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Insecticidal and Antitumour Properties of Coral Epibiotic Bacteria from Gulf of Mannar, Southeastern India

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The discovery of novel chemical classes has been in decline for the past two decades, the need to exploit new resources in search for effective chemicals with novel mechanism of actions is imperative. Marine bacteria are such a resource yet to be tapped, and the potential it offers is vast. The antitumour assay (artemia toxicity bioassay) developed by McLaughlin *et al.* is being used as a "bench top bioassay" by many researchers for the preliminary screening of bioactive compounds. In this study 94 Streptomyces strains isolated from different types of corals were screened for artemia and insecticidal activity. The lowest ED50 values were exhibited by 4 strains out of the 94 strains screened. Bacterial extracts exhibiting artemia toxicity were further screened for their insecticidal activity using two storage pests, Tribolium castaneum and Sitophilus oryzae. 59 strains exhibited activity either against Tribolium castaneum or Sitophilus oryzae or both.

Key words: Soft Corals, Antitumor Activity, Insecticidal Activity, Epibiotic Bacteria.

The taxonomic diversity of marine organisms is large as is their biochemical and behavioral diversity, so it probably should have come as no surprise that this chemical diversity was even found to contain pesticide substance close to DDT and Chlordane (Kennett, 1990). Microorganisms have been exceptionally rich sources of drugs, including antibiotics, immunosuppressants etc (Chellaram *et al*., 2011). However, these drugs have been produced from a very small range of world's microbial diversity (Chellaram *et al*., 2012). The capacity of Streptomyces to produce new compounds remains unsurpassed though members of other actinomycetes genera are becoming increasingly important as a source of novel products (Nolan and Cross, 1988). Actinomycetes have been found to be a unique source of commercially significant products and these organisms were best known as a source of novel antibiotics (Okami *et al*., 1979, Anbuselvi *et al*., 2009 and Chellaram *et al*., 2013).

In spite of the advances in computer assisted drug design in molecular biology and gene therapy there is still a pressing need for new drugs to counteract drug resistant pathogens, for instance, the mycobacterium that causes tuberculosis or multidrug resistant cancers or even disease states such as Alzheimer which is of pressing concern as the age demographics of the Western world change (Munro *et al*., 1999). Regarding marine natural products, in the area of cancer there have been valuable discoveries. Of the marine natural products or analogues that are currently under clinical investigation as potential new anticancer drugs, the marine alkaloid (Eteinascidin 743 (ET-743) is by far the most advanced compound. Marine microorganism has

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been reported to produce metabolites, which exhibit anticancer activity (Proksch *et al*., 2002; Chellaram *et al*., 2009). The novel cytotoxic macrolide and anticancer antibiotics produced by marine bacterium Micromonospora and Pelagiobacter variabilis respectively (Chimeno *et al*. 2000). Fenical, 1993 isolated an unidentified Streptomycetes producing cytotoxic agents, octalactins A and B from the surface of a gorgonian coral.

Insects and mites produce damage and loss of crops in both quantity and quality in various ways. These damages occur during plant growth, post harvest storage and transportation. The yield loss of crops by nibbling, injury, and growth retardation is estimated to be 20 - 30%. An additional 10% is lost during post harvest storage and transportation. The world market of pesticides has increased to a value of 1.1 billion dollars in 1960 and it increased to 23 billion US dollars in 1990 (Duke, *et al*., 1993). The artemia toxicity and pesticidal bioassay developed by McLaughlin *et al*.1991 is being used as a "bench top bioassay" by many researchers for the preliminary screening of bioactive compounds. The brine shrimp lethality or toxicity assay has the following advantages, it is rapid (24 hrs), inexpensive and simple (no aseptic techniques are required), utilizes a large number of organisms for statistical validations, requires no special equipment and relatively small amount of sample (20mg), and does not require animal serum as is needed for cytotoxicities. Until now very few works reported on the screening of marine bacteria for insecticidal activity. So in the present study, bacterial extracts exhibiting artemia toxicity were further screened and their insecticidal activity were tested using two storage pests, *Tribolium castaneum* and *Sitophilus oryzae.*

MATERIALS AND METHODS

In the initial screening for artemia toxicity with bacterial culture extracts, results were observed only in actinomycetes strains. So the screening was limited to the 94 Streptomyces strains isolated from soft corals, (*Lobophytu*m sp and *Sinularia* Sp) and Sea fans (Subergorgia suberosa and Junceella juncea), to save time and effort. As all the Streptomyces strains were isolated from Zobell Marine Agar plates, the broth culture of these strains were carried out in Zobell Marine broth.

