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The Haemolytic Effect of Aqueous Extract of Azadirachta Indica (Neem)

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Abstract

Medicinal plants have been in use from the ancient times till date. Most drugs used in Othordox medicines have their origin from medicinal plants. Azadirachta Indica (Neem) fresh leaves were sourced from Nsukka (Enugu State, Eastern Nigeria) and identified by Botanist at the department of Botany, University of Nigeria, Nsukka. The crude aqueous leaf extract was assayed to determine the phytochemical contents using appropriate methods. The subjects used for this study were between 20 and 30 years, which includes normal Haemoglobin genotype (Hb AA), sickle heterozygotes (Hb AS) and sickle homozygotes (Hb SS) individuals. The subject's blood samples were collected from University of Nigeria Teaching Hospital, Enugu and their genotypes duly confirmed. The haemolytic effect of Azadirachta Indica was determined using the subjects blood samples at different incubation period and at different concentrations of the plant extract.

This study showed that Azadirachta Indica has haemolytic effect which was more pronounced in genotype 'SS'. This observation is attributed to the fragility and instability of sickle haemoglobin. The haemolytic effect of A. Indica on the three haemoglobin genotypes was due to the presence of surface active substances such as saponin and tannin which are active phytochemicals detected in the aqueous leaf extract.

Keywords: Azadirachta Indica, Genotype, phytochemical, Haemolysis, fragility.

Introduction

The importance of medicinal plants to human health cannot be over-emphasized.Majority of the drugs used in othordox medicine are sourced from medicinal plants. Morphine from Opium, Quinine gotten from Cinchoma bark, Digotoxin is sourced from Digitalis leaf and several examples (Iwu 1993, Pamplona and Rogers, 2001, Mutiu et al 2015). Several scientific research works showed that most medicinal plants contain phytochemicals that had been proven to be therapeutic (Zafari, et al 2017, Ajazuddin and Shailendra, 2010, Ukoroije et al, 2018). The toxicity aspect of medicinal plant had been neglected although their potency is not disputable. *Azadirachta Indica* (Common name –Neem) belongs to the family of Meliaceae: it is planted for ornamental and medicinal purposes. *A. Indica* plant is used widely in Africa especially West Coast for the traditional treatment of Malaria and fever. The extracts of *A. Indica* had been shown to have antipyretic, analgesic and anti-inflammatory activities. The leaf decotion caused a fall in the parasite count in chloroquine-sensitive strains of *Plasmodium berghei* injected in mice and inhibited the growth of *P.falciparum*, The anti malaria activity has been estimated to be equivalent to half the therapeutic dose of chloroquine sulphate on dry weight basis (Iwu, 1993). An aqueous leaf extract of *A. Indica* was found to have cytotoxic effect (Chen et al, 2013). Some medicinal plants had been associated with haemolytic activity due to their constituent which include saponins, phenols (example tannins) and other lysine (Newall et al, 1997, Hoffbrand , et al, 2001). Haemolysis may occur as a result of either the action of lysine or action of surface active substances such as saponins and phenols (example tannin). The use of herbal medicine for the treatment

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of ailment is a common practice in Africa. However, It is not without limitation. There is always an error of ‘’no side effect syndrome’’ making people to take herbs without caution to the detriment of their health. The main objective of this research work is to investigate the haemolytic effect of the aqueous leaf extract of *A. Indica* and subsequently advise the people who patronize traditional medicine for several reasons on how best to use them.

1.0 Materials and methods

1.1 Materials

Blood samples from human subjects were used for this study. Individuals (females) with haemoglobin genotype AA (Normal homozyotes), Individuals (females) with HaemoglobinHb - Genotype AS (Sickle heterozygotes), Individuals (females) with haemoglobin genotype SS (Sickle homozygote) between the ages 20-30 years were used for this study. Blood specimen, 5.0ml were collected from each human subject with different haemogloblin genotypes HbAA, Hb AS and Hb SS and used for the research.

1.1.1 Material (Leaf Extract):

Azadirachta indica (Family Meliaceae) leaves were collected from Nsukka town (Eastern Nigeria)and were identified by Botanist in the department of Botany University of Nigeria Nsukka. The crude aqueous extracts (decotions) of these medicinal plants as being used by herbal practitioners were used since this is a toxicological study.

2.0 Methods:

2.1 Preparation of medicinal plant extracts

A decotion (herbal dose obtained by boiling of part of plants) of the medicinal plant *Azadirachtaindica* were prepared by boiling 175g of the leaf in 500ml of water. Thoroughly washed leaves were placed in a clean heat resistant container and 500ml of water was added. The boiling lasted for about 10 – 15 minutes on low heat. The resulting liquid was filtered through strain and used for the study.

