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Screening and Isolation of Associated Bioactive Microorganisms from *Fasciospongia cavernosa* from of Visakhapatnam Coast, Bay of Bengal

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Abstract: Nature, especially the marine environment, provides the most effective drugs used in human therapy. Among the metazoans, the marine sponges produce the most potent and highly selective bioactive secondary metabolites. These animals (or their associated symbiotic microorganisms) synthesize secondary metabolites whose activity and selectivity has developed during their long evolutionary history. During the course of exploitation of these resources two marine sponges, *Fasciospongia cavernosa doc var.brown* (dark brown) *Fasciospongia cavernosa doc var.yellow (yellow)* collected from the visakhapatnam coast of Bay of Bengal were investigated in order to assess the potential of these microorganisms for the production of antimicrobial compounds. The aqueous and organic extracts of both the sponges showed broad spectrum antibiotic activity. In this study a total of 178 microorganisms were isolated from different parts of two sponges and most of them from middle part of the sponge. The isolates were investigated in order to assess the potential of these microorganisms for the production of antimicrobial compounds. Testing for antimicrobial activities were performed against Gram-positive (*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus*) Gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris*), fungi (*Candida albicans, Aspergillus niger*) and 10 pathogenic organisms. Resulting mean diameter of inhibition zones revealed isolates B4 & B6 were the most potent of all the isolates. The present study has revealed the presence of high numbers of diverse culturable microorganisms associated with the marine sponges from Visakhapatnam Coast of Bay of Bengal as well as their potential to produce bioactive metabolites**.**

Keywords: Marine sponges, antimicrobial activity, bioactive metabolites.

Introduction

Marine sponges (Porifera) are primitive metazoans. Sponge species are predominantly marine and sessile. They produce a plethora of compounds that protect them from

predators and/or possibility from being infected and fouled by other marine organism[1]. Marine sponges are considered to be true "chemical factories" producing hundreds of unique chemical compounds, many of which have been isolated and their structure determined[2]. Despite the popular and somewhat overexploited statement that marine sponges are an important source of new bioactive compounds that may be used in various biomedical applications, so far only a few have found such an application[3]. Nevertheless, a large number of sponge secondary metabolites show interesting biological activities, for example calyculins from Discodermia calyx[4], discodermolide from D. dissoluta[5], latrunculins from Latrunculia magnifica[6,7,8,9], and spongistatins from Spongia sp. and Spirastrella sp.[10]. They are cytotoxic, inhibit cell proliferation and could be used as chemotherapeutics. These compounds differ structurally and act on different cytoskeletal elements, but have similar antiprolific and antitumoral activities. The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals, for development of new therapeutic agents[11] and the streptomyces species produce about 75% of commercially and medically useful antibiotics[12]. It is perhaps not surprising that novel marine bacteria are providing to be such a valuable source of new bioactive compounds[13,14] as bacteria systematic is providing a taxonomic road map to genes hence products, including the discovery of first-in-class drug candidates[15,16,17,18].

Cultivation of sponge associated microorganisms that produce bioactive compounds is the most direct method for large scale production of these chemicals[19]. By isolating and cultivating these metabolite producing microorganisms on artificial media we can obviate the need for large scale harvesting of natural sponge populations, with its environmentally and financially negative implications. The success of efforts to isolate sponge associated microorganisms that produce bioactive compounds is dependent upon a number of factors. Most significantly, the majority of environmental microorganisms, including those in sponges, have proven resistant to cultivation by standard techniques.

The main aim of this present study was isolation of marine sponge associated microorganisms from Visakhapatnam Coast of Bay of Bengal and to screen for their antagonistic activity against gram positive, gram negative bacteria, fungi, yeast and pathogenic microorganisms. The present study is the first of its kind which focuses on antimicrobial compounds from culturable microrganisms associated with two sponges viz. *Fasciospongia cavernosa doc var.brown* and *Fasciospongia cavernosa doc var. yellow* near the Visakhapatnam coast of Bay of Bengal.

