IN-VIVO ANTIMALARIAL STUDY OF PITC2 of *Pluchea indica* (L.) Less AGAINST *Plasmodium berghei* AND *Plasmodium yolli* MODEL

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Summary

The antimalarial properties of crude extract and PITC-2 a compound isolated from tissue cultured plant *Pluchea indica* traditionally used medicine in various regions of India was evaluated for antimalarial property. The air-dried powdered plant part (roots) was extracted, sequentially with solvents of increased polarity (methanol, butanol, petroleum-ether, and ethyl-acetate). The extract was then administrated orally into albino mice infected with malarial parasites at dose of 100 and 300 mg/kg/day keeping Chloroquine and Sodium artisunate as standard. Ethyl acetate insoluble fraction and isolated compound PITC2 prove significant antimalarial effects.

Key words: *Pluchea indica*; Antimalarial; Plasmodium berghei; PITC2; Plasmodium yolli

Introduction

Malaria is still a life threatening disorder even in this decade. Studies show that every year through out the world more then 300 million people suffer from malaria infections and millions of people die due to this disease (**1**).Due to the ongoing development of resistance of *P. falciparum* to conventional antimalarial drugs (2) has made malaria a major global problem and efforts to discover new agents against multi-drug resistant Plasmodium strains are long-term and essential tasks for researchers. Medicinal agents based on novel mechanisms of action are, therefore, required to overcome the out coming resistance and to control an ever-increasing number of epidemics caused by the malaria parasite (3). Till today nature is the ultimate source of antimalarial drugs. The first drugs Quinine to artemisinin (**Figure 1**) the latest drugs which are highly active against malaria were obtained from nature (4). More over another highly active compound Febrifugine (**Figure 2**) an alkaloid obtained from a Chinese plant root of *Dichroa febrifuga* Lour. (Chinese name: Cha´ng Shan). (5) Though it is not used as a drug due to its toxicity. In our continuing research efforts towards the development of new antimalarials we carry out a random screening of various plants for aforesaid activity. Here we report the antimalarial property of tissue cultured *P.indica* plant and its principal(PITC2) compound showing this properties.

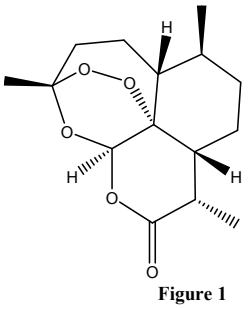


Figure 1

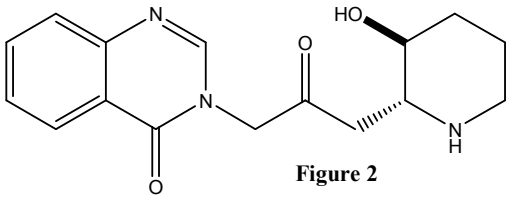


Figure 2

Materials and Methods

Plant material and Chemicals.
The plant material was collected from our university garden; the root was collected and washed with tap water followed by distilled water and shade dried. To carry out antimalarial activity study, we use Giemsa stain, Phosphate buffer saline, Sodium artisunate and Chloroquine as a standard drug.

Preparation of extracts and isolation of PITC2.
Shade dried roots of *Pluchea indica* were grinded. Crude plant extracts were prepared by submitting 650g of air-dried powdered plant material to a sequential sohxlet extraction procedure with 150-500 ml of methanol. A portion of the crude methanolic extract (A) was partitioned between n-butanol and water. The butanol and water fractions were separately evaporated and the crude residues were named as fraction B and fraction C respectively. The butanol fraction was then shaken with ethyl acetate to obtain an ethyl acetate soluble (D) and insoluble part (E). All the fractions (A to E) were separately evaporated to dryness and stored in the refrigerator as crude. We isolated the PITC2 (**Figure 3**) from ethyl acetate insoluble part, through column chromatography as per our reported methodology. (6)

ANTI-MALARIAL STUDY
To carry out the screening of the extracts and isolated compounds for their blood schizonticidal activity,(7) is based on a comparison of responses by groups of treated and control mice, six in each group, after infection with *P. berghei* and *P. yolli* [Obtained from ICMR, New Delhi, India]. It is possible to produce uniform disease using standard inoculums of *P. berghei*, and *P. yolli* (1×10^7 infected red cells) that is fatal to 100% of untreated animals, within 6-8 days, with a mean survival time of 6.2 days. Test animals (Swiss mice of either sex, approximately 29±2g and same age) were housed in metal-topped cages, given a standard laboratory diet and water *ad libitum.* A group of infected animals treated with Chloroquine diphosphate and sodium artisunate at dose levels 10 mg/kg/day for 4 days, producing definite increase in survival time is included as a positive control in every experiment in order to check the factors such as changes in the infectivity of the strain or in the susceptibility of the host or to detect technical errors. The test extracts and PITC2 were administered in graded doses of 100 and 300 mg/kg in every experiment. The compound PITC2 showing positive biological activity at 36 mg/kg were further selected for screening at lower doses. On day '0', groups of 6 mice each were inoculated intra peritoneal route with 1×106 infected erythrocytes in PBS solution from a donor mouse. 4hr later, mice were administered test compounds / Chloroquine / Sodium artisunate / vehicle, orally. A total of four doses were given orally on days D0, D1, D2, and D3. The thick and thin films blood smears (blood collected from tail vain) were made on day D4 and D7 were fixed with methanol and stained with 4% Giemsa at pH 7.2 for 45minutes and examined microscopically (**Figure 4)** in agreement with study results of Peters (8) One group was left untreated as a positive control. Three different fields were examined on each slide and the number of infected and uninfected red blood cells (RBC) counted and the mean taken. The experimental design was similar to the 4-day suppressive test (9).

