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Antibacterial Potential of Ethyl-Acetate Extracts of Marine *Streptomyces* spp. AIAH-10 against Drug Resistant *Escherichia coli*

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Authors' contributions

This work was carried out in collaboration between all authors. Author MAH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript and managed literature searches. Authors AKS, MSI, MAUI managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The rapid increase in drug resistance among bacteria is a public health challenge in the 21st century. To combat drug resistance, searching for new broad-spectrum antibiotics is urgent and marine microbial flora may be a potential source. The present study was designed to isolate marine actinomycetes strains (AIAH-01 to AIAH-29) from the soil sediments of different locations of the sundarbans mangrove forest, Bangladesh by serial dilution method. Primary screening of antimicrobial activity was performed by cross plate technique and AIAH-10 was undertaken for further analysis due to its potential activity against a number of pathogenic bacteria. On the basis of morphological, cultural, biochemical and cell composition analysis, AIAH-10 belonged to *Streptomyces* spp. Secondary metabolites of AIAH-10 exhibited significant activity (with zone sizes of 15 mm and 12 mm) against ciprofloxacin and azithromycin induced resistant *Escherichia coli* (*E. coli)* respectively (50 μg extracts/disc). Minimum inhibitory concentration (MIC) of extracts against

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ciprofloxacin and azithromycin induced resistant *E. coli* was found to be 16 and 32 μg/ml respectively. Time-kill profile of the extracts also revealed its considerable antimicrobial activity. Therefore it may be concluded that the marine *Streptomyces* spp. AIAH-10 isolated from the sundarbans mangrove forest, Bangladesh produces bioactive compounds that can be applied against resistant and multi-drug resistant pathogens.

Keywords: Mangrove forest; susceptibility; marine Streptomyces spp. antimicrobial activity; multidrug resistant susceptibility.

1. INTRODUCTION

Recently the world health organization (WHO) identified antimicrobial resistance as one of the greatest threats to human health, jeopardizing patient safety and public health. In recent years, indiscriminate use of antibiotics, pathogenic microorganisms are gaining resistance to the existing microbial agent [1]. Due to this high prevalence of antimicrobial resistance, WHO themed the 2011 world health's day "Antimicrobial resistance: no action today, no cure tomorrow". The prevalence of drug resistant among pathogenic strains is a threat to human health and can lead to high mortality rates in immune compromised, especially patients from AIDS [2].

To combat antibiotic resistance, researchers are currently engaged in studying marine bacteria as a source of potent antimicrobial compounds that could be biocidal to resistant species. It has been appraised that not more than 1% of desired chemicals from marine environments have been screened. However actinomycetes flora of marine environment, are poorly screened and only few researches are done pertaining to microorganism from mangroves forest [3,4]. Due to different environmental conditions of marine derived microorganisms from that of terrestrial ones, it is assumed that microorganisms isolated from marine locations may have different criteria from those of terrestrial environment and hence marine derived microorganisms might produce different types of bioactive compounds [5]. Now days, the researchers point out that, marine oriented microorganism may become a super recognized source of discovering novel antibiotic, anti-inflammatory, antifungal, antiviral, anticoagulant, cardiovascular and anticancer agent with distinguish structure and properties [6]. Moreover, marine oriented antibiotics are found to exhibit more efficient activity against pathogenic bacterial strains and yet have not any published data concerning drug resistant against them [7].

This study was performed to find out the potential susceptibility of ethyl acetate extracts of marine *Streptomyces* spp. AIAH-10 against ciprofloxacin and azithromycin induced resistant *E. coli* isolated from the sundarbans mangrove forest, Bangladesh.

2. MATERIALS AND METHODS

2.1 Collection of Marine Soil Samples

Soil samples were collected from the different locations of mangrove forest (Sundarbans), like Kochikhali, Jamtoplapoint, Tigerpoint, Dublarchor, Koramjol of Bangladesh to a depth of 15 cm. Sixteen soil samples were collected in properly labeled sterile plastic bags. The samples were dried in hot air oven at 121ºC for about 3 hours to remove spores from the vegetative cells [8] and stored at 4ºC till further processing.

