



ISSN: 2277- 7695

TPI 2014; 3(9): 12-16

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www.thepharmajournal.com

Received: 10-09-2014

Accepted: 13-10-2014

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Phytochemical analysis and evaluation of antimalarial activity of *Azadirachta indica*

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Abstract

Azadirachta indica is a very useful traditional medicinal plant in the sub-continent and each part of the tree has some medicinal properties. The plant is native to Asia, but has now naturalized in West Africa and is widely cultivated in Nigeria as an ornamental as well as medicinal plant. Fresh leaves of the plant were collected, dried, homogenized and extracted using 95% Ethanol, Methanol and Acetone. Phytochemical analysis gave positive results for steroids, triterpenoids, reducing sugars, alkaloids, phenolic compounds, flavonoids and tannins. This study aimed at screening the active components and the antibacterial effects of the Ethanol, Methanol and Acetone. Leaf extract of *Azadirachta indica* contains pharmacologically active constituents that may be responsible for its activity against *P. falcifarum in vitro* and *P. vivax in vivo* model. Therefore, the use of Neem plant in our community for treating diverse medical ailments especially infectious diseases is highly justified.

Keywords: *Azadirachta indica*, Phytochemical analysis, Antimalarial, *P. falcifarum* and *P. vivax*

1. Introduction

Azadirachta indica (Meliaceae) commonly known as neem is native of India and naturalized in most of tropical and subtropical countries is of great medicinal value and distributed wide spread in the world. The Chemical constituents contain many biologically active compounds that can be extracted from neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, Carotenoids, steroids and ketones. *Azadirachtin* is actually a mixture of seven isomeric compounds labeled as *azadirachtin* A-G and *azadirachtin* E is more effective [1]. Other compounds that have a biological activity are salannin, volatile oils, meliantriol and nimbin [2]. Neem leaf is effective in treating eczema, ringworm, acne, anti-inflammatory, antihyperglycemic properties and it is used to heal chronic wounds, diabetic food and gangrene developing conditions. It is believed to remove toxins from the body, neutralize free radicals and purify the blood. It is used as anticancer agent and it has hepato-renal protective activity and hypolipidemic effects [3].

Medicinal plants have been found useful in the cure of a number of diseases including bacterial diseases. Medicinal plants are a rich source of antimicrobial agents [4]. Almost every part of the tree is bitter and finds application in indigenous medicine. Neem extract has been reported to have antidiabetic, antibacterial and antiviral activity [5]. Almost every part of the tree has been in use since ancient times to treat a number of human ailments and also as a household pesticide. The extract from bark, leaves, fruits and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children [6]. Flavonoids, flavonoglycosides, dihydrochalcones, tannins and others are also important constituents of bark, leaves, fruits and flowers of neem. The biological activities and medicinal properties of neem have recently been reported [7].

Natural drugs have been a part of the evolution of human, healthcare for thousands of years. Nowadays nearly 88% of the global populations turn to plant derived medicines as their first line of defence for maintaining health and compacting diseases. One hundred and nineteen secondary plant metabolites derived from plants are used globally as drugs, 15% of all angiosperms have been investigated chemically and of that 74% of pharmacologically active plant derived components were discovered [8]. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, etc. which have been found *In-vitro* to have medicinal properties. Pharmacological studies have accepted the value of medicinal plants as potential source of bioactive compounds [9]. Phyto-chemicals from medicinal plants serve as lead compounds in antimicrobial discovery [10-12].

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2. Materials and Methods

2.1 Collection of plant materials

Leaves were collected from the *Azadirachta indica* tree from Barkatullah University campus. It was ensured that the plant was healthy and uninfected. Plant was authenticated by Prof. A.K. Pathak head of pharmacy department Barkatullah University, Bhopal. Specimen were also submitted for future reference. The leaves were washed under running tap water to eliminate dust and other foreign particles and to clean the leaves thoroughly and dried.

2.2 Preparation of leaf extracts

2.2.1 Ethanol Extract

Azadirachta indica leaves (100 g) were ground into fine powder using a stainless-steel grinder, and deep in 100% ethanol (200 mL) for overnight [13]. The ethanol fraction was separated using sterile muslin cloth and filter through sterile Whatman filter paper (No. 02). The filtered extract was concentrated by a rotary film evaporator.

