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RESEARCH ARTICLE

SEAWEED EXTRACTS IS AN ALTERNATIVE MEDICINE TO CONTROL *VIBRIO* SPP. ISOLATED FROM COMMERCIALLY IMPORTANT FIN FISHES OF GULF OF MANNAR REGION

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ABSTRACT

Disease has always been a great topic of concern in aquaculture industry. Antibiotic sensitivity, bacterial resistance is an issue that needs to be considered while treating the disease. Various steps were taken to find out possible alternative methods to minimize the antibiotics use in the aquaculture industry for disease management. Probiotics, feed additive and natural products such as herbals, sea weeds and good management practices in disease control have proposed for better production without having adverse effect. In the present study, five different *Vibrio* spp. isolated from four different marine fishes *Strongylura* spp (V1, V5), *Sphyraena* spp (V2), *Lethrinus* spp (V3), *Carangoides* spp (V4) are characterized by Random Amplification of Polymorphic DNA (RAPD) profile. Three primers (TW-3, RBA- 5, OPA-2) were used to distinguish *Vibrio* spp. by the RAPD profile analysis method. Among three primers RBA-5 showed fewer banding patterns range from 250 bp to 1000 bp. RAPD profiles were clearly distinctive between the five *Vibrio* isolates (V1-V5). Primer TW-3 and OPA-2 produced greater banding patterns between five *Vibrio* spp. The antibacterial activities of five important seaweeds namely *Sargassum wightii,* were screened against five different *Vibrio* spp isolated from commercially important marine fishes. The zone of inhibition in the crude extract of seaweeds *(Sargassum wightii, Caulerpa peltata*) was varied from against *Vibrio* spp. methanol extract of sea weeds shows maximum zone of inhibition against V3 strain and minimum zone of inhibition against V5 strain. The results clearly indicate that the seaweed compounds effectively inhibit the *Vibrio* spp.

INTRODUCTION

Consumption of fishery products is increasing day-by-day especially in developed countries which lead to higher demand. Disease is one of the major constraints for aquaculture production. Many pathogens like virus, bacteria and fungi are positively correlated with environmental factors especially high or low temperature and high stocking density. The combination of intensive culture methods and environmental changes, have meant that bacterial diseases caused by pathogens such as *Vibrio, Flavobacterium, Salmonella* and parasitic infections are common. In shrimp farming the *Vibrio* spp. are consider to be the most common and significant infection pathogen (Lightner, 1996, Vaseeharan *et al* 2003). Antibacterial drugs play an important role for controlling the bacterial, fungal and parasitic diseases. Antibiotics are used at the lowest possible effective dose for economic reasons, avoid side effects, and lessen environmental impact (Scott, 1993). Thus, fish treated by antibiotics and recovered from an infection may retain low numbers of the pathogen and act as carriers. This infection level can be sufficient to cause a further spread of the disease (Bullock, 1975; Busch, 1975), particularly in stressful situations (Hunter *et al*., 1980). Broad-spectrum antibiotics such as penicillins, chloramphenicol, gentamicin, sulfonamides are used to control the bacterial disease. Application of antibiotics through oral (Gravningen, 1998) and bath administered used for controlling the disease (Gagirgan and Tanrikul, 1998). Antibiotic sensitivity bacterial resistance is an issue that needs to be considered when choosing an appropriate antibiotic. Overdose of antibiotic usage also increases the resistance to the microbes. The evolving resistance of microorganisms to existing antibiotic results need for new antibiotics without harming the animals, humans and environment. Various alternative management steps are taken to control antibiotics use like application of probiotics, feed additive, natural products such as herbals, and good management practices in disease control. Seaweeds have been recognized as possible sources of bioactive compound and its produce a large selection of secondary metabolism characterized by a wide spectrum of biological activity (Chiheb *et al*., 2009). The alternative therapy of infectious disease by the use of antimicrobial drugs has assured limitations due to altering patterns of resistance in pathogens. Since these limitation commands for get better pharmacokinetic properties and which require continued research for the search of new antimicrobial compounds for the development of drugs. Seaweeds are used in traditional remedies in numerous parts of the world. The production of inhibitory substances by seaweed was notes as early as in 1971. Brown algal polyphenols Phlorotannins worked as antioxidants and antibacterial compounds (Kuda *et al*., 2007)

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has essential minerals are provided all dietary requirements to human from marine resources (Bergner,1997). Macro algae have broad range of biological activities such as antibiotics, antiviral, anti-tumor, anti-inflammatory (Scheuer, 1990). Antimicrobial activity of marine algae seaweed for antibacterial activity against fish pathogenic bacteria and could be an alternative to the commonly used antibiotics in aquaculture. Keeping above facts in mind the present study is to evaluate the antimicrobial activity of seaweeds against *Vibrio* spp. isolated from different commercially important fin fishes collected from Gulf of Mannar costal area of Tamilnadu, India.

