Inhibitory activity of Marine Actinobacterial Extracts against Dengue-2 Virus

Lavanya D., Radhakrishnan M. and Parthasarathy Krupakar*
Centre for Drug Discovery and Development, Col. Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Chennai, INDIA
*pkrupakar.cddd@sathyabama.ac.in

Abstract
Dengue is the major arthropod-borne viral disease in tropical and sub-tropical regions of the world. It has been estimated that India contributes about 34% of the global dengue infection of the world. To date, there is no clinically approved dengue antiviral for humans. Actinobacteria isolated form marine sources have been shown to produce chemically diverse metabolites. However, they are under investigated for anti-dengue viral activity. The present study was attempted to screen organic extracts of selected marine actinobacterial strains for anti-dengue viral activity. Marine sediment samples were collected from five places of three different East coastal locations (Thiruchendur, Pichavaram, Parangipettai) in Tamil nadu. Isolation of actinobacteria was performed using oat meal agar and cultured in ISP2 agar. The isolates were optimized for the production of metabolites in the solid medium. Crude extract was obtained using ethyl acetate precipitation method.

Crude extracts were subjected to drying and fractionation followed by evaluation of anti-dengue property by Plaque Reduction and Neutralization (PRNT) assay. Twenty four different marine actinobacteria were isolated and 14 extracts were obtained. Preliminary screening for viability of cells by the actinobacterial extracts were carried out using cytotoxicity assay in order to determine the minimal toxicity dosage (MNTD) for the antiviral screening followed by analyzing for anti-viral activity against Dengue-2 serotype by PRNT. Two marine actinobacterial strains whose extracts exhibited anti-dengue activity have been identified. Further testing of other extracts, optimizations for production, purification, characterization of the compounds are in progress.

Keywords: Dengue-2, Actinobacteria, Plaque Reduction Neutralization Assay, BHK-21.

Introduction

Dengue is the arthropod-borne viral disease in humans caused by Dengue virus. This RNA virus belongs to family *Flaviviridae* and genus Flavivirus with four serologically distinct serotypes [DEN-1, DEN-2, DEN-3, and DEN-4]. The serotypes differ at the amino acid level in the viral envelope proteins by 25% to 40% leading to high morbidity and mortality upon secondary infection by a different serotype other than the one which caused the primary infection.

At present there are 50–100 million cases every year and 40% of the world's population is at risk. An estimated 5,00,000 people with severe dengue requires hospitalization each year and 2.5% of affected people are dead.

The gene order of Dengue virus is C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The viral genome has a 5′ noncoding region (NCR), 3′ noncoding region (NCR), a short untranslated region at 5' end and a non-polyadenylated 3' terminus. The genome has three structural genes which code for the structural proteins capsid (C), membrane (prM) and envelope (E). The E glycoprotein and prM protein form the membrane proteins. E is the most highly conserved structural protein among the serotypes. The C or core protein interacts with viral RNA to form virion nucleocapsid which is surrounded by the lipid bilayer or envelope. Non-structural genes NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 play a role in viral replication and polypeptide processing.

Dengue virus is transmitted by *Aedes aegypti* and *Aedes albopictus*. Their major breeding sources are fresh water storage containers. Trans-ovarial transmission of all four dengue serotypes has been demonstrated in both *Aedes aegypti* and *Aedes albopictus* experimentally and from field collected mosquito larvae. Trans-ovarial transmission of dengue virus is a crucial etiological phenomenon responsible for persistence of virus during inter-epidemic periods. Dengue viruses are also maintained in nature by vertical transmission by *Aedes aegypti*[1].

Dengue virus causes Dengue Fever (DF), Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS)[2].

Dengue fever is an acute febrile illness defined by the presence of fever, retro-orbital or ocular pain, headache, rash, myalgia, arthralgia, leukopenia or 2 haemorrhagic manifestations. Anorexia, nausea, abdominal pain, and persistent vomiting may also occur but not in case of DF. Fever lasting from 2-7 days, haemorrhagic manifestation, thrombocytopenia (≤100,000 cells per mm3) and plasma leakage shown by hemoconcentration (an increase in haematocrit ≥20% above average for age or a decrease in hematocrit ≥20% of baseline following fluid replacement therapy), or pleural effusion, or ascites or hypoproteinemia are the symptoms of DHF. Rapid and weak pulse and narrow

pulse pressure (<20mm Hg), or age-specific hypotension and cold, clammy skin and restlessness occurs in cases of DSS.[3]

Present methods of diagnosis include polymerase chain reaction (PCR), IgM capture ELISA (MAC ELISA), IgG ELISA, NS1 ELISA, Plaque Reduction Neutralization Test and microneutralization PRNT, Rapid diagnostic tests, Complement Fixation Tests and Hemagglutination Inhibition Test.

