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In-vitro Assessment of Antimicrobial and Cytotoxic Activities of Crude Metabolites of DADA-1AIMR-24, a Bacterium Isolated from Soil in Bangladesh

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Abstract : The aim of this study was to screen antibacterial, antifungal and cytotoxic activities of crude metabolites of a bacterium DADA-1AIMR-24 isolated from soil in Rajshahi division, Bangladesh. The antibacterial activity of crude extracts was tested by agar well diffusion technique against *Bacillus cereus, Staphylococcus aureus* ATCC-259233*, Listeria monocytogenes, Agrobacterium spp.*, *Escherichia coli* FPFC-1407*, Shigella dysenteriae AL-35587, Shigella sonnei* and *Shigella boydii.* At the same time, antifungal activity was also conducted by disc diffusion method against *Aspergillus niger, Tichoderma herzanium, and Microphomina phaseolina.* The MIC values of the extract against *Bacillus cereus, Staphylococcus aureus* ATCC-259233*, E. coli* FPFC-1407*, Shigella dysenteriae AL-35587, Shigella sonnei, Shigella boydii* were 64, 64, 64, 64, 64 and 32 µg/ml respectively. The minimum bactericidal concentration (MBC) was also determined (Table 2). The MBC values of the extract were 128, 128, 128, 128, 128 and 64 µg/ml. As the MBC value was higher than the MIC value, it is clear that the extract was bacteriosatic not bactericidal. The approximate counts of bacteria in the MIC tubes were also determined (table 2). The brine shrimp lethality bioassay was conducted to determine the cytotoxic nature of crude extracts, which showed that the degree of lethality of extracts was directly proportional to the concentration (LC_{50} 21.55 µg/ml) compounds in the crude extract but due to low production of metabolites, we could not extract sufficient amounts as it took long time to produce metabolites. Further work is necessary to isolate and characterize the active compounds.

Keywords: Antimicrobial, Cytotoxic activity, Metabolites, Soil, Bangladesh

INTRODUCTION

From the beginning of civilization, natural products have played an important role in the treatment of human diseases. Natural products with medicinal value have come from various sources namely plants, microorganisms, marine organisms, and terrestrial vertebrates and invertebrates (Newman *et al.*, 2000, 2003). Among them, microorganisms have become a valuable source of producing secondary bioactive metabolites and led to the discovery of several important drugs. Microorganisms especially bacteria are the most promising sources of potent bioactive compounds (Lebar *et al.*, 2007; Fenical, W. 1993 and Laatsch, H. 2006). Bacteria producing secondary bioactive metabolites possess a wide range of biological activities such as antibacterial, antifungal, antitumor and antiviral (Schwartsmann *et al.*, 2001). Many bioactive compounds are used in the treatment of human and animal diseases (Takizawa *et al.*, 1993; Jha *et al.*, 2004).

But nowadays drug resistant and multidrug resistance of pathogens has become a global threat. Each year in the United States, more than 2 million people infect serious infections with bacteria that are resistant to one

or more of the drugs. At least 23,000 people die each year due to drug resistant infections. Many people die from other conditions that were created by drug resistant infection (Antibiotic resistance threat in the United States 2013). There is an increasing demand for bioactive compounds to treat drug resistant pathogens (Nathan, C. 2004 and Li et al., 2009). The increasing drug resistance to infectious pathogens shows that there is an emergency need for new and effective bioactive compounds with novel modes of action.

MATERIALS AND METHODS

Collection of the organism: The bacterial strain DADA-1AIMR-24 was isolated from a soil sample collected from Rajshahi, Bangladesh using isolation medium (starch casein agar nitrate medium). This strain showed a broad spectrum activity determined by plug technique and cross streaking method. For this reason, the bacterial strain DADA-1AIMR-24 was selected for further study. Then the culture was stored at 4^0C in yeast extract glucose agar slants and 20% w/v glycerol stock.