MATERIALS AND METHODS

Collection of fishes

Four different group of infected (based on external wounds) marine fish such as *Strongylura* spp , *Sphyraena* spp , *Lethrinus* spp, - *Carangoides* spp were collected from Memisal and Kattumavadi landing centers of east coast of India. The infected fish samples were immediately transported to the laboratory under control condition for further of bacteria.

Isolation, identification and molecular characterization of *Vibrio* spp.

The collected fish samples were aseptically excised and the gills, body surface were examined microscopically for the presence of bacteria. Spleen, gill, liver and kidney of the fishes were aseptically removed thiosulphate citrate bile salts sucrose (TCBS) selective media (Hi Media, Mumbai). After incubation for 24 hrs, Predominant bacterial colonies were sub cultured in the nutrient medium supplemented with NaCl to check purity of the isolate followed by biochemical analysis (Laurent, 2000). The selected five pathogenic *Vibrio* spp were cultured individually on luria broth at 37^{o}C for 18 hrs, before inoculation for assay. 100 µl of broth culture, which contain 10^{6}– 10^{8} number of bacteria per ml was added to agar medium (Hi-media) and swabbed on sterile Petri dishes and allowed for solidification. To differentiate bacterial strains molecular characterization RAPD profile was used. All the five *Vibrio* strains were used as target organism against extract.

DNA extraction and RAPD analysis

Collection and preparation of seaweeds

The various group of fresh marine seaweeds such as *Sargassum wightiia* and *Acanthopora spicefera* collected from the intertidal region of Mandapam (Gulf of mannar marine biosphere reserve) along Coast of India. The seaweeds were cleaned thoroughly with seawater to remove foreign materials (sand and epiphytes) and brought to the laboratory in plastic bags containing water to prevent evaporations. Seaweeds were shade dried and chopped in an electronic mixer to make powder.

Extraction

One gram of seaweed powder sample was suspended in 10 ml of methanol for 72 hrs at room temperature. After incubation the samples was centrifuged at 1200 rpm for 15 min. Extract was incubated for overnight for evaporation. The crude extracts were tested for their antibacterial activity against the *Vibrio* spp.

Antibacterial activity of seaweeds

The sterilized petriplates were poured with medium *Vibrio* spp. (10^{6}-10^{8} CFU/ml) were spread on the medium using sterilized " L "rod. The agar surface was allow to dry for five minutes then prepared the crude extract in Whatman No 1 sterile filter paper disc were impregnated. The impregnated dried discs were transferred into the plate with sterile forceps. Plates were stored in incubate after 24 hrs. Diameter of the zones of inhibition was measured in millimeters. Disc without act as a positive control.

Antibiotic sensitivity test

Five different type of *Vibrio* spp isolated from infected fishes were tested for their susceptibility to 10 different commercially available antibiotic disks obtained from Himedia (Bombay, India). Test the susceptibility pattern of antibiotics (ampicillin, ciprofloxacin, erythromycin, amikacin, ofloxacin, gentamicin, oxacillin, vancomycin) by the agar disk diffusion method (Bauer *et al.,* 1966). The standardized bacterial suspension was inoculated on to (Himedia, Mumbai) using sterile cotton swabs, allow drying for 10 min before placing the sensitivity discs. After incubation for 24 hrs, the plates were observed for growth. A clear zone around the disc was evidence for antibiotic resistance and susceptibility of the isolate. Diameter of the zones of inhibition was measured in millimeters. Muller agar plates without antibiotics served as control.

RESULTS

Isolation and identification of bacteria

Vibrio spp. were isolated from the commercially important fish samples were observed based on colony, morphological appearance as entire margin, circular form, convex elevation, consistency and different colony colour and the results are shown in (Fig.1).