Presently, there is no standard specific treatment for dengue fever. Since the treatment is based on alleviation of the symptoms, patients are prescribed paracetamol to bring down the fever and to reduce the joint pain. They are also advised to take plenty of fluids to overcome dehydration. Medical care by physicians and nurses experienced in the treatment of effects and progression of the disease can efficiently save lives. Nilavembu kudineer and *Carica papaya* extract tablets formulated by Siddha medical experts and given to Dengue infected patients for their recovery.

Components of nilavembu kudineer are *Andrographis Paniculata* (Nilavembu), *Plectranthus Vettiveroides* (Vilamichai Ver), *Vetiveria zizanioides* (Vetiver), *Zingiber Officinale* (Chukku), *Piper Nigrum* (Milagu), *Cyperus Rotundus* (Korai Kizhangu), *Santalum Album* (Santanam), *Trichosanthes Cucumerina* (Peyputtal) and *Mollugo Cerviana* (Parpadagam)[4]. The exact mechanism and role of these plant extracts in increasing platelets are unknown; however they are able to play a major role in decreasing the death rate due to dengue.

Infection by one of the four dengue virus serotypes has been shown to confer lasting protection against homotypic re-infection but only transient protection against a secondary heterotypic infection. Due to these dengue-specific complexities, vaccine development focuses on the generation of a tetravalent vaccine aimed at providing long-term protection against all virus serotypes. Additional challenges are posed by the lack of an adequate animal disease model and the resulting uncertainty around correlates of protection.

Actinomycetes are the filamentous bacteria resembling fungi. Some of the commonly isolated Actinomycetes from the soil are *Actinomyces, Streptomyces, Nocardia, Micromonospora* etc.[5] They produce over 5000 novel antibiotics such as *Actinomycin, Kanamycin Streptomycin, Vancomycin, Erythromycin* and *Tetracycline* etc. which have been proved to possess anti-bacterial and anti-fungal activities[6].

Understanding the vast potential of actinobacteria in this study, numerous metabolites produced by isolated actinobacteria were tested against DENV-2 serotype. Identification of the active compounds responsible for anti-dengue activity was also done.

Material and Methods

Sample collection and pre-treatment: Sediment samples were collected from 5 different locations in Thiruchendur, Pichavaram, Parangipettai marine environment in Tamil Nadu of East coastal region and stored at 4ºC till usage. Five grams of each of the sediment soil samples were weighed, dried in hot air oven at 55 ºC for 10minutes and added to 45 mL of sterile distilled H_2O.[7]

Isolation of Actinobacteria: The sample was further serially diluted using 9 mL sterile distilled water blanks up to 10 -5 dilution. 100 µL aliquot from 10-3, 10-4, 10-5 dilutions was taken and inoculated onto starch casein agar and Kuster's agar medium. All the plates were incubated at 28°C for one month. During incubation, colonies with suspected actinomycete morphology were selected and subcultured in ISP2 agar plates. Morphologically distinct actinobacterial colonies were selected and subcultured on ISP2 agar slants. All the cultures were preserved as slant stock as well as in 30% glycerol broth.

Characterization of Actinobacteria: Isolates were characterized based on their morphology, colour, consistency macroscopically and type of mycelium formation microscopically.

Preparation of extracts: Each of the actinomycete cultures was grown on five ISP2 agar plates and incubated at 28°C for 7-10 days for the production of bioactive metabolites. After incubation, the spores were scrapped and removed and agar sliced and dipped in ethyl acetate solvent. After 4-5 days, the agar was removed and solvent was dried and extracts were refrigerated and used for activity.

Cell line cultivation: BHK-21 cell line was received from NCCS, Pune. Sigma DMEM containing 10% FBS was used as growth medium for the cultivation of BHK-21 cell line. The cells were incubated at 37ºC in 5% CO_2 incubator.

Dengue-2 virus propagation: 100 µL of Dengue-2 virus was inoculated and allowed to infect 90% confluent cells in a T25 flask and incubated at 37ºC till complete cytopathic effect occurred. Then the infected cells were removed from the flask, collected in a centrifuge tube and centrifuged at 2000 rpm for 5 minutes. Then the supernatant was collected and filtered using 0.22 µm filter. The filtered supernatant was stored as aliquots of 1 mL at (-80 ºC).