2.2 Isolation of Soil Actinomycetes

Actinomycetes isolation from marine soil sediments was done by serial dilution and spread plate method [8]. One gram of processed soil sample was serially diluted in sterilized distilled water to obtain a concentration ranging from 10^{-1} to 10^{-6}. A volume of 100 μl of each dilution was transferred aseptically into an isolation medium (casein starch media), modified nutrient agar (MNA) and Kuster's medium (KA) which was supplemented with nystatine (25 μg/ml). The sample was spread uniformly on the plate by rotating it clockwise and anticlockwise direction. The plates were incubated at room temperature for 7 days [9]. The isolated actinomycetes were picked up using their organoleptic properties: dusky and powdery in nature with an earthy odor. The isolates were further subcultured (Yeast Extract Glucose Agar media) to obtain pure cultures. Pure isolates were kept at 4ºC for further studies.

2.3 Primary Screening (Cross Streak Method)

The primary screening of isolated actinomycetes strains was performed by single cross streak method on plates containing MNA media [10]. Isolated strains were inoculated in straight line on MNA plates and incubated for 7 days. Test bacteria were cross streak on the same plate in perpendicular manner. The plates were incubated at 37ºC for 24 hours. The plates were checked for the length of inhibition.

2.4 Taxonomic Investigation of the Potential Strain

2.4.1 Morphological characteristics

The isolated strain AIAH-10 having potent antibacterial activity was inoculated in different media stated in "International *Streptomyces* Project" (ISP) [11] and cultured for 8 days at 28ºC temperature. The colonies were observed under microscope and morphology was identified in respect to the color, size, nature of colony both aerial and reverse side [12].

2.4.2 Biochemical characterization

The ability of isolated strain to assimilate of various carbon sources viz., xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose as sources of energy was done following the method recommended by the International *Streptomyces* Project [11].

2.4.3 Generic investigation

The strain (AIAH-10) having tremendous antagonistic activity against the test bacteria was identified by using cell wall composition analysis like amino acids and cell sugars analysis [8].

2.5 Fermentation Process

The antagonistic actinomycetes isolate's suspensions were prepared by suspending a loopful of pure colony of AIAH-10 in 2 ml sterile normal saline, vortexed to homogenize and stored at 4ºC 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 suspension was cultured in 100 ml of modified nutrient (MN) broth in Erlenmeyer flasks. Flasks were kept on the shaker at a speed of 120 rpm at room temperature for 8 days. After successful fermentation, the medium was harvested and centrifuged to remove debris. Filtrate was preserved for further use.

2.6 Isolation of Metabolites

The metabolites were recovered from the harvested medium by solvent extraction technique. The cultured supernatant was extracted twice with equal volumes of ethyl acetate, shaken sufficiently for 1 hour in extracting funnel, vaporized to dryness in a rotary evaporator at 50ºC and dried in lyophilizer.

2.7 Collection of Resistant Species

Ciprofloxacin and azithromycin induced resistant *E. coli* was collected from Department of Pharmacy, University of Rajshahi, Bangladesh while pathogenic bacterial strains were collected from the "International Centre for Diarrheal Disease Research' Bangladesh (ICDDR'B).

2.8 Antibiogram

Induced resistant *E. coli* was screened for their sensitivity towards ciprofloxacin (30 µg/disc) and azithromycin (30 µg/disc) by disc diffusion method. Freshly cultured resistant *E. coli* was seeded unto plates (yeast extract glucose agar media) by using sterilized cotton swabs. The antibiotic discs were placed on the agar surface using a sterilize forceps. Plates were incubated at 37ºC for 24 hours. Plates were checked for zones of inhibition [13].

