Mosquito (Diptera: Culicidae) Larvicidal and Ovicidal Properties of Extracts from *Streptomyces vinaceusdrappus* (S12-4) Isolated from Soils[1]

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Abstract The actinomycete *Streptomyces vinaceusdrappus* (S12-4) was isolated from soil samples collected from the Venganayakkan region in Western Ghats of Tamil Nadu, India. The strain was identified using 16S rRNA sequencing, and a phylogenetic tree was constructed using MEGA4 software with sequences submitted to GenBank. In laboratory bioassays, crude extracts of secondary metabolites of the actinomycete demonstrated larvicidal and ovicidal activity against *Culex quinquefasciatus* Say and *Anopheles stephensi* Liston, but not against *Aedes aegypti* L. These results suggest that metabolites of *S. vinaceusdrappus* S12-4 may have value in development of alternatives to conventional chemical insecticides for control of mosquito populations.

Key Words *Streptomyces vinaceusdrappus*, *Anopheles stephensi*, *Culex quinquefasciatus*, actinomycetes metabolites, biological activity

The actinomycetes (Actinobacteria: Actinomycetales) are Gram-positive filamentous microbes and are sources of a large proportion of natural antimicrobial drug compounds (Ishida et al. 1965, Valanarasu et al. 2008). More than 80% of the biologically active secondary metabolites derived from soil actinomycetes are from the genus *Streptomyces* (Demain 2006, Edwards 1992, Vining 1992). Actinomycetes play an important role in biological control of agricultural and mosquito insect pests through the production of secondary metabolites with insecticidal properties (Bream et al. 2001, Sundarapandian et al. 2002). Such metabolites are being increasingly studied for potential development as insecticides (El-Khawagh et al. 2011). Thus, the study reported herein was aimed at isolating the actinomycete *Streptomyces vinaceusdrappus* (S12-4) from soil samples from Western Ghats in India and assessing extracts for their larvicidal and ovicidal properties against the

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mosquitoes *Culex quinquefasciatus* Say, *Anopheles stephensi* Liston, and *Aedes aegypti* L.

Materials and Methods

Isolation and characterization of *S. vinaceusdrappus* (S12-4). Soil samples were collected from the Venganayakkan region of Western Ghats of Tamil Nadu, India, and transported aseptically to the laboratory using sterile plastic containers. *Streptomyces* v*inaceusdrappus* (S12-4) which, through preliminary screening was shown to be biologically active against mosquito larvae, was isolated from the soil samples by drop-plating serial dilutions of the samples onto Actinomycetes Isolation Agar in petri dishes. The dishes were incubated at 28°C for 10–14 d (Valanarasu et al. 2009). The pure isolate was characterized morphologically and physiologically as per *Bergey's Manual of Systematic Bacteriology* (Locci 1989) at various pH levels, sodium chloride (NaCl) concentrations, temperatures, and on various growth media for differing lengths of incubation for optimal production. The more biologically active isolates were selected for biochemical characterization using a biochemical kit (KB014 HiAcinetobacter Identification Kit, HiMedia Laboratories, Mumbai, India) according to the manufacturer's protocol.

Secondary metabolite extraction. Five liters of the International Streptomyces Project No. 2 (ISP-2) broth in an Erlenmeyer flask were inoculated with an active isolate of S12-4 and incubated at 28°C for 10–14 d. The culture was then centrifuged at 3,293 $\times$ *g* for 15 min to separate the biomass and supernatant. The biomass was immersed in methanol and the supernatant was mixed in an equal volume of ethyl acetate. The solvent was separated using a separating funnel and concentrated with a vacuum rotary evaporator at 50°C. The resultant extract was transferred to a sterile vial at −20°C (Saravanakumar et al. 2012).

Antibiotic sensitivity. A slightly modified disc diffusion method as described by Yao (2002) was used to assess antibiotic sensitivity of isolate S12-4. The isolate culture was grown on slants of ISP-2 agar at 28°C for 2–4 d. Aliquots of these cultures were mixed in 5 ml sterile water (Waksmans 1961) and, within 1 h, the suspension was drop-plated and uniformly spread on the surface of ISP-2 agar in petri dishes and allowed to air-dry for 15 min. Commercially prepared discs of 37 antibiotics were placed on the surface of the agar and removed after 30 min at room temperature allowing for diffusion into the medium. These plates were incubated at 28°C for 2–3 d. Zones of inhibition were measured and recorded in millimeters.

