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The Effect of *Azadirachta indica* (Neem Tree) On Human Plasmodiasis: The Laboratory Perspective.

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Herbal remedies are still sought by a cross section of Nigerians in both rural and urban areas for various ailments. Crude extracts of *Azadirachta indica* leaves (both aqueous and methanolic) were prepared using the methodology of Obi and Makinde. Doubling dilutions were made from the stock solution to give serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. Aliquots of 25 µl of each dilution (concentration) were dispensed into wells of 96 sterile microtitre plates, one concentration per row of 12 wells. The activity of both the aqueous and methanolic extracts of *Azadirachta indica* was investigated against 18 isolates. The calculation for the IC_{50} was not feasible owing to the fact that the stock (undiluted) concentration only had a 34.4% inhibition of schizont maturation for the aqueous while the methanolic extract on the other hand had a 43.4% inhibition of schizont maturation. The results however indicate that *Azadirachta indica* leaves may have the active ingredient against *Plasmodium falciparuim*. This could be extracted, studied and formulated into doseable anti-malarial drugs.

Keywords: *Azadirachta indica*, Herbal remedies, Plasmodiasis.

INTRODUCTION

The "neem" tree *Azadirachta indica* (Dogonyaro in local parlance) has been described as early as 1830 by De Jussieu as was reported by Banerjee *et al.,* (2002).

Medicinal plants are part of human society to combat diseases, from the dawn of civilization. *Azadirachta indica* is well known in India and its neighbouring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. Every part of the tree has been used as traditional medicine for house – hold remedy against various human ailments, from antiquity (Chopra *et al.,*1958; Chatterjee and Pakrashi, 1994).

It has been reported that neem seed and leaf extracts are effective against malarial parasites. And that component of the alcoholic extracts of leaves and seeds are effective against both chloroquine – resistant and sensitive strains of malaria parasite (Banerjee *et al.,* 2002). In 1998, Dhar *et al*., reported that neem seed extract and its purified fractions have been shown to inhibit growth and development of asexual stages of drug – sensitive and resistant strains of the human malaria parasite of *Plasmodium falciparum*.

The use of herbs in traditional medicine has been going on unabatedly in Africa for centuries (Agomo, 1991). This could be due to the prevailing socio-economic factors and has continued to generate increased conflict between orthodox and traditional medicine practitioners as regards the efficacy and scientific basis for such use of herbs. One of such herb is the more popular *Azadirachta indica* (Dogonyaro).

Herbal remedies are still sought by a cross section of Nigerians in both rural and urban areas for various ailments. For, in spite of ready availability of various orthodox anti-malarial drugs in Nigerian market, there is an up-surge in the search for herbal remedies by a cross section of both literate and illiterate populations living in rural and urban centers for this serious endemic disease (Agomo, 1991). It is this renewed interest and claims by herbal practitioners that the herbs are even more effective than orthodox medicine that stimulated this aspect of investigation. The additional factor of failure of some of the conventional anti-malarial drugs leading to the development of resistance by the malaria parasite to these drugs is equally a boaster effect.

In Nigeria, some scientists have carried out series of investigations to establish the authenticity of claims by

traditional healers that *Azadirachta indica* is effective against malaria. In 1978, Ekanem found *Azadirachta indica* effective against malaria, whereas other workers (Makinde and Obi, 1985; Obi and Makinde, 1985; Odetola and Bassir, 1986) observed little or no chemosuppression when the leaf extract was used either alone or in combination with other medicinal plants.

MATERIALS AND METHODS

Study Area

The Federal Capital Territory (FCT), Abuja

Sample Sites

Three representative hospitals within the FCT – National Hospital, Abuja, Wuse General Hospital, and the Asokoro General Hospital, were the study sites.

Collection and Processing of Pre-culture Blood Specimens

Fresh blood samples already collected from patients for the diagnosis of malaria from the three representative hospitals were used following permission by the respective medical laboratory authorities.

Pre-culture thick and thin films were prepared for each blood sample and treated as urgent cases, using the Giemsa staining technique. Stained films were examined microscopically using the oil immersion objective lens (100 x)

Subject Selection

This was based on exclusion and inclusion criteria set for the research. Results obtained from the processed pre-culture thick and thin films formed the basis for the criteria.

Exclusion Criteria

Patients' blood samples were considered inadequate for the study if microscopy results of Giemsa stained films had a falciparum parasitaemia (in a thick film) of less than two trophozoites in every high power microscope field (< 1000 parasites/µl of blood). Patients with mixed infections were also excluded from the study.

