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Antiplasmodial activity of traditional polyherbal remedy from Odisha, India: Their potential for prophylactic use

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ABSTRACT

Objective: To evaluate the potential prophylactic activity of traditional polyherbal remedy against malaria.

Methods: A traditional polyherbal remedy against malaria from Odisha, India was evaluated for its potential prophylactic activity using *in vitro* hepatic cell lines assay and the murine malaria system *Plasmodium yoelii yoelii/Anopheles stephensi*.

Results: The polyherbal extract inhibited the *Plasmodium yoelii* hepatic stages *in vitro* (IC_{50} 0.74 mg/mL), a therapeutic index of 9.54. In mice treated with the aqueous extract (2000 mg/kg/day), peak parasitaemia values were 81% lower in the experimental 2.35% ± 0.14% as compared to controls 12.62% ± 0.52% ($P < 0.001$), suggesting significant prophylactic activity.

Conclusions: The observations provide a proof of concept for a traditional malaria prophylactic remedy used by tribal populations in India.

1. Introduction

Malaria, a disease caused by the parasite of the genus *Plasmodium*, remains a major public health problem in tropical and sub-tropical countries. It is the most important parasitic disease in humans with an annual incidence of 198 million cases and over 584 000 deaths annually [1]. Nearly half (273 million) of the high-risk population outside Africa reside in India. India contributes over one fifth (22.6%) of clinical episodes of *Plasmodium falciparum* (*P. falciparum*) and 42% of episodes of *Plasmodium vivax* globally [2,3] and around 200 000 persons die annually from malaria [4]. Nearly two-thirds of the malarial episodes and the majority of malarial deaths in India are observed in five states in India: Odisha, Chhattisgarh, Madhya Pradesh, Jharkhand and West Bengal [5]. The situation of malaria is aggravated by an increased prevalence of drug-resistant *P. falciparum*, which poses a threat not only to those living in endemic areas, but also to the millions of non-immune travellers who visit the endemic areas [6]. There is a need for safe and effective new drugs for both treatment and prevention of malaria [7].

As in almost all tropical endemic countries, malaria in India affects particularly people living in rural, remote areas, where most often affordable modern drugs are not available and where poor health care infrastructure cannot assure prompt and appropriate treatment. Thus, a majority of the population still relies on the use of traditional herbal medicines for the management of malaria. A recent ethnobotanical survey conducted by our team revealed that in Odisha, India, there is a rich repertoire of traditional herbal remedies used for malaria prevention [8]. One polyherbal remedy (coded as TPMP74) was selected for further investigation, as it is widely used by many traditional healers to provide protection specifically against malaria during the transmission season as these plants are

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commonly available in the local areas and are affordable to the population of malaria endemic areas. The selection of one over another traditional medicine was based on its perceived value to prevent, rather than treat the malaria infection because drugs which are able to provide prophylactic cover by inhibiting the liver stages have some advantages over chemotherapeutic drugs [9]. The plants used in TPMP74 are known for their antimalarial activity at blood stage.

Given the popular use of this polyherbal remedy as a malaria prophylactic drug, information on its efficacy and safety was sought from *in vitro* and *in vivo* studies to explore the potential activity against the *Plasmodium* hepatic stages and further to identify the novel prophylactic drug of malaria.

2. Materials and methods

2.1. Plants and its extract preparation

The plants of TPMP74 were collected in Koraput District of Odisha State, India, during September, 2011, by a taxonomist who was conversant with the flora of the area. The plants were verified and authenticated by a botanist and voucher specimens were deposited at our institute. TPMP74 was standardized and quality control tests were done as per the standard guidelines [10]. The shade dried plant parts were chopped into small pieces and reduced to coarse powder. The aqueous extract was prepared by soaking 320 g of the powdered plant material with 2.5 L of distilled water using the Soxhlet apparatus (Borosil Glass Works Ltd. India). The extract was concentrated at 60 °C using Rotavapor rotary evaporator and then freeze-dried to obtain the crude extract. The percent yield of the extract was 11% w/w. The dried extract (brown solid) was stored in air tight vial. The extract was subsequently reconstituted in culture medium (*in vitro*) and in distilled water (*in vivo*) at appropriate concentrations as necessary.

2.2. In vitro assay

2.2.1. Cells and parasites

HepG2-A16/hCD81-EGFP cells were cultured in Dulbecco modified Eagle medium (DMEM), supplemented with 10% foetal calf serum, 2 mmol/L L-glutamine, 100 units/mL of penicillin and 100 μg/mL of streptomycin (all from Invitrogen, France) [11,12]. *Plasmodium yoelii yoelii* (265 BY strain) (*P. yoelii yoelii*) sporozoites were obtained from the dissection of infected *Anopheles stephensi* mosquito salivary glands on 14th day after their infective blood meal.

