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RESEARCH ARTICLE

IN VIVO ANTIMALARIAL ACTIVITY OF *STREPTOMYCES* SP AGAINST *PLASMODIUM BERGHEI* IN MICE MODEL

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ABSTRACT

Treatment of malarial infection has becoming extremely challenging due to widespread resistance of the parasite towards available antimalarial drugs. In the present study, we investigated the antimalarial activity of the ethanolic extracts of *Streptomyces* sp (KMA08) in *Plasmodium berghei* infected mice. The antimalarial activities during early and established infections were evaluated using Peters' 4-day suppressive test method. Different concentration of the crude extracts were orally administered to screen for their antimalarial activities. The extracts significantly inhibited parasitemia dose-dependently. Each extract showed variable level of parasitemia suppression in dose related manner. The ethanolic extract of *Streptomyces* sp was found to produce 47.5% (30ml/kg) parasite suppression. The test substance observed to be safe with no toxicity on the mice even at 30 ml/kg. Results showed that significantly improved the suppression of parasitemia. The results of the present work supported the use of the *Streptomyces* sp against malaria and confirmed the antimalarial activity. Moreover, antimalarial compounds can be isolated and tested against human malaria parasite in the future.

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INTRODUCTION

Malaria is a treatable mosquito borne disease, whose main victims are children under five years of age in Africa. According to the latest WHO estimates, there were about 219 million cases of malaria in 2010 and an estimated 600,000 deaths. Africa is the most affected continent, about 90 % of all malaria deaths occur there. In 2012 malaria caused an estimated 627000 deaths (with an uncertainty range of 473,000 to 789,000) mostly among African children (WHO, 2012) Malaria is the worldwide most important parasitic disease with an incidence of almost 300 millions clinical cases and over one million deaths per year. There were an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years. 109 countries were endemic for malaria in 2008, 45 within the WHO African region WHO, 2008 (Winstanley, 2000). Malaria can be, in certain epidemiological circumstances, a devastating disease with high morbidity and mortality demanding a rapid and comprehensive effort. The search for new anti-malarial agents is therefore crucial in the effort of combating the disease. Southeast Asia has been reported to have the greatest problem of drug resistance against malaria. There is evidence of resistance against all anti-malarial drugs including

mefloquine, halofantrine and even quinine (Kondrashin *et al.*, 1992) This situation is worsened with the emergence of chloroquine- resistant strains of *Plasmodium falciparum*, the malaria parasite responsible for most of death cases every year. Therefore, there is an urgent need to discover and develop new, effective and safe drugs for the treatment of this disease (Min Chung 2007). This has led to attempts to discover other antimalarial agents, mainly from microbial sources. Among the microorganisms, *Streptomyces* were the most studied group because of their capacity to produce novel bioactive secondary metabolites. Despite their importance in soil ecology, the role of actinomycetes as potential antibiotic producers became apparent in 1940, following the discovery of actinomycin (Waksman *et al.*, 1940), and was fully realized by the 1980s, when actinomycetes accounted for almost 70% of the world's naturally occurring antibiotics (Okami *et al.*, 1988). Although the exploitation of marine *Streptomyces* as a source for novel secondary metabolites is in its infancy, the discovery rate of novel secondary metabolites from marine *Streptomyces* has recently surpassed that of their terrestrial counterparts, as evident by the isolation of many different diverse structures in the past few years (Fiedler *et al.*, 2005, Jensen *et al.*, 2008). Furthermore, very few of these marine compounds have been tested against malaria parasites (Prudhomme *et al.*, 2008 and Isaka *et al.*, 2002). The aim of this study to investigate the antimalarial potential of mangrove *Streptomyces* from

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mangrove soil, whereby, an *in vivo* of the antiplasmodial properties of the *Streptomyces* species were determined.

MATERIALS AND METHODS

Isolation of *Streptomyces* species

Soil samples were collected from four different area of Pichavaram mangrove, Tamilnadu, India. The soil suspension (0.5 ml) was spread on the starch casein agar plates. The plates were incubated for 4 weeks at 30°C. The *Streptomyces* sp colonies grown on Petri plates were counted at regular intervals. All the morphologically different *Streptomyces* colonies were sub-cultured.

Solvent extraction of *Streptomyces* species

To the culture filtrate, equal volume of various solvents (n-butanol, ethanol, ethyl acetate, petroleum ether, chloroform, benzene and xylene) were added separately and centrifuged at 5000rpm for 10min to extract the active compound (Sambamurthy and Ellaiah, 1974). Active extract selected for the further studies.