Cytotoxicity assay of the actinomycetes extract: The extracts were processed for cytotoxicity assay by XTT assay method as follows. BHK-21 cells were seeded in 96 well plate and incubated for 24 hours at 37ºC in 5% CO_2 incubator. The electron mediator reagent and activator reagent were mixed in equal volume and stored at -20ºC till usage. After 24 hours, 100 µL compounds were diluted in plain medium and 100 µL from each dilution was added to the respective wells. Triton-X was added as negative control. The mixture was incubated at 37ºC in 5% CO_2 incubator for

24 hours. After incubation, activated XTT reagent was added and absorbance was measured in ELISA reader at 450nm. Results were interpreted with controls.

Screening for anti-dengue activity by Plaque Reduction Neutralization Test: BHK-21 cells were seeded in 12 well plate and incubated for 24 hours at 37ºC in 5% CO_2 incubator. Virus stock was taken from (-80ºC) and thawed quickly and serially diluted in medium without FBS. The diluted virus samples were added to the wells respectively and virus control and cell control were maintained. The plate was incubated for 1 hour at 37ºC in 5% CO_2 incubator by rocking the plate at 15mins interval. After incubation, the inoculum was removed and 0.5% of CMC with 2% DMEM was overlaid and incubated at 37ºC in 5% CO_2 incubator for 2-3 days. After the observation of plaques CMC was removed and fixed using 10% formaldehyde and stained using crystal violet stain for 3 to 5 mins.

The plaques were counted and viral titre was determined. In another 24 well plate, BHK-21 cells were seeded and incubated for 24 hours at 37ºC in 5% CO_2 incubator. 100 µL of virus of least dilution determined using plaque assay was inoculated with different dilutions of compounds and incubated. Plaque reduction was identified and compound activity was observed.

Results

Isolation of actinobacteria from marine sediments: Isolates from different marine ecosystems were cultured. The isolated colonies were observed for colour, morphology, consistency, pigment formation, presence of mycelia and spore formation. Among 24 isolates, colonies identified were creamy white-3, grey-9, white-5, yellow-1, brown-4 and pink-1 as represented in the table 1. Among isolates, 18 colonies were powdery and 6 colonies were leathery. Some colonies showed reverse side pigment and some showed surface pigment. Aerial and surface mycelia were observed in all the 24 colonies. Colonies were isolated and recovered by streaking on ISP2 plates (Figure 1). Further the colonies were streaked in ISP2 slants and stored in 4ºC and prepared for glycerol and ISP2 stocks for storage at (-80ºC).

Extract preparation: Among 24 isolated colonies, 14 extracts were obtained by the ethyl acetate precipitation method. The extracts prepared were dried and refrigerated till usage. The extracts were evaluated for concentration as mentioned as mentioned in the table 2 and diluted in 100% DMSO.

Cytotoxicity assay of the actinomycetes extract: To standardise the minimal toxic dosage of the actinobacterial extract, the extracts were processed for cytotoxicity assay by XTT assay method. The processed 14 extracts showed cell toxicity at various levels. Among the 14 extracts, MAE7 and MAE13 extracts showed less toxic effect to the cells. Based on the toxicity test, the MAE7 and MAE13 showing minimal toxicity for mammalian cells were processed for PRNT assay.

Plaque Reduction Neutralization Test: Upon allowing infection of the virus at different dilutions in the cell line and subsequent incubation, plaques were formed and counted. Plaque forming units of 12 plaques were identified in the dilution 10-6 (Figure 2). By this, PRNT was processed for two compounds that showed minimal toxicity in cell line. The MAE7 and MAE13 extracts when inoculated with 10-6 dilution of Dengue-2 virus and incubated, showed antiviral activity against dengue-2 virus. Based on the reduction of plaques, antiviral activity of MAE7 and MAE13 was determined as shown in the figure 3. Hence, the compounds MAE7 and MAE13 are the entry inhibitors of the Dengue virus-2.

