RESEARCH ARTICLE

Anti-tuberculous, phytotoxic and insecticidal activities of secondary metabolites obtained from aspergillus and penicillium species isolated from soil

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Abstract

Objectives: To identify biological activities of secondary metabolites obtained from penicillium and aspergillus species.

Method: The experimental study was conducted from December 2014 to November 2015 and comprised aspergillus and penicillium species that were isolated from the top soil of Khyber Teaching Hospital, Peshawar, and Mian Rashid Hussain Shaheed Memorial Hospital, Pabbi, both in the Khyber Pakhtunkhwa province of Pakistan. To produce secondary metabolites, the species were grown in Czapek yeast broth. Fungal metabolites were extracted from the broth medium and were checked for anti-tuberculous, phytotoxic and insecticidal activities. Anti-tuberculous activity was checked against M.TB strains. Phytotoxicity was tested against Lemna minor plant, while insecticidal activities were performed against tribolium castaneum and rhyzopertha dominica.

Results: Secondary metabolites of aspergillus were active against Mycobacterium tuberculosis whereas those of penicillium showed no significant inhibitory activity ($p>0.05$). Penicillium metabolites at 1000 µg/ml concentration showed significant (>80%) phytotoxic activity. Aspergillus metabolites showed good (60-80%) insecticidal activity against tribolium castaneum and low (20-40%) activity against rhyzopertha dominica. Penicillium metabolites showed moderate (40-60%) activity against tribolium castaneum and low (20-40%) activity against rhyzopertha dominica.

Conclusion: Secondary metabolites of both fungi contained some biologically active compounds. These metabolites could be further investigated for isolation of bioactive substances in purified form.

Keywords: Aspergillus, Penicillium, Anti-tuberculous, Phytotoxic, Insecticidal, Secondary metabolites. (JPMA 68: 1024; 2018)

Introduction

New medicines are obtained from natural resources i.e. plants, fungi etc. which have low toxic effect and are more effective for treatment of various infections. Recently, interest in the therapeutic value of natural products has increased.[1] Natural products have been the most effective source of potential drug leads.[2]

Fungi are a large and diverse group of organisms. After insects, fungi are the largest group and essential part of tropical ecosystems throughout the world.[3] Fungi live in both terrestrial and aquatic environments such as in soil, water, air, and internally or externally on both animals and plants.[4]

Fungi readily synthesise a broad range of natural products often called secondary metabolites. These metabolites are of therapeutic, industrial and agricultural value. Some natural products synthesised by fungus are toxic like mycotoxins while some are beneficial like antibiotics.[5]

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Recently, aspergillus and penicillium species have been found as good sources of new biologically active secondary metabolites.

Penicillium is an anamorphic fungus that lives in a wide range of terrestrial habitats. It has about 200 described species. Several species live in soil while some are food borne pollutant or food constituents added in cheese and sausages preparation.[6,7]

Many aspergillus species produce biologically active secondary metabolites e.g. terpenoids, polyketides and xanthones which have antifungal, antibacterial and cytotoxic activities.[8,9] The genus aspergillus contain more than 180 species which live in wide range of habitats.[10] Polyketides isolated from an endophytic fungus i.e. aspergillus fumigatus inhibit growth of mycobacterium tuberculosis (M.TB).[11] TB, a dangerous bacterial infection globally, is caused by M.TB and affects millions of people. Most of the cases arise in highly populated countries like Pakistan, Bangladesh, Indonesia, India and China. The development of M.TB drug-resistant strains is a serious public health problem worldwide. New medicines obtained from natural resources might be a good option to address the issue of drug resistance.

Microorganisms, mostly bacteria and fungi, produce many biologically active compounds called secondary metabolites which perform various important biological activities. The current study was planned to identify biological activities of secondary metabolites.

Materials and Method

This experimental study was conducted from December 2014 to November 2015, and comprised soil samples that were acquired from Khyber Teaching Hospital, Peshawar, and Mian Rashid Hussain Shaheed Memorial Hospital, Pabbi, both in the Khyber Pakhtunkhwa province of Pakistan. Surface level soil samples were collected in polyethylene bags and shifted to Biotechnology and Microbiology Laboratory, Centre of Biotechnology and Microbiology, University of Peshawar. The study involving clinical isolates was approved by the institutional ethics committee.

