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Cytotoxic Potential of Secondary Metabolite Isolated from Soil Fungi

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Abstract

Microorganism is of great promise in the search for new chemical molecules of pharmaceutical interest. Hence discovery of novel microbial metabolites through fermentation is one of most interesting field in research. At present study we isolated one of the fungi from soil. The fungal culture was further grown by solid state fermentation using wheat bran as medium. The fermented culture was extracted by using organic solvent, ethyl acetate. we successfully purified one the metabolite by using column chromatographic technique. the purity was confirmed by LC-MS. The purified compound showed 50% cytotoxicity (CC_{50}) against Vero cells at 332.66 µM and Madin Darby canine kidney (MDCK) cells at 21.8 µM. The anti viral potential of isolated compound was carried out using the plaque reduction assay method against Herpes simplex virus-1 and Influenza A virus. The compound tested did not show any significant antiviral activity. Further analytical studies are required to establish its molecular structure and structure based docking studies may help to find its pharmacological potentials.

Key words: Microbial metabolite, antiviral activity, cytotoxicities, Herpes simplex virus-1 and Influenza A

INTRODUCTION

Natural products are sources of greater structural and molecular diversity[1]. It has always been an attractive field of drug research because many of the wonder drugs of today like penicillin, morphine and quinine etc. are all derived from natural products.

Microorganisms are valued for the production of a number of biologically active substances[2] and secondary metabolites like antibacterial, antifungal, antiviral and antitumor substances. Since the discovery of penicillin, there has been tremendous work on the microbial metabolites as antibiotics, which has translated into an unparalleled commercial success. Many pharmaceutical/biological agents are known to inhibit specific enzymes and that several diseases are associated with abnormal enzyme activities, the concept of enzyme specific "target enzyme inhibitors" has yielded exceptional pharmaceutical compounds of microbial origin. There has been a continued demand for newer agents for the health benefits.

On the other hand, bioactive compounds isolated from natural biological sources offer a vast and unexplored diversity of chemical structures, unmatched by even the biggest combinatorial databases[3]. Solid-state fermentation (SSF) can be defined as the fermentative process in which the microorganisms grow in solid materials without free water[4]. In this process are used microorganisms, mainly filamentous fungi, due to their ability to grow on the support/substrate used. Microorganisms have the ability to utilize various substrates as a consequence of the diversity of their biological and biochemical evolution[5]. Actinomycetes and fungi, of all microorganisms studied, have been found to be the most prolific producers of secondary metabolites.

MATERIALS AND METHODS:

Isolation of soil fungi:

1.0 g soil sample was added into 250 mL conical flask with 99 mL sterilized water and glass beads, thoroughly stirred for 20 min to obtain 10^{-2} g/mL soil suspension. Then the soil suspension was diluted to 10^{-3}~10^{-6} g/mL. The concentration of 10^{-4}, 10^{-5} and 10^{-6} g/mL diluted solution were smeared on the PDA substrate and cultivated upside down under 37°C for 2~3 days. Furthermore, the single colony was separated and inoculated on plate medium cultivated for another 2~3 days. Repeating the separation and purification process until the single colony was obtained. The pure colony was inoculated on the test tube slant preserved at 4°C until use.

Fermentation of isolated fungal culture:

The isolated pure culture (to be identified) was subjected to solid state fermentations. Briefly the culture was grown on potato dextrose slants for 8 days to achieve complete sporulation. A loopful of spores were scraped and transferred into 4 number of 250-ml Erlenmeyer flasks each containing 100 ml of sterilized potato dextrose broth in aseptic condition. Then the flasks were kept for fermentation at room temperature in a shaker incubator. After 4 days, developed pellets were used for solid state fermentation in 250-ml Erlenmeyer flasks. After 8 days, fermented medium were extracted using 200 ml of ethyl acetate. The ethyl acetate extract was filtered through handmade filter paper and then using Whatman no 1 filter. The clear filtrate was concentrated using rotary evaporator and subjected to purification by column chromatographic technique and successfully purified one the metabolite (Figure 1).

The melting point of purified compound was determined by introducing a small amount into a capillary tube, then placed this and thermometer in a heating bath (Thiele tube), and observed the temperatures at which melting begins and is completed.

Figure 1: Purified fungal secondary metabolite.

Biological Activity Of The Purified Compound

The most widely used methods for the initial screenings of fungal extracts to evaluate their antiviral activity are the plaque reduction assay[7-9] and cytopathic effect (CPE) assay[10].

Cytotoxicity profile of purified metabolite in Vero cells (CPE-based) :

African green monkey kidney cells was used to establish cytotoxicity (Vero cells, CCL-81TM strain, ATCC). 1 µM solution (stock solution) was prepared by diluting using Minimum Essential Media (MEM) and 10-400 µM concentration range was used. Each concentration of Purified compound was added in triplicate to the Vero cells ($1*10^4$/well) in a 96-well microtitre plate and kept for incubation at 37 ºC. Cells were observed after every 24 hours for 3 days.

Cytopathic effect (CPE) based observation for 72 hours under the microscope, followed by crystal violet staining. The plate was read at 490 nm using ELISA reader (BioTek, ELx800, USA). Percentage cytotoxicity was calculated using the formula:

Percentage of cell cytotoxicity = [AC – AT / AC] ×100

where AC = Absorbance of cell control and AT = Absorbance of test wells.

Any change in the morphology of the treated cells compared to untreated cells (control) indicated cytotoxicity due to the samples. Concentrations at which toxicity was evident by microscopic observations were recorded.

The CC_{50} value (concentration of test compound required to reduce the cell viability by 50%) was calculated as compared with untreated control cells. The concentration of purified compound showed 50% cytotoxicity (CC_{50}) in Vero cells was 332.66 µM. Based on this assay, the non-cytotoxic range of BCP was obtained for further antiviral screening against Herpes simplex virus-1by plaque reduction assay.

