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ORIGINAL ARTICLE **OPEN ACCESS**

Inhibitory Potential of Actinomycetes isolated from mangrove *(Avicennia marina)* against selected bacterial species

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ABSTRACT
Mangrove is excellent and underexploited ecosystem with unique microbial diversity for discovery of new and chemically diverse antimicrobial compounds. The main objective of the study was to explore the therapeutically important actinobacterial resources from mangrove soil and gain into the diversity and novelty of cultivable actinobacteria. A total 140 actinomycetes isolates were isolated from the mangrove region of South Gujarat (Surat and Navasari) and were identified by morphological studies. And Actinomycetes isolates were further screened based on the antimicrobial activity. Agar well diffusion method used for antimicrobial activity of the crude extract against four test pathogens Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes and Staphylococcus aureus. The Actinomycetes isolate J-20 was found to be more effective against all four pathogens.
Keywords: Mangroves, Actinomycetes, inhibitory potential, *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes*, *Staphylococcus aureus*

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INTRODUCTION

Currently, microbial diseases mainly antibiotic resistance is increasing by year and they becoming a big risk to health and becoming a global challenge to public health [1, 2]. The development of multi drug resistance due to the extreme use of antibiotic by human and animals (3). However, in this situation new types of drugs are limited and scientists are stimulated to the situation as 'Bad bugs, No drugs' to find novel sources for new drugs (1). In 2017, World Health Organization (WHO) by member states made a request to developed a global priority list (PPL) of multi drug resistant bacteria to help the development of natural sources with different mechanism of action effective as antibiotic treatments. In 2017, World Health Organization (WHO) made a request to the member states to develop a global priority list (PPL) of multi drug resistant bacteria in order to help in the development of effective antibiotics (1, 4). For search of novel drug and bioactive compound ministry of Earth Science, government of India has also focused on the studies related to 'Drugs from the sea' (5). Normally microorganisms produce bioactive secondary metabolites that are important for their defense mechanism (3). Of such organisms, actinomycetes are the strongest producers of secondary metabolites that play a major role in agricultural, pharmaceutical and industrial applications (6). Actinomycetes are filamentous Gram-positive bacteria with high G+C content (2). It was reported that more than 70% of the total naturally obtained antibiotics are isolated from, mainly two genera *Streptomyces* and *Micromonospora* of actinomycetes (7). After decades of isolating antagonistic substances or compounds of therapeutic importance from actinomycetes from soil environment it is increasingly becoming difficult to discover novel secondary compounds from actinomycetes. Now a days, researchers are more focused on extreme habitats such as, desert (8-10), marine (11-13), and mangroves (2,14-19) for prospecting of novel compounds. Since microbes present in such environments have developed a unique defense mechanism against stress and evolved adaptive biosynthetic pathways for synthesizing new secondary metabolites (1). Mangroves are the world's one of the most productive ecosystems in spite of extreme salinity and moisture (20) Mangrove's ecosystem is also unique as it is mainly situated at the interphase between the terrestrial and the marine ecosystems. They are known to harbor rich and diverse group of microbes that produce novel metabolites (2). Mangroves are the important coastal ecosystems that support wide range of animals, plants and large group of microbes. This diversity, especially microbial diversity is the main key for the ecosystem to maintain food chain and the ecological balance (21) Mangrove ecosystems are rich in nitrogen, sulfur and