Fungal strains were isolated on a potato dextrose agar (PDA) medium. It was prepared by adding 4gm of potato starch, 20gm of dextrose and 15gm of agar in 1L distilled water. The medium and plates were autoclaved at 15psi for 15min. Sterilised medium was poured into sterile plates and allowed to cool in a laminar flow hood (LFH). Serial dilutions of soil samples were prepared. Sample (100 µl) of each dilution was added to sterile plate using sterile pipette under aseptic conditions and the plates were incubated at 28°C for 10 days. Fungal growth was visible on PDA medium after 10 days of incubation. Further purification to isolate single fungal strain was carried out by sub-culturing individual colonies on fresh PDA medium.

A light microscope (Olympus, CH20Bi MF200) was used to observe fungal morphology. Different characteristics like colour, texture, hyphae structure, spores arrangement and pigmentation of the colonies were examined to identify penicillium and aspergillus species.

Czapek yeast broth (CYB) nutrient medium for the growth of fungi was prepared using three constituent solutions i.e., A, B, and C. Solution A was prepared by dissolving 20g of sodium nitrate (NaNO3), 5g of potassium chloride (KCL), 5gm of Epsom salt (MgSO4.7H2O and 0.1g of Ferrous sulfate heptahydrate (FeSO4.7H2O) in 500mL of distilled water. Solution B was prepared by dissolving 10g of dipotassium phosphate (K2HPO4) in 500mL of distilled water. Solution C was prepared by dissolving 1g of Zinc Sulfate Heptahydrate (ZnSO4.7H2O) and 0.5gm of copper (II) sulfate pentahydrate (CuSO4.5H2O) in 100mL of distilled water. CYB nutrient medium was constituted by mixing 5g of yeast extract, 30g of saccharose, 50mL solution A, 50mL solution B and 1mL solution C in 1 Litre distilled water.

For the production of secondary metabolites, penicillium and aspergillus species were grown in CYB medium. A small fragment of fungal inoculum was inoculated into sterile 250mL Erlenmeyer flasks containing CYB medium and the flasks were further incubated at 28°C for 14 days in shaking incubator at 150rpm.

After an incubation period of 14 days, secondary metabolite extraction was carried out. About 200-500 µL of 40% hydrochloric acid (HCl) was added to the fungal culture. After this, the culture was blended using a blender. An equal volume of ethyl acetate was added and the culture was mixed for 40min. This culture slurry was filtered through cheese cloth. The filtered medium was added to a separating funnel and allowed to stand for 10min that resulted in two layers. The organic layer containing metabolites of interests was separated from the aqueous phase and washed with 2M brine solution to purify further. To remove remaining traces of water from the organic layer, anhydrous sodium sulphate (Na2SO4) was added to it. Finally, the isolated metabolites were then concentrated by rotary evaporator at 45°C and 150rpm. The isolated metabolites were screened for three types of activities: anti-tuberculous, phytotoxic and insecticidal activity. All the activities are categorised in four groups; low (20-40%), moderate (40-60%), good (60-80%) and significant (>80%) activity.

Penicillium and aspergillus species were screened for three types of activities: anti-tuberculous, phytotoxic and insecticidal activity. Anti-tuberculous activity was checked against M.TB strains. Phytotoxicity was tested against Lemna minor plant, while insecticidal activities were performed against tribolium castaneum and rhyzopertha dominica.

Anti-tuberculous assay was performed at Provincial TB Reference Laboratory (BSL-3), Khyber Pakhtunkhwa, Pakistan. Appropriate biosafety practices were followed when performing anti-tuberculous activity. Clinical isolates of M.TB and H37RV reference strain were used in this assay. The strains were acquired from Provincial TB Reference Laboratory, Khyber Pakhtunkhwa, Pakistan. M.TB was cultured using BACTEC MGIT 960 system. Anti-tuberculous assay was performed with slight modifications of the reported procedure.[12] Secondary metabolites extracted from fungal mycelia were screened for anti-tuberculous activity. Nutrient media plates were prepared by pouring digested media into petri plates. Solidified nutrient media plates were incubated overnight at 37°C for sterility test. The plates passing the sterility test were inoculated with a fresh culture of M.TB to get a lawn of bacteria. Wells were made in agar medium with the help of 6mm sterile borer. The stock solutions of test compounds at a concentration of

