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In vitro Antimicrobial Activities and Cytotoxicity of Ethyl Acetate Extract from *Streptomyces maritimus*

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Abstract: Ethyl acetate extract from new actinomycetes, *Streptomyces maritimus*, showed good antibacterial and antifungal activities against a total of 14 bacteria (5 Gram positive plus 9 Gram negative) and 8 fungi. The Minimum Inhibitory Concentrations (MIC) were determined and found to be 16 μg mL^{-1} against *Bacillus subtilis*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Aspergillus flavus* while 32 μg mL^{-1} against *Salmonella typhi*, *Candida albicans* and *Aspergillus niger*.

Key words: *Streptomyces maritimus*, antimicrobial activity, cytotoxicity

INTRODUCTION

The frequency of life threatening infections such as diarrhea, tuberculosis, acute respiratory tract infections, cancer and recently AIDS caused by various pathogenic microorganisms is increasing worldwide especially in developing countries and is becoming an important cause of morbidity and mortality in immunocompromized patients (Black *et al.*, 1982; Walsh and Warren, 1974; Pelczer, 1986). Although huge numbers of antibiotics have been discovered, these antibiotics are developing resistance to the pathogenic organism day by day.

The growth in antibiotic usage globally has been paralleled by the ability of bacteria to resist being killed by these agents and has resulted in a steady decline in the number of effective antibiotic each year. Spontaneous mutations of bacterial DNA, which confers resistance to a particular class of antibiotic, are estimated to occur in every year 10^5-10^{10} generations (Scientific American. Com, 2005). These novel genes can then shared with other bacteria following transfer of plasmids by conjugation, transformation or transduction. As its most extreme, the acquisition of antibiotic resistance genes has resulted in at least four species of bacteria for which there are no effective forms of conventional therapy available. Methicillin resistant *Strephylococcus aureus* (MRSA), Vancomycin Resistant Entarococcus feaculis (VRE), *Mycobacterium tuberculosis* and *Pseudomonus aeruginosa* dubbed superbugs by the media and between them accounts for the deaths of many people in the world (Sanderson, 1984). In order to combat these infections, new antibiotics with high potent will need to be developed.

Among the antibiotics developed from the microorganisms, the actinomycetes, particularly the genus *Streptomyces*, is reported to produce a number of well known antibiotics (Dictionary of Natural Products (DNP) CD-ROM, 2001; ISIC database, 2004) including streptomycin, neomycin, tetracycline and chloramphenicol (Waksman and Woodruff, 1940; Dienstag and Nue, 1972; Argoudelis *et al.*, 1987; Jabbar *et al.*, 1998; Biswas *et al.*, 2000; Jabbar *et al.*, 1999) from antagonistic organism, by screening a number of soil sample in Bangladesh, we isolated an actinomycetes, *Streptomyces maritimus* from a soil sample collected in the region of northern parts of Bangladesh (Sayeed, 2004). We herein report the antimicrobial activities of the ethyl acetate extract from its cultural broth against a number of bacteria (both Gram positive and Gram negative) and fungi.

MATERIALS AND METHODS

The present study was carried out during January-June 2004 in Pharmaceutical Microbiology Laboratory, Department of Pharmacy, Faculty of Science, University of Rajshahi, Rajshahi-6205, Bangladesh.

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Organism: The organism was isolated from soil sample, collected from a cultivated land of Northern part of Bangladesh, at a depth of 1 m; by using crowded plate technique (Hammond and Lambert, 1978). The organism was identified as *Streptomyces maritimus* strain BD26T (GenBank accession number AF233338) previously described (Piel *et al.*, 2000; Xiang and Moore, 2003) on the basis of its similar morphological, physiological, biochemical, antimicrobial activities and 16S rDNA sequencing analysis data (Sayeed, 2004).

Extraction: The maximum secretion of metabolites from the strain was found at the 14th day of incubation in yeast extract sucrose agar (alkaline pH8.6) medium at 37.5°C by maintaining all the physicochemical factors at optimum level for the culture (Sayeed, 2004). The extraction of the metabolites was carried out by ethyl acetate on the basis of best solubility and maximum antimicrobial activities. The solvent, ethyl acetate was evaporated using a rotary evaporator at 40°C under reduced pressure. On average, 1 L of culture filtrate gave 2.5 mg of crude extract.