other nutrients that provide rich source for growth (22). Mangrove ecosystems have the potential to become a new source for identification of diverse and novel actinomycetes (23). Mangrove environments were reported as the novel source of actinomycetes (6). Mangroves in India are distributed highly and rich in biodiversity. They cover an area of about 4639 sq/km that include mangrove rich states include west Bengal, Gujarat, Orissa, Andhra Pradesh, Tamil Nadu, Andaman and Nicobar Island, Kerala, Goa and Maharashtra (24). In the western region Gujarat has the largest area of mangrove and are divided into mainly five major area which include the Gulf of Kutch, the Gulf of Khambhat, the Saurashtra Coast, the South Gujarat Coast and the Rann of Kutch (25). Further, Gulf of Kutch, Saurashtra and South Gujarat (including areas of Dumas, Dandi and Umbhrat) have 15.6%, 0.3% and 12.6% of the total mangrove cover of the Gujarat state (25) Studies on actinomycetes from mangroves of Gujarat have largely focused on the Gulf of Kutch and studies on actinomycetes of south Gujarat mangroves are scanty. Researchers have reported that most of the actinomycetes isolates from mangrove environment showed antibacterial activity against various tested pathogens (2, 14-18) and actinomycetes isolates were also shown to have promising broad spectrum antimicrobial activity (26). The present study aimed to evaluate actinomycete isolates from different mangrove s located in South Gujarat (Surat and Navsari) region of Gujarat, India for their antibacterial activity against human pathogens.

MATERIAL AND METHODS

Isolates

This is study is a part of a large experimental study. In total 127 isolates were tested for their efficacy against the tested microorganisms. These isolates were isolated from three different actinomycetes media and from various dilutions as mentioned in Desai & Nallanchakravarthula (2021) from mangrove tree species *Avicennia marina* of south Gujarat region, India.

Test Microorganisms

The test microorganisms used in the present study were obtained from microbial type culture collection (MTCC) Chandigarh, India. The isolates were tested against members that are known to act as human pathogens *Escherichia coli* (MTCC-1687), *Klebsiella pneumoniae* (MTCC-4031), *Enterobacter aerogenes* (MTCC-111) and *Staphylococcus aureus* (MTCC-96).

Screening of actinomycetes for antibacterial activities

The antibacterial screening was performed on Mueller Hinton Agar (MHA) by cross streak method (4), (27). In primary screening all the 127 isolates were tested against *Escherichia coli* (MTCC-1687). MHA plates were inoculated with actinomycetes isolates by a single streak in center of the plate and incubate at 28°C for 7 days and then plates were inoculated with test pathogens by perpendicular single streak on to the actinomycetes strain and incubated for overnight at 37°C. Then the inhibition of the test pathogen was observed by absence of growth of test pathogen around actinomycetes strain and results were recorded (28). The results were presented as in the following manner '+ = weak activity', '+ + = moderate activity', and '+ + + = good activity' (23).

Screening by well diffusion method

The overnight grown test microorganism was evenly spread (200µl) using a spreader on to the MHA plate and dried for 30 min in aseptic conditions. The isolates were inoculated in starch casein broth and kept on a shaker. After 96 hours, the culture broth was centrifuged at 10,000 rpm for 10 min and the supernatant was used for secondary screening (28). The well was prepared in agar plates using a sterile cork borer (6 mm diameter). A 100 µl volume of supernatant from each isolate was inoculated into the well carefully and allowed for 30 minutes for diffusion. Later the plates were incubated for 24 h at 37°C and the zone of inhibition around the well was measured. Starch casein broth was used as control and streptomycin used as reference control (28)(29). Of these the isolates that showed inhibition of more than five millimeters was considered for screening against the other test isolates.

Statistical analysis

All the analysis was done in triplicates and the statistical significance ($p \leq 0.05$) was analyzed by PAST (ver 4.06b). NMDS was also drawn by PAST and the 2D stress value (≤ 0.1).