3mg/mL were prepared in Dimethyl sulfoxide (DMSO) and 100µL of these stock solutions was loaded into respective wells. Streptomycin and DMSO were used as positive and negative control respectively. Plates were sealed with tape. After three days of incubation at 37°C, zone of inhibition was noted in millimetre and the zone of inhibition in percentage was determined using following formula.[13]

% Zone of Inhibition = Zone of inhibition of sample / zone of inhibition of standard x 100

Lemna minor plant was used to screen the phytotoxic activity of the test samples following an earlier research.[14] Stock solutions (20mg/ml) of metabolites of aspergillus and penicillium species at concentration of 10 µg/ml, 100 µg/ml and 1000 µg/ml were added to petri plates. To evaporate the organic solvent, the plates were left at room temperature. After evaporation of organic solvent, 20ml of E-media was added to all plates. Sixteen healthy Lemna minor plants were chosen and placed carefully in respective plates. Plates containing E-media and plants were kept for 7 days in growth chamber at 27°C. After 7 days, phytotoxic activity was observed.

The insects Rhyzopertha dominica and Tribolium castaneum were used to screen insecticidal activity of the test samples following protocol from an earlier research.[15] For stock solution preparation, 200mg of test sample was dissolved in 3ml methanol. Filter paper was cut according to the size of petri plate and positioned in the plate. Samples stock solutions were added to plates. To evaporate organic solvent, the plates were left at room temperature. Ten healthy insects (rhyzopertha dominica and tribolium castaneum) were chosen and placed carefully in respective plates containing test samples and control. For positive control permethrin which is an insecticidal drug was used while for negative control methanol was used. Plates were kept at 27°C for 1 day. After 24 hours, the mortality percentage was calculated with the help of following formula.[13]

$$\text{Insecticidal activity} = \frac{\text{100 - (No. of insects alive in test}}{\text{No. of insects alive in control}} \times 100)$$

Results

The zones of inhibition against M.TB observed for secondary metabolites isolated from aspergillus and penicillium were 25mm and 1mm, respectively. The positive control streptomycin created 40mm zone of inhibition. Secondary metabolites from aspergillus were found to have a significant inhibitory effect, i.e., 62.5% inhibition (Figure).

1000 µg/ml concentration of Penicillium metabolites showed high phytotoxic activity while 10 µg/ml and 100 µg/ml concentration showed low phytotoxic activity. However, aspergillus metabolites at 10 µg/ml and 100 µg/ml showed low activity and moderate activity at 1000 µg/ml (Table-1).

Aspergillus metabolites showed good insecticidal activity against tribolium castaneum and low activity against rhyzopertha dominica. Penicillium metabolites showed

Table-1: Lemna minor phytotoxic activity of the fungal extracts.

Name of plant	Concentration of sample (µg/ml)	Percentage of dead plants Penicillium crude extract	Aspergillus Crude extract
Lemna minor	10	37.5 %	12.5%
	100	37.5%	18.75%
	1000	81.25 %	50%

Table-2: Insecticidal activity of secondary metabolites of Penicillium and Aspergillus species.

Fungal Extract	Type of insect	Mortality %
Penicillium	Tribolium castaneum	50%
Aspergillus	Tribolium castaneum	80%
Penicillium	Rhyzopertha dominica	30%
Aspergillus	Rhyzopertha dominica	30%