3.0 Phytochemical analysis of *Azadirchta indica* plant extract.

Standard phytochemical methods (Harbourne, 2000) were used to test for the presence of alkaloids, flavonoids, glycosides, cardiac glycosides, cyanogenic glycosides, anthracene glycocides, proteins, carbohydrates, reducing sugars, saponins, steroidal aglycine, anthraquinone, tannins, TLC Techniques

The TLC method was used for the detection of the various secondary metabolites in the crude extract of *A. indica* (Fresh and Dry Leaves). The samples of the plant extract were spotted 2cm from the base of the plate using a capillary tube and allowed to dry before developing in appropriate solvent system in chromatographic tank. (Alkaloids-methanol-conc NH_4 OH (200:3) and n-Bu0H-aq Citric acid (870ml:4.8g citric acid in 130ml of H_20), flavonoids: - system A (forestall conc HCI-H0Ac-H_20, 3:30:10), System B (BAW, n-Bu0H-H0AC- H_20, 4:1:5 Top layer) System C (15% H0Ac) and system D (n-Butano-methanol-H20, 8:1:1), Saponins:- System A (Chloroform –methanol-water, 13:7:2 lower layer) and system B (chloroform-ethyl acetate 1:1), Sugars:- System A (n-

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butanol, acetic acid-ether-water 9:6:3:1) and system B (butanol-acetone-H_20 4:5:1, top layer), Terpenoids:- Benzene-chloroform 1:1, Glycosides:- System A (chloroform-methanol, 9:1) and system B (ethyl acetate-methanol-water 16:1:11), Vitamin C:- System A (water) system B (ethanol) and system C (ethanol-10% acetic acid, 9:1).

4.0 Methods for Haematological Studies:

4.1 Genotype determination (Dacie and Lewis, 1999)

Genotypes were determined using cellulose acetate electrophoresis for separation of haemoglobins.

Principle: Haemoglobin when placed in an electric field will migrate to one of the electrodes. The difference in charge distribution and molecular weight of the haemoglobin at different pH Value is used in the separation of the haemoglobin.

Study of haemolytic activity of plant extracts *Azadirachta indica* (Spirichev, et al, 1989).

The method is based on the measurement of light absorption of exoerythrocytic haemoglobin. This test was carried out on all the subjects with the plants extracts at incubation period of 2 hours, 12 hours, 24 hours and 48 hours.

The plant extract was serially diluted and the dilutions of ½, 1/6, and 1/64 were also used to study the haemolytic activity of *A. indica* at incubation period of 2 hours.

Calculation was carried out from the equation

$$X=E1(100)/E3$$

Where x = the degree of heamolysis caused by the plant extract

Where E1 = The absorbance measured for plant extract containing sample

Where E3 = The absorbance measured for control sample.

5.0 Data Analysis

Data were analyzed using the SPSS version 7.5 software packages. Mean values (SD) experiments with duplicate samplings were taken for analysis. Differences between groups were assessed by one-way Anova while differences within were assessed by student t-test. The acceptance level of significance was $p<0.05$

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Results

Phytochemical characteristics of plant extract. *Azadirachta indica*

The result of the phytochemical screening of A. indicashowed the following constituents (Table 1) Aqueous extracts of both the fresh and dry leaves of A. indica were found to contain reducing sugars, flavonoids, saponins, anthroquinones, tannins, carbohydrates, cyanogenic glycosides, cardiac glycosides and vitamin C (vitamin C was not detected in dry leaves extract).

The result of phytochemical screening (TLC method) of *Azadirachtaindica*plant extract:

The extracts of *Azadirachta indica* (both fresh and dry leaves) showed presence of flavonoids (Qucertin, 3 – rutinoside and 3 - Rhamnoside), Terpenoids, saponins, sugars (fructose, Rhamnose, Xylose and Mannose) and ascorbic acid (Vitamin C was not detected in dry leaves)

The haemolytic effect of the *Azadirachta indica* plant extract on Hb AA, Hb AS and Hb SS erythrocytes at different incubation periods of 2, 12, 24 and 48hours:-

The study of haemolytic activity of *Azadirachta indica* plant extract on different Hb-genotypes at different incubation periods showed heamolysis (invitro) significantly higher ($p<0.05$) in the genotype Hb-SS when compared to that of Hb AS and Hb AA individuals. (Figure 1) The Haemolysis observed in Hb AS is insignificantly higher ($p>0.05$) than that of the Hb AA individuals. Generally, there was a significant increase ($p<0.05$) in the haemolytic activity as the incubation period increases from 2 hours to 48 hours for all the Hb-genotypes study.