Parasites and Inoculum

P. berghei were used to assess the *in vivo* intrinsic antimalarial activity. The strain was originally obtained from the National Institute of Malaria Research, Delhi. Parasite strain was maintained by serial passage of blood from mice to mice. A standard inoculum of 1×10^7 of parasitized erythrocytes from a donor mice in volumes of 0.1ml was used to infect the experimental animals intraperitoneally. The parasite has been maintained at the School of Life Science, Karpagam University, Coimbatore.

Test animals

Male Swiss albino mice weighing between 27–30g were used for this study (Peters *et al.*, 1975). The animals were fed Standard mice cubes and clean drinking water. Animals were caged in groups of five. The animals were housed in the Animal House in Karpagam University, Coimbatore.

Experimental Design

This study is animal based experiment where a total of 25 mice for each experiment were randomly assigned in to five groups for microbial extract with five mice per group (Table 1). Three groups of mice received the extracts at 10, 20, and 30 ml/kg respectively. The other two groups of mice were treated by 20 ml chloroquine and normal saline at dose of 20 ml/kg respectively.

Table 1. Experimental design of the study

Treatment group	Number of mice	Extract / drug dose ml/kg
i	5	10 extract
ii	5	20 extract
iii	5	30 extract
iv	5	20 chloroquine
v	5	20 normal saline

Acute Toxicity Tests

The oral acute toxicity of the ethanolic extract was estimated in albino mice (27 - 30g) by medium lethal dose (LD50) described by Lorke's method (Lorke, 1983). A total of fifteen albino mice were employed, acclimatization period of 24 h was allowed. The extract was administered orally at doses of 10, 20 and 30 ml/kg to three groups of 5 animals each received respectively. The animals were monitored for 24 h and number of deaths per group recorded. The mice were observed for gross behavioural changes such as feeding, hair erection, lacrimation, mortality and other signs of toxicity manifestation (Pillai, 1984).

Test on early malaria infection (Peters 4-day suppressive test)

Twenty five mice were divided into five groups of five mice each were inoculated with the parasite at the commencement of the experiment (day 1). Group 1- 3 mice received 10, 20 and 30ml extract/kg body weight respectively. While the 4th group which served as the positive control received 20ml chloroquine/ kg body weight, mice in 5th group received 20ml normal saline and served as the negative control. On the fifth day (i.e., day 5) two drops of blood samples from the animals' tail vein were taken and transferred on slides, thus, making thin film from each mice and staining with Giemsa stain, Then, each stained slide was examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract. The average percentage (%) parasitaemia could be evaluated as

$$\text{Average \% Suppression} = \frac{\text{Average Parasitaemia in negative control} - \text{Average Parasitaemia in drug treated group}}{\text{Average parasitaemia in negative control}}$$

Evaluation of schizontocidal activity in established infection (Rane test)

Rane test was employed to evaluate schizontocidal activity of extract in established infection. On the first day (D0), standard inoculum of 1×10^7 *P. berghei* infected erythrocytes were injected intraperitoneally into mice (Ryley *et al.*, 1970). Seventy two hours later, the mice were divided into five groups of five mice each. Different doses of ethanolic extract (10, 20 and 30 ml/kg/day) were administered orally to these groups. Chloroquine (20 ml/kg/day) was given to the positive control group and an equal volume of normal saline to the negative control group. The drug/extract was given once daily for 5 days. Thin blood smears were prepared from tail of each mouse for 5 days, to monitor the parasitaemia level.

Determination of Mean Survival Time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period (Maje *et al.*, 2007). The mean survival time (MST) for each group was calculated as:

$$\text{MST} = \frac{\text{Sum of survival time of all mice in a groups (days)}}{\text{Total numbers of mice in that group}}$$

Statistical analysis

Data obtained by this study were analyzed using SPSS (version 16, 2004). The Student's t-test and ANOVA (one- or two-way) were used to test the differences between groups. Differences between means at 5% level ($P \leq 0.05$) were considered significant.

Determination of Body Weight and Temperature

The body weight of each mice in all the groups was taken before infection (day 0) and on day 4. The rectal temperature of the mice was measured with a digital thermometer before infection, three hours after infection and then daily up to day 4 to see the effect of the extracts on body temperature.