Antimicrobial assay: Test pathogenic microorganisms employed for *in vitro* antimicrobial assay were obtained from the Institute of Nutrition and Food Science (INFS), University of Dhaka and International Center for Diarrhea Disease and Research, Bangladesh (ICDDRB) Dhaka. The antimicrobial assay of ethyl acetate extract was performed against a various pathogenic bacteria (Gram positive and Gram negative) and a number of fungi by standard disc diffusion technique (Bauer *et al.*, 1966; Barry, 1980). The sample solution of the extract to be tested was prepared by dissolving a definite amount of material in ethyl acetate to attain the desired concentrations (200, 20 μg^{-1} disc for fungi and 100,30 μg^{-1} disc for bacteria). Sample solutions of desired concentrations were applied on the sterilized filter paper discs (5mm in diameter) with the help of a micropipette in an aseptic condition and allowed to leave these discs for a few minutes in the aseptic hood for complete removal of the solvent. To compare the antibacterial and antifungal activities, kanamycin (30 μg disc^{-1}) and nystatin (20 μg disc^{-1}) were used as standard antibiotics respectively. As a negative control, a blank disc impregnated with solvent followed by drying off was used.

A total of five Gram positive and nine Gram negative bacteria (Table 1) and four pathogenic fungi (Table 2) were used in this antimicrobial screening. Briefly, in this study, the test discs, standard discs and blank discs were placed in a petridish with a particular bacteria or fungi and then left in a refrigerator at 4°C for 12-18 h in order to diffuse the material from the discs to the surrounding media in the petridishes. The petridishes were then incubated at 37°C for overnight to allow the bacterial growth and 48-72 h for fungal growth. The antibacterial and antifungal activities of the extract were then determined by measuring the respective zones of inhibition in mm.

The Minimum Inhibitory Concentrations (MICs) of the crude extract against *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae*, *Candida albicans*, *Aspergillus flavus* and *Aspergillus niger* were determined by serial dilution technique (Reiner, 1982; Noble *et al.*, 1977).

Table 1: Antibacterial activity of the ethyl acetate extract

	Diameter of zone of inhibition (mm)		
Test organisms	100 μg disc^{-1}	30 μg disc^{-1}	Kanamycin 30 μg disc^{-1}
Gram positive bacteria			
Staphylococcus aureus	17	11	20
Streptococcus-β-haemolyticus	18	9	19
Bacillus megaterium	19	14	21
Bacillus subtilis	18	11	16
Sarcina lutea	17	8	18
Gram negative bacteria			
Salmonella typhi	25	10	18
Shigella dysenteriae	20	9	19
Shigella shiga	20	13	22
Shigella flexneri	20	12	18
Shigella sonnei	18	8	19
Shigella boydii	24	13	30
Escherichia coli	23	12	24
Pseudomonas aeruginosa	29	12	31
Klebsiella species	24	9	24

Table 2: Antifungal activity of the ethyl acetate extract

	Diameter of zone of inhibition (mm)	
Test fungus	ethyl acetate extract (200 μg disc^{-1})	Nystatin (20 μg disc^{-1})
Candida albicans	20	22
Aspergillus fumigatus	15	19
Aspergillus flavus	18	23
Aspergillus niger	20	24
Epidermophyton floccosum	14	21
Trichoderma species	18	30
Fusarium species	17	26
Bipolavis species	15	24

Cytotoxicity screening: The cytotoxity activity of the ethyl acetate extract was determined by brine shrimp lethality bioassay (Mayer *et at.*, 1982; Mclaughlin and Anderson, 1988; Persoone, 1988; Mclaughlin, 1992). In this method, the eggs of the brine shrimp, *Artemia salina* Leach, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched for 48 h to mature shrimp. Thirty eight grams of sea salt was weighed, dissolved in 1 L of distilled water, filtered off and was kept in a small tank. The eggs were then added to the divided tank. Constant oxygen supply was provided and temperature (37±1°C)

was maintained for 48 h to hatch and mature the shrimp called as nauplii (larvae). The test sample extract was prepared by dissolving them in DMSO (not more than 50 μL in 5 mL solution) plus sea water (3.8% NaCl in water) to attain concentrations-5, 10, 20, 40 and 80 μg mL^{-1} A vial containing 50 μL DMSO diluted to 5 mL was used as a control. Then about 10 brine shrimp nauplii were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 h was counted. The findings were presented graphically by plotting log of concentration versus percentage of mortality of nauplii from which LC_{50} was determined by extrapolation (Goldstein *et al*., 1974).