Inclusion Criteria

Patients' blood samples were considered adequate for the study if microscopy results of Giemsa stained thick films had mono-infections with *Plasmodium falciparum* and asexual parasitaemias in excess of 1000 parasites, but less than 80,000 parasites per µl of blood (more than one trophozoite in every high power microscope field - > / = +++)

Counting Parasite Numbers

The following two methods are commonly used:

► Estimating parasite numbers/µl of blood from the thick film

► Estimating parasite numbers/µl of blood by counting parasites against white cells.

For the purpose of this research, we used the first method.

Estimating Parasite Numbers/µl of blood from the thick film

This was carried out by multiplying the average number of parasites per high power field (100 x objective) by 500. Between 10-50 fields (depending on parasitaemia) was examined to determine the average number of trophozoites per high power field (HPF). Ten fields are sufficient when the parasite density is high.

The factor of 500 was proposed by Greenwood and Armstrong (1991). They calculated that 5 – 8 µl is the volume of blood required to make a satisfactory thick film and that the volume of blood in one HPF (100 x objective) of a well-prepared thick film is about 0.002µl. Therefore the number of parasites per HPF multiplied by 500 gives the estimated number of parasites/µl of blood. This method, Greenwood and Armstrong found to be more accurate and quicker than counting the parasites against white cells.

Crude extracts of *Azadirachta indica* leaves (both aqueous and methanolic) were prepared by us in the laboratory using the methodology of Obi and Makinde (1985).

Preparation of crude extracts of *Azadirachta indica* leaves

In previous studies, some scientists prepared *Azadirachta indica* extract by employing the methods used by the local traditional healers. Scientific experiments need to be standardized and repeatable. Hence in this research, we employed the methodology by Obi and Makinde (1985) for both the aqueous and methanolic (alcoholic) extracts.

Aqueous Extract

123g of *Azadirachta indica* leaves were washed, dried, and boiled with 750 ml of distilled water for 30 minutes. The liquid was drained off and re-boiled to

reduce the final volume to 150 ml (stock aqueous extract). The stock extract was stored at 4°C in a refrigerator while not in use.

For Use

Doubling dilutions were made with sterile distilled water to have

1: 2; 1:4; 1:8; 1:16; 1:32 and 1:64 dilutions.

Methanolic Extract

123g of *Azadirachta indica* leaves were washed, dried, and boiled with 750 ml of methanol for 30 minutes. The liquid was drained off and made to a final volume to 150 ml (stock alcoholic extract). The stock extract was stored at 4°C in a refrigerator while not in use.

For Use

1. Doubling dilutions were made with methanol to have 1:2; 1:4; 1:8; 1:16; 1:32; and 1:64 dilutions.
2. Positive patients' sample having mono-infections with *Plasmodium falciparum* and asexual parasitaemias in excess of 1000 parasites, but less than 80,000 parasites per µl of blood.
3. RPMI – 1640 medium (product number R6504), purchased from and supplied by the Vector Control Research Unit, Universiti Sains Malaysia (USM) under the auspices of the Communicable Disease Surveillance and Response (CDR), WHO, 1211, Geneva, 27, Switzerland.
4. Methanol
5. Giemsa stain
6. Microscope slide with frosted end.

In vitro Microtest Technique

The drug susceptibility of *Plasmodium falciparum* was determined using the World Health Organization (WHO) standardized in-vitro microtest system used by Wernsdorfer (1980) which was developed by Rieckmann *et al*.,(1978); adapting the methodology used for cultivation of *Plasmodium falciparum* (Trager and Jensen, 1976). The technique involves quantitating schizont maturation following cultivation of infected erythrocytes in plates charged with defined quantities of drug.

Drug Preparation

Azadirahta indica leaves were obtained from Kubwa, a satellite town in Bwari LGA of FCT, Abuja and the pre-dosed plates were prepared after extraction following the method of Obi and Makinde (1985). Pre-dosed plates of both the aqueous and methanolic extracts of *Azadirachta indica* leaves were prepared from the stock. Doubling dilutions were made from the stock solution to give final test dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. Aliquots of 25µl of each dilution (concentration) were dispensed into the wells of 96-well sterile microtitre

plates, one concentration per row of 12 wells. The first row was the control, containing extract – free culture medium, 25µl per well. The charged plates were dried in an incubator at 37°C overnight, covered with a lid and stored at 4°C.

In-vitro Microtest

Following centrifugation of infected blood samples at low speed (200 rpm) the plasma was discarded and the erythrocytes (packed cells) were washed three times in culture medium (RPMI – 1640 with L-glutamine, without sodium bicarbonate) at 2000 rpm for 5 minutes. 100µl of washed infected erythrocytes was then mixed aseptically with 900µl of complete culture medium (1:10) and 50µl of the blood-medium mixture (BMM) was pipette into each well of the pre-dosed plates.