2.9 Antimicrobial Activity

The antimicrobial activity of secondary metabolites of AIAH-10 against ciprofloxacin and azithromycin resistant *E. coli* was carried out by the disc diffusion method [13]. The bacterial culture was maintained in nutrient broth. From this 100 µl suspension of each culture was uniformly distributed on plates containing yeast extract glucose agar media. The test sample impregnated discs (50 µg/disc) were placed gently on solidified agar plates which were seeded with the organism to ensure contact with the media, by means of a sterile forceps. The plates were kept at 4ºC for 4 hours for proper diffusion of sample to the surrounding area. Then, the plates were incubated at 37ºC for 24 hour. At the end of the incubation period, the zones of inhibition were measured by scale.

2.10 Determination of Minimum Inhibitory Concentration (MIC)

Sterile nutrient broth tubes containing different dilutions of extract (0.125 to 512 μg/ml) were specifically inoculated with 60 μl of ciprofloxacin and azithromycin resistant *E. coli* inoculums (10^7cfu/ml). The tubes were incubated aerobically at 37ºC for 18-24 h. Two control (tube containing the growth medium, saline and the inoculums) tubes for each organism were maintained. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes were regarded as MIC [14].

2.11 Determination of Time-kill Profile

The method of Spangler [15] was adopted for the time-kill assay against ciprofloxacin and azithromycin resistant *E. coli*. Viable counts of the test bacteria were first determined. Initial inocula of 10^5 to 10^6cfu/ml were prepared from 100 µl aliquots of strain in normal saline and this was verified by performing colony count. 80 µl suspension of selected test bacteria was added to 10 ml of MN broth in McCartney bottles containing known concentration (relative to MIC) of each of the extracts. Time kill assay of extracts against ciprofloxacin and azithromycin resistant *E. coli* bacteria were determined using the following extract concentrations: MIC, 2 × MIC, 3 × MIC and 4 × MIC and the reactants bottles were incubated in a rotary incubator at 37ºC. The time kill kinetics were determined at 0, 3 and 6 h. Approximately 80 μl of *E. coli* suspension was withdrawn at the appropriate time interval and transferred to 4.5 ml of MN broth medium containing 2-4% sodium thioglycolate to minimize the action of the extracts carry-overs. Approximately 100 µl aliquots of each dilution were plated out for viable counts by pour plate technique. Each experiment was done in duplicate, and the mean of two almost identical results was calculated. Only plates yielding 30 to 300 colonies were selected for counting. Data were analyzed by expressing the reduction of growth as the Log_{10} colony forming unit per milliliter (cfu/ml). For the Time-kill end point determination; bacteriostatic activity was defined as a reduction of 0 to 3 Log_{10}cfu/ml, and bactericidal activity was defined as a reduction of ≥ 3 Log_{10}cfu/ml at 3 and 6 h compared to that at 0 h [15]. McCartney bottles containing broth and test organism without extract were used as control in each experiment.

3. RESULTS

A total of 29 actinobacterial colonies were isolated from different marine sediments and soil samples of Sundarbans (Bangladesh) surroundings. They were designated as AIAH-01 to AIAH-29. The isolates AIAH-01 to AIAH-12 were isolated from the soil of Dublarchor while the isolates AIAH-13 to AIAH-19 from the soils of Kochikhali and the isolates AIAH-20 to AIAH-29 from the soils of Koromjol. It is shown in the Table 1.

Among the 29 isolates, 73.53% exhibited different ranges of antimicrobial activity during primary screening (Table 2). The isolated strain, AIAH-10 showing promising broad spectrum activity against different pathogenic organisms was selected for further study.

The strain AIAH-10 was identified by morphological and biochemical test. The entire report was shown in Tables 3 and 4. Furthermore, generic investigation was done for the isolate. Results are shown in Table 5. Findings of this study suggested that the strain belongs to *Streptomyces* spp. and designated as *Streptomyces* spp. AIAH-10.