Fermentation and extraction: The small scale liquid fermentation is the widely used method for the production of secondary metabolites (Shepherd *et al*., 2010; Demain *et al*., 1999). Fermentation was carried out in yeast-extract glucose broth medium for obtaining sufficient amount of active secondary metabolites. The maximum secretion of metabolites from the strain DADA-1AIMR-24 was found at the 10^{th} day of incubation in Yeast extract glucose broth medium at 37.5°C (Sayeed, 2004). The metabolites were extracted from the fermentation broth using ethyl acetate solvent on the basis of best solubility and antimicrobial activities. The ethyl acetate was evaporated under reduced pressure in a rotary vacuum evaporator at 45^0C until a greenish solid mass was obtained (Suthindhiran *et al*., 2009). After evaporation of ethyl acetate, the solid crude extract was collected from rotary evaporator. Then the crude extract was subjected to screening for antibacterial, antifungal and cytotoxic activities.

Antibacterial activity: The antibacterial activities of the crude extract of DADA-1AIMR-24 were determined by disc diffusion method (Bauer *et al*., 1966). Nutrient agar was sterilized in a flask at 121^0C and cooled to 35-45 °C and then poured into sterilized petridishes with a diameter of 90 mm. The filter paper discs (5 mm in diameter) were impregnated with the crude extract at 100 µg/disc and then placed onto the previously inoculated agar plates with the test bacteria. Kanamycin was used as standard (30 µg/disc). The petridishes were kept at 4 °C for 2 h. Then the plates were incubated at 37 °C for 24 hrs to allow the growth of test bacteria. The diameter of the zone of inhibition was measured using a millimeter scale.

Antifungal activity: The antifungal activities of the crude extract were also tested by disc diffusion method against test fungi (Vinayaka *et al*., 2009). In this study, 48 hours old potato dextrose broth cultures of test fungi were swabbed uniformly on solidified sterile potato dextrose agar plates using sterile cotton swab. Then, disc of 5 mm diameter was impregnated with crude extract and nystatin at 100 µg/disc and 30 µg/disc respectively, and then placed onto the previously inoculated potato dextrose agar plates with the test fungi. After that the plates were incubated at 37 °C for 24 hours and finally the zone of inhibition around the disc was measured with a millimeter scale.

Minimum inhibitory concentration (MIC) determination: The MIC of the extract was determined (Table 2) by serial tube dilution method (Reiner 1982; Tyler *et al*., 1988) against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Shigella dysenteriae, Shigella sonnei* and *Shigella boydii.* The minimum bactericidal concentration (MBC) was also determined by sub-culturing the contents of the tubes of MIC after adding 5 ml of nutrient broth medium.

Test organisms: In this study, we used eight test bacteria namely *Bacillus cereus*, *Staphylococcus aureus* ATCC-259233, *Listeria monocytogenes, Agrobacterium spp., Escherichia coli* FPFC-1407*, Shigella dysenteriae* AL-35587*, Shigella sonnei and Shigella boydii.* The test fungi were *Aspergillus niger, Tichiderma viridae and Microphomina phaseolina*. The test bacteria were previously collected from International Centre for Diarrheal Disease Research, Bangladesh by Pharmacy Department at Rajshahi University and the test fungi were

collected from "Botany Department" at Rajshahi University. All reference bacterial and fungal cultures were sub-cultured on nutrient agar and potato-dextrose agar media, respectively. The bacterial slants were incubated overnight at 37ºC, and the fungal slants were incubated for 48 h at 37ºC.