The plates were incubated at 37°C for 24-30 hours (depending on schizont maturation) using the candle jar method, 5% CO_2 (Trager and Jensen, 1976). All the susceptibility assays for each concentration were done in duplicate. After incubation, the plates were allowed to stand for 30 minutes in a semi-vertical position (about 45° inclined). The supernatant was then removed, erythrocytes resuspended in the remaining fluid, and a thick blood film was made from each well. The blood smears were air-dried for 24 hours and stained with 3% Giemsa stain for 30 – 45 minutes. The stained thick films were examined with the oil immersion objective (100 x). The number of schizonts with three or more nuclei out of 200 asexual parasites (i.e., schizonts and trophozoites) was counted. For an acceptable test, schizont maturation in the control (well A) must be 10% or more (i.e., 20 schizonts with three or more nuclei per 200 asexual parasites). The counts read in the drug wells were then expressed as a percentage of the control.

Interpretation and Reporting of Test Reports

For data analysis, a simple evaluation from a table showing the original data and those derived after standardizing for control was used.

Schizont growth (formation) at 8 pmol/well (1.6µmol/l) or more for chloroquine, 256 pmol (51.2µmol/l) or more for quinine, 4 pmol (0.8µmol/l) or more for monodesethylamodiaquine (amodiaquine) were considered as threshold for in vitro *Plasmodium falciparum* resistance (WHO, 2001). Schizonts growth in

the leaf extracts of *Azadirachta indica* were correlated against Chloroquine, Quinine, and Monodesethylamoiaquine as stated above. The aqueous and methanolic extracts were also correlated against their activities.

Calculation of IC_{50}

The IC_{50} (50% inhibitory concentration) represents the concentration at which 50% of the isolates were inhibited from maturing to schizonts. The IC_{50}s for the individual isolates were determined by the linear extrapolation method described by Freese *et al*., (1993).

RESULTS

A total of 22 falciparum isolates were collected and cultured, but only 18 isolates were evaluated. The reasons for discarding the other tests were contamination and failure of schizonts to mature satisfactorily. The drug susceptibility data are presented for both the aqueous and methanolic extracts as shown below.

***Azadirachta indica* (aqueous extract)**

The activity of aqueous extract of *Azadirachta indica* was investigated against the 18 isolates. The calculation for the IC_{50} was not feasible owing to the fact that the stock (undiluted) concentration only had a 34.4% inhibition of schizont maturation.

Table i: Results of invitro microtests with *Azadirachta indica* (aqueous extract)

Extract concentration	No. (%) of isolates Totally inhibited	% inhibition of schizont maturation
1 : 64 dil	0 (0 %)	0.0 %
1 : 32 dil	0 (0 %)	0.0 %
1 : 16 dil	0 (0 %)	0.0 %
1 : 8 dil	0 (0 %)	2.70%
1 : 4 dil	0 (0 %)	8.77%
1 : 2 dil	0 (0 %)	15.81%
Stock extract	0 (0 %)	34.33%

***Azadirachta indica* (methanolic axtract)**

The activity of methanolic extract of *Azadirachta indica* was investigated against the 18 isolates. The calculation for the IC_{50} was not feasible owing to the fact that the stock (undiluted) concentration only had a 43.37% inhibition of schizont maturation.

Table ii : Results of invitro microtests with *A. indica* (methanolic extract)

Extract concentration	No. (%) of isolates totally inhibited	% inhibition of schizont maturation
1 : 64 dil	0 (0 %)	0. 0 %
1 : 32 dil	0 (0 %)	0. 0 %
1 : 16 dil	0 (0 %)	3.47%
1 : 8 dil	0 (0 %)	8.93%
1 : 4 dil	0 (0 %)	14.50%
1 : 2 dil	0 (0 %)	23.01%
Stock extract	0 (0 %)	43.37%

DISCUSSION

The activity of aqueous extract of *Azadirachta indica* was investigated against 18 isolates. The calculation for the IC_{50} was not feasible owing to the fact that the stock (undiluted) aqueous concentration only had a 34.4% inhibition of schizont maturation. The methanolic extract on the other hand had a 43.4% inhibition of schizont maturation.

The un-impressive performance of both the aqueous and methanolic extracts of *Azadirachta indica* against *Plasmodium falciparum* is in line with results of previous studies. Though in 1978, Ekanem reported that *Azadirachta indica* was effective against malaria, other workers (Makinde and Obi, 1985; Obi and Makinde, 1985; Odetola and Bassir, 1986) observed otherwise. These previous research results indicate little or no chemo-suppression when the leaf extract was used either alone or in combination with other medicinal plants.

In conclusion, the results of this study revealed a high level inactivity of extracts of *Azadirachta indica* against *Plasmodium falciparum*. The results however indicate that *Azadirachta indica* leaves may have the active ingredient against *Plasmodium falciparum*. This could be extracted, studied and formulated into doseable anti-malarial drugs.

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