

EVALUATION OF THE ANTIMALARIAL POTENTIAL OF *STREPTOMYCES* SP.

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ABSTRACT

Objective: Isolate, screen and identify *Streptomyces* sp. from mangrove soil from pichavaram, Tamil Nadu, India, and study the molecular identification of selected *Streptomyces* sp. and check the antimalarial activity for the purified compound.

Methods: The 16S rRNA secondary structure and the restriction sites of KMA08 were predicted using Genbank online software, respectively. Antiplasmodial activity of the 80% Ethyl acetate extract of *Streptomyces* sp. against chloroquine-sensitive *Plasmodium berghei* in mice using the 4 days suppression test was conducted. A total of 30 mice assigned to 5 groups of 6 animals each were infected with chloroquine-sensitive (*P. berghei*) intraperitoneally. The Ethyl acetate extract (10, 20, and 30 ml/kg), standard drug (chloroquine, 10 mg/kg) and distilled water were administered orally daily for the treatment period. Percent Parasitemia was determined on the 5th day from Giemsa stained smears obtained from tail vein and percent parasitemia suppression was calculated. Daily measurement of rectal temperature was also taken while body weight and packed cell volume were recorded on day 0 and 5.

Results: Results showed the extract produced a dose-dependent reduction in parasite density compared to the control group. Percent parasitemia calculation revealed 21.3, 65.3, and 80.5% inhibition at 10, 20, and 30 ml/kg of the extract, respectively.

Conclusion: The study revealed the present work indicated that *Streptomyces* sp. has as promising antiplasmodial activity against chloroquine sensitive *P. berghei* in a dose-dependent. As part of the drug discovery process, these promising finding may contribute to the medicinal and pharmaceutical field for malarial treatment.

Keywords: Actinomycetes, Sequence, Antiplasmodial, Parasitemia, Malaria.

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INTRODUCTION

About 300–500 million clinical cases and 1.2–2.8 million deaths due to malaria occur each year [1,2]. The rising problems of resistance to the classical drugs (chloroquine and sulfadoxine pyrimethamine) [3,4] and the problem of recrudescence of artemisinin stress the 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 antimicrobial agents is, therefore, crucial in the effort of combating the disease so need to look for new antimalarial agents [5]. Southeast Asia has been reported to have the greatest problem of drug resistance against malaria [6].

This situation is worsened with the emergence of chloroquine-resistant strains of malaria parasite responsible for most of the death cases. Therefore, there is an urgent need to discover and develop new, effective and safe drugs for the treatment of this disease [7]. This has leads to attempt to discover other antimalarial agents, mainly from microbial sources. microorganisms, *Streptomyces* due to their capacity to produced novel bioactive secondary metabolites.

Among the microorganisms, *Streptomyces* were the most studied group due to 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 [8], and was fully realized by the 1980s, when actinomycetes accounted for almost 70% of the world's naturally occurring antibiotics [9]. 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 [6].

Furthermore, very few of these marine compounds have been tested against malaria parasites [7,8]. The aim of this study to investigate the antimalarial potential of mangrove *Streptomyces* from mangrove soil, whereby, an *in vivo* of the antiplasmodial properties of the *Streptomyces* species was determined.

METHODS

Isolation of Actinomycetes

Soil samples were collected from four different area of Pichavaram mangrove, Tamil Nadu, India. The soil suspension (0.5 ml) was spread on the starch casein agar plates. The plates were incubated for 4 weeks at 27°C. The *Actinomycetes* sp. colonies grown on Petri plates were counted at regular intervals. All the morphologically different *Actinomycetes* sp. colonies were subcultured [10].

Identification of isolated cultures

Purified isolates of actinomycetes were identified using morphological and cultural characteristics by the methods as described Bergey's manual. The morphology of the spore-bearing hyphae with the entire spore chain, the structure and arrangement of the spore chain with the substrate and aerial mycelium of the actinomycetes were examined using slide culture technique and identified. After growth, the slide cultures were examined under light microscope [11].

