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Cytotoxicity Assay of Secondary Metabolites produced from Mould Fungi: Penicillium spp

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Abstract: Emerging infectious diseases and the increase in incidence of drug resistance among pathogenic bacteria have made the search for new antimicrobials inevitable. Despite the increased knowledge of microbial pathogenesis and application of modern therapeutics, the morbidity and mortality associated with the microbial infections remains high. Therefore, there is a demand in discovering novel strategies and identify new antimicrobial agents to develop the next generation of drugs or agents to control microbial infections. Fungi produce a wide range of secondary metabolites with high therapeutic value as antibiotics, cytotoxic substances, and insecticides compounds that promote or inhibit growth and are gaining importance for their biotechnological applications. The present research work deals with the synthesis of secondary metabolites from one mould fungi, *Penicillium* spp culture filtrate and to evaluate the effect of bioactive compounds against selected bacteria pathogens. The antimicrobial activities of compounds were also tested by adding 15µl of compounds in antibiotic discs. The cytotoxicity studies of crude bioactive compound from *Penicillium* spp showed that L2 had the least inhibition on cell viability and a drastic decrease in the cell viability was recorded when the concentration of crude extract U3 and U4 increased beyond 50ug/ml.
Keywords, *Penicillium* spp, Bioactive compounds, Antimicrobial activity and Cytotoxicity.

Introduction

The increased use of antibacterial and antifungal agents in recent years has resulted in the development of resistance to these drugs. The significant clinical implication of resistance has led to heightened interest in the study of antimicrobial resistance from different angles. Endophytes provide an abundant reservoir of bioactive metabolites for medicinal exploitation and an increasing number of novel compounds are being isolated from endophytic fungi. The metabolite produced by the endophytic fungus could be an alternative source of antimicrobial agents against clinical pathogens. Fungi are common in nature and consider as good natural sources for antimicrobial agents[1,2]. So far, several antibiotics have been discovered from the secondary metabolites produced by fungi and actinomycetes[3,4]. Nevertheless, during the last decade, research interests were focused on the bioactive compounds due to an increase of microbial resistance to the available drugs, hence, more fungi needs to be examined for new antimicrobial agents[5]. Most soil fungi are regarded as, decomposing organic matter and contributing to nutrient cycling. Also, recognized as prolific secondary metabolite producers, fungi have provided several bioactive compounds and chemical models currently used as pharmaceutical. Soils are traditionally the main source of fungal genetic resources for bioprospection programs[6]. The array of alkaloids and other chemicals synthesized by the endophytes endow the plant with more resistance to nematodes, insect herbivores and livestock are gaining importance for their biotechnological applications[7].*Penicillium* is a large anamorphic (asexual state) ascomycetous fungal genus with widespread occurrence in most terrestrial environments. This genus comprises more than 200 described species and many

are common soil inhabitants, as well as food borne contaminants or food ingredients used in the preparation of cheese and sausages[8,9]. *Penicillium* spp produce diversified array of active secondary metabolites, including antibacterial, antifungal, immune suppressants, cholesterol-lowering agents and also potent mycotoxins[10,11,12]. Thousands of *Penicillium* isolates have probably been screened in bio-prospecting programs since the discovery of penicillin and new bioactive metabolites continue to be discovered from these fungi nowadays[13,14,15,16], indicating their current importance as sources of high amounts of novel bioactive molecules to be used by pharmaceutical industry[17]. The present study is an attempt to produce secondary metabolites (SM) from mould fungi; *Penicillium* spp viz., *P. chrysogenum* and *P. citrinum* and to test its (SM) antibiosis nature against pathogenic bacteria with cytotoxicity assay.

Materials and methods

Extraction of Bioactive Compounds

Penicillium spp were cultured in the Sabouraud Dextrose Broth and allowed to grow up to 25 days. After the complete color change, the broth was decanted slowly and subjected to mix with ethyl acetate and chloroform at equal ratio (1,1) in a separating funnel. With a continuous shaking for five minutes the separating funnel was kept at stand in still condition and slowly the lower part containing the bioactive compounds was collected in a separate conical flask. The separated compound was kept in the rotary evaporator for extracting the bioactive compounds.

Antimicrobial activity Test

Disc diffusion method was used for checking the antimicrobial activity of bioactive against bacterial pathogens and also to confirm the dose dependant concentration. The zone of inhibition was measured and compared with the control in its raw form.