The haemolytic effect of the various concentration of *Azadirachta indica* plant extracts on Hb AA, Hb AS and Hb SS erythrocytes after 2 hours incubationperiods:

The study of haemolytic activity of *Azadirachta indica* plant extract on different Hb genotypes at different concentrations after an incubation period of 2hours showed a significant decrease ($p<0.05$) haemolysis (invitro) as the concentration decreased from ½ to 1/64 for all the Hb-genotypes (Figure 2).The haemolysis observed for the Hb-Genotype SS for all the concentrations of plant extract was significantly higher ($p<0.05$) when compared with Hb AA and Hb AS individuals.

Discussion:

The result of the phytochemical studies showed constituents (Table 1) which agreed with the report of other researchers who have done phytochemical studies on *Azadirachta indica*(Nair, et al 1997, Pamplona and Roger, 2000, Distasi et al, 2002) Vitamin C was detected in *Azadirachta indica* extract (only in the fresh leaves but not in the dry leaves) using TLC method. The absence of vitamin C in the dry leaves extract of *Azadirachta indica* indicated that the drying of the leaves depletes the vitamin c content in the fresh leaves.

The extract of *Azadirachta indica* showed haemolytic activities at different concentrations (Figure 1) and at different incubation periods (Figure 2) which agreed with the study done earlier by Nohl and Klanu, (1998). The haemolysis observed in Hb Genotype

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SS is significantly higher ($p<0.05$) when compared to Hb AS and Hb AA erythrocytes. These results will be attributed to the fact that the sickle erythrocytes are more fragile than normal ones (Tse and Lux, 1999, Tanner and Anstel 1999, Hebbel, 1990). Hence there was accelerated auto-oxidation and heme loss due to instability of sickle haemoglobin (Hebbel et al, 1988). The haemolytic effect of *Azadirachta indica leaf extract was attributed to the saponin and tannin constituents which are surface active substances capable of causing haemolysis. This observations was in conformity with the earlier research done by Cristy and Hawes where haemolytic anaemia was observed after ingestion of Neem (Azadirachta indica) tea (Cristy and Hawes, 2013).*

Conclusion

The results of this studies showed that *Azadirachta indica* plant extract has haemolytic effect (invitro) on all the haemoglobin genotypes, being significantly higher, ($p<0.05$) in Hb SS. This observation is attributed to the fragility and instability of sickle haemoglobin. The saponin and tannin constituents of *A. Indica* leaf extract are responsible for the haemolytic effect observed on all the haemoglobin genotypes used for this study. Therefore this herbal medicine should be taken with caution in low doses.

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Table 1: Phytochemical analysis of Azadirchta Indica (Chemical methods)

TEST	PLANTS EXTRACTS	
	A	B
Vitamin C	Nil	+ve
Biuret test	Nil	Nil
Million test	Nil	Nil
Reducing sugar test	+ve	+ve
Flavonoids with $ALCl_3$	+ve	+ve
Flavanoids with dil Ammonia	+ve	+ve
Saponins-Emulsion	+ve	+ve

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Alkaloids (Mayer's reagent	Nil	Nil
Alkaloids (Wagner's reagent)	Nil	Nil
Saponins Stable	+ve	+ve
Alkaloids (Picric acid)	Nil	Nil
Alkaloids (Draugendroff's reagent)	Nil	Nil
Anthraquinone	+ve	+ve
Tannins with lead sub acetate solution	+ve	+ve
Tannins with ferric chloride	+ve	+ve
Molish test	+ve	+ve
Anthracene glucoside	Nil	Nil
Steroidal aglycone	+ve	+ve
Cardiac glycosides	+ve	+ve
Cyanogenic glycosides with feeling's solution	+ve	+ve
Cyanogenic glycosides with dil H_2S0_4	+ve	+ve

A = A. Indica (Dry leaf extract)

B = A.Indica (Fresh leaf extract)

