ORIGINAL ARTICLE

Cytotoxicity Studies of *Mortierella alpina* AIB RR2 Extracts on Hela and a549 Cell Lines

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Abstract

Aim: The aim is to understand any possible cytotoxic activity of *Mortierella alpina* extract against carcinoma cell lines and possible interaction with normal cell lines. **Materials and Methods:** The extract of *M. alpina* at varying concentrations was incubated with the normal Vero cell lines and also the carcinoma cell lines HeLa and A549, after which 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay was carried out to determine the viable cell. **Results and Discussion:** The cytotoxic activities of *M. alpina* extract were quite strong against both HeLa and A549 carcinoma cell lines with inhibitory concentration 50 values of 106 and 116 µg/mL, respectively. However, the cytotoxic concentration 50 value of the fungal extract against the normal Vero cell line was 2674 µg/mL, which shows the very low toxic activity of the fungal extract against the normal cell lines. Further, the therapeutic index of *M. alpina* against HeLa and A549 cell lines was found to be 25.22 and 23.02, respectively. **Conclusion:** The present investigation indicates the probable use of *M. alpina* extracts in the treatment of carcinoma.

Key words: A549, HeLa, *Mortirella alpina,* 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay, therapeutic index

INTRODUCTION

Mortirella alpina, a filamentous fungus generally found in saprophytic soil and has oleagenic properties, is a widely distributed psychrophilic Mortierellae.[1-3] This fungus with a rosette globe pattern under cultural conditions is used extensively to produce omega fatty acids mostly under submerged fermentation. *M. alpina* is considered to have a promising future in the development of production related to omega fatty acid, especially in the omega 6 group.[4] Due to the presence of important secondary metabolites, this oleaginous microorganism (psychrophilic filamentous) is presently highly considered for large-scale production of arachidonic acid (ARA).[4,5]

Microbial metabolites have a long history of usage as therapeutic agents. Microbial metabolites used as therapeutic agents include antibiotics, enzymes, cholesterol-lowering metabolites, anticancer agents, and immunosuppressants.[6] Although the number of potent and active metabolites discovery has seen a growing trend, the requirement for novel metabolites is growing at a faster pace due to the increasing trend in untreatable diseases including cancer. Further, any major discoveries have to be confirmed not only for its efficacy but also for its safety before introducing it into a treatment regime. This is because of the fact that the active compound as well as any minor metabolite associated with the fungus might has some toxic effects on the normal functioning of cell and tissues.[7] Preliminary safety studies are generally conducted on cell lines isolated from healthy mammalian tissues, whereas the effectiveness and potential of the active component to treat various diseased conditions are tested on specific affected cell lines including carcinoma cells.[8,9]

According to the WHO, 8.8 million deaths reported in 2015 worldwide due to different types of cancer.[10] Chemotherapy

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is one of the few potential and effective treatment strategies available,[11] with effectiveness mainly restricted to the preliminary stages. Functional foods capable of supporting health and preventing and protecting the biological system[12] from fatal diseases such as cancer are the need of the hour. *M. alpina,* which has been considered as a gifted microorganism to produce the conditionally essential fatty acid ARA, with numerous role in the physiological system including development and prevention of age-related brain and cardiovascular development as well as cognitive functions,[4,13] still more need to be exploited for its numerous other potential roles in promoting health and preventing diseases. Due to the proven role of ARA in improving and preventing various degenerative diseases and also due to a long history of fungal metabolites in preventing cancer and related diseases, *M. alpina* can be a potent candidate to be evaluated for its anticancer properties. The present study is focussed on the cytotoxic and anticancer properties of a fungal isolate *M. alpina* AIB-RR2.