Insects and bioassays. *Culex quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* larvae were obtained from colonies maintained at the Entomology Research Institute, Loyola College, Chennai. The colonies were maintained at 75–85% relative humidity and 27 $\pm$ 1°C and on a light phase of 11 $\pm$ 0.5 h. Larvae were fed with dog food and brewer's yeast in a 3:2 ratio.

Larvicidal activity of the crude extract of isolate S12-4 was evaluated using a slightly modified method of the World Health Organization (2005). Concentrations of 62.5, 125, 250, and 500 ppm were prepared using 0.5% dimethyl sulfoxide (DMSO). Third-instar larvae were placed in each suspension, with 20 larvae per replicate and 5 replicates per concentration and mosquito species. DMSO and water alone, each without the extract, served as controls. Numbers of dead larvae

Table 1. Morphological characterization of the active isolate of *S. vinaceus-drappus* (S12-4) on various culture media.

	Character					
Medium	Aerial Mycelium	Substrate Mycelium	Soluble Pigment	Colony Margin	Growth*	Gram Stain
ISP-2	Whitish gray	White	—	Filaments	+++	+
ISP-4	Black	White	—	Filaments	+++	+
ISP-6	White	Yellow	—	Filaments	+++	+
ISP-7	—	—	—	—	—	—
YPG	Yellowish gray	White	—	Filaments	+++	+
MNGA	White	Yellow	—	Filaments	+++	+
M3	Light orange	Orange	—	Filaments	+++	+
AIA	Whitish gray	White	—	Filaments	+++	+
SCA	Whitish gray	Yellow	—	Filaments	+++	+
BENNET	Whitish gray	White	—	Filaments	+++	+

* Growth = no soluble pigment.

were counted 24 h after exposure. Mortality data were corrected using Abbott's (1925) and were subjected to probit analysis (US EPA Probit Analysis software, version 1.5) to estimate concentration–mortality response to the S12-4 extract.

Ovicidal activity was evaluated using modified methods of Elango et al. (2009). The concentrations were prepared as previously described with the same treatments and numbers of replicates. Twenty newly deposited eggs of three mosquito species were placed in the treatment suspensions. Eggs were monitored using stereomicroscope (Wild M7S TYP 308700, Switzerland). The number of unhatched versus hatched eggs was tabulated at 120 h after initial exposure to the treatments. Unhatched eggs were identified as those with unopened opercula.

Molecular analyses. DNA of the S12-4 isolate was extracted using the HipurA Streptomyces DNA purification kit-MB 527-50 pr (HiMedia) according to the manufacturer's guidelines. The primers 27F-5′AGAGTTTGATCMTGGCTCAG3′ and 1492R-5′TACGGYTACCTTGTTACGACTT3′ were used to amplify the 16S ribosomal sequence from the genomic DNA in a thermal cycler as follows: initial denaturation for 3 min at 94°C, 35 cycles of denaturation 1 min at 94°C, annealing at 54°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min and then held at 4°C. The polymerase chain reaction (PCR) products were confirmed with 1% agarose gel electrophoresis stained by ethidium bromide (Farris et al. 2007, Valanarasu et al. 2008). The confirmed PCR product was sequenced using the di-deoxy chain termination method in an Applied Biosystems Automated Sequencer (Synergy Scientific Services, Chennai, India). The sequences were

Table 2. Antibiotic sensitivity of *S. vinaceusdrappus* (S12-4) as determined by disc diffusion method on ISP-2 agar.

