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ANTIMALARIAL POTENTIAL OF NEEM LIMONOIDS AGAINST *PLASMODIUM FALCIPARUM*

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Objective: The antiplasmodial activity of pure neem limonoids (azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin) was evaluated in vitro against the chloroquine resistant and-sensitive strains of *P. falciparum*

Methods: Six neem limonoids (purity > 99%) namely azadirachtin, salannin,deacetylgedunin, gedunin, 17- hydroxyazadiradione and deacetylnimbin were sent from M. Ishida, Central Research Laboratories, Taiyo Kagaku Co. Ltd., Japan. They were dissolved in isopropanol and different concentrations such as 25, 50 and 75 µg/ml were prepared by dilution with isopropanol tested against *P. falciparum*.

Results: The Inhibitory Concentration (50) obtained in vitro on human malaria strain ranged from sensitive 1.37%; resistance 3.13% at 75 µg /ml with Azadirachtin. From the six limonoids Azadirachtin, salannin and deacetylgedunin showed high inhibitory activity at all doses, while the rest of the neem limonoids were less active, and were only biologically active at high doses. By synchronization ring stage of parasites suggested as the susceptible target stages to various neem compounds. In addition, all the maturation stages of gametocytes were also killed by various neem fractions tested. The anti-plasmodial effect of neem components was also observed on parasites previously shown to be resistant to other anti-malarial drugs, i.e. chloroquine and pyrimethamine suggesting a different mode of action.

Conclusion: These findings raise the possibility of developing azadirachtin-based compounds as antimalarials with transmission-blocking potential, as well as permitting the further study of structure-activity relationships in these compounds.

INTRODUCTION

Malaria is still an ever-continuing epidemic that claims thousands of lives in Africa each year and the majority of the malaria deaths are due to *Plasmodium falciparum* [1]. A new map of areas where this parasite has been reported, including risk areas, has been drawn [2]. These grim statistics could become even worse if resistance to the existing antimalarial drugs develops further [3]. The spread of multidrug-resistant *Plasmodium* strains has raised the need to search for new drugs or new combinations in order to circumvent this phenomenon. True "new compounds" are difficult to elaborate de novo, while natural products from many plants represent a remarkable and low explored source in the field to treat malaria with miscellaneous success. Two plants have yielded antimalarial drugs that are highly effective against multidrug-resistant *P. falciparum* parasites: Cinchona tree bark, from which quinine and quinidine were isolated, and *Artemisia annua*, from which Chinese scientists have isolated artemisinin (qinghaosu) [4]. Hence, a renewed interest has been shown by various workers world over to the feasibility of use of environment friendly and generally least toxic herbal extracts from plants.

The Neem tree (*Azadirachta indica*) is traditionally labeled as "The Village Pharmacy" on account of its multifaceted healthful properties. Its biological properties range from immunomodulatory and anti-inflammatory effects to antimicrobial and pesticidal attributes. The

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leaves and seeds of *A. indica* yield limonoids with wide biological applications. These have antibacterial, antimalarial activity and have been traditionally used in the management of skin conditions such as eczema, psoriasis and certain fungal infections. Limonoids are described as modified triterpenes, having a 4, 4, 8 trimethyl-17furanyl steroid skeleton (Fig.1). Arrangements of sub-groups and ring structures within this basic building block provide a host of characteristics that have generated interest in this plant product [5].

Recent studies have shown effect of neem limonoids on lactate dehydrogenase (LDH) of the rice leaffolder, *Cnaphalocrocis medinalis* [6]. Antimalarial Activity of methanolic leaf extract of *Piper betle* L against *Plasmodium berghei* with *(*50–400 mg/kg) [7]. Effect of neem limonoids on the malarial vector *Anopheles stephensi* Liston [8]. Antiplasmodial Activity of Lignans and Extracts from *Pycnanthus angolensis* [9]. In vitro antiplasmodial and and cytotoxicity activities of some selected medicinal plants from Kenya and Cuban [10, 11]. Indeed, the vast majority of the existing antimalarial chemotherapeutic agents are based on natural products, and this fact anticipates that new leads may certainly emerge from the tropical plant sources, since biological chemodiversity continues to be an important source of molecular templates in the search for antimalarial drugs. In the search for neem limonoids with anti-malarial properties, the authors have conducted a screening for neem limonoids with activity against the malaria parasite, *P. falciparum*. For this purpose, the neem limonoids were sent from M. Ishida, Central Research Laboratories, Taiyo Kagaku Co. Ltd., Japan was evaluated for antiplasmodial activity performed in 96-well tissue culture plates as described [12] with modifications reported [13]. The present study would be useful in promoting research aiming at the development of new agent for parasite control based on plant source. In view of the recent increased interest in developing plant-based insecticides as an alternative to chemical insecticides, this study therefore, aims to find out neem limonoids possess anti-malarial effects against the laboratory malaria model, *P. falciparum*.