Results and Discussion

Studies have been published on the potential antagonistic abilities of actinomycetes isolated from mangrove environments (30–32). Studies have been reported that *Avicennia marina* (mangrove plant species) was shown to harbor antagonistic actinomycetes (33,34), but very few studies have reported from the mangroves of South Gujarat region. In the initial screening, the isolates from 'Junagam' sample (J isolates) were shown to have highest number of organisms of antagonistic potential in comparison with others, indicating there was an effect of site of the isolates with antagonistic potential (Table 1). Similar result was also shown by Mitra et al., (35) and proposed that such effect could be attributed to the large number sea tides that detected in such sites, in comparison with the sites that gave isolates with low

antagonistic potential. In the primary screening against *E.coli* (cross streak and well difussion method) out of 127 morphologically different isolates 21 (16%) showed promising. Similarly, Zainal Abidin et al., (6) showed that only 12% of the total actinomycete isolates (140 isolates) that were isolated from mangrove sediment samples showed inhibition against *E coli*. Out of the 63 isolates only 11% (7 isolates) were shown to be of antagonistic potential against *E coli* (32). In our study the tested 21 isolates showed lowest average ZOI in comparison with the other test microorganisms. In the above studies, isolates that were isolated from mangrove environment showed less inhibitory potential against *E coli* in comparison with the other tested organisms, for this reason in the present study we first screened against *E coli*. Thereafter, in the well diffussion method (Figure 1) these 21 isolates also showed more than 6 mm inhibition (6.7±0.4 to 22.3±0.5) against *E.coli* and were selected for the secondary screening (others showed less than 5mm). The isolates 'VI-3' (20.3±0.5), 'VA-15' (20.4±0.5) and 'J-20' (22.3±0.5) showed highest amount of inhibition in both the methods against *E coli*. These 21 isolates were further tested against other test organisms *Staphylococcus aureus*, *Enterobacter* and *Klebsiella pneumoniae* by cross streak and well diffusion methods. In a previous study, the actinomycetes isolates that showed inhibition against two test microorganisms were selected for the secondary screening (36). There was an effect of isolate and as well as the test organism on the inhibition potential. For example, 'D11' isolate was able to moderately inhibit *E. coli*, *Enterococcus* Spp. and *Klebsiella pneumoniae* but weakly inhibited *S. aureus*. But whereas, Isolate 'V8' there was a weak inhibition of *E. coli* but moderate inhibition of *S. aureus* and *Enterococcus* Spp. and *K pneumoniae*. Similar results were reported by other studies (31, 36). During the secondary screening of the 21 isolates against the four tested bacteria, overall ZOI was recorded with a minimum of 6 mm and a maximum of 24 mm. The overall average ZOI was recorded for all the tested isolates and 'J20' (23 mm) and the minimum was recorded by 'VM6' (8 mm). Similarly, with the organisms tested *K. pneumoniae* gave the highest amount of ZOI (13 mm), followed by both *Enterobacter* Spp. and *S aureus* (12mm) and lowest was in *E coli* (11 mm). Individually, the maximum ZOI was recorded with the 'J20' isolate against the *Enterobacter* Spp. (24 mm) and minimum was recorded in the 'VM6' (6 mm) with *K. pneumoniae*. Based upon ZOI, the NMDS analysis (Figure 2) showed the top three isolates 'J20', 'VA15' and 'VI3' have formed a clear grouping compared with the other isolates, when tested for the tested organisms *S aureus* and *E coli* formed a clear grouping. There was no clear picture for Gram positive and negative organisms. Compared with control (Streptomycin) there was a statistically significant increase in the ZOI by 'J20' isolate in all the tested organisms except *Enterobacter* Spp. (25% in *S aureus* (p=0.003), 10% in *E coli* (p=0.018), 21% in *K. pneumoniae* (p=0.015) and 5% in *Enterobacter* Spp. (p=0.057)). Isolate 'VA15' (25% in *S aureus* (p=0.003) and 22% in *K. pneumoniae* (p=0.008)) and 'VI3' (16% in *S aureus* (p=0.009)) gave statistically significant increased ZOI with respect to control results. Remaining all other isolates either gave more or less ZOI than streptomycin control. With respect to *Enterobacter* test organism, there was no isolate that gave a statistically significant difference but gave more or equal ZOI to the streptomycin control. Studies have been scanty and reported on the inhibitory potential comparison of a control antibiotic (especially streptomycin, that is known to be produced from an actinomycete) with the antagonistic potential of the isolated actinomycetes, but studies have been published showing that antibiotics were used as a control (1, 37). Native microbial communities that are known to have antagonistic potential such as actinomycetes have to adapt to the harsh environments such as mangroves, thereby improving their chances for the production of novel metabolites and mechanisms to counter various threats. Hong et al., (22) has suggested that the isolates from the mangrove environments that are known for their salinity and tidal gradients may be the future source for discovering novel actinomycetes and as well as novel bioactives.