Antibiotic (Concentration)	Diameter (mm) of Zone of Inhibition*	Antibiotic (Concentration)	Diameter (mm) of Zone of Inhibition*
Cephaloridine (30 μg)	35	Nalidixic acid (10 μg)	28
Clavulanic acid (30 μg)	R	Carbenicillin (100 μg)	R
Rifamycin (30 μg)	R	Spectinomycin (20mg)	R
Cephalothin (30 μg)	R	Tetracyclin (10 μg)	R
Ticarcittin (75 μg)	R	Aureomycin (20mg)	R
Oxacillin (1 μg)	R	Actidione (30 μg)	R
Penicillin (10 units)	R	Neomycin (30 μg)	33
Amikacin (30 μg)	38	Kanamycin (30 μg)	R
Cefotaxime (30 μg)	R	Amoxicillin (30 μg)	R
Gentamycin (10 μg)	21	Erythromycin (15 μg)	R
Ampicillin (25 μg)	R	Ciprofloxacin (5 μg)	R
Norfloxacin (10 μg)	20	Doxycycline (30 μg)	19
Imipenem (10 μg)	R	Nystatin (50 μg)	R
Vancomycin (30 μg)	17	Menicycline (30 μg)	20
Polymyxin (300 units)	R	Linezoid (15 μg)	35
Streptomycin (30 μg)	34	Azithromycin (15 μg)	8
Trimoxazole (25 μg)	R	Clarithromycin (15 μg)	28
Ketoconazole (30 μg)	14	Cotromoxazole (25 μg)	R
Fluconazole (30 μg)	R		

* R = Resistant.

identified and compared to the reference species of actinomycetes closely related to the genomes in the database using the National Center for Biotechnical Information (NCBI) Basic Local Alignment Search Tool (BLAST) (http:www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree for isolate S12-4 was constructed with the neighbor-joining method using MEGA4 software. The sequence was submitted to the GenBank, NCBI (Saltau and Nei 1987).

Gas chromatography–mass spectrometry analysis. The S12-4 isolate extract was subjected to gas chromatography–mass spectrometry (GC-MS) analysis on GC-MS-5975 (Agilent, Palo Alto, CA), column DB 5 ms Agilent, dimension length = 30.0 m, internal diameter = 0.2 mm, film thickness = 0.25 μm, with a temperature program of 70–300°C, 10°C /min, injection temperature = 240°C,

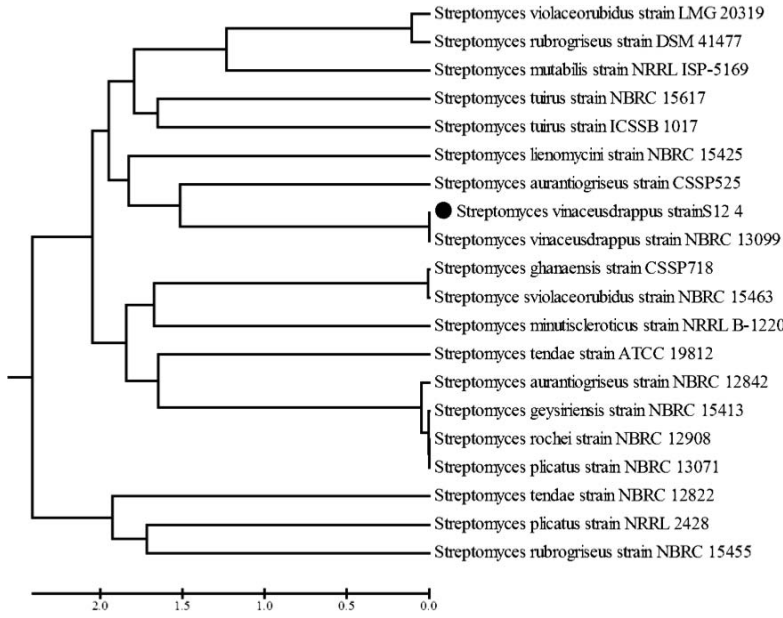

Fig. 1. Phylogenetic tree indicating the taxonomic position of *S. vinaceus-drappus* (S12-4).

carrier gas = helium, flow rate =1.51 ml/minute, equipped with GC-MS NIST-II library.