Brine shrimp lethality bioassay: In this study, brine shrimp lethality bioassay (Mayer *et al.*, 1982; Mclaughlin and Anderson, 1988; Mclaughlin, 1992; Persoone, 1988) technique was used to determine the cytotoxic prpperties of the crude extracts. *Artemia salina*, the brine shrimp were used to determine the cytotoxicity of the crude extract obtained from DADA-1AIMR-24 (Ullah *et al.,* 2013). The eggs were collected from "Biochemistry and Molecular Biology Department" at Rajshahi University, Bangladesh and hatched in seawater. A continuous oxygen supply was maintained throughout the process. In this study, mature nauplii were used. After 36-48 hours, the phototropic shrimps were collected by pipette. 10 mg of crude extract was accurately weighed and dissolved in 1 ml DMSO. Thus the concentration of 10 mg/ml was obtained and used as stock solution. Then the different concentrations of extract (10, 20, 40, 80, 100 µg/ml) were made. For each concentration, vials containing the same volume of DMSO plus seawater were used as control. Then 10 brine shrimp nauplii were applied to each of all experimental vials and control vial. The vials were then incubated at 25^0C and surviving shrimps were counted after 24 hours with the help of magnified glass. Finally, from this data, percentage of lethality of the brine shrimp was calculated and LC_{50} of the sample was also determined.

RESULTS

Fermentation and Extraction: In this study, a total of twenty-six bacterial strains were isolated from soil of Rajshahi, Bangladesh. Among them DADA-1AIMR-24 was selected due to its strong antimicrobial activities. The small scale liquid fermentation was carried out in yeast extract glucose broth medium because the isolate DADA-1AIMR-24 gave relatively greater production of secondary metabolites in this medium than others.

Antimicrobial Activity: The crude extract isolated from DADA-1AIMR-24 showed potent antimicrobial activity against test organisms (Table 1). The result shows that the crude extracts are more active against gram negative bacteria than gram positive bacteria. The MIC and MBC values of the extracts against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Shigella dysenteriae, Shigella sonnei and Shigella boydii* were determined (Table 2). The extract was also active against *Aspergillus niger, Tichoderma viridae, Tichoderma herzanium, Microphomina phaseolina* and the minimum antifungal concentration values were 16,16,8 and 32 µg/ml respectively.

Brine Shrimp Lethality Test: Brine shrimp lethality bioassay was conducted to determine the cytotoxicity of crude extract (Table 4). Crude extract showed different mortality rate at different concentration. With the increasing of concentration of test sample, the mortality rate of brine shrimp larvae was increased. 100% mortality rate of nauplii was recorded at the concentration above 100 gµ/ml. The median lethal concentration (LC_{50}) of the extract was found to be 21.56 µg/ml.

Table 1. Antibacterial activity of crude extracts against a series of test bacteria.

Test organisms	Zone of inhibition (in mm)	
	Crude extract (100µg/disc)	Kanamycin (30 µg/disc)
Gram positive bacteria		
Bacillus cereus	30	20
Staphylococcus aureus	15	12
Listeria monocytogenes	10	11

Agrobacterium spp.	30	20
Gram negative bacteria		
Escherichia coli	25	16
Shigella dysenteriae	30	20
Shigella sonnei	30	20
Shigella boydii	25	15

