**Title**

Isolation and characterization of antiprotozoal compound-producing *Streptomyces* species from Mongolian soils

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**Abstract**

Natural resources are recognized as important sources of potential drugs for treating various infections, and microorganisms are a rich natural source of diverse compounds. Among the world's microorganisms, actinomycetes, which are abundant in soil and marine, are the well-known producers of a wide range of bioactive secondary metabolites and antibiotics. In the present study, four actinomycetes (samples N25, N6, N18, and N12) were isolated from soil samples in Mongolia. Phylogenetic analysis of these isolates revealed that they share the highest similarity with *Streptomyces canus* (N25), *S. cirratus* (N6), *S. bacillaris* (N18) and *S. peucetius* (N12), based on 16S rRNA gene sequencing. Crude extracts were obtained from them using ethyl acetate, and the crude fractions were separated by thin layer chromatography. The fractions were then evaluated for their cytotoxicities and their anti-*Toxoplasma* and antimalarial activities *in vitro*. The *S. canus* (N25) crude extract was selected for further chemical characterization based on its antiprotozoal activities. Using liquid chromatography-high resolution mass spectrometry, phenazine-1-carboxylic acid (PCA) was detected and identified in the active fractions of the metabolites from strain N25. We next confirmed that commercially available PCA possesses antiprotozoal activity against *T. gondii* ($IC_{50}$: 55.5 μg/ml) and *Plasmodium falciparum* ($IC_{50}$: 6.4 μg/ml) *in vitro.* The results of this study reveal that soil actinomycetes are potential sources of antiprotozoal compounds, and that PCA merits further investigation as an anti-protozoal agent.

**Keywords** Phenazine-1-carboxylic acid; *Toxoplasma gondii; Plasmodium falciparum; Streptomyces* sp.; Actinomycetes ·Mongolia

## 1. Introduction

Apicomplexan parasites including *Plasmodium falciparum* and *Toxoplasma gondii* are globally distributed protozoan parasites that cause human infectious diseases such as malaria and toxoplasmosis, respectively. *Plasmodium* is an intracellular protozoan parasite and the causal agent of malaria, is restricted to tropical and subtropical regions of the globe. Malaria is a major life-threatening disease and was responsible for over 216 million cases and over 445,000 deaths worldwide in 2016 [1]. Millions of people are infected by malaria parasites annually, and many of them, especially children, die from these infections. A vaccine against malaria is not yet available, and chloroquine, sulfadoxine, pyrimethamine and artemisinin-based combination drugs are the currently available antimalarial agents. However, drug resistance has been documented for all antimalarial drug classes [2].

Toxoplasmosis is a zoonotic disease caused by *T. gondii*, an intracellular protozoan parasite. This common protozoan infection, which has a worldwide occurrence, can infect humans and all other warm-blooded animals [3]. The recommended therapies against toxoplasmosis are sulfadiazine, pyrimethamine, and spiramycin. However, their ability to control toxoplasmosis is limited and significant side effects have been reported with standard treatments based on them [4,5]. This situation necessitates the discovery and development of new, effective drugs against toxoplasmosis.

Natural products derived from plants and microorganisms are recognized as an essential source of antibiotics and bioactive compounds. Among the microorganisms, actinomycetes are a vital group of naturally occurring antibiotic producers. They are recognized for their wide range of biological activities and have been shown to have antibacterial [6,7], antifungal [8,9],

antiparasitic [10], antiviral, antitumor, immunostimulatory, immunosuppressive, herbicidal [11] and enzyme inhibitory properties [12].

Actinomycetes characterized by a complex life cycle belonging to the phylum *Actinobacteria*, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain Bacteria [13]. The *Streptomyces* species are filamentous Gram-positive bacteria and belonging to the class of *Actinomycetes* and phylum of *Actinobacteria,* more than 550 *Streptomyces* species have been described [14]. They are widely distributed in terrestrial and aquatic ecosystems and are abundant in soil. This bacterial group is well known for their ability to produce important bioactive compounds. Indeed, approximately 7,630 bioactive metabolites are known to be produced by only *Streptomyces* species [15].

Mongolia is one of the largest landlocked countries in the world and its extreme and variable climate has preserved ecosystems with rich microbial biodiversity [16]. Recently, *Streptomyces* species have been found in soil and potential sources of bioactive secondary metabolites explored by researchers in Mongolia [17,18]. In the present study, Mongolian soil-isolated actinomycetes isolates were assessed for their anti-*Toxoplasma* and antimalarial activities.

## 2. Material and methods

### *2.1. Isolation of actinomycetes*

Soil samples were collected from Khentii and Uvurkhangai provinces and from around Ulaanbaatar, Mongolia's capital city. The samples were obtained by removing a 5 to 10 cm

depth of the loose surface litter layer. The samples were dried, and appropriate aliquots of the diluted samples were prepared in saline solution and then spread on Gauze's synthetic no. 1 media (Soluble starch: 20 g, $KNO_3$: 1 g, NaCl: 0.5 g, $MgSO_4 \cdot 7\ H_2O$: 0.5 g, $K_2HPO_4$: 0.5 g, $FeSO4 \cdot 7\ H_2O$: 0.01 g, Agar: 15 g, Distilled water: 1L, pH 7.4). Single actinomycetes colonies were purified by re-streaking onto agar plates containing the same media, after which they were stored as slants in vials at 4 ˚C. The Microbial Synthesis Laboratory of the Institute of General and Experimental Biology, Mongolian Academy of Sciences screened the actinomycetes from the soil samples for bioactive compounds, and four isolates (N6, N12, N18, N25) were selected for evaluation in this study.

*2.2. Phylogenetic analysis*

Genomic DNA was extracted using the QIAamp DNA blood mini kit *(Qiagen, Hilden, Germany)* according to the manufacturer's instructions. The 16*S* rRNA gene from *Streptomyces* sp. was polymerase chain reaction (PCR)-amplified using 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′), 1492R (5′-TAC GGC TAC CTT GTT ACG ACT T-3′), and 330F (5′-CGG CCC AGA CTC CTA CGG GAG GCA GCA-3′) universal primers [19]. The PCR products from the isolates were purified using a PCR product purification kit (Qiagen, Hilden, Germany). The PCR amplicons were confirmed as authentic 16*S* rRNA products via sequencing on the 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). The resultant sequences were analyzed by utilizing the Molecular Evolutionary Genetics Analysis 10.0 package with computing platform software (MEGA X) [20]. The sequences from the isolates were compared with the sequences in the GenBank database (www.ncbi.nlm.nih.gov).