2.2.2 Acetone Extract

For preparation of Acetone extract ground plant sample (100 g) was added in Acetone respectively (200 ml each case) and left for overnight at room temperature [14]. The extracts were separated using sterile muslin cloth and filter through sterile Whatman filter paper (No. 2) and then concentrated in vacuum at 40-50 °C using a rotary evaporator.

2.2.3 Methanol Extract

100 grams of dried plant material was extracted with 100 ml of methanol kept on a rotary shaker for 24 h. Thereafter, it was filtered Whatman filter paper (No. 2) and then concentrated in vacuum at 40-50 °C using a rotary evaporator and centrifuged at 5000 g for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fifth of the original volume. It was stored at 4 °C in airtight bottles for further studies.

2.3 Phytochemical Analysis

The extracts were analyzed by the following procedures.¹⁵To test for the presence of the alkaloids, saponins, tannins, Terpenoids, flavonoids, glycosides, volatile oils and reducing sugars

2.3.1 Saponins

Saponins were detected using the froth test. 1g of the sample was weighed into a conical flask in which 10ml of sterile distilled water was added and boiled for 5 minutes. The mixture was filtered and 2.5 ml of the filtrate was added to 10 ml of sterile distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

2.3.2 Tannins

To a portion of the extract diluted with water, 3-4 drops of 10% ferric chloride solution is added. A blue color is observed for gallic tannins and green color indicates for catecholic tannins.

2.3.3 Reducing Sugars

To 0.5 ml of plant extracts, 1ml of water and 5-8 drops of Fehling's solution was added and heated over water bath. Brick red precipitate indicates the presence of reducing sugars.

2.3.4 Glycosides

25 ml of dilute sulphuric acid was added to 5ml extract in a test tube and boiled for 15 minutes, cooled and neutralized with 10%NaOH, then 5ml of Fehling solution added. Glycosides are indicated by a brick red precipitate.

2.3.5 Alkaloids

2 ml of extract was measured in a test tube to which picric acid solution was added. An orange coloration indicated the presence of alkaloids.

2.3.6 Flavonoids

4ml of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and orange color for flavones.

2.3.7 Volatile oils

2ml of extract was shaken with 0.1ml dilute NaOH and a small quantity of dilute HCl. A white precipitate is formed if volatile oils are present.

2.3.8 Terpenoids

Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoid.

2.4 Parasites culture *in vitro*

Fresh infected blood samples were obtained for clinical isolation of *Plasmodium falciparum* from Malaria unmulan kendra (Bhopal, M.P) and from People's hospital, Bhopal. Enquiry was made on drug intakes of the patients to select those who had taken any antimalarial drug. Giemsa stained thin smear were examined for Plasmodium species identification. Samples having co infection were discarded.

2.4.1 Preparation of host erythrocytes

Human erythrocytes for parasite culture are prepared by drawing blood into heparin-treated tubes and washing several times in RPMI 1640 medium to separate the erythrocytes from the plasma and buffy coat. Separation can be achieved by centrifuging the blood at 3500 rpm for 8 minutes at 4°C in a swing-out rotor (centrifuged machine). Leukocyte-free erythrocytes are typically stored at 50% hematocrit (i.e. 1 volume of malaria culture media for 1 volume of packed erythrocytes, corresponding to approximately 5×10^9 cells ml⁻¹).

2.4.2 Malaria Culture Media

RPMI 1640 medium containing L-glutamine (High Media), 25 mM HEPES (CDH), 10 µg ml⁻¹ gentamicin (CDH), 0.225% NaHCO₃ (CDH). Medium is adjusted to a pH of 7.3 to 7.4. Once media was prepared media was filtered through 0.22 µm syringe filters then stored in air tight plastic bottles, at 4-8 °C till further use. During culture maintenance media was supplemented with 5% fresh human serum (O+ blood group).