MATERIALS AND METHODS

All the media chemicals including Potato Dextrose Agar, Potato Dextrose Broth (PDB), glucose, and polysorbate 80 were purchased from HiMedia (Mumbai, India). Cell lines HeLa, A459, and Vero cell lines were purchased from the National Center for Cell Science, Pune, cell culture medium, Dulbecco's Modified Eagle's Medium - high glucose (AL111, HiMedia), Adjustable Multichannel Pipettes and a Pipettor (Eppendorf, Germany), fetal bovine serum (RM10432, HiMedia), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reagent (5 mg/mL) (4060 HiMedia), dimethyl sulfoxide (DMSO) (PHR1309, Sigma), Camptothecin (C9911, Sigma), D-PBS (TL1006, HiMedia), 96-well plate for culturing the cells (From Corning, USA), T25 flask (12556009, Biolite - Thermo), 50 mL centrifuge tubes (546043 TARSON), 1.5 mL centrifuge tubes (TARSON), 10 mL serological pipettes (TARSON), 10–1000 µL tips (TARSON), 96-well enzyme-linked immunosorbent assay (ELISA) plate reader or spectrophotometer capable of measuring the absorbance (ELX-800 Biotek), inverted microscope (Biolink), and 37° C incubator with humidified atmosphere of 5% CO_2 (Heal Force, China) were the reagents and materials used in the present study.

Preparation of fungal extract

M. alpina AIB RR2, obtained from Mysore, Karnataka, and characterized using internal transcribed spacer sequencing, was grown on a PDB medium for 7 days at 25° C. On completion of incubation, mycelia were harvested and mixed with ethyl acetate and kept for vigorous shaking for 1 h. On completion of extraction, the supernatant was collected and concentrated under vacuum using a rotary evaporator at 40°C and dried under nitrogen. The concentrate was stored at −80°C for future experimentation.[14]

Cytotoxicity analysis of the selected strain by MTT assay

About 200 µL of the culture of suspension cell (cell density 20,000 cells per well) was seeded into a 96-well plate (ELISA plate) and was allowed to grow for about 12 h. After completion of incubation, appropriate concentrations of the test agents ranging from 0.1 to 1000 µL were added to the plates and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. After completion of the incubation period, the ELISA plates were removed from the CO_2 incubator and spent media were washed with D-PBS. MTT reagent was added to this to a final concentration of 0.5 mg/mL of total volume, and the plate was wrapped with aluminum foil to avoid exposure to light and incubated for 3 h. On completion of incubation, the excess MTT reagent was washed and then added 100 µL of DMSO as a solubilizing agent with intermittent gentle stirring in a gyratory shaker. The controls included were medium control (medium without cells), negative control (medium with cells but without the experimental extract), and positive control (medium with cells and with 50 µM of CPT/75 µM of H_2O_2). The absorbance was read on an ELISA reader at 570 nm with 630 nm used as reference wavelength. The percentage inhibition was calculated using the following equation:

$$\% \text{ Cell inhibition} = \frac{(\text{Absorbance value of control} - \text{Absorbance value of sample})}{\text{Absorbance value ofcontrol}} \times 100$$

The inhibitory concentration (IC_{50}) value which denotes the inhibition of 50.0% of the cancer cell growth and cytotoxic concentration (CC_{50}) which denotes the inhibition of 50.0% of the healthy cell growth was determined using linear regression equation, i.e. y = mx+b, with y = 50, and m, x and b values were derived from the viability graph. The therapeutic index (TI) value was derived[15,16] by the following equation:

$$TI \frac{CC_{50}}{IC_{50}}$$

RESULTS

In the present study, the *in vitro* cytotoxic effects of *M. alpina* AIB-RR2 extracts on HeLa and A549 cells were assessed. The cytotoxicity of the fungal extracts was also investigated against the normal Vero cell line [Figure 1]. It was found that *M. alpina* AIB-RR2 exerts a concentration-dependent antiproliferative effect on the tested carcinoma cell lines. The extract showed an exponential increase in cytotoxic activity against both HeLa and A549 cells [Figure 2 and 3]. The IC_{50} value of the extract against HeLa cells was 106 µg/mL [Table 1]. A similar result was also found against A549 cells, which showed the IC_{50} value of 116 µg/mL. In both the cytotoxic activity graphs [Figure 4 and 5], slope showed a very high correlation between increasing concentration of the fungal extract and corresponding decrease in cell viability as