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Figure 1

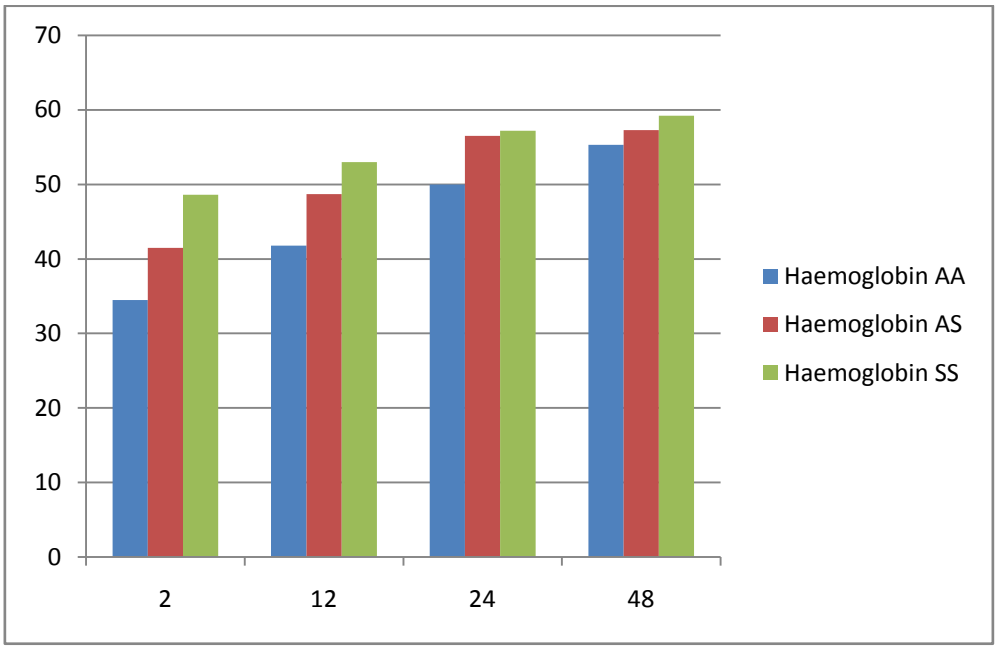

Fig. 1: Histogram of haemolytic activity of *azadirachataindica plant* extract on different haemoglobin genotypes aa, as, and ss at different incubation periods of two, twelve, twenty-four and forty eight hours

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Figure 2

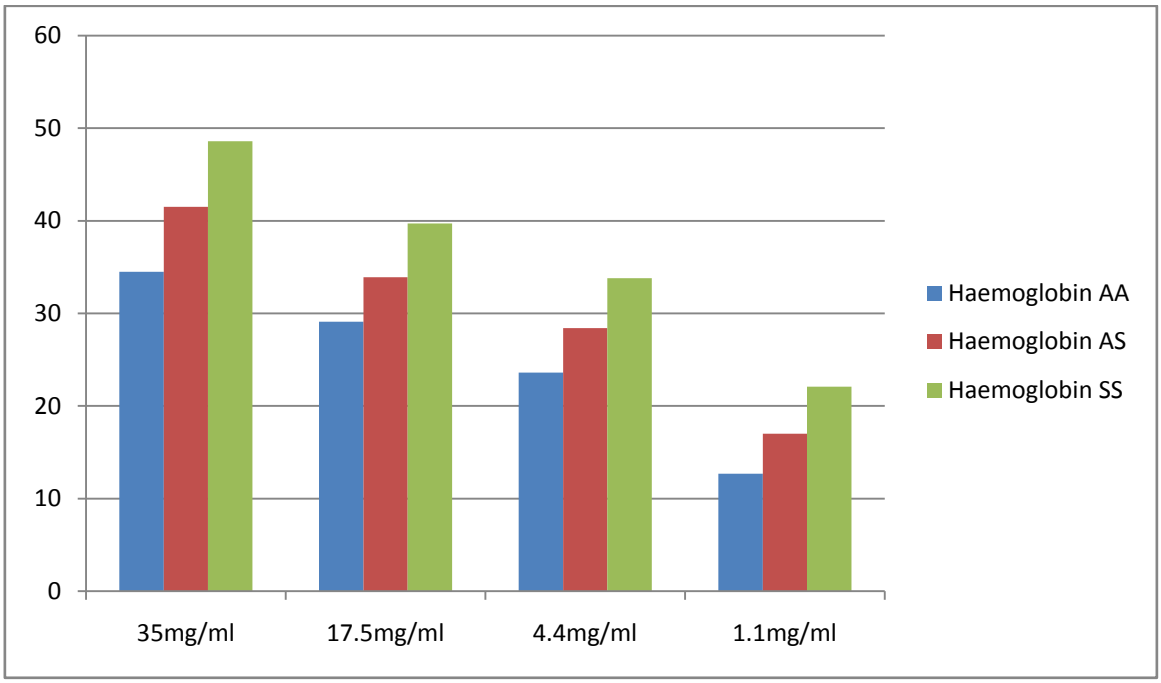

Fig 2: Histogram of the haemolytic activity of a. indica plant extract on the different hb genotypes aa, as, and ss at different concentrations 35mg/ml, 17.5mg/ml (1/2), 4.4mg/ml (1/16) and 1.1mg/ml (1/64) at incubation period of 2hrs

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