CONCLUSION

Due to the increasing antibiotic resistance and frequent disease outbreaks. Mangroves are less explored regions for novel antimicrobials. The present study details about the antagonistic potential of actinomycetes isolated from *Avicennia marina* (a mangrove tree species). The present study indicates that the zone of inhibition is affected both the test isolate and actinomycete isolate that was tested for antagonism potential. 'J20' was found to show a significant inhibition potential in comparison with streptomycin (control in this study). These isolates can be used for bioprospecting of antimicrobials that can be used against drug resistant organisms or pathogenic members of these groups. But such studies should also be further validated by pharmacological studies.

Table: 1 Antagonistic potential of isolates against various test organisms (cross streak method).

S.no	Isolate name	*Escherichia coli*	*Staphylococcus aureus*	*Enterococcus Spp.*	*Klebsiella pneumoniae*
1	D 11	++	+	++	++
2	D 14	++	++	++	++
3	D 21	++	+	+	+
4	D 22	++	++	+	+
5	J 2	++	++	+	+
6	J 8	++	+	++	++
7	J 12	+	+	+	+
8	J 14	+	+	+	+
9	J 16	++	+	+	+
10	J 17	++	+	++	++
11	J 18	+	+	+	+
12	J 20	+++	+++	+++	+++
13	VA 8	+	++	++	++
14	VA 13	++	+	+	+
15	VA 15	+++	+++	+++	+++
16	VI 2	+	+	+	+
17	VI 3	+++	+++	+++	+++
18	VM 1	++	++	++	++
19	VM 5	+	+	+	+
20	VM 6	+	+	+	+
21	VM 12	++	+	+	+