RAPD

Genomic DNA from five different *Vibrio* spp was used for RAPD PCR. Three primers (TW-3, RBA -5, OPA-2) were used to distinguish the *Vibrio* spp. strains by the RAPD method. The results shows that each *Vibrio* spp isolated from different fishes produced unique RAPD profile and indicated that isolates are genetically varied. All the primer showed reproducible bands pattern on five *Vibrio* spp. Among three primers RBA- 5 showed band patterns from 250 bp to 1000 bp. Primer TW-3 and OPA -2 produced greater banding patterns between five *Vibrio* spp. **(**A, B, C) RBA- 5 primers produced one bands nearly Primer TW-3 showed long for all the *Vibrio* spp.

Activity of seaweeds

Activity of methanol extract were tested against five different isolates of *Vibrio* spp. by the agar disc diffusion method. *Sargassum wightii* shows inhibitory activity against all the five *Vibrio* spp. However the maximum zone of inhibition

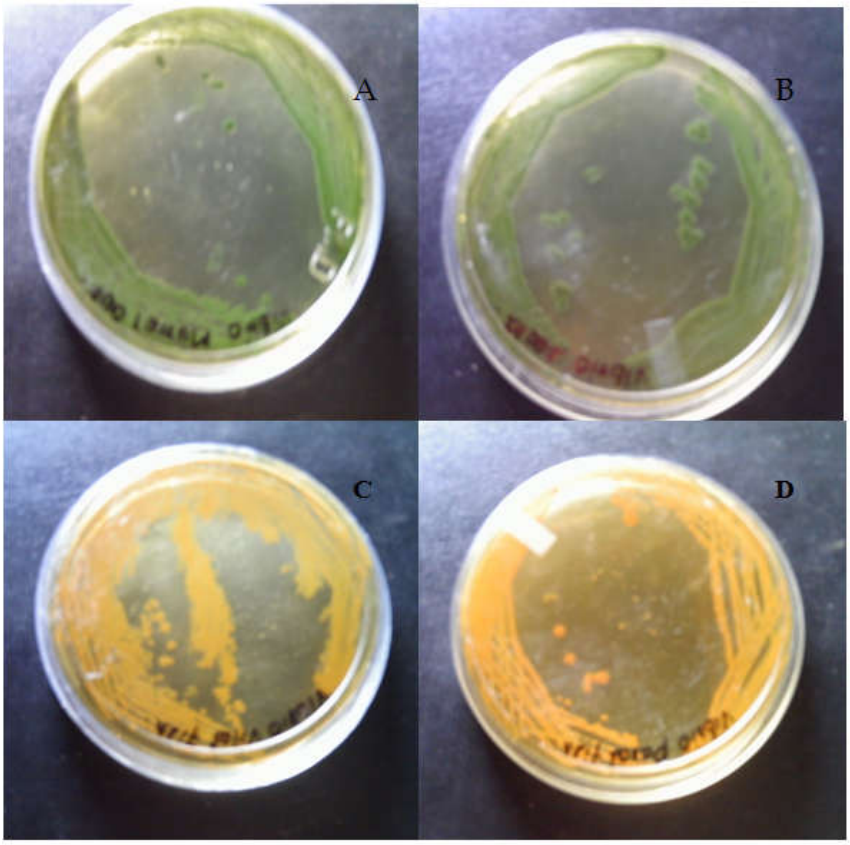

Fig 1. ***Vibrio*** **spp gut sample from fishes,** ***Strongylura*** **spp. (A),** ***Sphyraena*** **spp. (B),** ***Lethrinu*** **spp. (C) and** ***Carangoides*** **spp. (D).**