Figure 1: Recovery of marine actinobacterial isolates on ISP2 medium

Table 1
Morphological identification of isolated Actinobacterial colonies from soil

S.N.	Sample source	Index No	Microscopic identification						
			Growth	Consistency	Aerial Mass Colour	Reverse Side Pigment	Surface Pigment	Aerial Mycelia	Surface Mycelia
1.	Thiruchendur	MAE1	good	powdery	Creamy white	nil	nil	+	+
2.	Thiruchendur	MAE2	good	powdery	grey	nil	nil	+	+
3.	Thiruchendur	MAE3	good	powdery	grey	nil	nil	+	+
4.	Thiruchendur	MAE4	good	powdery	grey	nil	nil	+	+
5.	Thiruchendur	MAE5	good	powdery	grey	nil	nil	+	+
6.	Thiruchendur	MAE6	good	powdery	grey	nil	nil	+	+
7.	Pichavaram	MAE7	good	leathery	orange	orange	nil	+	+
8.	Pichavaram	MAE8	good	powdery	white	Pale yellow	nil	+	+
9.	Pichavaram	MAE9	Moderate	leathery	yellow	white	nil	+	+
10.	Pichavaram	MAE10	good	leathery	brown	nil	nil	+	+
11.	Parangipettai	MAE11	good	Leathery	brown	nil	nil	+	+
12.	Parangipettai	MAE12	good	Powdery	white	nil	nil	+	+
13.	Parangipettai	MAE13	good	Powdery	white	nil	nil	+	+
14.	Parangipettai	MAE14	Moderate	powdery	grey	nil	nil	+	+
15.	Parangipettai	MAE15	good	powdery	pink	nil	nil	+	+
16.	Parangipettai	MAE16	good	powdery	white	nil	nil	+	+
17.	Parangipettai	MAE17	good	powdery	creamy white	pale yellow	nil	+	+
18.	Parangipettai	MAE18	good	powdery	creamy white	nil	white	+	+
19.	Parangipettai	MAE19	good	leathery	brown	nil	nil	+	+
20.	Parangipettai	MAE20	good	leathery	brown	nil	nil	+	+
21.	Parangipettai	MAE21	good	powdery	grey	nil	nil	+	+
22.	Parangipettai	MAE22	good	powdery	grey	nil	nil	+	+
23.	Pichavaram	MAE23	good	powdery	grey	nil	nil	+	+
24.	Parangipettai	MAE24	good	powdery	white	nil	nil	+	+
25.	Pichavaram	MAE25	good	powdery	grey	nil	nil	+	+
26.	Pichavaram	MAE26	good	powdery	grey	nil	nil	+	+
27.	Pichavaram	MAE27	good	powdery	grey	nil	nil	+	+

Table 2
Marine Actinobacterial extracts concentration derived by ethyl acetate extraction method

S.N.	Index no	Source of the sample	Concentration of the extract
1.	MAE3	Thiruchendur	0.0618g
2.	MAE5	Thiruchendur	0.0861g
3.	MAE6	Thiruchendur	0.1369g
4.	MAE7	Pichavaram	0.0612g
5.	MAE8	Pichavaram	0.0067g
6.	MAE12	Parangipettai	0.0179g
7.	MAE13	Parangipettai	0.0323g
8.	MAE16	Parangipettai	0.0203g
9.	MAE17	Parangipettai	0.0519g
10.	MAE18	Parangipettai	0.0185g
11.	MAE21	Parangipettai	0.0327g
12.	MAE22	Parangipettai	0.0334g
13.	MAE23	Pichavaram	0.0266g
14.	MAE24	Parangipettai	0.0192g

Table 3
Growth and morphological pattern of Actinomycetes isolated from different rare ecosystems

Characteristics		MAE7	MAE13
Micromorphology	Aerial mycelium	+	+
	Substrate mycelium	+	+
	Fragmentation	-	-
Cultural characteristics	Colony consistency	Leathery	Powdery
	Aerial mass colour	Orange	White
	Reverse side pigment	Orange	-
	Soluble pigment	-	-
Physiological Characteristics	ISP1	-	-
	ISP2	+	+
	ISP3	-	-
	ISP4	+	+
	ISP5	-	-
	ISP6	+	+
	ISP7	+	+
Carbon utilization	Arabinose	+	+
	Xylose	+	+
	Inositol	+	+
	Mannitol	+	+
	Fructose	+	+
	Rhamnose	+	+
Enzyme production	Asparagine	+	+
	Glutamine	+	+
pH tolerance	5	-	-
	6	-	-
	7	+	+
	8	+	+
	9	+	+
	10	+	+
	11	+	+
	0	+	+
	1	+	+
NaCl tolerance (%)	2	+	+
	3	+	+
	5	-	-

