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Original article

Evaluation of antifungal, hemolytic and cytotoxic potentiality of ethyl acetate crude extracts of a novel marine *Streptomyces* spp. AIAH-10

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ABSTRACT

To face newly generated diseases, searching for new, safe and effective bioactive molecules is urgent and marine microbial flora may be a potential source. The present study was designed to isolate marine microorganisms (AIAH-1 to AIAH-29) from the soil of mangrove forest Sundarbans, Bangladesh by serial dilution method using isolation media. Among them, AIAH-10 was selected for further study due to its promising antibacterial activity (done by streak plate and plug technique method) against a series of pathogenic bacteria. On the basis of morphological, cultural and biochemical analysis, the strain AIAH-10 belongs to Streptomyces spp. Secondary metabolites of the strain was obtained by small scale fermentation process. Antifungal activity of the extracts was performed by disc diffusion method. The crude ethyl acetate extract (50 µg/disc) showed significant antifungal activity against Aspergillus niger, Candida albicans and Saccharomyces cerevaceae (14, 12, 10 mm zone of inhibition respectively). No hemolytic activity was found of the extracts towards the human erythrocytes. During cytotoxic study against brine shrimp nauplii (Artemia salina), a dose dependent mortality rate was observed. A 100% larva mortality rate was

recorded in 40 µg/ml and more where as LC_{50} was found to be 6.61 µg/ml.

1. Introduction

Marine habitat has been proven as an outstanding and fascinating resource for innovating new and potent biomolecules producing microorganisms that has not been properly screened (Uzzal et al., 2015). It is reported that not more than 1% of the bioactive compounds from marine ecosystem have been isolated (Haque et al., 2014). Exploration of unscreened marine locality has led to the discovery of hundreds of biologically active compounds. Compared with terrestrial organisms, the secondary metabolites produced by marine organisms have broader bioactivities, more novel & unique structures owing to the complex living circumstance and diversity of species (Carte, 1996 and Schwartsmann et al., 2001). Moreover, marine derived antibiotics are more efficient at fighting against pathogens over terrestrial bacteria and have not developed any resistant against them (Jensen et al., 2005). A number of marine organisms including actinomycetes, which live in marine environment, are inadequately understood and only few reports are available pertaining to marine oriented microorganisms (Siva, 2011). The actinomycetes are an important part of the microbial community in the soil environment, responsible for degradation and recycling of natural biopolymers, such as cellulose, lignin and chitin (Semedo et al., 2001). The species belonging to the genus Streptomyces constitute 50% of the total population of soil actinomycetes and are well known for producing a variety of bioactive secondary metabolites including antibiotics, immunomodulators, anticancer & antiviral drugs, herbicides, and insecticides (Rahman et al., 2010). Although thousands of antibiotics have been isolated from Streptomyces, these represent only a small fraction of the repertoire of bioactive compounds produced (Berdy, 1995 and Watve et al., 2011). So, still there is a chance of discovery of new Streptomyces species producing novel compounds from this genus. From the soil of mangrove forest Sundarbans, Bangladesh, previously we discovered several new species of actinomycetes (e.g., Smithella propionica, Syntrophus aciditrophicus) as well as some novel bioactive compounds having significant biological activities (Sarker et al., 2015). Recently, we reported the isolation and characterization of a marine Streptomyces spp. and the initial screening showed interesting antibacterial and insecticidal activities (Haque et al., 2014).

In this study, we report the antifungal, hemolytic and brine shrimp lethality bioassay of the crude extract obtained from the solid fermentation media of Streptomyces sp. AIAH-10.

2. Materials and methods

2.1. Collection of marine soil samples

Marine soil samples were collected from the marine sediments and different locations of mangrove forest (Sundarbans), like Kochikhali, Jamtoplapoint, Tigerpoint, Dublarchor, Koramjol of Bangladesh, from the layers beneath the upper surface to the 1.5cm depth during March 2012. Samples were collected in plastic bag with proper labeling. Sixteen soil samples were collected and allowed to dry in hot air oven at 121°C for about 3 hours to remove spores from the vegetative cells (Karthik et al., 2010) and stored at 40C till further processing.

2.2. Isolation of soil actinomycetes

Actinomycetes isolation from marine soil sediments was performed by serial dilution and spread plate method. One gram of previously processing soil sample was serially diluted in sterilized distilled water to get a concentration ranging from 10-1 to 10-6. A volume of 80µl of each dilution was transferred aseptically to isolation medium (casein starch glucose media) which was supplemented with nystatin (25µg/ml) to remove the growth of fungi. The sample was spread uniformly to the sterile plate by rotating it clockwise and anticlockwise direction. The plates were incubated at room temperature for 7 days (Sivakumar et al., 2005). The actinomycetes strains were picked up as they are dusky and powdery in nature as well as earthy odor. The isolated strains were further subcultured on the respective media in order to obtain pure culture. Pure isolates were maintained at 4ºC in refrigerator for further studies.