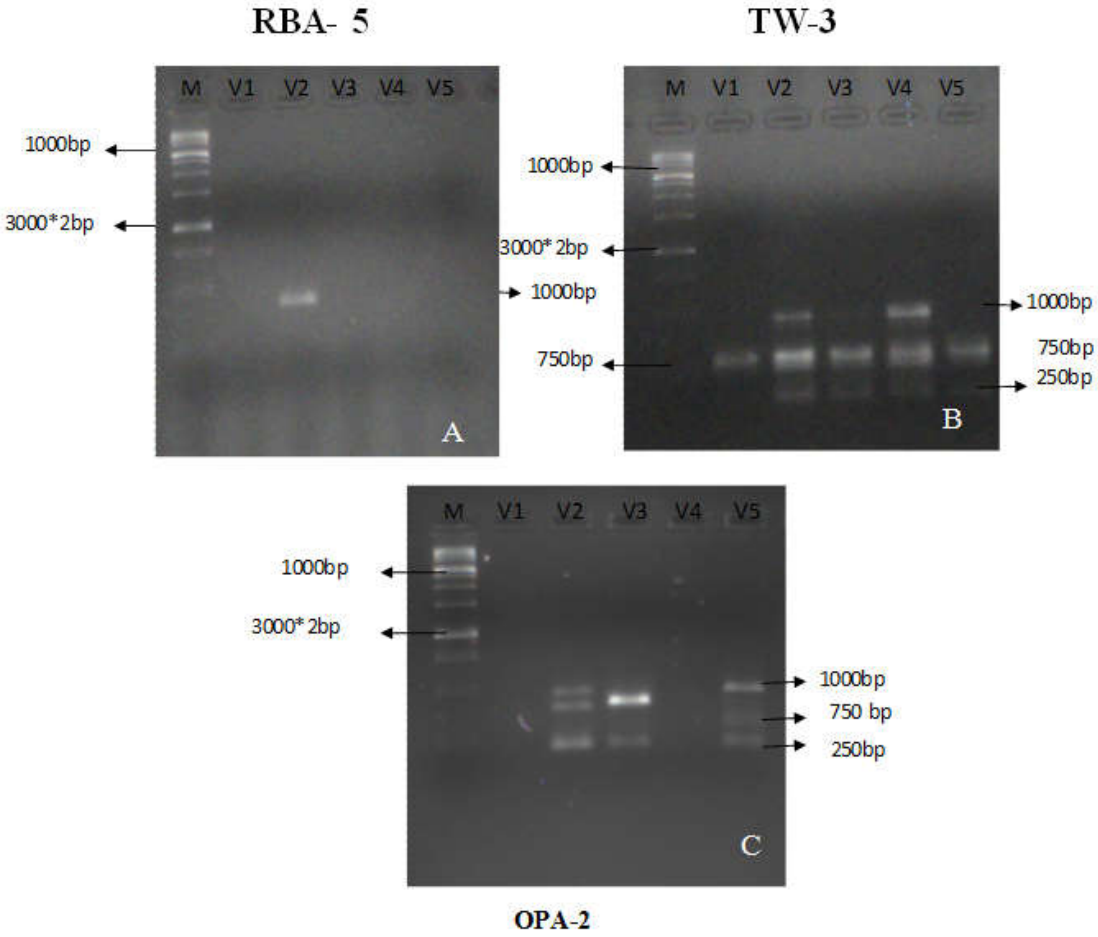

Fig 2. RAPD profile with random pirmer (A- RBA-5), (B- TW-3), (C-OPA-2), (V1-*Strongylura* spp.,V2-*Sphyraena* spp., V3- *Lethrinus* spp., V4- *Carangoides* spp., V5- *Strongylura* spp.)

21 mm was in against V3 and minimum zone of inhibition was V4 (11 mm). *Caulerpa peltata* did not show inhibitory activity against V5 and V4. Did not show inhibitory activity against V4, V5, V2 and V5 respectively. *Vibrio* -V5 shows maximum resistance to the majority of the seaweed extracts.

Antibiotic susceptibility test

The antibiotic susceptibility test against five different isolates of *Vibrio* spp. are summarized. The maximum zone of inhibition was notice (32 mm) in ofloxacin against V2 and minimum susceptibility was observed in gentamicin, amikacin against V1, V3 and V5 respectively. V4 develops the maximum resistance against the maximum antibiotics (Ciprofloxacin, erythromycin, ampicillin, oxacillin and vancomycin). Amikacin shows antibiotic activity against all the five *Vibrios* spp. zone of inhibition varied from 21mm. Oxacillin and vancomycin did not shows activity against all the *Vibrios* and erythromycin shows activity only in V3.

DISCUSSION

The pathogenesis of *Vibrio* spp has been reported in capture fisheries and aquaculture throughout the world (Peggy and Reed *et al*., 1996). Several reports have evaluated the pathogenicity of environmental isolates of *Vibrio harveyi* in aquaculture farms (Harris, 1999; Pizzutto, 1995; Ruangpan *et al*. 1999). During the few decades large amount of money was spent on antibiotics to control fish diseases. However, it gives adverse effect to the environment, human food consumption and antibiotic resistant, in addition to that rejection of container due to antibiotic contaminations.

