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Therapeutically Active Marine Sponge Isolated from Rameshwaram Thoppu Kaadu Theevu against MDR Pathogen *Lactobacillus acidophilus* Isolated from Childhood Caries

S. Vijayalakshmi[1,*], A. Mohankumar[2]

[1]HOD i/c, PG and Research Department of Zoology, Vivekanandha College of Arts and Sciences for Women (Autonomous), Tiruchengode - 637 205, Tamilnadu, India
[2]Assistant Professor, PG and Research Department of Zoology, Chikkanna Govt. Arts College, Tirupur – 641 602, Tamil Nadu, India

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Abstract Dental illness is a foremost health problem upsetting childhood, and this action severely causes sickness which is caused by emerging pathogen *Lactobacillusacidophilus*. So plaque samples were collected from diverse dental clinics around Tirupur Dt. In this study, 20 MDR isolates were isolated and identified by biochemical analysis. Additionally, the decay pathogen *Lactobacillus acidophilus* was assayed against Levofloxacin, Moxifloxacin, Clindamycin, Vancomycin, Ampicillin, Chloramphenicol, Tetracycline and Novobiocin. Among these strains 0%, 0%, 26%, 34%, 22%, 2%, 16%, 16% and 6% were found to exhibit resistance to four groups of antibiotics. Currently, the extension of resistance to contemporary antimicrobials has demand to search for innovative anti-therapeutic agent. Moreover, several studies mostly deal with marine sponge to explore the therapeutic drug and this potential role in the manufacture of metabolites is becoming a driving area for exploring a rich source of novel compounds that are of prospective interest to mankind for diagnosis of tooth decay. Different concentration of sponge 50µg, 100µg, and 150µg were prepared with DMSO, and well diffusion assay was used. The result indicated that the maximum and minimum inhibition of 22 mm and 16 mm was observed against the isolate LAVG07 and LAVG08. This research proved that the marine sponge extract shows the prominent antimicrobial activity against decay causing organism and it may be broadcast to be a successful approach to come to blow promising multi challenging clinical drugs in the future.

Keywords Dental Caries, *Lactobacillus acidophilus*, Antibiotic Susceptibility, Plasmid, Marine Sponge

1. Introduction

Childhood caries is a mainly common dental illness and prevalent human disease which involves the mineralized tissue of the teeth, namely enamel, dentin and cementum. Tooth decay is a multifaceted process surrounded by numerous circuitous factors which is difficult to understand the direct causes and the bacteria take over on tooth surfaces and cause a noticeable decrease of *p*H in the occurrence of a sweetie subtract, and accordingly stimulate tooth decay. More than a few molecular techniques expand for the innovation and quantification of microbes on the

basis of difference in DNA and RNA that are uncomplicated quick in finding and specifically responsive and also poor dental and oral health affects the quality of children's life [1].

Many studies have demonstrated that in dental caries, the pathogenicity of plaque is related to species present. Dental decay could be defined as the soft deposited that form the biofilm complex adhering to the tooth exterior in the oral hole [2]. It is the major problem in dentistry disturbing the human beings. This illness mostly affected the developing countries. Vulnerability to dental caries varies among persons is teeth of certain individuals do not decay in the existence of ecological conditions that normally are linked with decay microorganisms thought to be conscientious for caries infection which includes *Actinomyces* odontolyticus, *Streptococcus* spp., and *Lactobacillus* spp., are the major expansion of decay caries [3].

Presently, the kids getting high level units of penicillin antibiotic every day for acute fever have been accounted to practice decline in the occurrence of tooth decay up to 56% as evaluated with their unprocessed relatives [4]. Resistance derived in exacting place can enhance worldwide, in some victims in a matter of weeks. Moreover, present drugs have been exhibited ineffectively for microorganisms [5]. Development of resistance pathogens among the bacteria is an urgently needing new therapeutic drug to treat childhood decay [6].

Marine diversity is larger than terrestrial diversity. Therefore, it is believed that the marine environment holds the treasure for nature products which may be significant for healthcare today. Presently many active compounds of naval origin with diverse geographically active compounds have been isolated and developed as new bio-drugs [7]. Furthermore, therapeutic bio-medically active compounds recently recommended from associated organism especially sea plants and animals [8]. For the reason that, low content of energetic separate elements in aquatic flora and fauna in addition to the control of bio resource contribute, more and more scientists have been alert on marine microbes as sustainable resources especially in flora and fauna [9].