Statistical analysis: Statistical analyses of the antibacterial and antifungal activities of compounds with different concentrations of each (20, 30, 100 and 200 μg $disc^{-1}$) was performed using Kruskal-Wallis test (Debnath and Shil 2001). Individual antibacterial and antifungal activity differences of the tested compounds was examined using post hoc Nemenyi's test following Kruskal-Wallis test. A significance level of 5% was considered as significance ($p<0.05$) in all cases. Probit analysis (Finney, 1971) was used to determine the LD_{50} values from the mortality data using Probit software. The cytotoxicity of the novel ethyl acetate extract was compared with the standard gallic acid and also with the anticancer agent vincristine sulfate. Determination of LD_{50} by probit analysis allowed the ranking of the extract with respect to their biocidal activity.

RESULTS

The results of antibacterial and antifungal screenings in terms of zone of inhibition in mm are presented in Table 1, respectively. All the Gram positive and Gram negative bacteria showed a remarkable sensitivity towards the crude extract. However, better antibacterial activities were observed against *Staphylococcus aureus*, *Streptococcus*-β-*haemolyticus*, *Salmonella typhi*, *Shigella dysenteriae*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Klebsiella* species. Among the fungi tested the crude ethyl acetate extract showed good antifungal activities against *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* and *Trichoderma* species.

The Minimum Inhibitory Concentrations (MICs), observed by serial dilution technique (Reiner, 1982) were found to be 16 μg mL^{-1} against *Bacillus subtilis*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Aspergillus flavus* while 32 μg mL^{-1} against *Salmonella typhi*, *Candida albicans* and *Aspergillus niger*. However, further work is necessary in order to isolate active antimicrobial compounds from this organism.

The cytotoxicity activity of the extract and standard, vincristine sulfate on brine shrimp nauplii is presented in Table 3 and 4. The 50% mortality (LC_{50}) of the extract and standard, vincristine sulfate was found to be 14.125 and 0.758 μg mL^{-1}. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality (Goldstein *et al*., 1974) was plotted on the graph paper and the result was obtained by extrapolation from the graph. Although there was no mortality in the control group, the test sample showed different mortality rate at different concentrations, which was found to increase with increasing concentration of the sample. It is evident that the test material was moderately lethal to brine shrimp nauplii but less toxic than the standard compound (Biswas *et al*., 2002). The cytotoxic action of a drug is exhibited by disturbing the fundamental mechanisms concerned with cell

Table 3: The cytotoxic effect of ethyl acetate extract and ampicillin trihydrate

Test sample	Conc. (μg mL^{-1})	Log of Conc.	No. of Napulii taken	No. of Napulii dead	No. of Napulii alive	% of mortality	LC_{50} μg mL^{-1}
Ethyl acetate extract	5	0.699	10	1	9	10	14.125
	10	1.0	12	3	3	25	
	20	1.301	10	4	6	40	
	40	1.604	11	8	6	73	
	80	1.903	14	14	0	100	
	0.625	-0.202	10	5	5	50	
Vincristine sulfate	1.25	0.097	10	6	4	60	0.758
	2.5	0.398	10	7	3	70	
	5.0	0.699	10	8	2	80	
	10	1.0	10	9	1	90	
	20	1.301	10	10	00	100	

Table 4: The results of cytotoxic effect of ethyl acetate extract and standard vincristine sulphate and gallic acid

Test samples	LC_{50} (μg mL^{-1})	95% confidence limit (μg mL^{-1}) Lower	Upper	Regression equation	χ^2 (df)
Ethyl acetate extract	14.125	11.94	14.67	Y = 3.878+2.368 X	3.30 (2)
Vincristine sulfate	0.758	0.57	0.82	Y = 3.163+2.989 X	0.62 (2)

growth, mitotic activity, differentiation and function (Goodman *et al*., 1980). Although the exact mechanism of cytotoxic action of this drug could not be explained by these preliminary tests, it may be commented that the results obtained indicate that the compound may be a safe and effective antibiotic. However, further tests to assess the antimicrobial and cytotoxic effectiveness of the extract are needed to establish the compound as a potent antibiotic.