Cytotoxicity studies for crude bioactive compound

A fungal crude extract of bioactive compound was extracted by using rotary evaporation evaporator and was assayed for cytotoxicity activity by using the MTT assay.

a) Cytotoxic assay

b) Cell culture and Cytotoxic activity

The mouse cell line L929 (fibroblast) was used in this study, to measure the *in vitro* inhibitory effects of the compounds using MTT assay. Mouse fibroblast cells was purchased from National Centre for Cell Science (NCCS), Pune and maintained at 37°C in a humidified atmosphere containing 5% of CO_2 using a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) at pH 7.2. Penicillin, Streptomycin and Gentamycin were used as antibiotic mixture. Cell culture reagents was purchased from (Himedia) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt (MTT) from SRL. On the first day, cells were detached by trypsinization and seeded in a 24-well plate at a concentration of 1×10^5 cells / ml. After 24 hours, the medium was decanted. Solutions of the crude extract namely the lower phase (L1, L2, L3, L4 of 20mg/ml) and upper phase (U1, U2, U3, U4 of 10mg/ml) obtained after rotary evaporation was prepared in water. The final concentrations (10µg/ml, 25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml) were achieved by direct dilution into the medium. The samples was added and incubated for 24 hours. After the interval, 100µl of the MTT solution (2mg/ml in medium) was added to each well after decanting the medium and the cells was incubated for an additional 3 hours. After the formation of formazan crystals, 100 µL of cell lysis buffer was added and incubated for 4 hours. Cell viability was determined by absorbance measurements at 570nm taken in Elisa Reader. The crude extract of *Penicillium* spp comprising upper and lower part was assessed for cytotoxic activity. The results of the cytotoxicity of the crude extract of *Penicillium* was interpreted by calculating the percentage of viability using the formula, **% viability = $(A_{test}/A_{control})*100$.**

Results and Discussion

For the production of secondary metabolites, the *Penicillium* spp culture broth was subjected to chloroform and ethyl acetate at equal ratio of the filtrate in a separating funnel which showed the clear separation of the bioactive compound[20]. Slowly the lower part containing the bioactive compounds was collected in a separate conical flask and kept in the rotary evaporator for extracting the bioactive compounds.

The present work especially on fungi like bacteria and actinomycetes are important group of microorganisms in our environment that have served as a rich source of secondary metabolites like bioactive compounds and commercial antibiotics was carried out to derive high commercial value bioactive compounds and antibiotics [21].

Antimicrobial activity Test

The bioactive compounds were treated against the pathogens viz., *Staphylococcus aureus*, *E. coli* and *Proteus vulgaris* and zone of inhibition measured which was compared with standard antibiotic discs (Table 1). The dose dependant concentration confirm that 15 µl showed the zone of inhibition was more in *E. coil* followed by *Staphylococcus aureus* and *Proteus vulgaris*. But in all the cases the pathogens did not respond to the antibiotic disc Penicillin G. The maximum zone of inhibition was recorded by *Staphylococcus aureus* (18mm) followed by *E. coli* (15mm), and *Proteus vulgaris* (10mm). The zone of inhibition was increased when 15µl of compounds added with standard antibiotic discs (tetracycline, vancomycin,ampicillin, amoxicillin and penicillin G) (Table 1). Production of some biologically active secondary metabolites from marine-derived fungus, Varicosporina ramulosa was done by Atalla and his coworkers (2008) and they found the SM derived by them was suitable as antibiotics for different pathogens[19].

Table 1: Comparison of bactericidal efficacy of drugs with bioactive compounds against pathogens.