A phylogenetic tree was then constructed using the maximum-likelihood method based on the Tamura-Nei model [21].

*2.3. Fermentation and compound extraction*

In this study, the strains were grown on ISP 2 (International Streptomyces project-2) broth at 28°C for 7 days as a production media for obtaining crude extracts. The fermentation was carried out with 250 ml of ISP 2 media in a 500 ml Erlenmeyer flask at 28°C for 7 days with shaking (200 rpm). The supernatant and mycelia from each sample was separated by centrifugation (2,236 × *g*, 10 min). Each supernatant was extracted with an equal volume of ethyl acetate and evaporated using the Ren-1000 rotary evaporator at 50°C (IWAKI, Shizuoka, Japan). Crude extracts were obtained from four *Streptomyces* strains for antiprotozoal testing.

*2.4. Fractionation and compound identification*

Fractionation of the compounds was carried out on thin layer chromatography (TLC) plates (Silica gel 60, $F_{254+366}$, 2 mm, EMD Millipore, Billerica, MA, USA). Chloroform and methanol (9:1 ratio) were used as the solvent system to fractionate the crude extracts. The fractions were scraped from each TLC plate, and ethyl acetate was added to the scrapings. The silica was then removed by centrifugation. The purified fractions were further subjected to liquid chromatography-high resolution mass spectrometry (LC-HRMS) to identify candidate compounds.

*2.5. LC-HRMS analysis*

LC-HRMS was performed using the following conditions: high-performance liquid chromatography (HPLC) was carried out with the UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) using a SPELCO Ascentis Express C18 column (2.1 mm i.d. × 50 mm, Sigma-Aldrich, St Louis, MO, USA) at 40°C. The mobile phase consisted of 0.1% HCOOH aq. (A) and 0.1% HCOOH-$CH_3CN$ (B), with a gradient of 95% A to 0% A (0 to 5 min) and 0% A (5 to 8.5 min). The flow rate was 0.4 ml $min^{-1}$. MS data acquisition was performed on the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using positive electrospray ionization with a spray voltage of 4 kV and a capillary temperature of 350°C. The MS range was scanned from m/z 100 to 2000 (resolution 15,000), and the MS data analyses were performed by Xcalibur 2.2 (Thermo Fisher Scientific).

*2.6. Chemicals*

Resistomycin (BioViotica, Dransfeld, Germany), amphomycin (Bioaustralis, Smithfield NSW, Australia), bafilomycin A1 (Sigma-Aldrich) and tylosin phosphate (LKT Laboratories, St. Paul, MN, USA) were prepared in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution, followed by storage at −30°C until use. Phenazine-1-carboxylic acid (PCA) was prepared from a 10 mg/ml stock solution (Toronto Research Chemicals, North York, Canada) in DMSO. All the crude extracts and fractions were also prepared in DMSO.

*2.7. In vitro anti-Toxoplasma activity*

Human foreskin fibroblast (HFF) cells were grown in 96-well plates (100 µl cell suspensions containing $1\times10^5$ cells/ml) in Dulbecco's Modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Nichirei Bioscience,

159 Tokyo, Japan). The HFF cells were incubated at 37°C in a 5% $CO_2$ atmosphere for 48 h before
160 they were infected with 100 µl of the green fluorescent protein-expressing *T. gondii* RH strain
161 (RH-GFP) [22] at $5\times10^4$ tachyzoites per well, with incubation for 4 h. The extracellular
162 parasites were aspirated and new media (100 µl/well) was added before adding the extracts and
163 compounds to the 96-well plates. The crude extracts and/or compounds (100 µl/well) were
164 added to the infected HFF cells after 4 h infection and then incubated for a further 72 h. The
165 fluorescence intensity of RH-GFP was measured by a microplate reader (SH-900, Corona,
166 Ibaraki, Japan). The percentage inhibition of parasite growth (RH-GFP) was calculated by
167 utilizing the following formula: [(average fluorescence intensity of GFP with medium alone)
168 − (the fluorescence intensity of GFP treated with either of the extracts or compounds) / (average
169 fluorescence intensity of GFP with medium alone)] × 100 [23].
170
171 *2.8. In vitro antimalarial activity*
172 *P. falciparum* (3D7 strain) cultures were maintained using human erythrocytes (1%
173 hematocrit) in RPMI 1640 medium (Sigma–Aldrich). *P. falciparum* was synchronized to the
174 ring stage by sorbitol lysis to >90% purity. The crude extracts and compounds with the desired
175 concentrations were then added to the 96-well plates (50 µl per well). The infected erythrocytes
176 (50 µl per well, parasitemia: 0.5%, hematocrit: 2%) were added and incubated at 37°C for 24
177 h [24]. Parasite growth was determined by adding 100 µl of lysis buffer with SYBR Green I
178 stain (SYBR® Green I Nucleic Acid Stain 10,000×, Lonza Rockland, Rockland, ME, USA).
179 The relative fluorescence inhibition values of the wells were determined using a fluorescence
180 plate reader (Fluoroskan Ascent, Thermo Fisher Scientific, Vantaa, Finland) at 485–518 nm
181 excitation and emission wavelengths. Parasite growth inhibition percentages were calculated

using the following formula: [(average excitation and emission wavelengths for *P. falciparum* with media) − (average excitation and emission wavelengths for *P. falciparum* with either of the extracts or compounds) / (average excitation and emission wavelengths for *P. falciparum* with media)] × 100, as described above. The uninfected erythrocytes and background fluorescence signals from the drugs were subtracted [25].

*2.9. In vitro cytotoxicity assay*

HFF cells in DMEM supplemented with 10% FBS were added to 96-well plates (cell suspensions, $1 \times 10^5$ cells/ml) and incubated at 37°C with 5% $CO_2$ for 48 h. The compounds or the crude extracts were prepared in DMSO and tested at 100 μg per ml, the highest final concentration. Cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added to the culture after 72 h of incubation and the cell viability was measured at 450 nm using the MTP-120 microplate reader (Corona, Ibaraki, Japan). HFF cell viability (%) was expressed as [(the absorbance of cells treated with the extracts or compounds / (the absorbance of cells cultured with medium alone) × 100] [23].