2.3. Primary screening of isolates for antibacterial activity (cross streak method)

Primary screening of the isolated actinomycetes was performed by cross streak method on modified nutrient agar (MNA) plates (Santina et al., 2009). The actinomycetes isolates were inoculated in straight line on MNA plates and incubated for 7 days. Pathogenic bacterial strains were cross streak on the same plate in perpendicular manner. The plates were incubated at 37°C for 24 hours and examined for the length of inhibition.

2.4. Characterization of potential strain

The isolated strain AIAH-10 having potent antibacterial activity was subjected for morphological, cultural and biochemical study to identify the strain.

2.5. Fermentation and isolation of secondary metabolites

The antagonistic actinomycetes isolate's suspensions were prepared by suspending a loopful of pure colony in 2 ml sterile normal saline, vortexed to homogenize and stored at 4°C until ready for use. This suspension was used as Streptomyces inoculants in all cultivations. Fermentation for production of bioactive metabolites was done as described by Umasankar 2010 with modification. 100 µl isolate suspension was inoculated in 100 ml of modified nutrient (MN) broth in Erlenmeyer flasks. Flasks were lodged on the flask shaker at a speed of 120 rpm at room temperature for 8 days. After fermentation, the medium was harvested and centrifuged to remove growth and debris. The bioactive compounds were recovered from the harvested medium by solvent extraction method. The culture supernatant was extracted twice with equal volumes of ethyl acetate (1:1 v/v), shaken vigorously for 1 hour in a solvent extraction funnel, vaporized to dryness in a rotary evaporator at 50°C and dried in lyophilizer.

2.6. Collection of test species

Three pure fungal strains Aspergillus niger, Saccharromyces cerevaceae and Candida albicans were collected from ICDDR'B. The eggs of the brine shrimp, Artemia salina, were collected from an aquarium shop, Dhaka, Bangladesh.

2.7. Antifungal Activity

Antifungal activity was done by disc diffusion method (Iqbal et al., 2004). The fungi cultures were maintained in Sabouraud's dextrose broth. From this100 µl suspension of each culture was uniformly distributed on Sabouraud's dextrose agar (SDA) plates. Sterile filter paper discs containing crude extracts (50 µg/disc) and standard Nystatin discs (30 µg/disc) were placed on the surface of Sabouraud's dextrose agar (SDA) plates. The plates were prepared at 4°C for 4 hour to diffuse the extracts and nystatin to the surrounding media. Then, the inoculated plates were incubated at 25oC for 48 hour. At the end of incubation period, the zones of inhibition were measured.

2.8. Hemolytic activity

Hemolytic activity was carried out by using blood agar plate method. The ethyl acetate extract was used to detect the hemolytic activity. The blood agar plates were prepared by adding human blood (5%) to blood agar base. Wells were punched on the blood agar surface by using a gel borer. The ethyl acetate extracts were prepared 1000 µg/ml concentration and a volume of 100 µl was transferred aseptically into the well. Then plates were incubated at 37°C for 12 hrs. The plates were then examined for the zone of hemolysis (Sathish et al., 2011).

2.9. Cytotoxic profile

Brine shrimp lethality bioassay (Jaki et al., 1999 and Mayer et al., 1982] is a recent development in the assay procedure of bioactive compounds which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS, etc) of the compounds. In brief, the eggs of brine shrimp, Artemia salina were hatched in seawater. Ten mature larvae (nauplii) were kept in glass vials containing 10 ml of seawater. The extracts dissolved in DMSO (10 mg/ml) were applied to the nauplii in each vial. However, not more than 50 µl of DMSO was added to the vials containing the shrimps. For each concentration, vials containing the same volume of DMSO plus seawater and shrimps were used as control. After 24 h, the vials were observed for mortality with the help of magnified glass. The number of survived nauplii in each vial was counted and from this data, the percentage of lethality of the brine shrimp nauplii was calculated. The findings were presented

graphically by plotting concentration versus percentage of mortality of nauplii from which LC50 was determined by extrapolation.

3. Results and discussions

Total 29 actinomycetes were isolated from different marine soil samples of Sundarbans (Bangladesh) surroundings. Colonies of actinomycetes in isolation plate are shown in figure1. These were designed as AIAH-1 to AIAH-29 (Table 1).