Percent parasitemia was calculated according to the following formula;

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

Where PRBC: Parasitized red blood cells; RBC: red blood cells

Average percentage of parasitemia suppression was calculated according to the following formula (10)

$$\text{Average percentage of paristemia} = \frac{\text{Av.\% parasitaemia in control - Av.\% parasitaemia in test}}{\text{Av.\% parasitaemia in control}} \times 100$$

The minimum dose that completely suppressed parasitemia on days D_4 and D_7 was termed as Effective suppressive dose (ESD), and the minimum dose that cleared the parasitemia for up to next 30 days was termed as therapeutically effective dose (TED).
Acute toxicity study was conducted for the active extracts (Single dose 2000mg/kg) using the method of Weil.(11) Albino mice weighing 20-25 g of either sex were divided in to groups of five mice each. Each group received the test compounds orally. Signs of toxicity were observed for the first two hours and in two hours interval for six hours. Mortality was assessed after 24hours.

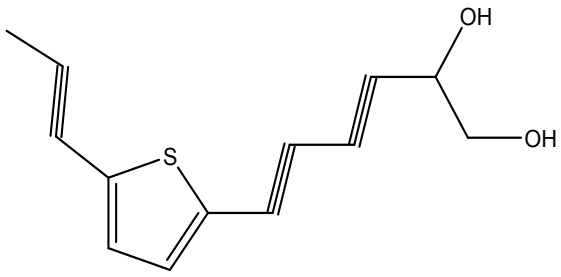


Figure 3

Results and discussion

Assessment based anti malarial study indicate *P.indica* possesses anti malarial property (**Table 1**). The reduction in the level of parasitemia in the blood was significantly higher for the group treated with *control* than for those treated with *P.indica* methanoloc extract. (**Figure 4a** and **4b).**

Table 1: Antimalarial property of various fraction/extracts of *P.indica*

ID	Extracts	Mice (six mice for each dose) alive up to D_7 after 4 days Treatment *(P. berghei)*		Mice (six mice for each dose) alive up to D_7 after 4 days Treatment *(P. yolli)*	
		100mg/kg/day×4, oral	300mg/kg/day ×4, oral	100mg/kg/day ×4, oral	300mg/kg/day ×4, oral
P.imdica	MeOH-Ext (A)	3/6	4/6	2/6	3/6
	MeOH-Ext (A)	0/6	2/6	1/6	4/6
	Aqueous-Ext (C)	0/6	0/6	0/6	0/6
	Aqueous-Ext (C)	0/6	0/6	0/6	0/6
	ethyl acetate soluble (D)	0/6	0/6	0/6	0/6
	ethyl acetate soluble (D)	0/6	0/6	0/6	0/6
	ethyl acetate insoluble part (E)	1/6	2/6	2/6	2/6
	ethyl acetate insoluble part (E)	3/6	2/6	1/6	3/6
	PITC2	3/6	4/6	4/6	4/6
	PITC2	2/6	5/6	4/6	4/6
	Control	0/6	0/6	0/6	0/6
	Standard	6/6	6/6	6/6	6/6
	Chloroquine	6/6	6/6	--	--
	Na-artisunate	--	--	6/6	6/6

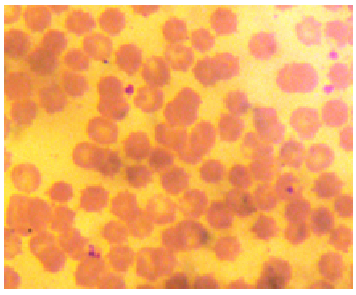
But aqueous fraction dose not show any significant anti malarial property. Further fractionation and subsequent antimalarial evolution indicate the Ethyl acetate insoluble fraction shows prominent anti malarial property. The active principal of *P.indica* was isolated from this fraction produced satisfactory antimalarial effect (**Figure 4b)** in compare to methanolic extract. PITC2 contains thiophene ring associated with three conjugated triple bond with one primary and one secondary hydroxyl group (**Figure 4c)**.