The metabolites were tested for susceptibility against ciprofloxacin and azithromycin induced resistant and sensitive *E. coli* strain. Resistance of the strain was confirmed by antibiogram test with ciprofloxacin and azithromycin and negligible zone of inhibition was observed. Antimicrobial potentiality of the crude extracts (50 μg/disc) against azithromycin and ciprofloxacin induced resistant and sensitive *E. coli* strain was recorded to be 12 mm (for azithromycin resistant *E. coli*), 15 mm (for ciprofloxacin resistant *E. coli)* and 24 mm (sensitive *E. coli)* respectively. The result is shown in the Table 6 and Fig. 1.

Table 1. Collection site, depth and number of actinomycetes colonies per gram of soil

Collection site	Depth of the soil	Number of colony/gm of soil	Isolate strains
Dublarchor	8 inches	1.2×10^4	AIAH-01 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

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

ID number of the isolates	*Streptococcus agalactiae*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Shigella dysenteriae*	*Shigella sonnei*	*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-03	-	-	-	-	-	-	-
AIAH-04	-	-	-	-	-	-	-
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-11	-	-	-	-	-	-	-
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-18	-	-	-	-	-	-	-
AIAH-19	-	-	-	-	-	-	-
AIAH-20	-	04±0.31	06±0.11	02±0.21	03±0.25	06±0.17	08±0.29
AIAH-21	-	-	-	-	-	-	-
AIAH-22	15±0.37	19±0.33	12±0.51	11±0.32	10±0.14	10±0.28	12±0.16
AIAH-23	-	-	-	-	-	-	-
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

Table 3. Growth and cultural 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

Table 4. Assimilation of carbon sources by the strain AIAH-10

Carbon source	Growth	Pigments production
D-Glucose (Positive control)	+++	++
D-fructose	+	+
Ethyl Cellulose	+	-
D-Xylose	-	-
Lactose	+	+
Sucrose	+	+
Mannitol	+++	++
Inositol	++	+
L-Rhamnose	++	+
No carbon (Negative control)	-	-

Legend; '+++'= strongly positive assimilation, '++'= moderately positive assimilation, '+'= low assimilation and '-'= negative assimilation

Minimum inhibitory concentration was determined by broth dilution method. The MIC values of the extracts against ciprofloxacin and azithromycin induced resistant and sensitive *E. coli* were found to be 16 μg/ml (for ciprofloxacin resistant *E. coli*), 32 μg/ml (for azithromycin resistant *E. coli*) and 2 μg/ml (sensitive *E. coli*) respectively. The result is presented in the Table 7.

Time-kill profile of the metabolites against azithromycin and ciprofloxacin induced resistant *E. coli* strain is shown in Table 8. The metabolites exhibited varying degrees of bacteriostatic activities depending on their potency. A significant decrease in mean of viable count of test bacteria was observed at each time interval. Results are presented as Log_{10} cfu/ml.

Mean Log reduction in viable cell count for the metabolites ranges between 0.31 Log_{10}, 0.62 Log_{10}, 0.86 Log_{10} and 1.23 Log_{10} cfu/ml after 3h interaction in MIC, 2×MIC, 3×MIC and 4×MIC, respectively and between 0.45 Log_{10}, 0.73 Log_{10}, 0.97 Log_{10} and 1.36 Log_{10}cfu/ml after 6h interaction in MIC, 2×MIC, 3×MIC and 4×MIC, respectively (against azithromycin resistant *E. coli*).

Table 5. Cell wall amino acids and whole sugars content of AIAH-10 isolate

Cell wall composition	Possible results
LL-DAP	Present
Meso-DAP	Absent
Glysine	Present
Whole cell sugars	Absent
Wall type	I

Table 6. Antimicrobial activity of the extracts against azithromycin and ciprofloxacin induced resistant *E. coli*

Name of antibiotic	Zone of inhibition(in mm)		
	Azithromycin resistant *E. coli*	Ciprofloxacin resistant *E. coli*	Sensitive *E. coli*
Metabolites	12±0.15	15±0.23	24±0.51
Ciprofloxacin	8±0.31	7±0.26	22±0.18
Azithromycin	8±0.57	10±0.21	25±0.57