Table 1

Collection site	Dept of sample	Number of colony/gm of soil	Isolate strains
Dublarchor	8 inches	1.2×10^4	AIAH-1 to AIAH-12
Kochikhali	6 inches	0.7×10^4	AIAH-13 toAIAH-19
Koromjol,	1 feet	1.0×10^4	AIAH-20 to AIAH-29

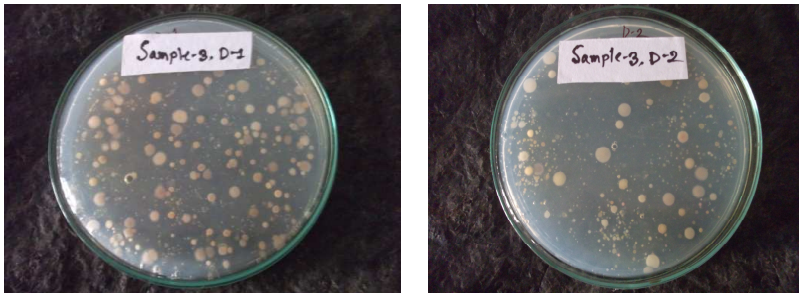

Fig. 1. Colonies of actinomycetes appeared on the dilution plates using the marine soil sample.

Among the 29 isolates, 73.53% exhibited antimicrobial activity during primary screening (Table 2). The isolate AIAH-10 showing promising broad spectrum activity against different pathogenic organisms (Figure 2) was selected for further study. The potential strain AIAH-10 was identified by morphological, cultural and biochemical study. The complete data was reported in Table 3 and 4. Findings of this study suggested that the strain was belonged to Streptomyces spp.

Table 2
Diameter of length of inhibition (in mm) of the isolates against pathogenic bacteria.

ID number	*Streptococcus agalactiae*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Shigella dysenteriae*	*Shigellasonnei*	*Agrobacterium*
AIAH-01	15±0.12	22±0.13	16±0.17	20±0.27	20±0.32	30±0.16	21±0.14
AIAH-02	02±0.31	05±0.26	04±0.14	02±0.16	-	-	04±0.37
AIAH-05	21±0.34	28±0.21	19±0.17	27±0.34	30±0.11	22±0.23	20±0.41
AIAH-06	10±0.24	12±0.41	07±0.23	09±0.34	15±0.27	-	12±0.24
AIAH-07	-	-	-	-	-	-	-
AIAH-08	13±0.14	14±0.32	15±0.38	12±0.17	16±0.52	10±0.27	12±0.51
AIAH-09	-	-	-	-	-	-	-
AIAH-10	32±0.23	37±0.29	30±0.15	30±0.53	35±0.17	28±0.33	25±0.27
AIAH-12	06±0.37	-	-	-	03±0.19	06±0.15	02±0.31

AIAH-13	16±0.18	11±0.48	14±0.19	12±0.41	02±0.23	10±0.29	08±0.54
AIAH-14	-	-	-	-	-	-	-
AIAH-15	13±0.14	15±0.21	10±0.23	05±0.17	06±0.39	04±0.52	10±0.41
AIAH-16	-	-	-	-	-	-	-
AIAH-17	05±0.41	23±0.14	05±0.39	07±0.62	02±0.69	03±0.49	-
AIAH-20	-	04±0.31	06±0.11	02±0.21	03±0.25	06±0.17	08±0.29
AIAH-22	15±0.37	19±0.33	12±0.51	11±0.32	10±0.14	10±0.28	12±0.16
AIAH-24	11±0.43	02±0.51	03±0.37	05±0.12	06±0.34	02±0.44	03±0.18
AIAH-25	10±0.16	05±0.57	05±0.42	02±0.26	-	06±0.31	07±0.31
AIAH-26	16±0.25	08±0.32	05±0.68	07±0.43	02±0.28	03±0.11	-
AIAH-27	-	02±0.21	01±0.29	02±0.19	-	02±0.16	-
AIAH-28	-	-	-	-	-	-	-
AIAH-29	28±0.19	41±0.42	37±0.41	30±0.33	25±0.43	31±0.28	27±0.53

Legend, The values expressed as mean ± SEM of 3 -4 experiments. " – " indicates no inhibition

