before use.

Preparation of plant extract

Two hundred grams (200g) of pulverized dried powdered leaves of *Azadirachta indica* were macerated with 500 mL of n-hexane in an air-tight bottle and stood with occasional shaking for 24 hours at room temperature. The extracts were filtered using Whatman No.1 filter paper to remove extractable substances. The extract was then evaporated with rotary evaporator and the dried extracts were stored at room temperature in two different sterile containers.

Phytochemical analysis of plant extract of *Azadirachta indica* was carried out by the standard methods as previously described.[16]

Ethical approval

The study was approved by the Ethical Committee on Human Research of the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria, with Approval No.

FUT/SOBS/BCH/COM.1/016/2019. The study conforms with the Helsinki Declaration on Medical Research on Human Subjects.

Blood Sample Collection and Evaluation of Anti-Platelet Aggregation Activity

Six milliliters (6mL) of venous blood sample type O positive (O^+) were collected from fifty eligible female student volunteers between the ages of 20-23 years and who were from the same ethnic group were recruited. The volunteers were selected based on the administration of consent form and questionnaires and those who were within our inclusion criteria, which include those who were healthy, without any known sickness such as HIV, tuberculosis, malaria, hepatitis B, diabetes and who neither had eaten food nor been on medication. The essence and procedures of the study were thoroughly explained to the volunteers and only those who consented in writing in our questionnaire participated in the study. The blood sample was dispensed into an ethylenediamine tetra acetic acid (EDTA) anticoagulant-containing sample bottle. Platelet-rich plasma for each assay was resuspended in tyrod buffer (pH adjusted to 7.4 with 0.25 M HCl). Aggregation of platelets was induced by 0.08 mL $CaCl_2$ at a final concentration of 2µM. The choice of the concentrations used for plant extract was based on the result of earlier studies.[17]To determine the *in vitro* antiplatelet aggregation property, different concentrations (4, 2,

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0.5 mg/ mL) of plant extract were added to the platelet suspension for one minute, incubated at 37°C for five minutes in order to mimic the normal human body temperature before treating with platelet aggregating agents. Aspirin at 75 mg was used as standard. Two milligram per milliliter of aspirin was prepared by dissolving 75 mg of aspirin in 37.5 mL of distilled water. Distilled water was used as a negative control and another test tube marked untreated. Six test tubes were labeled 4mg/mL, 2 mg/mL, 0.5mg/mL, aspirin, untreated and negative control respectively and each containing 3mL of the platelet suspension (blood). All the samples in the test tubes were incubated at 37°C in water bath for 5 minutes. After five minutes, 0.8mL of the extract from 4mg/mL, 2mg/mL, 0.5mg/mL, aspirin, and distilled water respectively was gently collected and added to the incubated samples but for the test tube and was marked untreated, no extract was added.[18]

Platelet aggregation was induced by adding 0.08 mL of calcium chloride ($CaCl_2$) (0.25 M) solution to each of the samples in the test tubes and then incubated again at 37°C for 30 minutes. After incubation, 1mL of the liquid portion of each test tube was transferred to another test tube and labeled. Each test tube was diluted with 40mL of distilled water and mixed with slight agitation. A spectrophotometer was prepared using distilled water as a control. 2 mL of the sample from each diluted test tube was gently transferred into a cuvette and then read spectrophotometrically at 280 nm and the absorbance of each test tube labelled.

Mean absorbance reading

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

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

Percentage Inhibition of Platelet Aggregation

The mean absorbance readings were then used to calculate the percentage inhibition of platelet activity with the succeeding formula:

$$\%\ \text{Inhibition of platelet aggregation} = \frac{\text{Absorbance untreated} - \text{Absorbance treated}}{\text{Absorbance untreated}}$$

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

Percentage antiplatelet activity

The percentage antiplatelet activity of the test solutions and controls were compared.

$$\text{Percentage antiplatelet activity (\%)} = \frac{\%\ \text{Inhibition of test solution}}{\%\ \text{Positive control}} \text{X } 100$$

Statistical analysis

Data generated were analyzed using one-way Analysis of Variance (ANOVA). Significant inferences were adjudged at a 95% confidence level.