2.2.2. In vitro assay for cell viability

HepG2-A16/hCD81-EGFP cells were seeded in 96-well plates at the concentration of 25×10^3 cells per well in 200 μL medium. After incubation of the cells for 24 h at 37 °C, various concentration range of 0.039–20 mg/mL (half dilution) of plant extracts were added, which were changed daily. After 72 h of drug exposure, the viable cells were determined by MTT reduction assay [13]. In brief, MTT was dissolved in phosphate-buffered saline (1× solution) at 5 mg/mL and diluted with DMEM (without foetal calf serum) until the final concentration was 0.5 mg/mL. MTT solution (100 μL) was added to each well. After incubation for 4 h, cell culture medium was removed carefully and 100 μL dimethyl sulfoxide and ethanol (1:1) was added to each well and mixed thoroughly to dissolve the dark purple crystals. Optical density change in viable cells was quantified by measuring the optical density at 540 nm on a FlexStation (Molecular Devices Ltd., UK). According to 50% toxic concentration (TC_{50}), the drug concentration at which a 50% reduction of MTT incorporation was observed, as compared to that incorporated in the control cultures (control wells consisted on untreated cells, they only received culture medium), was derived from three independent experiments in which each concentration was tested in triplicate.

2.2.3. In vitro assay for P. yoelii yoelii liver stage inhibition

HepG2-A16/hCD81-EGFP cells were seeded in 96-well plates at a density of 25×10^3 cells per well. After 24 h of incubation, cells were pre-treated with the various concentrations (0.039–5 mg/mL, half dilution) of plant extracts and the freeze-dried sample was solubilised in DMEM supplemented as described above. After 48 h of incubation, cells were inoculated with 15000 sporozoites of *P. yoelii yoelii*. The treatment was continued from the time of the infection and renewed 3 h later, after sporozoite penetration into hepatic cells, and at 24 h post-infection. Sporozoite-inoculated culture plates were centrifuged for 10 min at 2000 r/min at 4 °C in order to enhance the infection rate. Culture medium containing the appropriate drug concentration was changed daily until 48 h. Parasite quantification was done by immunofluorescence analysis following the fixation of cultures with cold methanol. Intracellular parasites were stained with a mouse polyclonal serum raised against *Plasmodium* heat shock protein 70, followed by goat anti-mouse Alexa Fluor 488 conjugate (Invitrogen, Molecular Probes) and cell nuclei were stained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (Invitrogen) [14]. Parasite numbers were counted under a fluorescence microscope with a 200× magnification. According to IC_{50} values, the drug concentration at which a 50% reduction in the number of parasites was observed, as compared to the number in the control culture medium, was derived from three independent experiments in which each concentration was tested in triplicate wells.

2.3. In vivo assay

2.3.1. Animals

The female animals (Swiss albino mice) used for these experiments were obtained from Janvier Labs (Centre d'élevage Roger Janvier, France). The animals were allowed to acclimatize for 7 days before the commencement of the experiment. The study was carried out in strict accordance with the recommendations in the guide of the Care and Use of Laboratory Animals of the European Union “European Directive 86/609/EEC” and of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Pierre and Marie Curie, Paris 6, France (Permit number: 75–1087).

2.3.2. Acute toxicity test

The LD_{50} of the plant aqueous extract was tested to determine the safety of the agent using Lorke's method [15]. Twelve mice were randomized into four groups of three mice per cage and were administered 1500, 2500 and 5000 mg/kg (0.5 mL) of the extract orally and the fourth group received 0.5 mL of

distilled water to serve as control. The mice were observed for manifestation of physical signs of toxicity such as writhing, decreased motor activity, paw licking, decreased body/limb tone, respiration distress and death in the 4th hour and subsequently for 4 days.

2.3.3. In vivo assay for activity on P. yoelii yoelii infections

The mice were randomly divided into four groups of six mice and administered orally with either 500, 1000, 2000 mg/kg/day of the TPMP74 aqueous extract which were safe dosages or distilled water 0.5 mL to the control group for three consecutive days (Day 0–Day 2). On the fourth day, the mice were challenged by retro-orbital injection with 5000 sporozoites of *P. yoelii yoelii*. The parasitaemia levels were assessed in Giemsa-stained thin films made from tail-blood of each mouse for 72 h after inoculation. The percentage of parasitaemia was determined by counting the number of the parasitized red blood cells out of 1000 erythrocytes in random fields of the microscope.