Molecular taxonomy

Isolation of DNA and polymerase chain reaction (PCR) amplification

The isolated genomic DNA was amplified using two primers 16S forward primer (5'-AGAGTRTGATCMTYGGCTWAC-3') and 16S reverse primer (5'-CGYTAMCTTWTACGRCT-3'). The final volume of reaction mixture of 100 µl contained DNA 1 µl, 16S forward primer 400 ng, 16S reverse primer 400ng, dNTPs (2.5 mM each) 4 µl, 10× Taq DNA polymerase assay buffer 10 µl, and Taq DNA polymerase enzyme (3 U/µl) 1 µl and water × µl. All PCR reagents were produced by chromous biotech. 10 µl of sequencing reaction contain PCR reaction mix 4 µl, template (100 ng/ul) 1 µl, primer (10 pmol/λ) 2 µl, and Milli Q water 3 µl. The amplification was performed on Eppendorf Thermocycler 96, according to the following promoter: An initial denaturation step at 96°C for 10 s followed 25 amplification cycles of 96°C for 1 min, 96°C for 10 s, 50°C for 5 s, and a final extension step of 60°C for 4 min. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet fluorescence after ethidium bromide staining.

Sequence similarities and phylogenetic analysis

Nucleotide sequence of the 16S rRNA of strain KMA08 was determined and compared for similarity level with the reference species of actinomycetes contained in genomic database bank. The BLAST program (<http://www.ncbi.nlm.nih.gov/blast>) was employed to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software [12]. The phylogenetic tree was constructed using the BioEdit Mega5.5 software.

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) was added separately and centrifuged at 5000 rpm for 10 min to extract the active compound [13]. Active extract selected for the further studies.

Experimental animals and parasite preparation

Swiss albino mice (8–12 weeks, weighing 25–37 g) of either sex bred at the animal house of Karpagam Academic of Higher Education Coimbatore were used. The animals were kept at room temperature and were exposed to a 12-h light/dark cycle. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline [9]. The study was completed as per the guidelines of CPCSEA, Government of India, after approval from IAEC (IAEC no: KU/IAEC/Project 132. (21.09.2013)). For *in vivo* antimalarial assays of *Streptomyces griseus* extract, the mouse infective chloroquine-sensitive strain of *Plasmodium berghei* maintained at Karpagam Academic of Higher Education. The parasites were maintained by serial passage of blood from infected mice to non-infected ones on weekly basis.

Animal management and grouping

A slightly modified method described by Peters and Robinson, 1992 was used for this test. The mice were divided into five groups of six each and which were all infected with malaria parasites. The negative control was treated with the normal saline used for reconstitution, whereas positive control was treated with chloroquine 20 mg/kg (CQ10). The normal saline, the *Streptomyces* extract and the standard drug were administered orally. The dose level of the extract was selected to be 10 ml/kg, 20 ml/kg, and 30 ml/kg for mice after having considered the safety of the extract based on the information obtained from the toxicity study [14].

Inoculation of mice

Chloroquine-sensitive strain of *P. berghei* was obtained from the National Institute of Malaria Research, Delhi. Albino mice previously infected with *P. berghei* having variable parasitemia were used as a donor. The parasitemia of the donor mice was first determined [15]. The donor mice were then sacrificed by head blow, and blood was collected by cardiac puncture into heparinized vacutainer tube having 0.5% trisodium citrate added as an anticoagulant. The blood was then diluted with physiological saline (0.9%). The dilution was made based on the parasitemia of the donor mice and the red blood cell (RBC) count

of normal mice (4.5×10 RBC/ml) [16,17] in such a way that 1ml blood contains 5×10⁷ infected erythrocytes. Each mouse was administered intraperitoneally with 0.2 ml of this diluted blood which contains 1×10⁷ *P. berghei* infected erythrocytes. To avoid variability in parasitemia, the blood collected from all donor mice was pooled together. The parasite was maintained by weekly passage to the mice.