Results and Discussion

The phytochemical assessment of the leaf extract of *Azadirachta indica* revealed the presence of major components of phytochemicals which are saponins, steroids, cardiac glycosides and alkaloids which appeared in very high concentration; terpenoids and phenols appeared in moderate concentrations, while flavonoids and tannins appeared in low concentrations (Table 2). However, 4.0mg/mL and 2.0mg/mL of the leaf extract on percentage inhibition of the platelet aggregation showed a significant ($p<0.05$) effect on the red blood cells of individuals with blood group O positive (O^+) when compared to aspirin while that of 0.5mg/mL showed slight effect compared to aspirin on the red blood cells of the same blood group (Table 1). This agrees with the[17] who studied an overview of medicinal plants as potential anti-platelet agents and showed that the presence of these phytochemicals in *Azadirachta indica* leaf extract are responsible for its antipletelet activity. This could be traced to the presence of alkaloids, tannins, phenols and flavonoids and it is supported by the statement that, medicinal plants are rich source of alkaloids having antipletelet and anticoagulant activities.[19] These phytochemicals might have inhibited the pathway that leads to the synthesis of thromboxane by inhibiting the enzymes cyclooxygenase. This could be attributed to the presence of flavonoid in the leaf extract of *Azadirachta indica*. This is in agreement with the report of,[20] affirmed that flavonoids are secondary metabolites associated with platelet activity inhibition. The action is due to several reasons, such as binding to the membrane surface receptor, modifying its protein structures thereby disrupting its physical integrity,[20] This result also corroborates with the reports of,[21] who stated that, flavonoids are the major phytochemical component that is responsible for the antipletelet aggregation activity in *Azadirachta indica* leaves.

Table 1: Antiplatelet aggregation, % inhibition of *A. indica* n-hexane leaf extract

Concentration (mg/mL)	Absorbance value	% inhibition	% antiplatelet
4 mg/mL extract	0.176	69.05	170.53
	0.202	60.92	151.42
	0.194	62.61	158.51
2 mg/mL extract	0.261	59.40	146.70
	0.293	43.32	107.68
	0.259	50.10	126.84
0.5 mg/mL	0.331	41.73	103.06
extract	0.344	33.46	83.17
	0.340	34.48	87.29
Aspirin	0.338	40.49	100.00
(2 mg/mL)	0.309	34.48	100.00
	0.314	39.50	100.00
Distilled water	0.479	15.67	38.70
	0.485	6.19	15.38
	0.499	3.85	9.70
Untreated	0.568	0.00	0.00
	0.517	0.00	0.00
	0.519	0.00	0.00

Table 2: Phytochemical composition of *Azadirachta indica* n-hexane leaf extract

Phytochemicals	*Azadirachta indica*
Saponins	+
Flavonoids	+
Tannins	+
Steroids	+
Alkaloids	+
Cardiac glycosides	+
Terpenoids	+
Phenols	+

Key: - = absent; + = present

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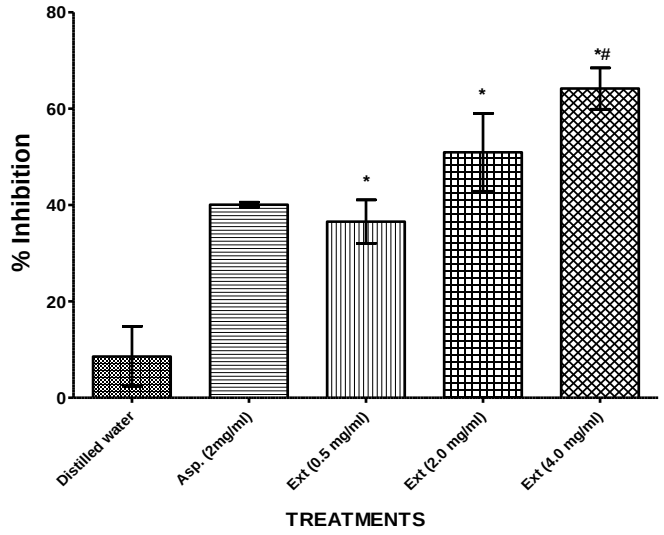

Figure 2: Percentage inhibition of platelet aggregation by *Azadirachtaindicaextracts*.