Preparation of crude extracts

The bacteria were cultured in five 250 ml conical flasks each containing 200 ml of Zobell Marine broth. Initially a seed culture of 100ml was cultured in a rotary shaker (290 rpm) for 7 days in room temperature and 10 ml of this was added to the bacterial cultures in the flasks and cultured in the shaker for 7 to 10 days at 290 rpm at room temperature. Extraction of the culture broth was carried out by liquid-liquid extraction method outlined by Gailliot, 1998 with slight modification. The cultures were pooled in a "I -lit beaker and equal volume of ethyl acetate was added and stirred in a magnetic stirrer for 30mins. The broth and solvent phase (ethylaceate phase) was removed in a separating funnel, and the water phase discarded. The ethylaceate phase was filtered through a Whatman no. 1 filter and concentrated by evaporation. The residue obtained was weighed in an electronic balance and used for the assay.

Brine Shrimp Lethality Bioassay

The brine shrimp lethality bioassay was carried out following the method outlined by McLaughlin *et al*., 1991. The filtered seawater (35ppt) was taken in a small tank and commercially procured Artemia cysts were added to one side of the divided tank and a lamp was placed on the other side to attract hatched nauplii through perforations in the tank. The cysts were allowed to hatch as nauplii. 20 mg of test sample was weighed and 2 ml solvent was added. From this solution 500, 50 and 5 ml were transferred to vials corresponding to 1000, 100 and 10 µg /ml (test concentrations). Three vials for each concentration were prepared. The solvents were evaporated overnight. Ten numbers of the hatched out nauplii were added to each vial, and the volume was adjusted with seawater to 5 ml/vial and after 24 hrs the numbers of survivors were recorded. The data were analyzed with Finney computer program to determine ED50 values.

Insecticidal activity

Insecticidal activity was tested against two storage pests Tribolium castaneum and Sitophilus oryzae by dissolving the weighed crude extract in acetone in 3 different concentrations, 0.5, 1 and 2 mg/ml, following Direct Spray technique.

Direct Spray technique

10 test insects were placed inside the petridish and 5 ml quantity of different concentration of the extract was sprayed over the insects using a sprayer. The petridish was covered and survivors were recorded after 24 hrs. All experiments were carried out in triplicates and a control was also maintained without the extract.

RESULTS AND DISCUSSION

A total of 94 Streptomyces strains were screened for Artemia lethality and subsequently insecticidal activity against Tribolium castaneum and Sitophilus oryzae and 76 were found active. The lowest ED50 values were exhibited by the strains SH6, GE13, SCU 13, and STCL7 (6.112ug/ml), (Table 1). The 76 strains exhibited Artemia lethality was further screened for their insecticidal activity. 59 strains exhibited activity either against Tribolium castaneum or Sitophilus oryzae or both. The strains that exhibited high toxicity against Artemia were also found to possess potential insecticidal activity. The lowest ED50 values for Tribolium castaneum ranged from 431.120 µg /ml to 588.717 µg /ml. For Sitophilus oryzae low ED50 values ranged from 329.686 µg /ml to 588.717 µg /ml. (Table 2). Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Thus, in vivo lethality in simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products (McLaughlin *et al*., 1991 1991 and Chellaram *et al*., 2012))