Suppressive test (4 day)

The method described by Trager and Jensen [18] with slight modification was used. Briefly, 3 h after inoculation of the parasite, the mice in the three treatment groups were administered with the extracts in doses of 10, 20, and 30 ml/kg for 4 consecutive days. Two control groups were used in the experiment, the negative control treated with normal saline for 4 consecutive days while the positive control administered chloroquine a standard antimalarial drug at 10 mg/kg/day. The drug, the normal saline and the extracts used in this study were administered by oral route with the aid of an oral gavage. Treatment was continued for 4 days: [19] Parameters rectal temperature was measured daily starting from day 0 (before infection and also after infection) till day 4 while body weight and packed cell volume (PCV) were taken at day 0 and day 5 using Wintrobe's method [20]. Then, parasitemia was measured on the 5 days.

Evaluation of schizontocidal activity in established infection (Rane test)

Rane test was employed to evaluate schizontocidal activity of extract in established infection [21]. On the 1st day (D0), standard inoculum of 1×10⁷ *P. berghei* infected erythrocytes were injected intraperitoneally into mice (Ryley *et al.*). 72 h later, the mice were divided into five groups of six mice each. Different doses of microbial extract (10, 20, and 30 ml/kg/day) were administered orally to these groups. Chloroquine (10 mg/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 parasitemia level.

Determination of body weight and temperature

The body weight of each mouse in all the groups was taken before infection and after infection. The rectal temperature of the mice was measured with a digital thermometer before infection, 3 h after infection and then daily up to day 4 to see the effect of the extracts on body temperature.

PCV measurement

PCV was measured to predict the effectiveness of the test extracts in preventing hemolysis resulting from increasing parasitemia associated with malaria, using Wintrobe method [22].

Parasitemia measurement

Blood smears from tail were applied on Menzel-Glaser microscope slides (Germany), fixed with absolute methanol and stained with 10% Giemsa stain at pH 7.2 for 15 min [23]. The number of parasitized RBC was counted using Phase contrast microscopy with an oil immersion nose piece of 100× magnification power. Two fields were counted for each slide, the average was taken and percentage parasitemia was determined by analysis of variance (ANOVA) calculation [24,25].

Statistical analysis

The values were expressed as mean ± SEM and p<0.05 was considered significant.

The statistical significance was assessed by one-way ANOVA followed by Tukey's comparison test for determining significance using computerized GraphPad Prism version 4.0, GraphPad software, U.S.A. The analysis was performed with 95% confidence interval [26].

RESULTS AND DISCUSSION

Isolation and molecular characterization

The soil samples collected from different areas of pichavaram, actinomycetes isolates were obtained in pure form and analyzed for their antibacterial activities. Totally 23 isolates were found to be Gram-positive and showed branched mycelium in their cell morphology.

Three isolates which exhibited activity against the pathogens were selected for the further studies. According to Bergey's manual of determinative bacteriology and the laboratory manual for identification of actinomycetes, the isolates were identified as *Streptomyces* sp (Fig. 1).

16S rRNA sequencing is a powerful tool for rapid identification and phylogenetic analysis of *Streptomyces* species. The obtained 16S rRNA nucleotide sequence was compared with available 16S ribosomal sequences in the NCBI database using BLASTN (Fig.2). This isolate KMA08 has been enrolled into a *Streptomyces* sp. and was found to be closely related to *Streptomyces* strain with 99% sequence similarity. Hence, it was designated as *Streptomyces* sp. The submitted nucleotide sequence was provided a GenBank accession number KF913719. A fast minimum evolution analysis based on 16S rRNA gene sequences revealed that the isolate KMA08 identified as *S. griseus*.

In vivo antimalarial activity of *S. griseus*

The crude extract of *S. griseus* displayed a very good activity against *P. berghei* (Table 1). The comparison analysis indicated that only two dose levels (20 ml/kg and 30 ml/kg) of the extract showed statistically significant ($p < 0.001$) difference on the 5th day parasitemia compared to the negative control. The 10 ml/kg dose level, however, showed a statistically non-significant reduction. Percentage inhibition analysis indicated that inhibition was dose-dependent with 21.3, 65.3, and 80.5% inhibition by *Streptomyces* sp. 10 ml, 20 ml, and 30 ml of the extracts, respectively, for the 4 day suppressive test ($p < 0.001$ in the latter two doses) when compared the negative control. When compared among themselves, 20 and 30 ml/kg showed a significant ($p < 0.001$) percent parasitemia suppression than 10 ml. CQ10 had shown a statistically significant ($p < 0.001$) inhibition compared to control as well as other doses with 10 and 20 ml/kg. All the values are presented in Table 1.