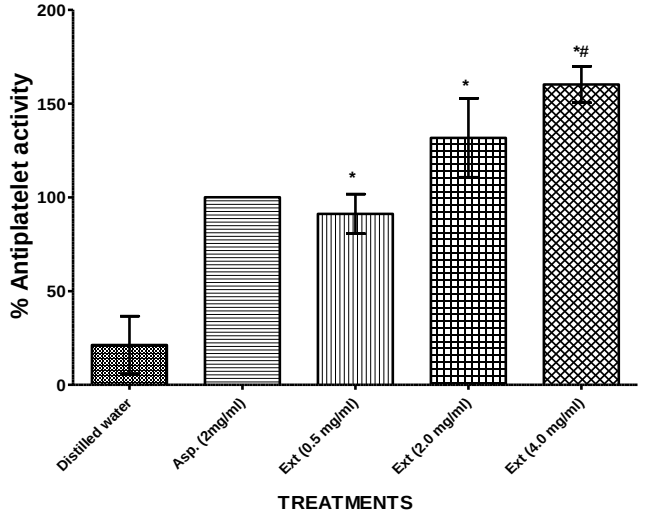

Figure 3: Percentage antiplatelet activity of Azadirachtaindican-hexane leaf extract.

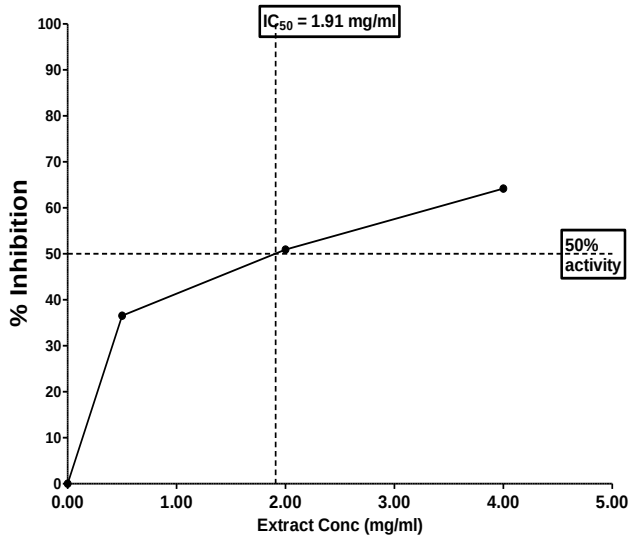

Figure 4: Inhibitory concentration (IC_{50}) of *Azadirachta indica* extract against platelet aggregation.

Other possible inhibition could be the inhibition of the platelet membrane receptor by the phytochemicals acting as antagonist blocking the collagen receptor, the adenosine diphosphate receptor; glycoprotein Ellb/llla receptor thus inhibiting the activation of the platelet from secreting adhesive substance p-selectin, which possibly leads to the aggregate formation.[22]Furthermore, flavanoids and tannins antiplatelet activity could be attributed to the increased production of prostacyclin by endothelian cells and its decreasing aggregation via synthesis of cAMP and the increased concentration of cAMP thereby, inhibiting the expression of platelet GP11b/111a receptor, thus supported by the reports by.[23]

The percentage inhibition of platelet aggregation by the extract showed a significant ($p<0.05$) effect on group O positive blood. There was a significant ($p<0.05$) increase in percentage inhibition by the plant extract which exerted 55% at 2 mg/mL higher when compared to aspirin which gave 40% (Figure 3). This study corroborates the report of [24]reported that *Psophocarpus tetragonolobus* (L.) Dc pod extract inhibited platelet aggregation at 4 mg/mL concentration. Also, percentage antiplatelet activity of the extract was found to be 130% at 2.0mg/mL when compared to that of aspirin which exerted 100%antiplatelet inhibition at the same concentration. As the concentration of the extract increased, the percentage antiplatelet inhibition increased, indicating that the effect of the extract is concentration dependent (Figure 3). This also agrees with the reports of,[24]who reported that pod extract of *Psophocarpus tetragonolobus* (L) Dc pod extract on percentage antiplatelet inhibition was concentration dependent (Figure 4). The results from our study show that at 50%, the concentration gave 1.91 mg/mL, indicating that the extract is effective for platelet inhibition *in vitro*. This indicates that *Azadirachta indica* leaf could be included among medicinal plants with high antiplatet potential with less harmful effect.

Conclusion

From the phytochemical assessment and *in-vitro* antiplatelet aggregation of n-hexane leaf extract of *Azadirachta indica* on blood group O positive, it can be concluded that *Azadirachta indica* leaf extract proved to have high percentage *in-vitro* aggregation inhibition activity of human plateletdue to the presence of important phytochemicals (flavonoids, tannins, phenols and alkaloids) thus, could be used for the production of novel platelet inhibition and antiplatelet drugs. However, the mode of action and efficacy of *Azadirachta indica* leaf extract for the above actions need further investigation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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