Frequently, the studies in accord with the production of secondary inhalation are becoming drive area for doing research; particularly sponges that belong to the phyla Porifera have been documented as novel bio-compounds to human beings [10]. Today the production of novel drugs using marine animals is an easy and inexpensive tool to act as better biological compounds [11].

Recurrently, the sponges with source of bio-medically active products are also producing potential sources of unique bioactive metabolites and many of these compounds are valuable for medicinal uses [12]. Moreover, the possibility of associated bacteria present in phylum Porifera is able to construct high biological energetic drugs to cleave the pathogenic microbes. So this research will be a successful approach to move towards dental caries in the future.

2. Materials and Methods

2.1. Sample Collection

Tooth plaques samples were collected from Fen Dental Clinic in Tirupur, Tamilnadu, in the period of March to April 2021, at the age group of six to seventeen years using sterile forceps. In this study, 1% concentration of saline solution was used as transporting medium. After reaching to the laboratory the samples incubated at 37ºC over night for isolation of decay pathogens.

2.2. Isolation and Identification of *Lactobacillus acidophilus* from Dental Plaque Samples

In this research, isolation of *Lactobacillus acidophilus* was done by selective MRS agar. Further the extraction of isolates grown in MRS broth 37°C for overnight. After, the identification of *Lactobacillus acidophilus* was done by Bergeys manual of systematic bacteriology and genotypic characterization.

2.3. Antibiotic Susceptibility Testing by Disc Diffusion Test

In this study, AST was done by the diffusion method. To take 0.1ml overnight cultures of*L. acidophilus* swamped over the prepared Mueller Hinton Agar plates overload were exhausted off and allowed to dry in a temperate incubator for about 15 to 20 min. Antibiotic permeate disc of known concentration were placed in the plates and incubated for 37°C, 24 hours. After incubation, inhibition of zone was deliberate using standard measuring scale.

2.4. Isolation of Plasmid by Boiling Preparation Method

In this study plasmid was extracted by Rapid Boiling Preparation Technique (Holmes and Quigley, 1981; Riggs and McLachlan, 1986) [13, 14].

2.5. Invitro Isolation of Extract from Marine Sponge against *Lactobacillus acidophilus*

2.5.1. Collection of marine sponge

Marine *Halicona fibulata* (Fig. 1) were collected from Gulf of Mannar, Thoppu Kaadu Theevu, and South East Coast of India. Specimen were sealed and preserved in 75% methanol in ice box (-20°C) for further laboratory analysis.

2.5.2. Identification of Sponge

The classification of sponges is quite difficult especially

as it is primarily based on the physical observation of the animals (shape, color and type of spicules). Spicules are an important part of the sponge skeleton because of their diversity in size and shape, constituting recognizable characters for the identification of sponges.

2.5.3. Identification of Sponge

In this study the complete identification of marine sponge *Halicona fibulata* was done by CMFRI (Central Marine Fisheries Research Institute).

2.5.4. Laboratory Analysis

In this research 10 gram of sponge was suspended in 50 ml of DMSO solution in reagent bottle. It was placed in dark room at 24 – 48 hrs. Finally end of the incubation hours the sample was used to analyze the antibacterial activity against test pathogen *Lactobacillus acidophilus.*

2.5.5. Antibacterial activity of sponge against dental pathogen

The antibacterial activity of the sponge extract was performed by using well diffusion method. The Petri plate containing 20 ml of Mueller Hinton agar was spreader with the *Lactobacillus acidophilus.* The sponge extract con. 50µg, 100µg and 150µg were prepared with DMSO. The different concentrations of sponge were screened against above 50% resistant isolates of *Lactobacillus acidophilus.* After solidification process to make well using cork borer then the sponge extract with the different concentration (50µl, 100µl and 150µl) added on each well. After incubation at overnight the inhibition zone was recorded.

3. Results and Discussion

A total 20 isolates of *Lactobacillus acidophilus* were isolated from the samples. The ECC causing predominant pathogen *Lactobacillus acidophilus* strains was confirmed by comparing the results with standard test methodology.