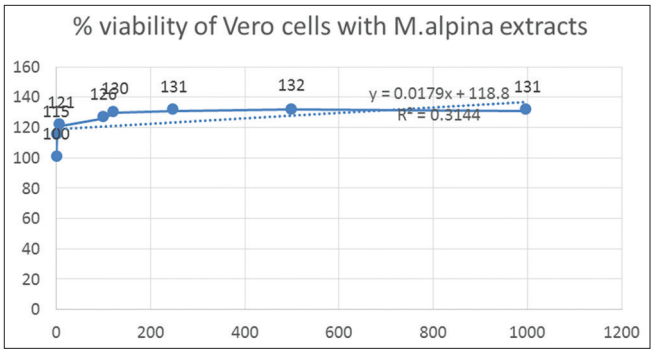

Figure 1: Concentration versus % viability with Vero cells

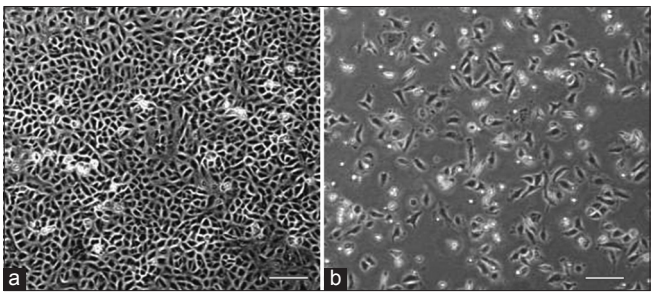

Figure 2: Microscopy of cytotoxicity analysis in A549 cells using dimethyl sulfoxide extract of *Mortierella alpina* (a) at 1.0 µg and (b) at 125 µg

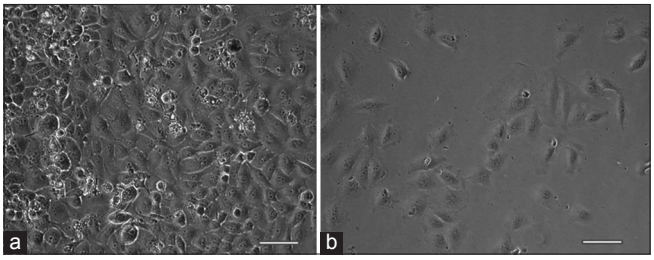

Figure 3: Microscopy of cytotoxicity analysis in HeLa cells using dimethyl sulfoxide extract of *Mortierella alpina* (a) at 1.0 µg and (b) at 125 µg (*Scale Bar = 100 µm)

depicted by the R^2 value of 0.977–0.971 with HeLa and A549 cells, respectively.

Further, the non-inhibitory activity of the extract against the healthy Vero cell lines was very evident with a CC_{50} of 2674 µg/mL. Furthermore, the TI value which is the ratio of CC_{50} and IC_{50} was recorded at 25.22 and 23.05 with HeLa and A549 cells, respectively.

DISCUSSION

The increasing number of cancer cases around the world is demanding for more improved strategy for prevention as well as treatment of this dreaded disease. Approaches, which are more than just a treatment mode rather which may be included as a nutritional supplement in our regular diet, can be a better strategy to prevent carcinogenesis.