The present study focuses on effective alternative control measures for *Vibrio* spp. Infection in commercially important fishes by sea weed extracts and activity was compared with commercially available antibiotic to evaluate the effectiveness. The RAPD (Random amplification of polymorphic DNA) method has been widely used in the development of molecular diagnostic techniques for bacteria, because it is a powerful tool that allows comparative analysis of genomes between different isolates of the same species by establishing distinct molecular markers (Akopyanz *et al*. 1992; Mukhopadhyay *et al*. 2001). The result of RAPD profiles obtained using a10-mer Universal random primer for isolated *Vibrio* spp, significant genetic variability among the isolates was confirmed. Three universal random primers (TW-3, RBA -5, OPA-2) showed band ranged from 250 bp -1000 bp. The results shows that each *Vibrio* spp isolated from different fishes produced unique RAPD profile and indicated that isolates are genetically diverse (Fig. 2. A, B, C). In this study we compare the effectiveness of seaweed extract with the antibiotics by evaluating the, sensitive and interrelate pattern of antibiotics such as ciprofloxacin, erythromycin, ampicillin, amikacin, ofloxacin, oxacillin vancomycin.

Antibiotics like oxacillin and vancomycin did not shows activity against all the *Vibrios* and erythromycin show activity against *Vibrio* V3. The result confirms that *Vibrio* spp. exhibits high resistance to the commercial available antibiotics. Among the five species, V4 shows resistance against maximum antibiotics. The results correlate with the earlier studies in India, chlorophenicol, erythromycin, and streptomycin were used to combat the antibiotic resistant Vibrios (Karunasagar *et al.,* 1994), farmers in Thailand tend to use kanamycin and carbenicillin in aquaculture (Nakayama *et al*., 2006). Different types of antibiotics were used for controlling the of *Vibrio* spp. in aquaculture. However application of antibiotics in aquaculture industries varied between the countries for controlling the bacterial diseases for an example, nitrofurantoin, novobiocin, and sulfonamide were applied in shrimp culture to the *Vibrio* treatment in Taiwan, China (Liu *et al*., 1997). In the present study, most of the *Vibrio* spp. isolated from the commercially important fishes showed multiple resistances against the antibiotics. Now a day's antibiotic sensitivity and bacterial resistance is a major issue and that needs to consider. Farmers and researchers are seriously thinking about the alternative innovative measures to control *Vibrio* spp in aquaculture to avoid effect to the human health and environment from the antibiotic usage. Commercial aquaculture farmers are attempt to control *Vibrio* spp by organic based compounds like herbal, plant based products and probiotics. Very few farms are attempt with seaweeds for the control of pathogenic bacteria without knowing the scientific reasons. Seaweeds may be used effectively because of its availability, cost effective and has potentially active against the disease. To support the farmer's activity our attention has been directed towards extracts and biologically active compounds isolated from seaweeds. In the present study *Sargassum wightii* were tested for the antimicrobial activity against the isolated *Vibrio* spp. *Sargassum wightii* shows inhibitory activity against all the five *Vibrio* spp. However the maximum zone of inhibition 21 mm was in *Heliminia floressia* against V3 and minimum zone of inhibition was in V4 (11 mm). *Caulerpa peltata* did not shows inhibitory activity against V5 and V4 did not shows inhibitory activity against V4,V5,V2,V5 respectively. *Vibrio* V 5 shows maximum resistance to the majority of the seaweed extracts (*Acanthopora spicefera*) (Fig. 3).

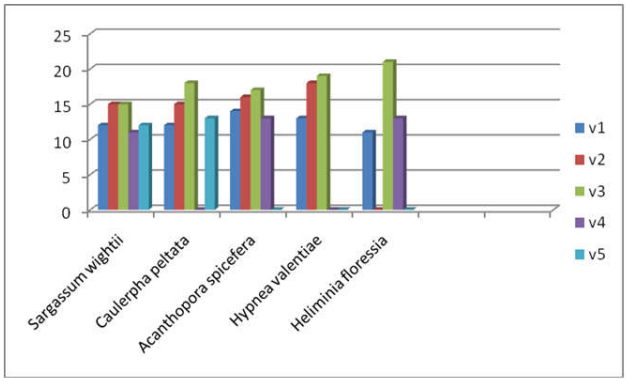

Fig 3. Extract inhibitory activity against *Vibrio spp.* isolated from *Strongylura* spp. (V1, V5), *Sphyraena* spp. (V2), *Lethrinus spp.* (V3) and *Carangoides* spp (V4)