Materials and Methods

Sponge Collection

The sponges were collected by SCUBA diving at depths of 3–20 m in the Bay of Bengal near Visakhapatnam coast (GPS: 24˚21.432 N; 28˚72.725 E) of Andhra Pradesh, INDIA. The sponge samples soon after collection was transferred to a sterile polyethylene bag and transported under frozen condition to the laboratory for the isolation of associated microbes. On reaching the laboratory, the invertebrate was thawed and cut aseptically into small pieces (2 × 2 cm) using a sterile scalpel at upper , middle and lower parts of the sponges. The pieces were freed from adhering particles by vortexing twice for 20 seconds with 2 mL of sterile seawater. The seawater was decanted and replaced with methanol, which was once again replaced with sterile seawater with continued vortexing between washings. Finally, samples in sterile seawater were homogenized using sterilized mortar and pestle in a Laminar flow hood. The remaining sponge samples were used to check the biological activities of the sponges.

Sample preparation

The remaining sponge samples were lyophilized and dried weight was determined. The total material was divided into two parts; one part for aqueous extraction, the other subjected to extraction with organic solvents. The total mass of freeze-dried sponge samples was within the range from 0.29 g to 15.5 g.

Aqueous extraction

One half of total lyophilized mass of each sponge specimen was homogenized, dissolved in 10 mL of deionized water and extracted for 12 hours with constant shaking (400 rpm at 4 °C) followed by centrifugation (15,000 rpm at 4 °C). Supernatants were removed and was stored in aliquots of 1 mL at -20 °C. The dry weight of each sample was determined using 500 µL of each sample which was placed for 30 min into an oven and dried at 120 °C. The dry weight was expressed in mg/mL. The protein concentration of the extracts were estimated using Lowrys method[20].

Organic solvent extraction

One half of each total lyophilized sponge body mass was macerated and divided into three parts which were placed into three labeled tubes (A, acetone; B, butanol; M, methanol, all solvents were from Merck, INDIA). To the each tube, the solvent was added in a way that its volume was about 1 cm above the sample. Tubes were sealed with metal stoppers and parafilm and were shaken overnight at 37 °C. The extracts were filtered and the remaining material was subjected to repeated extraction for 3 hours at 37 °C with constant shaking. Both filtrates were combined and put into Erlenmayer flasks. The solvents were evaporated, and each of the resulting supernatants resuspended in 2 ml of 96% ethanol (Merck, INDIA).

Isolation of Associated Microbes:

The homogenate was serially diluted up to 10−3 dilutions and then spread plated on Zobell Marine Agar (ZMA) plates which contained cycloheximide (100 µg/mL), as an antifungal agent for the isolation of bacteria. The plates were incubated at 37°C till visual growth of culture was observed. Single bacterial colonies were isolated on the basis of distinct colony morphologies from the Zobell Marine Agar (ZMA) plates. Actinomycete Isolation Agar (AIA), Marine Agar (MA) and Glycerol Asparagine Agar (GAA) were used for the isolation of actinobacteria. All media were supplemented with 0.2 µm pore size filtered cycloheximide (100 µg/mL), nystatin (25 µg/mL) and nalidixic acid (25 µg/mL) to facilitate the isolation of slow-growing actinobacteria. Cycloheximide and nystatin inhibit fungal growth, while nalidixic acid inhibits many fast-growing Gram-negative bacteria. Czapek –dox agar, Potato dextrose agar (PDA) & Yeast peptone dextrose agar (YPDA) supplemented with Rifampicin (25 µg/mL) is used to isolate fungi and yeast respectively. All media contained Difco Bacto agar (18 g/L) and were prepared in 1 L artificial sea water (NaCl 234.7 g,MgCl2.6 H2O 106.4 g, Na2SO4 39.2 g, CaCl2 11.0 g, NaHCO3 1.92 g, KCl 6.64 g, KBr 0.96 g, H3BO3 0.26 g, SrCl2 0,24 g, NaF 0.03 g and ddH2O to 10.0 L). The inoculated plates were incubated at 30 °C for 6–8 weeks. Colonies were selected on the basis of uniqueness relative to other plates and ease to select single colonies. Distinct colony morphotypes were picked and re-streaked until visually free of contaminants. Isolates were maintained on ZMA, AIA, PDA & YPDA agar slants at 4 °C until use.

