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IN VITRO ANTI-MALARIAL ACTIVITY OF RHIZOME EXTRACTS OF *CURCUMA* SPECIES

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ABSTRACT

Malaria is a major parasitic disease that is transmitted to humans by Anopheles mosquitoes and is responsible for deaths each year in many tropical and subtropical regions. The aim of this study was to assess the efficacy of Chloroquine in the treatment of Plasmodium vivax malaria in Curcuma species. Malaria parasite (Plasmodium vivax) was used in this study. Parasitemia was cultured in RPMI 1640 culture medium (with 10% human serum and gentamycin 2µg/ml) at 37°C in 5% CO_2 incubator. To test the anti-malarial activity of plant extracts we used the susceptibility micro assay technique. Ethanolic extracts of *Curcuma caesia, Hedychium coronarium,* and *Curcuma longa* showed significant parasitaemia inhibitions ranging from 5.8-75.6%, 2.2-29%, 2-29.8% against the blood stage Chloroquine resistant P. vivax with negligible toxicity effect to parasitaemia cells in vitro. The extracts belonging to six plant species were able to perturb the growth of Chloroquine resistant P.vivax effectively. The findings justified the bioassay guided fractionation on these plants for the search of potent anti-malarial compounds or formulation of standardized extracts which may enhance the anti-malarial effect in vitro.

KEY WORDS: **Anti-malarial, Chloroquine, Plasmodium vivax, Curcuma species, parasitaemia inhibitions.**

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INTRODUCTION

Mosquitoes can transmit more diseases than any other group of arthropods and affect millions of people throughout the world. WHO has declared the mosquitoes as "public enemy number one"s due to lack of novel insecticides, high cost of synthetic insecticides, concern for environmental sustainability, harmful effect on human health, and other non-target populations, their non biodegradable nature, higher rate of biological magnification through the ecosystem, and increasing insecticide resistance on a global scale[2,3] Plants have always been the main source for the search of new anti-malarial drugs. Until the year of 2003, 1277 plant species from 160 families have been published by 33 tropical countries for their use in treatment of malaria and fevers]. The use of plants for the treatment of malaria extends over at least three continents, included several countries in Africa, in the Americas and in Asia [5]*Curcuma longa* rhizome has been traditionally used as antimicrobial agent as well as an insect repellant [6]. Since many modern drugs such as quinine and artemisinin originate from plants, it is essential that other medicinal plants which have folklore reputation for anti-malarial properties were investigated, in order to establish their safety and efficacy, and to determine their potential as sources of new anti-malarial drugs [7] It was generally understood that Chloroquine could be trusted to produce a clinical, parasitological cure of an acute attack of vivax malaria. However, high levels of Chloroquine resistance have since been documented in western Oceania and Indonesia [8-10]. This approach recently led to a morphological P. vivax drug-sensitivity test using a more elaborate evaluation system that allows for morphological growth assessment without requiring fully synchronized samples [11][12]. In this study, six medicinal plants (Table-1) with traditional claims were screened for their anti-plasmodial activity against the malaria parasite, Chloroquine (CQ) resistant P. vivax in vitro.

Table 1
Medicinal plants in Zingiberaceae with traditional claims

Scientific Name (Species)	Family Name	Local Name	Common Name	Parts Used
Curcuma longa	Zingiberaceae	Pasupu	Turmeric	Rhizomes
Curcuma amada	Zingiberaceae	Mamidi Allam	Mango Ginger	Rhizomes
Curcuma caesia	Zingiberaceae	Nalla Pasupu	Black Turmeric	Rhizomes
Hedychium coronarium	Zingiberaceae	Dumpa Rashtram	Ginger Lilly	Rhizomes
Curcuma zedoaria	Zingiberaceae	Kakoramu	White Turmeric	Rhizomes
Curcuma aromatic	Zingiberaceae	Kasthura Pasupu	Aromatic Turmeric	Rhizomes

MATERIALS AND METHODS

Collection of plants and Extract preparation

The plant materials used in present study was collected from (Gudala, Allavaram and Amalapuram) Andhra Pradesh. Freshly collected plant materials were dried under shade and the dried material was milled to obtain a coarse powder. The powdered material was extracted in a Soxhlet apparatus for 6 hrs successively with Ethanol and it was concentrated to dryness under vacuum by using Rota-vapor.