Figure 1. Sample Collection - Marine sponge *Halicona Fibulata*

The antibiotic vulnerability patterns were resolute by using antibiotic disc and using standard guidelines of Kirby – Bauer (1979) disc diffusion method: Ampicillin (10mcg), Chloramphenical (10mcg), Tetracycline (30mcg), Vancomycin (30mcg), Novobiocin (30mcg), Levofloxacin (5mcg), Clindamycin (10mcg) and Moxifloxacin (5mcg).

Totally 8 antibiotic discs were used for this assay, among that Strain No. LAVG07 and LAVG08 showed maximum resistant of 50% and the antibiogram was VA – C - AMP- CD and NV – VA – AMP - CD was recorded. Strain LAVG01 and LAVG02 showed minimum resistant of 12.5% and the antibiogram VA and AMP was recorded.

In this study, as per anti-biogram recorded the resistance was found in Ampicillin (22%), Chloramphenical (2%), Tetracycline (16%), Vancomycin (34%), Novobiocin (6%), Levofloxacin (0%) and Clindamycin (26%), Moxifloxacin (0%). Two strains LAVG07 and LAVG08, showed more than 50% percentage frequency among the 20 isolates of *Lactobacillus acidophilus* (Table 1 & 2).

Table 1. Antimicrobial susceptibility pattern of *Lactobacillus acidophilus*

S. No	Antimicrobial agent	Susceptibility pattern	*Lactobacillus acidophilus.*
1.	Levofloxacin	S R	20 0
2.	Novobiocin	S R	17 3
3.	Moxifloxacin	S R	20 0
4.	Vancomycin	S R	3 17
5.	Tetracycline	S R	20 0
6.	Chloramphenical	S R	19 1
7.	Ampicillin	S R	9 11
8.	Clindamycin	S R	7 13

Table 2. Resistant pattern of *Lactobacillus acidophilus*

S. No	Strain. No	LE	NV	MO	VA	T	C	AMP	CD
1.	LAVG01	S	R	S	R	S	S	R	R
2.	LAVG02	S	R	S	R	S	S	R	R
3.	LAVG03	S	S	S	R	S	R	R	R
4.	LAVG04	S	S	S	R	S	S	S	R
5.	LAVG05	S	S	S	R	S	S	R	S
6.	LAVG06	S	S	S	R	S	S	S	R
7.	LAVG07	S	R	S	R	S	S	R	R
8.	LAVG08	S	S	S	R	S	S	S	R
9.	LAVG09	S	S	S	**S**	S	S	S	S
10.	LAVG10	S	S	S	R	S	S	R	S
11.	LAVG11	S	S	S	**S**	S	S	R	S
12.	LAVG12	S	S	S	**S**	S	S	R	R
13.	LAVG13	S	S	S	R	S	S	S	R
14.	LAVG14	S	S	S	R	S	S	R	R
15.	LAVG15	S	S	S	R	S	S	S	S
16.	LAVG16	S	S	S	R	S	S	R	R
17.	LAVG17	S	S	S	R	S	S	S	S
18.	LAVG18	S	S	S	R	S	S	S	R
19.	LAVG19	S	S	S	R	S	S	R	S
20.	LAVG20	S	S	S	R	S	S	S	R

Multiple Antibiotic Resistance (MAR) index was calculated according to the formula: (Table 3)

$$\text{MAR index for isolates} = \frac{\text{No. of antibiotics resistant to the isolates}}{\text{No. of antibiotics} \times \text{No. of isolates}}$$

The greatest MAR index 0.50 recorded by LAVG07 and LAVG08 and Least MAR index 0.125 was exhibited by LAVG01 and LAVG02 (Table 3).

Table 3. Multiple antibiotic resistant phenotypes among *Lactobacillus acidophilus*

S. No	Strain. No	Resistant pattern
1	LAVG01	VA
2	LAVG02	AMP
3	LAVG03	VA,CD
4	LAVG04	VA,AMP
5	LAVG05	CD,AMP
6	LAVG06	VA,AMP,CD
7	LAVG07	VA,C,AMP,CD
8	LAVG08	NV,VA,AMP,CDs

VA – Vancomycin, AMP – Ampicillin, CD – Clindamycin, C – Chloramphenical, NV – Novobiocin

Strains which showed more than 50% resistance was taken for isolation of plasmid, by boiling preparation method, two fragments were obtained from the strains: LAVG07 and LAVG08 but all the strains were plasmid born *Lactobacillus* spp. 100bp DNA ladder (MEDOX, Chennai), was used to know the molecular weight of the strain, it showed 1000bp and 1500bp.