Early malaria infection or Peters 4 days suppressive activity test for the Ethyl acetate extract of *Streptomyces* produced a dose-dependent suppression activity and was shown in Table 1. The highest suppression of parasitemia was observed at the dose of 30 ml/kg body weight of mice. Percentage suppression was observed to increase as extract concentration increased. After 4 days treatment with the different extract doses, the mean parasitemia of the test groups ranged from 32.6 ± 1.0 , 25.4 ± 0.6 , and $10.0 \pm 0.3\%$ while the corresponding value of the negative control group being $62.4 \pm 1.2\%$. The mice treated with CQ were completely free from the parasites on day 4. The antimalarial activity produced by the extract was statistically significant ($p < 0.05$) when related to control.

The result of the *in vivo* evaluation of the *Streptomyces* sp. extract on established infection showed a slight increase in suppressive activity. The extract was marginally active at 10 ml/kg per day and active at 20 and 30 ml/kg per day doses, respectively (Table 2). The mice that received 10 mg/kg of chloroquine per day, however, showed 100% suppression. The antimalarial activity produced by the extract was statistically significant ($p < 0.05$) when related to control. All the values are presented in Table 2.

Weight of *P. berghei* infected mice before and after treatment in the 4 day suppressive test

A body weight changes were observed between pre- and post-treatment days in all groups of mice treated with a crude extract of *S. griseus*. Treatment with crude extract of *S. griseus* prevented loss of weight associated with the increase Fig. 3 in parasitemia level at all the three dose levels compared to the negative control. However, the increase in body weight was found to be dose-dependent, the highest increment being caused by 10 ml/kg followed by 20 ml/kg and the least was by 30 ml/kg. There were no detectable differences in preventing weight reduction associated with increasing parasitemia between different doses of the extract as well as between the extract and standard. In the 4-day suppressive test, all extract treated groups prevented body weight loss as compared to negative control. However, there were no significant differences in weight gain among extract treated groups.

The test extracts of *S. griseus* KF913719 prevented a loss of body weight in infected mice with increasing parasitemia. The comparison analysis indicated that the extracts significantly prevented weight loss at all dose levels compared to the controls. However, the increase in body weight was not found to be dependent on dose levels.

The effect of the test extract on body temperature of *P. berghei* infected mice was also observed (Table 3). Only the 10 ml/kg and 20 ml/kg dose level showed a statistically significant difference on body temperature change when compared to negative control ($p < 0.01$) after the 4 days suppressive test. The analysis showed that at 20 ml/kg and 30 ml/kg doses, the CQ10 mg/ml and the negative control there was a reduction in rectal temperature. In all these groups, there was a relatively comparable non-preventive effect when compared to each other although the result was statistically significant. In the 10 ml/kg, however, the rectal temperature seems to be maintained with only a slight rise at day 4 when compared to the day before infection. All the values are presented in Table 3.

The analysis of PCV pre- and post-treatment, and an examinations of the rectal temperature indicated that the ethyl acetate extract of *S. griseus* was significantly prevent the reduction in PCV and body temperature, although, somewhat lesser percentages of reduction and significant preventive effects were observed, compared to the controls and dose levels.

Table 1: Effects of ethyl acetate extract of *Streptomyces* sp. on 4 day suppressive test

Treatment	% Parasitemia	% inhibition
Normal saline 20 ml/kg (negative)	62.4±1.2	0
CQ 10 mg/kg (control)	0.0	100
Extract 10 ml/kg	32.6±1.0	21.3
Extract 20 ml/kg	25.4±0.6	65.3
Extract 30 ml/kg	10.0±0.3	80.5

Values are mean±SEM; one-way ANOVA followed by Tukey's multiple comparison tests. SEM: Standard error of mean, ANOVA: Analysis of variance

Table 2: Effects of ethyl acetate extract of *Streptomyces* sp. on established malaria infection

Treatment	% Parasitemia	% inhibition
Normal saline 20 ml	63.5±1.0	21.3
CQ 20 ml	1.00±0.0	100
Extract 10 ml	35.6±1.0	40.3
Extract 20 ml	25.4±0.6	50.3
Extract 30 ml	10.0±0.3	80.5



Fig. 1: Colonies after 1-week inoculation of *Streptomyces* sp. on Starch casein agar

There was no statistically significant difference in the mean PCV on days 0 and 4 indicating that the extracts prevented significant PCV reduction (Table 4).