*2.10. Statistical analysis*

GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was used for analysis of the $IC_{50}$ values from the percentage inhibition of the parasites. The final mean $IC_{50}$ values were calculated based on at least three independent experiments, and the data represents the mean ± SD.

205 ## 3. Results

206 ### *3.1. Isolation and identification of actinomycetes*

207 In the present study, four actinomycetes (N6, N12, N18, N25) were isolated from four
208 different soil samples in Mongolia. The 16S rDNA gene sequences for these actinomycetes and
209 related organisms were retrieved from the GenBank database (www.ncbi.nlm.nih.gov).
210 Comparisons of the GenBank gene sequences with those from the four isolates revealed that the
211 isolates belong to the *Streptomyces* genus. The phylogenetic analysis revealed the following:
212 isolate N25 (1,490 bp, GenBank accession number: MK509920) shares the highest nucleotide
213 sequence identity (99%) with *Streptomyces canus* (NR_043347.1), strain N6 (1,489 bp,
214 GenBank accession number: MK509917) shares 100% nucleotide sequence identity with *S.*
215 *cirratus* (KR085952.1), strain N12 (1,492 bp, GenBank accession number: MK509918) shares
216 100% nucleotide sequence identity with *S. peucetius* (NR_024763.1), and strain N18 (1,492 bp,
217 GenBank accession number: MK509919) shares 99% nucleotide sequence identity with *S.*
218 *bacillaris* (NR_041146.1) (Fig. 1).

219

220 ### *3.2. In vitro anti-Toxoplasma and antimalarial activities of antibiotics derived from*
221 *Streptomyces spp.*

222 Some well-known antibiotics derived from various *Streptomyces* species were tested *in*
223 *vitro* against *T. gondii* and *P. falciparum* (Table 1). Kanamycin and resistomycin did not inhibit
224 the growth of *T. gondii* or *P. falciparum*. However, amphomycin and tylosin phosphate
225 inhibited *P. falciparum* growth with $IC_{50}$ values of 9.3 µM and 182.7 µM, respectively.
226 Bafilomycin A1 was also active against *P. falciparum* ($IC_{50}$: 34.1 nM), but its selectivity index
227 (SI) was quite low (SI: 1.0). Furthermore, amphomycin, tylosine phosphate and bafilomycin

A1 at highest concentration 100 µM did not induce hemolysis (Table S1). On the other hand, amphomycin, tylosin phosphate and Bafilomycin A1 did not inhibit the growth of *T. gondii*.

### *3.3. In vitro anti-Toxoplasma and antimalarial activities of the crude extracts*

To identify novel compounds with anti-*Toxoplasma* and antimalarial activities in the *Streptomyces* species present in our isolates, crude extracts from these isolates were tested (Table 2). The *S. bacillaris* (N18) and *S. canus* (N25) crude extracts both exhibited significant antiprotozoal activities against *T. gondii* (RH-GFP) and *P. falciparum* (3D7), respectively. However, the crude extracts from *S. cirratus* (N6) and *S. peucetius* (N12) were less effective in this regard. N18 inhibited *P. falciparum* (3D7) growth ($IC_{50}$ of 156.9 ng/ml; SI: 2.01) and inhibited *T. gondii* growth ($IC_{50}$ of 91.8 ng/ml; SI: 3.4), but cytotoxicity was observed against the HFF host cells ($IC_{50}$ of 316 ng/ml), but not in erythrocytes. While the $IC_{50}$ value of the N25 extract against *P. falciparum* was 4.9 µg per ml, the extract was also active against *T. gondii* ($IC_{50}$ 19.2 µg/ml), with no apparent cytotoxicity noted in the HFF host cell line and the erythrocytes (Table S1).

### *3.4. In vitro anti-Toxoplasma and antimalarial activities of TLC fractions from S. canus (N25)*

Analysis of the *S. canus* (N25) crude extract revealed ten fractions that produced single bands on the TLC plate (Fig. S1). After purifying the compounds, their cytotoxicities and their anti-*Toxoplasma* and anti-malarial activities were evaluated. Two of the ten fractions (Fractions 8 and 9) possessed antiprotozoal properties against *T. gondii* and *P. falciparum*, respectively (Table 3). Fraction 8 possessed antiprotozoal activity against *T. gondii* ($IC_{50}$: 2.6

µg/ml; SI: 53.4) and *P. falciparum* ($IC_{50}$: 4.8 µg/ml; SI: 28.9), but fraction 9 possessed the highest activity against *T. gondii* ($IC_{50}$:122.2 ng/ml, SI: 239.7) and *P. falciparum* ($IC_{50}$:163.8 ng/ml, SI: 178.8). Moreover, fractions 8, 9 and 10 did no show apparent cytotoxicity on the human erythrocytes (Table S1).

### 3.5. *Identifying the chemical components in the fractions displaying antiprotozoal activity*

The crude extract (90 mg) from *S. canus* (N25) was run on preparative silica gel TLC plates and developed with $CHCl_3$: MeOH (9:1) as the mobile phase to produce ten fractions (F1 to F10). The active fractions, F8 and F9, had a retention factor (Rf) of 0.76 and 0.91 to 0.86, and were extracted with a mixed solution of $CHCl_3$: MeOH (2:1) to yield 2 mg and 4 mg of the active compound, respectively. The active compound was showed single peak on silica gel TLC (hexane:ethyl acetate=1:9, Rf 0.9) and HPLC (Rt: 2.95 min). From LC-HRMS analysis; 225.0661 $[M+H]^+$ (calculated for $C_{13}H_9N_2O_2$: 225.0659) and 239.0820 $[M+H]^+$ (calculated for $C_{14}H_{11}N_2O_2$: 239.0815), PDA-UV spectrum and compared with standard compound, the active compounds were considered to be 1-phenazinecarboxylic acid (PCA) (Fig.2) and its metabolite PCA methyl ester, respectively (Fig. 3).

### 3.6. *In vitro anti-Toxoplasma and antimalarial activity of PCA*

Commercially available PCA ($C_{13}H_8N_2O_2$: 224.21) was tested against *T. gondii* ($IC_{50}$: 55.5 µg/ml, 247.3 µM) and *P. falciparum* ($IC_{50}$: 6.4 µg/ml, 28.6 µM) *in vitro*. No cytotoxic effects of the candidate compound were observed in the HFF host cells (Fig. 4) and the human erythrocytes (Table S1)..