DISCUSSION

Compared to standard antineoplastic agents such as cisplatin, doxorubicin, mitoxantrone and vinblastine were found to exhibit higher cytotoxicity in renal cell carcinoma (Kurbacher *et al*., 1994). Therefore it is of our interest to explore some novel compound as potent cytotoxic agents which might come as potent anticancer agent in clinical trials. In the present investigations we found the ethyl acetate extract with potent antimicrobial agent and had moderate cytotoxicity. The different LC_{50} values for the extracts indicated the different mode of actions of their cytotoxicity. Further investigations are required to explore the exact mechanism of their cytotoxic properties which may be helpful for to explore new type of potent cytotoxic agent (s) with the hope of adding new and alternative chemotherapeutic agent (s) in clinical implications.

The ethyl extract displayed poor antibacterial activity at the concentration of 30 μg $disc^{-1}$, but gave promising activity at concentrations of 100 μg $disc^{-1}$. The MIC values of this complex against the tested organisms indicated their noticeable antibacterial and antifungal potencies compared with standard antibiotic, kanamycin and nystatin respectively. The mechanism of biocidal activity of these compounds may be due to oxidative DNA damage as the previous reports (Vijayalakshmi *et al*., 2002; Joudah *et al*., 2002). The different antibacterial activity of the complexes indicated their different mechanism of biocidal property and further studies are required to explore the exact mechanism of antibacterial potency (Domarle *et al*., 1998).

It was concluded that the extract possesses substantial antimicrobial activity with a minimum inhibitory concentration and moderate cytotoxicity. Further, acute toxicity and other pharmacological tests are necessary to utilize the extract as a potential therapeutic agent.

REFERENCES

Argoudelis, A., D.L. Baczyneky, M.T. Duo, A.L. Laborde, O.K. Sebek, S.K. Truesdel and F.B. Shilliday, 1987. *In vitro* studies of arginomycin and its biodegradation products. J. Antibiotics, 40: 750-760.

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by standardized single disc method. Am. J. Clin. Pathol., 44: 493-496.

Barry, A.L., 1980. Procedure for Testing Antimicrobial Agents in Agar Media. In: Antibiotic in Laboratory Medicine. Lorin, V. (Ed.), Williams Wilkin's Co. Baltinore, USA., pp: 1-23.

Biswas, M.H.U., A.R.M.R. Amin, M.A. Isalm, C.M. Hasan, K.R. Gustafson, M.R. Boyd, L.K. Pannell and M.A. Rashid, 2000. Monocillinols A and B, novel fungal metabolites from a *Monocillium* sp. Tetrahedron Lett., 41: 7177-7180.

Biswas, M.H.U., S.A.H. Zakaria, A. Farooque, C.M. Zakaria, S.M. Zakir, S. Sadik and S.M.A. Bhuiyan, 2002. *In vitro* antibacterial and cytotoxic activity of a benzene sulfonic acid derivative complex compound. Bangladesh Pharm. J., 1: 43-46.

Black, R.E., K.D. Brown, S. Becker and M. Yunus, 1982. Longitudinal studies of infectious diseases and physical growth of children in rural Bangladesh. Am. J. Epidemiol., 115: 305-314.

Debnath, S.C. and R.N. Shill, 2001. An introduction of the theory of statistics. Jahangir Press, Dhaka, Bangladesh, pp: 499-503.

Dictionary of Natural Products (DNP) CD-ROM, 2001. Version 9: 2. Chapman and Hall-CRC, Boca Raton, USA.

Dienstag, J. and H.C. Nue, 1972. *In vitro* studies of tobramycin and aminoglycoside antibiotic. J. Antimicrob. Agents Chemother., 1: 41-45.

Domarle, O., G. Blampain, H. Agnaniet, T. Nzadiyabi, J. Lebibi and J. Brocard, 1998. *In vitro* antimicrobial activity of a new organometallic analogue: Ferrocene-chloroquine. Antimicrobial Agents and Chemother., 42: 540-544.

Finney, D.J., 1971. Probit Analysis, 3rd Edn., Cambridge, University Press, UK., pp: 18-37-77.

Goldstein, A., L. Arnow and S.M. Kalkan, 1974. Principles of Drug Action. 2nd Edn., Wiley Biomdical Health Publication, pp: 376-381.

Goodman, L.S., A.G. Gilmann and A. Gilmann, 1980. Antiproliferative Agents and Immunosuppressive Drugs. The Pharmacological Basis of Therapeutics. Macmillan Publishing Co., Inc., USA,. 6th Edn., pp: 1299-1313.

Hammond, S.M. and P.A. Lambert, 1978. Antimicrobial actions. Edward Arnold Ltd. London, pp: 4-9.