Results

Streptomyces vinaceusdrappus (S12-4) was isolated from soils collected from the Venganayakkan region of Western Ghats, Tamil Nadu, India. It was subsequently subcultured on ISP-2 medium, which proved to be a good substrate for the production of the S12-4 isolate with larvicidal properties. Growth of the isolate was inhibited at 70°C and at 10% (w/v) NaCl. Growth was observed in a temperature range of 20–60°C and a pH range of 4–12. Morphological characterizations on various growth media are summarized in Table 1.

Biochemical analysis revealed the presence of citrate, lysine, arabinose, malonate, urease, glucose, rhamnose, mannitol, inositol, sorbitol, sucrose, xylose, lactose, arginine, adonitol, and salicin. Antibiotic sensitivity tests demonstrated that among the 37 antibiotics tested, only 14 inhibited *S. vinaceusdrappus* growth (Table 2). The S12-4 isolate (Accession No. KT827088) showed 99% homology to *S. vinaceusdrappus* strain NBRC 13099 16S ribosomal RNA gene partial sequence (NR_112368.1). This similarity was corroborated by the phylogenetic tree constructed using MEGA4 software (Fig. 1).

Concentration–mortality responses at 24 h after exposure varied with the species of mosquito (Table 3). There was no larvicidal or ovicidal activity of the isolate S12-4 extracts against *Ae. aegypti*. Yet the median lethal concentration

Table 3. Concentration–mortality response of *An. stephensi* and *Cx. quinquefasciatus* larvae and eggs to ethyl acetate and methanol extracts of *S. vinaceusdrappus* (S12-4).

Species/Stage	Extract Solvent	LC_{50} (ppm)	95% Confidence Limits	Intercept (± SE)	Slope (± SE)	χ^2
An. stephensi larvae	Ethyl acetate	141.49	121.76–162.63	0.14 ± 0.49	2.25 ± 0.22	2.7*
An. stephensi larvae	Methanol	138.20	119.16–158.47	0.05 ± 0.50	2.31 ± 0.22	4.2*
Cx. quinquefasciatus larvae	Ethyl acetate	170.55	149.28–194.58	0.44 ± 0.51	2.43 ± 0.22	1.7*
Cx. quinquefasciatus larvae	Methanol	180.51	157.86–206.59	0.39 ± 0.51	2.39 ± 0.22	1.8*
An. stephensi eggs	Ethyl acetate	168.62	52.82–492.79	2.2 ± 1.4	3.2 ± 0.6	11.8
An. stephensi eggs	Methanol	171.20	198.3–256.21	1.1 ± 0.5	2.6 ± 0.2	1.5*
Cx. quinquefasciatus eggs	Ethyl acetate	224.53	48.58–556.70	1.8 ± 1.4	3.0 ± 0.6	11.7
Cx. quinquefasciatus eggs	Methanol	223.90	197.85–255.40	1.1 ± 0.5	2.6 ± 0.2	1.1*

* Level of significance ≤ 0.05.

Table 4. GC-MS analysis of ethyl acetate extract of *S. vinaceusdrappus* (S12-4).

Retention Time (min)	Area (%)	Compound
14.611	32.36	Pentanedioic acid
21.945	4.26	Unidentified
22.005	3.61	Unidentified
22.347	1.51	Phthalic acid, ethyl isopropyl ester
22.834	1.25	1,2-Benzenedicarboxylic acid, bis-methyl ester
22.946	17.03	Unidentified
23.048	1.24	Adipic acid, 2,6-dimethoxyphenyl heptyl ester
23.117	16.01	Unidentified
23.330	20.31	Dibutyl phthalate
23.493	2.43	Di-*sec*-butyl phthalate

(LC_{50}) of the S12-4 ethyl acetate extract against *An. stephensi* larvae was 141.49 ppm while the LC_{50} of the methanol extract was 138.20 ppm. The LC_{50} of the ethyl acetate extract of S12-4 against *Cx. quinquefasciatus* larvae was 170.55 ppm while the methanol extract LC_{50} was 180.51 ppm. The LC_{50} of the ethyl acetate extract against the eggs of *An. stephensi* was 168.62 ppm while the LC_{50} for the methanol extract was 171.20 ppm (Table 3). The LC_{50}s for *Cx. quinquefasciatus* eggs were 224.53 ppm with the ethyl acetate extract and 223.40 ppm for the methanol extract.