MATERIALS AND METHODS

Neem limonoids

Six neem limonoids (Fig.1) (purity > 99%) namely azadirachtin, salannin, deacetylgedunin, gedunin, 17- hydroxyazadiradione and deacetylnimbin were sent from M. Ishida, Central Research Laboratories, Taiyo Kagaku Co. Ltd., Japan. They were dissolved in isopropanol and different concentrations such as 25, 50 and 75 µg/mL were prepared by dilution with isopropanol.

Parasite sample collection

Malaria positive blood samples (from patients) were collected from Kovai Medical Centre and Hospital, Coimbatore, Tamilnadu-641046, India. The samples are collected in EDTA tubes and stored at 4^{O}C.

Staining and visualizing of parasites

Microscopic diagnosis of malaria is performed by preparing blood smear. The prepared blood smear was stained with Leishman stain (0.15%) to visualize the malarial Parasite. On light microscopic examination of the slide, the ring stage of the parasite was very clearly visualized after staining.

Culture of Parasites

Parasites are grown in human erythrocytes in a settled layer of cells in RP-C: RPMI – Complete. Incomplete medium (RP-I) is prepared by dissolving 16.2 g of powdered RPMI 1640 (supplemented with L-glutamine and 25 mM HEPES buffer but without sodium bicarbonate. Complete medium (RP-C) is obtained by adding 4.2 mL of 5% sodium bicarbonate solution and 5 mL of 8% Albumax stock solution per 100 mL of RPI.Parasites from cultures are added to the freshly washed erythrocytes to give a starting parasitemia between 0.1–1.0%.The cultures are provided appropriate atmosphere using the candle-jar method with 1% O_2, 5% CO_2, and 94% N_2 with 24-h medium changes, requiring subculture by addition of fresh erythrocytes every 4–5 days [14].

In vitro antiplasmodial assay

Twofold serial dilutions of test samples dissolved in sterile methanol were placed in microtiter plates and diluted with culture medium (RPMI 1640 plus 10% human serum). The malaria infected blood suspension (0.5–1% Parasitaemia, 2.5% haematocrit) was added to the wells to give a final volume of 100 ml. Chloroquine was used as positive control and uninfected and infected erythrocytes without any treatment were included as negative controls. The plates were incubated at 37^{O}C and after 24 and 48 h the culture medium was replaced with fresh medium and fresh erythrocytes with or without test samples. After incubation for 24hrs, Leishman stained thick blood smears were prepared for each well, and the percentage of inhibition of parasite growth was determined under microscope by counting the number of ring stages with two lobes by dividing the slide into three parts in comparison of parasites with that of control wells.

Average percentage of parasitaemia =

$$\frac{\text{Av. \% parasitemia in control} - \text{Av. \% parasitaemia in test}}{\text{Av. \% parasitaemia in control}}$$

RESULTS AND DISCUSSION

Neem is derived from the neem tree *A. indica* A. Juss. (Meliaceae), and its primary insecticidal component is the tetranortriterpenoid azadirachtin and other limonoids. The effect of neem limonoids, azadirachtin, salannin, deacetylgedunin, gedunin, 17- hydroxyazadiradione and deacetylnimbin showed reasonably good activities towards the target strains. It inhibited (Reduced the number of infected parasite cells) the sensitive strain and resistant strain range at IC_{50} of Azadirachtin (1.37; 3.13%) Salannin (4.27; 6.23%), Deacetylgedunin (8.37; 9.13%), Gedunin (12.13; 13.17%), 17- Hydroxyazadiradione (17.37; 19.23) % and Deacetylnimbin (25.53; 27.3%) at 75 µg /ml

Table.1 In *vitro* antiplasmodial activity of Neem limonoids against *P. falciparum* clinical isolates

Treatment (µg/ml)	*Parasitemia inhibitory concentration (%)	
	Sensitive	Resistance
Azadirachtin		
25	3.43±0.06	5.23±0.06
50	2.43±0.06	4.37±0.12
75	1.37±0.12	3.13±0.12
Salannin		
25	7.27±0.12	8.07±0.12
50	5.33±0.06	7.37±0.06
75	4.27±0.05	6.23±0.06
Deacetylgedunin		
25	10.27±0.12	11.23±0.06
50	9.4±0.14	10.43±0.06
75	8.37±0.12	9.13±0.06
Gedunin		
25	15.23±0.06	17.27±0.05
50	13.3±0.1	14.4±0.1
75	12.13±0.12	13.17±0.13
17-Hydroxyazadiradione		
25	23.23±0.15	24.23±0.13
50	20.53±0.12	22.47±0.12
75	17.37±0.13	19.23±0.15
Deacetylnimbin		
25	31.38±0.1	34.13±0.56
50	28.43±0.58	30.33±0.56
75	25.53±0.58	27.3±0.1
Chloroquine (control)		
5	1.0±0.0	2.26±0.13

* = Values were presented as Mean ± SEM, n= 5

(Table 1). Significant inhibition (desirable inhibition when compared with that of the parasites in the control) rates were observed after the treatment of neem limonoids. Lowest inhibition rate (sensitive 31.38; resistance 34.13%) was observed in parasites at 25µg/ml with Deacetylnimbin. Our results show the potent parasitic inhibitors were Azadirachtin, Salannin, Deacetylgedunin, and Gedunin. These four compounds have some common structural features such as furan ring and an, β-unsaturated ketone in their A-ring (fig 1). Azadirachtin is by far the most potent plasmodial inhibitor among all the limonoids, being more than five times as effective as the least potent malarial inhibitor, gedunin.

