

# Antimicrobial and Anticancer Activities of Ethyl Acetate Extract of Co-culture of *Streptomyces* sp. ANAM-5 and AIAH-10 Isolated From Mangrove Forest of Sundarbans, Bangladesh

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## ARTICLE INFO

### Article history:

Received on: 16/11/2015

Revised on: 10/12/2015

Accepted on: 30/12/2015

Available online: 27/02/2016

### Key words:

Mixed fermentation,  
*Streptomyces* sp., MIC,  
MFC, anticancer activity.

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## ABSTRACT

In this study we investigated the antimicrobial and anticancer activities of ethyl acetate extract of co-culture of *Streptomyces* sp. ANAM-5 and AIAH-10 isolated from soil of mangrove forest Sundarbans, Bangladesh. The antimicrobial activity of ethyl acetate extract was determined using broth-dilution method against *Candida albicans*, *Saccharomyces cereviceae* and *Aspergillus niger* whereas anticancer activity was evaluated against Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice with the dose of 50 mg/kg and 100 mg/kg body weight (i.p). The Minimum Inhibitory Concentrations (MIC) of ethyl acetate extract was found 32µg/ml against *Candida albicans* while 64 µg/ml against *Saccharomyces cereviceae* and *Aspergillus niger*. The antineoplastic activity of the crude extract was increased in dose dependent manner with a significant value (p<0.01). Bacterial crude extract enhanced the mean survival time (MST) of tumor bearing mice at 71.79% and maximum cell growth inhibition was found 75.75 % with dose of 100 mg/kg body weight (i.p.). Our study revealed that ethyl acetate extract of co-culture of *Streptomyces* sp. ANAM-5 and AIAH-10 is an excellent source of antimicrobial and anticancer compounds which may become helpful to treat infections and cancer.

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## INTRODUCTION

Since the ancient time people utilize different natural sources for the cure of diseases. Among such natural sources microbial secondary metabolites have been considered one of the powerful resources for drug discovery owing to their diverse biological activities. Actinomycetes are a group of gram-positive bacteria, widespread in nature, playing a significant role in the field of biotechnology, because they are the prolific producers of antibiotics, anti-tumor agents, immunosuppressive agents and enzymes (Blunt *et al.*, 2006; Mann, 2001; Selvam *et al.*, 2011). The marine environment could be an interesting source of the rare bacterial groups and biologically active molecules (Asha *et al.*, 2006; Munro *et al.*, 1999; Pelaez, 2006; Pomponi, 1999). Members of the actinomycetes living in the marine environment are poorly understood and only few reports are available

(Vikineswary *et al.*, 1997; Rathna and Chandrika, 1993; Bredholt *et al.*, 2008). Compared with terrestrial organisms, the bioactive molecules produced by marine microorganisms have more novel and unique structures due to their complex living environment and diversity of species (Carte, 1996; Schwartzmann *et al.*, 2001). Recently, the frequency of finding a new compound from actinomycetes is decreasing (Berdy, 2005). However, recent reports by some scientists have revealed a new dimension in producing new bio-active compounds by co-culturing microorganisms. In modern microbiology, single-strain culture is the standard method for cultivating microorganisms. However, owing to the absence of interacting microorganisms that are present in the natural environment, the growth conditions in a flask culture are significantly different from those in the natural environment. The members of the order Actinomycetales, especially the genus *Streptomyces*, produce a number of antibiotics and other bioactive natural products. The genomic analysis of some *Streptomyces* strains revealed the presence of biosynthetic gene clusters for about 30 secondary metabolites (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008).

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However, some of these secondary-metabolite genes are not expressed in fermentation culture (Onaka *et al.*, 2011). Microbial competition for limiting natural resources within a community is thought to be the selective force that promotes biosynthesis of antimicrobial compounds (Slattery *et al.*, 2001). So, co-culture of microorganisms can be used as a promising approach to discover bioactive secondary metabolites from actinomycetes. In recent year there are several reports on co-culture of microorganisms. Co-culture of *Streptomyces endus* S-522 and *T. pulmonis* produced a new antibiotic, alchivemycin A (Onaka *et al.*, 2011). Recently, a new antibiotic-antitumor compound, glionitrin A was isolated from co-culture of the marine-derived fungus *Aspergillus fumigatus* and the marine-derived bacterium *Sphingomonas* sp. (Park *et al.*, 2009). Co-culture of the fungus *Aspergillus fumigatus* with the bacteria *Streptomyces peucetius* led to the production of N,N'-((1Z,3Z)-1,4-bis(4-methoxyphenyl) buta-1,3-diene-2,3-diyl) diformamide, which exhibited significant activity against several cell lines (Karina *et al.*, 2011).