Statistical comparison of the effect of the extract on the study parameters (weight, PCV, and rectal body temperature) among groups at a fixed time and over time, have shown that extract administration did not cause significant change on any of these parameters. The present determination of antimalarial effect of the *S. griseus* extract is additional proof of its medicinal values, and it can, thus, be assumed that the extract can be safe at the dose levels used in the study [27]. According to Saxena *et al.* [27] and Ramazan *et al.* [28], several classes of secondary metabolites are responsible for the antimalarial activity, the antimalarial activities exhibited by this extract may also be due to the presence of other active compounds.

An *in vivo* antiplasmodial activity can be classified as moderate, good and very good if an extract displayed a percent parasite suppression equal to or >50% at a dose of 30, 20 and 10 ml/kg body weight per day. Based on this classification, the extract of *S. griseus* exhibited a good antiplasmodial activity. Anemia, hypoglycemia, body weight loss, and body temperature reduction are the general features of malaria-infected mice body weight loss is one feature of rodent malaria infections [29]. The result of the present work, however, showed only a statistically non-significant slight gain in body weight of crude extract administered of *P. berghei* infected mice. This could possibly be due to depressing effect

of the crude extract on feed intake/appetite. Mice treated with crude extract of *S. griseus* showed a lower body weight pattern as compared with the non-treated ones which are in agreement with this result.

According to Taylor and Hurd [30], the effect of rodent malaria on PCV as measured by hematocrit was parasite-induced fall down to 42–43%, which occurred approximately 48 h post-infection. *P. berghei* infected mice suffer from anemia due to RBC destruction, either by parasite multiplication or by spleen reticuloendothelial cell acts as the presence of many abnormal RBC stimulates the spleen to produce many phagocytes [31]. The significant decrease in PCV could be attributed to the presence of antinutritive factors in the crude extract. *Plasmodium* infection is correlated with the incidence of the high destruction of red blood cells, hence anemia which could be life-threatening [32]. In this study, the extract prevented significant PCV reduction in a dose-dependent manner. A decrease in the metabolic rate of infected mice occurred just before death and was accompanied by a corresponding decrease in internal body temperature [33]. In this study, however, the extract prevented significant body temperature reduction in a dose-dependent manner. This could be attributed to the effect of the extract as it may have a hypothermic effect on the treated mice.

CONCLUSION

The extract used in this study showed considerable antimalarial activities that are worth to be investigated. Further studies are

Table 3: Rectal temperature of *P. berghei* infected mice before and after treatment in the 4 days suppressive test

Group	Before infection	3 h after infection	D-2	D-3	D-4	% change
Normal saline (10 ml/kg)	35.12±0.47	36.66±0.36	37.09±0.22	38.12±0.37	38.76±0.25	-0.3
CQ (10 mg/kg)	35.84±0.42	37.22±0.54	37.12±0.65	37.6±0.46	36.50±0.22	-0.33
Extract (10 ml/kg)	35.27±0.18	37.18±1.30	37.54±0.13	37.22±0.10	37.6±0.23	0.57
Extract (20 ml/kg)	35.43±0.15	37.65±0.15	37.97±2.28	37.38±0.1	37.32±0.08	-0.26
Extract (30 ml/kg)	36.22±0.33	36.39±0.26	37.160±0.31	36.53±0.18	36.1±0.33	-3.14

Values are represented as mean±standard error of mean. Statistical analysis performed using one-way analysis of variance followed by turkey test. *P. berghei*: *Plasmodium berghei*

Table 4: PCV of *P. berghei* infected mice before and after treatment in the 4 days suppressive test