Brine shrimp nauplii have been used previously in a number of bioassay systems McLaughlin *et al*., (1991) have developed this Brine shrimp lethality assay and found a positive correlation between brine shrimp toxicity and cytotoxicity and isolated a number of novel antitumour and pesticidal natural products using this bioassay (Alkofahi *et al*., 1989 and Prem Anand *et al*., 2011). In marine environment, the actinomycetes clearly predominate (Fenical, 1993) and many novel compounds have been reported from marine Streptomyces (Takahashi *et al*, 1989).Around 140 compounds have been reported from marine Streptomyces sp. until 1999 (Dobler *et al*., 2002). Dobler *et al*. (2002) observed that every third out of 500 streptomyces strains showed interesting properties in any respect and they isolated 50 new compounds from 100 selected strains. In the present study, 94 Streptomyces strains isolated from various marine sources were screened for Brine shrimp lethality. As the initial screening with other bacterial strains gave negative results, and Streptomyces sp are potential producers of bioactive compounds, they were chosen for the study.

In the Artemia toxicity assay, four strains exhibited low ED50 values. The SCU13 isolated from sea urchin exhibited the lowest ED50 value of 2.507 µg /ml. The other 3 strains SH6, GE13 and STCL 7 exhibited ED50 values of 6.112 µg /ml. The SH6 strain was isolated from a sponge, GE 13 from gastropod egg and STCL7 from a coral. These isolates seem to be potential source of antitumor compounds as it has already been proven the relationship between Artemia toxicity and cytotoxicity. The structurally novel alkaloid Altemicidin, which is produced by a marine strain of Streptomyces sioyaensis SA-1758, this alkaloid, was detected by screening cultures for toxicity against the common brine shrimp Artemia salina (Fenical, 1993). Osterhage (2001) screened marine derived fungal strains for antitumor activity using Artemia toxicity assay. Tan *et al*., (2000) isolated Hermitamides A and B, toxic malyngamide type natural product from marine cyanobacterium Lyngbya majuscule using Artemia toxicity bioassay. Further studies dealing with mass culture and extraction of the active compound may provide novel anticancer antibiotics from these Streptomyces strains.

Most of the strains exhibiting low ED50 for Artemia toxicity were also found to exhibit low ED50 value for insecticidal activity. 18 and 8 strains were found to be highly active against the pests Sitophilus oryzae and Tribolium castaneum respectively, out of which, 7 strains exhibited low ED50 values for both the pests. From the marine source insecticidal compounds have been isolated form macroalgae, sponge, coral and parts of mangrove tree. Kabaru and Gichia (2001) screened different parts of the mangrove tree Rhizophora mucronata for insecticidal activity and found that the extract of bark and pith exhibited high toxicity to Artemia salina larvae. Works related to

insecticidal activity of marine bacteria not encountered by the author after a through literature search. But in the terrestrial side, the microbial metabolite tetranactin was discovered in the 1970s and later proved to be the first useful pesticide (Tanaka and Okuda, 1992). Today five microbial products such as tetranactin, milbemycin, destomycin, hygromycin B and ivermectin are