In the present study, we report the minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and anticancer activity of a crude ethyl acetate extract obtained from the mixed fermentation of two marine *Streptomyces* sp. ANAM-5 and AIAH-10.

## MATERIALS AND METHODS

### Isolation and selection of marine actinomycetes

In this study, soil samples were carefully collected from the different location of mangrove forest Sundarbans, Bangladesh (August 2011-March 2012). Isolation of bacteria from these soil samples were done by following spread plate technique (Bernard, 2007). The collected soil samples were dried in a hot air oven at 60-65°C for about three hours to reduce the number of bacteria in the soil other than actinomycetes. For the isolation of pure actinomycetes, serial dilutions of marine soil suspensions were prepared by ten-fold dilution methods (Nonomura and Ohara, 1969).

In this method, 1 g of dried soil was suspended in 9 mL sterile water and consecutive serial dilutions were made by transferring 1 mL of aliquots to 2<sup>nd</sup> test tube containing 9 mL of sterile water and in this way dilutions up to 10<sup>-4</sup> were prepared. Uniform suspensions of the samples were made by using vortex. An aliquot of 0.1 mL of each dilution was taken and spread evenly over the surface of starch-casein-nitrate-agar medium supplemented with cycloheximide (100 µg/mL) on Petri dishes. Incubation at 30°C for seven days results in the formation of actinomycetes colonies that were recognized by their characteristic appearance.

The purified actinomycetes were preserved on yeast-extract-glucose-agar slants at 4°C. Then by using streak plate technique (Alcamo and Pomerville, 2004) on yeast extract-glucose-agar medium, all of the pure actinomycetes were preliminary screened for antimicrobial activity. Among them,

ANAM-5 and AIAH-10 were selected for this research work based on primary screening of antimicrobial activity and metabolite production.

### Fermentation and extraction of secondary metabolites of co-culture of ANAM-5 and AIAH-10

The fermentation of marine actinomycetes in liquid media is very important for the generation of secondary bioactive molecules and small scale liquid fermentation is the most useful fermentation process (Demain and Davies, 1999). Yeast-extract glucose broth media (Yeast extract 0.25 g/100 mL; Glucose 0.5 g/100 mL) was used to obtain sufficient quantity of the active secondary metabolites from mixed fermentation of ANAM-5 and AIAH-10. A loopful of the organisms of ANAM-5 and AIAH-10 were transferred from preserved slant culture to 100 mL flasks containing 60 mL sterilized yeast-extract glucose broth media (seed culture). The flasks were shaken in a rotary shaker at 220 rpm at 31°C for 2 days. 20 mL inocula of ANAM-5 and 20 mL inocula of AIAH-10 were mixed in a 500 mL conical flask containing 200 mL sterilized yeast-extract glucose broth media (co-culture). 40 mL inocula of ANAM-5 and AIAH-10 were cultured separately in 500 mL conical flasks containing 200 mL sterilized yeast extract glucose broth media (control). The flasks were then shaken on a rotary shaker (220 rpm) at 31°C for seven days. After the seven days the contents of the flasks were filtered through sterile Whatman filter paper no. 1 aseptically and the filtrate was partitioned with ethyl acetate. The ethyl acetate fraction was evaporated under reduce pressure in a rotary vacuum evaporator at 45°C until areddish orange solid mass was obtained (Haque *et al.*, 2015). After evaporation of ethyl acetate, the weight of the solid extract was determined (crude extract). The crude extract was subjected for the evaluation of antifungal and anticancer activities.