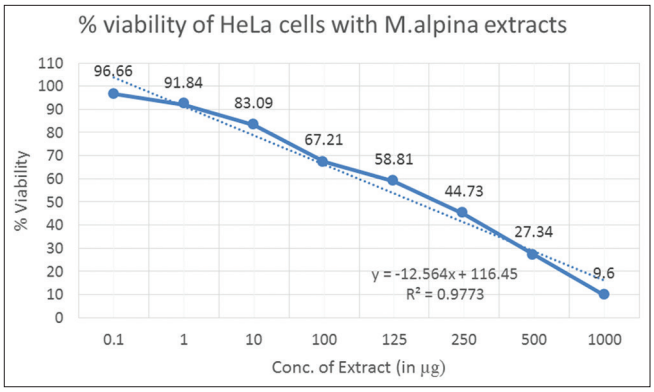

Figure 4: Concentration versus % viability with HeLa cells

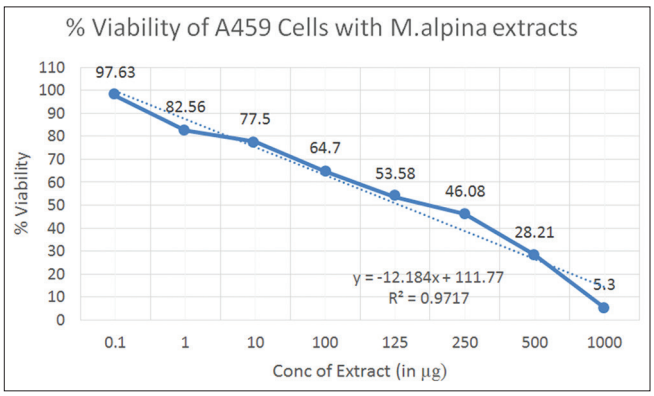

Figure 5: Concentration versus % viability with A459 cells

Table 1: Cytotoxicity analysis in HeLa cells using DMSO extract of *M. alpine*

Cell line	IC_{50} values (in µg/mL)	CC_{50} value (in µg/mL)	TI
HeLa	106		25.22
A549	116		23.05
Vero		2674	16.901

M. alpine: *Mortierella alpine*, DMSO: Dimethyl sulfoxide, IC: Inhibitory concentration, CC: Cytotoxic concentration, TI: Therapeutic index

Fungal extracts including *Saccharomyces cerevisiae* and the endophytic fungus *Annona muricata* extracts have been demonstrated previously to possess anticancer activity in cancer cell lines *in vitro*.[14,17] The present study reports the anticancer activity of the oleaginous fungus, *M. alpine,* which is also having one of the most efficient ARA-producing capabilities. The extracts of *M. alpina* which has been previously reported to have multiple biological activity including antioxidant and antibacterial activity[18] showed a good inhibitory effect on the cervical cancer cell line HeLa, as well as on the adenocarcinoma cell line A549. Further, the low IC_{50} of the extract against both the cell lines highlights the potential of the extracts as a possible therapeutic agent. The IC_{50} values obtained in the present study were in line with the maximum limit set for crude extracts at 30 mg/mL by the National Cancer

Institute.[19] This was ascertained by *in vitro* study carried out on the normal kidney Vero cell lines in which the extracts showed CC only at a very high concentration which was quite promising. Furthermore, the TI value reaffirmed the possible therapeutic ability of *M. alpina* AIB-RR2 extracts. As a result, it is predicted that this extract may possess significant therapeutic characteristics for cancer. At relatively low concentrations, this extract was able to inhibit HeLa cells and A549 cells and this inhibition was in a dose-dependent manner. This important finding gives a hope that *M. alpina* RRB_2 active materials can be useful in cancer. Further detailed studies needed to be conducted on the extracts of *M. alpina* to identify the potent anticancer molecules so as to develop specific extraction procedures as well as biotechnological approaches to improve the production of the target metabolite. Fungus produces numerous secondary metabolites, many of which are found to have multiple biological activities, which includes anticancer activities also. Some of the anticancer compounds produced by fungus include anguidine and rhizoxin which act at molecular level including protein synthesis inhibition and inhibition of microtubule assembly. Referring to the above-detailed anticancer secondary metabolites produced by filamentous fungi including *Fusarium* and *Rhizopus* spp., further detailed investigation needs to be conducted at the molecular level, including secondary metabolite separation and structural characterization to identify the target molecule. Further cellular level studies also need to be carried out to identify the specific possible mechanisms of action of the anticancer molecule produced by *M. alpina*, which might include inhibition of protein synthesis, inhibition of DNA synthesizing enzymes, and inhibition of angiogenesis. Furthermore, rigorous biotechnological studies have to be undertaken to identify and explore specific production, extraction, and separation procedures to improve the production of the target metabolite.

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