ISIC Database, 2004. Institute for Scientific Information, UK. Available on-line through Web of Science at: http://wos.mimas.ac.uk/>.

Jabbar, A., A.P. Shresta, M.A. Rashid, M. Shammem and S. Yahada, 1998. Dehydroaltenusinic acid-a novel microbial metabolites from *Streptomyces* sp. Nat. Prod. Lett., 12: 311-316.

Jabbar, A., A.P. Shresta, C.M. Hasan and M.A. Rashid, 1999. Anti-HIV activity of dehydralterusin, a metabolites from *Streptomyces* sp. Nat. Prod. Sci., 5: 162-164.

Joudah, L.S., Moghaddas and R.N. Bose, 2002. DNA oxidation by peroxo-chromium(v) species: Oxidation of guanosine to guanidinohydantoin. Chem. Commun., 21: 1742-1743.

Kurbacher, C.M., W. Nagel, P. Mallmann, J.A. Kurbacher, G. Sass, H. Hubner, P.E. Andreotti and D. Krebs, 1994. *In vitro* activity of titanocenedicholoride in human renal cell carcinoma compared to conventional antineoplastic agents. Anticancer Res., 14: 1529-1533.

Mclaughlin, J.L. and J.E. Anderson, 1988. Brine shrimp and crown gall tumors; simple bioassays for the discovery of plant antitumor agents. Proceedings NIH workshop. Bioassays for Discovery of Antitumor and Antiviral Agents from natural Sources, Bethesda, pp: 18-19-220.

Mclaughlin, J.L., 1992. Bench-top bioassays for the discovery of bioactive compounds in higher plants. Brenesia, pp: 220.

Meyer, B.N., N.R. Ferrigni, J.E. Putnam, L.B. Jacobsen, D.E. Nichols and J.L. Mclaughlin, 1982. Brine Shrimp: A convenient bioassay for active plant constituents, Plant. Medica., 45: 31-34.

Noble, M.D. and R.B. Sykes, 1977. G 7063-2 a new nitrogen containing antibiotic of the epoxydon group isolated from fermentation broth of a species of *Streptomyces.* J. Antibiotics, 30: 4.

Pelczer, J., C.S. Chan and R. Kreig, 1986. Microbiology 5th Edn., McGrow Hill Book Company, pp: 511-515.

Persoone, G., 1988. Proceeding of the International Symposium on Brine Shrimp, Artemia salina, Vol. 1-3. Universa Press, Witteren, Belgium.

Piel, J.C. Hertweck, P.R. Shipley, D.M. Hunt, M.S. Newman and B.S. Moore, 2000. Cloning, sequencing and analysis of the enterocin biosynthesis gene cluster from the marine isolate *Streptomyces maritimus*: Evidence for the derailment of an aromatic polyketide synthase. J. Chem. Biol., 7: 943-55.

Reiner, R., 1982. Detection of antibiotic activity. Antibiotics, An Introduction. Roche Scientific Service, Switzerland, 2: 21-25.

Sayeed, M.A., 2004. Studies on Marine Bacterium, *Streptomyces maritimus*; Characterization of *Streptomyces tendae* and a new Streptomyces species, *Streptomyces chittagongensis*. M. Pharm. Thesis, University of Rajshahi, Baangladdesh.

Sanderson, P.J., 1984. Common bacterial pathogens and resistance to antibiotics. Br. Med. J., pp: 638-639.

Scientific American. Com: http://www. sciam.com/article. cfm. New Antibiotics Successful against Superbugs, August 30, 2005.

Vijayalakshmi, R.V., Subramanian and B.U. Nair, 2002. A study of the interaction of Cr (III) complexes and their selective binding with B-DNA: A molecular modeling approach. J. Biomol. Struct. Dyn., 19: 1063-1071.

Walsh, J.A. and K.S. Warren, 1974. Selective Primary Health Care: An interim strategy for disease control in developing countries. N Eng. J. Med., 301: 367-370.

Waksman, S.A. and H.B. Woodruff, 1940. Bacteriostatic and bactericidal substance produced by a soil Actinomyces. Proc. Soc. Exp. Biol. Med., 45: 609-614.

Xiang, L. and B.S. Moore, 2003. Characterization of Benzoyl Coenzyme A Biosynthesis Genes in the Enterocin-Producing Bacterium *Streptomyces maritimus*. J. Bacteriol., 185: 399-404.