Antibacterial activity assay for sponge extracts

Antibacterial activity was tested by means of a standard agar plate diffusion assay. The Gram positive *Bacillus subtilis* and the Gram negative *Escherichia coli* bacterial strains (obtained from the microbial type culture collection, Chandigarh, MTCC) were

used. Precultured bacteria (grown in Luria broth media, Himedia) were used for the inoculation of Luria broth agar plates in a final concentration of 5×10^5 cells/L. Four holes (1 cm in diameter) were made into each agar plate and filled with 100 µL of unheated or heated samples in the case of aqueous extracts. In the case of organic extracts to the each hole 100 µL of ethanol-dissolved acetone, butanol or methanol samples were added. The fourth hole was used for the control and was filled with ethanol. The inhibition zone for each sample was determined after the overnight incubation of plates at 37°C. The minimal inhibitory concentrations (MIC) were determined for each extract and expressed as ng/mL.

Antimicrobial activity of associate microbes

Primary screening: The antimicrobial activity was determined by perpendicular streak method on nutrient agar (NA) by in vitro screening of isolates for antagonism. NA plates were prepared and inoculated with isolates by a single streak of inoculum in the center of the petridish. After 24 hours of incubation at 37^0C the plates were seeded with test organisms by a single streak at a 90° angle to isolates. The microbial interactions were analyzed by the determination of the size of the inhibition zone.

Secondary screening: The promising isolates from primary screening were subjected to secondary screening by submerged fermentation. Fresh and pure culture of each strain from the primary screening will be inoculated in broth and incubated at different temperatures accordingly on a rotary shaker at 120rpm for 24-72 hrs. Growth of the organism in the flask was confirmed by the visible pellets, clumps or aggregates and turbidity in the broth. Contents of flasks will be centrifuged for 15 minutes at 7000rpm to separate the pellet and the supernant. Pellets and supernatants were prepared on the day of use. Each resultant supernatant was decanted on to a sterilized tube. The supernatant will be used for the determination of antimicrobial activity against the standard test organisms. The antimicrobial activity was determined by agar well method[21] in NA plates. 50µl of supernatants were then loaded into their respective wells. The plates were sealed and incubated at 37^0C for 24 hours (for actinomycetes and fungi at room temperature). Five millimeters was subtracted from the obtained diameters of complete inhibition. The isolates exhibiting zones of inhibition against any test bacteria were chosen. Triplicates of each were made, and the diameters of the inhibition zones were again measured.

Results & Discussion

In the course of Digging oceans for novel drugs two marine sponges were collected from the Visakhapatnam Coast of Bay of Bengal (GPS: 24˚21.432 N; 28˚72.725 E) and they were identified as *Fasciospongia cavernosa doc var. brown* (dark brown) *Fasciospongia cavernosa doc var. yellow (yellow)* (**Fig 1**). The diversity and biological activity of sponges and bioactive metabolite production capability of sponge associated microorganisms were assayed. As shown in **Table 1** a total of 91 microorgamisms from *Fasciospongia cavernosa doc var. brown* and 87 microorganisms from *Fasciospongia cavernosa doc var. yellow* were isolated. Most of the microorganisms were associated in the middle part of the sponge which may be due to escape from the environmental and biological hurdles. The results indiacted that the collected sponges were higly diverse in microbial association. There are more number of bacteria were isolated from both the sponges compared to fungi, yeast and actinomycetes. The isolates were designated according to the sponge name and variety, the position in the sponge from where it is isolated, type of the isolate like bacteria, fungi, yeast, actinomycetes and the number of strain in each type (**Table 2**). For example ScbmB-4 is a fourth bacteria isolated from middle part of *Fasciospongia cavernosa do var.brown.*