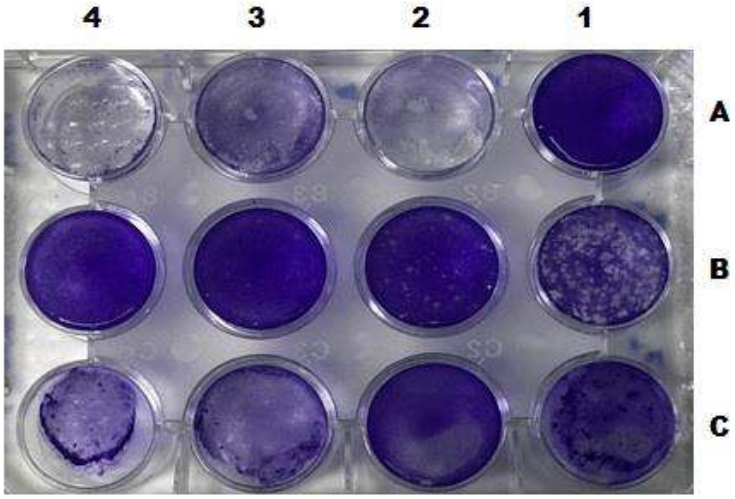

Figure 2: PRNT for Dengue-2 obtained in the virus dilution 10-6.
(A1-cell control, A2-virus control, B4- MAE7, C2- MAE13)

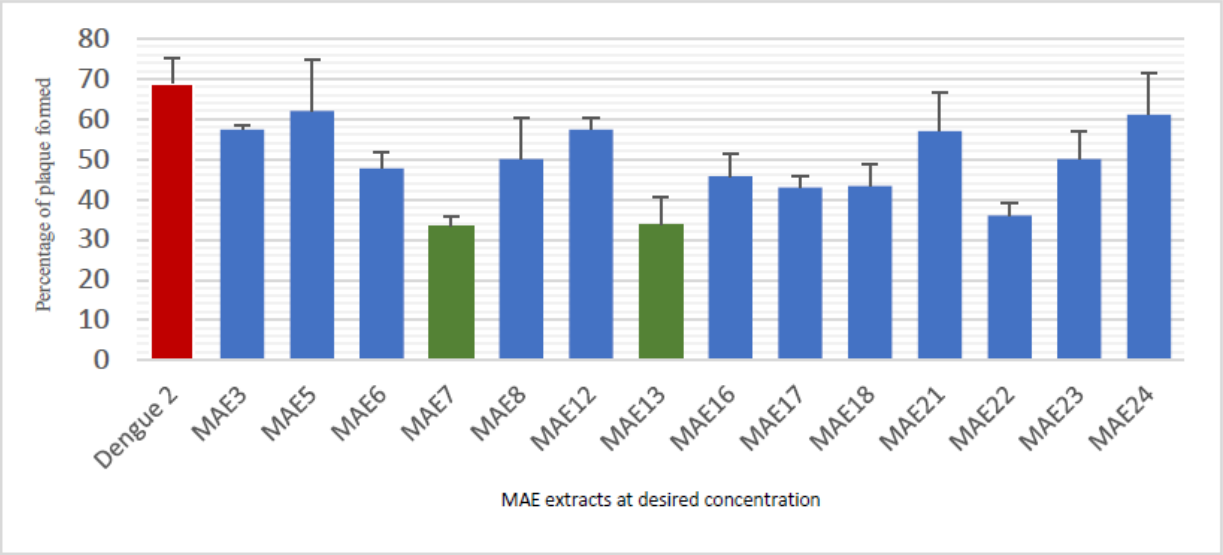

Figure 3: Graph showing PRNT results of 14 MAE extracts determined by PRNT. MAE7 and MAE13 (shown in green) showing entry inhibition against Dengue-2 virus

Figure 4: Schematic representation of MAE 7 strain

Sequencing: PCR amplification of 16s rRNA gene of Actinomycetes was done for the two potential strains showing anti-viral activity of Dengue-2. Base pair sequence of the amplified gene of MAE7 is 1458bp and MAE13 is 1465bp *Streptomyces sp*. Then the sequences were submitted in GenBank and accession numbers were obtained. *MAE7 (MK764920)* is 100% closely related to *Streptomyces coelicolor A3(2) (AL939108)*, 31% similarity to *Streptomyces olivaceus KLBMP 5084 (CP016795)* (Figure 4) and 35% related to *Streptomyces parvulus 2297 (CP015866). Streptomyces sp MAE13 (MK764921)* is 99% homologous to *Streptomyces sp. SR3-82 (LC128346)* (Figure 5).