The mean percentage suppression of parasitaemia was calculated as:

$$\text{Suppression}\ (\%) = \frac{\text{Parasitaemia in control} - \text{Parasitaemia in treated}}{\text{Parasitaemia in control}} \times 100$$

2.4. Statistical analysis

In *in vitro* study, TC_{50} and IC_{50} were calculated by nonlinear regression using log (inhibitor) *vs.* normalized response (variable slope) on the GraphPad Prism 5.00 software. Each TC_{50} and IC_{50} were expressed as a mean between three independent experiments, followed by the standard deviation of the mean. In *in vivo* study, mean of the percentage of parasitaemia was calculated for each group. Percentage reductions of parasitaemia in the treatment groups against control group were calculated. Kruskal and Wallis test was used to determine the effect of polyherbal extract on the median parasitaemia. Further, *post-hoc* test (multiple comparisons) was conducted to demonstrate if there was any significant difference in median parasitaemia for each group comparison. All the statistical analyses were done using SPSS 18.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. In vitro assay for cell viability

MTT test showed a cellular toxicity above the concentration of 0.63 mg/mL (starting from 4% at 1.25 mg/mL). TC_{50} value was (7.03 ± 0.21) mg/mL, with the highest toxicity (98%) reached at the highest dose tested (20 mg/mL).

3.2. In vitro antimalarial activity against liver stage of P. yoelii yoelii

The activity of the aqueous extract was assessed in a standard bioassay of cultured HepG2-A16/hCD81-EGFP cell line infected with *P. yoelii yoelii* sporozoites. The extract was found to be active against the *P. yoelii yoelii* liver stages. Parasite inhibition was observed in a dose-dependent manner, with an IC_{50} of (0.73 ± 0.11) mg/mL (number of schizonts in control vials: 230 ± 19). It was important to note that the hepatic forms were completely eliminated at concentrations of 2.5 mg/mL or greater. Figure 1 shows the graph representing the toxic and inhibitory concentration of the drug.

3.3. In vivo acute toxicity test

There were no manifestations of physical signs of toxicity or any mortality in any group of mice after oral administration of the aqueous extract even at the higher dose of 5000 mg/kg, signifying that the oral LD_{50} was >5000 mg/kg. Thus the experimental doses used (500, 1000 and 2000 mg/kg) were within safe margins.

3.4. In vivo prophylactic activity

The aqueous extract exerted a dose-dependent prophylactic activity resulting in significant ($P < 0.001$) reduction of parasitaemia in extract-treated groups when compared to controls. A suppression of parasitaemia of 65.3%, 73.8% and 81.4% was recorded for the corresponding dose of extract (500, 1000 and 2000 mg/kg/day, respectively) (Table 1) at Day 9 post-infection, which corresponded to the maximum level of parasitaemia reached all along the course of the infection in the mice (peak parasitaemia). The Kruskal and Wallis test followed by *post-hoc* tests showed a significant differences between the treated and control groups (Figure 2). Importantly, a delay of 1 day was observed in the appearance of parasitaemia in all treated mice (6 days), compared to all controls (5 days) which in term of parasite liver infection was considered to denote a major liver burden decrease. This showed the effectiveness of TPMP74 extract in the mouse model against the *P. yoelii yoelii* hepatic infection.

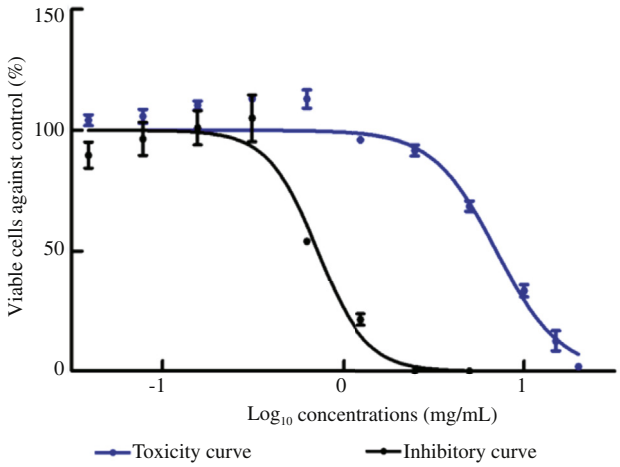

Figure 1. *In vitro* assay for *P. yoelii yoelii* liver stage inhibition.

Table 1
Prophylactic effect of the polyherbal decoction on mice infected with *P. yoelii yoelii*. %.