### Determination of minimum inhibitory concentration (MIC)

MIC was determined by serial tube dilution technique or turbidimetric assay against *Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevaceae* (Reiner, 1982). The test organisms were obtained from Pharmacy department, University of Rajshahi, Bangladesh. The crude extract was serially diluted in yeast-extract peptone dextrose (YPD) broth media. Various concentrations of the extract were prepared from the stock solution and 60 µl of properly diluted inoculums was added to the broth containing the extracts. The tubes were incubated aerobically at 35°C for 24-48 hours. Positive controls were prepared with respective organisms in the same culture media without the extract. After incubation, the tube with least concentration of extract showing no growth was taken as the MIC value for the respective organism.

### Determination of Minimum fungicidal concentration (MFC)

The minimum fungicidal concentration (MFC) is determined by sub-culturing the contents of the tubes of MIC showing no growth onto Sabouraud dextrose agar plates and

examining for fungal growth. If growth of fungi is observed in the Sabouraud dextrose agar plates, it indicates the presence of fungistatic agent and in this case the  $MFC > MIC$ . No growth of fungi in the plates, indicates the presence of fungicidal agent and in this case,  $MIC = MFC$ . The MFC was the lowest drug concentration that showed either no growth or fewer than three colonies (approximately 99 to 99.5% killing activity).

### Determination of anticancer activity

#### Cell growth inhibition

In vivo cancer cell growth inhibition was carried out by following the Sur *et al.*, 1994 method. Protocol used in this study for the use of mice as animal model for cancer research was approved from the Institute of Biological Sciences, University of Rajshahi, Bangladesh. The initial inoculums of EAC cells was kindly provided by the Indian Institute of Chemical Biology (IICB), located at Kolkata, India. To determine the cell growth inhibition of the bacterial metabolite, four groups of Swiss albino mice (6 in each group) weighing 20-25g were used. For therapeutic evaluation  $136 \times 10^4$  EAC cells in every mouse were inoculated into each group of mice on day "0". Treatments were started after 24 hours of tumor inoculation and continued for five days. Group one to two received the test compound at the doses of 50 mg/kg (i. p.), and 100 mg/kg (i. p.). Group three received standard bleomycin at the dose of 0.3 mg/kg (i. p.) and group four was used as control. Mice in each group were sacrificed on day six and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable tumor cells per mouse of the treated group were compared with those of control. The cell growth inhibition was calculated using the following formula:

$$\% \text{ Cell growth inhibition} = \left(1 - \frac{T_w}{C_w}\right) \times 100$$

Where,  $T_w$  = Mean of number of tumor cells of the treated group of mice and  $C_w$  = Mean of number of tumor cells of the control group of mice.

#### Survival time of EAC cell bearing mice

Survival time of mice bearing with EAC cells treated with crude ethyl acetate extract was determined by following Sur *et al.*, 1994 method. Four groups of Swiss albino mice (6 in each group) were used. For therapeutic evaluation  $136 \times 10^4$  EAC cells per mouse were inoculated in to each group of mice on day 0. Treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Tumor growth were monitored and host survival was recorded and expressed as mean survival time in days and percent increase of life span was calculated by using the following formula:

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{total number of mice}}$$

$$\text{Percent increase of life span (ILS)\%} = \left[ \frac{\text{MST of treated group}}{\text{MST of control group}} \right] - 1 \times 100$$

### Statistical analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean  $\pm$  SD ( $n=3$ ). Probability (P) value of 0.05 or less ( $P < 0.05$ ) was considered significant.

## RESULTS

### Antimicrobial activity

MIC of crude bacterial metabolite was determined by broth dilution method against three pathogenic fungi *Candida albicans*, *Saccharomyces cerevaceae* and *Aspergillus niger*. The approximate count of fungi in 60 $\mu$ l broth of *Candida albicans*, *Saccharomyces cerevaceae* and *Aspergillus niger* were  $0.17 \times 10^7$ ,  $0.13 \times 10^7$  and  $0.15 \times 10^7$  respectively. The results of MIC determination against fungi are given in the Table 1. The outcome of the result is promising. The MIC value of crude bacterial metabolite varies in between 32-64  $\mu$ g/ml. The lowest MIC value 32 $\mu$ g/ml was found against *Candida albicans*, whereas MIC value for *Saccharomyces cerevaceae* and *Aspergillus niger* was found to be 64  $\mu$ g/ml. From the result of MFC, it is evident that the extract is fungistatic not fungicidal. When the effect of the extract was removed with plating fresh media the organisms revived and were grown in culture media. The minimum fungicidal concentration ranges in between 128-256  $\mu$ g/ml. This value of MIC was lower than the values of MFC.