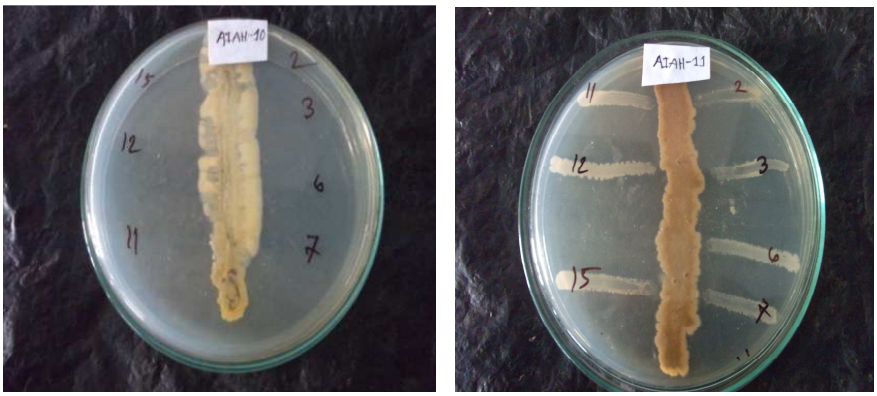

Fig. 2. Antibacterial activities of the isolates (against Streptococcus agalactiae (2), Bacillus cereus (3), Pseudomonas aeruginosa (6), Escherichia coli (7), Shigella dysenteriae (11), Shigella sonnei (12) and Agrobacterium (15)) through single line streaking technique.

Table 3
Growth and Characteristics of strain AIAH-10 on different media.

Medium	Growth	Aerial mycelium	Reverse mycelium	Pigmentation
Trypton–yeast extract agar(ISP-1)	+	Light brown	Light yellow	+
Yeast-extract-malt extract agar (ISP- 2)	++	Yellowish brown	Darkish pink	++
Oatmeal agar (ISP -3)	++	Yellowish gray	Dark yellowish orange	++
Inorganic salt-starch agar (ISP- 4)	+++	Grayish yellow	Yellowish white	+++
Glycerol-asparagine agar (ISP-5)	++	Grayish brown	Pinkish white	++
Tyrosine agar(ISP-7)	++	Light gray	Dusky yellow	+
Yeast-extract glucose agar (YEGA)	+++	Yellowish orange	Dark gray	+++

Legend, '+++'=High growth, '++'=Moderate growth and '+'=Low growth.

The antifungal activity of the ethyl actate extracts from marine Streptomyces sp. AIAH-10 was determined at a concentration of 50 µg/disc against number of fungi and was found to be 14, 10 and 12 mm against Aspergillus niger, Saccharromyces cerevaceae and Candida albicans respectively (Table 5). It was 18-19 mm for Nystatin (30 µg/disc) as standard.

Table 4

Phenotypic properties of AIAH-10 and a reference strain (Xu *et al.,* 2004).

Properties	Results of AIAH-10	Reference (KM-4927^T)
Spore chain	Flexuous	Spiral
Substrate mycelium color	Grayish yellow	Gray
Liquefaction of gelatin	-	-
Hydrolysis of starch	+	+
Decomposition of cellulose	+	ND
Nitrate reduction	+	+
NaCl tolerance	2-4%	ND
Melanoid production	+	ND
Sucrose	+	±
D-Manitol	+	-
Inositol	+	+
Optimum growth temperature	32-41°C	ND

Note, +, positive or utilized; -, negative or not utilized; ±, ambiguous; ND, not determined.

Table 5

Antifungal activity for the extracts of AIAH-10.

Test organisms	Zone of inhibition(in mm)	
	Nystatin (30µg/disc)	Extracts (50µg/disc)
Aspergillus niger	19 ± 0.52	14 ± 0.32
Saccharromyces cerevaceae	19 ± 0.57	10 ± 0.15
Candida albicans	18 ± 0.45	12 ± 0.24

Legend, the values expressed as mean ± SEM of 3 -4 experiments. " – " indicates no inhibition.

The ethyl acetate extracts did not show any hemolytic activity when tested against human erythrocytes. The results of the brine shrimp lethality bioassay are shown in Table 6.Test sample showed different mortality rate at different concentration. The mortality rate of brine shrimp nauplii was found to be increased with the increasing of concentration of the sample. The larval mortality was recorded as 100% in 40 µg/ml and higher concentrations. The median lethal concentration (LC50) of the extract was found to be 6.61 µg/ml (Figure 3).