## 4. Discussion

Natural products play a vital role in the discovery and development of new and effective drugs against various human infections [26]. Most *Streptomyces* species have the ability to produce various bioactive secondary metabolites with antibacterial, antitumor, antifungal, and anti-parasitic properties, as well as producing enzyme inhibitors. Widely distributed across ecosystems and Gram-positive bacteria, Streptomycetes are important sources of natural antibiotics [15].

Previous studies have reported on compounds isolated from *Streptomyces* spp. with potential antimalarial activities such as gancidin W [27], trioxacarcins [28], metacycloprodigiosin [29] and munumbicins [30]. Other antiparasitic compounds isolated from *Streptomyces* spp. (i.e., valinomycin, staurosporine and butenolide) are reportedly active against *Leishmania major*, *Trypanosoma brucei* and other *Trypanosoma* spp. [31]. Furthermore, spiramycin, a macrolide antibiotic produced by *S. ambofaciens* [32], was reported to be effective against acute toxoplasmosis and was able to prevent vertical transmission of the parasite [33]. In the present study, we isolated and identified four *Streptomyces* species from Mongolian soil samples. Based on their molecular characterizations, the isolates were identified as *S. canus* (N25), *S. cirratus* (N6), *S. bacillaris* (N18) and *S. peucetius* (N12). We also evaluated the efficacies of some well-known antibiotics (e.g., amphomycin, kanamycin, and resistomycin) against *T. gondii* and *P. falciparum in vitro*, which are derived from the identified strains. *S. canus* produces several antibiotics including telomycin [34], amphomycin [6], amphomycin analogues [35], kanamycin [35] and resistomycin and tetracenomycin D, which are antifungal metabolites [36]. Here, the antibiotic amphomycin which is a bacterial

297 cell wall inhibitor [37] was also found to exhibit antimalarial activity against *P. falciparum*
298 ($IC_{50}$: 9.3 µM), without any general cytotoxicity being observed. This is the first report on the
299 antimalarial activity of amphomycin. The bafilomycin analogs, which were obtained from a
300 marine-derived *S. bacillaris* actinomycetes bacterium, have been shown to have autophagy
301 inhibitory activities [38,39]. Moreover, an $IC_{50}$ value of 0.041 ± 0.010 µg/ml was reported in
302 a previous study exploring the *in vitro* antimalarial activity of bafilomycin A against *P.*
303 *falciparum* (K1 strain) [29]. In the present study, bafilomycin A1 also exhibited *in vitro*
304 antimalarial activity against *P. falciparum*, this time against the 3D7 strain, with an $IC_{50}$ value
305 of 34.1 nM (Table 1). However, cytotoxicity was observed in the host mammalian HFF cells
306 with an $IC_{50}$ value of 33.1 nM. In contrast, anti-*Toxoplasma* activity was not observed with
307 these antibiotics (> 100 µM).

308 The crude extracts from all the strains (N6, N12, N18, N25) were then evaluated to
309 identify novel compounds with antiprotozoal activities. The individual crude extracts from N12
310 and N6 did not inhibit parasite growth *in vitro* (>100 µg/ml). Furthermore, the crude extract
311 from N18 was able to inhibit parasite growth ($IC_{50}$: 91.8 ng/ml for *T. gondii*, $IC_{50}$: 156.9 ng/ml
312 for *P. falciparum* $IC_{50}$: 316 ng/ml for HFF cells). However, the cytotoxic effect of the N18
313 extract on the host cells prohibited further assessment of its antiprotozoal activity (SI: 3.4 for
314 *T. gondii*, SI: 2.0 for *P. falciparum*) (Table 2). Consequently, because of its lower cytotoxicity
315 (>100 µg/ml), only the N25 extract ($IC_{50}$: 19.2 µg/ml for *T. gondii*, $IC_{50}$: 4.9 µg/ml for *P.*
316 *falciparum*) was fully evaluated chemically.

317 LC-HRMS is a powerful tool used for the identification of bioactive molecules [40]. In
318 our study, LC-HRMS was utilized to confirm the identity of the compound in the N25 extract
319 as PCA and its delivative, PCA methyl ester. PCA and its derivatives comprise a large group

of heterocyclic nitrogen-containing compounds produced by bacteria such as *Pseudomonas* spp. and *Streptomyces* spp. [42]. PCAs display a broad-spectrum of biological properties including antifungal [43], anticancer [44] and antimalarial activities [45,46]. PCA, a precursor of other phenazine derivatives, is a well-known antibiotic with a broad spectrum of biological properties, and is also known as tubermycin B or shenqinmycin. Tubermycin B, an antibiotic produced by *P. aeruginosa*, possesses antifungal activity [47]. Shenqinmycin is also an antifungal compound [48]. Previous studies have reported on a wide range of activities for PCA including antifungal [49,50], anti-bacterial, and anti-cancer [51]. PCA has previously been found to be a product of *S. kebangsaanensis* [52] and *S. anulatus* [53]. The present study is the first to report on PCA and its metabolite being identified in *S. canus* to date.

Another study reported that PCA can react with $O_2$ to generate cell-damaging reactive oxygen species [53]. To the best of our knowledge, antiprotozoal activities against toxoplasmosis and malaria have not been reported yet for PCA. Furthermore, the present study has shown that PCA derived from *S. canus* exhibited antiprotozoal activities against *T. gondii* ($IC_{50}$: 55.5 µg/ml) and *P. falciparum* ($IC_{50}$: 6.4 µg/ml) *in vitro.* This result indicates that *S. canus* (N25) is a potential antibiotic producer with anti-protozoan properties.

## Conclusion

Our study has reported on the antiprotozoal activities of *S. canus* (N25) derived from Mongolian soil samples against *T. gondii* and *P. falciparum.* That two previously known compounds, PCA and amphomycin, are produced by *S. canus* and also possess anti-*Toxoplasma* and antimalarial properties *in vitro*, is a new finding. Our results suggest that *S.*

*canus* isolated from Mongolian soil may be a potential source of new treatments for human parasitic protozoan infections.

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**Conflict of interest**

All authors have declared no conflict of interest.