Parasite culture

Malaria infection is initiated when an infected Anopheline mosquito injects sporozoites into the human host during a blood meal. After injection, sporozoites enter the bloodstream and go to the liver, initial replication (exo-erythrocytic schizogony) in the liver takes place, the parasites undergoes asexual multiplication in the erythrocytes (erythrocytic schizogony). Enter schizonts, and merozoites are released where they invade hepatocytes and develop into exo-erythrocytic forms. Malaria parasite (Plasmodium vivax) was used in this study. Parasitemia was cultured in RPMI 1640 culture medium (with 10% human serum and Gentamycin 2µg/ml) at 37°C in 5% CO_2 incubator.

Determination of in vitro Anti-plasmodial Activity of Extracts

Susceptibility micro assay technique was used to test the anti-malarial activity of plant extracts [[314]. Plasmodium vivax was continuously maintained in culture, by the method of Trager W, Jensen was used in this assays [15]. Plant extracts were dissolved in ethanol and diluted with RPMI 1640 culture medium, with 10% human serum, to prepared stock solutions (5 mg/ml with 1% ethanol). These solutions were sterilized and used to prepare a series of concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 , 3.125 and 1.562 µg /ml. Chloroquine was used as control and negative controls were prepared with the culture medium and 0.1% ethanol. 50µl of test sample and 100µl of 2% parasitemia was added to each test well of the microplate. Three duplicate assays were carried out per parasite line; microtitre plates were placed in a CO2 incubator at 37 °C for 48 hr; thick films were prepared and stained with Giemsa, showed schizont formation, parasitaemia were scored by light microscopy. Screened 25 microscopic fields per well (200 visual fields/ slide). Since the number of RBC/microscope field has been estimated to be 200/field, the percent parasitaemia can be calculated from a total of 40,000 RBC/ culture

$$\frac{\text{\% of Parasitemia in control - \% of Parasitemia in sample}}{\text{\% of Parasitemia in control}} \times 100$$

The concentration, which inhibited growth by 50% (IC50) in comparison to control wells, was estimated by linear interpolation between drug concentrations values [16].

RESULTS

Anti-malarial activity of plant extracts in combination with Chloroquine

The predominant stage was found under microscopic examination was the ring stage. The average parasitaemia for the standard Chloroquine (no serum added) was determined to be 48.5% (Table 2) while the lowest parasitaemia found was from *Curcuma caesia* 24.4% representing the *Curcuma* species with highest percent in inhibition of parasite invasion. At the standard Chloroquine (control), the lowest Conc. of 1.562 resulted in highest parasitaemia inhibition was found to be *Curcuma caesia (5.8%)* and the with lowest parasitaemia inhibition was reported in *Curcuma amada* (0.8%). At the standard Chloroquine (control), the highest Conc. of 400 resulted in highest parasitaemia inhibition was found to be *Curcuma caesia* (75.6%) and with the lowest parasitaemia inhibition was reported in *Hedychium coronarium* (29%).

Anti-malarial activity of plant extracts alone

A graph is plotted between Chloroquine c*oncentration and percentage* inhibition activity of *Curcuma species* Extract on Parasitized RBC of P. vivax infected malaria patient shown in figure-1 and Tables (Table-2 and Table-3). Ethanolic extracts from 6 *Curcuma* species showed significant parasitaemia inhibitions ranging from 5.8-75.6%, 2.2-29%, 2-29.8%. These are *Curcuma caesia, Hedychium coronarium, Curcuma longa.* In contrast other extracts, *Curcuma amada*, *Curcuma zedoaria, Curcuma aromatic* had non-significant parasitaemia inhibitions which ranged from 0.8-29.1%, 1.8-30%, 1-58%. Among the *Curcuma* species the only extract that showed sustained parasitaemia inhibition is *Curcuma caesia.*

Dose Response of Curcuma species on Malaria Parasite (Plasmodium vivax)

Table 2
Dose response on C. caesia, C. amada and C. longa

	Standard (Chloroquine)		*Curcuma caesia*		*Curcuma amada*		*Curcuma longa*	
Conc. In µg/ml	% of Parasitemia	% of Parasitemia Inhibition	% Parasitemia	% Parasitemia Inhibition	% Parasitemia	% Parasitemia Inhibition	% Parasitemia	% Parasitemia Inhibition
1.562	48.5	51.5	94.2	5.8	99.2	0.8	98	2
3.125	36	64	90.1	9.9	97.1	2.9	94	6
6.25	28.1	73.9	83.3	16.7	94.4	5.6	91.2	8.8
12.5	11.8	88.2	71.7	28.3	89.3	10.7	87.1	12.9
25	3.3	96.7	68.5	31.5	85.5	14.5	86.2	13.8
50	1.2	98.6	50.1	49.9	81.1	18.9	79.2	19.8
100	0.75	99.25	46.5	53.5	77.5	22.5	75.3	24.7
200	0.01	99.89	38.2	61.8	75.2	24.8	71.2	28.8
400	0	100	24.4	75.6	70.9	29.1	70.2	29.8