2.4.3 Parasite Culture Conditions

P. falciparum asexual blood stage parasites are propagated at 37 °C in RPMI 1640 media at 3-5% hematocrit in a reduced oxygen environment (e.g. a custom mixture of 5% CO₂, 5% O₂ and 90% N₂). Culture can be maintained in petri plates of 2ml

capacity in candle lid jar with petri plate containing sterile water on the bottom of jar to increase humidity and minimize desiccation [16]. These chambers can be suffused with the low O₂ gas and maintained at 37 °C in an incubator designed to minimize temperature fluctuations. Depending on the line, parasites typically propagate 3-8 fold every 48 hr, thus care must be taken to avoid parasite cultures attaining too high a parasitemia (i.e. percentage of erythrocytes that are parasitized) for healthy growth. Most lines grow optimally at 0.5 – 4% parasitemia. Parasites are most suitable for drug assays when they are 2-5% parasitemia, and mostly ring stages with few or no gametocytes. Fresh medium with uninfected fresh human red blood cells were added to culture plate on daily basis to maintain *Plasmodium* culture, and the mean parasitemia was calculated and expressed as follows.

$$\% \text{ Parasitemia} = \frac{\text{Total number of PRBC}}{\text{Total number of RBC}} \times 100$$

PRBC= Parasitized red blood cells; RBC = Red blood cells

2.4.4 Compounds

Compounds were dissolved in 100% dimethyl sulfoxide (DMSO) and stored at –20 °C. Particle size of insoluble compounds can be reduced by sonication. For the drug assays, serial drug dilutions (either 2× or customized) were made in medium and added to culture plates at 100 ml per well. Drugs were added to 3-12 (test samples), with 1 and 2 reserved for cells with medium without compound. All drugs are typically tested in duplicate or triplicate for parasite line. Once completed, plates were placed into their own modular chamber, gassed and placed at 37 °C. These plates should be set up not more than two hours prior to addition of the parasites.

2.4.5 Drug Assay Conditions

Parasites are diluted to a 2× stock consisting of 0.6% to 0.9% parasitemia (depending on the growth rate of the line) and 3.2% hematocrit in medium, and 100 µl added per well already containing 1 ml of medium with or without compound (present at different concentrations). *Plasmodium* were then subjected to sorbitol treatment for synchronized growth before subjected to assay. Plates are then incubated in a gassed modular chamber at 37 °C for 24 hr to 48 hrs [17]. Percentage parasitemia suppression was calculated according to the following formula.

$$\% \text{ Parasitemia suppression} = \frac{\text{Av \% Parasitemia in negative control group} - \text{Av \% Parasitemia in study group}}{\text{Parasitemia in negative control group}} \times 100$$

Av=Average

2.4.6 Determination of LD₅₀

LD₅₀ was calculated by percent mortality values converted to probit value plotted on probit values against log doses, LD₅₀ value corresponds to probit 5.18 Percentage reductions are used to plot percentage inhibition of growth as a function of drug concentrations. LD₅₀ values are determined by linear regression analyses on the linear segments of the curves (LD₉₀) values can also be determined by curve-fitting and can provide a useful measure of variation between experiments).

Assays are typically repeated on two or three separate occasions.

3. Result and Discussion

The Phytochemical test was done to find the presence of active chemical constituents such as glycosides, alkaloids, tannins, flavonoids, terpenoids, saponin, reducing sugar and volatile oil. The phytochemical analysis of extracts using Acetone, Ethanol and Methanol was showed in Table - 1. From the phytochemical analysis reducing sugar were found in *Azadirachta indica* in the solvents such as Acetone, Ethanol and Methanol. The Ethanol extract showed the presence of flavonoids, saponins, tannin, reducing sugar were found in presence of Ethanol extract. Reducing sugar, glycosides were observed only in Acetone extract. In all extracts found glycosides except in Ethanol extract. Saponin were observed in the Acetone and Ethanol extract, Terpenoids were observed only Methanol extract, the Acetone, Ethanol and Methanol extract showed the absences of alkaloid and volatile oil. The preliminary phytochemical tests performed were of qualitative type and from the phytochemical investigations it was observed that alkaloids, tannins, flavonoids, terpenoids, saponins Glycoside and compounds reducing were present in the extracts. The findings of the preliminary Phytochemical investigations and the results of anti-plasmodium activity were depicted in the respective Tables (table 1 and table 2). This findings conforms to the report of in which similar constituents was found to exhibits antiprotozoal and antibacterial activities.¹⁸ In present study the LD₅₀ for Ethanol extract 7.52 (µg ml⁻¹), Methanol extract 6.76 (µg ml⁻¹), Acetone extract 5.96(µg ml⁻¹) respectively against *Plasmodium in vitro* analysis. Flavonoid has also been reported to have greater potential benefit to human Health [19]. Extracts from Nigerian neem leaves (*Azadirachta indica*) have been earlier reported to have anti-malarial activities [20], but Udeinya *et al.* demonstrated that acetone/water mixture is a more efficient solvent than water alone for the extraction of anti-malarial activity from Nigerian neem leaves [21-23]. This activity has been reportedly retained by IRAB [22, 23]. Its anti-malarial activity has been reported to be superior to chloroquine [24], gametocytocidal [22, 23] and schizonticidal [24] against falciparum malaria parasite. Indeed Anyaehie (2009) reported anti-pyrexial activity among Nigerians where malaria remains the commonest cause of Fever.