Extracts	Doses	Parasitaemia (mean ± SD)	Suppression
Aqueous extract	500 mg/kg	4.38 ± 0.87	65.3
	1000 mg/kg	3.30 ± 0.33	73.8
	2000 mg/kg	2.35 ± 0.34	81.4
Control (distilled water)	0.5 mL	12.62 ± 1.27	

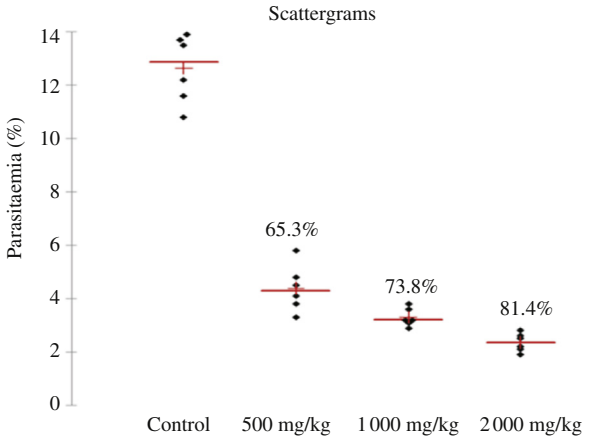

Figure 2. *In vivo* assay for activity on *P. yoelii yoelii* infections. Control *vs*. treated 500 mg/kg: $P = 0.021$; Control *vs*. treated 1000 mg/kg: $P = 0.020$; Control *vs*. treated 2000 mg/kg: $P = 0.021$; Treated 500 mg/kg *vs*. treated 1000 mg/kg: $P = 0.061$; Treated 500 mg/kg *vs*. 2000 mg/kg: $P = 0.021$; Treated 1000 mg/kg *vs*. 2000 mg/kg: $P = 0.020$.

4. Discussion

This study demonstrated the malaria prophylactic potential of the polyherbal remedy used by traditional healers in Odisha, India, in the murine malaria parasite *P. yoelii yoelii*. The prophylactic assay determined that the traditional remedy was able to reduce parasitaemia by about five times the values recorded in control mice.

The TPMP74 extract was found to be active against the *P. yoelii yoelii* liver stages with parasite inhibition in a dose-dependent manner [IC_{50} of (0.73 ± 0.11) mg/mL]. MTT test showed a cellular toxicity above the concentration of 0.63 mg/mL *i.e.* $IC_{50} > TC$.

The aqueous extract tested in this study has shown 73.8% reduction of parasitaemia at 1000 mg/kg body weight and parasitaemia reduction has increased to 81.4% at 2000 mg/kg body weight. The increased reduction of parasitaemia may be due to higher dosage of the polyherbal drug or potential synergistic effect or potential additive effect of the plants which need to be determined in the future experiments. Although there are some single plant formulations, traditionally, combinations of plants have been commonly used and it is important to understand the logic or science behind the combinations of these formulations.

In addition to a five times reduction in peak parasitaemia in treated as compared to control mice, a one-day delay was observed for the first day of patency in the treated mice (6 days *vs*. 5 days in the controls-after sporozoites inoculation). Because of the exponential increase of the asexual parasites every 24 h, such a delay could reflect a dramatic reduction and/or delay in merozoites emerging after liver stage development [16]. In order to establish whether this effect was due to an inhibitory activity against the hepatic parasite rather than an additional activity on the blood stages alone, we assayed the formulation against the *in vitro* hepatic stages. This clearly demonstrated that the remedy was active at inhibiting the development of the *P. yoelii yoelii* livers stages with an IC_{50} of (0.73 ± 0.11) mg/mL and a therapeutic index of 9.63 which compares favourably with that of primaquine (5.25) [17]. It should be noted that the traditionally administered formulation was not toxic to mice when administered at the highest concentration tested of 5000 mg/kg body weight which signifies the oral LD_{50} was >5000 mg/kg as per guidelines of Organization for Economic Co-operation and Development [18].

Generally, in malaria drug discovery studies, emphasis is given to routine screening for erythrocytic (blood) phase which is relatively less expensive and easier to carry out compared to pre-erythrocytic stage investigations and clinically more demanding for the treatment of malaria. However, screening of drugs for pre-erythrocytic phase is important to develop novel malaria prophylactic drugs. Traditionally, the selected polyherbal drug is exclusively used for the prevention of malaria; hence it was preferred to determine the antiplasmodial activity of this drug using *in vitro* and *in vivo* methods. This is the first study where these two methods have been used to study a traditionally used polyherbal malaria prophylactic drug in its crude form.

The polyherbal extract was shown a good potential as malaria prophylaxis against rodent parasites. This needs to be further explored on human primary hepatocytes infected with *P. falciparum*. This can also be tested on *P. falciparum in vivo* in humanized mice (engrafted with human hepatocytes). Performing isobolograms of the effect of the different plant constituents when associated together will help in understanding the synergistic or additive value of each plant material. Positive controls need to be used in the future studies.

Traditional herbal remedies are widely available and commonly used as malaria prophylaxis by the population of Odisha, India. In malaria endemic areas, community based trials should be encouraged to verify the prophylactic activity which may contribute to reduce the malaria burden. In conclusion, the results of this study showed that the traditional herbal remedy possesses antiplasmodial activity which justifies the development of further clinical trials in order to establish its efficacy as a malaria prophylaxis.

Conflict of interest statement

We declare that we have no conflict of interest.

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