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549 **Figure legends**

550 **Fig. 1**. Phylogenetic tree for the N25, N6, N12, N18 isolates based on 16S rRNA gene
551 sequences. The maximum-likelihood method based on the Tamura-Nei model was used.
552 Bootstrap values are based on 1,000 replicates. The 16S rRNA gene sequence from
553 *Actinomadura hibisca* (NR_042031) was used as the outgroup.

554

555 **Fig. 2.** (A) Structure of 1-phenazinecarboxylic acid. (B) LC-HRMS analysis of the active
556 compound in F8. The upper plot shows the total ion chromatogram (TIC), total scan
557 photodiode array chromatogram and extracted ion chromatogram from *m/z* 225.0661±5
558 ppm (*m/z* 225.0650-225.0672) (shown in order from the top). The middle and lower plots
559 represent the ultraviolet (UV) spectrum and mass spectrum, respectively, each with a
560 retention time (Rt) of 2.95 min.

561

562 **Fig. 3.** (A) Structure of 1-phenazinecarboxylic acid-methyl ester. (B) LC-HRMS analysis
563 of the active compound in F9. The upper plot shows the total ion chromatogram (TIC),
564 total scan photodiode array chromatogram and extracted ion chromatogram from *m/z*
565 239.0819±5 ppm (*m/z* 239.0807-239.0831) (shown in order from the top). The middle and
566 lower plots represent the ultraviolet (UV) spectrum and mass spectrum, respectively, each
567 with a retention time (Rt) of 3.09 min.

568

569 **Fig. 4.** (A) Anti-*Toxoplasma* activity of phenazine-1carboxylic acid (PCA) based on the *T.*
570 *gondii* RH-GFP strain. The half maximal inhibition concentration ($IC_{50}$) was 55.5 µg/ml.

571 (B) Antimalarial activity of PCA based on the *P. falciparum* 3D7 strain. The $IC_{50}$ value
572 was 6.4 μg/ml. Data represent the mean values ± SD for three independent experiments.
573

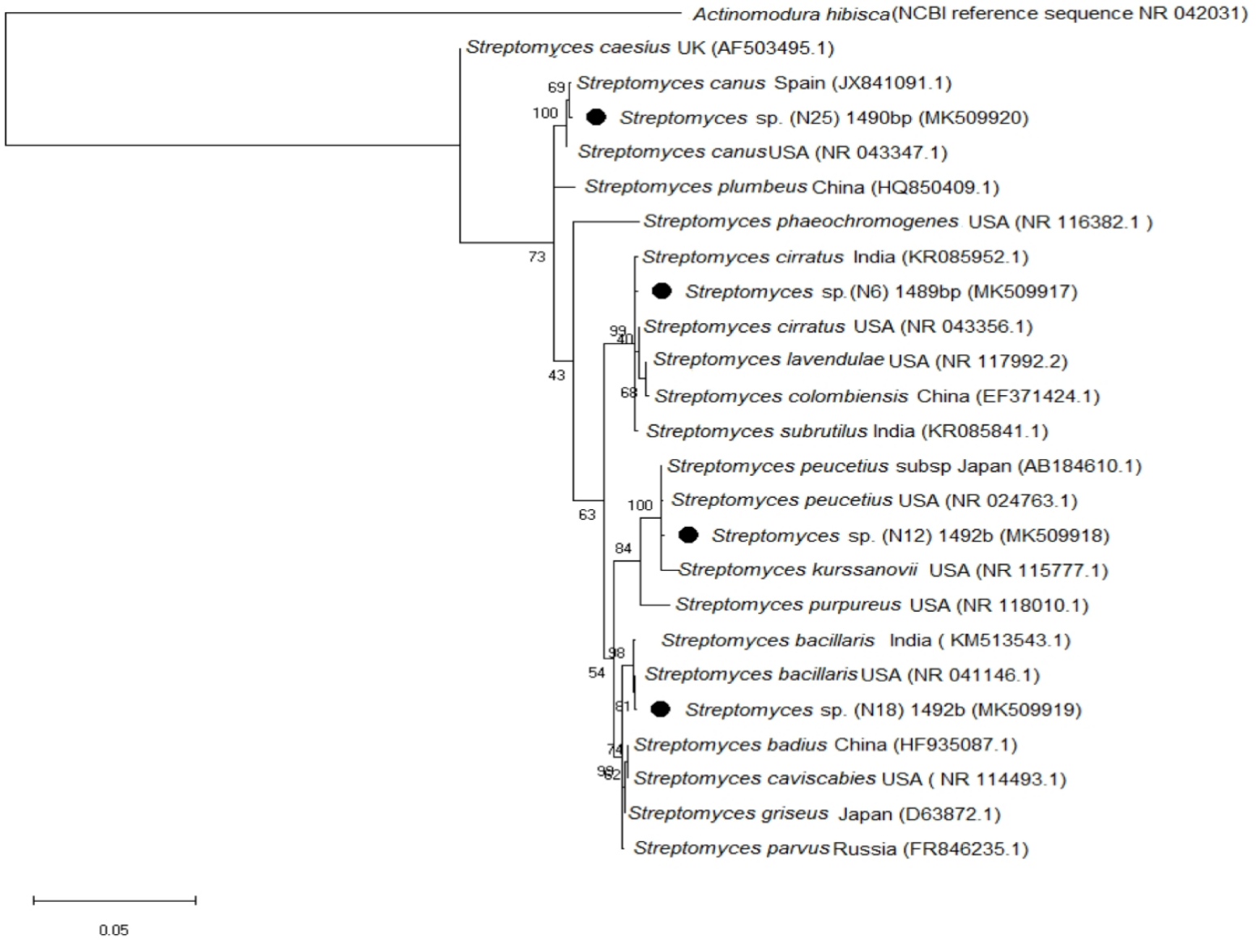


Fig. 1. Pagmadulam et al.