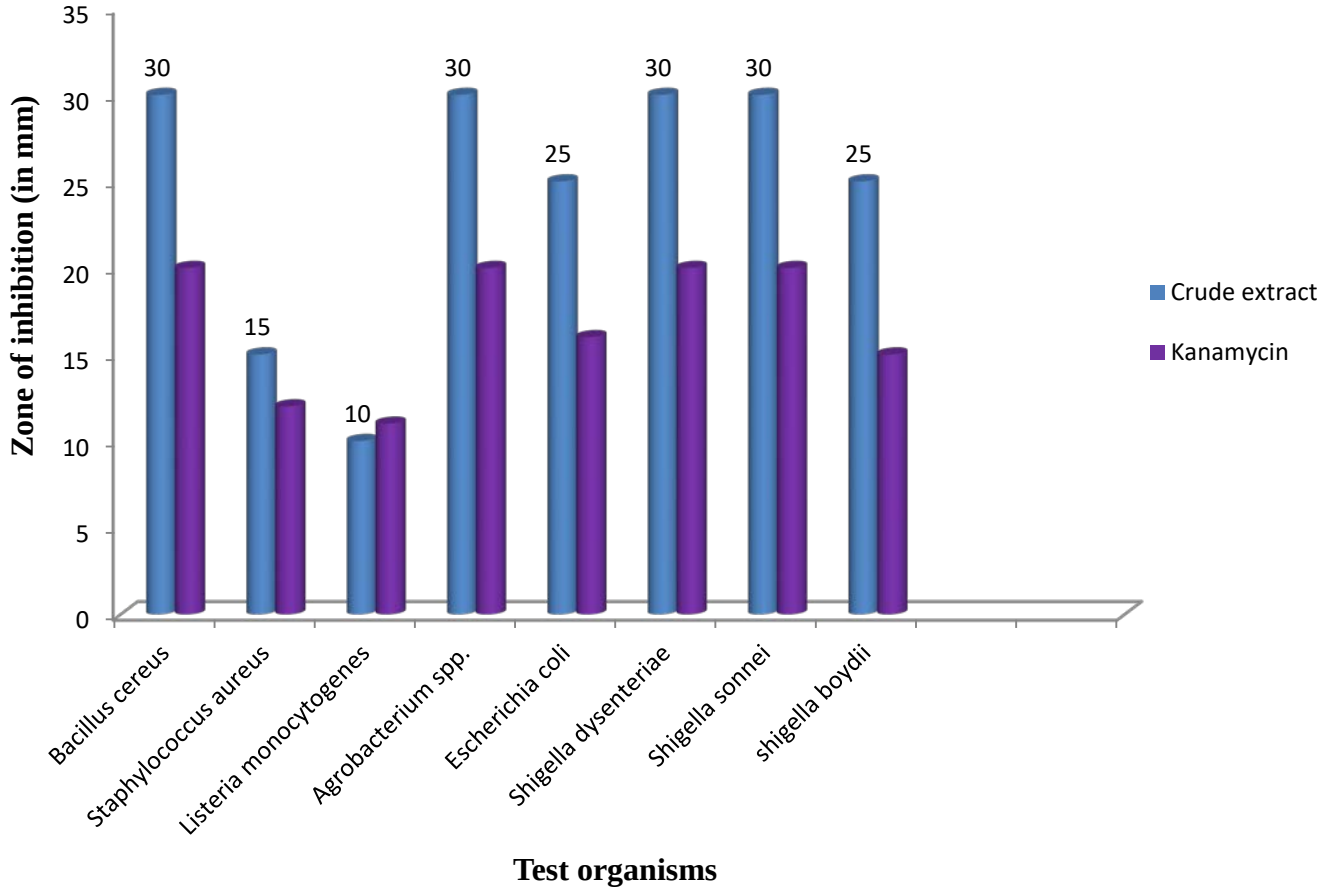

Fig. 1. Antibacterial activity of crude extracts against different test organisms.

The concentration of Kanamycin and crude extract were 30 µg/disc & 100 µg/disc respectively.