As shown in **Table 3**, the antibacterial activity of the aqueous and organic extracts of both the sponges showed broad spectrum antimicrobial activity which indicate the presence of polar and non polar bioactive compounds existence. Stock concentrations of aqueous extracts were from 5.85 to 95.40 mg/mL (proteins from 0.46 to 44.18 mg/mL). Among the organic extracts the most active samples were butanolic extract (MIC 20ng/mL) from *Fasciospongia cavernosa doc var. brown* and Methanolic extracts (MIC 30ng/mL). from *Fasciospongia cavernosa doc var.Yellow.* The microorganisms associated with sponges were investigated in order to assess the potential of these microorganisms for the production of antimicrobial compounds. All the isolates were subjected to antimicrobial activity against, *Pseudomonas aeruginosa , Escherichia coli*, *Proteus vulgaris Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Candida albicans, Aspergillus niger* and 10 pathogenic microorganisms using cross streak method. As shown in **Table 4** twenty two bacteria, eight fungi, one yeast, six actinomycetes from *Fasciospongia cavernosa doc var. brown* and fifteen bacteria, five fungi one yeast and five actinomycetes were found to be bioactive among all the strains isolated from *Fasciospongia cavernosa doc var. yellow* sponges. Bioactive isolates from primay screening were further subjected to secondary screening using submerged fermentation. The isolates B4, B-6, B-16, B-23 among all bacteria are promising showing broad spectrum antimicrobial activity against most of the gram positive and gram negative bacteria, fungi and Yeast. These bioactive isolates showed inhibition zone of 18 mm or above for most of the test organisms. Resulting mean diameter of inhibition zones revealed, isolates B4 & B6 were the most potent of all the isolates. The B4 and B-6 isolates were obtained from middle part of the *Fasciospongia cavernosa doc. var brown* . Eight fungal isolates among 28 from *Fasciospongia cavernosa doc var. brown* and five among 16 from *Fasciospongia cavernosa doc var. brown* showed antimicrobial activity. Among 29 actinomycetes isolated from two sponges 11 Isolates showed antimicrobial activity against one or more of the test pathogens. Most of the bioactive actinomycetes among isolated strains were white in color which reflects the streptomyces population among bioactive actinomycetes in nature. Two isolates were red and Two isolates are blue and one isolate is black in appearance. The actimomycetes A-2, A-4, A-7 and A-15 were very promising among all actinomycetes isolates and they were white in color. Altogether, the results indicated that the natural marine environment is also good sources for isolation of novel varieties of antagonistic microorganisms.

Discussion

Microorganisms play a central role in sponge biology, as they are associated with many sponges either extracellularly, intracellularly or both. They also serve as food source for the host. Isolation is a mandatory approach to obtain novel microbes and also for evaluating their biochemical characteristics to understand the ecophysiological and environmental functions *vis a vis* their potential applications[22]. The discovery of enormous microbial diversity in marine sponges provides unprecedented research opportunities. Increased metabolic capabilities of sponge-associated bacteria were directly correlated with increased levels of potentially available nutrients in the sponge. Sponges filter seawater and accumulate copious amount of organic matter within the choanocytic chambers along with bacteria[23].

It has been reported that the production and potential of bio active compounds by different microorganisms can be strongly influenced by the source of isolation. The first detailed study of antibiotic producing marine bacteria was done[24.] Since then there are several reports showing their antagonistic effect against human pathogens[25]. Although, the antibiotic activity of marine bacteria is well-known and has been demonstrated in a number

of studies[26],[27,28,29] the vast diversity of microorganisms in the marine niches [Austin 1989], continue to yield many novel bioactive compounds. Hence, exploration of biotechnological potentials of microbes associated with invertebrates still remains a very important and untapped resource. From all these observations the bacterial isolates from the sponges are found to be most prolific marine producers of novel compounds.

It has been estimated that over 99% of the marine sponge-associated microbes have yet to be cultured in the laboratory with bacteria isolated from the sponges containing diverse Bacillus species being one of the most divergent forms [30]. Among all isolates bacillus species was found to be predominant in symbiotic association with sponge. Many members of the bacillus group continue to be dominant bacterial workhorses in microbial fermentation for the production of novel proteins[31].

The present study has revealed the presence of high numbers of diverse culturable heterotrophic bacteria, fungi yeast and actinomycetes in association with sponges producing bioactive compounds which are industrially and medically useful. These results illustrate just how challenging it may be to culture microbial symbionts from invertebrates which are responsible for production of novel pharmaceutically important compounds. In particular, the development of new and innovative cultivation strategies holds great potential for accessing the microbial lineages that are so far under represented in culture. Mass fermentation of sponge associated microorganisms can provide a renewable resource of antibiotics and harnessing these microbes for other metabolites as well as conserving the natural population of sponges. Further studies of taxonomic characterization and purification of compounds are underway.

Table 1. Microorganisms isolated from various parts of the sponges.

Sponge species	**Part of Sponge**	**No. of microorganisms bacteria isolated**				**Total no. of Microorganisms**
		Bacteria	*Fungi*	*Yeast*	*Actinomycetes*	
Fasciospongia cavernosa doc var. brown	Lower	10	6	1	3	91
	Middle	25	12	2	6	
	Upper	18	5	1	2	
	Total	53	23	4	11	
Fasciospongia cavernosa doc var. yellow	Lower	15	3	2	4	87
	Middle	19	8	3	8	
	Upper	12	5	2	6	
	Total	46	16	7	18	

Table 2. Designations of Bacteria, Fungi, Yeast and Actinomycete isolated from sponges.