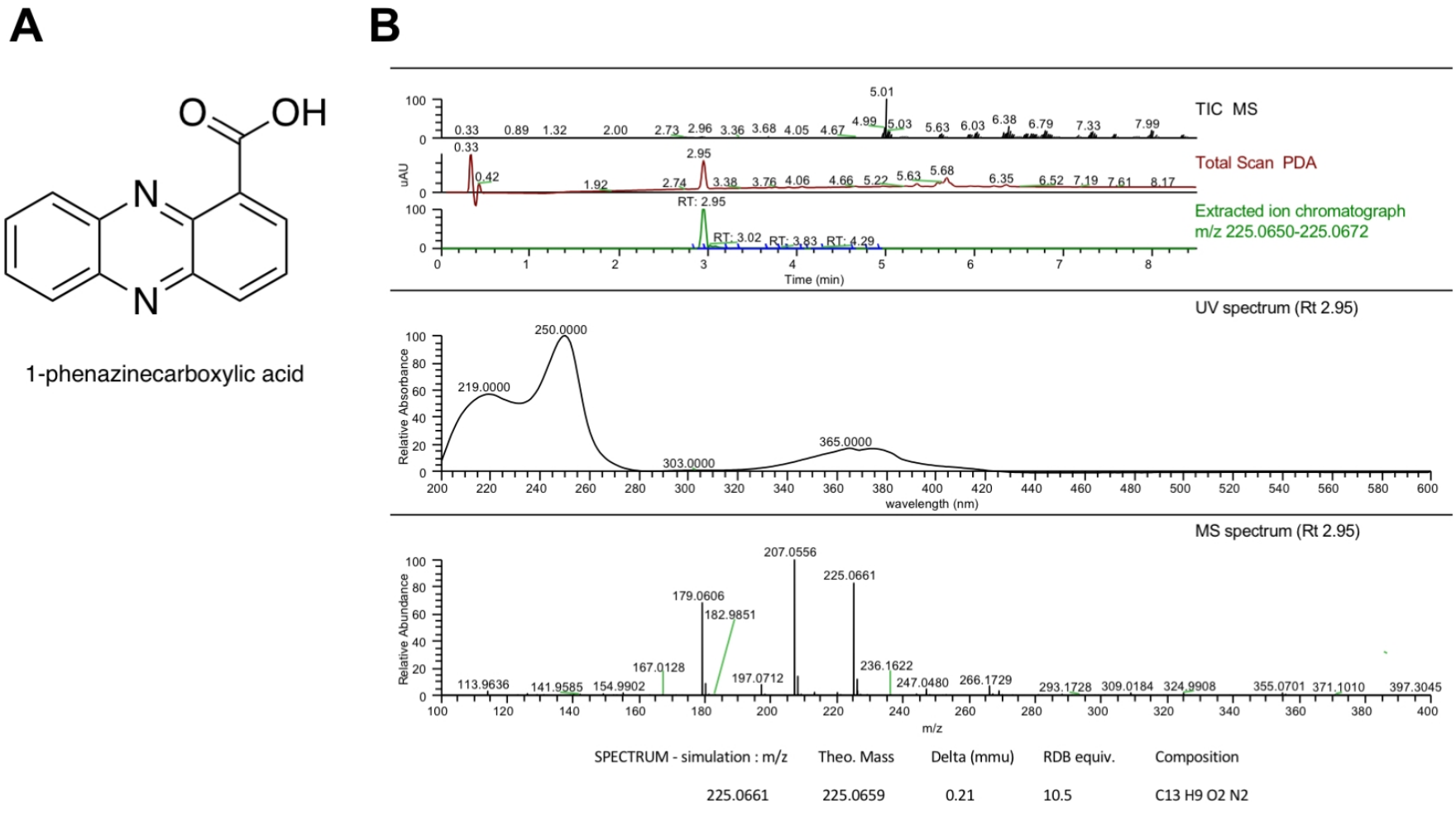


Fig. 2. Pagmadulam et al.