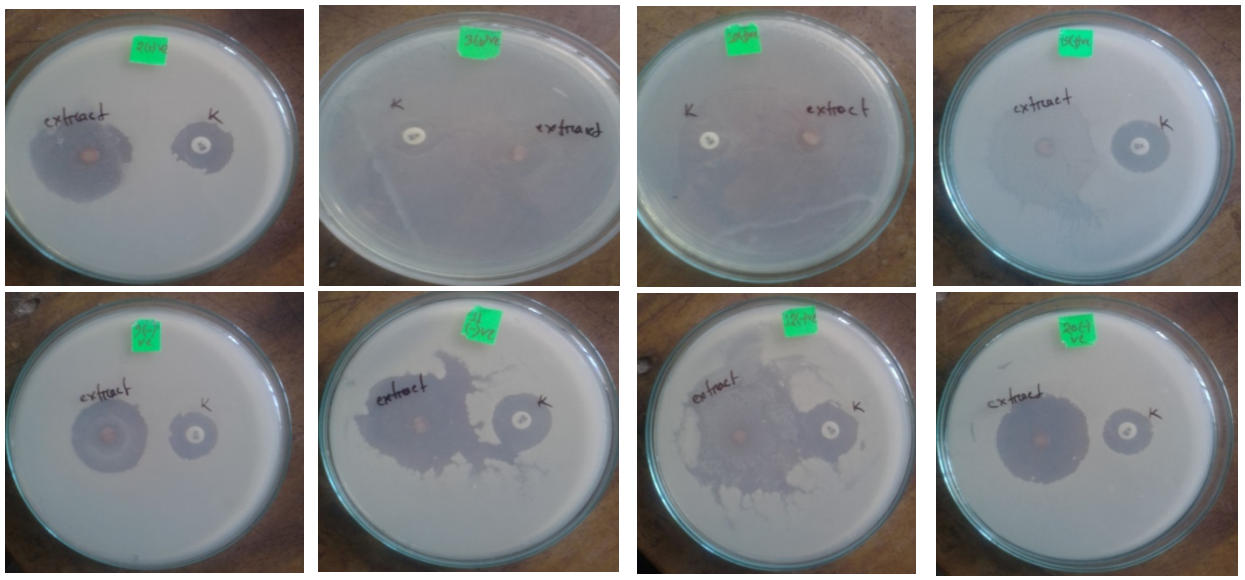

Fig. 2. Antibacterial activity test of crude extracts against A. *Bacillus cereus,* B. *Staphylococcus aureus,* C. *Listeria monocytogenes,* D. *Agrobacterium spp.* E. *Escherichia coli,* F. *Shigella dysenteriae,* G. *Shigella sonnei*, H. *Shigella boydii*.

Table 2. Results of MIC and MBC Values (in µg/ml)

Name of bacteria	Approximate count in 60 µl at 12 hr fresh culture	MIC values of crude extract (µg/ml)	MBC values of crude extract (µg/ml)
Bacillus cereus	0.35×10^6	64	128
Staphylococcus aureus	0.30×10^6	64	128
Escherichia coli	0.40×10^6	64	128
Shigella dysenteriae	0.25×10^6	64	128
Shigella sonnei	0.21×10^6	64	128
Shigella boydii	0.19×10^6	32	64

Table 3. Antifungal screening of crude extracts of DADA-1AIMR-24.

Test fungi	Zone of inhibition (in mm)	
	100 µg extract/disc	Nystatin 30 µg/disc
Aspergillus niger	10	14
Tichoderma herzanium	10	10
Microphomina phaseolina	10	10

The result shows that the crude extracts have antifungal activity against *Aspergillus niger, Tichoderma herzanium, and Microphomina phaseolina.*

Table 4. The result of brine shrimp lethality bioassay of crude extracts of DADA-1AIMR-24.

Group	Conc. of sample (µg/ml)	LogC	No. of nauplii added	No. of death in each vial			Average Number of death	Mortality (%)	$LC_{50.0}$(µg/ml)
				1	2	3			
Control	20µl DMSO	0	10	0	0	0	0.00	0.00	0.00
Crude Extract	2.5	0.3979359	10	1	2	1	1.33	13.3	21.55582
	5	0.6989628	10	2	1	2	1.67	16.7	
	10	0.9999897	10	3	2	2	2.33	23.3	
	20	1.301017	10	4	3	4	3.67	36.7	
	40	1.602043	10	7	8	6	7.00	70.0	
	80	1.90307	10	8	8	9	8.33	83.3	
	100	1.999979	10	9	9	9	9.00	90.0	