Treatment	Pre-treatment PCV	Post-treatment PCV	Percentage change %
Negative control (10 ml/kg)	51.55±1.51	44.85±1.62	-9.87
CQ (10 mg/kg)	59.65±1.25	25.08±0.66	-3.30
Extract (10 ml/kg)	41.43±1.66	35.4±0.63	2.55
Extract (20 ml/kg)	40.2±1.35	29±0.66	-0.97
Extract (30 ml/kg)	45.78±1.34	27.43±1.45	-4.89

Values are represented as mean±standard error of mean. Statistical analysis performed using one-way analysis of variance followed by turkey test. *P. berghei*: *Plasmodium berghei*, PCV: Packed cell volume

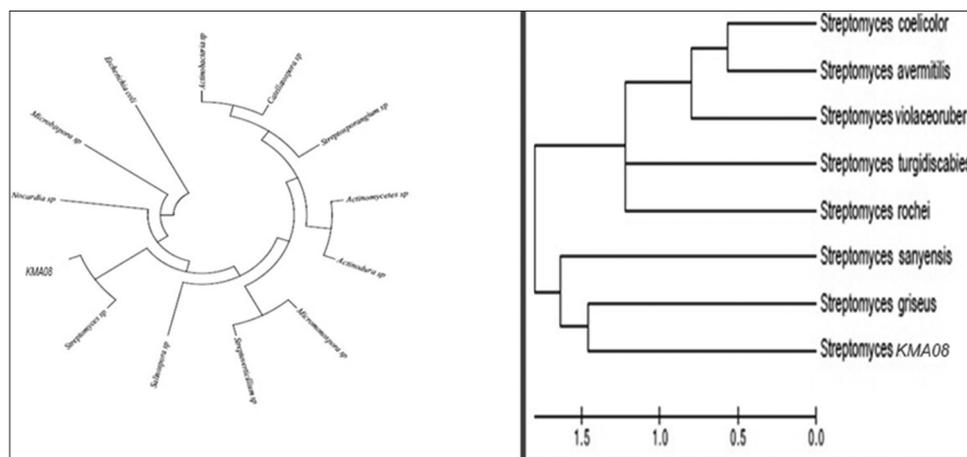


Fig. 2: Phylogenetic analysis of *Streptomyces griseus* KF913719 (KMA08)

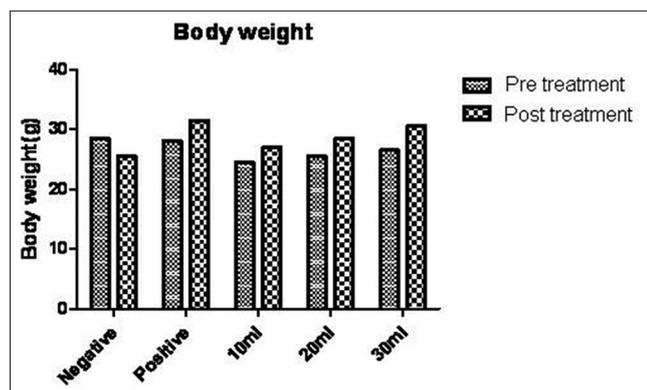


Fig. 3: Body weight of *Plasmodium berghei* infected mice before and after treatment in the 4 days suppressive test

suggested to elucidate their antimalarial properties and also to isolate the active substance(s) responsible for their antimalarial activity. The current antimalarial drugs have their own problems to prevent and treat human malaria. The results of this study indicated no toxicity in mice with an even high dose of *S. griseus* KF913719 solvent extract, which confirm its lowest effects. The results of the study indicated that *in vivo*, Ethyl acetate extract of *S. griseus* KF913719 displayed a very good activity against the *P. berghei* malaria parasite. Rivo *et al.* [34] observed that ethyl acetate extracts of *Streptomyces* sp. showed higher antiplasmodial activity. Kondrashin *et al.* [35] in their study showed ethyl acetate extracts of *Streptomyces* sp. had maximum plasmodial activity. However, although the results clearly indicated that the test extract of *S. griseus* KF913719 significantly prevented weight loss at all dose levels compared to the control, the increase in body weight is not consistent with increasing dose of the extracts. It may possibly indicate the appetite suppressive effects of the extracts, which would reasonably increase with increasing dose.

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