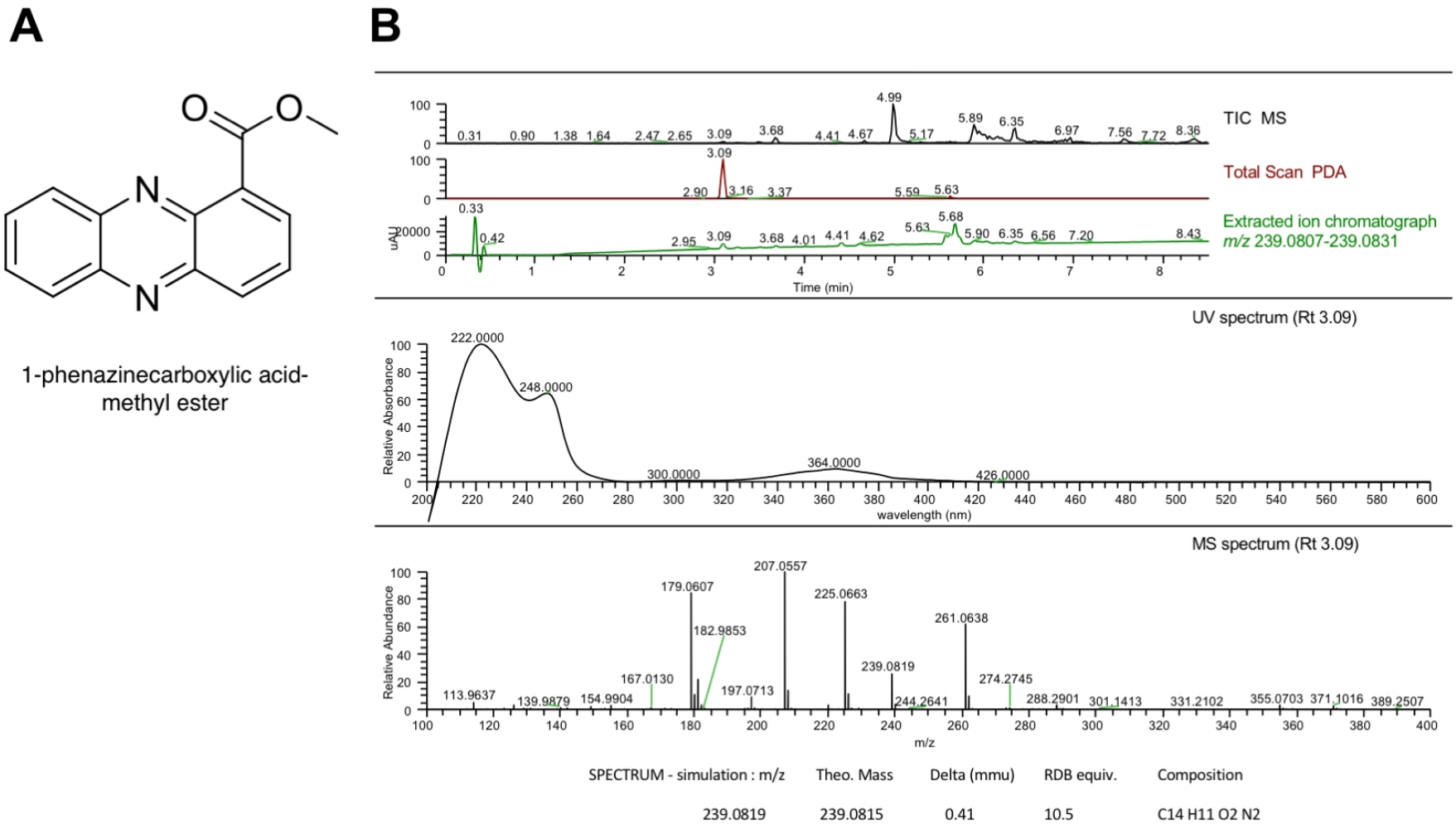


Fig. 3. Pagmadulam et al.

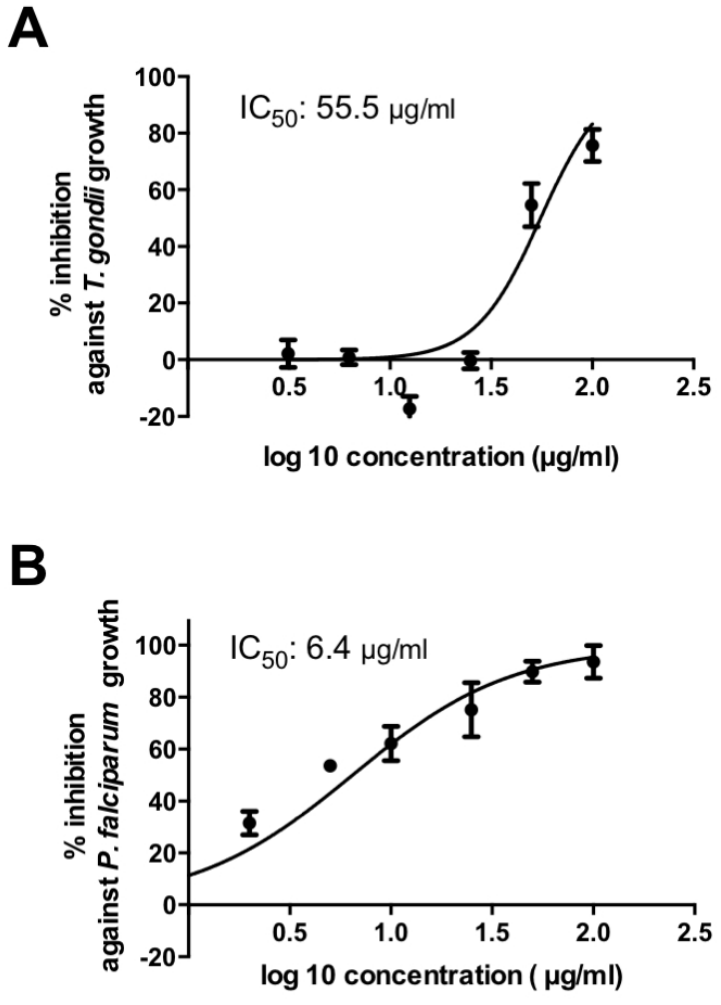


Fig. 4. Pagmadulam et al.