Based on the results of the GC-MS analysis, the ethyl acetate extract was composed of 10 compounds, with pentanedioicacid (32.36%), dibutyl phthalate (20.31%), and an unidentified compound (17.03%) being the most abundant (Table 4). The methanol extract was composed of 27 compounds, with *n*-hexadecanoic acid (23.59%) being the predominant component (Table 5).

Discussion

Certain species of actinomycetes have insecticidal properties, are environmentally friendly and, therefore, have potential for development as alternatives to conventional chemical insecticides. In our study, we successfully isolated the actinomycete *S. vinaceusdrappus* (S12-4) from soils collected in the Venganayakkan region of Western Ghats, Tamil Nadu, India. Our subsequent study of the S12-4 isolate demonstrated that extracts collected with either ethyl acetate or methanol solvents were biologically active against larvae and eggs of *An. stephensi* and *Cx. quinquefasciatus* and effectively expands the number of *Streptomyces* isolates with activity against mosquito species. These extracts had no activity against *Ae. aegypti* larvae or eggs in our study. Larvicidal activity also has been reported for ethyl acetate extracts of *Streptomyces* sp. KA13-3 and *Nocardiopsis* sp. KA25-A against *Cx. quinquefasciatus* (Rajesh et al. 2015), actinomycetes isolates SA-

Table 5. GC-MS analysis of the methanol extract of *S. vinaceusdrappus* (S12-4).

Retention Time (min)	Area (%)	Molecule Name
16.115	0.92	Unidentified
18.235	0.42	Unidentified
20.424	0.64	Tridecanoic acid
20.817	2.39	Tetradecanoic acid
21.552	1.07	Methyl 13-methyltetradeconate
21.638	1.53	Methyl 9-methyltetradeconate
21.928	5.44	Pentadecanoic acid
22.014	12.16	Unidentified
22.552	1.19	1-Docosanol, methyl ester
22.612	4.64	Hexadecanoic acid
22.800	0.95	Methyl 2-hydroxy-pentadecanoate
22.980	23.59	*n*-Hexadecanoic acid
23.108	4.16	Unidentified
23.245	2.10	Unidentified
23.339	10.28	*n*-Hexadecanoic acid
23.621	1.34	Hexadecanoic acid, 15-methyl-methyl ester
23.706	1.73	Hexadeconic acid, 14-methyl-methyl ester
23.826	2.11	Hexadecanoic acid, 2-hydroxy-methyl ester
23.954	2.71	Heptadecanoic acid
24.040	6.39	Heptadecanoic acid
24.185	3.19	Unidentified
24.279	1.40	Unidentified
25.134	1.44	Hexadecanoic acid, 1,1-dimethylethyl ester
25.467	0.90	Unidentified
27.904	1.08	Unidentified
28.357	3.59	Phthalic acid, di(2-propylphenyl) ester
30.443	2.63	Unidentified

10BC, SA-9K, and SA-9L against *Cx. quinquefasciatus* (Anwar et al. 2014), *Streptomyces* sp. VITSTK7 against *Cx. quinquefasciatus* and *An. subpictus* Grassi (Thenmozhi et al. 2013), *S. gedanensis* (LK-3) against *Cx. quinquefasciatus* and *Cx. gelidus* Theobald (Karthik et al. 2011), and actinomycetes strain LK1 against

An. stephensi and *Cx. tritaeniorhynchus* Giles (Loganathan et al. 2013). Karthik et al. (2011) also found ovicidal activity of extracts from LK-1 and LK-3 against *Cx. gelidus* and *Cx. tritaeniorhynchus*.

Based on these results, the *S. vinaceusdrappus* S12-4 isolate should be further explored as a source of biologically active compounds for development as mosquito larvicides and ovicides. Our characterization of this isolate in terms of antibiotic sensitivity (Table 2), chemical composition (Tables 4, 5), and phylogeny (Fig. 1) serves as a foundation for these further studies.

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