Pathogens	Bioactive compound Samples	Antibiotics without[$] and with bioactive compounds				
		Tetracycline	Vancomycin	Ampicillin	Amoxicillin	Penicillin-G
S. aureus	Antibiotics[$]	28	16	6	11	6
	P. chrysogenum	31	18	11	14	8
	P. citrinum	29	19	11	13	9
E. coli	Antibiotics[$]	19	10	6	6	6
	P. chrysogenum	24	7	9	9	10
	P. citrinum	22	10	10	11	7
P. vulgaris	Antibiotics[$]	25	15	9	10	6
	P. chrysogenum	29	19	12	15	10
	P. citrinum	27	21	10	11	9

Cytotoxicity studies for crude bioactive compound from *Penicillium* spp

The cytotoxicity assay showed the reduced cell viability at higher concentration (100µg) of the crude extract (Table 1). Five concentrations tested was found varying in the range 10µg/ml to100µg/ml and it was determined that at the concentration of 100 µg/ml, the crude extract U1, U3 and U4 inhibited 50% cell viability in L929 cells. In this assay, L2 showed the least inhibition on cell viability. There is a drastic decrease in the cell viability when the concentration of crude extract U3 and U4 increased beyond 50ug/ml (Table 1). The CC50% of HeLa cells was 7100µg/ml after 48 hrs. Other extracts, aqueous, ethanolic crude extracts and secondary metabolites extracts (rutin and alkaloids) of mature fruit of *C.spinosa* caused less inhibition activity on the growth of Hep-2 and HeLa tumor cell lines. The CC50% for all these extracts were more than 10000 µg/ml[18]. Biological evaluation of the two compounds towards 6 different types of tumor cell lines showed weak effect of compound 3 at different concentrations on the viable cell count of the different tumor cell lines. While compound 10 showed different activities against the viable cell count of the 6 different tumor cell lines. It kills 50% of the viable infected liver and lung cells at concentrations equal to 99.7 µg/mL,74.9µg/mL, respectively. Compound 10 can be recommended as new anticancer compounds[18,19].

Table 2: Evaluation of viability of L929 cell line challenged with Bioactive compounds of *Penicillium* spp using MTT assay.

Bio-active Compound of *Penicillium* spp	**Various Concentration of Bio-active compounds (µg/ml)**				
	10µg/ml	**25µg/ml**	**50µg/ml**	**75µg/ml**	**100µg/ml**
L1	95.43±0.94	93.58±1.08	92.60±2.46	91.73±0.79	81.31±2.9
L2	95.43±0.65	90.14±0.29	88.09±1.16	79.92±0.65	76.23±0.50
L3	98.81±0.50	93.37±1.08	89.01±0.43	77.25±2.54	69.60±1.16
L4	90.67±2.86	86.8±2.1	80.41±0.9	76.89±6.9	60.89±8.7
U1	92.62±5.81	90.92±6.04	88.65±3.47	57.20±3.30	55.43±4.4
U2	100.34±0.82	95.31±1.82	78.86±8.08	67.64±1.27	65.65±1.27
U3	97.80±2.93	96.33±1.47	71.43±1.43	43.27±3.50	42.27±2.36
U4	96.41±1.04	93.07±1.61	67.72±1.69	57.20±2.41	47.05±3.05

Similar studies would help in order to prompt the pharmaceutical companies to look upon fungi as prolific resources of secondary metabolites which would be used as drugs to combat human diseases.

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Declaration of Interest

We have no conflict of interest.