**Table 1:** MIC and MFC of the ethyl acetate extract against *Candida albicans*, *Saccharomyces cerevaceae* and *Aspergillus niger*.

Name of fungi	MIC ( $\mu$ g/ml)	MFC ( $\mu$ g/ml)
<i>Candida albicans</i>	32	128
<i>Saccharomyces cerevaceae</i>	64	256
<i>Aspergillus niger</i>	64	256

### Anticancer activity

#### EAC cell growth inhibition

Effects of the crude ethyl acetate extract on EAC cell growth on day six after tumor transplantation are shown in the Table 2. Treatment with bacterial metabolite resulted in maximum cell growth inhibition at the doses 100 mg/kg (i.p.) and 50 mg/kg (i.p.), as evident from 75.75% and 42.49% reduction of tumor cells respectively. Treatment at the dose of 0.3 mg/kg with bleomycin showed cell growth inhibition by 89.57%.

**Table 2:** Effect of the ethyl acetate extract on EAC cell growth inhibition.

Name of experiment	Dose in mg/kg/day (i.p.)	Number of EAC cells in mouse on day 6 after tumor cell inoculation	% of cell growth inhibition
Control (EAC cell bearing mice)	-	$(1.93 \pm 0.12) \times 10^7$	-
Bleomycin	0.3 mg/kg	$(0.20 \pm 0.03) \times 10^{7**}$	89.64
Crude extract	50 mg/kg	$(1.11 \pm 0.18) \times 10^{7*}$	42.49
	100 mg/kg	$(0.468 \pm 0.05) \times 10^{7**}$	75.75

Number of mice in each case ( $n=6$ ); the results were shown as mean  $\pm$  SD. Where significant values are  $*p < 0.05$  and  $**p < 0.01$ .

### Survival time of EAC cell bearing mice

All anticancer drugs show a significant effect on survival time of EAC cell bearing mice. The effect of bacterial metabolite at different doses has been summarized in Table 3. It has been observed that tumor induced mice treated with the test compound at doses 50 mg/kg (i. p.) and 100 mg/kg (i. p.) resulted in, increase of life span significantly, which were 49% and 71.79% respectively, when compared to that of control mice. Thus the survival time was found to be increased when the dose of the test compound was increased. Standard bleomycin increased life span by 82% when compared to control.

**Table 3:** Effect of bacterial metabolites on survival time of EAC cells bearing mice.

Name of experiment	Dose in mg/kg/day (i.p.)	Mean survival time in days	% Increase of life span (% ILS)
Control (EAC cell bearing mice)	–	19.5 ± 1.20	–
Bleomycin	0.3 mg/kg	35.5 ± 0.32	82.00***
Crude extract	50 mg/kg	29.0 ± 0.61	49.00*
	100 mg/kg	33.5 ± 0.52	71.79**

Number of mice in each case (n=6); the results were shown as mean ± SD. Where significant values are \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001.

### DISCUSSION

Marine soil is an inexhaustible resource that has been poorly explored. It is estimated that less than 1% of potentially useful chemicals from marine environment has been screened so far, with microbial products representing approximately 1% of the total number (Neidelman and Laskin, 2000). In this study, we co-cultivate two marine bacteria isolated from soils of Sundarbans, the largest tidal halophytic mangrove forest in the world to explore bioactive compounds and evaluation of their biological activities. In recently, several studies have been conducted on co-culture of microorganisms as a powerful experimental tool for inducing new bioactive compounds or enhancing the production of existing compounds.