Table 1. Artemia toxicity of marine *Streptomyces strains*

Strains	Control	1000 ug/ml	100 ug/ml	10 ug/ml	ED50 value
SA1	10	0	0	0	0
SA4	10	10	7	0	66.792
SB4	10	10	8	0	54.585
SC8	10	0	0	0	0
SD9	10	10	6	1	63.065
SE4	10	8	3	0	253.277
SF1	10	3	1	0	3976.818
SG6	10	2	0	0	8255.043
SH6	10	10	8	6	6.112
SJ7	10	3	1	0	3976.818
AA12	10	2	0	0	8255.043
AB12	10	10	7	5	13.440
AD9	10	5	2	0	896.053
AE7	10	0	0	0	0
AE8	10	4	1	0	1756.398
AF3	10	10	6		26.089
AF11	10	5	1	0	1025.445
AF14	10	3	1	0	3976.818
AG 11	10	0	0	0	0
AG3	10	10	6	3	36.670
AH7	10	10	8	5	11.160
AH11	10	2	0	0	8255.043
AH16	10	4	1	0	1756.398
AI4	10	10	8	5	11.160
AI6	10	0	0	0	0
AI12	10	7	3	0	337.362
AK14	10	4	1	0	1756.398
AK17	10	0	0	0	0
CA1	10	3	1	0	3976.818
CA10	10	7	3	1	299.479
CB9	10	4	1	0	1756.398
CB15	10	0	0	0	0
CC6	10	6	2	0	581.238
CC7	10	10	6	3	36.670
CC12	10	10	7	4	20.978
CC13	10	0	0	0	0
BFA10	10	8	3	0	253.277
BFA20	10	3	1	0	3976.818
BFA22	10	10	7	5	13.440
BFB1	10	0	0	0	0
BFB13	10	10	8	4	17.205
BFB18	10	5	2	0	896.053
BFB19	10	10	8	5	11.160
BFB20	10	3	0	0	2950.232
BFC15	10	0	0	0	0
BFC20	10	5	2	0	896.053
BFC21	10	0	0	0	0
BFC22	10	2	0	0	8255.043
SM4	10	7	4	0	272.363
SM21	10	10	7	5	13.440
SM23	10	5	1	0	1025.445
SM24	10	9	6	3	42.986
SCU8	10	0	0	0	0
SCU12	10	7	3	0	337.362
SCU13	10	10	9	7	2.507
SUR9	10	4	1	0	1756.398
SUR10	10	8	2	0	312.553
JF1	10	10	8	4	17.205
JF4	10	2	0	0	8255.043
GMA6	10	0	0	0	0
GMA7	10	10	6	4	26.089
GMB8	10	2	0	0	8255.043
GMB10	10	8	3	0	253.277
GMB12	10	10	6	4	26.089
GMC10	10	10	7	5	13.440
ASA7	10	10	6	2	48.795
ASA8	10	4	1	0	1756.398
ASB3	10	0	0	0	0
ASB4	10	2	0	0	8255.043
ASC5	10	4	1	0	1756.398
CE11	10	0	0	0	0
CE14	10	5	1	0	1025.445
GE2	10	8	3	0	253.277
GE10	10	0	0	0	0
GE13	10	10	8	6	6.112
GE14	10	10	6	3	36.670
BCL5	10	2	0	0	8255.043
BCL10	10	6	1	0	581.238
BCL14	10	0	0	0	0
SCL10	10	5	1	0	1025.445
SCL15	10	9	3	1	153.804
SCL18	10	3	0	0	2950.232
STCL9	10	2	0	0	8255.043
STCL17	10	10	8	6	6.112
STCL20	10	10	7	5	13.440
OBSA12	10	10	8	4	17.205
0BSA17	10	9	5	2	76.151
0BSA18	10	0	0	0	0
0BSB9	10	10	8	4	17.205
SPE4	10	7	3	1	299.479
SPE5	10	8	2	0	312.553
SPE9	10	9	3	1	153.804
SQE8	10	10	7	4	20.978
BFC17	10	10	7	5	13.440