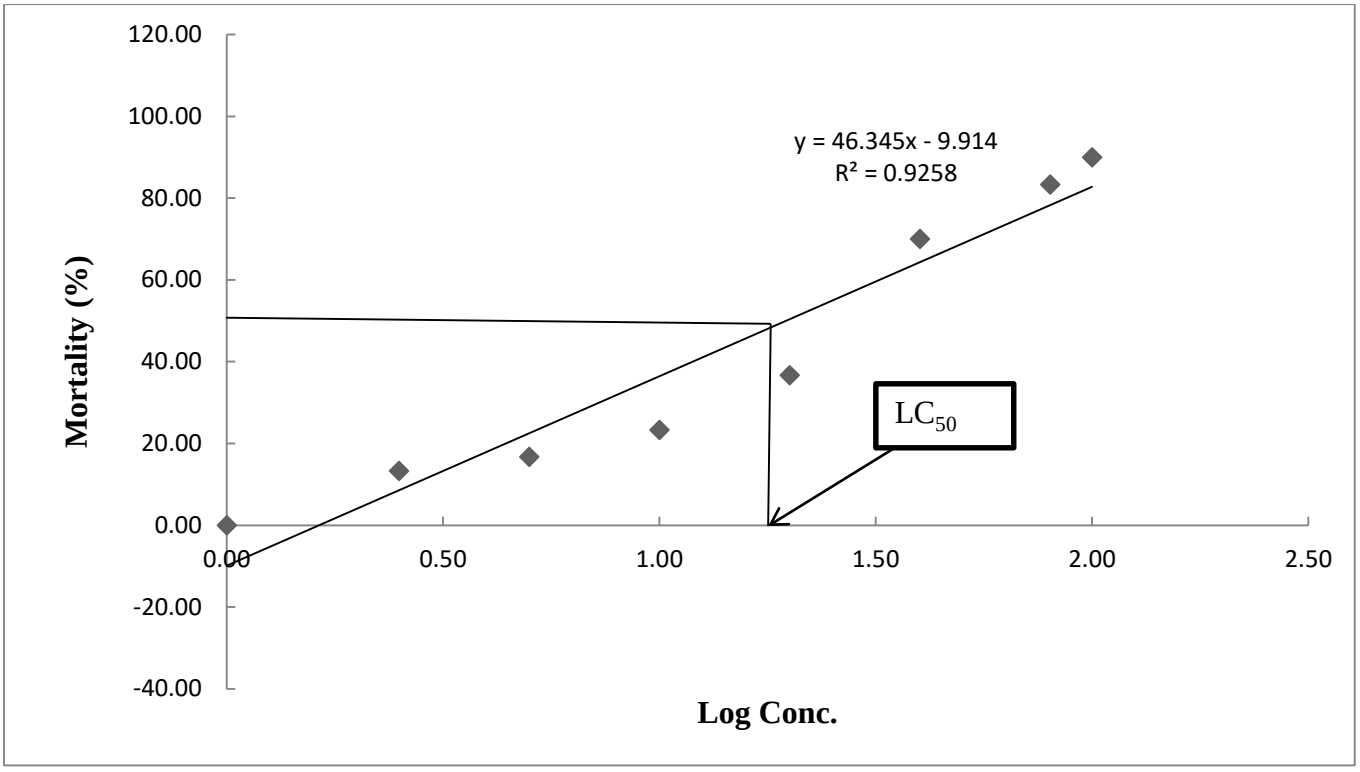

Fig. 3. Determination of LC_{50} of crude extracts against brine shrimp larvae.

Discussion

This study was designed with the intention of discovery of bacterial strain producing bioactive metabolites. The extract of this strain possesses strong antimicrobial and cytotoxic activity. The potential cytotoxicity of crude extract indicates that new bioactive compounds may present the extract having antitumor of anticancer activities. Due to low production of crude extract, we could not separate the active components. However, further works are necessary for increasing the yield of secondary metabolites. Finally, from the current study, it could be noted that a vast stretch of bioactive compounds producing bacteria present in the soil of Bangladesh that is still unexplored. Extensive study should be carried out to explore more bioactive compounds from this potential source of bacterial strain.

Conclusion

Drug resistance is one of the major problems for treating infectious diseases with presently available antibiotics. The U.S centre for disease control and prevention (CDC) considered antibiotic resistance as one of the world's most pressing public health problems. Many known pathogenic organisms become drug resistance due to known or unknown causes. It is believed that the misuse and overuse of antibiotics are the major causes of emergence of antibiotic resistance in human medicine. This study suggests that the antimicrobial activities of crude extracts of DADA-1AIMR-24 are most encouraged due to strong inhibition of pathogenic organisms. However, further study should be done to isolate the active compound responsible for above activities.

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