In order to induce the accumulation of novel compounds through fungal-fungal co-cultivation, Zhu *et al.*, 2011 demonstrated that a new alkaloid designated as aspergicin (1), together with a previous secondary metabolite, neaspergillilic acid (2), and a common compound, ergosterol (3), were isolated from the mixed cultured mycelia of two marine-derived mangrove epiphytic *Aspergillus* sp. fungi. Aspergicin and neaspergillilic acid were evaluated for their antibiotic potential towards the Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Bacillus dysenteriae*, *Bacillus proteus* and against the Gram-negative *Escherichia coli*. Aspergicin exhibited the MIC of 15.62 µg/mL against *B. subtilis*, whereas the MIC of neaspergillilic acid was in the range of 0.49–15.62 µg/mL against all tested bacteria (Zhu *et al.*, 2011).

A new xanthone derivative, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether (1), was isolated from the mixed fermentation broth of two mangrove fungi (strain No. K38 and E33) isolated from the South China Sea coast. Primary

bioassays showed that compound 1 has mild antifungal activity against *Gloeosporium musae* and *Peronophthora cichoralearum* (Li *et al.*, 2011). In our study, it was found that the crude extract obtained from mixed cultivation of marine *Streptomyces* sp. ANAM-5 and AIAH-10 exhibited the MIC in the range of 32–64 µg/ml against pathogenic fungi which was similar to previous studies. This MIC value indicates that this extract has moderate antifungal activity.

In our previous studies, mixed fermentation of two mangrove endophytic fungi (strains Nos. 1924 and 3893) isolated from the South China Sea resulted in the formation of a novel 1-isoquinolone analog designated as marinamide (A) and its methyl ester (B) (Zhu and Lin, 2006). When tested for their cytotoxic effects on HepG2, 95-D, MGC832 and HeLa cells, IC50 values for marinamide (A) were in the nanomolar range whereas those of marinamidemethylether (B) were in the low micromolar range (Zhu *et al.*, 2013). In 2001 Cueto *et al.* (2001) showed that co-culture of the marine-derived fungus *Pestalotia* sp. with a likewise marine-derived unidentified Gram-negative bacterium of the genus *Thalassospira* (CNJ-328) led to the production of the new antibiotic, pestalone. Evaluating of the antibacterial activity of pestalone against MRSA and against vancomycin-resistant *Enterococcus faecium*, MIC values of 37 ng/mL and 78 ng/mL were obtained whereas the GI<sub>50</sub> of pestalone when measured in the NIH human tumor cell line screen amounted to 6.0 µM (Cueto *et al.*, 2001).

The potency of crude bacterial metabolite as anticancer agent has been judged by measuring cell growth inhibition and enhancement of life span of the EAC cell bearing mice. The efficiency of crude extract was compared with standard anticancer drug, bleomycin at the dose of 0.3 mg/kg (i. p.). Bacterial metabolite inhibits the cell growth rate effectively, 75.75% inhibition has been achieved at dose 100 mg/kg (i. p.) which is quite comparable to that of bleomycin (0.3 mg/kg). Crude bacterial extract also increased the life span of tumor bearing mice effectively.

The potency has been found to be increased with the enhancement of dose. In present study, 71.79% increased life span of tumor bearing mice at dose 100 mg/kg (i. p.) were observed with co-cultured bacterial crude metabolite. Further enhancement of the life span is therefore expected with still higher doses.

### CONCLUSION

Co-culture of bacteria could be used as an alternative way for the discovery of new antimicrobial and anticancer compounds. Although few anticancer drugs are available for the treatment of cancer but new anticancer drugs are urgently needed due to harmful adverse effects of the existing drugs. In our study we reported that our crude metabolite obtained from co-culture of two marine *Streptomyces* sp. ANAM-5 and AIAH-10 has excellent antifungal and anticancer activities. This extract might be used in infectious and cancer diseases and further research will be done for the isolation of pure compounds.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## ACKNOWLEDGEMENT

The authors would like to acknowledge the support received from Microbiology Research Laboratory, Faculty of Science, Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh.

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### How to cite this article:

Haque MU, Rahman MA, Haque MA, Sarker AK, Islam MAU. Antimicrobial and Anticancer Activities of Ethyl Acetate Extract of Co-culture of *Streptomyces* sp. ANAM-5 and AIAH-10 Isolated From Mangrove Forest of Sundarbans, Bangladesh. *J App Pharm Sci*, 2016; 6 (02): 051-055.