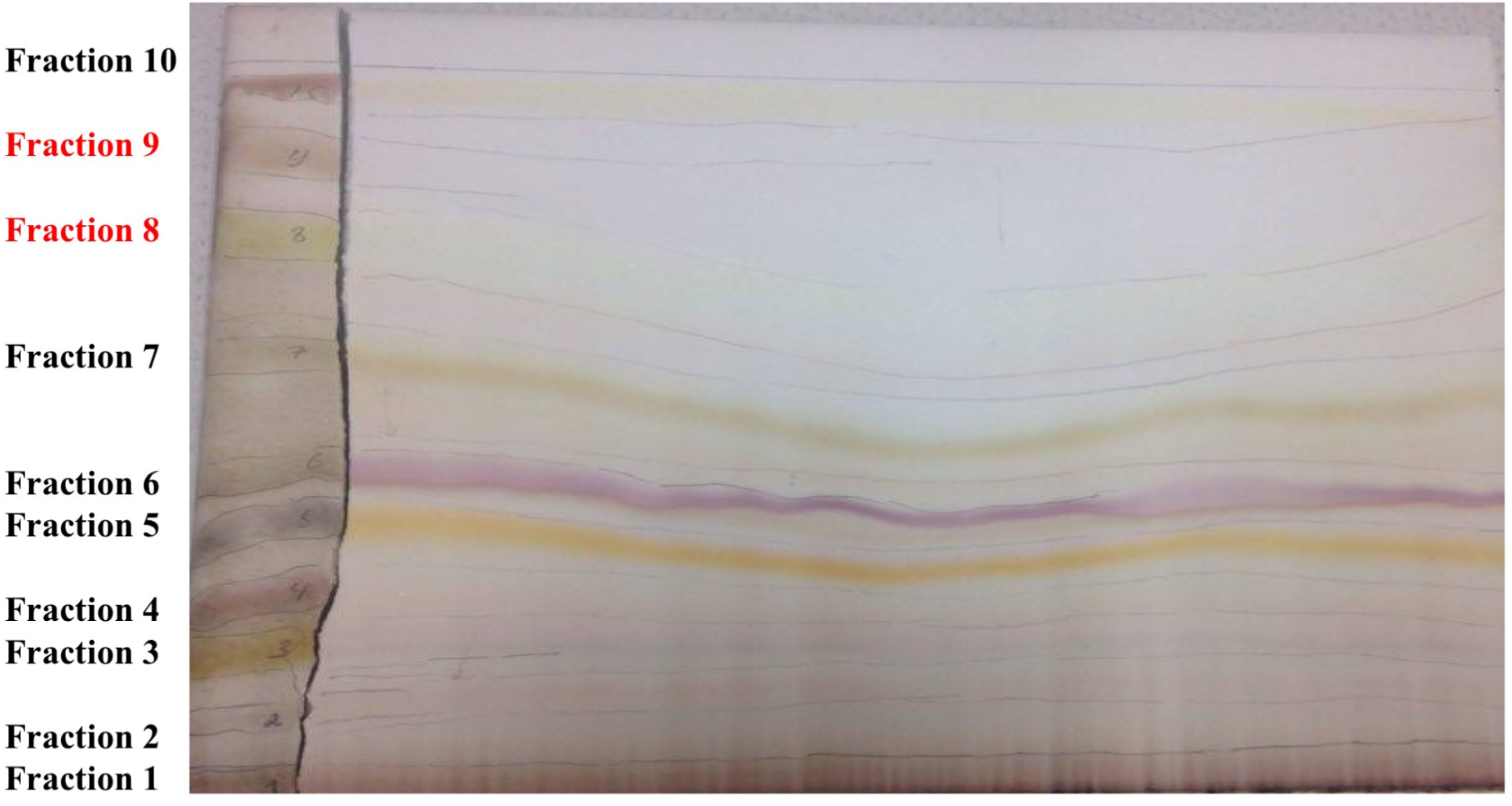


Fig. S1. Pagmadulam et al.

Table 1.

Anti-*Toxoplasma* and antimalarial activities of antibiotics derived from *Streptomyces* species.

| Antibiotics | Actinomycetes | $IC_{50}$ for *T. gondii* | $IC_{50}$ for *P. falciparum* (Selectivity index) | $IC_{50}$ for HFF cells |
|---|---|---|---|---|
| Amphomycin [7] | *Streptomyces canus* | >100 μM | 9.3 μM | >100 μM |
| Kanamycin [35] | *Streptomyces canus* | >100 μM | >100 μM | 11.5 μM |
| Resistomycin [36] | *Streptomyces canus* | >100 μM | >100 μM | 1.2 μM |
| Tylosine phosphate [14] | *Streptomyces cirratus* | >100 μM | 182.7 μM | >100 μM |
| Bafilomycin A1 [39] | *Streptomyces bacillaris* | >100 μM | 34.1 nM (1.0) | 33.1 nM |

All IC50 values were calculated based on three independent experiments.

Table 2

Anti-*Toxoplasma* and antimalarial activities of crude extracts from four actinomycetes (N6, N12, N18, N25) isolates.

| Actinomycetes | $IC_{50}$ for *T. gondii* (Selectivity index) | $IC_{50}$ for *P. falciparum* (Selectivity index) | $IC_{50}$ for HFF cells |
|---|---|---|---|
| N6 | >100 µg/ml | >100 µg/ml | >100 µg/ml |
| N12 | >100 µg/ml | >100 µg/ml | >100 µg/ml |
| N18 | 91.8 ng/ml (3.4) | 156.9 ng/ml (2.0) | 316 ng/ml |
| N25 | 19.2 µg/ml | 4.9 µg/ml | >100 µg/ml |

All IC50 values were calculated based on three independent experiments.

Table 3

Anti-*Toxoplasma* and antimalarial activities of the TLC fractions from *Streptomyces canus* (N25).

| **Fractions** | **$IC_{50}$ for *T. gondii* (Selectivity index)** | **$IC_{50}$ for *P. falciparum* (Selectivity index)** | **$IC_{50}$ for HFF cells** |
|---|---|---|---|
| 1 | >50 µg/ml | >50 µg/ml | >100 µg/ml |
| 2 | >50 µg/ml | >50 µg/ml | >100 µg/ml |
| 3 | >50 µg/ml | >50 µg/ml | >100 µg/ml |
| 4 | >50 µg/ml | >50 µg/ml | >100 µg/ml |
| 5 | >50 µg/ml | >50 µg/ml | >100 µg/ml |
| 6 | >50 µg/ml | >50 µg/ml | >100 µg/ml |
| 7 | >50 µg/ml | >50 µg/ml | >100 µg/ml |
| 8 | 2.6 µg/ml (53.4) | 4.8 µg/ml (28.9) | 138.9 µg/ml |
| 9 | 122.2 ng/ml (239.7) | 163.8 ng/ml (178.8) | 29.3 µg/ml |
| 10 | >50 µg/ml | 18.4 µg/ml | >100 µg/ml |

>50 µg/ml: No activity at 50 µg/ml; the highest dose tested.

The IC50 values of fractions 8, 9, and 10 were calculated based on three independent experiments.

Table S1

Hemolysis rate (%)

| Drugs | Concentrations | Hemolysis rate (%) |
|---|---|---|
| Amphomycin | 100 μM | 0.41 |
| Bafilomycin A1 | 100 μM | 7.78 |
| Tylosine phosphate | 100 μM | N.D. |
| Crude extract N18 | 100 μg/ml | 3.01 |
| Crude extract N25 | 100 μg/ml | 0.41 |
| Fraction 8 | 100 μg/ml | N.D. |
| Fraction 9 | 100 μg/ml | 0.1 |
| Fraction 10 | 100 μg/ml | 6.02 |
| PCA | 100 μg/ml | N.D. |

N.D.: Below detection limit

PBS and RBC lysis buffer (0.83% $NH_4Cl$; 0.01 M Tris-HCl, pH 7.2) are used for negative and positive controls, respectively.

**Procedure**: Hemolysis assay was performed as reported previously with minor modification (Evans et al., 2013). Each drug, extract and fraction was prepared in PBS as designed concentration and then 3% of erythrocyte suspension in PBS was added. The sample were incubated at 37ºC for 3 h and then the mixtures were centrifuged at 1,300 × *g* for 5 min. A 100 μl of supernatant was transferred into 96-well plate. The absorbance values of supernatants were determined with microplate reader at 540 nm. The hemolysis rate of erythrocytes was calculated by using the following formula.

$$\text{Hemolysis rate} = (A_{\text{sample}} - A_{\text{negative control}})/(B_{\text{positive control}} - B_{\text{negative control}}) \times 100\%$$

**Reference:** B.C. Evans, C.E. Nelson, S. S. Yu, K.R. Beavers, A.J. Kim, H.LI, H.M. Nelson, T.D. Giorgio, C.L. Duvall, *Ex vivo* red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs, J Vis Exp. 73 (2013), e50166, doi: 10.3791/50166.