+ = weak activity; + + = moderate activity; and + + + = good activity

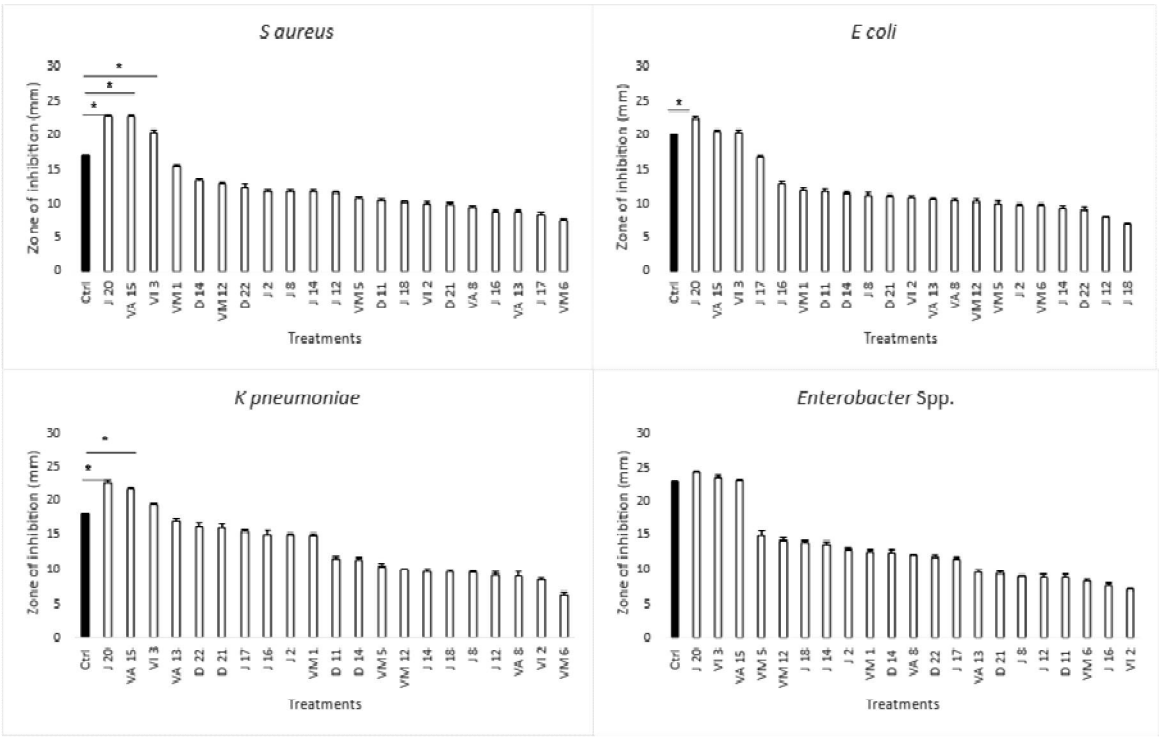

Figure 1. Zone of inhibition (mm) observed with four test organisms (*Staphylococcus aureus*, *E coli*, *Enterobacter* Spp. and *Klebsiella pneumoniae*) by actinomycetes isolates ('*' represents statistically significant ($p \leq 0.05$) difference between control (streptomycin) with actinomycete isolates zone of inhibition).

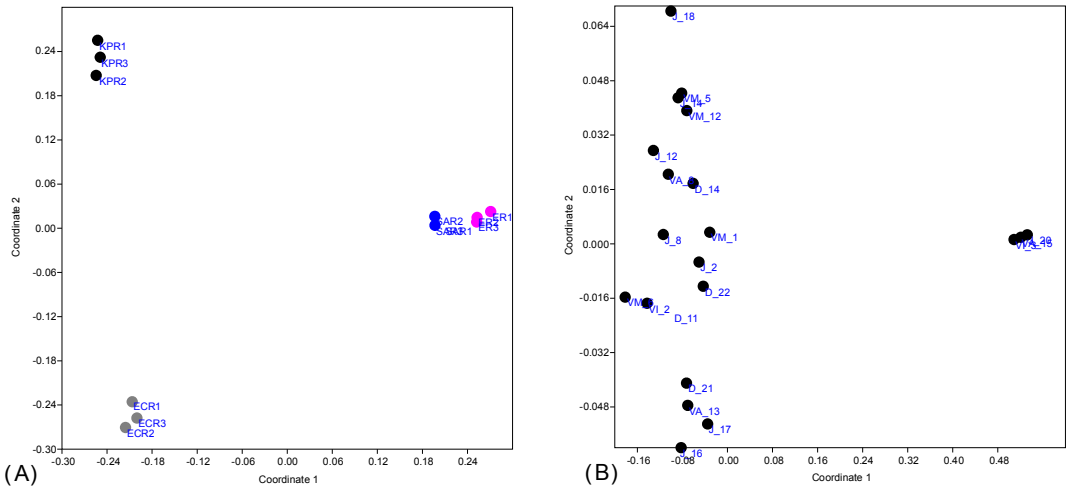

Figure 2. Non-Metric dimensional scaling (NMDS). (A) Test organisms ('R' replicate) (B) Twenty-one isolates that were tested for inhibition. *Escherichia coli* (EC, gray), *Klebsiella pneumoniae* (KP, black), *Enterobacter aerogenes* (E, pink), *Staphylococcus aureus* (SA, blue)

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CONFLICT OF INTEREST
We declare no conflict of interest.

AUTHOR'S CONTRIBUTION
Aneree Desai has performed the work, did the analysis and written the manuscript. Srivathsa Nallanchakravarthula designed the study, did the analysis and written the manuscript.

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