Legend; The values expressed as mean ± SEM of 3 -4 experiments

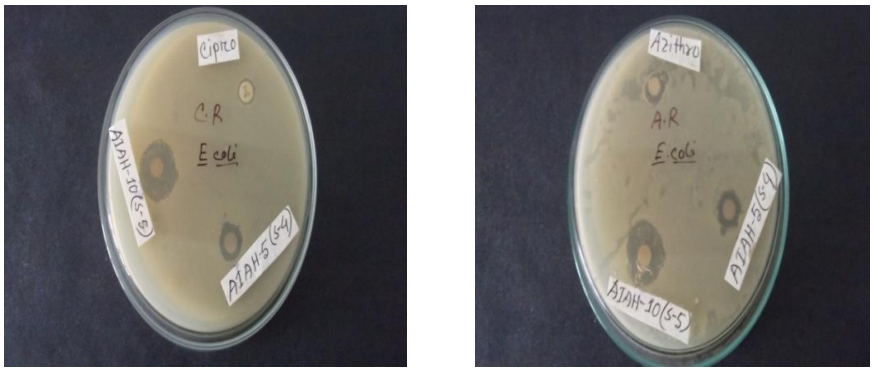

Fig. 1. Antimicrobial activity of the extracts against ciprofloxacin and azithromycin induced resistant *E. coli* respectively

Table 7. MIC of the extracts against ciprofloxacin and azithromycin resistant and sensitive *E. coli*

E. coli strain	MIC (μg/ml)	MBC (μg/ml)
Ciprofloxacin resistant	16	32
Azithromycin resistant	32	128
Sensitive	2	8

Table 8. Time-kill assay of metabolites against azithromycin and ciprofloxacin induced resistant *E. coli*

Name of the compound	*E coli* strain	MIC (Log_{10})		2×MIC (Log_{10})		3×MIC (Log_{10})		4×MIC (Log_{10})	
		3h	6h	3h	6h	3h	6h	3h	6h
Extracts	Azithromycin resistant	0.31	0.45	0.62	0.73	0.86	0.97	1.23	1.36
	Ciprofloxacin resistant	0.63	0.72	0.89	1.04	1.21	1.44	1.71	1.8

Mean Log reduction in viable cell count for the metabolites ranges between 0.63 Log_{10}, 0.89 Log_{10}, 1.21 Log_{10} and 1.71 Log_{10}cfu/ml after 3h interaction in MIC, 2×MIC, 3×MIC and 4×MIC, respectively and between 0.72 Log_{10}, 1.04 Log_{10}, 1.44 Log_{10} and 1.8 Log_{10}cfu/ml after 6h interaction in MIC, 2×MIC, 3×MIC and 4×MIC, respectively (against ciprofloxacin resistant *E. coli*).

4. DISCUSSION

From seven soil samples, 29 pure strains were isolated and screened for antibacterial activity. Among them 79.31% isolates inhibited the test bacteria with variable activity-low, moderate to high, when the strains were checked for primary antibacterial screening by cross streaking method. Inhibition zones of the extracts of this species against ciprofloxacin and azithromycin induced resistant *E. coli* was found as 15 and 12 mm respectively on 50 μg extracts per disc whereas MIC value was recorded as 16 and 32 μg/ml respectively. Time-kill profile of the extracts suggests the potential activity of the extracts against the antibiotic resistant *E. coli* strain. To best of our knowledge no work has been carried out against ciprofloxacin and azithromycin induced resistant *E. coli*, although some research were done against multi-drug resistant *Staphylococcus aureus* and some other pathogenic bacteria [16-19].

5. CONCLUSION

The *Streptomyces* spp. AIAH-10 isolated from sundarbans mangrove forest is a potential source of antibiotic molecules.

COMPETING INTERESTS

Authors have declared that no competing of interests exists.

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