Sponge Species	Collection Part	Total no. of Microorganisms	Designation
Fasciospongia cavernosa doc var. brown	Lower	20	Scb lB/ScblF/ScblY/ScblA
	Middle	45	ScbmB/ScbmF/ScbmY/ScbmA
	Upper	26	ScbuB/ ScbuF/ ScbuY/ ScbuA
Fasciospongia cavernosa doc var. yellow	Lower	24	Scy lB/ScylF/ScylY/ScylA
	Middle	38	ScymB/ScymF/ScymY/ScymA
	Upper	25	ScyuB/ ScyuF/ ScyuY/ ScyuA

Table 3. Minimal Inhibitory Concentrations per mL of aqueous and organic Extracts.

Sponge	Aqueous extract	Organic extract		
		Acetone	Butanol	Methanol
Fasciospongia cavernosa doc var. brown	80µg	40ng	20ng	50ng
Fasciospongia cavernosa doc var. yellow	60 µg	60ng	20ng	30ng

Table 4. List of bioactive actinomycetes from sponges.

Sponge Species	No. of bioactive strains among total isolates			
	Bacteria	Fungi	Yeast	Actinomycetes
Fasciospongia cavernosa doc var. brown	53/22	23/8	4/1	11/6
Fasciospongia cavernosa doc var. yellow	46/15	16/5	7/1	18/5

Table 5. Antimicrobial activity of promising isolates.

Test Organism	Test isolates										
	B-1	B-3	B-4	B-6	B-16	B-23	F-3	A-2	A-4	A-7	A-15
Gram negative bacteria											
Pseudomonas aeruginosa MTCC 424	7	-	22	21	17	10	19	12	-	16	15
Escherichia coli MTCC 443	6	7	20	18	10	3	12	18	14	-	14
Proteus vulgaris MTCC 1771	-	-	16	15	-	20	16	-	17	15	20
Gram positive bacteria											
Staphylococcus aureus MTCC 96	4	17	18	18	9	18	8	16	11	17	17
Bacillus subtilis MTCC 441	-	11	13	15	-	16	-	6	13	16	-
Bacillus cereus MTCC 430	-	9	22	20	-	15	-	5	16	12	-

Fungi & Yeast											
Candida albicans MTCC 227	12	8	16	14	12	16	4	12	16	11	16
Aspergillus niger MTCC 1344	10	6	12	17	12	15	-	6	12	14	18
Pathogenic organisms											
Pseudomonas sp1	9	-	20	18	19	9	18	9	-	18	15
Pseudomonas sp2	7	-	19	21	17	12	16	13	-	16	14
Vibrio	9	10	16	14	9	19	13	20	17	-	8
E.coli	6	8	18	15	11	-	9	16	13	-	16
Aeromonas sp1	6	8	18	16	13	-	17	16	16	12	5
Aeromonas sp2	4	7	16	16	12	4	16	16	16	14	5
Micrococcus	-	13	22	22	-	18	-	-	19	10	18
Staphylococcus aureus	6	16	17	19	7	16	6	14	8	17	16
Candida albicans	12	8	16	14	12	16	4	12	16	11	16
Candida tropicans	10	6	12	17	12	15	-	6	12	14	18

A B

Figure 1. A. Fasciospongia cavernosa doc brown, **B**. Fasciospongia cavernosa doc yellow.