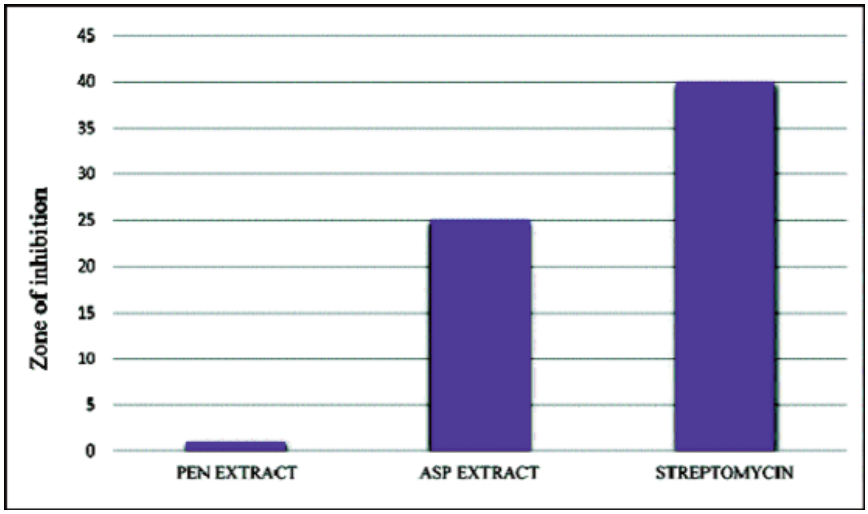

Figure: Growth Inhibition: Anti-tuberculous activities of Penicillium (3 mg/mL), streptomycin (1 µg/mL) and Aspergillus extract (3 mg/mL) against M. tuberculosis.

moderate activity against tribolium castaneum and low activity against rhyzopertha dominica (Table-2).

Discussion

Globally, human health is exposed to different types of contagious diseases. The increasing rate of drug resistance to infectious agents demand for the production of novel and effective biologically active compounds with novel modes of action.

Fungi are important organisms that readily synthesise several bioactive compounds often called secondary metabolites which have therapeutic value. Antimicrobial activity of large number of fungal extracts and extracellular products have been found, mostly isolated from penicillium and aspergillus species. From the time when penicillin was discovered, it is well-known to produce secondary metabolites with various biological activities i.e. antifungal, antibacterial, immunosuppressant and fungal toxins.[16,17]

Humans are exposed to various types of infectious diseases. The growing rate of drug resistance to infectious agents demands the development of novel and more effective compounds with newer modes of action. Many fungal extracts and extracellular products especially associated with penicillium and aspergillus species have been found to show antimicrobial activity. In this study, secondary metabolites from aspergillus showed anti-tuberculosis activity whereas those from penicillium showed no anti-tuberculosis activity. The findings of this study are in agreement with related work done previously. Secondary metabolites such as polyketides isolated from aspergillus fumigatus were found to inhibit the growth of M.TB.[11] Secondary metabolites from endophytic fungus such as penicillium sclerotiorum and aspergillus aculeatus were shown to have anti-tuberculosis activity.[18] Nigrosporin and 4-deoxybostrycin isolated from a marine fungus were screened against M.TB and were found to exhibit good activity, even better than 1st line TB drugs.[19]

Several studies have reported the phytotoxic and insecticidal activity of plant crude extract. A little knowledge is available about phytotoxic and insecticidal activity of fungal extract. A similar type study used ethyl acetate extract and n-Hexane fraction (100 µg/ml) of aspergillus species that inhibited Lemna minor growth by 65% and 85%, respectively while ethyl acetate extract and n-Hexane fraction (100µg/ml) of penicillium species inhibited Lemna minor growth by 56.66% and 30%, respectively.[20] In our study 10 µg/ml and 100 µg/ml concentration of Penicillium metabolites inhibited Lemna minor growth by 37.5% while 1000 µg/ml concentration of penicillium extract inhibited Lemna minor growth by 81.25%. However, Aspergillus at 10µg/ml and 100 µg/ml concentration inhibited Lemna minor growth by 12.5% and 18.75% respectively and at 1000 µg/ml concentration by 50%.

Penicillium, aspergillus, rhizopus, fusarium, amanita, syncephalastrum, tolypocladium, paecilomyces and monilia species have been found to produce secondary metabolites which are lethal to mosquitoes.[21,22] The current research also studied the metabolites of penicillium and aspergillus were screened for their insecticidal activity. Metabolites from aspergillus showed best activity (80%) against tribolium castaneum while penicillium showed moderate activity. Both fungal metabolites showed low activity against rhyzopertha dominica.

The current study was limited to three type of activities: anti-tuberculous, phytotoxic and insecticidal activity. Other important biological activities could be performed. Animal model studies could be carried out to further confirm the effectiveness of these metabolites. Uncharacterised fungal metabolites were checked for biological activities. Secondary metabolites of both fungi contain some biologically active compounds, hence these extracts could be further investigated to isolate active ingredients in pure form.

Conclusions

Extracts of aspergillus species showed the potential of inhibiting the growth of M.TB while extracts of penicillium species were not effective against M.TB. Phytotoxic and insecticidal activities of metabolites were good. Further studies could be carried out to purify the active ingredients to be used in the formulation of effective drugs.

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Conflict of Interest: None.

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