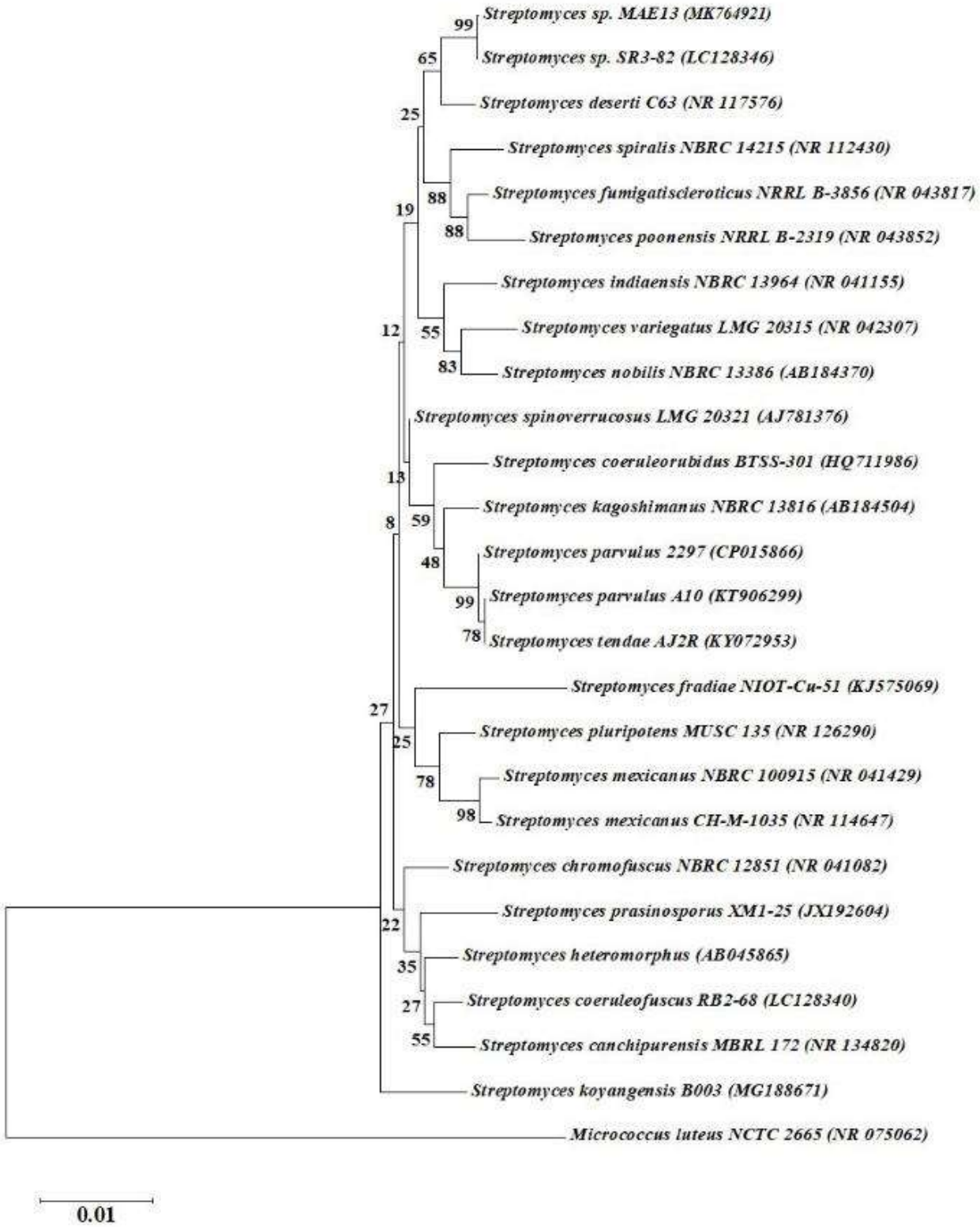

Figure 5: Schematic representation of MAE 13 strain

Discussion

The present need for novel, effective and safe antivirals for the treatment of Dengue is significant due to the absence of clinically approved anti-Dengue medication. Marine actinobacteria are considered to be gold mines with respect to their anti-bacterial and anti-viral properties due to the versatile secondary metabolites produced by them. Previous reports suggest that marine actinobacteria have been demonstrated to produce many different kinds of biochemical compounds which were proven to be effective in inhibiting human pathogenesis by pathogens at different sites. They have the capability of producing pharmacologically significant metabolites which can be exploited for effective therapeutics[8].