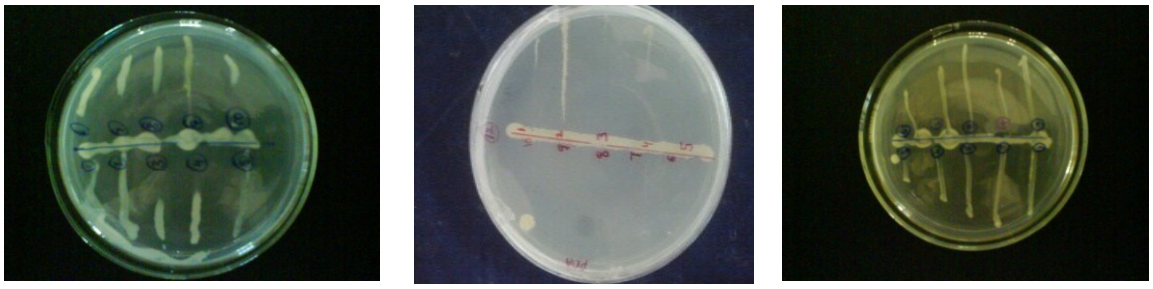
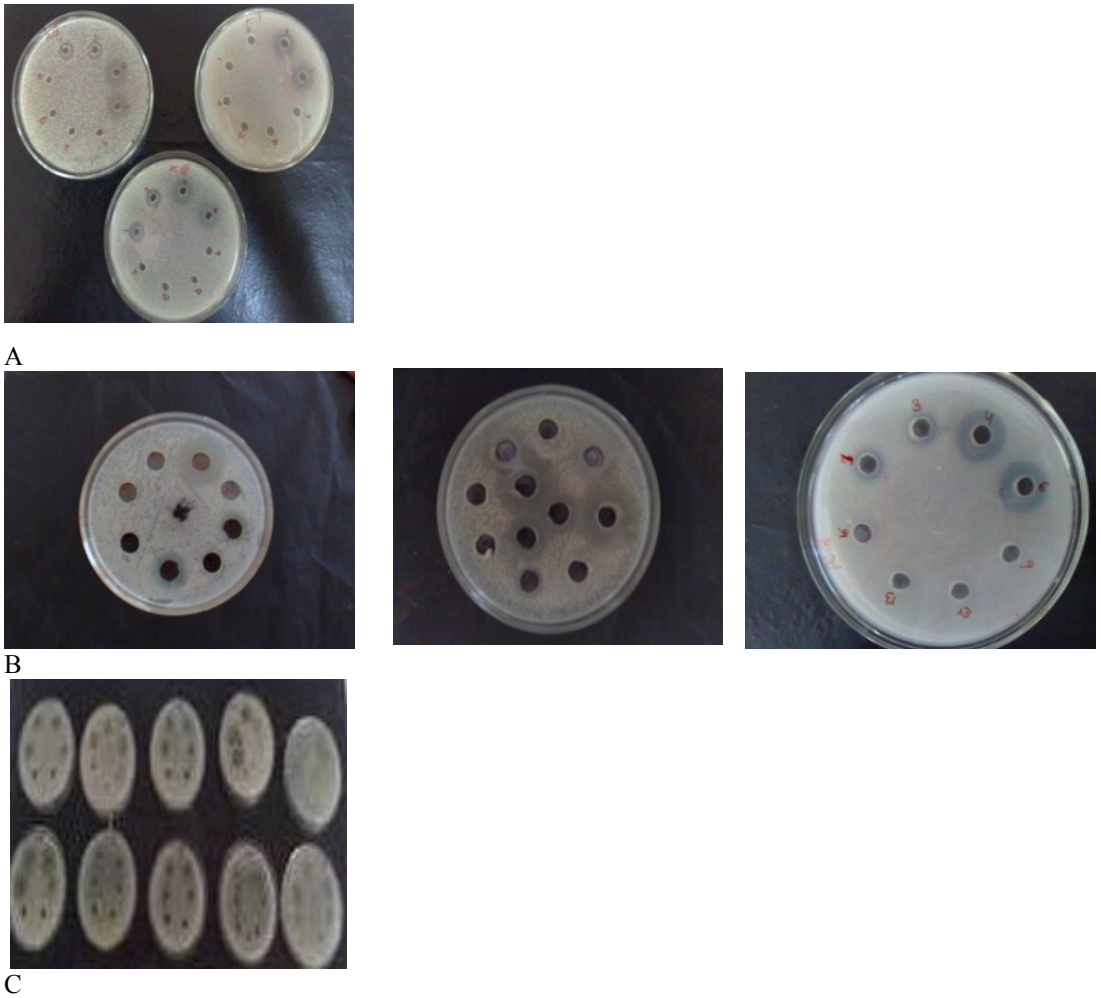


Figure 2. Primary screening (cross streaking) results of isolates.



A

B

C

Figure 3. Secondary screening results of promising isolates against (Gram positive bacteria) Gram Negative bacteria and C (pathogenic organisms).

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