4. Discussion

Actinomycetes are one of the major groups of organisms present in both terrestrial and marine environment (Imada et al., 2005). In present study, actinomycetes were isolated from the soils samples of mangrove forest sundarbans, Bangladesh using isolation media. The isolation media contains starch and casein as sole carbon and energy sources. Only organisms capable of degrading these complex polymers (mostly molds and Streptomyces) are able to grow (Sharmin et al., 2013). Among 29 isolated strains (AIAH-1 to AIAH-29), the strain AIAH-10 was selected for further study due to its potent antibacterial property against a series of pathogenic bacterial strains. On the basis of cultural, morphological and biochemical properties, AIAH-10 was characterized as Streptomyces spp. Similar procedure was followed for the identification of Streptomyces in "International Streptomyces Project" (Nonomura, 1974). Previous works on novel antibiotics reported that a high proportion of organisms possessing antimicrobial activity belong to the genus Streptomyces (Demain et al., 2009 and Ceylan et al., 2008)

Table 6

The results of brine shrimp lethality bioassay of the crude extract of Streptomyces spp. AIAH-10

Group	Conc.of sample (µg/ml)	LogC	No. of nauplii added	No. of death in each vial			Average No. of death	Mortality (%)	Corrected Mortality (%)	LC_{50}(µg/ml)
				1	2	3				
Control	20µl	0	10	0	0	0	0	0	0	0
Extracts	1.25	0.09	10	2	2	1	1.66	16.6	16.6	6.61
	2.5	0.39	10	3	2	2	2.33	23.3	23.3	
	5	0.69	10	4	3	5	4.33	43.3	43.3	
	10	1	10	6	5	6	5.33	53.3	53.3	
	20	1.30	10	9	9	8	8.66	86.6	86.6	
	40	1.6	10	10	10	10	10	100	100	
	80	1.9	10	10	10	10	10	100	100	

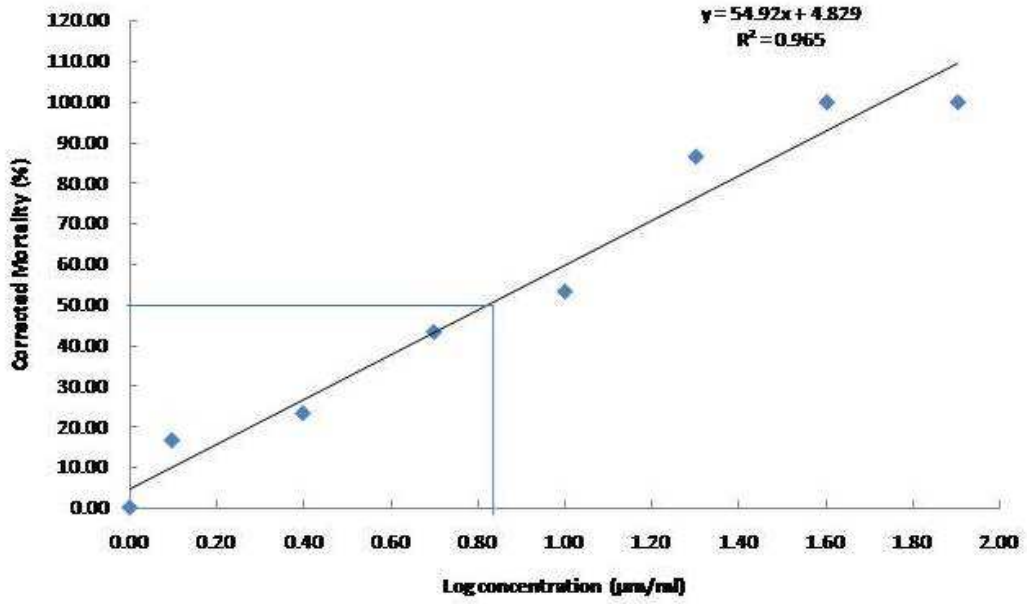

Fig. 3. Determination of LC50 by correlating between concentration of the crude extract and percentage of mortality of brine shrimp nauplii.

The crude extracts exhibited significant antifungal activity against the pathogenic fungi where as previous study (Abdul Alim et al., 2006) reported that actinomycetes spp. exhibited 12, 10, and 10 mm of zone of inhibition to Aspergillus niger, Saccharromyces cerevaceae and Candida albicans respectively that was less to compared our findings . The extracts did not show hemolytic activity to the human erythrocytes therefore it could be considered as safe for human use. In brine shrimp lethality bioassay, it was found that the extracts exhibited dose dependent activity and medial lethal concentration (LC50) was found to be 6.661 µg/ml. In earlier studies (Ruhul et al., 2003), the reported value was 17.78 µg/ml. Compare to other studies, our isolated Streptomyces spp. exhibited more antifungal and cytotoxic activity.

5. Conclusion

Therefore it can be concluded that the marine Streptomyces spp. AIAH-10 may be an interesting source for obtaining molecules. However further study is required to determine the structure of responsible compound and to identify the strain to its species level sequencing of 16S rDNA.

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