References

1. Abad, M.J., Ansuategu, I. M., Bermejio, P., Active antifungal substances from natural sources. Arkivoc. 2007, 2, 116-145
2. Muhsin, T.M., Al-Duboon, A.A., Khalaf K.T. (2011). Bioactive compounds from a polypore fungus Ganoderma applanatum (Pers ex Wallr.) Pat. Jordan Journal of Biological Sciences, 20121, 4, 205-212.
3. Hugo, W.B. and Russell, A.D., Pharmaceutical microbiology 4th P ed. Blackwell Scientific Publication, Oxford, London. 1987.
4. Anke, T., Basidiomycetes, A source for new bioactive metabolites. Prog. Ind. Microbiol., 1989, 27, 51-66.
5. Tawfik M. Muhsin and Halla M. Mohammad, Preliminary screening of bioactive metabolites from three fungal species of Drechslera isolated from soil in Basrah, Iraq, Journal of Basrah Researches ((Sciences)), 2012, 38(2), 44-53
6. Adrio, Jose, L. Demain, L. Arnold, Fungal biotechnology. International Microbiology, 2003, 6(3), 191-199.
7. Ahmed F. Sahab, Antimicrobial Efficacy of Secondary Metabolites of Beauveria Bassiana Against Selected Bacteria and Phytopathogenic Fungi, J. Appl. Sci. Res., 2012, 8(3), 1441-1444.
8. Pitt, John I. A laboratory guide to common Penicillium species. 3rd Ed. Australia, Food Science Australia Publishers, 197 p. ISBN 064303949X. 2000.
9. Frisvad, Jens C. and Samson, Robert A. Polyphasic taxonomy of Penicillium subgenus Penicillium, A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Studies in Mycology, 2004, 49, 1-174.
10. Rancic, Ana, Sokovic, Marina, Karioti, Anastasia, Vukojevic, Jelena and Skaltsa, Helen. Isolation and structural elucidation of two secondary metabolites from the filamentous fungus Penicillium ochrochloron with antimicrobial activity. Environmental Toxicology and Pharmacology, 2006, 22(1), 80-84.
11. Lucas, Esther M.F., Castro, Mateus C.M. and Takahashi, Jacqueline A. Antimicrobial properties of sclerotiorin, isochromophilone VI and pencolide, metabolites from a Brazilian cerrado isolate of Penicillium sclerotiorum Van Beyma. Brazilian Journal of Microbiology, 2007, 38(4), 785-789.
12. Nicoletti, Rosario, Lopez-Gresa, Maria Pilar, Manzo, Emiliano, Carella, Angela and Ciavatta, Maria Letizia. Production and fungitoxic activity of Sch 642305, a secondary metabolite of Penicillium canescens. Mycopathologia, 2007, 163(5), 295-301.
13. Larsen, Thomas Ostenfeld, Lange, Lene, Schnorr, Kirk, Stender, Steen and Frisvad, Jens Christian. Solistatinol, a novel phenolic compactin analogue from Penicillium solitum. Tetrahedron Letters, 2007, 48(7), 1261-1264.
14. Ge, Hui Ming, Shen, Yao, Zhu, Chun Hua, Tan, Shu Hua, Ding, Hui, Song, Yong Chun and TAN, Ren Xiang. Penicidones A-C, three cytotoxic alkaloidal metabolites of an endophytic Penicillium spp Phytochemistry, 2008, 69(2), 571-576.
15. Takahashi, Jacqueline A., Castro, Mateus C.M., Souza, Giovanni G., Lucas, Esther M.F., Bracarense, Adriana A.P., Abreu, Lucas M., Marriel, Ivanildo E., Oliveira, Mariana S., Floreano, Mônica B. and Oliveira, Thays S. Isolation and screening of fungal species isolated from Brazilian cerrado soil for antibacterial activity against Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Streptococcus pyogenes and Listeria monocytogenes. Journal de Mycologie Médicale, 2008, 18(4), 198-204.

16. Lucas, Esther M.F., Castro, Mateus C.M. and Takahashi, Jacqueline A. Antimicrobial properties of sclerotiorin, isochromophilone VI and pencolide, metabolites from a Brazilian cerrado isolate of Penicillium sclerotiorum Van Beyma. Brazilian Journal of Microbiology, 2007, 38(4), 785-789.
17. Philippe P et al., Novel antimicrobial secondary metabolites from a Penicillium spp isolated from Brazilian cerrado soil Electronic Journal of Biotechnology, 2009, 12(4), 15-20.
18. Asaad Abdulwahed B. AL-Asady, Khesar H. Khalil and Sa'adi Saleh M. Barwari, Cytotoxic and Cytogenetics Effects of Aqueous, Methanolic and Secondary Metabolites Extracts of Capparis spinosa on Tumor Cell Lines in vitr, Jordan Journal of Biological Sciences, 2012, 5(1), 15-30
19. Atalla, M. Mabrouk., Zeinab, H. Kheiralla., Eman, R. Hamed., Amani, A. Youssry and Abeer, A. Abd El Aty, Production of some biologically active secondary metabolites from marine-derived fungus Varicosporina ramulosa, Malaysian Journal of Microbiology, 2008, 4(1), 14-24
20. Sanglier, J. J., H. Haag, T. A. Huck and Fehr T., Review of actinomycetes compounds, 1990-1995. Exp. Opin. Invest. Drugs, 1996, 5, 207-223
21. Okami, Y. and Hotta, Search and discovery of new antibiotics, In, M. Goodfellow, S.T. Williams and M. Mordarski (ed.) Actinomycetes in biotechnology, Academic Press Inc., San Diego, California, Pp 33-67, 1988.

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