Table 2. Insecticidal activity of marine *Streptomyces* strains

Strain	Control	Mortality						ED50	
		T. castaneum			*S. oryzae*				
		0.5	1	2	0.5	1	2	*T. castaneum*	*S. oryzae*
		(mg/ml)			(mg/ml)				
SA4	10	0	0	0	0	1	3	0	3030.468
SB4	10	0	1	2	0	1	3	4758.507	3030.468
SD9	10	0	0	0	0	1	2	0	3030.468
SE4	10	0	2	4	1	2	5	2320.906	2106.891
SF1	10	0	0	0	0	1	3	0	3030.468
SG6	10	0	0	0	0	0	0	0	0
SH6	10	3	5	8	5	8	10	903.697	516.796
SJ7	10	0	0	0	0	1	2	0	3030.468
AA12	10	0	0	0	0	1	3	0	3033.291
AB12	10	3	6	8	5	8	10	822.171	516.796
AD9	10	0	1	2	0	2	4	3030.468	2319.542
AE8	10	0	0	0	0	1	2	0	3030.468
AF3	10	3	6	8	5	7	9	822.171	517.976
AF11	10	0	0	0	0	2	4	0	2319.542
AF14	10	0	0	0	0	0	0	0	0
AG3	10	0	1	2	0	3	5	3030.468	1829.880
AH7	10	4	7	9	6	8	10	3030.468	431.120
AH11	10	0	0	0	0	1	2	0	3033.291
AH16	10	0	0	0	0	2	4	0	2319.542
AI4	10	4	8	10	5	8	10	588.717	516.796
AI12	10	0	0	0	1	3	4	0	2602.109
AK14	10	0	0	0	0	1	2	0	3030.468
CA1	10	0	1	2	0	2	4	3030.468	2319.542
CA10	10	0	2	3	0	1	2	3071.386	3030.468
CB9	10	0	0	0	0	1	2	0	3030.468
CC6	10	0	0	0	0	0	0	0	0
CC7	10	1	3	5	2	5	7	1930.590	1107.669
CC12	10	4	7	9	5	8	10	3030.468	516.796
BFA10	10	0	0	0	0	1	2	0	3030.468
BFA20	10	0	0	0	0	0	0	0	0
BFA22	10	4	6	8	4	8	10	701.279	588.717
BFB13	10	5	8	10	6	9	10	516.796	422.054
BFB18	10	0	0	0	0	0	0	0	0
BFB19	10	3	6	8	4	8	10	822.171	588.717
BFB20	10	0	0	0	0	0	0	0	0
BFC17	10	5	8	10	6	8	10	516.796	431.120
BFC20	10	0	0	0	0	1	2	0	3030.468
BFC22	10	0	0	0	0	0	0	0	0
SM4	10	0	0	0	0	2	3	0	3071.386
SM21	10	5	7	9	5	8	10	517.976	516.796
SM23	10	0	0	0	0	1	2	0	3030.468
SM24	10	0	1	2	0	3	5	3030.468	1829.880
SCU12	10	0	0	0	0	1	2	0	3030.468
SCU13	10	6	8	10	7	10	10	431.120	360.627
SUR9	10	0	0	0	0	2	3	0	3071.386
SUR10	10	0	1	2	0	0	0	3030.468	0
JF1	10	4	7	9	5	8	10	3030.468	516.796

Continue Table 2.

JF4	10	0	0	0	0	0	0	0	0
GMA7	10	0	1	2	1	3	5	3030.468	1930.590
GMB8	10	0	0	0	0	1	2	0	3030.468
GMB10	10	0	0	0	0	0	0	0	0
GMB12	10	1	3	4	2	4	6	2602.109	1425.967
GMC10	10	6	8	10	6	9	10	431.120	422.054
ASA7	10	0	1	2	0	0	0	3030.468	0
ASA8	10	0	0	0	0	0	0	0	0
ASB4	10	0	0	0	0	0	0	0	0
ASC5	10	0	0	0	0	0	0	0	0
CE14	10	0	0	0	0	1	2	0	3030.468
GE2	10	0	2	3	0	0	0	3071.386	0
GE13	10	5	8	10	7	10	10	516.796	360.627
GE14	10	0	1	2	0	2	4	3030.468	2319.542
BCL5	10	0	0	0	0	0	0	0	0
BCL10	10	0	1	2	0	1	2	3030.468	3030.468
SCL10	10	0	0	0	0	0	0	0	0
SCL15	10	0	2	4	0	0	0	2319.542	0
SCL18	10	0	0	0	0	0	0	0	0
STCL9	10	0	0	0	0	0	0	0	0
STCL17	10	6	9	10	7	9	10	422.054	329.686
STCL20	10	4	6	8	5	8	10	701.279	516.796
OBSA12	10	3	6	8	4	7	9	822.171	822.171
0BSA17	10	0	0	0	0	2	4	0	2319.542
0BSB9	10	3	6	8	5	8	10	822.171	516.796
SPE4	10	0	1	2	0	2	4	3030.468	2319.542
SPE5	10	0	0	0	0	0	0	0	0
SPE9	10	0	2	4	0	3	5	2319.542	1829.880
SQE8	10	4	7	9	5	9	10	822.171	496.243

being used as pesticides in agriculture and all these compounds have been isolated from terrestrial *Streptomyces* sp. (Mishima, 1983). As the potential for terrestrial Streptomyces sp. for the production of insecticidal compounds have been well established, further works regarding mass culture and isolation of active compounds from these marine Streptomyces sp. may be productive.

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