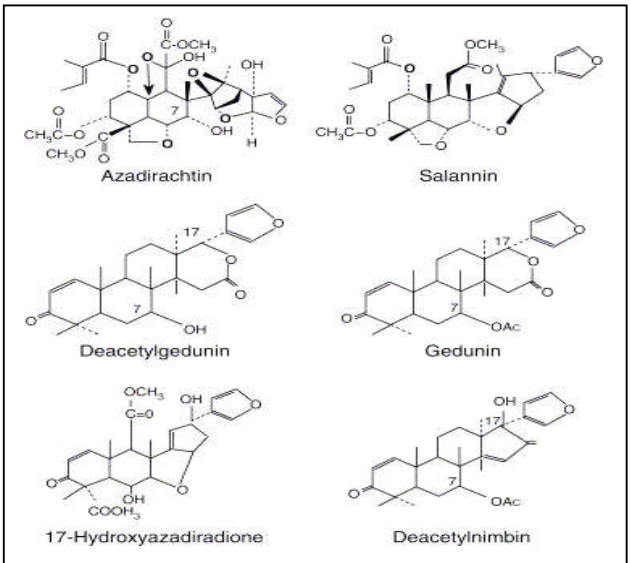

Fig.1 Structure of neem limonoids tested against *P. falciparum*

According to MacKinnon et al [15] the leaf extract containing the limonoid gedunin was examined for antimalarial activity using in vitro tests with two types of *P. falciparum*, one sensitive to chloroquine (W2) and one chloroquine resistant (D6). They state that the extract was found to be more effective against the W2 than the D6 clone, suggesting there is no cross-resistance to chloroquine. Isolated gedunin was more potent than chloroquine against W2. The effects of azadirachtin, salannin, nimbin and 6-desacetylnimbin, isolated from seed kernels, have been studied on ecdysone 20-monooxygenase (E-20-M) activity using various preparations of *Drosophila melanogaster*, *Aedes aegypti or Manduca sexta*. They were incubated with radiolabelled ecdysone and increasing concentrations of the compounds. All were found to inhibit, in a dose-dependent fashion, the E-20-M activity in all three insect species. The concentration of compounds required to produce 50% inhibition ranged from 2 x 10~5 tolxlO-3M [16].

Plants serve as best remedy for malaria since time immemorial beginning from chincona. For example screening the anti - malarial activity of crude extracts is a first step in the isolation of new molecules with potent activity [17, 18]. In addition a new Xestoquinone isolated in Vanuatu from a marine sponge (*Xestospongia sp.*) exhibited also an interesting inhibitory activity on plasmodium growth with an IC_{50} value of 3 Mm [19]. Furthermore, the ethanol extract from the leaves of *Cassia occidentalis*, showed a high *in vitro* antimalarial activity against a *P. falciparum* chloroquine-sensitive strain (IC_{50}<3 µg/mL) [20]. Antimalarial activity of *Morinda lucida* exhibited MIC of 0.6mg/mL and *Alstonia boonei* MIC 0.2mg/mL [21]. The antiplasmodal

effect demonstrated by the ethanolic stem bark extract of *Faidherbia albida* [22]. In vitro antiplasmodial activity of 200 extracts prepared from 50 traditional medicinal plants of Western Ghats, India of which 14.5 % extracts showed significantly high, 26.5% significantly good, and 14.0% moderate activity against CQR *P. falciparum* [23]. In another investigation involving ten plants from the southern India, excellent in vitro antimalarial activity in ethyl acetate extract of *Phyllanthus emblica* leaves and in methanol extract of *Syzygium aromaticum* flower buds was recorded against CQS as well as CQR *P. falciparum* strains [24]. In vitro antiplasmodial activity of ethanolic extracts of mangrove plants from South East coast of India against chloroquine-sensitive *P. falciparum* [25].

In conclusion, the present investigation revealed an efficient invitro antimalarial compounds, the neem limonoids which were traditionally used in chemotherapy of *P. falciparum* in human. So the traditional use of plants to treat malaria is based on a real anti- parasitic activity. These neem limonoids may therefore serve as effective alternatives to conventional anti-protozoan that able to assess the parasite life phase.

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