The medical application of sponges is gaining popularity with an increasing number of sponge based on therapeutics currently in clinical development to cure various diseases. Different concentrations of sponge 50µg, 100µg, and 150µg were prepared with Dimethyl sulphoxide (DMSO), and well diffusion method was used; Different concentrations of sponge were impregnated into well on the seeded Mueller Hinton Agar (MHA) media. After incubation over the 24hrs the zone of inhibition was recorded.

The strains LAVG07 and LAVG08 which showed more than 50% resistant against 8 antibiotics were used to test against sponge.

Among the three concentrations of sponges tested against two strains, the highest zone and lowest zone 22mm and 16mm were recorded in the strain LACVG07 and LACVG08 (Table 4), (Fig. 2).

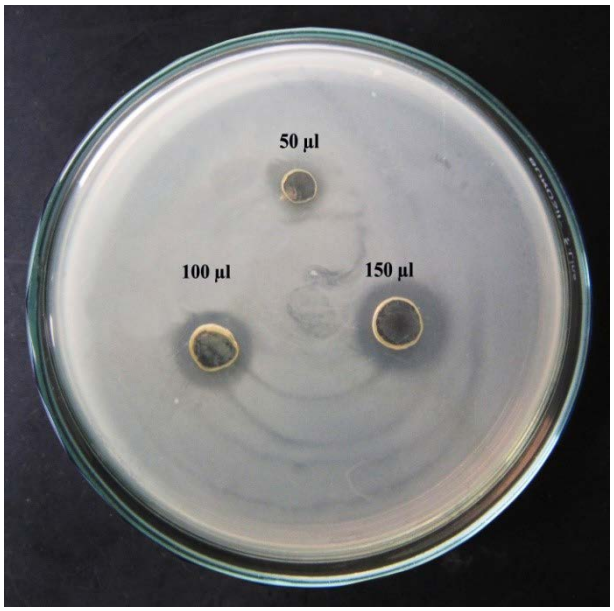

Figure 2. Antimicrobial activity of marine sponge against *Lactobacillus acidophilus*

Table 4. Antibacterial activity of the sponge against on 50% resistant *Lactobacillus acidophilus*

S. No	Strain. No	Sponge concentration (50µg)		
		50µl	100µl	150µl
1	LAVG07	9mm	19mm	22mm
2	LAVG08	10mm	14mm	16mm

Strains which showed more than 50% resistance was taken for isolation of plasmid, by boiling preparation method, two fragments were obtained from the strains: LAVG07 and LAVG08 but all the strains were plasmid born *Lactobacillus* spp. 100bp DNA ladder (MEDOX, Chennai), was used to know the molecular weight of the strain, it showed 1000bp and 1500bp.

The medical application of sponges is gaining popularity with an increasing number of sponge (Fig. 1) based on therapeutics currently in clinical development. At presently therapeutic nature of the sponge extract is ability to degrade the pathogenic microorganisms. In this present study proved the different concentrations of sponge 50µg, 100µg, and 150µg were prepared with Dimethyl sulphoxide (DMSO), and well diffusion method was used; different concentrations of sponge were impregnated into well on the seeded Mueller Hinton Agar (MHA) media. After incubation over the 24hrs the zone of inhibition was recorded.

The strains LAVG07 and LAVG08 which showed more than 50% resistant against 8 antibiotics were used to test against sponge.

Among the three concentrations of sponges tested against two strains, the maximum zone of inhibition (22 mm) was observed against the isolate LAVG07 at 150µl. The minimum zone (16 mm) was recorded in the strain LACVG08 (Table 4), (Fig. 2).

The scientist Ehab Essa Kheadr state that the following antibiotics which includes Ampicillin, Penicillin, Novobiocin, Chloramphenicol, Erythromycin and Nisin A were used against the *Lactobacillus* spp. and were resistant to Vancomycin, Paramycin, and Streptomycin etc. [15]. In their study the researcher Ehab Essa Kheadr among the different antibiotic usage the more resistant observed in Vancomycin, Paramycin, and Streptomycin antibiotics. But in present study the strain *Lactobacillus acidophilus* exhibit 2% of resistance showed by Chloramphenicol, 34% resistance to Vancomycin, 22% resistance showed Ampicillin, 6% resistance showed Novobiocin and Tetracycline showed 16% of resistance.