RESULTS AND DISCUSSION

Ethanol extract of the *Streptomyces* sp were tested for their toxicity against Swiss albino mice and for their antimalarial activity against *P. berghei* in mice. No toxicity symptom was observed with the selected dosage level (Table 2).

Table 2. Acute toxicity of the ethanolic extracts of *Streptomyces* sp

Dose ml/kg	Mortality	Toxic symptoms
	Death/Treatment	
0	0/5	None
10	0/5	None
20	0/5	None
30	0/5	None

The extracts were nontoxic to test mice, as they did not show signs of acute toxicity within 24 hours at the doses of 20 ml/kg and 30 ml/kg. Changes in general behaviours, variations in body weight and mortality are critical for the evaluation of the effect of a compound on test animals, since such changes are often the first signs of toxicity (Carol 1995). The main observed behavioural signs of toxicity were asthenia, piloerection, ataxia, anorexia, urination, diarrhoea, lethargy and coma. Jutamaad *et al.* (1998) reported that oral administration is about 100 times less toxic than the intraperitoneal. Early malaria infection or Peters four days suppressive activity test for the ethanol extract of *Streptomyces* produced a dose dependent suppression activity (Table 3).

Table 3. Effects of ethanolic extract of *Streptomyces* sp on early malaria infection

S.No	Treatment	Doses (ml/kg/day)	Average parasitemia in percentage	% of suppression
1	Extract	10	32.6±1.0	21.3
2	Extract	20	25.4±0.6	39.3
3	Extract	30	12.0±0.3	47.5
4	Chloroquine	20	1.0±0.0	100
5	Normal saline	20	52.4±1.2	0

The highest suppression of parasitaemia was observed at the dose of 30 ml/kg body weight of mice. Percentage suppression was observed to increase as extract concentration increased. After four days treatment with the different extract doses, the mean parasitaemia of the test groups ranged from 12.0±0.3% to 32.6±1.0% while the corresponding value of the negative control group being 52.4±1.2%. The mice treated with chloroquine were completely free from the parasites on day four. The antimalarial activity produced by the ethanolic extract was statistically significant ($P < 0.05$) when related to control. Rivo *et al.* (2013) reported that increase of inhibition level of parasitemia for all treatment groups with increasing doses of therapy. This inhibition level is significantly different as compared to positive control. Percentage of inhibition showed significant differences between control and group treated with metabolite extract of *S.hygroscopicus* 2600 µg/kgBW. An increasing dose of extract of *S. hygroscopicus* followed by an increasing of inhibition in parasite growth ($r=0,850$). The result of the *in vivo* evaluation of the *Streptomyces* sp extract on established infection showed the extract was marginally active at 10 ml/kg per day and active at 20 and 30 ml/kg per day doses, respectively (Table 4).

Table 4. Effects of ethanolic extract of *Streptomyces* sp on established malaria infection

S.No	Treatment	Doses (ml/kg/day)	Average parasitemia in percentage	% of suppression
1	Extract	10	32.6±1.0	21.3
2	Extract	20	25.4±0.6	39.3
3	Extract	30	12.0±0.3	47.5
4	Chloroquine	20	1.00±0.0	100
5	Normal saline	20	52.4±1.2	0

The mice that received 20ml of chloroquine/kg per day however showed 100% chemosuppression. The antimalarial activity produced by the extract was statistically significant ($P < 0.05$) when related to control. (Sayed *et al.*, 2002) reported that the marine sponges associated microbial alkaloids possess antiplasmodial activity. These findings could encourage the marine derived compounds for the antiplasmodial drug development. The biochemical constituent analysis of potential extracts showed the presence of reducing sugars and alkaloids. The mode of action could be due to the inhibition of *P.falciparum* merozoites invasion into the erythrocytes. (Adams *et al.*, 2005).

Mean Survival period of mice treated with ethanolic extract

The mean survival period of the Swiss albino mice treated with the extract in established infection during a period of 28 days showed that as the dose increase, the survival time reduce (Table 5).

Table 5. Mean Survival period of mice treated with ethanolic extract

Dose of extract (ml/kg/day)	Survival time (days)
10	10
20	13
30	16
Chloroquine	23
Normal saline	5

While mice treated with chloroquine at 20ml/kg per day survived for 23 days, mice treated with the extract at 10, 20 and 30ml/kg per day survived for only 16, 13 and 10 days respectively. The animals in the negative control group, which were treated with normal saline, were found to have mean survival time of 5 days only.

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