Cytotoxicity profile of BCP in MDCK cells (CPE-based):

FR-58- Madin Darby canine kidney (MDCK) cells, (London line, IRR.) to establish cytotoxicity of purified compound.

Stock solution 1 mM of purified compound was prepared by diluting with Dulbecco‟s Minimum Essential Media (DMEM) from purified compound-DMSO (10 mg in 1 ml) solution and 1 - 100 µM concentration range was used.

Each concentration was added in triplicate to MDCK cells ($1.5*10^4$/well) in a 96-well microtitre plate and kept for incubation at 37°C. Cells were observed after every 24 hours for 3 days.

Cytopathic effect (CPE) based observation for 72 hours, followed by crystal violet staining. The plate was read at 490 nm using ELISA reader (BioTek, ELx800, USA). Percentage cytotoxicity was calculated using the formula:

Percentage of cell cytotoxicity = [AC – AT / AC] ×100

Where AC = Absorbance of cell control and AT = Absorbance of test wells

Any change in the morphology of the treated cells compared to untreated cells (control) indicated cytotoxicity due to the samples. Concentrations at which toxicity was evident by microscopic observations were recorded. The CC_{50} value (concentration of test compound required to reduce the cell viability by 50%) was calculated as compared with untreated control cells. The concentration of purified compound showed 50% cytotoxicity (CC_{50}) in MDCK cells was 21.8 µM. Based on this assay, the non-cytotoxic range of BCP was obtained for further antiviral screening against Influenza A (H1N1) virus by plaque reduction assay.

Anti-viral activity:

Assessment of antiviral activity by Plaque reduction assay against Herpes simplex virus-1. (Standard strain - International Reagent Resource- IRR).

A non-cytotoxic concentration range of BCP (usually between 1/10 and 1/5 of CC_{50} value against ATCC) was prepared for testing antiviral activity.

The assay was carried out in 12-well plates. Virus titer of 100 Plaque forming units (PFU) was used to induce infection (Virus control). Drug treatment schedule was designed according to post-infection treatment (drug added after 1 hour of virus adsorption).

Three non-cytotoxic concentrations of compound (30, 60 and 120 µM) were tested. Standard antiviral drug, acyclovir was used as a positive control at a concentration of 17.17 µM (EC50 value determined by plaque reduction assay).

Post virus adsorption, acyclovir and test compounds were added at their specific concentrations and incubated for 1 hour. Then, the first overlay was added to the cells. After 24 hours, a second overlay was added. The next day plaques were counted in each group and analyzed by statistical tools.

Acyclovir significantly (*p<0.001*) reduced HSV-1 plaque number by 43% in Vero cells. All three concentrations of purified metabolites tested reduced the plaque numbers compared to virus control. However, the difference was not statistically significant and showed no antiviral activity against HSV-1 by plaque assay-based antiviral method.

Assessment of antiviral activity by Plaque reduction assay against Influenza A (H1N1) pdm09 (Standard strain - International Reagent Resource).

A non-cytotoxic concentration range of purified metabolite (usually between 1/10 and 1/5 of CC50 value) was prepared for testing antiviral activity. The assay was carried out in 12-well plates. Virus titer of 100 Plaque forming units (PFU) was used to induce infection (Virus control). Drug treatment schedule was designed according to post-infection treatment (drug added after 1 hour of virus adsorption). Three dilutions of test compound, 1.25, 2.5 and 5 µM were tested. Standard antiviral drug, oseltamivir was used as a positive control at a concentration of 10.81 µM (EC50 value determined by plaque reduction assay). Post virus adsorption, standard drug and test compounds were added at their specific concentrations and incubated for 1 hour. Then, the first overlay was added onto the cells. After 37 hours, a second overlay was added and plaques were counted after 7 hours in each group and analyzed statistically.

Oseltamivir significantly (*p<0.001*) reduced the Influenza A (H1N1) virus plaque number by 62% in MDCK cells. All three concentrations of BCP tested reduced the plaque numbers compared to virus control. However the difference was not statistically significant and showed no antiviral activity against Influenza A (H1N1) virus by plaque assay-based antiviral method.

RESULTS

Isolation of secondary metabolite:

The soil born fungi isolated (to be identified) was subjected to solid state fermentation. We used wheat bran as solid medium. The ethyl acetate extract was subjected to isolation of secondary metabolites by column chromatography and successfully isolated one secondary metabolite. The purity was confirmed by LC-MS and melting point determination. The purified compound was melted sharply at 242 to 246 ^{0}C.

Cytotoxicity and Antiviral activity:

The cytotoxicity and antiviral potential of the isolated compound were performed at Manipal Institute of Virology (MIV), MAHE, Madhav Nagar, Manipal, Karnataka-576104. The results reveal that concentration of isolated compound showed 50% cytotoxicity (CC_{50}) in Vero cells (ATCC) was 332.66 µM and MDCK cells was 21.8 µM.

All the three concentrations (30, 60 and 120 µM) of purified metabolites tested reduced the plaque numbers compared to HSV-1 virus control. However the difference was not statistically significant. A similar result was found against Influenza A (H1N1) virus at concentrations 1.25, 2.5 and 5 µM. The isolated compound is not showed antiviral activity against HSV-1 and Influenza A (H1N1) by plaque assay-based antiviral method.

CONCLUSION

The fungal secondary metabolites may hold potential pharmacological activities. At present we isolate one of the secondary metabolites from fungi (to be identified). The antiviral potential of the isolated metabolite was performed against HSV-1 and Influenza A (H1N1) virus and not showed antiviral activity. Further studies are required to establish its molecular structure. The structure-based docking studies to may help to find its pharmacological potential.

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