The present research found that the cariogenic strain of *Lactobacillus acidophilus* was showed resistance against Clindamycin, Tetracycline, and Vancomycin. This result was similarly matched with the investigator [16] conclude that the *Lactobacillus* species intrinsic resistant to commonly exhibiting antibiotics such as Clindamycin, Tetracycline and Vancomycin, also the gene that inducing the resistant characteristics in bacteria that exhibiting resistant mechanism with the gene that is Tet (W, L, M), Erm (B) and Van (A) were also detected in strains of dairy origin of *Lactobacillus.*

A hundred caries samples are from kids of different sexes and age group. The author gradually isolated *S. mutans*: 45.6%, *Lactobacillus* spp.: 41.2% and *S. aureus*: 13.2%. Their studies the researcher conclude that the antibiotic Ceftriaxone, Ciprofloxacin, Perfloxacin and Chloramphenicol are the most successful besides the caries-inducing organisms with a standard vulnerability series of 76.1% to 92.2% [17]. This result mismatched with the present invent found that the isolated 20 *Lactobacillus* spp. from clinical dental samples all are exhibiting 0% resistant and it was fully confirmed the microbe exhibiting susceptible to all eight antibiotics tested. This research proved the isolated cariogenic strains were recorded the dissimilar results against the pathogenic one.

The author studied the probiotic strains that were isolated from fermented milk product. Each isolate was tested against the antimicrobial susceptibilities and also the organism resistant mechanism was detected by extraction of plasmid [18]. The probiotic organism resist against eight antibiotics which includes the Kanamycin, Ampicillin, Vancomycin, Rifampin, Trimethoprim, Bacitracin, Penicillin and Streptomycin, was assessed by disk diffusion technique. Among these strains 20-100% was found to be exhibit an important quantity of resistance to Kanamycin (20%), Trimethoprim (20%), Rifampicin (60%), Kanamycin (70%), Ampicillin (90%) and Penicillin (100%). Further, the curing of plasmid were performed for the seven probiotic isolates and their results indicate that the cured 7 derivatives loss of plasmid except other 2 strains which was partially cured and also effective in nature. This research indicated the Vancomycin resistance is chromosomally encoded and not transmissible because

of intrinsic resistance.

Novelist reports that the isolate of the plasmid DNA *Lactococci* and *Lactobacilli* uses a simple, and rapid plasmid mini-prep procedure [19]. It gives the high yield product and can be done on overnight MRS broth culture in their study. Their results represent that the both plasmids from *Lactobacillus* spp. produce the 70kb size of the plasmid. But in this study, the typical isolation of plasmid DNA was done by boiling preparation method which exhibits the 1000bp and 1500bp.

Marine sponge isolated was collected from Gulf of Mannar. This sponge was identified by morphological characters and taken to antibacterial activity by using Muller Hinton agar.

Recurrently, the sponge *Halicona fibulata* was prospective manufacturer of pharmaceutically active metabolites suggested by researcher [20]. The novelist states that it remains an exciting source of new antidrug with better activity than some antibiotics. The similar results was observed in this study found that sponge could be used as an effective antibacterial agent against dental pathogens. In this revision marine sponge was used as antibacterial activity against the *Lactobacillus* spp. isolates isolated from different dental clinics. Totally 2 isolates LAVG07 and LAVG08 of *Lactobacillus* spp. were tested against different sponge concentration (50µl, 100µl and 150µl). Among the concentrations, the maximum zone of inhibition was rapidly increased from 50µl - 150µl concentration of sponge. Among two isolates tested, LAVG07 showed maximum zone (22 mm) compared than LAVG08 isolate.

4. Conclusions

Tooth decay is a major chronic disease of kids. It affects people of both genders in all age groups. The infection mainly caused by acidogenic *Lactobacillus* is common bacteria in ECC life. At present numerous researches focused on extraction of bio-medically active therapeutic extract from sponge *Halicona fibulata* and it has been recognized as specific compounds to eradicate the pathogenic disease causing organism. Hence, the present study chosen marine sponge and it showed outstanding antibacterial activity against tooth decay pathogen. So the research concluded that the marine sponge contains rich sources of pharmacological dynamic compounds that can potentially be used as the medicine to cure human diseases in the future.

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