Table 3
Dose response on *H. coronarium, C. zedoaria* and *C. aromatic*

	Standard (Chloroquine)		*Hedychium coronarium*		*Curcuma zedoaria*		*Curcuma aromatic*	
Conc. In µg/ml	% of Parasitemia	% of Parasitemia Inhibition	% Parasitemia	% Parasitemia Inhibition	% Parasitemia	% Parasitemia Inhibition	% Parasitemia	% Parasitemia Inhibition
1.562	48.5	51.5	97.8	2.2	98.2	1.8	99	1
3.125	36	64	95.1	4.9	94	6	98	2
6.25	28.1	73.9	96	4	95	5	94	6
12.5	11.8	88.2	89	11	91	9	84	16
25	3.3	96.7	85	15	87.4	12.6	80	20
50	1.2	98.6	81.2	18.8	83.1	16.9	73	27
100	0.75	99.25	78	22	80	20	67	33
200	0.01	99.89	74	26	75	25	59	41
400	0	100	71	29	70	30	42	58

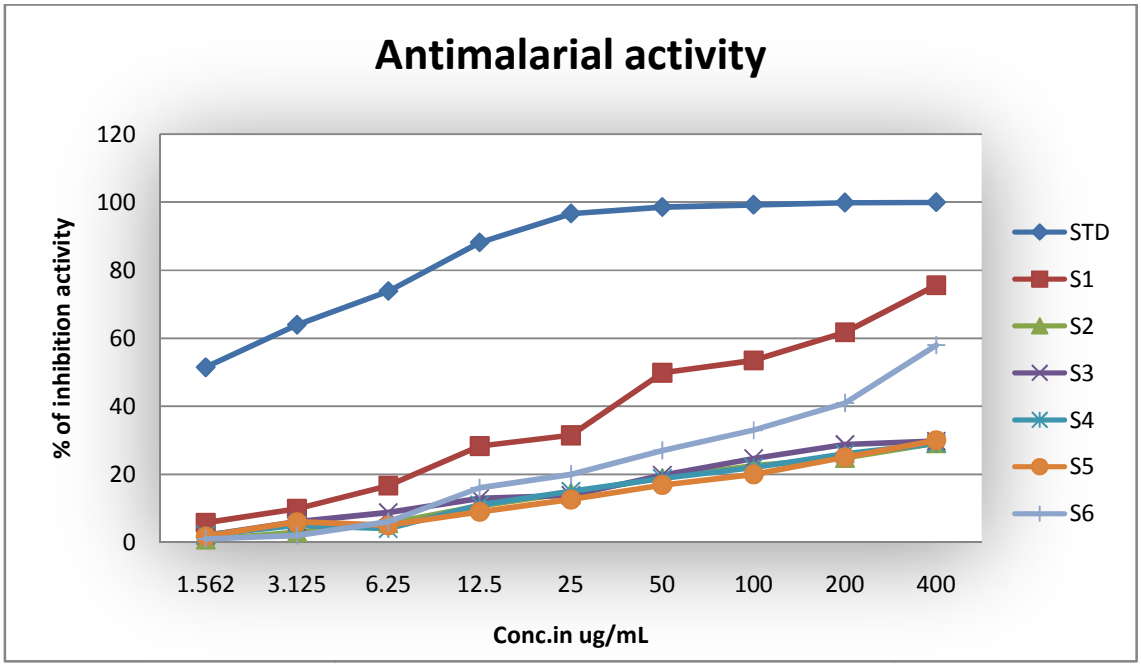

Figure 1
Percentage inhibition activity of *Curcuma species* Extracts on Parasitized RBC of P. vivax infected malaria patient. Key: STD –Chloroquine, S1- *Curcuma longa*, S2- *Curcuma amada*, S3- *Curcuma caesia*, S4- *Hedychium coronarium*, S5- *Curcuma zedoaria*, S6- *Curcuma aromatic*.