Based on the result of the present study it may be inferred that *Sargassum wightii,* have a potent antimicrobial activity to control bacterial pathogens. Marine algae have received comparatively less bioassay attention. On the contrary, there are a number of seaweeds with economic potential (Critchley *et al*., 1998). Methanol extraction appears more effective, particularly in terms of antimicrobial activity than *n*-hexane and ethyl acetate (Febles *et al.,* 1995). The crude extract with methanol was prepared it was evident from the experience of the previous reports. However, the fact that the use of organic

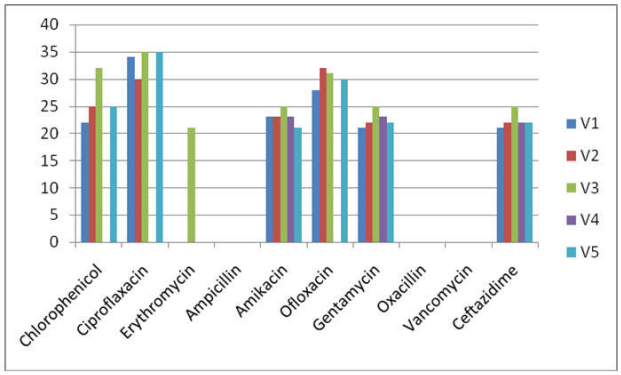

Fig 4. Antibiotic activity against *Vibrio* spp isolated from *Strongyluragu* spp (V1), *Sphyraena* spp (V2) , *Lethrinus nebulosus* (V3), *Carangoides* spp (V4) and *Strongyluragi* spp (V5).

solvents always provides a higher efficiency in extracting antimicrobial activities, as compared to water extraction (Rosell and Srivastava, 1987). Antimicrobial assay of the methanolic extracts of five different seaweeds showed the presence of biologically active compounds. Maximum activity was observed in extraction from the *Sargassum wightii,* the antimicrobial assay of the methanolic extracts of all the five seaweeds tested showed varying degrees of antibacterial activity. The complexity of antimicrobial properties in seaweeds is due to their multiple inhibitory properties. In several cases, different substances have been found in the same seaweed (Olesen *et al*., 1963). Various bacterial species were found to differ in susceptibility to the methanol extract of different seaweeds, with the gram positive organisms being generally more susceptible than the gram negative bacteria (Rao and Parekh, 1981; Pesando and Caram, 1984; Reichelt and Borowitzka, 1984). Macro algae have broad range of biological activities such as antibiotics, antiviral, anti-tumor, anti-inflammatory (Scheuer, 1990). There are research studies on antimicrobial activity of marine algae from Turkey (Haliki *et al*., 2005; Tuney *et al*., 2006, 2007; Karabay-Yavasoglu *et al*., 2007). Results showed that the difference in the activity of different seaweeds showed promising alternative to control pathogenic *Vibrio* spp. from fish samples. In concluded from this study in aquaculture practices the extract usage is one of the alternate approach to control pathogenic *Vibrio* spp. and leads to disease free environment to protect fishes from diseases in eco friendly approach.

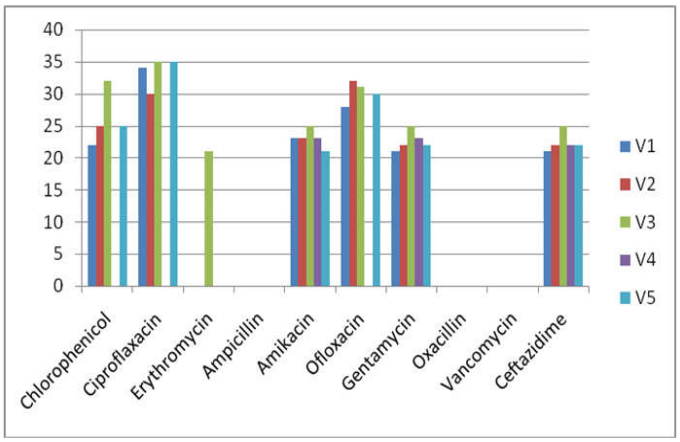

Fig 4. Antibiotic activity against *Vibrio* spp isolated from *Strongyluragu* spp (V1), *Sphyraena* spp (V2) , *Lethrinus nebulosus* (V3), *Carangoides* spp (V4) and *Strongyluragi* spp (V5).

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