Owing to the promising potential of marine actinobacteria, soil samples from Thiruchendur, Pichavaram and Parangipettai in Tamil Nadu, India were collected. 24 actinomycetes colonies were isolated and their range of different colours and morphology was observed. There were several different colours of isolated colonies including creamy white, white, grey, pink, yellow, orange and brown.

The colonies were observed to be powdery or leathery indicating characteristic morphology of actinomycetes. Of the 24 colonies isolated, 3 of them produced reverse side pigment and one produced surface pigment. Aerial mycelia and substrate mycelium were found in all the 24 isolated colonies. 14 colonies were chosen from which extracts using different solvents were prepared. The colonies chosen were named MAE1-14.

The marine actinobacterial extracts prepared from the isolated colonies were used for evaluation of anti- Dengue-2 activity using Plaque reduction neutralization test (PRNT). The PRNT results showed that the crude extracts from MAE7 and MAE13 had potent anti-Dengue-2 activity. 16S rRNA sequencing MAE7 and MAE13 was performed to study their relationship with the actinomycetes family. There are several novel anti-virals having been discovered from marine species such as Clathsterol from Red Sea Sponge which inihibited RT of HIV and Calyceramides obtained from marine sponges which displayed anti-influenza virus activity[9]. This supports our finding of marine actinobacteria producing compounds with anti-viral activity.

Conclusion

The crude extracts obtained from marine actinobacteria MAE7 and MAE13 isolated from mangroves of Tamil Nadu demonstrating anti-Dengue-2 activity. Further separation of individual components of the bioactive extracts can yield information about specific fractions responsible for the inhibitory activity of Dengue-2 and thereby pave way for potential anti-Dengue drug development.

Acknowledgement

The authors would like to thank sincerely to the funding agency, Department of Science and Technology (DST-SERB) (File No: YSS/2015/001854) for the financial support and also to Sathyabama Institute of Science and Technology for providing laboratory facility. Sincere thanks also to the King Institute of Preventive Medicine and Research, Guindy for providing us with virus samples and Dr. R. Balagurunathan, Professor and Head of microbiology department, Periyar University for guiding us in the area of Actinobacteria research.

References

1. Ferreira-de-Lima V. H. and Lima-Camara T.N., Natural vertical transmission of dengue virus in Aedes aegypti and Aedes albopictus: a systematic review, *Parasites & Vectors*, **11(1)**, 77 **(2018)**

2. Gubler D.J. and Roehrig J.T., Arboviruses (Togaviridae and Flaviviridae), *Topley and Wilson's Microbiology and Microbial Infections*, **1**, 579-600 **(1998)**

3. Christian G.J., Subramanian M., Periyasami D., Manickavasakam K., Gunasekaran P., Sivasubramanian S. and Nijavizhi M., Protective Effect of Polyherbal Siddha Formulation-Nilavembu Kudineer against Common Viral Fevers Including Dengue-A Case-Control Approach, *International Journal of Pharmaceutical Sciences and Research*, **6(4)**, 1656 **(2015)**

4. Hong K. et al, Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China, *Marine Drugs*, **7(1)**, 24-44 **(2009)**

5. Radhakrishnan M., Balagurunathan R., Selvakumar N., Doble M. and Kumar V., Bioprospecting of marine derived actinomycetes with special reference to antimycobacterial activity, *IJMS*, **40(3)**, 407-410 **(2011)**

6. ul Hassan S.S. and Shaikh A.L., Marine actinobacteria as a drug treasure house, *Biomedicine & Pharmacotherapy*, **87**, 46-57 **(2017)**

7. Uzair B., Mahmood Z. and Tabassum S., Antiviral activity of natural products extracted from marine organisms, *Bio Impacts*, **1(4)**, 203 **(2011)**

8. Adams B. and Kapan D.D., Man bites mosquito: understanding the contribution of human movement to vector-borne disease dynamics, *PloS One*, **4(8)**, e6763 **(2009)**

9. Basavaraj K.N., Chandrashekhara S., Shamarez A.M., Goudanavar P.S. and Manvi F.V., Isolation and morphological characterization of antibiotic producing actinomycetes, *Tropical Journal of Pharmaceutical Research*, **9(3)**, 231-236 **(2010)**