DISCUSSION

Malaria is a global disease that is predominant in the tropics and caused by blood parasites, *Plasmodium species.* The female anopheles mosquito transmits these parasites to humans. Malaria has a great morbidity and mortality than any other infectious diseases of the world. The need for a malaria vaccine is imperative because the global burden of the disease is increasing due to drug resistance, resistance of mosquitoes to insecticides, ineffective control measures, re-emergence of the disease, and increased tourism. Malaria vaccine research has investigated several vaccine candidates in clinical trials but with disappointing results in low titers and poor efficacy. Nine out of ten cases of this disease occur in this region and record over one million deaths annually [17]. High mortality rate is recorded in children and pregnant women. Although rarely lethal, P. vivax malaria is responsible for an important morbidity, mainly in children and in pregnant women; the infection has been associated with low birth weight [18]. Chloroquine is the drug of choice for P. vivax malaria, although in recent years resistance to this drug has been demonstrated in several countries. Resistance of P. vivax to Chloroquine was first reported from Papua New Guinea in 1989 [19] and in India [20]; Singh [21]. Our aim was to better document the therapeutic response of P. vivax to Chloroquine and therefore to assess whether the drug was still sufficiently effective. Thick and thin smears were stained with Giemsa 10% (pH 7.2) for 20 min. Determination of the Plasmodium vivax was done on a thin smear. Samples for genomic analysis and Chloroquine measurements were collected separately on Whatmann N_3 filter paper, air-dried and stored in the dark in sealed bags at room temperature. The blood spots were extracted for approximately 1 h using 2.0 ml perchloric acid (0.3 M), 1.0 ml acetonitrile and 5.0 ml phosphate buffer (pH 2; 0.05 M) contains internal standard Positive P. vivax cases should be treated with Chloroquine in full therapeutic dose of 25 mg/kg divided over three days. Vivax malaria relapses due to the presence of hypnozoites in the liver. The relapse rate in vivax malaria in India is around 30%. For its prevention, primaquine may be given at a dose of 0.25 mg/kg daily for 14 days under supervision. Primaquine is contraindicated in G6PD deficient patients, infants and pregnant women. Patient should be advised to stop primaquine immediately if he develops symptoms like dark colored urine, yellow conjunctiva, bluish discoloration of lips, abdominal pain, nausea, vomiting etc. and should report to the doctor immediately. Parasitemia of all groups were monitored, and growth inhibition calculated, as shown earlier. The standard deviation values of parasitaemia and weight were determined using the Microsoft Excel® 2002. The percentage parasitaemia relative to the number of days post infection was evaluated using the Graph Pad Prism 4 version. The difference in Chloroquine levels with that found in other studies could also possibly be explained by differences in procedures used by different laboratories. The present study has identified the anti-plasmodial activity in selected plant extracts by Susceptibility micro assay technique. Six plants (79%) were identified to possess promising anti-malarial properties in their Ethanolic extracts. Theoretically, the purpose of using this extraction technique is to extract specific classes of Phytochemical

constituents from non-polar compounds to polar compounds [22]. In another point of view, the majority of these plants also possessed at least 1% of CQ anti-plasmodial activity indicating the potential of these plants to be the source of anti-malarial candidates. In this study, three medicinal plant extracts had evident anti-plasmodial activity with Chloroquine resistant P. vivax. These are *Curcuma caesia, Hedychium coronarium, Curcuma longa.* The Curcuma plant species such as C. zedoaria, C. longa, C. aromatica and others were well studied for their anti-parasiticidal properties [23]. The results of the in vitro tests with plant extracts against Chloroquine resistant P. vivax are presented in Table 2.

CONCLUSION

This study has attempted to highlight medicinal plant claimed to be used or associated with malaria therapy. These medicinal plants may probably contain yet undiscovered anti-malarial properties, which can serve as a template for the production of cheap anti-malaria drug. Although Chloroquine remains the first-line drug for P. vivax infections in this area, regular monitoring is needed to detect further development of parasite resistance to manage the current burden of malaria in endemic areas, alternative methods such as insecticide spraying, insecticide-treated bed nets, long-lasting insecticidal nets and combination drug therapies should be used to their maximum. Malaria is a prominent, vector-borne parasitic disease and severe public health problem globally, and is especially prevalent in poor, developing countries. There is a great need to create a low cost human malaria vaccine with the elimination of laborious purification techniques and technical skills. Six extracts derived from *Curcuma caesia* (Rhizome), *Hedychium coronarium* (Rhizome), *Curcuma longa* (Rhizome) showed the best anti-plasmodial activities against the blood stage Chloroquine resistant P. vivax with no toxic effects. Ethno pharmacological approach appears to be a promising way to find plant metabolites that could be used as templates for designing new derivatives with improved properties.

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