Table 2: % parasitemia after 4 days Treatment by various extract/fraction of *P.indica*

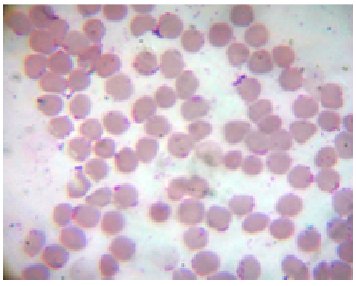
Sl No:	Extract	Dose	% parasitemia after 4 days Treatment *(P. berghei)*	% parasitemia after 4 days Treatment *(P. yolli)*
1	MeOH-Ext(A)	100	3.01±0.47	4.01±1.2
2	MeOH-Ext(A)	300	2.63±0.16	3.09±0.94
3	Aquous-Ext(C)	100	6.2±0.65	6.8±0.36
4	Aquous-Ext(C)	300	6.13±0.95	6.6±0.21
5	Ethyl acetate soluble (D)	100	5.96±0.83	6.2±0.44
6	Ethyl acetate soluble (D)	300	5.463±0.64	6.2±2.5
7	Ethyl acetate insoluble part (E)	100	1.46±0.36	2.3±0.75
8	Ethyl acetate insoluble part (E)	300	1.023±0.21	2.1±0.69
9	PITC2	10	1.012±0.31	1.8±0.21
10	PITC2	50	1.011±0.12	1.6±0.36
11	Standard	--	--	--
12	Control	--	7	7
13	Chloroquine	5	0	--
14	Na-artisunate	5	--	0

The percentage of parasitemia (**Table 2**) further validates the antimalarial property of PITC2. The Aqueous extract is almost in active (%parasitemia 6.13±0.95 and 6.6±0.21) but ethyl acetate insoluble part possesses good anti malarial property (1.46±0.36 and 2.1±0.69). Based upon this point of evidence further isolation and investigating of the PITC2 proves the anti malarial property of *P.indica.* Further PITC2 is active upon Chloroquine sensitive and Chloroquine resistant strains of rodent Plasmodium species. This line of evidence clearly indicates this naturally occurring compound may show the anti malarial effect with some novel mechanism of action.

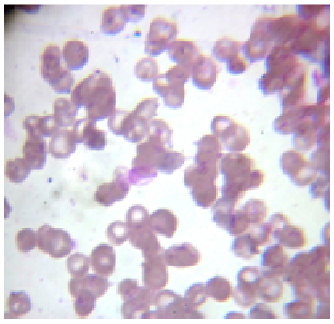
As our previous study indicates it is safe to use in biological system as it possess a wide range of therapeutic window PITC2 may be a potential lead to design a new drug against MDR malaria strains



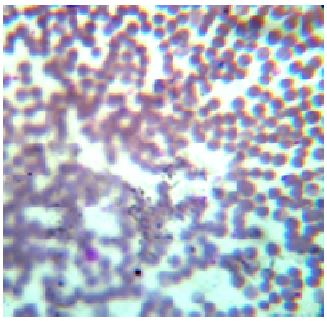
a) Control (no drug administered)



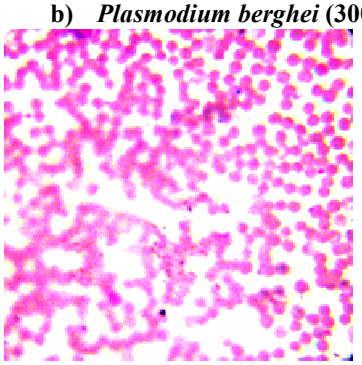
Standard drug (choloroquine)



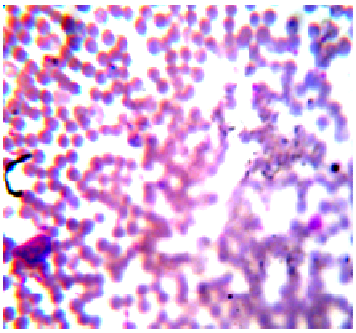
b) *Plasmodium berghei* (300mg kg$^{-1)}$



Plasmodium berghei (PITC 50mg kg^{-1})



c) Plasmodium yolli (300mg kg^{-1})



*Plasmodium yolli (*PITC 50mg kg^{-1})

Figure 4

Conclusion

A large number of antimalarial compounds with immense structural variety have been isolated from plants. However, despite advances made during the last decade, many of these compounds have been subjected to in vivo testing only, and in vitro results are still lacking. Furthermore, many of the compounds identified as antimalarials are also cytotoxic, and may not be suitable as drugs. Not withstanding these problems, plant metabolites will still play an important role in the development of a new generation of antimalarial drugs. We already undertook the study for discover the antimalarial mechanism of action of PITC2 and structural insight of this compound for antimalarial action for developing a potential lead compound against malaria disease.

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