4. Conclusion

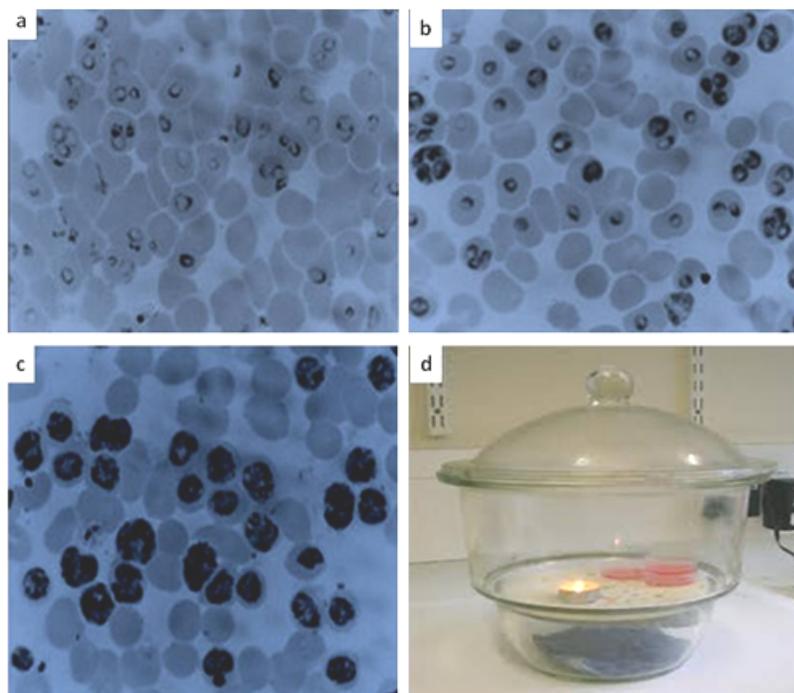
It may be concluded from this study that *Azadirachta indica* leaf extract has anti-plasmodium activity. It is expected that using natural products as therapeutic agents will probably not elicit resistance in pathogens. This can explain the rationale for the use of the plant in treating infections in traditional medicine. The plant could be a veritable and cheaper substitute for conventional drugs since the plant is easily obtainable and the extract can easily be made via a simple process of maceration or infusion. Development of modern non-toxic drugs from neem has earlier been suggested [1] and IRAB represents such dream. It is essential that research should continue to isolate and purify the active components of this natural herb and use in experimental animals.

Table 1: Showing Qualitative Phytochemical Analyses of Acetone, Ethanol and Methanol extracts of *Azadirachta indica* Leaf.

Solvents used for extraction	Alkaloid	Reducing sugar	Flavonoid	Saponin	Tannin	Volatile oil	Glycoside	Terpenoids
Acetone (A)	-	+	-	+	-	-	+	-
Methanol (M)	-	+	-	-	-	-	+	+
Ethanol (E)	-	+	+	+	+	-	-	-

Table 2: Showing LD₅₀ of Ethanol, Methanol and Acetone extracts of *Azadirachta indica*, against *Plasmodium falciparum* *in vitro* study

S. No	Extract	LD ₅₀ value ($\mu\text{g ml}^{-1}$)
1	Ethanol extract	7.52
2	Methanol extract	6.76
3	Acetone extract	5.96

**Fig 1:** Growth stages of *Plasmodium falciparum* under *in vitro* condition stained with giemsa stain at 100x (a, b, c). Candle lit jar for *Plasmodium* maintenance in laboratory (d).

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