10. Bandyopadhyay S., Lum L.C. and Kroeger A., Classifying dengue: a review of the difficulties in using the WHO case classification for dengue haemorrhagic fever, *Tropical Medicine & International Health*, **11(8)**, 1238-1255 **(2006)**

11. Chaturvedi U.C., Shrivastava R. and Nagar R., Dengue vaccines: Problems & prospects, *Indian Journal of Medical Research*, **121(5)**, 639 **(2005)**

12. Chaturvedi U.C. and Shrivastava R., Dengue haemorrhagic fever: A global challenge, *Indian Journal of Medical Microbiology*, **22(1)**, 5 **(2004)**

13. Crawford D.L., Lynch J.M., Whipps J.M. and Ousley M.A., Isolation and characterization of actinomycete antagonists of a fungal root pathogen, *Appl. Environ. Microbiol.*, **59(11)**, 3899-3905 **(1993)**

14. Gubler D.J., Nalim S., Tan R., Saipan H. and Saroso J.S., Variation in susceptibility to oral infection with dengue viruses among geographic strains of Aedes aegypti, *The American Journal of Tropical Medicine and Hygiene*, **28(6)**, 1045-1052 **(1979)**

15. Hung S.L., Lee P.L., Chen H.W., Chen L.K., Kao C.L. and King C.C., Analysis of the steps involved in dengue virus entry into host cells, *Virology*, **257(1)**, 156-167 **(1999)**

16. Kabilan L., Balasubramanian S., Keshava S.M., Thenmozhi V., Sekar G., Tewari S.C. and Satyanarayana K., Dengue disease spectrum among infants in the 2001 dengue epidemic in Chennai, Tamil Nadu, India, *Journal of Clinical Microbiology*, **41(8)**, 3919-3921 **(2003)**

17. Libraty D.H., Young P.R., Pickering D., Endy T.P., Kalayanarooj S., Green S. and Rothman A.L., High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever, *The Journal of Infectious Diseases*, **186(8)**, 1165-1168 **(2002)**

18. Pisano M.A., Sommer M.J. and Lopez M.M., Application of pretreatments for the isolation of bioactive actinomycetes from marine sediments, *Applied Microbiology and Biotechnology*, **25(3)**, 285-288 **(1986)**

19. Paramasivan R., Thenmozhi V., Hiriyan J., Dhananjeyan K.J., Tyagi B.K. and Dash A.P., Serological & entomological investigations of an outbreak of dengue fever in certain rural areas of Kanyakumari district, Tamil Nadu, *Indian Journal of Medical Research*, **123(5)**, 697 **(2006)**

20. Park K., Epidemiology of Communicable Diseases, In Park's textbook of Preventive and Social Medicine, M/s Bhanarsidas Bhanot, 19th ed., Jabalpur, India, 206-9 **(2007)**

21. Balagurunathan R., Masilamani S.M. and Kathiresan K., Bioprospecting of mangrove rhizosphere actinomycetes from Pitchavaram with special reference to antibacterial activity, *J Pharm Res*, **3(5)**, 909-911 **(2010)**

22. Manikkam R., Venugopal G., Subramaniam B., Ramasamy B. and Kumar V., Bioactive potential of actinomycetes from less explored ecosystems against Mycobacterium tuberculosis and other nonmycobacterial pathogens, *International Scholarly Research Notices*, **2014**, 1-9 **(2014)**

23. Kala R.R. and Chandrika V., Effect of different media for isolation, growth and maintenance of actinomycetes from mangrove sediments, *IJMS*, **22(4)**, 297-299 **(1993)**

24. Samuel P.P. and Tyagi B.K., Diagnostic methods for detection & isolation of dengue viruses from vector mosquitoes, *Indian Journal of Medical Research*, **123(5)**, 615 **(2006)**

25. Selvameenal L., Radhakrishnan M. and Balagurunathan R., Antibiotic pigment from desert soil actinomycetes; biological activity, purification and chemical screening, *Indian Journal of Pharmaceutical Sciences*, **71(5)**, 499 **(2009)**

26. Winkler G., Maxwell S.E., Ruemmler C. and Stollar V., Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization, *Virology*, **171(1)**, 302-305 **(1989)**

27. Radhakrishnan M., Vijayalakshmi G. and Venugopal Gopikrishnan J.J., Bioactive potential of actinobacteria isolated from certain under-studied regions in India, *Journal of Applied Pharmaceutical Science*, **6(08)**, 151-155 **(2016)**.

(Received 02nd March 2020, accepted 25th April 2020)